dCtBP mediates transcriptional repression by Knirps, Krüppel and Snail in the *Drosophila* embryo

Yutaka Nibu, Hailan Zhang, Ewa Bajor¹,
Scott Barolo², Stephen Small¹ and
Michael Levine³

Department of Molecular and Cellular Biology, Division of Genetics,
401 Barker Hall, University of California, Berkeley, CA 94720,

¹Department of Biology, 1009 Main Building, 100 Washington Square
East, New York University, New York, NY 10003-6688 and

²Department of Biology, Bonner Hall, 9500 Gilman Drive, UCSD,
La Jolla, CA 92093-0347, USA

³Corresponding author

e-mail: mlevine@uclink4.berkeley.edu

The pre-cellular *Drosophila* embryo contains 10 well characterized sequence-specific transcriptional repressors, which represent a broad spectrum of DNA-binding proteins. Previous studies have shown that two of the repressors, Hairy and Dorsal, recruit a common co-repressor protein, Groucho. Here we present evidence that three different repressors, Knirps, Krüppel and Snail, recruit a different co-repressor, dCtBP. Mutant embryos containing diminished levels of maternal dCtBP products exhibit both segmentation and dorsoventral patterning defects, which can be attributed to loss of Krüppel, Knirps and Snail activity. In contrast, the Dorsal and Hairy repressors retain at least some activity in *dCtBP* mutant embryos. dCtBP interacts with Krüppel, Knirps and Snail through a related sequence motif, PDXLSXX/H. This motif is essential for the repression activity of these proteins in transgenic embryos. We propose that dCtBP represents a major form of transcriptional repression in development, and that the Groucho and dCtBP co-repressors mediate separate pathways of repression.

Keywords: CtBP/*Drosophila*/embryo/Knirps/Krüppel/Snail

Introduction

Transcriptional repressors establish localized stripes, bands and tissue-specific patterns of gene expression in the pre-cellular *Drosophila* embryo (e.g. Rivera-Pomar and Jäckle, 1996; Dubnicoff *et al.*, 1997; Jimenez *et al.*, 1997; Nibu *et al.*, 1998; Poortinga *et al.*, 1998). Patterning of both the anteroposterior and dorsoventral axes depends on broadly distributed activators and localized sequence-specific repressors. For example, the maternal Dorsal gradient can activate *rhomboid* in both ventral and lateral regions of early embryos, but the Snail repressor keeps it off in the ventral mesoderm (Ip *et al.*, 1992). Similarly, the maternal Bicoid gradient can activate the *eve* stripe 2 enhancer in a broad anterior domain, but the Giant and Krüppel repressors restrict the pattern within sharp stripe borders (Small *et al.*, 1991, 1992).

Recent studies have identified two putative co-repressors in the early embryo, Groucho (Paroush *et al.*, 1994) and dCtBP (Nibu *et al.*, 1998; Poortinga *et al.*, 1998). Both proteins are encoded by maternally expressed genes, are ubiquitously distributed throughout the early embryo and are brought to the DNA template through interactions with sequence-specific regulatory factors. Groucho mediates transcriptional repression by Dorsal and Hairy. Dorsal is inherently an activator, but can recruit the Groucho co-repressor when it interacts with specific DNA-binding proteins located within the silencer elements of the *zen* and *dpp* genes (Dubnicoff *et al.*, 1997). Hairy represses pair-rule genes, such as *ftz* and *runt*, in early embryos, and later is involved in neurogenesis (e.g. Jimenez *et al.*, 1996). These functions of Hairy have been shown to depend on a specific sequence motif, WRPW, which is important for interactions with Groucho (Fisher *et al.*, 1996). The removal of maternal Groucho products results in complex patterning defects in mutant embryos, including disruptions in both segmentation and dorsoventral patterning (Paroush *et al.*, 1994; Dubnicoff *et al.*, 1997).

Recent studies have identified a second putative co-repressor in the early embryo, dCtBP (Nibu *et al.*, 1998; Poortinga *et al.*, 1998), which is the *Drosophila* homolog of the mammalian CtBP protein (e.g. Schaeper *et al.*, 1995; Turner and Crossley, 1998). CtBP attenuates transcriptional activation by the adenovirus E1A protein; it binds E1A through a specific sequence motif located near the C-terminus of E1A, P-DLS-K (Schaeper *et al.*, 1995; Sollerbrant *et al.*, 1996). This motif is conserved in two unrelated repressors in the *Drosophila* embryo, Snail and Knirps (Nibu *et al.*, 1998). Gene dosage assays are consistent with the occurrence of interactions between Knirps and dCtBP *in vivo*. Moreover, the P-DLS-K motif was shown to be important for the repression activity of a Gal4–Knirps fusion protein in transgenic embryos (Nibu *et al.*, 1998). The functional significance of the P-DLS-K motif in the Snail repressor currently is unknown. The Hairy repressor contains a divergent sequence, P-SLV-K, which raises the possibility that Hairy-mediated repression depends on both Groucho and dCtBP (Poortinga *et al.*, 1998).

In the present study, we analyze the expression of a number of target genes, both authentic and synthetic, in *dCtBP* mutant embryos to obtain evidence that the dCtBP co-repressor is essential for Snail function. Evidence is also presented that a third sequence-specific repressor in the early embryo, Krüppel, depends on dCtBP. The C-terminal repression domain of Krüppel contains a sequence (P-DLS-H) that is related to the P-DLS-K motif in E1A, Knirps and Snail; mutations in this sequence disrupt the repression activity of a Gal4–Krüppel fusion protein in transgenic embryos. Dorsal and Hairy retain at least some repression activity in *dCtBP* mutants, suggesting that they

Fig. 1. Altered patterns of segmentation gene expression in *dCtBP* mutants. Embryos were obtained from *dCtBP* germline mosaics and hybridized with digoxigenin-labeled antisense RNA probes. The staining patterns were visualized by histochemical staining, and the embryos are oriented with anterior to the left and dorsal up (except K and L). (A and B) *Krüppel* expression pattern in wild-type (A) and *dCtBP* mutant (B) pre-cellular embryos. Strong staining is observed in central regions, and a weak pattern is detected in head regions. As shown previously (Poortinga *et al.*, 1998), the *Krüppel* expression pattern is essentially normal in mutant embryos. (C and D) *knirps* expression pattern in wild-type (C) and *dCtBP* mutant (D) pre-cellular embryos. Staining is detected in both a band in the presumptive abdomen and an extended patch in antero-ventral regions. The *knirps* pattern is essentially normal in the mutant embryos (Poortinga *et al.*, 1998). (E and F) *giant* expression pattern in wild-type (E) and *dCtBP* mutant (F) pre-cellular embryos. *giant* is expressed in both anterior and posterior regions. The posterior band is expanded in mutant embryos (see brackets). A similar alteration in *giant* expression is observed in *Krüppel* mutants (Kraut and Levine, 1991). (G and H) *eve* expression pattern in wild-type (G) and *dCtBP* mutant (H) gastrulating embryos. There is a severe disruption in the *eve* pattern, with a loss of stripes 4–6 and a fusion of stripes 2 and 3. This altered pattern combines features seen in *knirps*⁻ and *Krüppel*⁻ mutants (see I and J). (I) *eve* expression pattern in *knirps*⁻ embryo. There is a loss of stripes 4, 5 and 6. (J) *eve* expression pattern in *Krüppel*⁻ embryo. Stripes 2 + 3 and 4–6 are fused into two broad bands. (K) *eve* stripe 2 expression in a wild-type, gastrulating embryo. A *lacZ* reporter gene driven by the minimal, 480 bp stripe 2 enhancer was visualized with a *lacZ* antisense RNA probe. (L) Same as (K) except that the stripe 2 reporter gene was crossed into a mutant embryo derived from a *dCtBP* germline clone. There is a posterior expansion of the stripe 2 pattern, which suggests a loss of *Krüppel*-mediated repression.

do not require dCtBP as a co-repressor. It would appear that the bulk of the patterning defects observed in *dCtBP* mutants can be attributed to the loss of Knirps, *Krüppel* and Snail activity. We suggest that Groucho and dCtBP mediate separate pathways of transcriptional repression.

