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Brinker requires two corepressors for maximal and versatile repression in Dpp signalling

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***decapentaplegic* (*dpp*) encodes a *Drosophila* transforming growth factor-β homologue that functions as a morphogen in the developing embryo and in adult appendage formation. In the wing imaginal disc, a Dpp gradient governs patterning along the anteroposterior axis by inducing regional expression of diverse genes in a concentration-dependent manner. Recent studies show that responses to graded Dpp activity also require an input from a complementary and opposing gradient of Brinker (Brk), a transcriptional repressor protein encoded by a Dpp target gene. Here we show that Brk harbours a functional and transferable repression domain, through which it recruits the corepressors Groucho and CtBP. By analysing transcriptional outcomes arising from the genetic removal of these corepressors, and by ectopically expressing Brk variants in the embryo, we demonstrate that these corepressors are alternatively used by Brk for repressing some Dpp-responsive genes, whereas for repressing other distinct target genes they are not required. Our results show that Brk utilizes multiple means to repress its endogenous target genes, allowing repression of a multitude of complex Dpp target promoters.**

Keywords: Brinker/CtBP/Dpp signalling/Groucho/transcriptional repression

Introduction

In multicellular organisms, the patterning of tissues depends on the intracellular integration and fine-tuning of multiple external signals, which evoke an assortment of cellular responses. Extrinsic cues are transduced by cytoplasmic effectors and, ultimately, information is relayed to the nucleus, where transcription factors are stimulated to either activate or repress target gene transcription. The transforming growth factor-β (TGF-β) superfamily members, which dictate a wide range of cellular outcomes such as proliferation, alteration of cell shape, apoptosis and cell fate specification, exemplify such signalling molecules (Raftery and Sutherland, 1999). One key *Drosophila* TGF-β homologue, encoded by the *decapentaplegic* (*dpp*) gene, has been shown to function as a long-range morphogen, specifying varied cell fates in a concentration-dependent manner (Lecuit *et al*., 1996; Nellen *et al*., 1996). In both embryonic and post-embryonic development, Dpp governs multiple patterning events, including the specification of cells along the dorsoventral (D/V) axis in the embryo and the patterning of the adult appendages (Podos and Ferguson, 1999). Thus, in the developing wing imaginal disc, *dpp* is expressed in a central narrow stripe of cells along the anteroposterior (A/P) compartment boundary, from where Dpp spreads towards the periphery, mediating both cell proliferation and A/P patterning (Burke and Basler, 1996; Lecuit *et al*., 1996; Nellen *et al*., 1996; Entchev *et al*., 2000; Teleman and Cohen, 2000).

A seemingly simple model provides a coherent framework for explaining how the external Dpp signal is transmitted by cytoplasmic components and how it brings about nuclear transcriptional outcomes: Dpp binds to a heteromeric type II/type I transmembrane serine/threonine kinase receptor complex, encoded by *thickveins* and *punt*, triggering the phosphorylation of Mad, the *Drosophila* receptor-specific Smad. Subsequently, phosphorylated Mad (pMad) associates with Medea, and the pMad–Medea complex enters the nucleus to activate Dpp-responsive genes (Raftery and Sutherland, 1999).

The recent cloning and characterization of *brinker* (*brk*), a resident Dpp target gene encoding a repressor protein that antagonizes Dpp-mediated activation (Campbell and Tomlinson, 1999; Jazwinska *et al*., 1999a,b; Minami *et al*., 1999), has enhanced our understanding of how Dpp is able to trigger specific target gene expression programs. Where Dpp signalling is active, the transcriptional regulator Schnurri (Shn) switches off *brk* expression, thus relieving a subset of Dpp-responsive genes from Brk repression (Marty *et al*., 2000). Consequently, *brk* is expressed in the ventrolateral regions of the embryo, abutting the dorsal *dpp* expression domain, whereas in the wing imaginal disc, *brk* is expressed at high levels only in the periphery of the disc, with its transcription diminishing towards the centre. Thus, two opposing and complementary gradients, i.e. activation mediated by Smads and repression by Brk, ensure that discrete thresholds for Dpp activity are attained. In this paper we explore the molecular basis underlying Brk repression.

Transcription factors negate gene expression in diverse manners (Mannervik *et al*., 1999). Some do so 'passively', by competing with, and occluding activators from binding to coincident *cis*-acting DNA elements. For other repressors, DNA binding is not sufficient. Rather, these repressors are fully reliant on tethered corepressors and act more instructively, by local 'quenching' of proximally-bound activators, interference at a distance with the basal transcription machinery or altering chromatin structure and organization (Johnson, 1995; Cai *et al*., 1996; Gray and Levine, 1996). To date, two prototypic *Drosophila*

corepressors have been characterized that seem to be markedly distinct from each other, i.e. where tested, one assists negative transcriptional regulators acting at short-range while the other supports long-range repressors (Zhang and Levine, 1999). Thus, the C-terminal binding protein (CtBP; Nibu *et al*., 1998a,b; Poortinga *et al*., 1998) is a corepressor that acts in conjunction with repressors obstructing the function of activators bound up to 150 base pairs away (e.g. Gray *et al*., 1994; Arnosti *et al*., 1996). In addition, the corepressor Groucho (Gro; Fisher and Caudy, 1998; Parkhurst, 1998; Chen and Courey, 2000) is required by repressors capable of hindering promoter function at long-range, shutting off transcription even over distances of up to several thousand base pairs (Paroush *et al*., 1994; Cai *et al*., 1996; Barolo and Levine, 1997; Dubnicoff *et al*., 1997).

In this study we show that Brk contains a functional repression domain that accommodates Gro and CtBP recruitment motifs, and that Brk interacts physically with these cofactors. Although other *Drosophila* repressors are known to possess more than one repressor domain (e.g. Arnosti *et al*., 1996; Keller *et al*., 2000; Kobayashi *et al*., 2001), the biological relevance of this feature has not yet been genetically addressed. Here we investigate the functionality of Brk's association with two corepressors and demonstrate that the mechanism of repression by Brk is dependent on promoter context. We show that Brk requires either or both Gro and CtBP for switching off some target genes, whereas for the silencing of others, it requires neither of these cofactors, presumably relying on its reported ability to outcompete activators from binding DNA. We surmise that the combinatorial use by Brk of these two corepressors provides a versatility that allows it to silence a variety of composite promoters in response to graded morphogenetic activity of Dpp.

Results

Dpp target genes are specifically repressed by overexpression of gro

Gro is ubiquitously expressed in the adult wing (Tata and Hartley, 1993) and mutations in *gro* have been identified in genetic screens for modifiers of various wing and eye phenotypes (e.g. Heitzler *et al*., 1996; Chanut *et al*., 2000), implicating Gro in advanced developmental stages. Indeed, Gro has been ascribed at least one specific role in the establishment of wing configuration, as a corepressor for the *Enhancer of split* basic-helix–loop–helix proteins acting downstream of *Notch* signalling in D/V wing patterning (Heitzler *et al*., 1996). To assess whether Gro also contributes in hitherto unrecognized ways to wing A/P axis formation, we analysed the expression of wing-patterning genes in marked clones of cells that either ectopically overexpress, or are mutant for, *gro* (Xu and Rubin, 1993; Pignoni and Zipursky, 1997; see Materials and methods). Overexpression of *gro* should enhance the silencing of genes normally repressed by Gro-dependent transcriptional regulators while, reciprocally, the loss of *gro* should result in derepression, and therefore in the ectopic induction of these genes.

