# CtBP-dependent activities of the short-range Giant repressor in the *Drosophila* embryo

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**There are at least three short-range gap repressors in the precellular *Drosophila* embryo: Krüppel, Knirps, and Giant. Krüppel and Knirps contain related repression motifs, PxDLSxH and PxDLSxK, respectively, which mediate interactions with the dCtBP corepressor protein. Here, we present evidence that Giant might also interact with dCtBP. The misexpression of Giant in ventral regions of transgenic embryos results in the selective repression of *eve* stripe 5. A *stripe5-lacZ* transgene exhibits an abnormal staining pattern in *dCtBP* mutants that is consistent with attenuated repression by Giant. The analysis of Gal4-Giant fusion proteins identified a minimal repression domain that contains a sequence motif, VLDLS, which is conserved in at least two other sequence-specific repressors. Removal of this sequence from the native Giant protein does not impair its repression activity in transgenic embryos. We propose that Giant-dCtBP interactions might be indirect and mediated by an unknown bZIP subunit that forms a heteromeric complex with Giant. We also suggest that the VLDLS motif recruits an as yet unidentified corepressor protein.**

There are at least three short-range gap repressors in the precellular embryo: Krüppel, Knirps, and Giant (1–3). These repressors direct multiple stripes of gene expression by interacting with defined enhancers within the complex cis-regulatory regions of pair-rule genes such as *eve* and *hairy* (e.g., refs. 4–7). Two of the repressors, Krüppel and Knirps, have been shown to interact with a common corepressor protein, dCtBP, which is maternally expressed and uniformly distributed throughout the early embryo (8). dCtBP lacks an intrinsic DNA-binding activity but can be recruited to the DNA template by interacting with a highly conserved peptide motif, PxDLSxR/K/H (8, 9). Mammalian CtBP proteins recognize the same motif and have been implicated as corepressors of a number of transcription factors, including E2F (10), Ikaros (11), and ZEB (12).

In the present study, we investigate the role of dCtBP as a potential corepressor of Giant, which is required for establishing the anterior border of *eve* stripe 2 and the posterior border of stripe 5 (5, 13). The Giant repressor exhibits many of the same properties as Krüppel and Knirps. In particular, Giant must bind within ≈100 bp of upstream activators or the core promoter to mediate transcriptional repression (3). However, previous studies raise the possibility that Giant might not require dCtBP, thereby suggesting an additional mechanism of short-range repression (8).

The Giant protein was misexpressed in ventral regions of transgenic embryos by using the *twist* PE enhancer (14). The *twi-giant* transgene is sufficient to repress the endogenous *eve* stripe 5 pattern, but not stripe 2. The dCtBP corepressor is required for repression because *dCtBP* mutant embryos exhibit an abnormal stripe 5 pattern and attenuated activity of the Giant repressor. However, Giant–dCtBP interactions may not be direct. The analysis of Gal4-Giant fusion proteins in transgenic embryos identified a minimal repression domain that contains a conserved sequence motif, VLDLS, which is related to the dCtBP interaction motif (PxDLSxR/K/H). Amino acid substitutions in this motif attenuate the activities of a Gal4-Giant fusion protein but do not impair the ability of the *twi-giant* transgene to repress *eve* stripe 5. We propose that Giant interacts with an unknown bZIP subunit, X, which in turn recruits dCtBP to the stripe 5 enhancer.

## Materials and Methods

**Gal4-Giant Fusion Proteins.** The KREG P-element vector was used to express various Gal4-Giant fusion proteins in central regions of transgenic embryos (8). This vector contains the Krüppel 5′ cis-regulatory region and the Gal4 DNA-binding domain (8). A 1.4-kb *Nde*I-*Sal*I cDNA fragment containing the entire *giant* protein coding sequence (448-aa residues) was cloned into the *Nde*I and *Xho*I sites of a modified pBluescript SK+ plasmid that contains *Kpn*I-*Nco*I-*Nde*I-*Xho*I sites at the original *Kpn*I-*Apa*I-*Xho*I sites. The initiation codon has the artificial *Nde*I site. Various portions of the *giant* coding sequence were generated by either PCR by using appropriate primers or restriction enzyme digestions and then cloned into the pBluescript SK+ plasmid. The different *giant* coding sequences were isolated as *Kpn*I-*Xba*I DNA fragments from the recombinant SK+ plasmid. These fragments were inserted in-frame into the *Kpn*I-*Xba*I sites of the KREG vector. A mutant form of the *giant* coding sequence, *giant 1–389 ΔDLS*, was mutagenized at amino acid positions 100–102 to convert the conserved DLS sequence within the VLDLS motif into alanines. The following mutagenic oligonucleotide was used:

CCTCTGCAGAGGTCCTGGCGGCCGCCCGTCGATG-TGACAGC. The underlined nucleotides indicate substitutions that create the three alanines. An internal deletion within the *giant* coding sequence, *giant Δ60–133*, lacks codons 60 to 133. This deletion was made with the QuickChange Site-Directed Mutagenesis kit (Stratagene). Two primers were used:

GGATCTGTACACGACCAGCAATCTTC and GAAGAT-TGCTGGTCGTGTACAGATCC

The single- and double-underlined sequences correspond to codons 59 and 134, respectively. A 680-bp *Bsr*GI-*Eco*47III fragment containing the DLS mutation or a 390-bp *Bsr*GI-*Stu*I fragment that lacks codons 60 to 133 was isolated and exchanged with the corresponding region of the normal *giant* coding sequence within the pBluescript SK+ recombinant plasmid containing a giant cDNA fragment spanning codons 1–389. *Kpn*I-*Xba*I fragments containing either the *giant 1–389 ΔDLS* or *giant Δ60–133* were inserted into the KREG expression vector.

