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Multiple modular promoter elements drive graded *brinker* expression in response to the Dpp morphogen gradient

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Morphogen gradients play fundamental roles in patterning and cell specification during development by eliciting differential transcriptional responses in target cells. In *Drosophila*, Decapentaplegic (Dpp), the BMP2/4 homolog, downregulates transcription of the nuclear repressor *brinker* (*brk*) in a concentration-dependent manner to generate an inverse graded distribution. Both Dpp and Brk are crucial for directing Dpp target gene expression in defined domains and the consequent execution of distinct developmental programs. Thus, determining the mechanism by which the *brk* promoter interprets the Dpp activity gradient is essential for understanding both Dpp-dependent patterning and how graded signaling activity can generate different responses through transcriptional repression. We have uncovered key features of the *brk* promoter that suggest it uses a complex enhancer logic not represented in current models. First, we find that the regulatory region contains multiple compact modules that can independently drive *brk*-like expression patterns. Second, each module contains binding sites for the Schnurri/Mad/Medea (SMM) complex, which mediates Dpp-dependent repression, linked to regions that direct activation. Third, the SMM repression complex acts through a distance-dependent mechanism that probably uses the canonical co-repressor C-terminal Binding Protein (CtBP). Finally, our data suggest that inputs from multiple regulatory modules are integrated to generate the final pattern. This unusual promoter organization may be necessary for *brk* to respond to the Dpp gradient in a precise and robust fashion.

KEY WORDS: Morphogen, Developmental patterning, Transcriptional regulation, *decapentaplegic*, *brinker*, *Drosophila*

INTRODUCTION

Dpp, the *Drosophila* homolog of vertebrate bone morphogenetic protein (BMP) 2/4, plays a vital role in patterning embryonic and larval structures. A gradient of BMP signaling is essential for specifying cell fates throughout the dorsal region of the embryo, while later in embryogenesis Dpp acts more locally to induce specific cell fates or tissues. During larval development, Dpp regulates growth and patterning in the imaginal discs. In the wing disc, a gradient of Dpp activity centered on the anteroposterior (A/P) compartment boundary controls cell fate, proliferation and survival. In adults, Dpp acts as a juxtacrine signal to maintain stem cell fates in the male and female germline (Parker et al., 2004; Raftery and Sutherland, 1999; Segal and Gelbart, 1985).

Dpp signaling is initiated by binding of the ligand to a complex of the type I and type II serine/threonine kinase receptors, Thickveins (Tkv) and Punt (Put), respectively. Activated Tkv phosphorylates the BMP-specific Smad Mothers against dpp (Mad), leading to its association with the co-Smad Medea (Med) and accumulation of the Mad/Med complex in the nucleus. Mad and Med-binding sites have been found in the promoters of many Dpp-responsive genes. However, two other transcription factors, Brinker (Brk) and Schnurri (Shn), also play essential roles in the regulation of most Dpp targets. Brk binds to the enhancers of Dpp target genes and functions as a constitutive repressor (Kirkpatrick et al., 2001; Rushlow et al., 2001; Saller and Bienz, 2001). Shn, a conserved protein with multiple zinc-finger DNA-binding domains, represses *brk* in regions where Dpp signaling is present (Marty et al., 2000; Torres-Vazquez et al., 2001). This repression is mediated by a Shn/Mad/Med (SMM) complex that antagonizes transcriptional activation by binding to a GRCGNC(N5)GTCTG motif (Gao and Laughon, 2006; Gao et al., 2005; Muller et al., 2003; Pyrowolakis et al., 2004). Thus, Dpp regulates its target genes through two mechanisms: directly by activating gene expression and indirectly by Shn-dependent repression of Brk.

Repression by Dpp results in an inverse gradient of Brk throughout development. Inputs from both Dpp and Brk are required for regulating growth and apoptosis, and for establishing the distinct thresholds that direct target gene expression in defined domains (Moser and Campbell, 2005; Muller et al., 2003). The contribution of *brk* to delimiting regions of gene expression has been best demonstrated for *spalt* (*sal*) and *optomotor blind* (*omb*), which are expressed in domains of high and low Dpp activity, respectively, and are differentially sensitive to repression by ectopic Brk (Jazwinska et al., 1999; Moser and Campbell, 2005; Muller et al., 2003). These essential roles for Brk underscore the importance of understanding the mechanism through which the Dpp gradient is interpreted to generate a graded *brk* expression pattern. The complex and dynamic expression pattern of *dpp* throughout development is mediated by multiple tissue and stage-specific enhancers distributed over ~50 kb (St Johnston et al., 1990; Stultz et al., 2006). By contrast, the similarly dynamic *brk* pattern results from two simple inputs – ubiquitous activation and spatially restricted Dpp-dependent repression mediated by the SMM complex (Muller et al., 2003). This work identified only a single region within the *brk* promoter that drives ubiquitous activation, and three repression elements that mapped as far as 3 kb away, suggesting a model in which SMM complexes act at long range to counteract activation.