In the wing imaginal disc, cells in the posterior compartment are programmed by the *engrailed* selector gene product to secrete Hedgehog (Hh), which induces *dpp* in a stripe of anterior cells along the A/P boundary. Dpp then acts as a long-range morphogen that governs patterning across the entire imaginal disc field (Podos and Ferguson, 1999). To determine whether Gro participates in the implementation of Hh signalling, we stained clones overexpressing *gro*, or clones that are homozygous for the strong gro^{E48} allele, for *dpp-lacZ* expression. In all clones, even those overlapping with the Hh activity domain, there are no noticeable alterations in the *dpp* expression pattern (Figure 1A; data not shown), indicating that Gro is not required downstream of Hh for *dpp* transcriptional regulation. In striking contrast, however, three distinct targets of the Dpp pathway, expressed either in the wing pouch (*optomotor-blind*; *omb* and *vestigial*; *vg*) or in the periphery of the wing disc (*brk*), are repressed in clones overexpressing *gro* (Figure 1B–D). Expression of *omb-lacZ* (Figure 1B), as well as that of a *lacZ* reporter driven by *vg*'s Dpp-responsive enhancer (*vgQ-lacZ*; Figure 1C), is completely abrogated in these clones, whereas expression of *brk-lacZ* is only reduced (Figure 1D; see below). All three Dpp targets are repressed in a cell autonomous manner, i.e. only in the clones but never in adjacent cells. These results, together with an extensive *gro* loss-of-function clonal analysis detailed below, implicate Gro specifically as a downstream effector of Dpp signalling.

Brk interacts physically with two corepressors, Gro and CtBP

Recent genetic and molecular studies have shown that *brk* encodes a repressor acting downstream of the Dpp pathway, which helps define the low end of the Dpp gradient (Campbell and Tomlinson, 1999; Jazwinska *et al*., 1999a; Minami *et al*., 1999). In particular, the Dpp targets *omb* and *vgQ* are both derepressed in brk^{-} mutant clones and in brk^{-} wing imaginal discs, suggesting that they are normally subjected to Brk repression (Campbell and Tomlinson, 1999; Jazwinska *et al*., 1999a; Minami *et al*., 1999). More directly, Brk binds to specific sequences within defined *omb* and *vgQ* enhancer elements, bringing about their silencing by outcompeting the Mad–Medea complex, or some other activator, from binding to overlapping DNA sites (Sivasankaran *et al*., 2000; Kirkpatrick *et al*., 2001).

That putative Brk target genes are repressed in clones of cells with increased *gro* dosage strongly suggests that Brk is a Gro-dependent repressor. Accordingly, Brk's proposed repression domain (RD) (Campbell and Tomlinson, 1999) harbours a potential Gro recruitment motif (FKPY), similar to the Gro-binding domains defined in the repressors Hairy (WRPW), Runt (WRPY) and Huckebein (FRPW), and identical to that in Even-skipped (Eve) (Paroush *et al*., 1994; Aronson *et al*., 1997; Goldstein *et al*., 1999; Kobayashi *et al*., 2001). It has been noted previously by others that Brk also contains a CtBP-binding domain (PMDLSLG; Jazwinska *et al*., 1999a). Below we show that Brk is in fact able to interact physically with both Gro and CtBP, and address the functional relevance of these associations to Brk's *in vivo* repressor capacity.

To demonstrate Brk's ability to associate with the two corepressors *in vitro*, we fused the protein's putative RD (amino acids 369–541) to glutathione *S*-transferase (GST), and incubated it with radioactively labelled Gro or CtBP (Figure 2; see Materials and methods). In GST pull-down

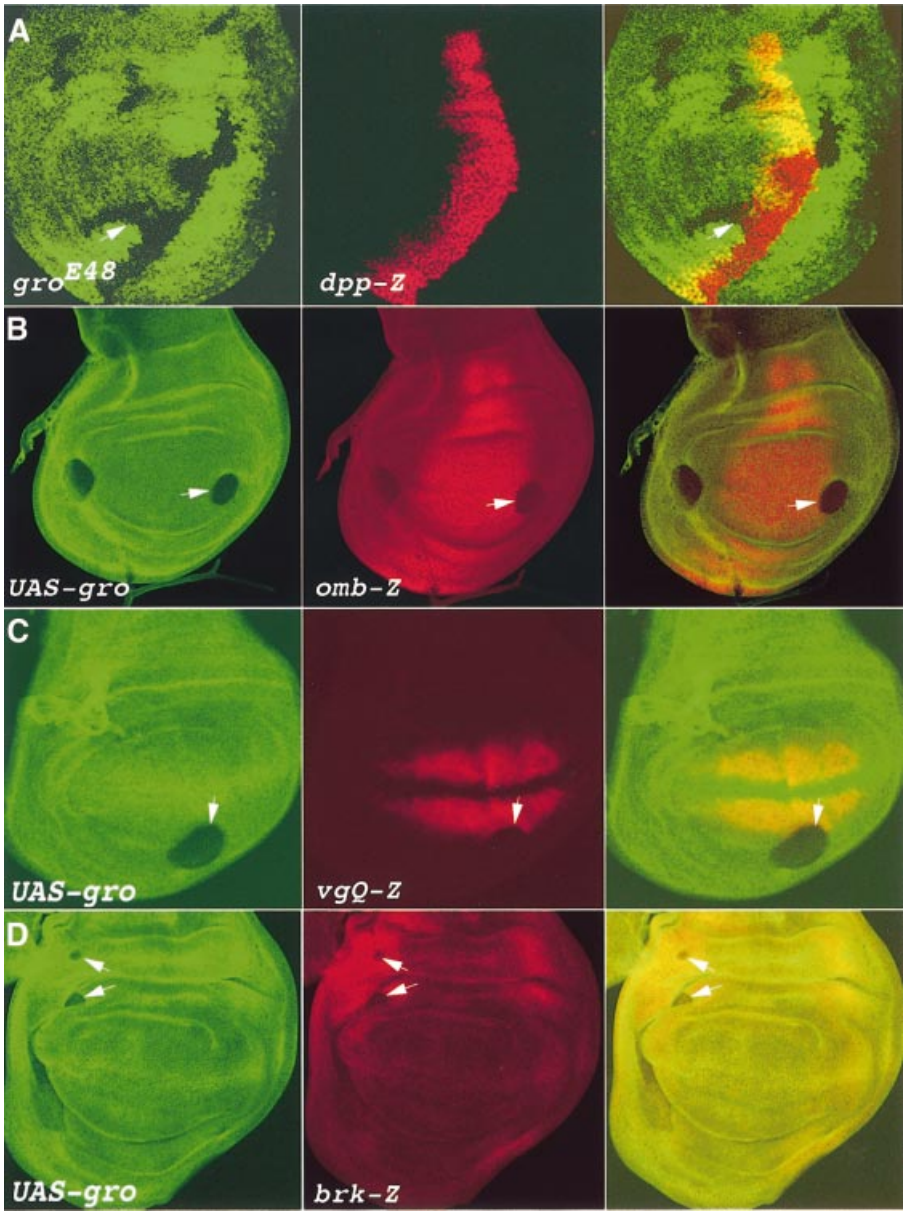

Fig. 1. Overexpression of *gro* does not affect *dpp* expression, but brings about repression of Dpp target genes. (A–D) Third instar larval imaginal wing discs, stained for the πMyc or CD2 markers (left) and for β-galactosidase (centre); merge, right. In these, and subsequent figures, anterior is to the left and dorsal up. (**A**) *dpp-lacZ* expression (centre, red) is unaffected by *groE48* mutant clones, marked by loss of the πMyc marker (left, green), or by *gro* overexpression (data not shown). In contrast, *gro* overexpression, in clones marked by loss of CD2 (left, green), leads to the complete repression of *omb-lacZ* (**B**) and *vgQ-lacZ* (**C**), and to a reduction in *brk-lacZ* (**D**) expression levels, in a cell-autonomous manner.

assays, Brk's RD (Brk^{RD}), but not GST alone, readily retains [^{35}S]methionine-labelled Gro (Figure 2A). To test further the specificity of this interaction, three mutant derivatives of the Brk^{RD}, fused to the GST moiety, were generated in which the Gro recruitment domain (Brk^{RDmutG}; FKPY to FEAY; Goldstein *et al*., 1999), the core of the CtBP-binding motif (Brk^{RDmutC}; DLS to AAA; Zhang and Levine, 1999) or both ($Brk^{RDmutC/G}$) were altered (Figure 2D). As shown in Figure 2A, Brk's binding to Gro is impaired by the modifications in the FKPY motif. Significantly, however, Gro associates with the GST–Brk^{RDmutC} construct as strongly as it does with the native GST–Brk^{RD} fusion. GST–Brk^{RD} also binds labelled CtBP *in vitro* (Figure 2B) and, although the binding of Brk to CtBP is weak in this assay, the specificity of the interaction is clearly evident: the association between the two proteins is abolished by mutations in the CtBP recruitment domain but is unaffected by alterations in the Gro recruitment motif (Figure 2B).