Results

Maternally encoded dCtBP products are distributed uniformly throughout early embryos (Nibu *et al.*, 1998; Poortinga *et al.*, 1998), and mutants derived from *dCtBP* germline clones exhibit altered patterns of segmentation, gene expression and severe patterning defects (Poortinga *et al.*, 1998). These mutants also possess disruptions in dorsoventral patterning. As a first step towards identifying the repressors that might require dCtBP as a co-repressor, we have analyzed the expression of a number of authentic and synthetic marker genes in mutant embryos derived from germline clones, which are homozygous for a P-induced mutation in *dCtBP* (see Materials and methods).

The role of dCtBP in segmentation

In situ hybridization assays suggest that the mutant embryos exhibit a severe reduction in, but not complete elimination of, *dCtBP* expression (data not shown). *dCtBP* mutant embryos exhibit essentially normal patterns of *Krüppel* (Figure 1B; compare with A) and *knirps* (Figure 1D; compare with C) expression (see Poortinga *et al.*, 1998). These results suggest that the Hunchback (Hb) repressor does not require dCtBP as a co-repressor, since

Hb is important for establishing the anterior borders of both the *Krüppel* and *knirps* expression patterns (Struhl *et al.*, 1992).

There is a substantial expansion of the posterior *giant* expression pattern in *dCtBP* mutants (Figure 1F; compare with E). A similar expansion was observed in *Krüppel*⁻ mutants (Kraut and Levine, 1991), thereby raising the possibility that *Krüppel*-mediated repression depends on dCtBP. Further evidence stems from the analysis of *eve*.

As shown previously (Poortinga *et al.*, 1998), there is a severe disruption of the *eve* expression pattern in *dCtBP* mutants (Figure 1H; compare with G). The altered pattern combines aspects of both *knirps* (Figure 1I) and *Krüppel* (Figure 1J) mutants (Frasch *et al.*, 1987). As in the case of *knirps*⁻ embryos, *dCtBP* mutants exhibit a severe reduction in *eve* stripes 4–6. Like *Krüppel*⁻ embryos, *dCtBP* mutants display fusions of stripes 2 and 3. This latter phenotype might result, in part, from a breakdown in the *Krüppel*-mediated repression of the *eve* stripe 2 enhancer (Stanojevic *et al.*, 1991). To test this idea, an *eve-lacZ* transgene containing the 480 bp minimal stripe 2 enhancer (Small *et al.*, 1992) was crossed into *dCtBP* mutant embryos (Figure 1L; compare with 1K). Expression directed by the transgene was detected by *in situ* hybridization using a *lacZ* antisense RNA probe. In *dCtBP* mutants, the posterior stripe 2 border expands into central regions, similar to that observed in *Krüppel* mutants (or when the *Krüppel*-binding sites in the stripe 2 enhancer are mutagenized; see Stanojevic *et al.*, 1991). These results suggest that both Knirps and *Krüppel* require dCtBP to

Fig. 2. Altered patterns of dorsoventral patterning genes in *dCtBP* mutants. Embryos are oriented with anterior to the left and stained after *in situ* hybridization with different digoxigenin-labeled antisense RNA probes. (A and B) *rhomboid* expression pattern in wild-type (A) and *dCtBP* mutant (B) pre-cellular embryos. *rhomboid* is normally expressed in two lateral stripes along the length of the embryo (A), but is derepressed in ventral regions in *dCtBP* mutants (B). This staining pattern is similar to that observed in *snail*⁻ mutants (C). (C) *rhomboid* expression pattern in a *snail*⁻/*snail*⁻ homozygote. Strong staining is observed in both lateral and ventral regions. (D) *snail* expression in a *dCtBP* mutant pre-cellular embryo. Staining is observed in ventral regions that normally invaginate to form the mesoderm. This staining pattern is similar to that observed in wild-type embryos and suggests that the derepression of the *rhomboid* staining pattern seen in *dCtBP* mutants (B) is not due to a loss in *snail* expression but, rather, results from a loss in Snail repressor function. (E and F) *sim* expression pattern in wild-type (E) and *dCtBP* mutant (F) pre-cellular embryos. *sim* is normally expressed in two lateral lines that coincide with the presumptive mesectoderm (E), but there is a severe derepression in the pattern in *dCtBP* mutants (F). This alteration in the *sim* pattern probably results from a loss in Snail repressor activity (see Kasai *et al.*, 1992).

function as repressors. In contrast, neither Hb nor Giant appear to require dCtBP; the latter repressor is required for establishing the anterior stripe 2 border (Small *et al.*, 1991, 1992), which is normal in *dCtBP* mutants (see Figure 1L).

Dorsoventral patterning

The Snail repression domain contains both a conserved copy of the P-DLS-K motif, as well as the slightly divergent sequence, P-DLS-R. Mutations in the former sequence attenuate the binding of Snail to a GST-dCtBP fusion protein (Nibu *et al.*, 1998). As a first step towards determining whether Snail requires dCtBP to mediate transcriptional repression *in vivo*, the expression patterns of different Snail target genes were examined in *dCtBP* mutant embryos.

rhomboid is expressed in lateral stripes that help specify ventral regions of the neurogenic ectoderm (Figure 2A; see Bier *et al.*, 1990; Ip *et al.*, 1992). It is repressed in the ventral mesoderm by Snail, and in *snail*⁻ mutants there is a severe derepression of the *rhomboid* staining pattern (Figure 2C; compare with A). A similar disruption of the pattern is observed in *dCtBP*⁻ embryos (Figure 2B). This is not due to the loss of Snail products, since *snail*

expression appears to be essentially normal in *dCtBP* mutants (Figure 2D). Thus, it would appear that Snail repressor activity depends on dCtBP.

Snail represses a number of neurogenic genes in the ventral mesoderm. Among these is *single minded* (*sim*), which specifies the mesectoderm at the ventral midline of advanced-stage embryos (Nambu *et al.*, 1991; Kasai *et al.*, 1992). *sim* initially is expressed in ventrolateral lines (Figure 2E) that coincide with the ventral-most cells of the presumptive neurogenic ectoderm. In *dCtBP* mutants, there is a severe derepression of the *sim* staining pattern (Figure 2F), again suggesting that the Snail repressor requires dCtBP as a co-repressor *in vivo*.