**The *twi-giant* Expression Vector.** *giant* coding sequences were expressed in ventral regions of precellular transgenic embryos by using two tandem copies of a modified twist PE enhancer, PEeEt (15). An FRT-stop-FRT cassette was inserted between the transcription start site and initiating ATG to circumvent dominant lethality and permit the isolation of transgenic lines (16). A 1-kb *Not*I-*Not*I DNA fragment containing two tandem copies of the *eve* stripe 2 enhancer was removed from a previously described pCasPeR transformation vector (17). This vector contains the *eve* stripe 2 enhancer positioned upstream of an

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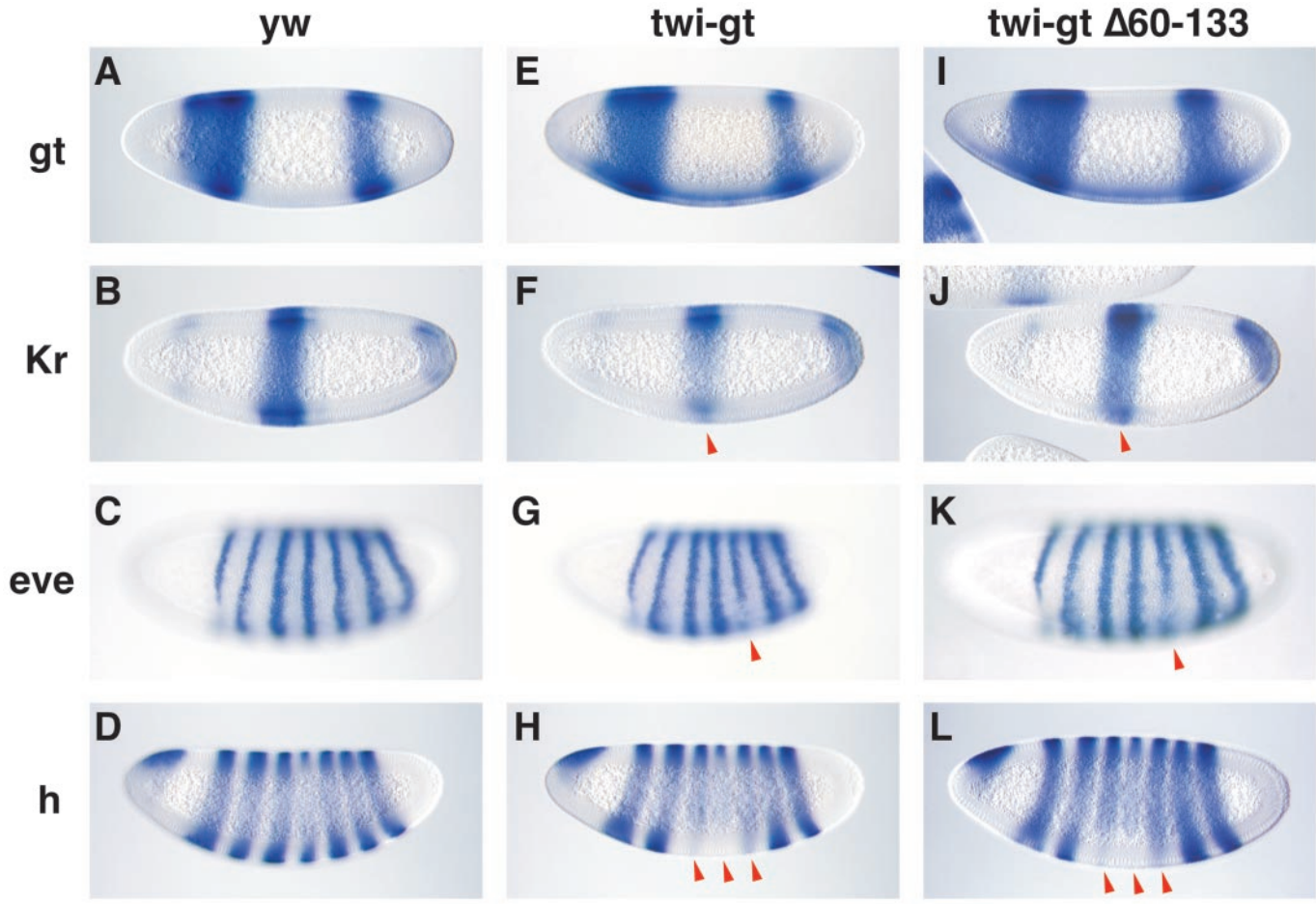