We confirmed Brk's ability to interact with Gro and CtBP, particularly the specificity of the associations, using the yeast two-hybrid system (Figure 2C; see Materials and methods). The full-length Brk, or only its RD portion, interacts strongly with Gro and pZP54 ($Gro_{251–719}$; Paroush *et al*., 1994), as with CtBP (Figure 2C; data not shown). Here too, mutations in one recruitment motif selectively obliterate the binding of Brk to only the single respective corepressor but not to the other.

Brk contains a functional repression domain that depends on corepressors

Brk has been reported to negate transcription by competing with activators, such as Mad/Medea, for overlapping DNA target sites, thereby preventing them access to target promoters (Sivasankaran *et al*., 2000; Kirkpatrick *et al*., 2001; Rushlow *et al*., 2001). Its direct interactions with Gro and CtBP, however, suggest that Brk acts in a more instructive manner. While in the former 'passive' mechanism Brk is expected to rely solely on its competitive DNA-binding activity, the latter 'active' mechanism predicts that it accommodates an innate RD that depends on the recruitment of corepressors.

To establish whether Brk contains a functional RD that can silence gene expression, separable from its DNA-binding domain, we employed an *in vivo* assay that relies on repression of the sex-determining *Sex-lethal* (*Sxl*) gene by ectopic expression of the pair-rule gene *hairy* (Parkhurst *et al*., 1990; Jiménez *et al*., 1997). *Sxl* is normally expressed only in female embryos whereas, in males, it is repressed by Deadpan (Dpn), an autosomally encoded Hairy-related repressor protein. When Hairy is expressed prematurely, under the *hunchback* (*hb*) promoter, it mimics Dpn's repressor function and eradicates *Sxl* transcription in the anterior of syncytial blastoderm female embryos. Because Sxl is essential for dosage compensation in females, this repression subsequently leads to female-specific lethality (Parkhurst *et al*., 1990). A form of Hairy, lacking its own RD, is inert in this assay. However, fusion of heterologous RDs to the truncated Hairy protein restores its ability to repress *Sxl* (Jiménez *et al*., 1997; Goldstein *et al*., 1999). Indeed, the equivalent expression of a *hb-Hairy-BrkRD* transgene results in an effective repression of *Sxl* in the anterior halves of female embryos (Figure 3A) and female-specific lethality ensues (Figure 3F; see Materials and methods). Thus, the region in Brk spanning the Gro- and CtBP-binding domains promotes potent repression in embryos.

The ability to selectively disrupt Brk binding to each individual corepressor allowed us to start exploring the dependence of its repressor potential on Gro and/or CtBP *in vivo*. As both Gro- and CtBP-mediated repression can be detected in the *Sxl*-repression assay (Jiménez *et al*., 1997), we fused truncated Hairy to the three derivatives of the Brk RD, mutated in the Gro, CtBP or both recruitment motifs (see Figure 2D) and placed them under *hb* promoter regulation. In female embryos expressing Hairy-Brk^{RDmutC}, *Sxl* is substantially repressed, although not as effectively as by Hairy-BrkRD (Figure 3B). Furthermore, this repression still leads to statistically significant female-specific lethality (Figure 3F; $p = 0.001$; see Materials and methods). Thus, blocking CtBP binding does not completely abolish activity of the Brk RD. In comparison, mutating the Gro recruitment domain causes only residual *Sxl* repression (Figure 3C) and no apparent female-specific lethality (Figure 3F). Finally, *Sxl* expression is seen

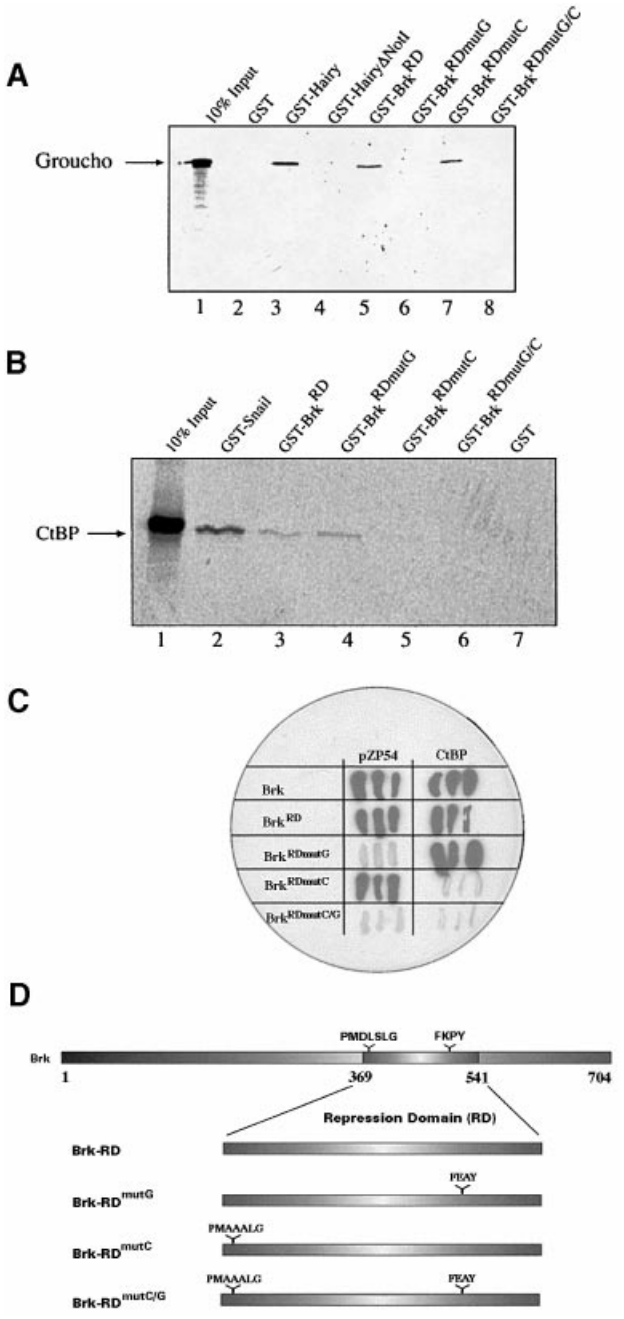

Fig. 2. Brk interacts physically with Gro and CtBP. (A and B) *In vitro* pull-down assays. ^{35}S-labelled Gro (**A**) or dCtBP (**B**) were incubated with GST–BrkRD derivatives immobilized on glutathione–agarose beads and, following washing, retained ^{35}S-labelled protein was subjected to SDS–PAGE (not shown) and autoradiography. (A) Gro binds specifically to GST–BrkRD (lane 5) and to GST–Brk^{RDmutC} (lane 7), but not to GST–Brk variants in which Brk's FKPY motif is mutated (lanes 6 and 8). GST–Hairy (lane 3) serves as a positive control, whereas Hairy lacking its C-terminal Gro-binding domain (HairyΔNotI, lane 4) and GST alone (lane 2) are negative controls. (B) CtBP binds specifically to GST–BrkRD (lane 3) and Brk^{RDmutG} (lane 4), although to a lesser extent than to GST–Snail (lane 2), an established CtBP partner (Nibu *et al*., 1998a). Mutating Brk's CtBP recruitment core motif abolishes CtBP binding (lanes 5 and 6). GST alone, lane 7. (A and B) 10% of input-labelled protein was run in lane 1. Arrows indicate positions of full-length Gro (A) and CtBP (B). (**C**) Yeast two-hybrid assay. Full-length Brk, or just its RD, interacts strongly in yeast with full-length Gro (not shown), with pZP54 ($Gro_{251–719}$) and with CtBP (white colonies indicate lack of interactions). Mutating either the Gro or CtBP recruitment motif in Brk's RD results in loss of interactions between Brk and the corresponding corepressor. (**D**) A schematic representation of Brk's RD (residues 369–541) and derived constructs. The Brk corepressor recruitment motifs, and respective mutant versions, are indicated.