Contrary to this model, we show that the *brk* promoter uses a much more intricate enhancer logic. We demonstrate that the 16 kb *brk* regulatory region harbors multiple modular elements along its length, each of which can independently drive a *brk*-like expression pattern. Analysis of individual modules reveals that they contain

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SMM sites closely linked to sequences that mediate activation. We show that the SMM complex represses adjacent activators through a distance-dependent mechanism that enables each module to respond autonomously to Dpp signaling. Thus, in the *brk* promoter, multiple SMM sites individually interpret the Dpp gradient and combined outputs from multiple modules generate the endogenous *brk* pattern. This unique architecture may be required to produce a robust and precise response to Dpp signaling.

MATERIALS AND METHODS

Drosophila strains and genetics

shn^{4738} and *brk*^{X47} have been described previously (Arora et al., 1995; Campbell and Tomlinson, 1999). *en-Gal4>UAS-TkvA* embryos were maintained at 18°C and shifted to 25°C at first instar. For *shn*^{4738} rescue, embryos received two 1-hour heat shocks at 37°C separated by 25°C rest periods (Yao et al., 2006). Homozygous mutants were identified by the absence of marked balancers. Embryo extracts from Myc-tagged UAS-ShnCT and UAS-ShnCT^{M} lines were used to monitor expression by probing western blots with anti-Myc antisera (9E10, Santa Cruz) and re-probing with anti-β-tubulin to confirm equivalent loading.

Promoter analysis and histochemistry

Fragments L3, L6, L7, L13, L13^{M3}, L13^{M4}, L13$^{M7/8/9}$ and L13$^{M3+M7/8/9}$ were cloned into C4PLZ (Wharton and Crews, 1993), while pCasPerhs43βgal (Thummel et al., 1988) was used for all other inserts. The eGFP-coding region, which does not block long-range repression (Barolo and Levine, 1997), was used as spacer DNA in L12+Spacer and multimerized module reporters. Multimerized modules were generated from L12 by sequential insertion of 180 bp spacers and additional modules in the same orientation with respect to the *lacZ* transcriptional start. The L12 SMM site lies at +97 bp from the 5′ end, and insertion of the 180 bp spacer ensures that each SMM site is separated by 768 bp. The end-points of fragments amplified to demonstrate the presence of modules in vivo are: module 3, –2608/-3188; module 4, –4864/5634; module 5, –6217/6990; module 7/8/9, –7798/8581; module 10, –13467/14212. Mutant constructs were generated using PCR and standard molecular techniques.

Embryos and imaginal discs were stained (Torres-Vazquez et al., 2000) or visualized by fluorescence microscopy using mouse anti-β-gal and goat anti-rabbit-Alexa488 antibodies (Molecular Probes). *omb-Gal4>UAS*-eGFP expression was visualized directly. Except in Fig. 5, reporter expression is from homozygous transgenic lines. Multiple lines were tested for each construct.

Identification of SMM motifs

Potential SMM sites in *brk* regions of *D. melanogaster, D. pseudoobscura* and *D. virilis* were identified by searching with MERmaid (http://opengenomics.org/mermaid) for close matches to the SMM consensus GRCGNC(N5)GTCTG (Pyrowolakis et al., 2004). *D. melanogaster* contains nine perfect matches and two sites that diverge at one nucleotide (#3 G**C**CGCC(N5)GTCTG at –3097 and #1 G**T**CGTC(N5)GTCTG at –271 bp). Reporters (L1 and L12) containing these and other divergent sites (Yao et al., 2006) are functional in vivo, suggesting a modified consensus of GNCKNC(N5)GTCTG (K=G/T). SMM sites matching this consensus were identified in the *Anopheles gambiae* promoter using Fly Enhancer (http://genomeenhancer.org/fly) (Markstein et al., 2002).

Biochemical assays

Co-repressor constructs for S2 cell expression were obtained from David Arnosti (dCtBP-2xFlag) and Albert Courey (Flag-Groucho). ShnCT sequences [residues 1892-2529 (Gao et al., 2005)] were subcloned into pAWM, containing an Actin5C promoter and C-terminal 6XMyc epitopes (a gift from T. D. Murphy). ShnCT^{M} was generated by PCR mutagenesis. Whole-cell extracts from S2 cells transfected with ShnCT or ShnCT^{M} and dCtBP or Gro were incubated with anti-Myc antisera and the immunoprecipitates run on 4-12% gradient SDS-polyacrylamide gels followed by visualization of interacting protein with anti-Flag antisera (M2, Sigma).