Synthetic transgenes

The preceding studies suggest that Krüppel, Knirps and Snail require *dCtBP*⁺ gene activity in the early embryo. More definitive evidence was obtained by analyzing the expression of synthetic transgenes in *dCtBP* mutant embryos (Figure 3). Each of the transgenes contains a modified form of the 700 bp *rhomboid* lateral stripe enhancer (NEE), which lacks the four native Snail repressor sites (Ip *et al.*, 1992). The enhancer directs equally strong expression in both lateral and ventral

regions due to the loss of the Snail sites. As shown previously (Gray *et al.*, 1994), two synthetic Snail sites positioned within 50 bp of the NEE activators restore repression in ventral regions, so that the reporter gene is expressed in lateral stripes, similar to the endogenous pattern (Figure 3A). However, the same transgene exhibits a derepressed staining pattern in *dCtBP* mutants, indicating a loss of Snail-mediated repression (Figure 3B). In these experiments, the modified enhancer was placed between two marker genes, *white* and *lacZ*, and transgene expression was monitored with a *white* hybridization probe.

To assess the importance of dCtBP in Knirps-mediated repression, a different version of the enhancer was analyzed that contains synthetic Knirps-binding sites in place of the Snail sites (see diagram below, Figure 3C and D). In wild-type embryos, Knirps represses the modified NEE so that the *white* expression pattern includes a gap in the presumptive abdomen where there are high levels of the Knirps repressor (arrowhead, Figure 3C). This gap is lost

in *dCtBP* mutants (Figure 3D), similar to the situation observed in *knirps*⁻ embryos (Arnosti *et al.*, 1996). These results suggest that dCtBP is required for Knirps-mediated repression of the modified NEE.

Insertion of Krüppel-binding sites in the NEE results in a central gap in the *white* expression pattern (Figure 3E). This gap is lost in *dCtBP* mutants (Figure 3F), which suggests that dCtBP is also required for Krüppel-mediated repression. Further evidence for this possibility stems from *in vitro* binding assays (Figure 3G). In these experiments, a full-length Krüppel protein was labeled with [³⁵S]methionine by *in vitro* translation, and mixed with a GST–dCtBP fusion protein. The wild-type protein binds to GST–dCtBP, but not to a GST control protein. Amino acid substitutions in the Krüppel P-DLS-H motif abolish dCtBP binding (Figure 3G).

dCtBP is not essential for Dorsal or Hairy repression

Dorsal and Hairy require Groucho to mediate transcriptional repression (Paroush *et al.*, 1994; Dubnicoff *et al.*, 1997). Hairy also contains a weak dCtBP interaction motif, P-SLV-K, and it has been suggested that Hairy-mediated repression depends on both Groucho and dCtBP (Poortinga *et al.*, 1998). To investigate this possibility, a synthetic Hairy target gene was analyzed in *dCtBP* mutants (Figure 4). The target gene contains a modified NEE with two synthetic Hairy repressor sites (Barolo and Levine, 1997). In wild-type embryos, the enhancer directs a pair-rule pattern of expression (Figure 4C) due to inter-

Fig. 3. Expression patterns of synthetic reporter genes in *dCtBP* mutants. Different *lacZ-white* reporter genes were introduced into *dCtBP* mutant embryos and stained after *in situ* hybridization with a *white* antisense RNA probe. Cellularized embryos are oriented with anterior to the left and dorsal up. (**A** and **B**) *white* staining patterns in a wild-type (**A**) and *dCtBP* mutant embryo (**B**). The reporter gene contains a modified *rhomboid* lateral stripe enhancer (NEE) that lacks the four native Snail-binding sites, but contains two synthetic sites flanking the four Dorsal activator sites (see diagram beneath the embryos). The synthetic sites mediate repression in ventral regions by Snail (arrowhead), so that *white* staining is restricted to lateral stripes (**A**). There is a severe derepression of the staining pattern in *dCtBP* mutants (**B**, arrowhead), suggesting the loss of Snail repressor activity. (**C** and **D**) *white* staining patterns in a wild-type (**C**) and *dCtBP* mutant (**D**) embryo. The modified NEE contains two synthetic Knirps sites in place of the Snail sites (see diagram). Normally, the enhancer is repressed in the presumptive abdomen by Knirps (**C**; arrowhead). This repression is lost in *dCtBP* mutants (**D**, arrowhead), which suggests a loss of Knirps repressor function. (**E** and **F**) *white* staining pattern in a wild-type (**E**) and *dCtBP* mutant (**F**) embryo. The modified NEE contains two synthetic Krüppel-binding sites in place of Knirps or Snail sites (see diagram). This enhancer directs a staining pattern with a broad gap in central regions in wild-type embryos (arrowhead, **E**). This gap coincides with regions containing high levels of the Krüppel repressor (see Figure 1). The gap is lost in *dCtBP* mutants (**F**, arrowhead), suggesting a loss of Krüppel repressor function. (**G**) GST pull-down assays. A full-length Krüppel protein was labeled with [³⁵S]methionine by *in vitro* translation (lane 1). It was incubated with a full-length GST–dCtBP fusion protein produced in bacteria, and the bound protein was recovered on glutathione–Sepharose 4B beads, and fractionated on an SDS–polyacrylamide gel. Krüppel does not bind the GST moiety (lane 2), but selectively interacts with the GST–dCtBP fusion protein (lane 3). For comparison, lane 1 contains 10% of the total amount of the ³⁵S-labeled Krüppel protein used in the binding reaction. Three amino acid substitutions in the P-DLS-H motif (PEDLSMH to AAALSMH) eliminate binding of the Krüppel protein to the GST–dCtBP fusion protein (lane 6; compare with lane 3). The binding assays were done essentially as described in Nibu *et al.* (1998).

Fig. 4. Hairy and Dorsal mediate repression in *dCtBP* mutants. Embryos were hybridized with various digoxigenin-labeled antisense RNA probes and are oriented with anterior to the left and dorsal up. (A and B) *hairy* expression pattern in wild-type (A) and *dCtBP* mutant (B) cellularized embryos. *hairy* is normally expressed in seven stripes and a small patch near the head (A). There is a severe disruption in the pattern in *dCtBP* mutants (B), similar to the altered *eve* pattern (see Figure 1H). There is a loss of *hairy* stripes 4–6 and a fusion of stripes 2 and 3 (B). (C and D) *white* staining pattern of a reporter gene containing a modified NEE. The enhancer contains two synthetic Hairy repressor sites in place of the Snail, Knirps and Krüppel sites used in Figure 3. The Hairy sites result in the periodic repression of the *white* staining pattern; the sites of repression coincide with the *hairy* stripes (C; compare with A). The *white* staining pattern is altered in *dCtBP* mutants (D), although there is repression in regions of residual *hairy* expression (arrowheads, D; compare with B). This suggests that Hairy can continue to function as a repressor in *dCtBP* mutants. (E and F) *zen* expression pattern in a wild-type (E) and *dCtBP* mutant (F) pre-cellular embryo. *zen* can be activated throughout early embryos, but is normally repressed in ventral and lateral regions by the maternal Dorsal nuclear gradient. This repression depends on Dorsal–Groucho interactions (Dubnicoff *et al.*, 1997). The broad dorsal on/ventral off *zen* expression pattern is not altered in *dCtBP* mutants (F), suggesting that Dorsal-mediated repression does not depend on dCtBP.

stripe repression by Hairy (Figure 4A). The same synthetic transgene directs an altered pattern of expression in *dCtBP* mutants (Figure 4D), whereby there are only three sites of repression rather than seven. These sites coincide with the abnormal *hairy* pattern observed in *dCtBP* mutants (Figure 4B), which results from disruptions in *Krüppel* and *knirps* activity. Instead of seven *hairy* stripes, there are only two stripes and a broad band, similar to the abnormal *eve* pattern (see Figure 1H). It would appear that the residual Hairy products continue to repress the modified NEE, although the repression may not be as robust as that observed in wild-type embryos.

