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Huckebein is part of a combinatorial repression code in the anterior blastoderm

Luiz Paulo Andrioli [a,b,*], Luciano Antonio Digiampietri [b,1], Lilian Ponce de Barros [b,1], Ariane Machado-Lima [b,1]

[a] Departamento de Genética e Biologia Evolutiva, Instituto de Biociências, Universidade São Paulo, R. do Matão, 277, Cidade Universitária, 05508-000, São Paulo, SP, Brazil
[b] Escola de Artes, Ciências e Humanidades, Universidade de São Paulo, R. Arlindo Béttio 1000, Ermelino Matarazzo, 03828-000, São Paulo, SP, Brazil

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ABSTRACT

The hierarchy of the segmentation cascade responsible for establishing the *Drosophila* body plan is composed by gap, pair-rule and segment polarity genes. However, no pair-rule stripes are formed in the anterior regions of the embryo. This lack of stripe formation, as well as other evidence from the literature that is further investigated here, led us to the hypothesis that anterior gap genes might be involved in a combinatorial mechanism responsible for repressing the cis-regulatory modules (CRMs) of *hairy (h), even-skipped* (eve), *runt (run)*, and *fushi-tarazu* (*ftz*) anterior-most stripes. In this study, we investigated *huckebein* (*hkb*), which has a gap expression domain at the anterior tip of the embryo. Using genetic methods we were able to detect deviations from the wild-type patterns of the anterior-most pair-rule stripes in different genetic backgrounds, which were consistent with Hkb-mediated repression. Moreover, we developed an image processing tool that, for the most part, confirmed our assumptions. Using an *hkb* misexpression system, we further detected specific repression on anterior stripes. Furthermore, bioinformatics analysis predicted an increased significance of binding site clusters in the CRMs of *h* 1, *eve* 1, *run* 1 and *ftz* 1when Hkb was incorporated in the analysis, indicating that Hkb plays a direct role in these CRMs. We further discuss that Hkb and Slp1, which is the other previously identified common repressor of anterior stripes, might participate in a combinatorial repression mechanism controlling stripe CRMs in the anterior parts of the embryo and define the borders of these anterior stripes.

Introduction

The segmentation gene cascade is responsible for the specification of the *Drosophila* anterior–posterior axis throughout the blastoderm stages, but with profound regional differences. In the prospective thorax and abdomen, the cascade has three hierarchical levels that are composed of gap, pair-rule and segment polarity genes, whereas in the anterior head region, pair-rule stripes are not formed (Cohen and Jurgens, 1990; Finkelstein and Perrimon, 1990; Grossniklaus et al., 1994). Pair-rule stripes of *hairy* (*h* 1, *h* 2), *even-skipped* (*eve* 1, *eve* 2), *runt* (*run* 1) and *fushi-tarazu* (*ftz* 1) are expressed in the transition zone between these two macro regions, which corresponds to the posterior head area that will give rise to the gnathal segments (mandibular, maxillary and labial).

The pioneer studies carried out with *eve* 2 led to a general model that explains the formation of a pair-rule stripe (Small et al., 1991, 1992). According to this model, a pair-rule stripe is the balance output of transcriptional factors that act on a cis-regulatory module (CRM) responsible for a stripe. The expression domains of transcriptional activators overlap and extend the area where a stripe is formed, and therefore, its CRM might be expressed in larger portions than the area that the stripe normally occupies. However, the limits of the expression domains of transcriptional repressors, one anterior of the stripe and another posterior the stripe, narrow the expression of the target CRM and set the anterior and posterior limits of a stripe.

For *eve* 2, it was originally shown that Hunchback (Hb) and Bicoid (Bcd) are activators, whereas Giant (Gt) and Krüppel (Kr) are anterior and posterior transcriptional repressors of the stripe, respectively (Small et al., 1991, 1992). However, further studies detected just minor derepression effects for *eve* 2 in a *gt-* genetic background (Andrioli et al., 2002; Wu et al., 1998). If Gt was its only anterior repressor, the activators Bcd and Hb, which are distributed up to the anterior tip, might activate this CRM throughout the anterior regions of the embryo. Thus, additional anterior repressors that regulate eve 2 were predicted. Moreover, these studies showed that ectopic Gt expression directly over *eve* 2 was not sufficient for the repression of this stripe (Andrioli et al., 2002; Wu et al., 1998). In agreement with that findings, the Sloppy-paired 1 (Slp 1) gap domain was shown to be another anterior repressor of *eve* 2 (Andrioli et al., 2002). However, this study indicated that Gt and Slp1 are still not sufficient to account for all aspects of the anterior repression of *eve* 2, and the prediction was

* Corresponding author at: Escola de Artes, Ciências e Humanidades, Universidade de São Paulo, R. Arlindo Béttio 1000, Ermelino Matarazzo, 03828-000, São Paulo, SP, Brazil. Fax: +55 11 2943 9076, +55 11 3091 7553.
E-mail address: lpma@usp.br (L.P. Andrioli).
[1] Fax: +55 11 2943 9076.

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that still other repressors probably work in combination with Gt and Slp1 to impede the expression of this CRM in different anterior sub-domains of the embryo.

In contrast to *eve* 2, little is known about the regulation of *h* 1, *eve* 1, *run* 1 and *ftz* 1, even though the CRMs for these stripes have been experimentally isolated (Cadigan et al., 1994; Calhoun and Levine, 2002; Fujioka et al., 1999; Howard and Struhl, 1990; Klingler et al., 1996; Riddihough and Ish-Horowicz, 1991). In these studies, the anterior borders of anterior-most stripes investigated in gap mutants did not show clear derepression effects. However, anterior stripes were detected with small patterning problems in *slp-* embryos, suggesting repressive roles for the gap domain Slp1 in the regulation of wild-type stripes (Andrioli et al., 2004). With respect to activators, genetic data showed that *bcd* and/or *hb* regulate *h* 1, *run* 1 and *eve* 1 (Fujioka et al., 1999; Klingler et al., 1993; Riddihough and Ish-Horowicz, 1991).

It is possible that the regulation of *h* 1, *eve* 1, *run* 1 and *ftz* 1 is not the result of the presence of several repressors working together, as described in the model proposed for *eve* 2. Nevertheless, we favor the hypothesis that these stripes are regulated in a similar fashion. Although the widespread activators Bcd and Hb are capable of transcribing anterior stripe CRMs in the anterior regions of the embryo, they do not always do so because of a local combinatorial repression mechanism. In this study, we show evidence that Huckebein (Hkb), in addition to the common repressor Slp1, is other repressor likely necessary to maintain the expression of *h* 1, *eve* 1, *run* 1 and *ftz* 1 CRMs restricted to the stripes' anterior borders. With genetic experiments and the use of an *hkb* misexpression system, we detected small deviations in the wild-type pattern of the anterior stripes that are consistent with Hkb repression roles. These results revealed that Hkb could be part of a combinatorial code mechanism that might be involved in the repression of an initial diffused pair-rule expression pattern that is detected before the striped pattern. Additionally, Hkb might contribute to defining the anterior border of the anterior-most pair-rule stripes.