In vitro DNA binding assays

Gel shift and supershift assays were performed as described (Yao et al., 2006). In competition experiments, excess unlabelled probes were used at 100× (GST-Med) or 500× (GST-Mad). The wild-type sequence 5′-TTCAAACG**CAGACAGCGCGGCGG**AGCGTCGA-3′ contains an SMM site (bold) that was abolished in the mutant oligo 5′-TTCAAACG**actca**AGCGC**ttatta**AGCGTCGA-3′.

RESULTS

The *brk* promoter contains multiple activator elements

To analyze the cis-regulatory properties of the *brk* promoter, we generated transgenic β-gal reporter lines containing a series of partially overlapping fragments spanning ~12 kb upstream of the coding region (Fig. 1). Reporters derived from at least four separate regions directed expression in the wing disc in a pattern resembling endogenous *brk*, i.e. low to no expression medially where Dpp is transcribed along the AP boundary, and high levels laterally where Dpp signaling is absent (Campbell and Tomlinson, 1999; Jazwinska et al., 1999; Minami et al., 1999). These four regions correspond to fragments L1, L2/L3, L5/L6 and L6/L7 (Fig. 1A). The patterns driven by individual fragments, although similar, were not identical, and could be distinguished based on the level of expression and the extent of repression in the center of the disc. Fragment L1 drove expression at extremely low levels relative to a control *brk*^{X47} enhancer trap that mimics the endogenous *brk* expression pattern (Campbell and Tomlinson, 1999) (Fig. 1B,C). The adjacent overlapping fragments L2 and L3 resolved similar patterns but were transcribed at high levels compared with L1 (Fig. 1D, data not shown). Fragment L5 drove strong expression and was excluded from only a narrow region along the AP boundary (Fig. 1F). Reporters L6 and L7 differed in the width of their expression domains relative to *brk*^{X47} and each other (Fig. 1G,H); additionally, L6 was much weaker. Not all promoter regions drove patterned expression; two transgenes L4 and L8 were inactive (Fig. 1E,I). All fragments that directed *brk*-like patterns in the wing disc also drove expression in stage 11 and older embryos in regions where endogenous *brk* is detected, suggesting that the regulatory elements are not tissue or stage specific (Fig. 1J,K,M,N). Fragment L4, which was inert in the disc, showed no embryonic expression (Fig. 1L). Prior to stage 11, *brk* expression is directly activated by the Dorsal morphogen through sites contained in L8 (Markstein et al., 2002). Consistent with this, the L8 reporter that was inactive in the disc drove embryonic expression prior to stage 11 (data not shown).

The unexpected finding that several non-overlapping fragments drive patterned expression indicates that, at a minimum, the *brk* promoter contains four independent activator elements. These results are in striking contrast to a previous study (based on analysis of a nested deletion series), which concluded that the *brk* pattern results from a balance between activation mediated through a single region that maps between –5 and –6 kb (E1), and Dpp-dependent repression mediated through three ‘silencer’ elements (r) located 0.2 to 3 kb away (Muller et al., 2003) (Fig. 1A).

A compact *brk* promoter element contains closely linked but separable sites mediating activation and repression

We next chose to delineate one cis-regulatory unit and study its composition and mechanism of regulation. We focused on the region of overlap between fragments L2 and L3. Analysis of three additional overlapping fragments (L9-L11) identified a region