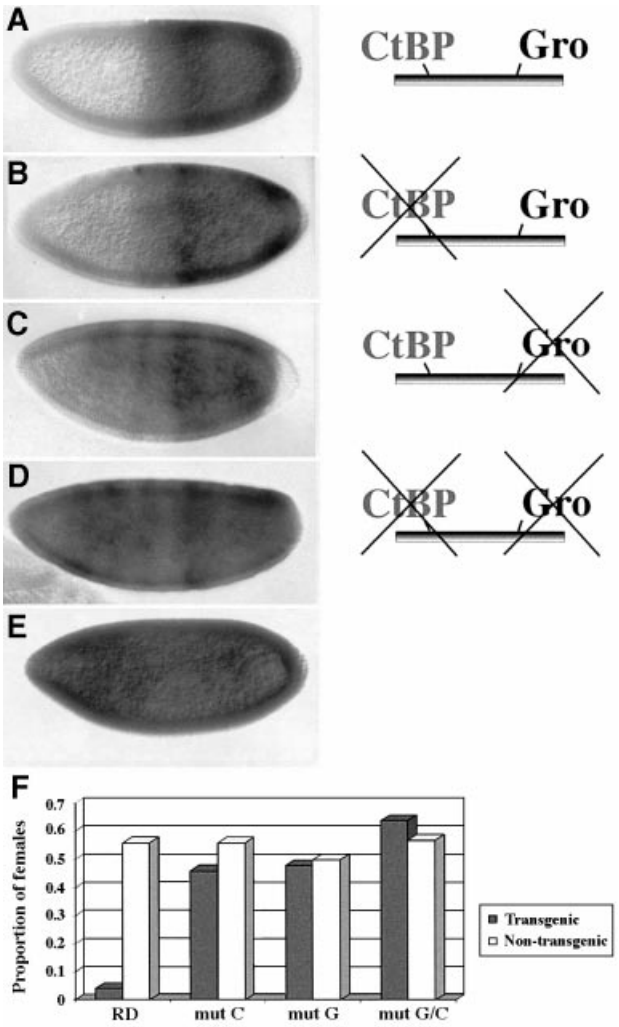

Fig. 3. Brk requires both Gro and CtBP for full repression of *Sxl*. (**A**) Expression of Hairy-BrkRD blocks *Sxl* expression in the anterior halves of female embryos and, consequently, strong female-specific lethality ensues (F). The ability of the BrkRD to repress *Sxl* in female embryos is compromised when the CtBP-binding motif is mutated (**B**) and is completely abolished by mutations in the Gro recruitment domain (**C** and **D**). Female-specific lethality is, correspondingly, affected (F). (**E**) Wild-type expression of *Sxl*. (A–E) Early *Sxl* expression was monitored using the *Sxl-Pe*:*lacZ* reporter strain (Estes *et al*., 1995). Equivalent results were obtained by staining embryos with a monoclonal antibody specific to the active form of Sxl. (**F**) The proportional number of transgenic (black) and non-transgenic (white) females of representative lines indicates the magnitude of female-specific lethality (see Materials and methods).

throughout female embryos expressing *hb-Hairy-Brk$^{RDmutC/G}$* (Figure 3D), and no female-specific lethality is observed (Figure 3F). Thus, Brk relies mainly on Gro for repressing *Sxl*. Nevertheless, since mutating the CtBP recruitment motif in Brk's RD attenuates *Sxl* repression (Figure 3B and F), we conclude that, for full potency as a negative transcriptional regulator, Brk requires both corepressors.

omb and spalt are repressed by Brk independently of Gro and CtBP

The above data indicate that the interactions between Brk and the corepressors Gro and CtBP are indispensable for maximal repression of *Sxl in vivo*. We next sought to establish whether Brk requires both cofactors for repression of its endogenous target genes. Below (Figures 4–7) we show that, for repression of distinct target genes, Brk requires Gro and/or CtBP differentially, presumably as a function of specific promoter topology and architecture (see Discussion).

Brk competes with an activator for binding to an *omb* wing enhancer (Sivasankaran *et al*., 2000), suggesting that, for this promoter, Brk should act independently of corepressors. Consistent with this, we find that *omb-lacZ* is not ectopically expressed in cells homozygous for *gro^{E48}* (hereafter referred to as *gro*$^{-}$ clones) (Figure 4A), nor is it affected by *CtBP* loss-of-function clones, generated using the *l(3)87De-10* allele (*CtBP*$^{-}$; data not shown), or by *CtBP*$^{-}$, *gro*$^{-}$ double mutant clones (Figure 4B). Thus, single and double mutant clones for *gro* and *CtBP* do not phenocopy the *omb* derepression seen in *brk*$^{-}$ clones (Campbell and Tomlinson, 1999; Jazwinska *et al*., 1999a; Minami *et al*., 1999), implying that Brk can repress *omb* even in the absence of these corepressors. Repression of the Dpp target gene *spalt* (*sal*; Nellen *et al*., 1996) is also independent of Gro and CtBP (data not shown). Nonetheless, in *gro* overexpression clones, *omb* is repressed (Figure 1B), suggesting that, even for the *omb* promoter, Gro reinforces Brk repressor function (see Discussion).

Brk requires Gro, but not CtBP, for repressing vgQ

To establish whether Brk represses *vgQ* via Gro, CtBP or both, we monitored *vgQ-lacZ* expression in *gro*$^{-}$ and *CtBP*$^{-}$ single, and *CtBP*$^{-}$, *gro*$^{-}$ double mutant clones. In this instance, we find a mandatory requirement for Gro, but not for CtBP; in *gro*$^{-}$ clones, *vgQ* is upregulated (Figure 5A). Importantly, as is the case for *brk*$^{-}$ clones (data not shown; Campbell and Tomlinson, 1999), the cell-autonomous upregulation of *vgQ* is seen only in *gro*$^{-}$ clones close to the periphery of the disc, suggesting that the observed effects are Brk dependent. In contrast, in *CtBP*$^{-}$ mutant clones *vgQ* expression is downregulated, in the Brk territory but also outside it, at the centre of the disc (Figure 5B), indicating that these effects are Brk independent and that CtBP is positively required for *vg* expression (see Discussion). *CtBP*$^{-}$, *gro*$^{-}$ double mutant clones show a composite effect: ectopic expression and upregulation of *vgQ* in clones in the *brk* expression domain, and a phenotype resembling that of *CtBP*$^{-}$ clones at the middle of the disc, where *brk* is not expressed (Figure 5C). Thus, Brk repression of *vgQ* is Gro- but not CtBP-dependent.