Results

Early activation of pair-rule genes in the anterior blastoderm

Genetic evidence indicated that the CRMs of *h* 1, *eve* 1, *eve* 2 and *run* 1 can be activated by Bcd and Hb (see Introduction). Furthermore, ChIP/chip experiments that were performed to map the genome-wide binding of transcription factors show consistent Bcd and Hb *in vivo* binding in the regions corresponding to the CRMs of *h* 1, *h* 2, *eve* 1, *eve* 2, *run* 1 and *ftz* 1 (Li et al., 2008; MacArthur et al., 2009 Supplementary Figs. S1–4). Accordingly, pair-rule genes are broadly expressed in the embryo as early as nuclear cycle 10, before the well-known striped pattern that is formed later at nuclear cycle 14 (Ingham et al., 1985; Klingler et al., 1993; Pritchard and Schubiger, 1996; Tsai and Gergen, 1994). In a population of embryos aged up to 120 min after egg deposition, the expression of *eve* is initially detected throughout the entire embryo, but soon after, it quickly and progressively retracts from both poles (Fig. 1A–C). However, an anterior stripe-like stain at the position corresponding to *eve* 1 remains and gets stronger (Fig. 1C–E). We also hybridized embryos of the same collection for *h*, *run* and *ftz*. Like *eve*, these other pair-rule genes are initially broadly expressed in the embryo, but their ubiquitous expression patterns progressively vanish from both poles with the exception of an anterior stain that remains at the position corresponding to stripe 1 of each of the seven-striped pattern of these genes (Supplementary Fig. S5; data not shown). Thus, it is possible that the maternal factors Bcd and Hb are able to activate anterior-most stripe CRMs throughout the head region and that these CRMs are then repressed by anterior zygotic gap proteins.

There are several gap genes that are expressed in the anterior regions of the embryo, including *hkb*, *tailless* (*tll*), *orthodenticle* (*otd*), *empty-spiracle* (*ems*), *btd* and *slp1* (data not shown). Compared to the other gap genes listed above, *btd* occupies the posterior domain in the head and overlaps or partially overlaps with most of stripe 1 (Fig. 1F; data not shown). Therefore, this expression pattern better fits an

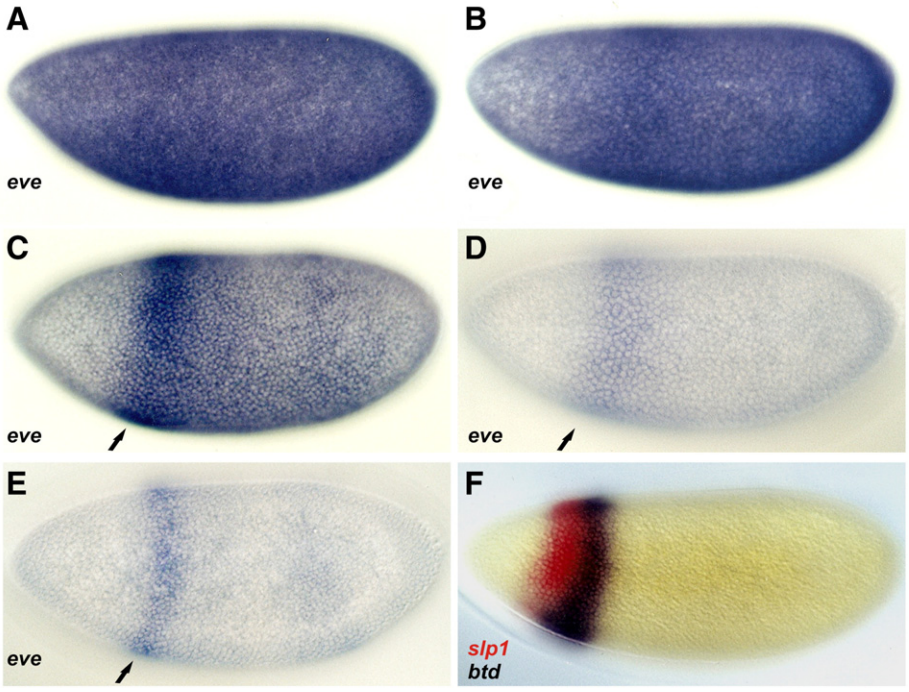


Fig. 1. Whole-embryo *in situ* hybridizations showing the expression of *eve* and the gap genes *slp1* and *btd*. Successive expression patterns of *eve* before the well-known seven-striped pattern is established (A–E). Initially, *eve* is detected all over the embryo (A), but then this ubiquitous expression pattern progressively disappears from both poles and moves towards the middle regions (B and C). Note that although most of the scattered detection vanishes, a stripe-like pattern remains and becomes darker at the position corresponding to *eve* 1 (indicated by arrows in C, D and E). (F) shows the relative positions of *slp1* and *btd* at early cycle 14. Unless otherwise mentioned, all embryos in this figure and in the following figures are displayed at the lateral view with the anterior portion of the embryo positioned towards the left. The dorsal side is upwards.

expected Btd-mediated activation role for these stripes. Indeed, *eve* 1 was detected as a weak stripe in a *buttonhead (btd)* mutant, which led to the suggestion that Btd is also an activator of this stripe (Vincent et al., 1997). Using a Position Weight Matrix (PWM) of Btd (Noyes et al., 2008) and the ClusterDraw program (Papatsenko, 2007), we were able to predict Btd binding sites in the CRMs of *h* 1 and *eve* 1 as well as *run* 1 and *ftz* 1 (Supplementary Figs. S1–4). Predictions of binding sites for Btd, Bcd and Hb performed on the whole locus of each of the investigated pair-rule genes showed highly significant clusters in the regions corresponding to the CRMs of *h* 1, *eve* 1, *run* 1 and *ftz* 1 (Table 1). Thus, Btd could be a putative activator of anterior-most stripes.

