Receptor tyrosine kinase signaling regulates different modes of Groucho-dependent control of Dorsal

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Transcriptional control of the *Drosophila* terminal gap gene *huckebein* (*hkb*) depends on Torso (Tor) receptor tyrosine kinase (RTK) signaling and the Rel/NFκB homolog Dorsal (Dl) [1–4]. Dl acts as an intrinsic transcriptional activator in the ventral region of the embryo, but under certain conditions, such as when it is associated with the non-DNA-binding co-repressor Groucho (Gro), it is converted into a repressor [5]. Gro is recruited to the enhancer element in the vicinity of Dl by sequence-specific transcription factors such as Dead Ringer (Dri) [6,7]. We examined the interplay between Dl, Gro and Dri on the *hkb* enhancer and show that when acting over a distance, Gro abolishes rather than converts Dl activator function. Reducing the distance between Dl- and Dri-binding sites, however, switches Dl into a Gro-dependent repressor that overrides activation of transcription. Both of the distance-dependent regulatory options of Gro – quenching and silencing of transcription – are inhibited by RTK signaling. These data describe a newly identified mode of function for Gro when acting in concert with Dl. RTK Dri-dependent recruitment of Gro to the enhancer.

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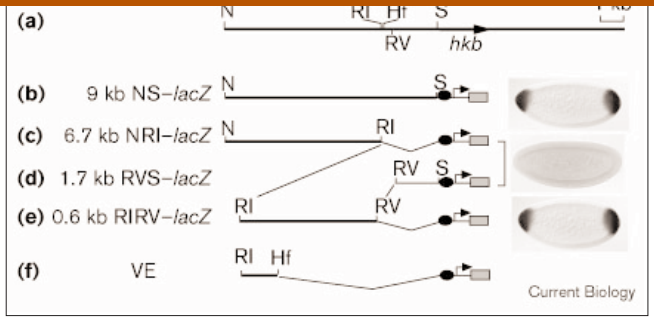
Results and discussion

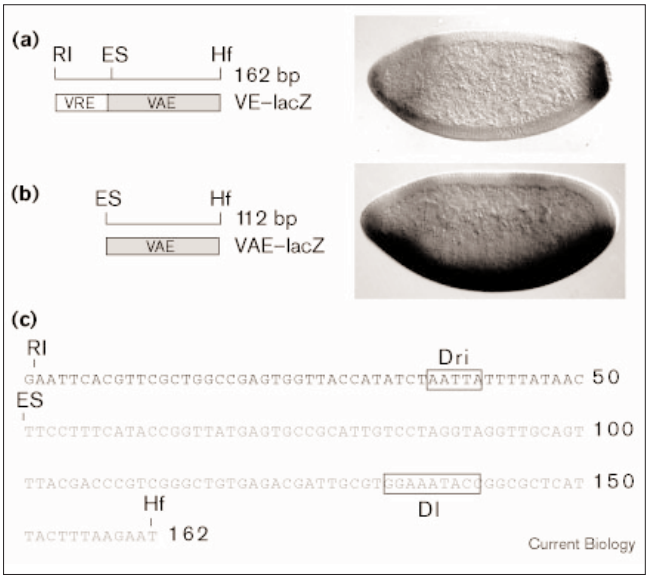
We identified the *cis*-acting element that mediates expression of the *Drosophila* gene *hkb*, which is necessary for terminal pattern formation and to size the mesoderm anlage in the blastoderm embryo [1,2]. Deletion analysis of this element (Figure 1a–f) revealed a 162 base pair (bp) sub-element (Figures 1f,2a) that integrates the activities of the Tor-dependent RTK signaling cascade [8] and the morphogen Dl [9] (see below). This element, termed *hkb* ventral element (VE; Figure 2a), comprises a 112 bp ventral activator element (VAE; Figure 2b) and a 50 bp ventral repressor element (VRE).