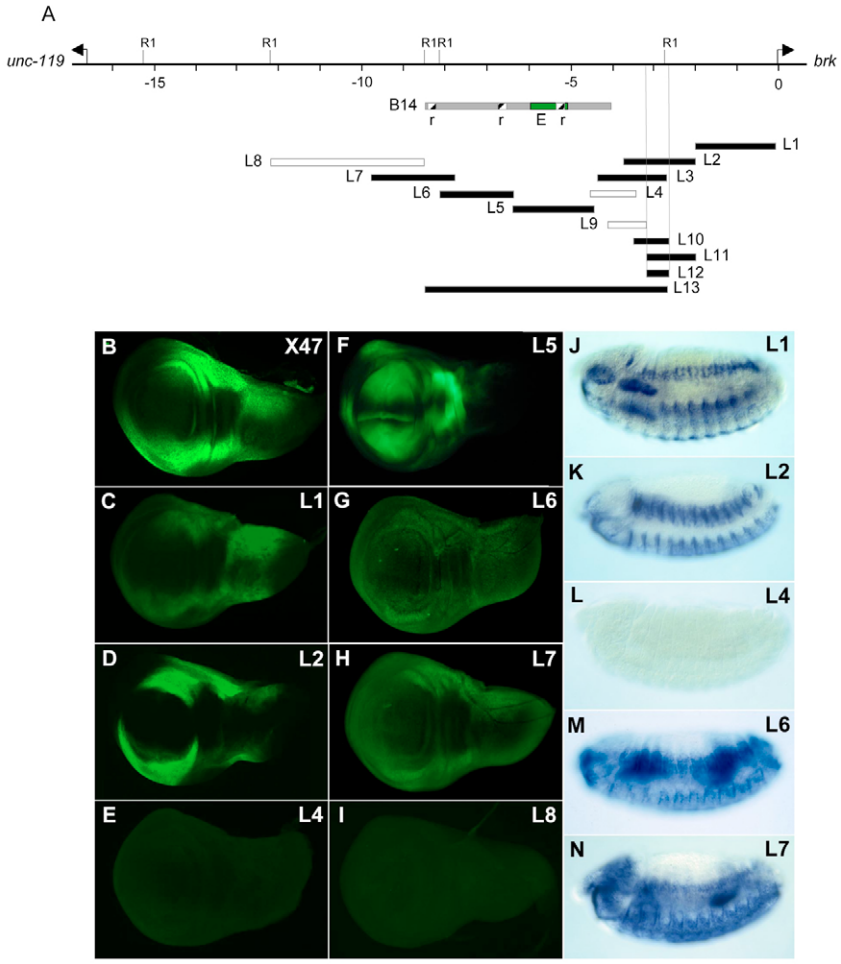

Fig. 1. The *brk* regulatory region contains multiple elements that mediate activation. (**A**) The *brk* promoter with arrows indicating transcription start sites for *brk* and *unc-119*. Scale is in kb and *Eco*RI sites (R1) are marked. Filled fragments drive expression in wing discs and embryos after stage 11; open fragments are transcriptionally inactive at these stages. The B14 construct of Muller et al. (Muller et al., 2003) is in gray, with 'r' signifying repressor elements and E the activator. (B-I) Reporter expression in wing discs oriented anterior upwards, ventral leftwards. (**B**) The brk^{X47} enhancer trap reproduces wild-type *brk* pattern. (**C**,**D**) L1 and L2 are non-overlapping promoter fragments that direct patterns resembling endogenous *brk*. L1 drives low-level expression and the image was enhanced by increased exposure time. (**E**) L4 does not display detectable expression. (**F**) L5 is expressed strongly and excluded from only a narrow central domain. The central stripe of expression corresponds to the AP compartment boundary, where pMad levels are reduced (Tanimoto et al., 2000). (**G**,**H**) L6 and L7 also drive laterally restricted expression. L6 directs expression at lower levels than L7, and was enhanced by increasing exposure time. (**I**) Expression of L8 cannot be detected. (J-N) Reporter expression in late stage 12/13 embryos, oriented laterally. (**J**,**K**) Both L1 and L2 reporters mimic *brk* expression in ventral and lateral stripes in the ectoderm. (**L**) No embryonic expression is detected with L4. (**M**,**N**) L6 and L7 reporters can be detected in a *brk*-like pattern in the ectoderm and midgut.

between the proximal end of L10 and the distal end of L11 that was crucial for expression (see Fig. 1A). A construct containing these 580 bases (L12) drove a *brk*-like expression pattern in both wing discs and embryos (Fig. 2A,B). To establish that the L12 reporter was Dpp responsive, we expressed a constitutively activated Tkv receptor (TkvA) in the posterior compartment of the wing disc using the *en-Gal4* driver. Ectopic activation of the Dpp pathway resulted in downregulation of reporter activity (Fig. 2C). Furthermore, the reporter was ubiquitously expressed in embryos mutant for *shn*, which is essential for Dpp-dependent repression (Fig. 2D). These results argue that the pattern driven by L12 is generated by repression of a ubiquitous activator in response to Dpp signaling.

The L12 reporter could contain either a composite activator/repressor element or separable activator and repressor sites that generate a *brk*-like pattern through a balance of their activities. Previous analysis of the *brk* promoter has shown that a GRCGNC(N5)GTCTG motif at –8.2 kb (silencer S) can assemble a Shn/Mad/Med complex and mediate transcriptional repression in response to Dpp signaling (Pyrowolakis et al., 2004). The GRCGNC sequence is bound by Mad while Med binds GTCTG, and the five-nucleotide spacer is crucial for recruitment of Shn to the complex (Gao et al., 2005; Pyrowolakis et al., 2004). We identified a single SMM site within L12 that diverges from the consensus at the second position (C rather than A/G, Fig. 3A). We tested whether this non-canonical motif in L12 could assemble a Shn/Mad/Med complex using gel-shift assays. Nuclear extracts from S2 cells transfected with Mad/Med or Shn/Mad/Med were incubated with oligos containing the L12 motif. Incubation with Mad/Med produced a slower migrating complex than was further retarded in the presence of Shn. Incubation with antibodies against epitope tags on Shn, Mad or Med resulted in supershifts, demonstrating that despite the divergent nucleotide, these proteins can form a complex at this site (Fig. 3B). We introduced mutations in the Mad and Med sites that disrupt binding by both proteins (Fig. 3C). A reporter containing the mutated Mad/Med sites ($L12^{M3}$) was ubiquitously expressed in both wing discs and embryos, demonstrating that the SMM motif in L12 is crucial for repression in vivo (Fig. 3D,E).