Hkb specifically represses anterior pair-rule stripes

Among anterior gap genes, *slp1* is a repressor that is necessary for the correct positioning of the anterior pair-rule stripes of *h*, *eve, run* and *ftz* (Andrioli et al., 2004). In *slp* mutant embryos, the anterior stripes *h* 1, *h* 2, *eve* 1, *eve* 2, *run* 1 and *ftz* 1 have anterior borders that are larger than wild-type and/or the whole stripe is shifted anteriorly. However, the new positioning of these stripes in the absence of Slp suggests that there are still other repressors responsible for setting these new anterior limits. We sought to investigate whether other anterior gap genes are involved in these regulations, and in this study, we investigated *hkb*.

Initially, we examined the striped patterns of *h*, *eve*, *run* and *ftz* in an *hkb* null mutant. We were able to identify *hkb*- embryos because of the posterior expansion of the *sna* domain, which reaches the posterior tip in this genetic background (Goldstein et al., 1999; Reuter and Leptin, 1994; Weigel et al., 1990). Thus, we observed small changes in the pattern of *eve* 1, and possibly *h* 1, but we were not able to detect changes in *run* 1 and *ftz* 1 in *hkb*- embryos (Supplementary Fig. S6). To confirm or disprove our assumptions, we developed an image processing tool to measure positional and shape changes of stripes in different genetic backgrounds (see Experimental procedures). We measured the distances from the anterior borders of the stripes to the anterior tip in wild-type and *hkb*- embryos, and we verified that this distance was significantly lower for *eve* 1 in *hkb*- embryos (Table 2). These data indicate a possible anterior repression role for Hkb on *eve* 1. We did not obtain such confident data for *h* 1 (Table 2). In an attempt to detect further deviations from the wild-type striped pattern, we made crosses with the parental stocks to obtain *slp*-; *hkb*- double-mutant embryos. As a result of these crosses, we observed embryos that had *h* 1, *h* 2, *eve* 1, *eve* 2, *run* 1 and *ftz* 1 stripes with larger anterior borders, and/or these stripes were shifted towards the anterior when compared to stripes in wild-type embryos (Fig. 2). Moreover, the measurements confirmed that the distances from the *h* 1, *eve* 1 and *run* 1 anterior borders to the tip were significantly lower in the double- mutant embryos even when compared to embryos of the *slp*- parental stock (Table 2). These increased effects detected in the double mutant embryos are consistent with Hkb repression acting on *h* 1, *eve* 1 and *run* 1.

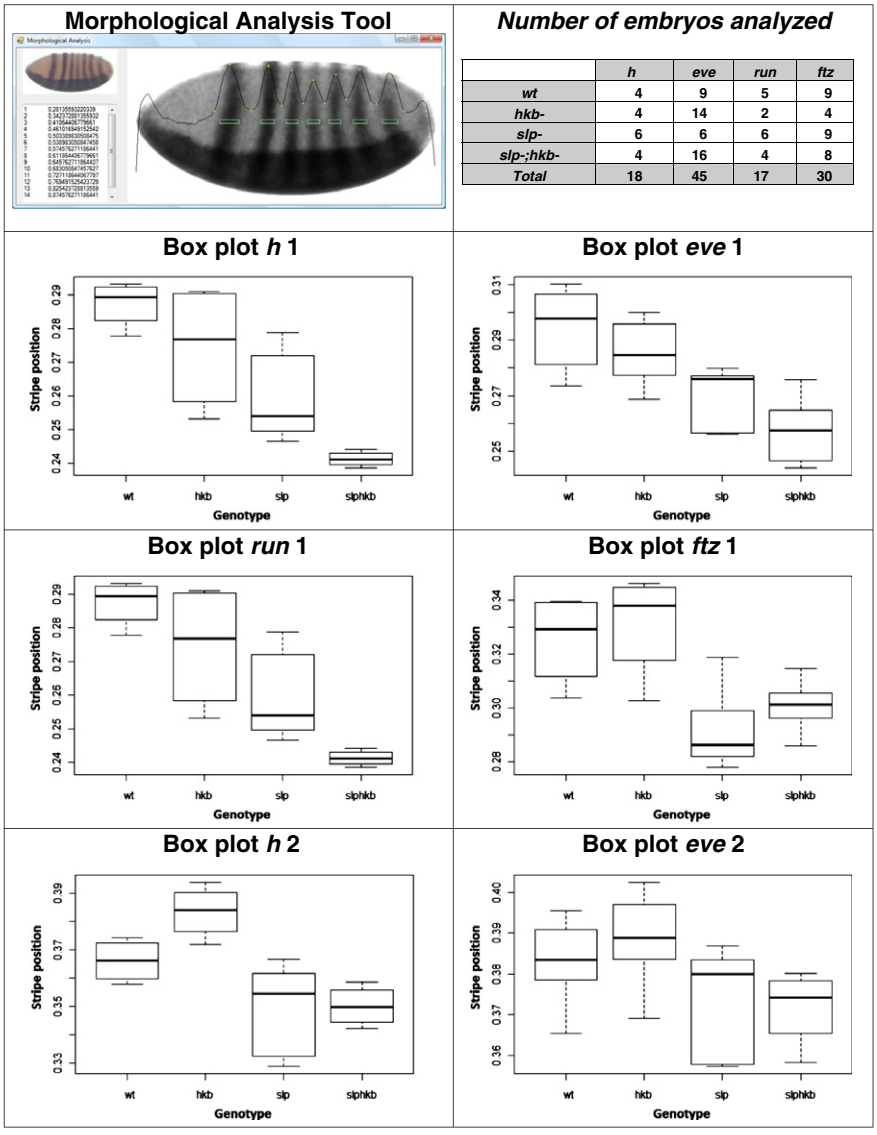
To further investigate Hkb activity in the regulation of *h*, *eve*, *run* and *ftz*, we cloned the *hkb* coding region into the *CasPeR>twi* vector (Nibu and Levine, 2001). This cloning strategy enabled us to express *hkb* in a ventral ectopic domain at a time when Hkb endogenous domains are simultaneously expressed (Fig. 3A). Embryos misexpressing *hkb* demonstrated clear repression effects on *h* 2, *eve* 1 and *run* 1, as well as a less noticeable effect on *ftz* 1 (Fig. 4). We also detected effects on *eve* 2, *run* 2 and *ftz* 2. Stripes *eve* 2, *run* 2 and *ftz* 2 were detected as partially repressed stripes that exhibited anterior ventral distortions. We could not conclude whether or not *h* 1 was repressed in these assays because this stripe normally retracts from the ventral regions, and we were not able to confirm increased *h* 1 retraction in transgenic embryos. However, we were able to test the ectopic expression in flies carrying an *h* 1 + 5 reporter construct. The reporter stripe 1 of this construct does not exhibit the normal ventral retraction like the endogenous *h* 1 stripe. Thus, we were able to detect weak ventral repression on *h* 1 when compared to control flies (Fig. 5B). We also tested an *eve* 1 + 5 reporter construct and detected the expected ventral repression on *eve* 1 (Fig. 5D).