The VAE contains a Dl-binding site [10], identified *in vitro* (Figure 2c), and mediates gene activation along the ventral side of the embryo (Figure 2b). VAE-mediated gene expression is absent in embryos lacking Dl activity (Figure 3a) and extends throughout *Toll*^{10b} mutants (Figure 3b), in which Dl is present in all nuclei of the embryo [11]. The expression pattern is not altered in embryos lacking *snail* and *twist*, the zygotic mediators of Dl [12]. It is also not affected in embryos that lack Tor or express constitutively active Tor^{Y9}, which causes RTK signaling throughout the embryo [13] (data not shown). In contrast, the VE (Figure 2a) fails to activate in the absence of Tor (Figure 3c) and mediates broad ventral expression in *tor*^{Y9} embryos (Figure 3d) not seen in the absence of Dl activity



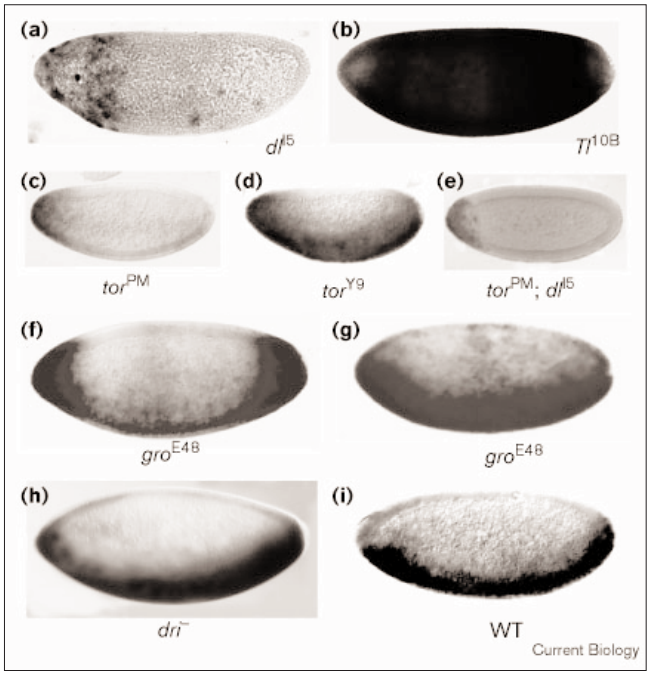
Dissection of the *cis*-acting control region of *hkb*. **(a)** The 14 kb transgene which rescues the *hkb* phenotype; the bold arrow represents the *hkb* transcript [1]. Restriction sites: Hf, *Hin*fI; N, *Not*I; RI, *Eco*RI; RV, *Eco*RV; S, *Sal*I. **(b–f)** Transgenes containing subfragments coupled to a *lacZ* reporter gene (grey box; left) and their expression pattern in the blastoderm embryo (right) as revealed by *in situ* hybridization with digoxigenin–UTP-labeled *lacZ* antisense [17]. Orientation of embryos: anterior left and dorsal side up. The fragment sizes and the restriction sites that identify the 5′ and 3′ ends are indicated. The fragments shown in (c,d) failed to drive gene expression, whereas fragments containing the region *Eco*RI–*Eco*RV (b,e) mediate *hkb*-like gene expression [1]. Thus, the *Eco*RI–*Eco*RV region is necessary and sufficient to drive *hkb*-like gene expression and includes the 162 bp VE ((f); see Figure 2). Genomic subfragments of the *hkb* promoter [1] were obtained by restriction digests or PCR amplification. For P-element-mediated transformation [23], DNA fragments were cloned into the shuttle vector pCaSpeR-*hs*43 [24]. At least two independent transgenic fly lines were examined.