Next, we generated a series of constructs to localize the sequences required for activation. A fragment lacking 161 nucleotides from the 3′ end (L12-a) drove expression in a pattern similar to L12, indicating that all sequences necessary for resolving pattern are present within this minimal fragment (Fig. 3F, compare with Fig. 2A). By contrast, deletion of 119 nucleotides from the 5′ end (L12-b and L12-c) resulted in ubiquitous expression in discs and embryos, consistent with the elimination of the SMM site at –3.1 kb (Fig. 3G, data not shown). Thus, sequences mediating activation lie within the central 303 bp region. Furthermore, a 5′ fragment containing only 187 nucleotides (L12-d) also directed a *brk*-like pattern in the wing disc, indicating that it retains most of the sequences required for activator function (Fig. 3H). In conclusion, the L12 fragment contains a compact module in which separable but adjacent elements direct ubiquitous expression and Dpp-dependent repression.

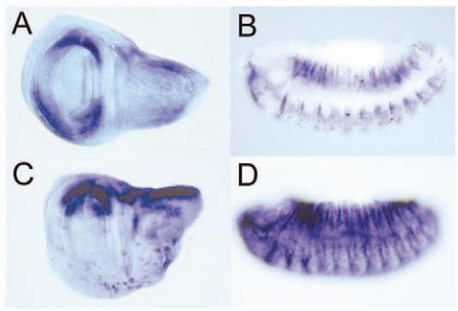

Fig. 2. A compact promoter fragment is sufficient to generate a *brk*-like pattern. (**A**,**B**) A 580 bp fragment (L12) located ~3 kb upstream of the transcription start drives expression in (A) wing discs and (B) embryos. (**C**) Upregulation of Dpp signaling by expression of TkvA using *en*-Gal4, results in repression of the L12 reporter in the posterior compartment. (**D**) L12 is derepressed in *shn*$^{-}$ embryos.

SMM-mediated repression is distance dependent

Transcriptional repression is thought to occur through at least four distinct mechanisms: inhibition of the basal machinery at the core promoter (silencing or direct repression), competition between activators and repressors for overlapping or shared binding sites (competition), recruitment of co-repressors that act over distances of 50-150 bases (short-range repression), or enlistment of a distinct set of co-repressors effective over distances of a kb or more (long-range repression) (reviewed by Arnosti, 2002; Courey and Jia, 2001). The initial characterization of the SMM site in silencer S suggested that it functions at long range to repress an activator located ~3 kb away (Muller et al., 2003). However, the proximity of sequences required for activation and repression in L12 suggested that repression by the SMM complex may be distance sensitive. To investigate this possibility, we increased the spacing between the SMM site and sequences necessary for activation, and examined whether reporter expression was altered. Insertion of a 380 bp neutral spacer adjacent to the SMM site in L12 resulted in widespread derepression throughout the wing pouch and the embryo (Fig. 3A,I; data not shown). These results indicate that the SMM complex uses a distance-sensitive, rather than a long-range, mechanism for repression.

The *brk* promoter contains multiple modular enhancers

If the SMM complex is effective only at repressing closely linked activators, we reasoned that other functional SMM sites should also be associated with activator-binding sites. As our promoter analysis detected multiple fragments capable of mediating activation independently (see Figs 1, 2), we searched the regulatory region to determine whether additional SMM motifs were located in transcriptionally active fragments. We found eleven sites (nine perfect matches and two that diverge at a single base) within 16 kb upstream of the transcription start site (Fig. 4; see Materials and methods). Remarkably, every fragment that drove a *brk*-like expression pattern (L1-L3, L5, L6 and L7) contained one or more SMM sites (see Fig. 1A). Conversely, fragments that lacked activator function (L4, L8 and L9) contained no SMM motifs, reinforcing the idea that repression and activation elements are closely linked. To test this hypothesis directly, we generated reporters containing selected SMM sites flanked by ~380 bp on either side, and examined whether they drove patterned expression (Fig. 4). We chose SMM sites #4, #5 and the #7/8/9 cluster, as regions containing these sites have been implicated in repression (Muller et al., 2003; Pyrowolakis et al., 2004) (see r sites in Fig. 1A); and site #10 because it is located near the *brk*^{X47} enhancer trap insertion at ~–14.0 kb (Campbell and Tomlinson, 1999). No activators have been mapped adjacent to site #5 and #7/8/9, providing a stringent test for the idea that SMM motifs and activation sequences are linked. Although site #4 is located within a 1 kb region (E1) required for activation (Muller et al., 2003), it has not been established whether the activator and SMM sequences are closely linked.