We used the misexpression system to investigate the effects of *hkb* on gap genes, but we only detected clear repression effects for the anterior *gt* domain (Fig. 3C). The previous observation that the expansion caused in the anterior *gt* domain is increased in *tll*-; *hkb*- double mutant embryos compared to the *tll*- embryos led to the hypothesis that Hkb is an anterior repressor of *gt* (Eldon and Pirrota, 1991). On one hand, the fact that *gt* was the only detected gap gene affected in the misexpression assays argues that Hkb has direct repression roles on *h* 1, *h* 2, *eve* 1, *run* 1 and *ftz* 1. On the other hand, these results could also explain the anterior torsions detected for *eve* 2, *run* 2 and *ftz* 2. Gt is a common anterior repressor of the anterior borders of these stripes (Ribeiro et al., 2010). Thus, the ventral repression of Gt in the misexpression assays probably allowed for the expression of these stripe CRMs in the anterior nuclei, although further expansions are probably repressed by other factors. At the same time, Gt repression could be required for the expansion of a posterior regulator of these stripes, thereby triggering the repression of target stripe CRMs in the more ventral posterior nuclei. This possibility was previously demonstrated for *eve* 2 (Wu et al., 1998). In the absence of Gt, Kr, which is the posterior repressor of *eve* 2, expands

Table 1
Predicted Bcd, Hb, Btd and Hkb binding sites in the stripe 1 and 5 CRMs of the pair-rule genes *eve*, *h*, *run* and *ftz*. For each of the following CRMs, *eve* 1, *eve* 5, *h* 5 (only), *h* 5 + 1, *run* 1, *ftz* 1 + 5 and *run* 5, this table shows the length of the CRM in base pairs, the number of predicted sites, the -logarithm of the p-value of the cluster detected in each one of these regions and the rank of the cluster (with rank 1 being the most significant of the whole gene locus). Predictions were done using 3 matrices of putative activators (Bcd, Hb, Btd) (white columns) or using the three activators and Hkb matrices (grey columns). The results are presented in order of cluster rank. The CRMs of *eve* 1 and *eve* 5 are adjacent regions and only one long cluster was predicted for Bcd, Hb, Btd motifs covering both CRMs. With the Hkb input, this cluster split in two. In the column labeled "number of sites" for Bcd, Hb, Btd and Hkb, the number in parentheses represents only the number of Hkb sites predicted.

CRM	Lenght	Number of sites		-log(cluster p-value)		Cluster rank	
		Bcd Hb Btd	Bcd Hb + Hkb Btd	Bcd Hb Btd	Bcd Hb + Hkb Btd	Bcd Hb Btd	Bcd Hb + Hkb Btd
eve 1	788	25	28 (4)	8.33	8.51	1	1
eve 5	1011	24	26 (5)	8.33	6.54	1	2
h 5	1674	46	49 (5)	6.39	6.02	2	2
run 1	1611	44	47 (15)	5.16	5.37	3	2
ftz 1 + 5	1254	20	23 (5)	4.87	4.81	3	2
h 5 + 1	1157	29	32 (10)	5.03	5.63	4	3
run 5	1336	20	25 (5)	3.81	3.99	11	10

Table 2
Morphological analysis of *h* 1, *h* 2, *eve* 1, *eve* 2, *run* 1 and *ftz* 1 stripe position. The distances between the anterior borders of anterior stripes and the anterior tip were measured in relation to the distance between the ends of embryos of different genotypes hybridized for *h*, *eve* or *run* and *ftz*. Upper on the left, this table presents a screenshot of the developed morphological analysis tool used to identify the position of the first stripe for all of the analyzed embryos. Upper on the left, there is the number of embryos used to measure stripes for each genotype. Below are the box plot diagrams for the analyzed set and the student's *t*-test probabilities. The value 1.99% in the *eve* 1 *t*-test table, for example, indicates the probability that there is no distinction between the stripe position in *slp*- and *slp-;hkb*- mutant embryos. Thus, the absence of Hkb in the double mutant embryos causes further anterior derepression of the CRM, which is consistent with the hypothesis that Hkb is a repressor for this stripe.



	h	eve	run	ftz
wt	4	9	5	9
hkb-	4	14	2	4
slp-	6	6	6	9
slp-;hkb-	4	16	4	8
Total	18	45	17	30