Negative autoregulation of brk requires either Gro or CtBP

omb and *vgQ* expression is completely shut off in clones of cells overexpressing *gro*, whereas that of *brk* is only reduced (Figure 1), suggesting that Brk might be repressing its own transcription via a negative autoregulatory loop (B.Müller and K.Basler, unpublished results). To establish whether, in negating its own expression, Brk is assisted by Gro and/or CtBP, we stained *gro*$^{-}$ and *CtBP*$^{-}$ single (Figure 6A and B), or *CtBP*$^{-}$, *gro*$^{-}$ double (Figure 6C) mutant clones for *brk-lacZ* expression. Figure 6A and B shows that *brk* is never ectopically expressed in any of the

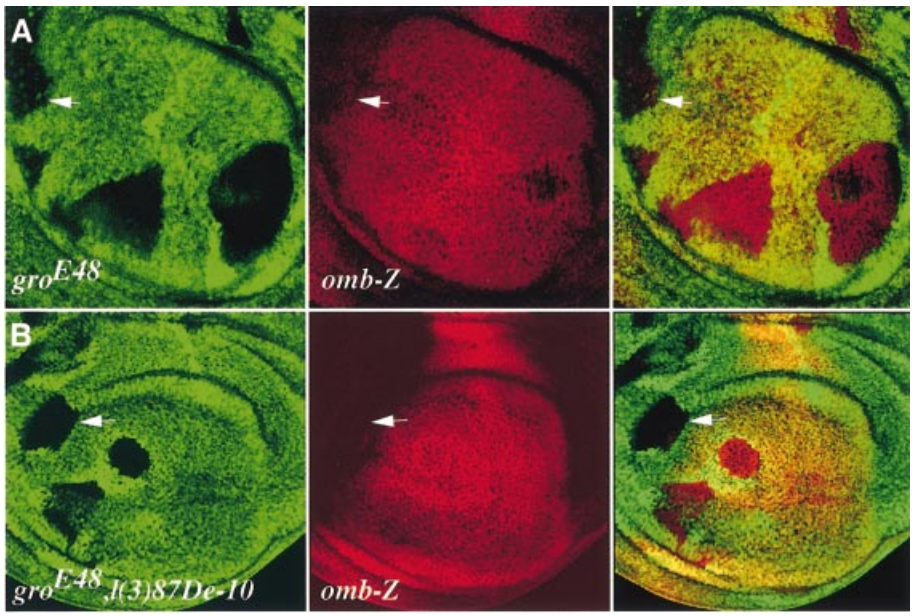

Fig. 4. Brk represses *omb* independently of Gro and CtBP. Wing imaginal discs, bearing *gro*⁻ single (**A**) or *CtBP*⁻, *gro*⁻ double mutant (**B**) clones, discernible by loss of the πMyc marker and by the appearance of a nearby twin-spot (left, green), do not show elevation of *omb-lacZ* expression (centre, red; arrows); merge, right.

single mutant clones, whereas ectopic *brk* expression is clearly observable in double mutant clones (Figure 6C). Thus, in the absence of one corepressor, repression is adequately mediated by the other, suggesting that negative autoregulation by Brk is robust, relying on either Gro or CtBP.

Strikingly, the effects on *brk* expression are seen only in double mutant clones found at the periphery of the disc, but not at the centre where Shn is active (Figure 6C; Marty *et al*., 2000), supporting the notion that the effects are, indeed, Brk- but not Shn-dependent. Furthermore, the fact that double mutant clones at the middle of the disc do not ectopically express *brk* suggests that Shn-mediated repression of *brk* transcription must be taking place even in the absence of both corepressors.

Corepressors are required for Brk repression in the embryo

In embryogenesis, Brk plays a comparable role to that in the wing disc, i.e. it blocks low- and intermediate-level Dpp target gene expression (Jazwinska *et al*., 1999b; Ashe *et al*., 2000; Rushlow *et al*., 2001; Zhang *et al*., 2001). We next wanted to establish whether, in the developing embryo, Brk is also reliant on corepressors for the silencing of its downstream targets. A direct assessment of Brk's dependence on Gro using germ-line clones devoid of maternal *gro* is hindered, however, by the prior requirement for Gro by Dorsal, which in embryonic D/V axis formation represses several Brk subordinate targets before *brk* is expressed and functional (Dubnicoff *et al*., 1997; Jazwinska *et al*., 1999b). For example, in *gro*⁻ germ-line clones Dorsal fails to repress *dpp* and *zerknullt* (*zen*), and these expand ventrally (Dubnicoff *et al*., 1997), making it impossible to distinguish between the loss of Brk activity from that of Dorsal's.

To be able to compare Brk's dependence on Gro and CtBP in the embryo, we undertook an alternative approach, of overexpressing full-length Brk in its native form or with its corepressor-binding domains mutated (Figure 2D), using *UAS-brk* transgenes driven by maternal *GAL4* (Brand and Perrimon, 1993). This experimental design is inapplicable for studying Brk's targets in the wing, as ectopic expression of *brk* prevents proliferation and survival of imaginal disc cells (data not shown), but is nevertheless effective in the embryo (Jazwinska *et al*., 1999b). Rushlow *et al*. (2001) have shown that ectopic Brk represses *zen* and *dpp* in mid- to late-cellularizing embryos but not earlier, so we analysed endogenous Brk targets in transgenic embryos at comparable stages of development. As shown in Figure 7, ectopic expression of all three mutant forms of Brk in embryos brings about repression of *zen* to the same extent as does native Brk (cf. Brk and $Brk^{mutC/G}$ in Figure 7A, right; Figure 7B). This result suggests that Brk represses *zen* independently of corepressors, in keeping with published reports showing that Brk acts on the *zen* promoter by competing with pMad over DNA-binding sites (Rushlow *et al*., 2001). In contrast, Brk requires corepressors for negating transcription of both *tolloid* (*tld*) and *dpp*. Thus, abolishing Brk's interactions with Gro (Brk^{mutG}), but not with CtBP (Brk^{mutC}), completely relieves *tld* repression (Figure 7A, centre; Figure 7C and D), indicating that Brk repression of *tld* is strictly Gro dependent, as is repression of *pannier* (*pnr*; Zhang *et al*., 2001). Similarly, *dpp* is repressed in embryos expressing Brk^{mutC}, but is still transcribed in embryos expressing Brk with its Gro recruitment motif mutated (Figure 7A, left; Figure 7E–G). In the case of *dpp*, however, CtBP must also be contributing to Brk repression, since the level of *dpp* expression is significantly lower in Brk^{mutG}-expressing embryos (marked by '**' in Figure 7A), in comparison with wild-type embryos or embryos expressing $Brk^{mutC/G}$ (cf. Figure 7F and G). We thus conclude that, for repression of *dpp*, Brk rests mainly on Gro, yet for maximal repressor activity it also requires