Strikingly, in all four cases, transgenic reporters containing SMM sites flanked by 380 bp on either side drove a 'lateral on/medial off' expression pattern in the wing disc (Fig. 4A-D). Thus, along with the enhancer elements identified in L12 (module 3; Fig. 4E), these data identify five separate examples where activator elements and SMM sites are closely linked to form compact regulatory modules. The expression patterns generated by individual modules share common features, but differ considerably in their level of expression and domain of repression. For example, module 4 shows only minimal repression along the AP boundary, compared with module 7/8/9, which contains three SMM sites and shows the broadest region of repression. Importantly, we find that the expression profiles of large fragments that contain a single module correspond closely with the pattern derived from the module itself (e.g. L5 and module 4, L7 and module 7/8/9; see Fig. 1). These similarities suggest that the crucial cis-elements responsible for the pattern are contained within the cognate module. In conclusion, these results establish that the *brk* promoter contains multiple discrete, compact regulatory modules, each of which can individually drive expression in a *brk*-like pattern.

The short-range co-repressor dCtBP contributes to SMM repression activity

We have shown that repression of adjacent activators in individual modules by the Shn/Mad/Med complex has a limited range. The ability of transcription factors to repress at short or long-range has been proposed to depend on their interaction with different classes of co-repressors. *Drosophila* C-terminal Binding Protein (dCtBP), a paradigmatic example of a short-range co-repressor, has been implicated in repression by the transcription factors Giant, Kruppel, Knirps and Snail (Arnosti et al., 1996; Hewitt et al., 1999; Keller et al., 2000). Analysis of the Shn sequence revealed that residues 1981-1985 (PMDLT; Fig. 5A) resemble the consensus CtBP interaction motif PX(D/N)LS (Aihara, 2006; Chinnadurai, 2002). This motif maps within a minimal Shn polypeptide (ShnCT, 1892-2529) that can complex with Mad/Med and repress *brk* transcription in vivo (Gao et al., 2005; Pyrowolakis et al., 2004). To determine whether Shn can recruit dCtBP, we tested its ability to form a complex in S2 cells. We observed a strong interaction between dCtBP and ShnCT (Fig. 5B). A mutated ShnCT^{M} protein, in which the PMDLT motif was replaced with Ala residues, failed to associate with dCtBP, demonstrating that this sequence is required for interaction. In control experiments, ShnCT failed to co-immunoprecipitate with the long-range co-repressor Groucho (Gro), consistent with the inability of the SMM complex to repress adjacent activators at long range (Fig. 5B).

We next examined the requirement for dCtBP in Shn-mediated *brk* repression in vivo. Ubiquitously expressed wild-type and mutant UAS-ShnCT transgenes were assayed for their ability to repress a *brk* reporter in *shn*$^{-}$ embryos. ShnCT repressed *brk-lacZ* in 74% of mutant embryos (Fig. 5C,D, n=140). By contrast, ShnCT^{M}, which lacks the dCtBP interaction motif, repressed *brk-lacZ* in only 16%