Figure 2



Deletion analysis of the VE region. **(a)** VE-dependent *lacZ* reporter gene expression in the transgenic blastoderm embryo. **(b)** Deletion of the VRE causes expression along the ventral side of the embryo, showing that the VE is bipartite; the VRE prevents ventral activation mediated by the VAE. Orientation of embryos: anterior left and dorsal side up. **(c)** Sequence of the VE (for its position within the *hkb* enhancer, see Figure 1). A Dri-binding site within the VRE and a Dl-binding site within the VAE are boxed. Binding sites were identified by *in vitro* binding studies (data not shown). RI, *Eco*RI; ES, PCR primer site used to amplify the VAE and VRE fragments; Hf, *Hin*fI. Methods are described in the legend of Figure 1.

Figure 3



VAE- and VE-dependent *lacZ* reporter gene expression in mutant embryos. Orientation of embryos: anterior left, dorsal side up. Wild-type embryos show VAE-dependent expression along the ventral side (see Figure 2b). **(a)** The ventral expression domain is absent in embryos obtained from dl^{5} homozygous females. Patchy anterior expression due to P-element vector of the overstained embryo was used as an internal staining control. **(b)** Embryo from a Tl^{10B} female showing ubiquitous VAE-mediated expression. **(c)** VE-mediated expression in an embryo from a female homozygous for the *tor* lack-of-function allele tor^{PM}. The anterior expression domain is probably due to *bicoid*-dependent activation as described elsewhere [4]. **(d)** VE-mediated expression along the ventral side of an embryo in which *tor* was ubiquitously active (tor^{Y9}). **(e)** VE-dependent ventral gene expression is absent in tor^{Y9}; dl^{5} double mutants. **(f,g)** *hkb* expression (f) and VE-mediated *lacZ* expression (g) in Gro-deficient embryos (gro^{E48} allele). Note the expanded expression domains and the expression along the ventral side of the embryo, which has not been reported before [14]. This apparent discrepancy is probably due to different staining sensitivities. **(h)** VE-mediated expression in a Dri-deficient embryo showing that ventral repression of Dl-dependent activation by Gro is mediated by Dri. **(i)** $VE^{\Delta Dri}$-dependent expression in a transgenic wild-type embryo, showing a pattern similar to that seen with VE-dependent expression in *dri* mutants. $VE^{\Delta Dri}$ was generated by site-directed mutagenesis (primers: GGCCGAGTGGTTACCATATCT-GCGCGTTTTATAACTTCCTTTCATACC and a primer with the reverse complement of the 162 bp sequence shown in Figure 2c). Embryos lacking maternal *gro* activity were generated by the ovoD–FLP–FRT system [25]. Transgene construction and *lacZ* expression analysis is described in Figure 1.

(Figure 3e). This indicates that VAE mediates transcriptional activation by Dl, that the VRE, which by itself fails to activate transcription (data not shown), is necessary to prevent Dl-dependent activation in the central region of the embryo, and that the activity of the unknown repressor, mediated by the VRE, is relieved by RTK signaling.

The evolutionarily conserved co-repressor Gro ([5–7] and references therein) [14] acts as a repressor of Dl activity, as both *hkb* expression and VE-driven gene expression expand along the ventral side of embryos lacking *groucho* (*gro*) activity (Figure 3f,g). However, VAE-driven gene expression (data not shown) and the terminal expression domains of *hkb* are not significantly affected by lack of Gro (Figure 3f; see also [14]). Thus, Gro functions as a repressor of VAE-directed, Dl-dependent transcriptional activation in the ventral region of the embryo and must act through the VRE.

Previous results have shown that Gro switches the transcriptional activator Dl into a potent silencer of trans-cription [5]. This requires the formation of a multiprotein repressor complex of which Dl and Gro are obligatory components [6]. Complex formation requires that Gro is recruited next to Dl by sequence-specific transcription factors such as Cut or Dri [6,7]. Figure 3g,h shows that lack of either Gro or Dri activity results in VE-driven gene expression along the ventral axis of the embryo, indicating that both factors are necessary for repression of Dl-dependent activation. We identified a single binding

site for Dri [15] in the VRE (Figure 2c). Replacement of 5 bp in this site (VE–^{DRI}) resulted in loss of repression in the central region of the embryo (Figure 3i), indicating that Dri is necessary for recruitment of Gro to the VE.

