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, PXDLSXK/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 fitz 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
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 repressors that might require dCtBP as a co-repressor, including Hunchback (Hb) with the repressors that might 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

**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
dCtBP is a co-repressor of Knirps, Krüppel and Snail

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 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-

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**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).
dCtBP is a co-repressor of Knirps, Krüppel and Snail

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 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. 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).
dCtBP is a co-repressor of Knirps, Krüppel and Snail

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 twist–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–Kruppel 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
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
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, homedomain, 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 Hucklebein 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 Hucklebein (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

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

<table>
<thead>
<tr>
<th>Family</th>
<th>Co-repressor</th>
<th>Range of action</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. AP axis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gap repressors</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hucklebein</td>
<td>zinc finger</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>Tailless</td>
<td>nuclear receptor</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>Krüppel</td>
<td>zinc finger</td>
<td>dCtBP</td>
<td>short</td>
</tr>
<tr>
<td>Knirps</td>
<td>nuclear receptor</td>
<td>dCtBP</td>
<td>short</td>
</tr>
<tr>
<td>Pair-rule (primary)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hairy</td>
<td>bHLH</td>
<td>Groucho (dCtBP)</td>
<td>long</td>
</tr>
<tr>
<td>Eve</td>
<td>homeodomain</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>II. DV axis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dorsal</td>
<td>rel</td>
<td>Groucho</td>
<td>long</td>
</tr>
<tr>
<td>Snail</td>
<td>zinc finger</td>
<td>dCtBP</td>
<td>short</td>
</tr>
</tbody>
</table>
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 mutated (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 1). 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 short-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, Hucklebein 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 flp-stop-flp 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 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 BamHI site to a fragment containing codons 1–93 of the gal4 coding sequence, followed by a polylinker from the pSCTEV gal43-Lf0-stop plasmid (Seipel et al., 1992). A BamHI–XbaI fragment containing the lacZ gene was excised from the vector and replaced with knirps–XbaI fragment 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 was 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 DR2.10. The stripe 2–stripe 3 reporter gene used in Figure 7 was prepared by removing the distal 2/1AAS 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 2/1AAS 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 was used in Figure 7E and F and 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). Thus, it is conceivable that the Groucho co-repressor mediates short-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, Hucklebein and Eve function as short-range or long-range repressors.
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–kniPS 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 kniPS, lacking the P-DLS-K motif, was prepared by PCR mutagenesis. A Clal–Xhol fragment containing codons 255–429 was annealed with the following four oligonucleotides: 1, TAAGCGGCCGCGCAAAAATCTGATGCG; 2, TAAGCGGCCGCGCATGCGCGAACGCTTCGGCATCATCG; 3, ATTAGGCCGCGGTGCCATGTACGAGGCGGCGGGG; and 2, GAAACC–

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(1)303463 used in our previous paper (Nibu et al., 1998). Males heterozygous for the dominant ovd 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 ovd 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 mps flies 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, kniPS and snail were used in the experiments shown in Figures 1 and 2. Two different Krüppel alleles were used, KrF and KrP. The KrP mutant was used in the experiment shown in Figure 11, similar results were obtained with the KrF. The snailP and kniPS alleles were used for the experiments shown in Figures 2C and 11, respectively. In all cases, embryos were collected from the balanced stocks, fixed and hybridized. The sma1 strain was obtained from the Bloomington stock center (#2311), while the KrF, KrP and kniPS strains were obtained from the Umea Drosophila stock center in Sweden (#41510, #39752 and #28844, respectively).

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