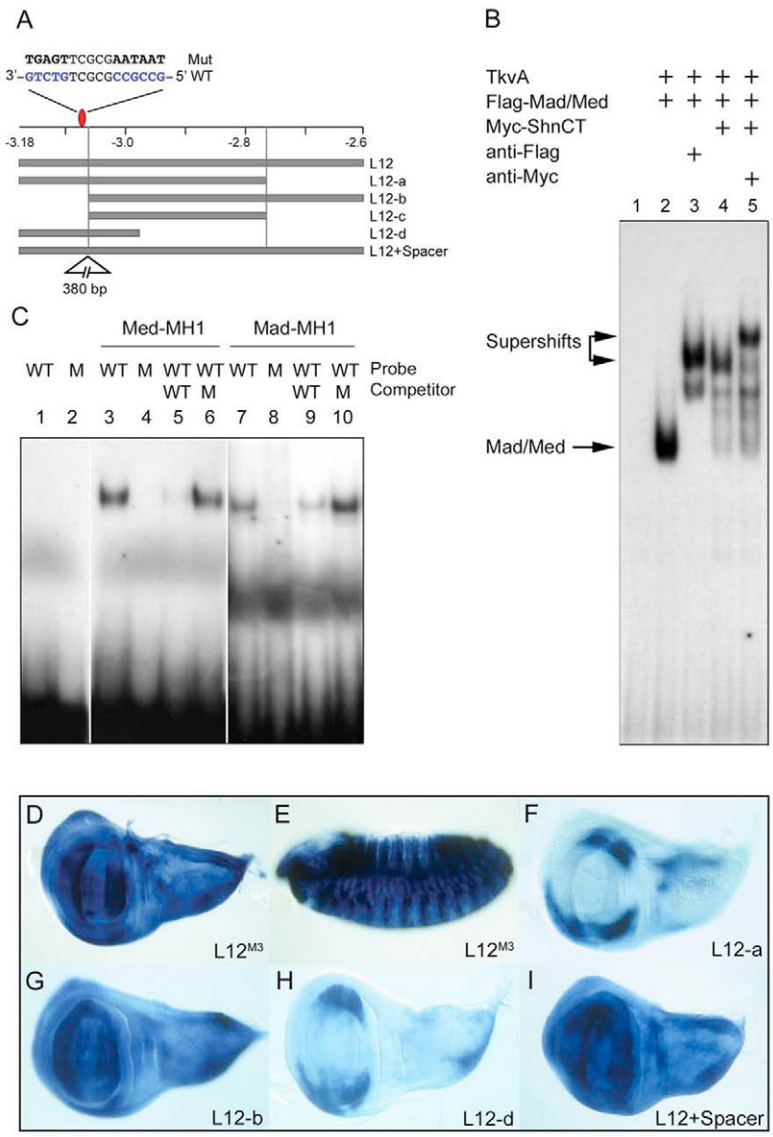

Fig. 3. A modular promoter element contains closely linked sites that mediate activation and Dpp-dependent repression. (**A**) Schematic showing L12 and derivatives. The red oval marks the SMM site containing C rather than A/G at position 2. The triangle in L12+Spacer marks the location of the insert. (**B**) Lysates from S2 cells transfected as indicated were used to gel-shift an oligo containing the SMM site. Lane 1 contains probe alone. The presence of Mad/Med results in a slower mobility complex (lane 2) that is further retarded by anti-Flag (lane 3) or Myc-ShnCT (lane 4). The Shn/Mad/Med complex is supershifted by incubation with anti-Myc (lane 5). (**C**) Wild-type (WT) or mutant (M) SMM oligos were incubated with GST-Mad or Med. Both proteins bind wild-type (lanes 3, 7) but not the mutant site (lanes 4, 8). Excess wild-type (lanes 5, 9), but not mutant, oligos (lanes 6, 10) block Mad/Med binding. $L12^{M}$ containing the mutant SMM site is ubiquitously expressed in (**D**) wing discs and (**E**) embryos. (F-I) Expression patterns of L12 derivatives in wing discs. (**F**) L12-a drives laterally restricted expression. (**G**) L12-b, which lacks the SMM site, is derepressed medially. (**H**) A 187 bp L12-d fragment drives *brk*-like expression, indicating the presence of closely linked SMM and activator sites. (**I**) Insertion of a 380 bp spacer between the SMM site and activator sequences (L12+Spacer) results in broad expression.

of *shn*$^{-}$ embryos (Fig. 5E, n=164). Equivalent levels of wild-type and mutant protein were detected on western blots of transgenic embryos (data not shown). Taken together, these data argue that interaction between Shn and dCtBP is biologically relevant, and that dCtBP contributes to the repressive function of the SMM complex in vivo.

The *brk* promoter integrates outputs from multiple modules

To gain insight into the logic of the multi-modular *brk* promoter architecture, we first examined the effects of changes in module copy number on the amplitude and spatial domain of reporter gene expression. Simplified promoters were constructed containing 1, 2 or 3 copies of module 3 (1×, 2× and 3×) with spacers to separate individual SMM sites by ~700 bp. The patterns driven by these *lacZ* reporters in wing discs were examined using *omb*>GFP expression to aid in comparison across samples (Fig. 6A-C). Confocal analysis of the intensity profile of the β-gal and GFP channels revealed that reporter expression levels in lateral regions of the disc increased with the number of modules. Interestingly, the domain of Dpp-dependent repression (i.e. sensitivity to Dpp activity) did not appear to change significantly with increased copy number, at least over a threefold range. These results suggest that one consequence of multiple modules in the endogenous *brk* promoter could be to help boost expression in lateral regions of the disc where Dpp signaling is absent.