The Dorsal protein requires *groucho*⁺ gene activity to repress the expression of *dpp* and *zen* (Dubnicoff *et al.*, 1997). To determine whether Dorsal-mediated repression also depends on *dCtBP*⁺ activity, *zen* expression was analyzed in *dCtBP* mutants (Figure 4F). There is no obvious change in the *zen* pattern as compared with wild-type embryos (Figure 4E). In both cases, *zen* exhibits a broad dorsal on/ventral off pattern, suggesting that the maternal Dorsal gradient can repress *zen* in ventral and lateral regions in both wild-type and mutant embryos.

In summary, the genetic analysis of *dCtBP* mutants suggests that Krüppel, Knirps and Snail depend on *dCtBP*⁺ activity, while Hairy and Dorsal continue to function as repressors in the absence of the dCtBP co-repressor. However, it is possible that the full repression activity of Hairy depends on both Groucho and dCtBP (see Discussion).

The P-DLS-K/H motif is essential for repression by Snail, Knirps and Krüppel

Previous studies have shown that a Gal4–Knirps fusion protein containing the C-terminal third of the Knirps protein (amino acid residues 255–429) can repress a modified *eve* stripe 2–*lacZ* reporter gene in transgenic embryos (Nibu *et al.*, 1998). The fusion protein contains the Knirps P-DLS-K motif, and mutations in this sequence (PMDLSMK to AAAASMK) inactivate its repression activity. These results suggest that dCtBP is an important component of Knirps-mediated repression, but do not exclude the possibility that additional sequences in Knirps are also important for repression.

To address this issue of sufficiency, the function of the

Fig. 5. The P-DLS-K motif is essential for Knirps-mediated repression. Cellularizing embryos were hybridized with mixtures of a digoxigenin-labeled *knirps* antisense RNA (red) and a fluorescein-labeled *eve* antisense RNA (black). They are oriented with anterior to the left and dorsal up. (A) Double staining pattern in a wild-type embryo. *eve* is expressed in a series of seven stripes, while *knirps* is expressed at the anterior pole and antero-ventral regions, as well as in a broad posterior band which encompasses *eve* stripes 4 and 5. (B) Same as (A) except that the embryo contains a transgene with the full-length *knirps* coding region placed under the control of the *eve* stripe 2 enhancer. As shown previously (Kosman and Small, 1997), the ectopic *knirps* stripe leads to the repression of *eve* stripe 3. (C) Same as (B) except that the *knirps* coding region was mutagenized to disrupt the P-DLS-K motif (PMDLSMK to AAAASMK). The ectopic *knirps* stripe does not cause an obvious change in the *eve* pattern; in particular, stripe 3 expression is normal. Transgenic strains that express higher levels of the mutagenized protein exhibit weak alterations in the stripe 3 pattern, suggesting that the mutant Knirps protein retains weak repressor activity (data not shown).

P-DLS-K motif was examined in the context of the full-length, wild-type protein (Figure 5). Knirps is normally expressed in two domains, one anterior to *eve* stripe 1 (Figure 5A) and the other in the presumptive abdomen, spanning *eve* stripes 4, 5 and 6. The posterior border of stripe 3 is thought to depend on repression by Knirps (Small *et al.*, 1996). As shown previously, ectopic expression of *knirps* with the *eve* stripe 2 enhancer results in the loss of stripe 3 expression (Figure 5B) and dominant lethality (Kosman and Small, 1997). It has been suggested that the endogenous stripe 3 pattern is repressed by the diffusion of ectopic Knirps products from stripe 2 (Kosman and Small, 1997). A mutant form of Knirps that lacks the

Fig. 6. The P-DLS-K and P-DLS-R motifs are essential for Snail-mediated repression. Cellularizing embryos were hybridized with digoxigenin-labeled *snail* or *rhomboid* antisense RNA probes and are oriented with anterior to the left and dorsal up. (A) *snail* expression pattern in a transgenic embryo that contains the *snail* coding region under the control of the *eve* stripe 2 enhancer. *snail* expression is observed both in ventral regions (the endogenous pattern) and in an ectopic stripe 2 pattern. (B) *rhomboid* expression pattern in the transgenic strain shown in (A). The ectopic *snail* stripe creates a gap (see arrow) in the *rhomboid* lateral stripes, but not in the aminoserosa pattern present in the dorsal-most regions of the embryo. This result is consistent with the notion that Snail is a direct repressor of the rhomboid NEE. (C) Same as (B) except that the *snail* coding region was mutagenized to disrupt both the P-DLS-K and related P-DLS-R motifs. The ectopic Snail stripe does not alter the *rhomboid* pattern, suggesting that the P-DLS-K and P-DLS-R motifs are essential for Snail-mediated repression. Similar results were obtained with a mutant Snail protein lacking only the P-DLS-K motif (H.Zhang, data not shown).

P-DLS-K motif does not repress stripe 3 expression (Figure 5C). The mutant protein is identical to native Knirps except for four changes in the P-DLS-K motif (PMDLSMK to AAAASMK). The mutant protein is expressed at the same levels as the wild-type protein (Figure 5C; compare with B, and data not shown), but does not mediate efficient repression. Moreover, while the ectopic expression of the wild-type Knirps protein results in embryonic lethality, transgenic strains that misexpress similar levels of the mutant protein are fully viable (E.Bajor and S.Small, unpublished observations). These