**Fig. 1.** Giant represses *Krüppel*, *eve*, and *hairy* expression. Wild-type and transgenic embryos were hybridized with the indicated digoxigenin-labeled antisense RNA probes (see labels to the left of *A–D*) and are oriented with anterior to the left and dorsal up. (*A*, *E*, *I*) *giant* (gt) staining patterns in precellular wild-type (*yellow;white*; *yw*; *A*), and transgenic embryos (*E* and *I*) that exhibit low levels of *giant* in ventral regions by using the modified *twi* enhancer. The wild-type and mutant *giant* RNAs (*twi-gt* and *twi-gtΔ60–133*) are expressed at comparable levels. All three strains exhibit strong staining in anterior and posterior regions, which represent the normal sites of *giant* expression. (*B*, *F*, *J*) *Krüppel* (Kr) staining patterns in wild-type (*B*) and transgenic cellularized embryos that express either the wild-type *giant* RNA (*F*) or the mutant RNA lacking the putative repression domain (*J*). Normally, *Krüppel* is uniformly expressed in dorsal and ventral regions (*B*). However, ectopic Giant leads to attenuated expression in ventral regions (arrowheads, *F* and *J*). Both forms of Giant are equally effective at repressing *Krüppel*. (*C*, *G*, *K*) *eve* staining patterns in wild-type (*C*) and transgenic embryos (*G*, *K*). Both *twi-giant* transgenes lead to the repression of *eve* stripe 5 in ventral regions (arrowheads, *G* and *K*). (*D*, *H*, *L*) *hairy* (h) staining patterns in wild-type (*D*) and transgenic embryos (*H*, *L*). Both *twi-giant* transgenes lead to the repression of *hairy* stripes 3, 4, and 5.

FRT-stop-FRT cassette. The 1-kb *Not*I-*Not*I fragment was replaced with a 0.5-kb *Not*I-*Not*I fragment containing two tandem copies of the PEeEt enhancer, which contains nucleotide substitutions that create optimal Dorsal operator sites and Twist bHLH E boxes (15). This enhancer directs expression in the ventral-most 22–26 cells, which includes the entire presumptive mesoderm and ventral regions of the neurogenic ectoderm. A 1.8-kb *Hin*dIII-*Eco*RI *giant* cDNA fragment that contains all 448 codons, as well as ≈40 bp of 5′UTR and ≈350 bp of the 3′ untranslated region, was cloned into a modified pBluescript SK+ plasmid, pB/Asc2, which contains two *Asc*I sites in place of unique *Hin*cII and *Sac*I sites in the polylinker. A 680-bp *Bsr*GI-*Eco*47III fragment containing the three alanines substitutions (in place of DLS; see above) or a 390-bp *Bsr*GI-*Stu*I fragment which lacks codons 60–133 was isolated and exchanged with the corresponding region of the wild-type giant sequence in the pB/Asc2 recombinant plasmid. Each of the three *giant* coding sequences was isolated as an *Asc*I-*Asc*I DNA fragment and inserted into the unique *Asc*I site within the 2xPEeEt expression vector. The *Asc*I site is located between the FRT-stop-FRT cassette and the 3′UTR from the *eve* gene.

***In Situ* Hybridization Assays and Fly Strains.** Two different *lacZ* reporter genes were used in this study. The *NEE.UAS-lacZ* gene (Figs. 2 *E* and *F* and 4 *D–H*) is described in ref. 8. The other reporter gene, *NEE.UAS-2xPE-lacZ*, contains a modified 300-bp *rhomboid* NEE placed upstream of the 2xPE *twist* enhancer (Fig. 4 *B* and *C*). The NEE was modified to include four gal4 UAS recognition sequences; a 340-bp CAT spacer DNA was inserted between the two enhancers. The following transgenic strains were used in these studies: RUCPT-3 and RUCPT-5 (*NEE.UAS-2xPE-lacZ*) and G18.2 and G18.3 (*NEE.UAS-lacZ*). Embryos were hybridized with digoxigenin-labeled RNA probes, as described in ref. 14.

## Results

Previous studies suggest that Giant might not require dCtBP to repress the anterior border of *eve* stripe 2 (8). This border usually appears normal in *dCtBP* mutants, although there may be variable expansions suggesting impaired Giant activity (data not shown). In contrast, the posterior border, which depends on the Krüppel repressor, exhibits a far more dramatic and consistent expansion (8). The analysis of *eve* stripe 5 regulation raises the