T-test -*h1*

	slp-	slp-;hkb-	wt
hkb-	21.94%	3.84%	27.09%
slp-		1.93%	0.23%
slp-;hkb-			0.04%

T-test –*eve1*

	slp-	slp-;hkb-	wt
hkb-	1.92%	0.00%	7.88%
slp-		3.07%	0.20%
slp-;hkb-			0.00%

T-test -*run 1*

	slp-	slp-;hkb-	wt
hkb-	26.16%	16.01%	85.63%
slp-		15.75%	0.50%
slp-;hkb-			0.25%

T-test -*ftz 1*

	slp-	slp-;hkb-	wt
hkb-	2.05%	4.92%	67.01%
slp-		14.23%	2.01%
slp-;hkb-			5.72%

T-test -*h2*

	slp-	slp-;hkb-	wt
hkb-	0.30%	0.16%	2.90%
slp-		95.81%	6.44%
slp-;hkb-			2.27%

T-test -*eve 2*

	slp-	slp-;hkb-	wt
hkb-	4.88%	0.00%	26.00%
slp-		68.40%	18.94%
slp-;hkb-			1.17%

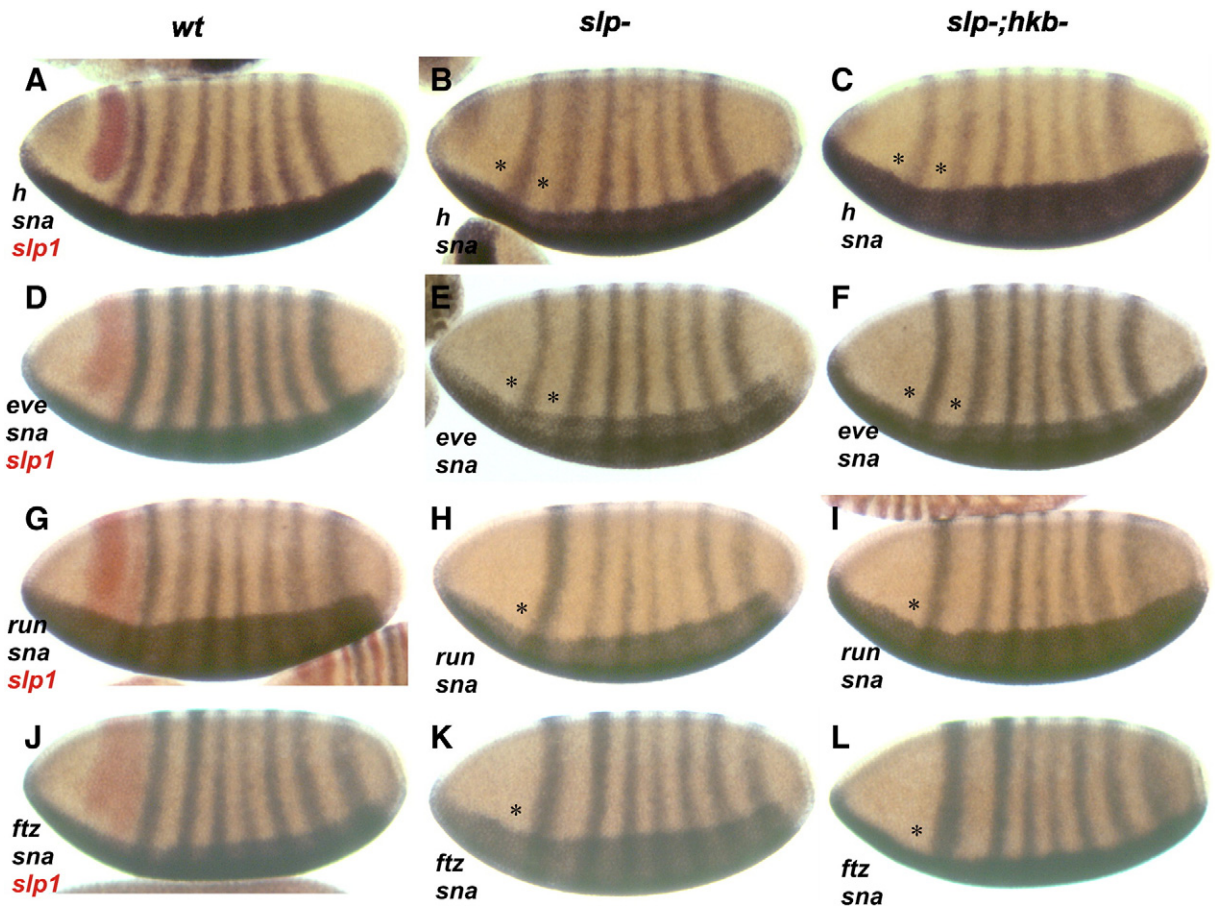


Fig. 2. Anterior pair-rule stripes exhibit increased derepression effects in *slp-;hkb-* embryos. Triple *in situ* hybridizations of the indicated pair-rule genes (black), *sna* (black) and *slp1* (red) shown in wild-type, *slp-* or *slp-;hkb-* embryos. Note the absence of *slp1* staining in *slp-* embryos as well as the posterior expansion of the *sna* ventral domain in *hkb-* embryos, which is a typical feature of this genetic background. The stripes marked with asterisks are *h* 1 and *h* 2 (B, C), *eve* 1 and *eve* 2 (E, F), *run* 1 (H, I) and *ftz* 1 (K, L). In *slp-;hkb-* embryos, all of these stripes exhibit increased deviation effects compared to wild-type embryos, and this deviation is statistically significant.

anteriorly and represses the posterior portions of the stripe. Taken together, the misexpression results are in agreement with the genetic results and reinforce an *hkb*-specific and direct regulation for the anterior-most pair-rule stripes.

With the bioinformatics analysis, we not only confirmed previous studies that predicted Hkb binding sites in the CRMs of the anterior-most stripes (MacArthur et al., 2009; Noyes et al., 2008), but we also examined this data (Table 1; Supplementary Figs. S1–4). Comparisons of the computational predictions of Bcd, Hb and Btd binding sites with predictions of Bcd, Hb, Btd plus Hkb binding sites also reinforce Hkb regulation of *h* 1, *eve* 1, *run* 1 and *ftz* 1 (Table 1). Binding site clusters predicted over the entire locus of each gene were ranked according to their significance values. The ranks of the clusters predicted in the stripe 1 CRMs were improved when the Hkb data was considered and, in the case of rank maintenance, the cluster significance was also improved. Therefore, such Hkb sites might not be spurious, and these predictions might indicate that Hkb indeed regulates *h* 1, *eve* 1, *run* 1 and *ftz* 1. Interestingly, the CRMs of *h* 5 + 1 and *eve* 5 also showed significant cluster values. These results could indicate that the physical link between stripe 1 and 5 CRMs could underlie the observed molecular regulatory constraints. It is also worth noting that the biological data from ChIP/chip experiments (Li et al., 2008; MacArthur et al., 2009) confirm Bcd, Hb and Hkb binding in the stripe 1 and 5 regions of *h*, *eve*, *run* and *ftz* (Supplementary Figs. S1–4). The only exception was *run 5*, on which no binding was detected. Indeed, the computationally predicted cluster in this region achieved rank 10, the least significant of all clusters predicted in the analyzed CRMs involving stripes 1 and 5 (Table 1).

Discussion

Our aim is to understand the mechanisms underlying the regulation of the anterior pair-rule stripes. We are testing the model that was first proposed for *eve* 2 regulation (Andrioli et al., 2002; Small et al., 1991, 1992). Transcriptional activators do not give enough patterning information, and the presence of repressors is instructive for determining the precise positioning of a particular stripe (Papatsenko et al., 2009). Our hypothesis is that transcription repressors could be working in a combinatorial manner to determine the correct positioning of the anterior stripes and prevent, in a spatial and temporal manner, the expression of stripe CRMs in the more anterior regions of the embryo by counteracting the activity of activators. There is plenty of evidence supporting this hypothesis, which we further confirmed in this study.