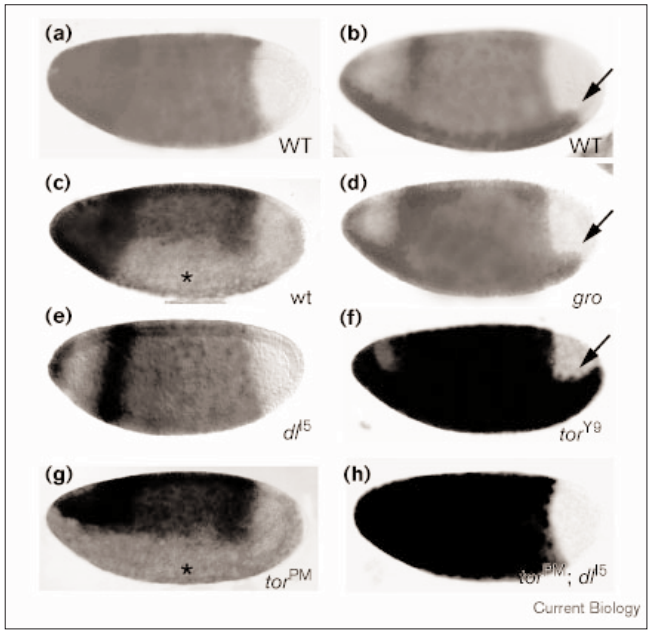
The VE differs from the *cis*-acting elements of the genes *zerknüllt* (*zen*) and *decapentaplegic* (*dpp*), which mediate long-range Dl-dependent transcriptional silencing by Gro [5–7]. In these elements, binding sites for Dri and Dl are directly adjacent, whereas in the VE they are some 90 bp apart (Figure 2c). This distance suggested the possibility that Gro cannot associate with Dl on the VE, implying that Gro must prevent Dl-dependent activation by a means other than formation of a long-range silencing complex, for example, by short-range quenching [7,16]. We tested this proposal by monitoring gene expression patterns directed by a *cis*-acting activator element of the gene *knirps* (*kni*-element) [17] to which the VRE, the VAE, the VE or molecularly defined variants of the VE were fused.

The *kni*-element drives gene expression throughout the embryo except in the posterior pole region (Figure 4a). It mediates activation in response to the transcriptional activators Bicoid (Bcd) and Caudal (Cad) [17] and acts in a Dl-independent fashion. Addition of the VRE to the *kni*-element did not cause ventral repression, nor did addition of the VE or the VAE (Figure 4b). This indicates that within the VE, Gro abolishes the activator function of Dl instead of converting Dl into a long-range repressor that interferes with transcriptional activation by Bcd and Cad.

To investigate whether this action of Gro on Dl is determined by the arrangement of Dri- and Dl-binding sites in the VE, we examined the transcription patterns driven by a modified VE–*kni*-element in which the normal distance of 91 bp between the binding sites (see Figure 2c) was reduced to 45 bp. This reduction resulted in Dl-dependent repression along the ventral side of wild-type embryos (Figure 4c). Repression was not observed in the absence of Gro (Figure 4d) or Dl (Figure 4e) or in embryos expressing the constitutively active Tor^{Y9} protein [13] (Figure 4f). In contrast, the repression domain expanded anteriorly in *tor* mutant embryos (Figure 4g), which lack RTK signaling [13], and was found to be Dl-dependent (Figure 4h). This suggests that the spatial arrangement of the Dl- and Dri-binding sites dictates the mechanism by which Gro and Dl act within the enhancer element. In one case, Dl is suppressed by Gro, in the other, Dl is converted into a potent silencer of transcription that can override activation by Bcd and Cad. Both modes of repression are controlled by Tor-dependent RTK signaling.