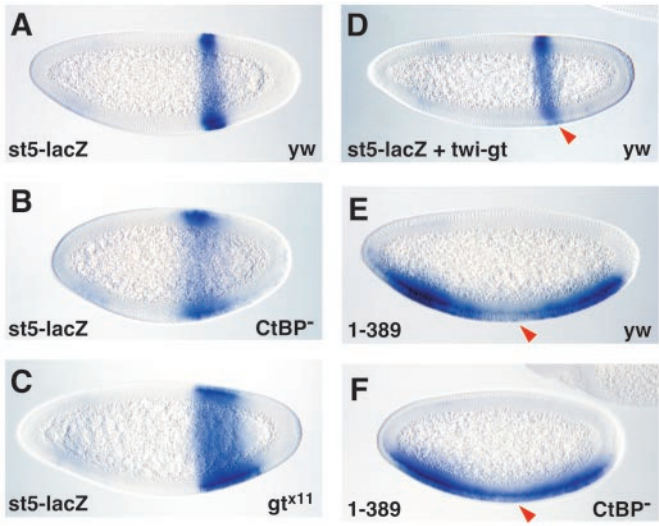


**Fig. 2.** dCtBP is required for the repression of the *eve* stripe 5 enhancer. Wild-type and mutant embryos were hybridized with a digoxigenin-labeled *lacZ* antisense RNA probe. The embryos in *A–D* contain an *eve stripe5-lacZ* transgene, whereas those in *E* and *F* contain a modified *rhomboid NEE.UAS-lacZ* transgene. (*A–D*) The *stripe5-lacZ* transgene exhibits a single stripe of gene expression in the presumptive abdomen of wild-type embryos (*A*). The pattern is expanded in embryos derived from *dCtBP*- germline clones (*B*). However, the expansion is not as severe as that observed in *giant* mutant embryos (*C*). (*D*) The *stripe5-lacZ* reporter gene is repressed in ventral regions (arrowhead) of transgenic embryos that contain the *twi-giant* transgene (compare with *A*). (*E*, *F*) *NEE.UAS-lacZ* staining patterns in a wild-type (*E*) and *dCtBP* mutant embryo (*F*). The central gap in the staining pattern is caused by a *Krüppel-Gal4-Giant* expression vector (see Fig. 4*A*). Repression activity is reduced in *dCtBP* mutants (*F*, arrowhead).

possibility that dCtBP might interact with Giant in posterior regions of early embryos (see below).

**Ectopic Expression in the Mesoderm.** Giant is expressed in anterior and posterior regions of precellular embryos (refs. 18 and 19; Fig. 1*A*). The *giant* coding sequence was attached to tandem copies of a modified *twist* enhancer, 2xPEeEt (summarized in Fig. 3*A*). The resulting *twi-giant* transgene is expressed in ventral regions (Fig. 1 *E* and *I*). This misexpression causes dominant lethality because of the repression of segmentation genes in the presumptive mesoderm (see below). Lethality was circumvented by the use of an FRT-stop-FRT cassette inserted between the transcription start site and *giant* coding sequence. The cassette was removed by excision with the flp recombinase in transgenic males that express flp in the sperm (17).

The *twi-giant* transgene causes attenuated expression of the Krüppel repressor in the ventral mesoderm (arrowhead, Fig. 1*F*; compare with *B*). This result is consistent with the documentation of mutually repressive interactions between Krüppel and Giant (20, 21). The *twi-giant* transgene also causes the repression of *eve* stripe 5 in ventral regions (arrowhead, Fig. 1*G*; compare with *C*). The stripe 2 pattern is not repressed; in fact, there might be a slight posterior expansion of the stripe in the ventral mesoderm because of the repression of Krüppel, which is responsible for establishing the posterior border. This failure to repress stripe 2 is consistent with the proposal that the anterior border depends on high concentrations of the Giant repressor or an unknown bZIP partner (21). In contrast, the *twi-giant* transgene is sufficient to repress *eve* stripe 5 (Fig. 1*G*), as well as *hairy* stripes 3, 4, and 5 (arrowheads, Fig. 1*H*; compare with *D*).

**Giant–CtBP Interactions Influence *eve* Stripe 5.** The 800-bp stripe 5 enhancer was recently identified in the *eve* 3′ flanking region (5).