Regarding activators, our computational analysis predicted Bcd, Hb and Btd binding sites as part of significant clusters in the anterior-most stripe CRMs (Table 1; Supplementary Figs. S1–S4). These predictions agree well with previous genetic data and *in vivo* DNA binding data from ChIP/chip experiments (Howard and Struhl, 1990; Riddihough and Ish-Horowicz, 1991; Klingler et al., 1996; Vincent et al., 1997; Fujioka et al., 1999; Li et al., 2008; MacArthur et al., 2009). Thus, Btd, and above all the widely spread maternal factors Bcd and Hb, might activate anterior stripe CRMs early in the anterior blastoderm. Alternatively, the early broad expression patterns of pair-rule genes could be under the control of dedicated CRMs, although no such elements have yet been reported. It is possible that other regulatory elements could contribute to the expression detected early in the anterior

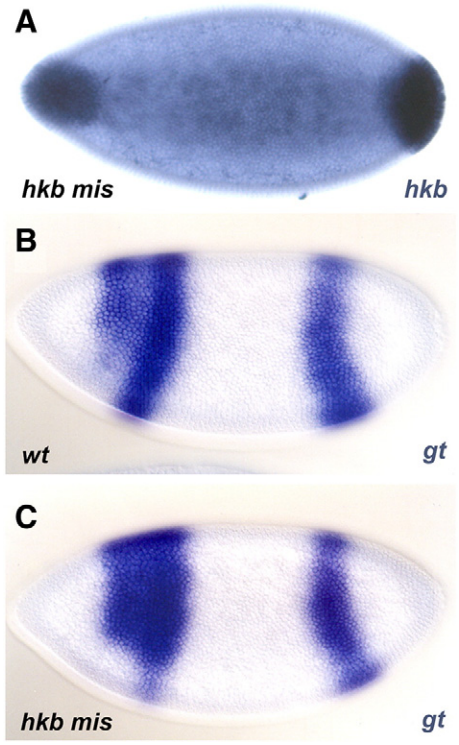


Fig. 3. The ectopic misexpression of *hkb* disrupts the *gt* anterior domain. *In situ* hybridization of *hkb* in a transgenic embryo that carries the misexpression construct reveals *hkb* endogenous expression domains at the poles and its ventral ectopic domain (A). In contrast to other images, this embryo is ventrally positioned. Note that in relation to the *hkb* endogenous domains, the ventral ectopic domain is weakly expressed. *In situ* hybridization of *gt* reveals *gt* expression domains in wild-type (B) or in transgenic (C) embryos. Note that the *gt* anterior domain is ventrally repressed in the embryo that misexpresses *hkb* (C).

blastoderm, for instance, the CRM responsible for the expression of *h* head patch or the CRMs responsible for *eve* 3, *eve* 5 and *h* 5, which were proposed to be activated by the maternal factor DSTAT (Drosophila Signal Transducer and Activator of Transcription), which is ubiquitously expressed in the embryo (Hou et al., 1996; Lardelli and Ish-Horowicz, 1993; Yan et al., 1996).

The expression of several gap domains covering all of the anterior regions of the embryo ahead of the seven-striped patterns is consistent with the expected subsequent local repression of pair-rule CRMs activated in the head region. Of these gap domains, Slp1 is a common repressor for anterior pair-rule stripes, but other repressors besides Slp1 were predicted to be necessary for correctly determining the borders of the anterior-most stripes (Andrioli et al., 2004). Here, we investigated *hkb*, which, in addition to *tll*, is the other major gap gene target of the Torso signaling regulation in the terminal system (Furriols and Casanova, 2003). In the anterior region, *hkb* is required for the proper formation of the foregut and midgut (Reuter and Leptin, 1994). Its domain at the anterior tip coincides with the region where the diffused early expression patterns of pair-rule genes first fade (compare Fig. 1B and Supplementary Fig. S5B, E, H with Fig. 3A). These observations are consistent with local repression roles of Hkb. However, we were not able to detect derepression of pair-rule genes in the anterior pole of *hkb*- embryos (Supplementary Fig. S6). One possibility is that the progressive non-detection of the expression of pair-rule genes might correspond to a failure in activation (Fig. 6A). In fact, Bcd activation was shown to be down-regulated by the Torso-signaling cascade at the anterior tip (Ronchi et al., 1993). Nevertheless, other data suggest that the Torso pathway might induce a repression mechanism at the anterior tip that would be parallel and redundant with Torso-induced inhibition of Bcd (Gao et al., 1996; Janody et al., 2000). Thus, one might predict that another repressor might still able to act on Hkb targets in the absence of Hkb protein (Fig. 6A).

Although we did not detect any pair-rule derepression in the anterior pole, we were able to detect subtle deviations in the positioning of *eve* 1 in *hkb*- embryos, which we confirmed by morphological measurements using our image processing tool (Supplementary Fig. S6; Table 2). We also detected enhanced derepression effects for all anterior-most stripes investigated in *slp-;hkb-* double-mutant embryos compared to the effects observed in *slp-* embryos; these results were statistically significant (Fig. 2; Table 2). With the *hkb* misexpression system, we detected repression effects for *h* 1, *eve* 1, *run* 1 and *ftz* 1 (Fig. 4). With the exception of *gt* repression, we did not detect any other gap domain disruption in these assays (Fig. 3; data not shown). These results strongly suggest direct repression by Hkb on the CRMs of these stripes. *In vivo* binding data confirms this possibility (Li et al., 2008; MacArthur et al., 2009; Supplementary Figs. S1–4). Moreover, with the bioinformatics analysis we verified that Hkb, along with putative activators, increased the already high

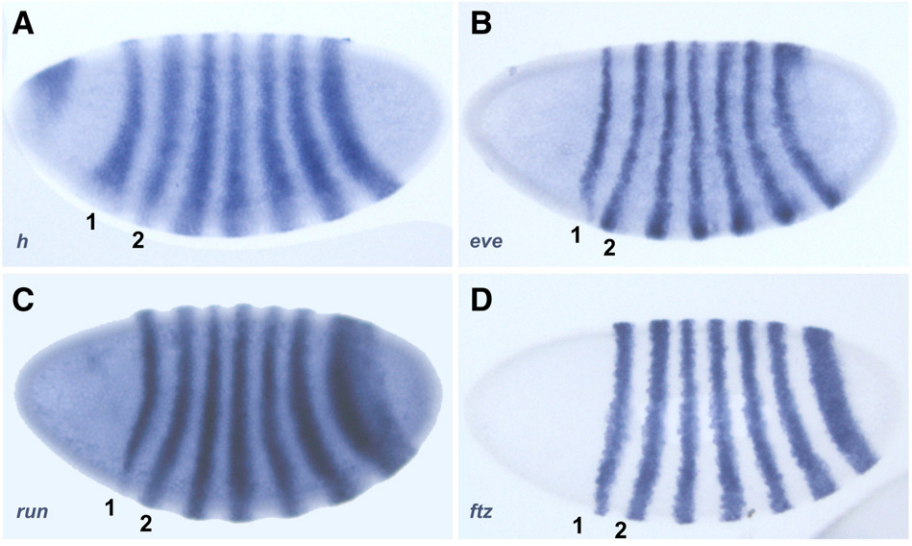


Fig. 4. The ectopic misexpression of *hkb* disrupts pair-rule striped patterns. Transgenic embryos expressing the *hkb* ventral ectopic domain hybridized for *h* (A), *eve* (B), *run* (C) and *ftz* (D). Note the major repression effects on *h* 2, *eve* 1 and *run* 1 and the less dramatic effect on *ftz* 1. The expression patterns of *eve* 2, *run* 2 and *ftz* 2 exhibit anterior torsions in their ventral parts, which are probably caused by the indirect effects of *hkb*, as discussed in the text.