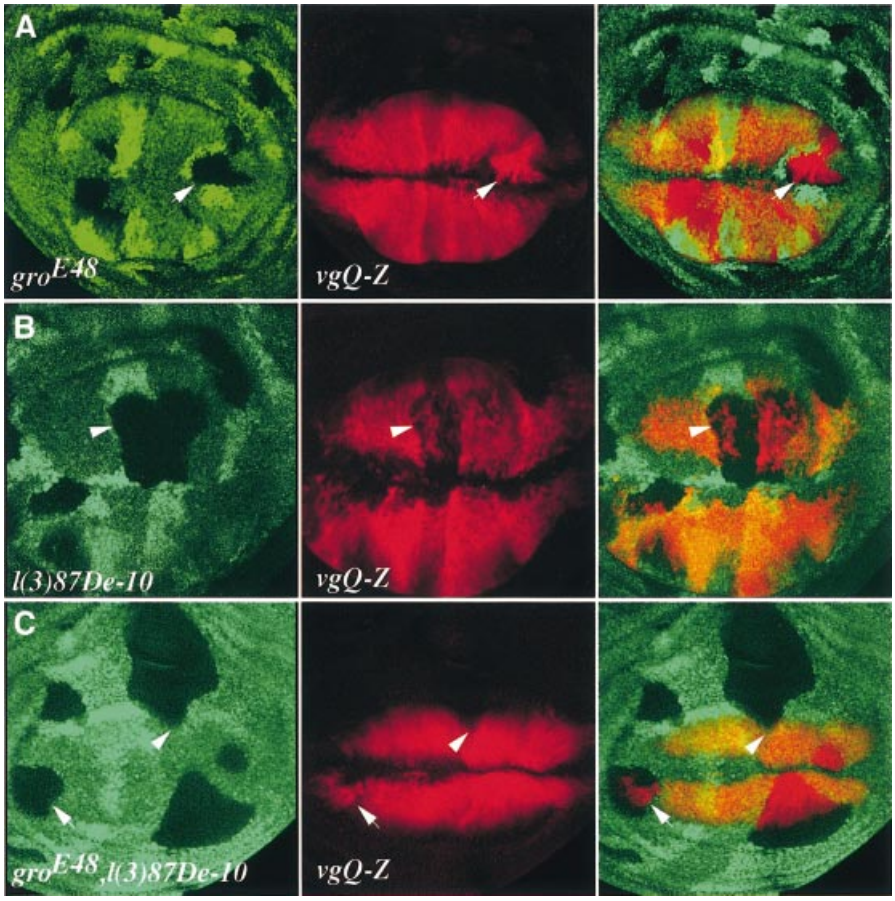

Fig. 5. Brk requires Gro, but not CtBP, for repressing *vgQ*. (**A**) Clones of cells mutant for *gro* and marked by the loss of πMyc (left, green) show ectopic *vgQ* expression (centre, red). In contrast, *CtBP*$^{-}$ clones show a reduction in levels of *vgQ* expression (**B**). Note that *gro*$^{-}$ clones show a phenotype only when in the periphery of the disc, as seen in *brk* mutant clones (Campbell and Tomlinson, 1999), while the downregulation of *vgQ* in *CtBP*$^{-}$ clones is observed regardless of their position, indicating that these effects are Brk independent. (**C**) A composite phenotype in *CtBP*$^{-}$, *gro*$^{-}$ double mutant clones resembles that of *gro*$^{-}$ in peripheral clones (arrow) and that of *CtBP*$^{-}$ in central clones (arrowhead).

CtBP. While these experiments were in progress, Zhang *et al*. (2001) reported that mutating Brk's Gro recruitment motif relieves *dpp* repression only partially, consistent with an additional input into *dpp* regulation. Our results suggest that this input might be in the form of Brk repression mediated by CtBP. In any case, our data indicate that Brk utilizes different means of repression for silencing its downstream targets in the embryo, as in the adult.

Discussion

Dpp activity converges on target genes as an assimilation of (i) transcriptional activation mediated by the pMad–Medea effector complex, and (ii) silencing induced by the sequence-specific repressor Brk (Podos and Ferguson, 1999). Thus, discrete thresholds induced by Dpp signalling output are generated in practice by a summation of two opposite and antagonistic gradients, with the holistic nature of the Dpp patterning process guaranteed by the fact that *brk* itself is a Dpp target. Here we have shown that Brk recruits two corepressors, namely Gro and CtBP, and that these cofactors are differentially required by Brk for repressing a subset of its targets while, for negating other promoters, they are entirely dispensable. Collectively, our results suggest that Brk utilizes multiple means to repress divergent Dpp-responsive genes in a promoter context-dependent manner.

Gro in adult patterning

The Gro corepressor participates in diverse embryonic developmental settings, such as neurogenesis, segmentation, sex determination and terminal patterning (Fisher and Caudy, 1998; Parkhurst, 1998; Chen and Courey, 2000). Previous work has also shown *gro* function to be critical for normal eye and wing patterning. In both these tissues, Gro is clearly involved in the Notch pathway, in silencing expression of proneural genes (e.g. Heitzler *et al*., 1996; Chanut *et al*., 2000). However, *gro* also contributes to organogenesis independently of the neurogenic pathway (de Celis and Ruiz Gomez, 1995; Heitzler *et al*., 1996). In this paper we implicate Gro as an effector of the Dpp pathway in patterning of the wing, specifically as a cofactor for the Brk repressor. Significantly, we also show CtBP to be required for full implementation of Brk repression.

Two additional points are implied indirectly from our data. First, the effects caused by overexpressing *gro* (Figure 1) suggest that although *gro* is uniformly expressed throughout the wing imaginal disc (Tata and

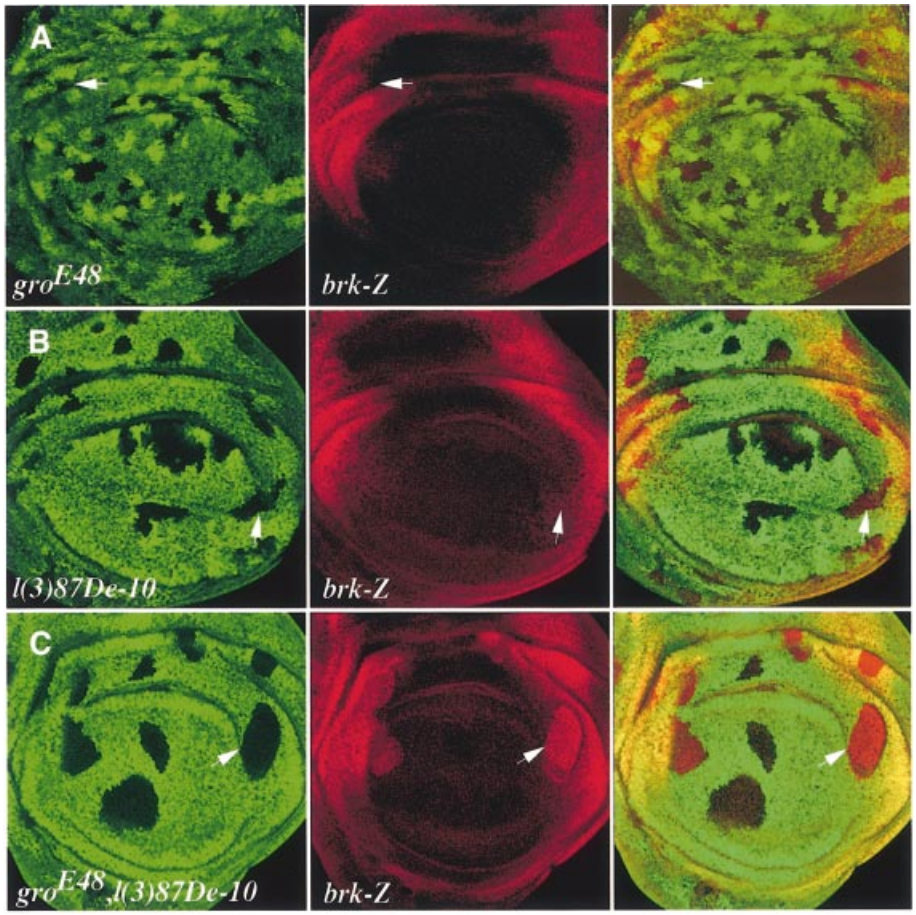

Fig. 6. Negative autoregulation of *brk* requires either Gro or CtBP. Single gro^{-} (**A**) or $CtBP^{-}$ (**B**) clones do not affect *brk* expression whereas $CtBP^{-}$, gro^{-} double mutant clones show ectopic *brk* expression (**C**). The phenotype in (C) is seen only in the periphery of the disc, suggesting that it is Brk dependent and Schnurri independent.

Hartley, 1993), its levels must be limiting. Secondly, as in the embryo, Gro is required in the adult only for a subset of repressors. *gro* is not involved in *dpp* transcriptional regulation, since the *dpp* pattern itself is unaffected by the absence of *gro* or by its overexpression, and Dpp target genes are repressed in *gro* overexpression clones even outside and away from the central *dpp* expression domain (Figure 1; data not shown). Thus, for Cubitus interruptus acting downstream of Hh and upstream of *dpp*, as for Shn acting upstream of *brk* (Figure 6), Gro (and CtBP) is non-essential.