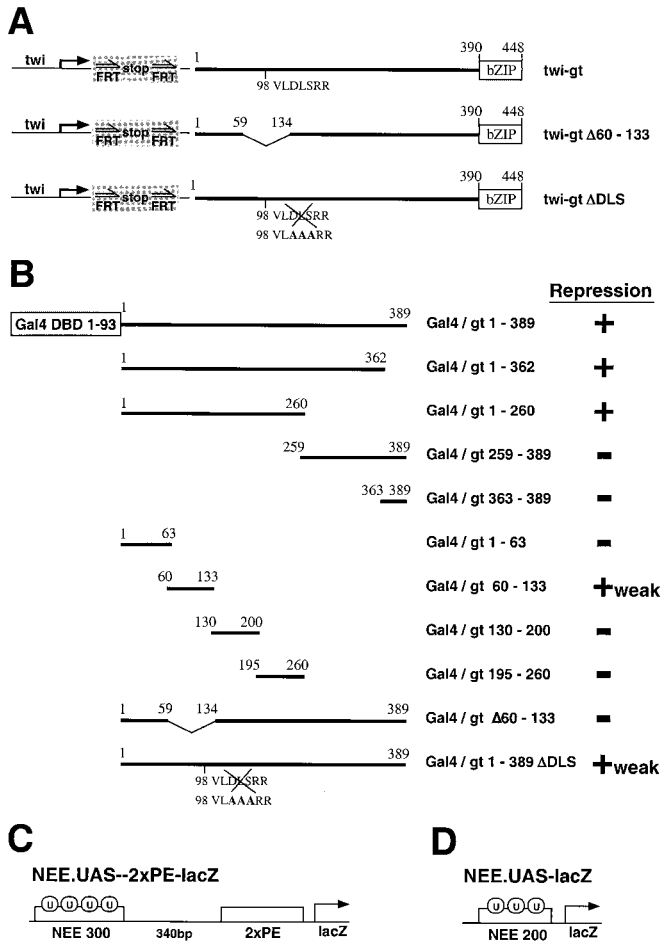


**Fig. 3.** Summary of expression vectors and reporter genes. (*A*) The Giant protein is composed of 448-aa residues, and includes a bZIP DNA-binding domain at the carboxy terminus (*Top*). Two tandem copies of a modified *twist* PE enhancer (PEeEt; ''twi'') were used to misexpress three different forms of the *giant* coding sequence in ventral regions of transgenic embryos: wild type, a mutant form lacking amino acid residues 60–133, and a mutant form containing alanine substitutions in the putative repression motif, VLDLS. Both mutant transgenes are as effective as the wild-type coding sequence in repressing *eve* stripe 5 and *hairy* stripes 3, 4, and 5. (*B*) Previous studies have shown that Giant repression activity is mediated by the first 389-aa residues in the absence of the bZIP domain. This extended region contains a sequence that is reminiscent of the dCtBP interaction motif, VLDLSRR starting at position 98. A variety of Gal4-Giant fusion proteins were expressed in central regions of transgenic embryos by using *Krüppel* 5′ cis-regulatory DNA. Mutations in the VLDLSRR motif impair the repression activity of an otherwise normal, ''full-length'' 1–389 Gal4-Giant fusion protein. Full repression activity is obtained with an N-terminal sequence spanning amino acid residues 1–260. Neither carboxyl-terminal peptide displayed repression activity in the transgenic embryo assays (see Fig. 4). The analysis of smaller amino-terminal peptides identified 60–133 as the minimal Gal4-Giant fusion protein that retained repression activity. Removal of this sequence from the full-length fusion protein eliminated repression activity. Alanine substitutions in the VLDLS motif attenuate the repression activity of a full-length Gal4-Giant fusion protein. (*C*) Reporter gene used to examine the short-range activities of Gal4-Giant fusion proteins. The *lacZ* reporter gene was placed under the control of two enhancers: a modified 300-bp *rhomboid* NEE lateral stripe enhancer placed upstream of two tandem copies of the 250-bp *twist* PE sequence. The NEE and PE enhancers are separated by a 340-bp spacer sequence. The NEE contains 4 UAS sequences (''u'') that permit binding of Gal4-Giant proteins. (*D*) The second reporter gene used for the analysis of Gal4-Giant fusion proteins contains a minimal 200-bp *rhomboid* NEE with three UAS sites.

When attached to a *lacZ* reporter gene, the enhancer is sufficient to direct an authentic stripe 5 pattern (Fig. 2*A*). The *twi-giant* transgene mediates repression of this stripe in ventral regions of