Fig. 7. The P-DLS-H motif is essential for Krüppel-mediated repression. Embryos expressing either wild-type or mutant forms of a Gal4–Krüppel fusion protein were hybridized with a *lacZ* antisense RNA probe, and are oriented with anterior to the left. (A and D) Summary of expression vectors and reporter genes used to analyze the Krüppel repressor. A Gal4–Krüppel fusion protein was expressed either in ventral regions of early embryos using the *twist* promoter region (A) or central regions using the *Krüppel* promoter (Kr 2.5) region (D). Two different reporter genes were used to monitor the activities of the fusion protein. A stripe 2–stripe 3 reporter gene contains Gal4-binding sites (U) near the distal stripe 2 enhancer. This reporter gene normally exhibits stripes of *lacZ* expression in both dorsal and ventral regions (see diagram in A). The other reporter gene contains a modified *rhomboid* NEE containing four Gal4-binding sites (U) placed upstream of the *twist* proximal enhancer (PE). This reporter gene normally exhibits both lateral lines of *lacZ* expression and a broad band of staining in the ventral mesoderm (see diagram in D). (B) Expression of the stripe 2–stripe 3 *lacZ* reporter gene in a transgenic embryo that contains the *twist*–Gal4/Krüppel vector. The Gal4–Krüppel fusion protein contains the C-terminal 101 amino acid residues from Krüppel. It binds to the Gal4 sites in the distal stripe 2 enhancer and represses the stripe 2 pattern in ventral regions (arrowhead). The stripe 3 enhancer is located nearly 1 kb downstream of the Gal4-binding sites and is not affected by the fusion protein. (C) Same as (B) except that the *Krüppel* coding sequence was mutagenized to disrupt the P-DLS-H motif. The mutant Gal4–Krüppel fusion protein does not repress the stripe 2 pattern (arrowhead). (E) Expression of the NEE–PE *lacZ* reporter gene in a transgenic embryo that contains the *Krüppel*–Gal4/Krüppel expression vector. The fusion protein is expressed in central regions of the embryo, binds to the Gal4 sites in the NEE, and represses the NEE-driven lateral lines (open arrowheads). The *twist* PE is located nearly 400 bp downstream of the Gal4 sites in the modified NEE and is not affected by the fusion protein. (F) Same as (E) except that the Gal4–Krüppel fusion protein contains point mutations in the P-DLS-H motif. This mutant protein does not repress the modified NEE in central regions (arrowheads).

results suggest that P-DLS-K represents the primary repression motif in the Knirps protein, although high levels of the mutant protein cause weak and variable disruptions in the stripe 3 pattern (data not shown).

Similar assays were used to assess the significance of the P-DLS-K and P-DLS-R motifs in the Snail repressor (Figure 6). The *eve* stripe 2 enhancer was used to mis-express *snail* in transgenic embryos (Figure 6A). *snail* is normally expressed in ventral regions where it helps establish the limits of the presumptive mesoderm by repressing various target genes such as *rhomboid* (see Figure 2). The ectopic *snail* stripe results in an abnormal *rhomboid* pattern (Figure 6B) that contains a gap in the vicinity of *eve* stripe 2 (arrow, Figure 6B; compare with A). This observation suggests that ectopic Snail products

bind to the endogenous *rhomboid* NEE and repress its transcription. Point mutations in the P-DLS-K and P-DLS-R motifs eliminate the repression activity of an otherwise normal stripe 2–*snail* transgene (Figure 6C). The mutant *snail* RNA is expressed at levels comparable with the wild-type RNA (data not shown). Additional studies indicate that mutations in the P-DLS-K motif alone, with P-DLS-R intact, result in only weak repression of the *rhomboid* pattern (H.Zhang, data not shown).

Tissue culture assays identified a repression domain in a C-terminal region of Krüppel (Hanna-Rose *et al.*, 1997). To assess the significance of the P-DLS-H motif contained in this domain, we analyzed the activities of a Gal4–Krüppel fusion protein that contains the C-terminal 101 amino acids residues from Krüppel. The chimeric coding

Fig. 8. dCtBP can mediate transcriptional repression when tethered to DNA. (A–D) Diagrams of the expression vector and *lacZ* reporter gene used in this analysis. The reporter gene (A) contains *lacZ* driven by a modified *rhomboid* NEE that contains three Gal4-binding sites (UAS), three Twist (t) activator sites and three Dorsal (dl) activator sites. This reporter gene is normally activated in the ventral mesoderm (see diagram in C). A Gal4–dCtBP fusion protein containing the full-length dCtBP sequence was expressed in central regions of transgenic embryos using the *Krüppel* promoter region (see diagram in D). (E) *lacZ* staining pattern obtained with the reporter gene in a wild-type embryo. Uniform staining is observed throughout the ventral mesoderm. (F) Same as (E) except that the *lacZ* reporter gene was crossed into a transgenic strain carrying the *Krüppel*–Gal4/dCtBP expression vector. The Gal4–dCtBP fusion protein mediates efficient repression of the reporter gene in central regions (arrowhead).

sequence was expressed in ventral regions of transgenic embryos under the control of the *twist* promoter region (Figure 7A). A *lacZ* reporter gene was introduced into embryos expressing this fusion protein (Figure 7B). It contains the *eve* stripe 2 and stripe 3 enhancers, and normally exhibits equally intense expression in both dorsal and ventral regions (data not shown). The distal stripe 2 enhancer contains two tandem Gal4-binding sites (UAS), and when the *lacZ* reporter gene is crossed into embryos expressing the *twist*–*gal4/Krüppel* vector, it is repressed in the ventral mesoderm (arrowhead, Figure 7B). The introduction of just three amino acid substitutions in the P-DLS-H motif (PEDLSMH to AAALSMH) eliminates the repression activity of the *Krüppel* fusion protein (Figure 7C). The same substitutions also eliminate interactions between *Krüppel* and dCtBP *in vitro* (see Figure 3G).

Similar results were obtained when the Gal4–*Krüppel* fusion protein was expressed in central regions of transgenic embryos using the *Krüppel* promoter region (Figure 7D). The *lacZ* reporter gene used to assess the activity of this expression vector contains a modified *rhomboid* lateral stripe enhancer placed upstream of the proximal enhancer from the *twist* promoter. Normally, the reporter gene is expressed in lateral lines (mediated by the modified *rhomboid* enhancer) and the ventral mesoderm (*twist* enhancer). However, there is a gap in the lateral lines when the reporter gene is crossed into embryos expressing the *Krüppel*–*gal4/Krüppel* expression vector (Figure 7E, arrowheads). This gap results from the binding of the Gal4–*Krüppel* fusion protein to UAS sites in the distal *rhomboid* enhancer. The gap is lost with a mutant fusion protein containing amino acid substitutions in the P-

DLS-H motif, thereby indicating the importance of this motif *in vivo*.

dCtBP functions as a co-repressor *in vivo*

We previously presented evidence that dCtBP mediates weak repression in transgenic embryos when it is tethered to DNA via the Gal4 DNA-binding domain (Nibu *et al.*, 1998). There are multiple dCtBP transcripts, and the one that was used encodes a ‘short form’ of the protein composed of 383 amino acid residues. The longest dCtBP coding region that has been characterized specifies a variant protein composed of 450–500 amino acid residues. The short and long versions have slightly different C-terminal sequences, and the long form also contains an insert of three amino acid residues in the N-terminal half of the protein. The following experiment was done to determine whether the full-length dCtBP protein mediates more efficient repression than the short form.