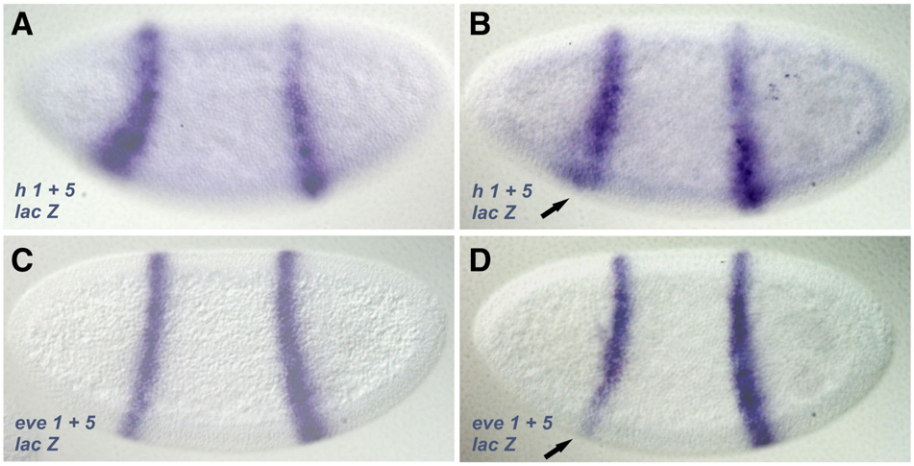


Fig. 5. The ectopic misexpression of *hkb* disrupts pair-rule CRMs. Transgenic embryos expressing the *hkb* ventral ectopic domain also carrying reporter constructs of *h* 1 + 5 *lac Z* (A and B) or *eve* 1 + 5 *lac Z* (C and D). *In situ* hybridization for the *β-galactosidase* reporter gene shows specific ventral repression of *h* 1 (B) and *eve* 1 (D), indicated by arrows.

significance values of predicted clusters for activators that match these stripe CRMs (Table 1). Therefore, the combined data suggest that Hkb acts as a repressor for a specific group of anterior pair-rule stripes.

These data also suggest that there is another possible mechanism underlying the repression that involves the activity of repressors further away from their original sources. One example of this mechanism is expression detected for the ectopic *hkb* domain (Fig. 3A), demonstrating that target CRMs are sensitive to Hkb-mediated repression even in the presence of low expression levels of Hkb. The prediction is that low concentrations of Hkb that have diffused away from its endogenous domain could still repress these CRMs. For this mechanism, repressors could fulfill additive repression roles at different anterior subdomains or even contribute to the definition of the anterior borders of stripes that are distantly positioned from where gap domains are detected (Fig. 6B). Thus, the increased derepression observed in *slp-; hkb-* embryos would be expected if a combinatorial additive mechanism existed in which each repressor had a small contribution to the overall repression. Following the same rationale, one can predict that at least one other repressor is still responsible for setting anterior border stripes in *slp-;hkb-* embryos.

The complexity of the regulation of genes involved in early patterning was postulated to be a condition that is necessary for sensing relatively small differences in the concentrations and combinations of many regulatory factors, which is likely the environment found in the syncytial blastoderm (Ochoa-Espinosa and Small, 2006). In agreement with that hypothesis, recent studies revealed that the protein gradients of factors such as Bcd and Dorsal alone are not sufficient to determine all of the spatial limits of target gene expression and that these gradients might combine with other factors to pattern the early embryo (Chopra and Levine, 2009; Löhr et al., 2009; Ochoa-Espinosa et al., 2009). In the head region, it has been suggested that Bcd and the terminal system-mediated activities interact at the level of the target CRMs to generate the proper patterning for the head region of the embryo (Löhr et al., 2009; Ochoa-Espinosa et al., 2009). In contrast to these two studies that focused on gap genes, our data shed light on a mechanism that is involved in the regulation of the anterior stripe CRMs, with the putative participation of *hkb*.

The correct positioning of the anterior pair-rule stripes must be a critical issue in the early developmental patterning of the fly. Even a slightly incorrect positioning of the anterior stripes, for instance, results in the non-formation of the mandibular segment in the *slp* null mutant (Andrioli et al., 2004). Thus, a complex repression mechanism is necessary to shape the stripes and to avoid inappropriate expression of their CRMs. Therefore, Hkb, Slp1 and other repressors are likely involved in a combinatorial repressive activity in the CRMs of the anterior stripes

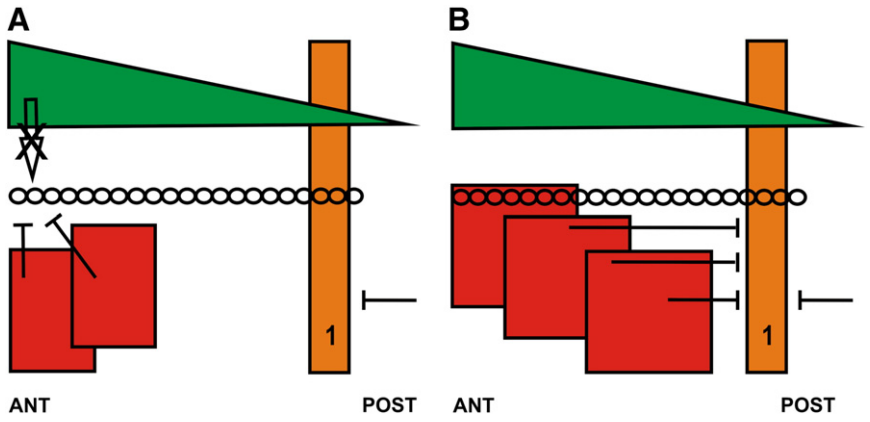


Fig. 6. Hkb is part of the anterior combinatorial repression mechanism that controls the CRMs of the anterior-most pair-rule stripes. Putative schematic representation of the activities of activators (green triangles) and of repressors (red rectangle) on a CRM of an anterior-most stripe (orange rectangle) relative to nuclei (represented by a row of circles) arranged in the anterior–posterior axis (ANT and POST, respectively) in wild-type embryos. (A) At the anterior tip, the crossed activation arrow indicates Bcd failure in the CRM activation, and the repression arrows indicate local repression by Hkb and an Hkb-redundant repressor as discussed in the text. (B) The repression activities of repressors (including Hkb and Slp1), additively contribute to the correct positioning of the anterior border of an anterior-most stripe. The posterior repression arrow indicates the repression activities responsible for setting the posterior border.