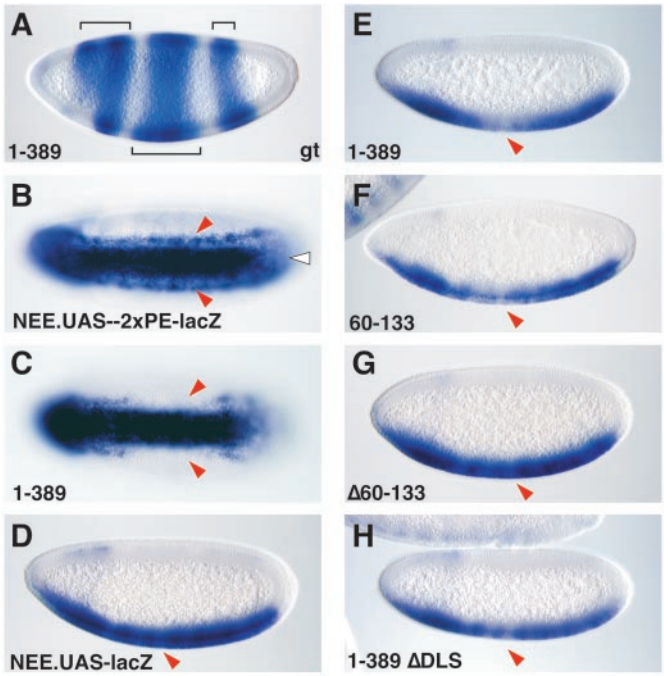


**Fig. 4.** Activities of *Gal4-Giant* fusion genes. Transgenic embryos contain either the *NEE.UAS-2xPE-lacZ* or *NEE.UAS-lacZ* reporter gene and different *Krüppel-Gal4-Giant* expression vectors. Embryos were hybridized with either a *giant* antisense RNA probe (*A*) or a *lacZ* probe (*B–H*). (*A*) *giant* staining pattern in a transgenic embryo that contains the *Krüppel-Gal4-Giant* (1–389) fusion gene. Staining is detected in anterior and posterior regions (*Upper*, brackets), which correspond to the endogenous pattern. The *Krüppel* transgene directs strong expression in central regions (*Lower*, bracket). (*B*) The *NEE.UAS-2xPE-lacZ* reporter gene in a wild-type embryo lacking any of *the Krüppel-Gal4-Giant* expression vectors. Staining is detected in the ventral mesoderm (white arrowhead) and in lateral lines in the neurogenic ectoderm (red arrowheads). (*C*) Same as *B*, except that the embryo also expresses the Gal4-Giant (1–389) fusion protein. The lateral staining pattern directed by the modified NEE is repressed in central regions (arrowheads). In contrast, staining directed by the *twist* enhancer (2xPE) is unaffected. (*D*) The *NEE.UAS-lacZ* reporter gene in a wild-type embryo lacking *Krüppel-Gal4-Giant* expression vectors. The *lacZ* reporter gene exhibits uniform expression in ventral regions. There is no gap in the center (arrowhead). (*E*) Same as *D*, except that the embryo also expresses the Gal4-Giant (1–389) fusion protein. There is a gap in central regions (arrowhead). (*F*) Same as *E*, except that the embryo expresses a Gal4-Giant fusion protein containing the minimal Giant repression domain (amino acid residues 60–133). There is a gap in the staining pattern in central regions (arrowhead). (*G*) Same as *D*, except that the embryo also expresses a mutant version of the full-length Gal4-Giant fusion protein with an internal deletion in the minimal repression domain (Δ60–133). There is no significant repression of *lacZ* staining in central regions (arrowhead). (*H*) Same as *D*, except that the embryo also expresses a mutant form of the Gal4-Giant fusion protein containing alanine substitutions in the VLDLS motif (see Fig. 3).

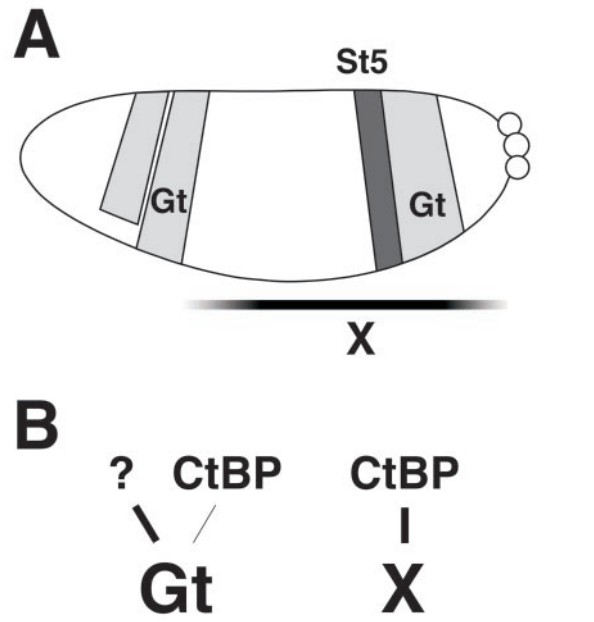


**Fig. 5.** Summary of Giant interactions. (*A*) Diagram of a cellularizing embryo showing the normal *giant* expression pattern (Gt) in anterior and posterior regions. The posterior pattern represses the *eve* stripe 5 enhancer (St5) to establish the posterior stripe border. It is possible that Giant interacts with an unknown bZIP subunit, X, that is distributed throughout the region where stripe 5 is expressed. (*B*) Giant might recruit an unknown corepressor, ''?'', via the VLDLS motif. It is possible that Giant also weekly interacts with dCtBP. The putative X subunit might strongly interact with dCtBP, so that mutations in the Giant repression domain do not impair the activity of Giant-X heterodimeric complexes.

early embryos (arrowhead, Fig. 2*D*). The stripe 5 staining pattern is altered in *dCtBP* mutant embryos (Fig. 2*B*). The stripe shifts toward the center of the embryo and the posterior border expands. This expansion is not as severe as that seen in *giant* mutants (ref. 13; Fig. 2*C*). Nonetheless, Giant repression activity seems to be impaired.

The activity of a Gal4-Giant fusion protein is also reduced in *dCtBP* mutants (Fig. 2*F*; compare with *E*). This fusion protein contains the yeast Gal4 DNA-binding domain (amino acid residues 1–93) and the entire non-DNA-binding domain (amino acid residues 1–389) of Giant. It was expressed in central regions of transgenic embryos by using the Krüppel 5′ regulatory region (see below). Repression was monitored with a *lacZ* reporter gene that contains a modified *rhomboid* lateral stripe enhancer (NEE) containing Gal4 (UAS)-binding sites (Fig. 3*D*). The Gal4-Giant fusion protein represses this *NEE.UAS-lacZ* reporter gene in central regions (arrowhead, Fig. 2*E*). This gap is reduced in *dCtBP* mutants (arrowhead, Fig. 2*F*).