In the *zen* and *dpp cis*-acting elements, Gro causes Dl-mediated long-range silencing [5,6,18]. Gro functions either by inhibiting the assembly and function of the core RNA polymerase II complex [7], by positioning nucleosomes over the core promoter [19] and/or by recruiting the histone deacetylase Rpd3 to the template, where the enzyme can modulate local chromatin structure [20]. In the VE, however, Gro only inhibits Dl-dependent activation without converting Dl into a repressor. The different modes of Gro function, that is, long-range silencing and short-range quenching [7,16], as shown here, are dependent on the distance between the Dl- and Dri-binding sites and/or their orientation on the enhancer, as shortening of the spacer distance converts the VE into a *dpp*- or *zen*-like element. This suggests that the way in which Gro regulates Dl activity depends on whether or not the two proteins can directly interact *in vivo*. Furthermore, both regulatory options of Gro on Dl are abolished by RTK signaling, a phenomenon which corresponds to the observation that Dl-dependent

Figure 4



Modes of Gro action on Dl are distance-dependent and regulated by RTK signaling. Embryo orientation: anterior is left and dorsal side up. **(a–c)** Transgenic *kni*-element-mediated expression [17] of a *lacZ* reporter gene in wild-type embryos. (a) Unmodified *kni*-element-mediated gene expression. Note the lack of expression in the posterior pole region [17]. (b) The *kni*–VAE fusion element results in an overlapping ventral expression domain (arrow). (c) Modified *kni*–VE fusion element (generated by PCR) in which the Dri- and Dl-binding sites are separated by 45 bp instead of 91 bp (see Figure 2c) mediates repression on the ventral side (asterisks). **(d–h)** Expression of the modified *kni*–VE fusion element in mutant embryos. (d) Repression is absent in embryos lacking Gro activity. Note the appearance of ventral expression (arrow). (e) Repression is absent in embryos lacking Dl activity. (f) Dl-dependent repression is absent in embryos expressing Tor^{Y9}, which causes ubiquitous RTK signaling activity [13]. Note the expansion of the ventral expression domain (arrow). (g) Repression (asterisks) is not affected by the lack of RTK signaling in *tor*^{PM} mutants, but expands anteriorly. (h) *tor*^{PM}; *dl*^{5} double mutants lack repression on the ventral side, indicating that repression is Dl-dependent. For methods see legends to Figures 1–3.

repression of *dpp* and *zen* is relieved by local Tor activity in the pole regions of the embryo [21]. RTK-dependent phosphorylation may therefore interfere with the binding of Dri to the DNA template, the recruitment of Gro, or with both. Phosphorylation of the vertebrate Gro homolog TLE1 has been demonstrated [22], and we have noted many potential phosphorylation sites in Dri. Thus, local RTK-dependent phosphorylation may render one or both factors inactive, preventing Gro-dependent repression of Dl in the termini of the wild-type embryo.

Our results establish that the cooperation between two maternal signaling systems, which determines the spatial limits of the *Drosophila* mesoderm anlage through *hkb* expression [1,2], is based on the management of the ubiquitously distributed factors Gro and Dri by local RTK signaling and that Gro can act through different modes on Dl. Lack of *dead ringer* (*dri*) activity did not result in an overt expansion of *hkb* expression on the ventral side of the embryo (data not shown). However, as has been observed for VE-dependent gene expression, it caused only weak defects in mesoderm formation as compared with Gro-deficient embryos or embryos which express *hkb* under the control of the VAE (data not shown). Thus, the interactions shown here represent only the Dri-dependent aspect of Gro's effect on *hkb* expression. The full picture of *hkb* control is likely to involve additional and redundantly acting factor(s) that recruit Gro to sites flanking the VE within the *hkb* control region.

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