(Fig. 6). Other experiments are necessary to test this hypothesis further and to reveal the underlying molecular mechanisms involved in this regulation.

Experimental procedures

Genetics

Flies from a y^1w^{67c23} stock were used as wild-type flies. In this study, we used flies with the mutant alleles $slp^{\Delta 34b}$ and hkb^{A32}. The $slp^{\Delta 34b}$ allele has a deficiency that removes both the *slp1* and *slp2* genes (Grossniklaus et al., 1992). The hkb^{A32} allele is a null mutant form of *hkb* (Casanova, 1990). We also used transgenic lines that express *lacZ* stripes under the regulatory regions for *h* 1+5 (Riddihough and Ish-Horowicz, 1991) and *eve* 1+5 (Fujioka et al., 1999).

In situ hybridization

Gene expression patterns were monitored by single, double or triple whole mount *in situ* hybridization experiments with antisense RNA probes (Kosman and Small, 1997). The embryos used in these experiments were usually between the ages of 2 and 4 h after egg-laying. To detect the early scattered expression patterns of pair-rule genes, we used embryos between 1 and 2 h old.

Ventral misexpression

We used the previously described *CasPeR>twi* (Nibu and Levine, 2001) to express *hkb* in an ectopic ventral domain. A 0.8-Kb *Bam*HI *Eco*RI fragment containing the *hkb* coding sequence was blunt-ended and cloned into the *CasPeR>twi* vector which had been previously opened with *Asc*I. Four transgenic lines were generated via microinjection and P-element-mediated transformation according to standard protocols (Small, 2000; Spradling, 1986). Misexpression was induced by generating males that contained the ectopic construct as well as the *β-tubulin-Flipase* transgene that is expressed during spermatogenesis (Struhl et al., 1993). These males were crossed with females of different genetic backgrounds and their embryos were collected for use *in situ* hybridization (Kosman and Small, 1997).

Computational sequence analysis

The CRMs responsible for *h* 1, *eve* 1, *run* 1 were defined by gene truncation experiments (Fujioka et al., 1999; Klingler et al., 1996; Riddihough and Ish-Horowicz, 1991), whereas *ftz* 1 was defined by analyzing a 10-kb region of the *ftz* locus for high-density clusters of binding sites (Calhoun and Levine, 2002). Interestingly, the regulatory regions for stripes 1 and 5 for these four pair-rule genes were isolated in adjoining or overlapping sequences. Therefore, stripe 5 sequences were also incorporated in our analysis. A detailed description of the sequences used in this study can be found in Ribeiro et al. (2010).

The prediction of binding sites was performed using the ClusterDraw webserver with default parameters (Papatsenko, 2007). This software receives as input a sequence and one or more position weight matrices (PWMs)in which the binding sites are predicted. The software also outputs the detected binding site clusters and their significance values (−log[p-value]). The clusters are ranked according to this significance value; the rank 1 cluster is the most significant. Therefore, we performed the analysis using the whole locus of each of the genes in order to obtain the cluster rank of the sites within CRMs 1 and 5. For each gene locus (*eve*, *h*, *run* and *ftz*), we performed two predictions: one using PWMs for three activators (Bcd, Hb and Btd) and another other using the same three PWMs plus the Hkb PWM. For each transcription factor, we chose the matrix derived from the largest number of sequences. The Bcd and Hb matrices were downloaded from the BDTNP project website (Berkeley Drosophila Transcription Network Project; Li et al., 2008), whereas the Btd and Hkb matrices were obtained from Noyes et al., 2008. The *eve*, *ftz*, *hairy* and *runt* DNA sequences with annotated regulatory regions were obtained from https://bspace.berkeley.edu/access/content/user/247388/data_04/appendix2.htm. Biological data from the ChIP/chip experiments describing the bound regions (at a 1% false discovery rate estimated by a symmetric-null test) were also downloaded from BDTNP website (Berkeley Drosophila Transcription Network Project; Li et al., 2008; MacArthur et al., 2009). Regions bound by each factor analyzed in this paper were added to the tables that show the computational predictions performed in this work.

Morphological image analysis

To measure the position of the embryos' stripes we developed a morphological analysis tool. This tool receives an image of an embryo as input and identifies the relative position of the borders of each stripe. The process of the identification of the stripe position is comprised of ten steps and is based on the color of the embryo images. In the first step, the image is converted to a matrix of bytes; the value of each cell in the matrix is calculated as an average value of the red and green color channels. In the second step, the image background is removed. In the third step, an increasing contrast algorithm is performed. In the fourth step, a low pass filter is executed over the full matrix in order to remove noise. In the fifth step, the anterior and posterior extremities of the embryo are identified. In the sixth step, the image is horizontally aligned from both ends. In the seventh step, a region of the middle of the embryo is identified and the histogram of this region is calculated. With this histogram, the peaks and valleys are identified. The peaks correspond to points inside the stripes and the valleys are points outside the stripes. A total of 15 or 17 peaks and valleys are expected in a given embryo. For example, the embryo in the screenshot from Table 2 contains seven peaks and eight valleys, which are highlighted as small rectangles in the image. Whenever the number of peaks plus valleys is different than the expected values, the tool executes an iterative process, step eight, which is composed of two tasks: performing a low pass filter in the histogram and calculating the new peaks and valleys. This iterative process is necessary to remove the remaining noise in the histogram. With the peaks and valleys calculated, the last step uses these values to identify the borders of each stripe.

The morphological analysis tool was used to identify the position of the anterior-most stripes of 61 embryos (18 stained for *h*, 26 for *eve* and 17 for *run*). Table 2 contains the *box plots* of the first stripe position for each gene. It is possible to observe the influence of different genetic backgrounds (*wt*, *hkb-*, *slp-* and *slp-;hkb-*) in the position of the first stripe. We were particularly interested in the comparison between the data of wt and *hkb-* and between *slp-* and *slp-*; *hkb-* embryos. Student's t-tests were calculated for each genotype in order to verify if this empirically observed influence has statistical significance. The t-tests values are shown in the bottom of Table 2. The null hypothesis for the *t*-test was that the studied genetic background has no influence in the position of the first stripe.

Supplementary materials related to this article can be found online at doi:10.1016/j.ydbio.2011.10.016.

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