Functional interactions between Brk and two corepressors

The Brk RD includes two distinct corepressor recruitment motifs, with which it recruits both Gro (this study; Zhang *et al*., 2001) and CtBP (this study) to target promoters. It is still unclear how, once tethered to DNA, Gro and CtBP elicit repression of activated and basal transcription. Both complex with histone deacetylases (Sundqvist *et al*., 1998; Chen *et al*., 1999), indicating that they suppress gene expression at least partly by influencing chromatin organization via histone deacetylation. CtBP probably also harbours a unique enzymatic activity (Nibu *et al*., 1998b; Poortinga *et al*., 1998). Whatever their precise molecular mode of action, the Gro (long-range) and CtBP (short-range) corepressors must be mediating repressor functions via separate mechanisms (Zhang and Levine, 1999). By acting with both, Brk uses multiple molecular means of repression, which should invigorate its regulatory activity.

Several *Drosophila* repressors have been shown genetically and molecularly to possess more than one discrete RD (e.g. Aronson *et al*., 1997; Keller *et al*., 2000; Kobayashi *et al*., 2001). What might be the biological significance of multiple RDs, or in the case of Brk two autonomous corepressor binding motifs, within a single negative transcriptional regulator? These domains could be acting jointly on each individual target gene, required both in tandem for full repressor competence (Kobayashi *et al*., 2001). Alternatively, each distinct RD could alone be mediating the full repression of a specific, distinguishable subset of targets. We have addressed this issue genetically, and find that Brk manifests full repression of some of its endogenous targets even in the absence of one corepressor or the other. Thus, for maintaining its negative autoregulation, Brk relies on the recruitment of either Gro or CtBP (Figure 6) whereas, for repressing *vgQ* (as well as *tld* and *pnr* in the embryo), Brk obligatorily rests on Gro, whether or not CtBP is present (Figures 5 and 7; Zhang *et al*., 2001). Importantly, for repressing *dpp* (and *Sxl*) in the embryo, Brk's two disparate corepressor recruitment domains are cooperatively required to promote maximal

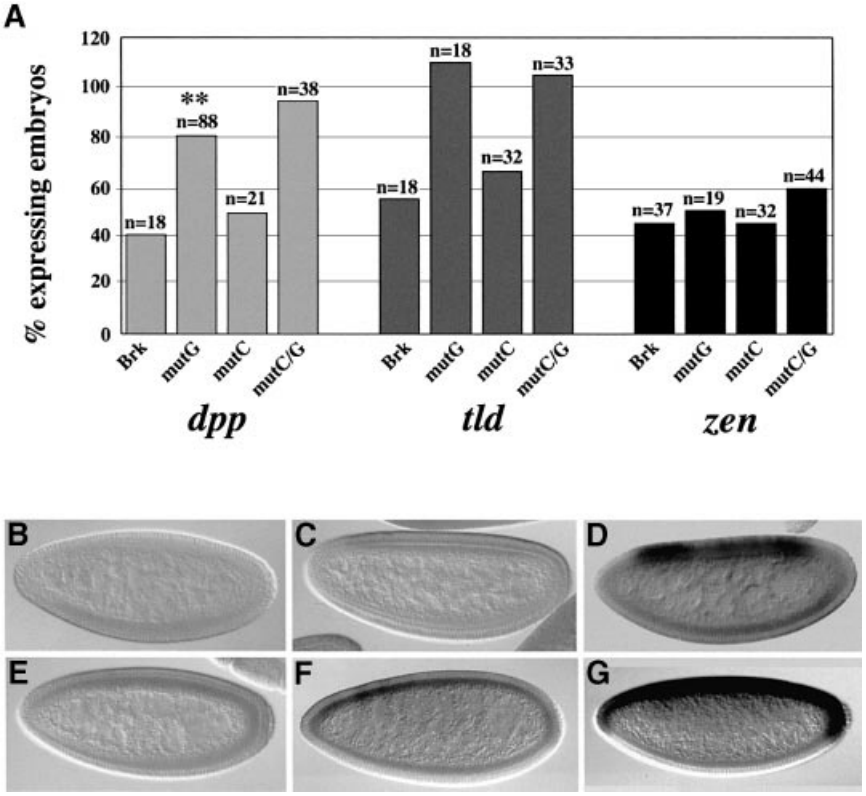

Fig. 7. Brk requires corepressors for its repressor function in the embryo. (**A**) Transgenic embryos overexpressing full-length Brk (from *UAS-brk* transgenes, driven by maternal Gal4^{VP16}), either in its native form or with its corepressor-binding domains mutated, were stained for the expression of *zen* (right), *tld* (centre) and *dpp* (left) during mid- to late-cellularization. The *Y*-axis designates the percentage of embryos expressing a given target gene, calculated relative to the number of expressing wild-type embryos, referred to as 100%. *n* = number of embryos, at the appropriate stage, that were scored. (B–G) Representative embryos. (**B**) UAS-Brk$^{mutC/G}$, stained for *zen*; (**C**) UAS-Brk^{mutC}, stained for *tld*; (**D**) UAS-Brk^{mutG}, stained for *tld*; (**E**) UAS-Brk^{mutC}, stained for *dpp*; (**F**) UAS-Brk^{mutG}, stained for *dpp*; (**G**) UAS-Brk$^{mutC/G}$, stained for *dpp*. Repression of *zen* is independent of corepressors (A and B) whereas that of *tld* is strictly Gro dependent (A, C and D). As for the silencing of *dpp*, Brk relies mainly on Gro (A and E–G). Nevertheless, since the level of *dpp* expression is significantly lower in Brk^{mutG}-expressing embryos (A, marked by '**'; F) when compared with wild-type embryos or with embryos expressing the Brk$^{mutC/G}$ transgene (G), CtBP must also be contributing to maximal Brk repressor ability.

repression (Figures 3 and 7). Thus, Brk requires Gro and CtBP differentially for repressing its endogenous target genes.

As a regulatory cofactor, CtBP is bifunctional in nature, acting either as a coactivator or as a corepressor (Phippen *et al*., 2000). In mediating Brk transcriptional silencing, CtBP seems to function as a dedicated corepressor; it can effectively mediate Brk autoregulation even when Gro is lacking (Figure 6), and it augments repression of some promoters for which Brk repressor activity is largely Gro dependent, such as *dpp* and *Sxl* (Figures 3 and 7). Notably, like Brk, the Hairy repressor also binds both CtBP and Gro; however, in this context CtBP plays an antagonistic role, suppressing Hairy's Gro-dependent repression (Zhang and Levine, 1999; Phippen *et al*., 2000). Given the Brk-independent reduction in *vgQ* expression observed in *CtBP*$^{-}$ clones (Figure 5B and C), we propose that, as with Hairy, in conjunction with some as yet unknown transcription factor CtBP participates as a coactivator in *vg* regulation.

Versatile employment of multiple repressor mechanisms by Brk in Dpp signalling

Gro and CtBP mediate gene silencing in qualitatively different ways (Zhang and Levine, 1999). Gro potentiates long-range repressors that function at a distance and that are able to block, in a dominant fashion, complex modular promoters consisting of multiple enhancer elements (Cai *et al*., 1996). In contrast, CtBP-dependent short-range repressors inhibit activators only locally, thereby permitting enhancer autonomy in a compound promoter (Gray *et al*., 1994; Nibu *et al*., 1998b). By virtue of its ability to recruit both Gro and CtBP, together with its capacity to outcompete pMad and other activators from binding DNA, Brk is competent to repress a multitude of complex Dpp target promoters, which receive positive inputs from manifold signalling pathways. We propose that, for promoters with low-affinity Mad-binding sites, the driving repressor force is direct competition between Brk and pMad for DNA binding, whereas for Dpp target promoters that contain high-affinity Mad-binding sites, corepressors are essential for mediating Brk repression. For this latter class of promoters, Brk relies on one or both of its cognate corepressors, depending on the particular promoter topology.