**Identification of a Minimal Repression Domain.** A transgenic embryo assay was used to identify sequences within Giant that are required for transcriptional repression (summarized in Fig. 3). Gal4-Giant fusion proteins were expressed in central regions of early embryos by using *Krüppel* 5′ regulatory sequences (Fig. 4*A*). The ''full-length'' Gal4-Giant fusion protein containing amino acid residues 1–389 from Giant mediates short-range transcriptional repression (Fig. 4*C*; compare with *B*). The *lacZ* reporter gene used in this experiment (Fig. 3*C*) contains two separate enhancers: a distal NEE with UAS sites that mediates expression in lateral lines in the ventral neurogenic ectoderm (red arrowheads, Fig. 4*B*) and the proximal 2xPE *twist* enhancer, which is active in the ventral mesoderm (white arrowhead, Fig. 4*B*). The Gal4-Giant fusion protein binds to the UAS sites in the distal NEE and causes a broad gap in the staining pattern in central regions (arrowheads, Fig. 4*C*). The binding of Gal4-Giant to the distal NEE does not alter the staining pattern directed by the proximal 2xPE *twist* enhancer because *lacZ* exhibits uniform expression in the ventral mesoderm. This result suggests that Gal4-Giant is a short-range repressor that acts only within the limits of the distal NEE.

The *lacZ* reporter gene used for most of the *in vivo* repression assays contains a single enhancer: a modified NEE with UAS sites that mediates expression in ventral regions of early embryos (Fig. 4*D*). Normally, this *lacZ* reporter gene is expressed uniformly along the anterior–posterior axis (the arrowhead in Fig. 4*D* indicates uniform staining in central regions). However, a

number of the Gal4-Giant fusion proteins cause a central gap in *lacZ* staining (Figs. 2*E* and 4*E*). The shortest Giant peptide that was found to mediate repression is 74-aa residues in length and encompasses amino acid residues 60–133 in the native Giant protein (summarized in Fig. 3; arrowhead, Fig. 4*F*). Removal of this peptide virtually abolishes the repression activity of an otherwise normal, full-length Gal4-Giant fusion protein (arrowhead, Fig. 4*G*).

The minimal repression peptide contains a sequence motif, VxDLSxR, that is related to the dCtBP interaction sequence, PxDLSxR (summarized in Fig. 3). Substituting the core DLS residues with alanines attenuates the repression activity of the full-length Gal4-Giant fusion protein (arrowhead, Fig. 4*H*; compare with *E*). This result raises the possibility that the VLDLS motif might participate in the binding of dCtBP. However, Giant–dCtBP interactions are barely discernible by *in vitro* binding assays, and it is unclear whether these interactions, if meaningful, depend on the VLDLS sequence (data not shown). Perhaps this conserved motif interacts with an unknown corepressor protein (see *Discussion*).

Removal of the minimal repression domain (amino acid residues 60–133), or mutations in VLDLS, do not significantly alter the repression activities of *twi-giant* transgenes (Fig. 1 *J–L*; data not shown). The mutant forms of the Giant protein continue to attenuate the ventral expression of *Krüppel* (Fig. 1*J*), *eve* stripe 5 (Fig. 1*K*), and *hairy* stripes 3, 4, and 5 (Fig. 1*L*). The differential requirement of the repression domain in the Gal4-Giant fusion protein (Fig. 4*G*) and the native Giant protein (Fig. 1 *J–L*) suggests that Giant might interact with an unknown bZIP subunit in posterior regions of precellular embryos (summarized in Fig. 5).

## Discussion

The minimal Giant repression domain spans amino acid residues 60–133. Alignment of this sequence with the *Drosophila* database identifies significant homology with the zinc finger repressor, Odd-skipped (Odd; ref. 22). Odd represses the expression of *engrailed* within the even-numbered parasegments and thereby defines which of the Ftz-expressing cells activate *engrailed* (23). Giant and Odd share the following sequence: VLDLSxxxxSxExP. A third transcriptional repressor in the early embryo, Tailless, also contains the VLDLS motif (24). Tailless is important for repressing segmentation gene expression in the anterior and posterior poles (25). It is unclear whether this sequence participates in Giant–dCtBP interactions, even though it is related to the dCtBP motif (PxDLSxR/K/H). Perhaps VLDLS helps recruit an unknown corepressor protein that mediates the residual repression activity of Gal4-Giant fusion proteins in dCtBP mutants (Fig. 2).