The full-length dCtBP-coding sequence was expressed as a Gal4 fusion protein and placed under the control of the 2.5 kb *Krüppel* promoter region, which directs expression in a broad central band in pre-cellular embryos (see Figure 1A and Figure 8D summary). A modified *rhomboid* NEE was used to monitor the activities of the Gal4–dCtBP fusion protein (described by Gray and Levine, 1996). This enhancer lacks Snail repressor sites and contains three UAS sequences (Figure 8A). Normally, the enhancer directs expression in ventral regions encompassing the presumptive mesoderm (Figure 8E). However, when crossed into transgenic embryos expressing the Gal4–dCtBP fusion protein, it is repressed in central regions (Figure 8F, arrowheads). These results suggest

Table I. Summary of repressors in the pre-cellular embryo

	Family	Co-repressor	Range of action	References
I. AP axis				
Gap repressors				
Huckebein	zinc finger	?	?	Bronner <i>et al.</i> (1992)
Tailless	nuclear receptor	?	?	Pignoni <i>et al.</i> (1990)
Hunchback	zinc finger	?	short?	Small <i>et al.</i> (1993)
Krüppel	zinc finger	dCtBP	short	Gray and Levine (1996)
Knirps	nuclear receptor	dCtBP	short	Arnosti <i>et al.</i> (1996)
Giant	bZIP	?	short?	Small <i>et al.</i> (1993)
Pair-rule (primary)				
Hairy	bHLH	Groucho (dCtBP)	long	Barolo and Levine (1997)
Eve	homeodomain	?	?	Frasch <i>et al.</i> (1987)
II. DV axis				
Dorsal	rel	Groucho	long	Cai <i>et al.</i> (1996)
Snail	zinc finger	dCtBP	short	Gray <i>et al.</i> (1994)

that the full-length dCtBP protein corresponds to a bona fide co-repressor.

Discussion

We have presented evidence that dCtBP functions as a co-repressor for three different sequence-specific repressors in the early embryo, Knirps, Snail and Krüppel. A related sequence motif is important for the binding of dCtBP: P-DLS-K (Knirps and Snail) and P-DLS-H (Krüppel). The analysis of various target genes, both authentic and synthetic, in *dCtBP* mutant embryos suggests that the dCtBP co-repressor is less critical for the activities of the Dorsal and Hairy repressors, which have been shown to interact with the Groucho co-repressor. We propose that dCtBP and Groucho mediate separate pathways of transcriptional repression.

Modes of repression

Table I lists 10 different well characterized repressors in the pre-cellular embryo. These represent a broad sampling of different DNA-binding proteins, including members of the Rel, homeodomain, zinc finger, bHLH, bZIP and nuclear receptor families. We have shown that three of these 10 repressors, Krüppel, Knirps and Snail, interact with the dCtBP co-repressor. However, it would appear that most or all of the remaining repressors do not require dCtBP (summarized in Table I). For example, the fact that the anterior border of *eve* stripe 2 is normal in *dCtBP* mutants suggests that the Giant repressor can function in the absence of dCtBP. Similar arguments apply to Hunchback and Dorsal. Evidence that Hairy does not require dCtBP stems from the analysis of a modified *rhomboid* lateral stripe enhancer containing synthetic Hairy repressor sites (Figure 4), although it is possible that full repression activity depends on both Groucho and dCtBP. It is possible that the Huckebein repressor does not require dCtBP since the *snail* expression pattern is repressed from the posterior pole in *dCtBP* mutants (data not shown); this repression has been shown to be mediated by Huckebein (Reuter and Leptin, 1994). Similarly, the Tailless repressor may not require dCtBP since the *eve* stripe 3/7 enhancer exhibits a normal stripe 7 pattern of expression in *dCtBP* mutants (data not shown); stripe 7 is lost in *tailless* mutants (Frasch and Levine, 1987; Steingrimsson *et al.*, 1991).

As discussed previously, at least two of the repressors,

Dorsal and Hairy, interact with the Groucho co-repressor. Due to the severe patterning defects observed in *groucho* mutant embryos (Paroush *et al.*, 1994), it is difficult to assess which of the remaining pre-cellular repressors also require the Groucho co-repressor. Removal of maternal Groucho products leads to the derepression of the *tailless* expression pattern, and a corresponding suppression of *Krüppel* and *knirps* expression in central and abdominal regions (Paroush *et al.*, 1997). Despite these severe patterning defects, it would appear that the Snail repressor does not require Groucho since *rhomboid* exhibits normal stripes of expression in *groucho* mutants (Dubnicoff *et al.*, 1997). We suggest that Groucho and dCtBP mediate different pathways of transcriptional repression. Hairy and Dorsal interact with Groucho but may not require dCtBP to mediate transcriptional repression *in vivo*. Conversely, at least one of the repressors that interacts with dCtBP, Snail, does not appear to require Groucho. We note, however, that a previous study identified dCtBP through the use of Hairy protein sequences in yeast two-hybrid assays (Poortinga *et al.*, 1998). It is therefore possible that dCtBP normally works together with Groucho to mediate transcriptional repression by Hairy.

As many as half of the pre-cellular repressors do not require either Groucho or dCtBP (see Table I). It is conceivable that some of the remaining repressors interact with co-repressor complexes containing histone deacetylases, which appear to represent a major mechanism of transcriptional repression in mammalian systems (reviewed by Pazin and Kadonaga, 1997). For example, the bHLH Mad-Max complex interacts with a Rpd3 histone deacetylase via interactions with the Sin3 adaptor protein, while nuclear receptor proteins such as the thyroid hormone receptor interact with histone deacetylases via N-Cor/Smrt proteins. Recent binding assays raise the possibility that mammalian Groucho proteins interact with histone H3 (Palaparti *et al.*, 1997), while mammalian CtBP interacts with the HDAC1 histone deacetylase (Sundqvist *et al.*, 1998). We note, however, that there is currently no genetic evidence for these interactions *in vivo*.

The mechanism by which dCtBP mediates transcriptional repression is unknown. However, the current study provides evidence against a previously proposed mechanism for Krüppel (Sauer *et al.*, 1995). We have shown that Krüppel activity is lost in *dCtBP* mutants (see Figures 1 and 3), and that the C-terminal region of the protein

contains an essential P-DLS-H repression motif (see Figure 7). Moreover, preliminary studies suggest that ectopic expression of the native Krüppel protein causes patterning defects in early embryos, which are reversed when the P-DLS-H motif is mutagenized (Y.Nibu, unpublished results). These results strongly suggest that Krüppel-mediated repression depends on the recruitment of the dCtBP co-repressor. The earlier study provided evidence that repression depends on direct interactions of Krüppel with the β -subunit of the TFIIE general transcription factor (Sauer *et al.*, 1995). It is conceivable that this mechanism of repression is employed in other tissues at later stages in the *Drosophila* life cycle, although we note that a recent study provides strong evidence that a mammalian Krüppel-like protein also employs a CtBP co-repressor (Turner and Crossley, 1998).

Short-range versus long-range repression

Previous studies suggest that repressors can be classified with regard to their range of action (Cai *et al.*, 1996; Barolo and Levine, 1997). Short-range repressors function over distances of <100 bp to quench either upstream activators or core components of the transcription complex (Arnosti *et al.*, 1996; Gray and Levine, 1996). In contrast, long-range repressors can inhibit the transcription complex over distances of >1 kb. For example, the ventral silencer element (VRE) from the *zen* promoter region keeps *zen* expression off in ventral and lateral regions in response to the maternal Dorsal gradient (Cai *et al.*, 1996). The VRE can repress the ventral expression of the heterologous *eve* stripe 2 enhancer even when it is positioned nearly 5 kb from the enhancer and target promoter (Cai *et al.*, 1996). It has been suggested that short-range repression can account for the autonomous activities of different enhancers contained within a complex, modular promoter region. A repressor bound to one enhancer does not interfere with the activators contained within a neighboring enhancer. For example, the binding of Krüppel to the *eve* stripe 2 enhancer (to form the posterior border) does not interfere with the expression of the stripe 3 enhancer (Small *et al.*, 1993).