Brk utilizes a self-reliant mechanism, which need not depend on tethered corepressors, by competing with activators over coinciding DNA-binding sites (Figure 8; Sivasankaran *et al*., 2000; Kirkpatrick *et al*., 2001; Rushlow *et al*., 2001). We find that, in the absence of

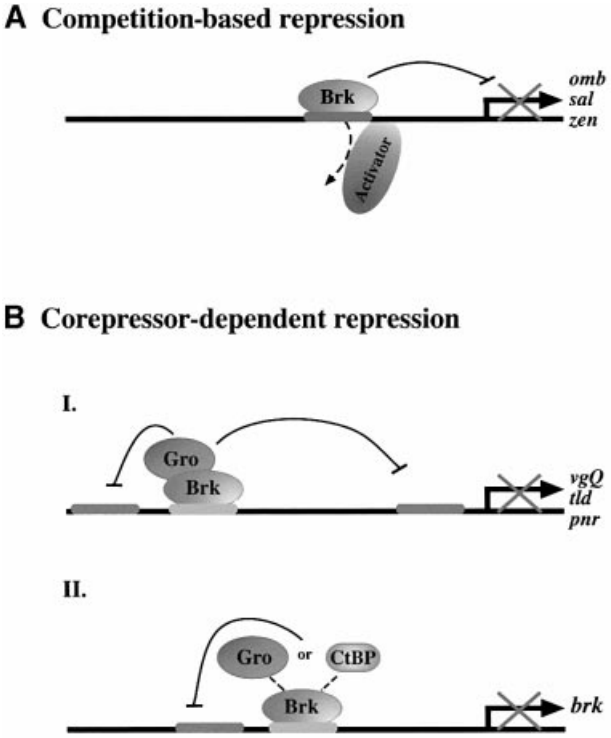

Fig. 8. Brk employs multiple repressor mechanisms in Dpp signalling. (**A**) Competition-based repression should suffice for promoters that bind Mad, or some other activator, at low affinity. Even for such promoters, recruitment of corepressors probably reinforces repression (see text). (**B**) For promoters with high activator-binding affinity, or when Brk-binding sites do not overlap with those of Mad, corepressors are employed to allow 'active' repression. Gro mediates dominant, long-range repression (B_I), whereas either Gro or CtBP act at short-range (B_{II}). Notably, Brk repression of *dpp* (and *Sxl*) is Gro dependent; however, for maximal repression CtBP is also required.

both Gro and CtBP, Brk represses not only *omb* and *zen*, but also *sal* (Figures 4 and 7; data not shown), suggesting that Brk-binding site(s) in the *sal* promoter overlap with those employed by activators. Transcription of both *sal* and *vgQ* requires activation by Mad (Kim *et al*., 1996; Nellen *et al*., 1996), yet, although both promoters are exposed to identical levels of pMad, the *sal* expression domain is spatially more restricted than that of *vgQ*, presumably because activation of *sal* requires higher levels of pMad than that of *vgQ*. Hence, 'passive' competition-based repression should efficiently block activation of *sal* but may not be sufficient for promoters like *vgQ*, which are activated even by low amounts of Mad. For silencing such promoters, alternative mechanisms such as recruitment of corepressors have evolved and are employed.

We find that Brk represses its distinct endogenous target genes by recruiting Gro and/or CtBP differentially (Figure 8). For the silencing of many target promoters, Gro alone is sufficient (*vg*, *tld* and *pnr*) but, for fully repressing others, Brk depends on both corepressors. Thus, in the case of *dpp* and *Sxl*, when CtBP is lacking, a decrease in Brk's overall repressor capacity is apparent and, in the absence of Gro, repression is almost completely impaired. Importantly, for negating its own transcription, Brk can utilize either corepressor.

The majority of activator and repressor binding sites in most Dpp-responsive enhancers have yet to be precisely mapped. We nevertheless propose that lengthy and complex promoters, which respond to several signalling inputs, will be found to be strictly silenced in a Gro-dependent manner. Thus, in repressing the *vgQ* enhancer, a composite *cis*-acting regulatory sequence with multiple elements that integrate information relayed by the *dpp*, *wingless* and EGF receptor signalling pathways, Brk is fully reliant on Gro, but not on CtBP. For other more simple promoters, short-range repression should be adequate and will be mediated by either corepressor, as exemplified by the robust Brk autoregulation, for which either Gro or CtBP is sufficient; CtBP and Gro are presumably interchangeable in this context, compensating for each other's absence.

Significantly, the overexpression of *gro* results in ectopic *omb* repression (Figure 1), suggesting that, even for promoters that are switched off in a 'passive', competitive manner, excess Gro can over-potentiate Brk-mediated negative transcriptional regulation. Thus, Gro and/or CtBP might reinforce Brk repression of those promoters on which it initially acts by competing with activators for binding to DNA (Sivasankaran *et al*., 2000; Kirkpatrick *et al*., 2001; Rushlow *et al*., 2001), via recruitment of histone deacetylases and alterations to chromatin structure, or by some other mechanism.

In summary, our data suggest that Brk uses multiple means to negate target gene expression, such as competition and the varied recruitment of long- and short-range corepressors. We propose that this versatility is, biologically, most significant given Brk's role in Dpp signalling, as it facilitates its negative regulation of diverse, complex Dpp target promoters.

Materials and methods

Fly culture

Flies were cultured and crossed on yeast–cornmeal–molasses–malt extract–agar medium at 25°C.

Generating clones

Clones of cells lacking functional *gro* (*gro*^{E48}), *CtBP* [*CtBP*$^{l(3)87De\text{-}10}$] or both were generated using Flp-mediated mitotic recombination (Xu and Rubin, 1993), and identified by the loss of πMyc and the concurrent appearance of a twin-spot. Marked clones of cells overexpressing *gro* were generated by crossing *UAS-gro* flies with *actin>CD2>Gal4* transgenic flies bearing an appropriate marker (Pignoni and Zipursky, 1997). After 2 days of egg laying, flies were removed and 2–3 days later larvae were heat-shocked (30 min at 34°C), dissected, fixed and stained.

In situ hybridization and antibody staining of Drosophila embryos

In situ hybridizations were performed as previously described (Goldstein *et al*., 1999). *Sxl* expression was monitored in three independent transgenic lines for each *hb-Hairy-Brk* variant, using the *Sxl-Pe:lacZ* reporter strain (Estes *et al*., 1995), and by staining with a monoclonal antibody specific to the active form of Sxl as described previously (Jiménez *et al*., 1997). Embryos were mounted in methacrylate (JB-4; Polyscience) and examined under Nomarski optics.

Calculating female-specific lethality

To establish whether female-specific lethality is caused by the expression of a given transgene, the Mantel–Haenszel test for association was used to determine whether the proportion of transgene-containing females differed from that of non-transgenic females. The expected proportion of females was calculated from the non-transgenic sibling group and female-specific lethality in a transgenic fly group was computed by the

following ratio: [(expected minus observed)/expected] female flies. *P*-values indicate that there is a significant association between the proportion of females and group (transgenic yes/no) in the *hb-Hairy-BrkRD* and *hb-Hairy-Brk^{RDmutC}* types, but not in the other two groups.

Plasmids

Molecular manipulations were conducted according to standard protocols. Constructs containing full-length Brk, or modified versions, were prepared by inserting fragments generated by standard PCR amplification into pBluescript (Stratagene) and, following sequencing, into appropriate vectors and sites. pGEX-Hairy and -HairyΔNot, pET-Gro and LexA-Gro have been described previously (Paroush *et al*., 1994). *hb-Hairy-BrkRD* and derivatives were constructed as described in Jiménez *et al*. (1997). The mutations in corepressor recruitment motifs were generated using Stratagene's QuikChange™ Site-Directed Mutagenesis Kit.

Yeast two-hybrid interaction assays

Yeast two-hybrid interaction assays were performed as described (Goldstein *et al*., 1999). *lacZ* reporter expression was analysed on appropriate indicator plates containing X-Gal.

In vitro GST pull-down assays

Pull-down assays were performed essentially as described (Goldstein *et al*., 1999), with the exception that for experiments using GST–CtBP, the pull-down buffer contained 20 mM HEPES pH 7.9, 0.2 M NaCl, 1 mM EDTA, 1 mM $MgCl_2$, 1 mM dithiothreitol, 0.5% NP-40 and 10% bovine serum albumin.

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