The low levels of Giant produced by the *twi-giant* transgene are sufficient to repress the endogenous *eve* stripe 5 pattern but not stripe 2. The failure to repress stripe 2 is consistent with previous studies, which suggested that Giant might interact with a localized "partner" in anterior regions of the early embryo (21). It is also possible that stripe 2 regulation depends on high concentration of the Giant protein. There are two alternative explanations for the sufficiency of low levels of Giant to repress stripe 5. First, the stripe 5 enhancer might contain optimal high-affinity Giant operator sites. Alternatively, Giant might interact with an unknown bZIP subunit, X, that is broadly expressed in the early embryo (summarized in Fig. 5).

We favor the second possibility, whereby Giant-X heterodimers regulate stripe 5 expression. Putative Giant operator sites in the stripe 5 enhancer lack obvious dyad symmetry, which might be expected for Giant-Giant homodimers. Moreover, the VLDLS motif is essential for the repression activity of Gal4-Giant fusion proteins (Fig. 4) but is dispensable in the context of the *twi-giant* transgene (Fig. 1). For example, a deletion that removes the entire minimal repression domain (amino acids 60–133) does not significantly impair the ability of a *twi-giant* transgene to repress *eve* stripe 5 and *hairy* stripes 3, 4, and 5 (Fig. 1). Presumably, Gal4-Giant fusion proteins function as homomultimers, so that mutations in the repression domain attenuate or eliminate activity. In contrast, the same mutations might not disrupt the activities of a heterodimeric Giant-X complex because of the ability of subunit X to recruit dCtBP (Fig. 5). Future studies will focus on the identification of subunit X and the corepressor(s) that interact with the conserved VLDLS motif.

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1. Gray, S. & Levine, M. (1996) *Genes Dev.* **10,** 700–710.
2. Arnosti, D., Gray, S., Barolo, S., Zhou, J. & Levine, M. (1996) *EMBO J.* **15,** 3659–3666.
3. Hewitt, G. F., Strunk, B. S., Margulies, C., Priputin, T., Wang, X. D., Amey, R., Pabst, B. A., Kosman, D., Reinitz, J. & Arnosti, D. N. (1999) *Development (Cambridge, U.K.)* **126,** 1201–1210.
4. Stanojevic, D., Hoey, T. & Levine, M. (1989) *Nature (London)* **341,** 331–335.
5. Fujioka, M., Emi-Sarker, Y., Yusibova, G. L., Goto, T. & Jaynes, J. B. (1999) *Development (Cambridge, U.K.)* **126,** 2527–2538.
6. Langeland, J. A., Attai, S. F., Vorwerk, K. & Carroll, S. B. (1994) *Development (Cambridge, U.K.)* **120,** 2945–2955.
7. La Rosee-Borggreve, A., Hader, T., Wainwright, D., Sauer, F. & Jäckle, H. (1999) *Mech. Dev.* **89,** 133–140.
8. Nibu, Y., Zhang, H., Bajor, E., Barolo, S., Small, S. & Levine, M. (1998) *EMBO J.* **17,** 7009–7020.
9. Nibu, Y., Zhang, H. & Levine, M. (1998) *Science* **280,** 101–104.
10. Meloni, A. R., Smith, E. J. & Nevins, J. R. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 9574–9579.
11. Koipally, J. & Georgopoulos, K. (2000) *J. Biol. Chem.* **275,** 19594–19602.
12. Postigo, A. A. & Dean, D. C. (2000) *Proc. Natl. Acad. Sci. USA* **97,** 6391–6396.
13. Small, S., Blair, A. & Levine, M. (1992) *EMBO J.* **11,** 4047–4057.
14. Jiang, J., Kosman, D., Ip, Y. T. & Levine, M. (1991) *Genes Dev.* **5,** 1881–1891.
15. Jiang, J. & Levine, M. (1993) *Cell* **72,** 741–752.
16. Struhl, G. & Basler, K. (1993) *Cell* 7 **2,** 527–540.
17. Kosman, D. & Small, S. (1997) *Development (Cambridge, U.K.)* **124,** 1343–1354.
18. Capovilla, M., Eldon, E. D. & Pirrotta, V. (1992) *Development (Cambridge, U.K)* **114,** 99–112.
19. Kraut, R. & Levine, M. (1991) *Development (Cambridge, U.K.)* **111,** 601–609.
20. Kraut, R. & Levine, M. (1991) *Development (Cambridge, U.K.)* **111,** 611–621.
21. Wu, X., Vakani, R. & Small, S. (1998) *Development (Cambridge, U.K.)* **125,** 3765–3774.
22. Coulter, D. E., Swaykus, E. A., Beran-Koehn, M. A., Goldberg, D., Wieschaus, E. & Schedl, P. (1990) *EMBO J.* **9,** 3795–3804.
23. Manoukian, A. S. & Krause, H. M. (1993) *Development (Cambridge, U.K.)* **118,** 785–796.
24. Pignoni, F., Baldarelli, R. M., Steingrimsson, E., Diaz, R. J., Patapoutian, A., Merriam, J. R. & Lengyel, J. A. (1990) *Cell* **62,** 151–163.
25. Yu, R. T., McKeown, M., Evans, R. M. & Umesono, K. (1994) *Nature (London)* **370,** 375–379.