Five of the 10 pre-cellular repressors have been examined with regard to range of action (see Table I). Two of the five, Dorsal and Hairy, function as long-range repressors (Cai *et al.*, 1996; Barolo and Levine, 1997), while the remaining three, Krüppel, Knirps and Snail, work over short distances (Gray *et al.*, 1994; Arnosti *et al.*, 1996; Gray and Levine, 1996). Thus, it is conceivable that the Groucho co-repressor mediates long-range repression, while dCtBP mediates short-range repression. There may be additional mechanisms of short-range repression. For example, Giant and Hunchback are likely to function as short-range repressors since they interact with specific enhancers in the modular *eve* promoter region. However, as discussed above, neither protein appears to require dCtBP to mediate transcriptional repression. It currently is unclear whether Tailless, Hückebein and Eve function as short-range or long-range repressors.

Materials and methods

P-transformation vectors and in situ hybridization

The P-element transformation vectors were injected into *yw* embryos, as described previously (e.g. Small *et al.*, 1992; Kosman and Small,

1997). Both the *knirps* and *snail* coding regions were placed under the control of the *eve* stripe 2 enhancer, which involved the use of a modified pCasPeR transformation vector containing two tandem copies of an augmented stripe 2 enhancer and an *frt-stop-frt* cassette (Kosman and Small, 1997). The stripe 2-*knirps* and stripe 2-*snail* vectors were introduced into males that express the yeast FLP recombinase under the control of a sperm-specific tubulin promoter (Kosman and Small, 1997). This results in the rearrangement of the expression vectors to produce active Knirps and Snail proteins in F₁ embryos. The stripe 2-*knirps* expression vector previously was shown to repress the expression of *eve* stripe 3 (Kosman and Small, 1997). Additional transgenic strains were obtained that contain point mutations in the P-DLS-K dCtBP-binding motif in the *knirps* coding region (Figure 5). The same stripe 2 expression vector was used to misexpress *snail* (Figure 6). Both the full-length, wild-type coding region, and a mutant derivative containing substitutions in the P-DLS-K and P-DLS-R motifs, were inserted into the stripe 2 pCasPeR vector.

Two different *gal4* expression vectors were used in this study, KREG and TWIG (Figures 7 and 8). The KREG vector is a derivative of pCaSpeR-AUG-bgal (Thummel *et al.*, 1988). It contains a 2.5 kb *EcoRI* fragment from the *Krüppel* promoter region (Ip *et al.*, 1991) placed upstream of the *eve* promoter, which extends from -42 bp upstream of the *eve* transcription start site and extends through codon 22 of the coding region. The resulting *Krüppel-eve* fragment was fused via a *Bam*HI site to a fragment containing codons 1-93 of the *gal4* coding sequence, followed by a polylinker from the pSCTEV gal93-LF0-stop plasmid (Seipel *et al.*, 1992). A *Bam*HI-*Xba*I fragment containing the *lacZ* gene was excised from the vector and replaced with *Kpn*I-*Xba*I fragments containing either *Krüppel* C-terminal sequences or the long form of *dCtBP*. These latter fragments were fused in-frame with the *gal4* 1-93 sequence in the transformation vector. The TWIG P-expression vector is described in Arnosti *et al.* (1996), where it is called 'pTwiggy'.

Several different *lacZ-white* reporter genes were used in this study. The modified *rhomboid* NEE derivatives containing synthetic Snail-, Knirps- or Krüppel-binding sites (Figure 3) are described in Gray *et al.* (1994), Gray and Levine (1996) and Arnosti *et al.* (1996). Specifically, lab stocks G8.7, G8.8 and G8.11 were used for the NEE.sna reporter gene (Figure 3A and B; Gray and Levine, 1996). Stocks A45 and A46 were used for the NEE.kni reporter gene (Figure 3C and D; Arnosti *et al.*, 1996). Stocks G5.3, G5.5 and G5.8 were used for the NEE.Kr reporter gene (Figure 3E and F; Gray and Levine, 1996). The *eve* stripe 2-*lacZ* reporter gene used in Figure 1K and L corresponds to laboratory stocks E278, E280, E282, E283 and E286 (see Small *et al.*, 1992). The modified NEE containing Hairy-binding sites (Figure 4) is described in Barolo and Levine (1997); this experiment involved the use of laboratory stock BR2.10. The stripe 2-stripe 3 reporter gene used in Figure 7 was prepared by removing the *eve* stripe 2/UAS sequence from the stripe 2-*lacZ* reporter gene described in Arnosti *et al.* (1996). It was used to replace the stripe 2 enhancer contained in the stripe 2-stripe 3 reporter gene described in Small *et al.* (1993); the distal stripe 2/UAS sequence was separated from the proximal stripe 3-*lacZ* sequence with a 750 bp spacer DNA from the GFP reporter gene (see Barolo and Levine, 1997). Laboratory stocks 2UG3-1, 2UG3-2 and 2UG3-5 were used for the experiments shown in Figure 7B and C.

The NEE-2XPE reporter gene used in Figure 7E and F was prepared with the 520 bp *twi* 2XPE DNA fragment described by Jiang *et al.* (1993), and a modified 300 bp *rhomboid* NEE, which contains four Gal4 UAS recognition sequences interspersed among the Dorsal and bHLH activator sites (see below). These fragments were inserted into the polylinker of the C4PLZ transformation vector (Wharton and Crews, 1993). A 340 bp CAT spacer DNA (Barolo and Levine, 1997) was inserted as a *Dra*I fragment between the distal NEE and the proximal 2XPE regulatory sequences. Laboratory stocks RUCPT-3, RUCPT-5 and RUCPT-8 were used for these experiments.

A *rhomboid* NEE containing three UAS sites was used for the experiments shown in Figure 8; laboratory stocks G18.2, G18.3 and G18.10 were used. This NEE-*lacZ* reporter gene is described by Gray and Levine (1996).

Embryos were collected from different wild-type, transgenic and mutant strains, and then fixed and hybridized as described by Jiang *et al.* (1991). Fixed embryos were hybridized with a variety of different digoxigenin-labeled antisense RNA probes. The *Krüppel*, *knirps*, *giant* and *eve* cDNAs that were used to produce these probes are described in Kraut and Levine (1991), Small *et al.* (1992) and Arnosti *et al.* (1996). The *rhomboid*, *snail* and *sim* cDNAs are described in Kosman *et al.* (1991), Ip *et al.* (1992) and Gonzalez-Crespo and Levine (1993). The *zen* probe is described in Cai *et al.* (1996), and the *lacZ* and *white*

antisense RNA probes used to detect the expression of the different synthetic reporter genes are described in Gray and Levine (1996). The double-staining experiment shown in Figure 5 involved the use of biotin-labeled and fluorescein-labeled probes, as described in Kosman and Small (1997).

Recombinant DNAs

The *eve* stripe 2-*knirps* expression vector used for the experiment shown in Figure 5B is identical to the one described in Kosman and Small (1997). A mutant form of *knirps*, lacking the P-DLS-K motif, was prepared by PCR mutagenesis. A *Clal*-*XbaI* fragment containing codons 255–429 was annealed with the following four oligonucleotides: 1, ACTGGTACCATCGATGCTGCCTGGAGG; 2, TAAGCGGCGCGGCTCCTTCTTGAGCGGAACGGTGG; 3, ATTGCGCGCGCTAGCATGAAGACCTCGCGGAGC; 4, AATTCTAGATTAGACACACACGAAATATCCCC. The underlined nucleotides in primers 2 and 3 indicate substitutions that create a unique *NotI* site within the dCtBP-binding motif. This converts the normal motif, PMDLSMK, into AAAASMK. This mutant form of *Knirps* fails to bind a GST-dCtBP fusion protein *in vitro* (Y.Nibu, unpublished results). The mutagenized *Clal*-*XbaI* *knirps* cDNA fragment was used to replace the normal sequence within the normal stripe 2-*knirps* expression vector (Figure 5C).

The *eve* stripe 2-*snail* expression vector contains the full-length *snail* coding region (Figure 6B). It was mutagenized to disrupt both the P-DLS-K and P-DLS-R motifs. This mutagenesis involved the use of the following mutagenic oligonucleotides: 1, GTTGGCCAGGATCA-GGCGCAGGCTGCAGCCCTGAAACGGGGTTCGC; and 2, GAAACCGGTTCCGAGGCTGAGGCTGCTGCAGTGCAGAAATGACATC. The underlined nucleotides in oligonucleotide 1 convert the PQDLSLK motif into AQAAALK, while the changes in oligonucleotide 2 convert the PEDLSVR motif into AEAAAVR. A DNA fragment containing this mutagenized *snail* sequence was used to replace the corresponding region of the wild-type sequence to produce the mutant stripe 2-*snail* P-expression vector used in the experiment shown in Figure 6C.

The C-terminal region of the Krüppel-coding sequence, codons 402–502, was cloned into the KREG and TWIG expression vectors (Figure 7), as follows. An 800 bp *SalI*-*EcoRI* fragment was isolated from a full-length Krüppel cDNA. This fragment contains codons 402–502 of the protein-coding region as well as the entire 3' untranslated region (3' UTR). It was cloned into the *XhoI*-*EcoRI* sites of a modified pBluescript SK(+) plasmid that contains *NcoI*-*NdeI*-*KpnI*-*XhoI* sites at the original *KpnI*-*ApaI*-*XhoI* sites. Codons 402–502 were isolated on a *KpnI*-*XbaI* fragment and inserted into the unique *KpnI* and *XbaI* sites of the KREG and TWIG P-expression vectors, which places the Krüppel-coding sequences in-frame with codons 1–93 of *gal4*.

A mutant form of Krüppel was used in the binding assay shown in Figure 3G and in the *in vivo* repression assays shown in Figure 7. A full-length, 2.1 kb Krüppel cDNA was cloned into the *PstI*-*EcoRI* sites of the pBluescript SK(+) plasmid (Stratagene, La Jolla, CA). This plasmid was used as a template to produce the ³⁵S-labeled Krüppel protein used in Figure 3G. A mutant form of this protein, KrüppelAPED (Figure 3G), was created by site-directed mutagenesis using the following mutagenic oligonucleotide: TGCATGCTCAAAGCGCGCCCTCGGT-TTGCTC. The underlined nucleotides indicate changes that convert the dCtBP-binding motif present in the C-terminal region of Krüppel, PEDLSMH, into the mutant sequence AAALSMH. These three amino acid substitutions eliminate binding of an otherwise normal Krüppel protein to the GST-dCtBP fusion protein (Figure 3G). This mutagenized Krüppel cDNA was digested with *SalI* and the 3' *Sal*-*EcoRI* fragment was inserted into the modified pBluescript plasmid, and inserted into the KREG and TWIG P-expression vectors as a *KpnI*-*XbaI* site, as described above.

A full-length, 2.6 kb dCtBP cDNA was isolated as a *BglII*-*BglII* fragment. It specifies a putative protein composed of 458 amino acid residues. The cDNA also contains a 100 bp 5' UTR, a 1 kb 3' UTR and polylinker sequences from the pACT *Drosophila* two-hybrid library (see Nibu *et al.*, 1998). The cDNA was inserted into the *SmaI* site of the pBluescript II KS(+) plasmid after insertion of a *BglII* linker sequence. A DNA fragment containing codons 1–13 was created with the following oligonucleotides: CATGGACAAAAATCTGATGATGCC-GAAGCGTTCGCGCAT and CGATGCGCGAACGCTTCGGCATCAT-CAGATTTTGTCCATGGTAC. The underlined nucleotides indicate the initiating methionine in the coding sequence. The resulting double-stranded DNA fragment contains a cohesive 5' sequence for the unique *KpnI* site in the KREG expression vector. The 3' sequence contains an authentic *Clal* site within the dCtBP-coding region. The remainder of

the dCtBP-coding region was cloned as a *Clal*-*XbaI* fragment into the KREG vector.

Fly stocks

The following strain was used to produce dCtBP germline clones: FRT82B-P1590/TM3, Sb (kindly provided by Norbert Perrimon). This dCtBP mutant is identical to the l(3)03463 used in our previous paper (Nibu *et al.*, 1998). Males heterozygous for the dominant *ovoD* allele (stock #2149; see Chou and Perrimon, 1996) were mated with females carrying the yeast Flp recombinase gene under the control of the *hsp70* promoter (stock #1970; Bloomington stock center). Double heterozygous males carrying *ovoD* and the Flp recombinase were mated with the dCtBP P1590 stock. Embryos were collected for 24 h, aged another 24 h and then heat-shocked on three successive days at 37°C for 3 h. The heat-shocked larvae were grown to adults, and virgin females were selected and mated with either FRT82B-P1590 males or *yw* males carrying a *lacZ* reporter gene. Embryos were collected from this final mating, fixed and hybridized with various digoxigenin-labeled RNA probes.

Null mutations in *Krüppel*, *knirps* and *snail* were used in the experiments shown in Figures 1 and 2. Two different *Krüppel* alleles were used, *Kr*¹ and *Kr*⁹. The *Kr*⁹ mutant was used in the experiment shown in Figure 1J; similar results were obtained with *Kr*¹. The *snail*¹⁸ and *knirps*⁹ alleles were used for the experiments shown in Figures 2C and 1I, respectively. In all cases, embryos were collected from the balanced stocks, fixed and hybridized. The *snail*¹⁸ strain was obtained from the Bloomington stock center (#2311), while the *Kr*¹, *Kr*⁹ and *knirps*⁹ strains were obtained from the Umea *Drosophila* stock center in Sweden (#41510, #39752 and #Z844, respectively).

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