The modules characterized in this study drive patterns that are similar but not identical to each other and to endogenous *brk*. To explore how these distinct patterns are integrated, we examined how a Dpp-insensitive (derepressed) module affects the transcriptional output of a larger promoter fragment that also contains several intact modules. We reasoned that if each module contributes independently to the final pattern, the mutant module lacking an SMM site should activate expression in the center of the disc, similar to the derepression it enables as an isolated module. For these experiments, we used the L13 fragment (–8.3 to –2.7 kb; see Fig. 1A) that contains at least four modules (modules 3, 4, 5 and 7/8/9). The wild-type L13 reporter drives expression in a pattern closely resembling endogenous *brk* (Fig. 7A). As shown previously, mutation of the lone SMM site in module-3 ($L12^{M3}$) resulted in loss of Dpp responsiveness and widespread activation throughout the wing pouch (Fig. 7B). We generated the identical lesion in L13. Surprisingly, the mutant reporter ($L13^{M3}$) showed no expression in the medial region of the wing disc where Dpp activity is high (Fig.

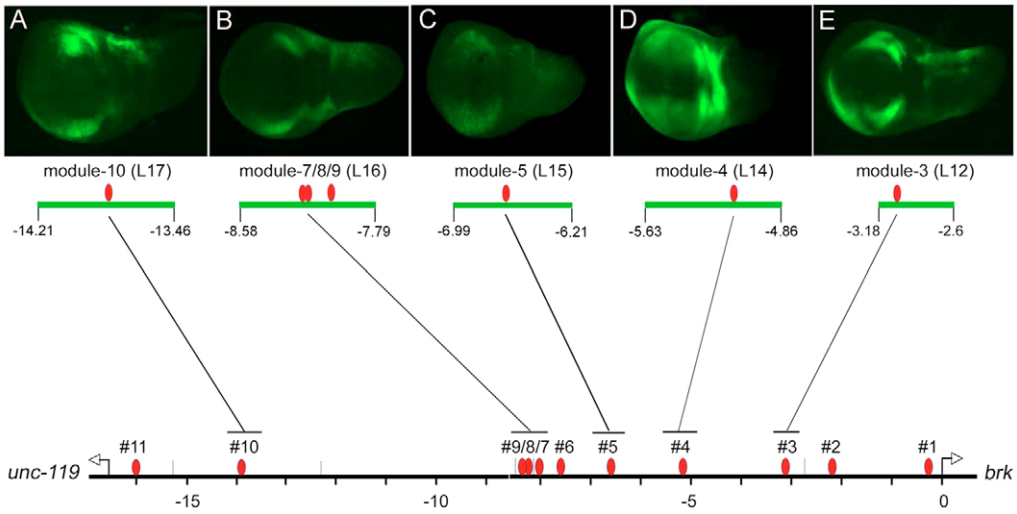

Fig. 4. The *brk* promoter contains multiple modular regulatory elements. (A-E) Transgenic reporters containing ~780 bp fragments (green bars), centered on SMM sites drive *brk*-like expression patterns in the wing disc. Red ovals mark the location of 11 predicted SMM sites (#1-11) within the *brk* promoter at –271, –2165, –3097, –5175, –6627, –7653, –8023, –8174, –8206, –13,833 and –15,983 bp. (**A-D**) Expression patterns derived from fragments L17, L16, L15 and L14 containing modules 10, 7/8/9, 5 and 4, respectively. Module 4 drives the highest levels of expression laterally. Module 5 drives weak expression (the image was enhanced by increasing exposure time). (**E**) Module 3 (L12) is shown for comparison.

7C). Double labeling with *omb*>GFP showed that the lateral domains of expression were slightly derepressed (see Fig. S1 in the supplementary material). The absence of medial derepression in $L13^{M3}$ is not due to the fortuitous presence of sequences immediately distal to L12, which act redundantly with the SMM site in module 3, as the same mutation in a larger fragment that contains 511 bp of additional sequence distal to the SMM site ($L10^{M3}$) also results in ubiquitous expression similar to $L12^{M3}$ (see Fig. S2 in the supplementary material). These results reveal that in a multi-modular promoter, the presence of wild-type Dpp-sensitive modules can significantly 'buffer' the impact of a mutant module in regions of high Dpp signaling.

To determine whether other Dpp-insensitive modules in the *brk* promoter are susceptible to buffering, we analyzed reporters in which SMM sites in two other modules (module-4 and module-7/8/9) were deleted in the context of the larger L13 fragment. We first examined the effects of the mutations in isolation ($L14^{M4}$ and $L16^{M7/8/9}$, respectively). In both cases, ubiquitous activation was observed throughout the central region of the disc (Fig. 7D,F). By contrast, the presence of a mutated module-4 in L13 ($L13^{M4}$) did not result in upregulation of expression in the center of the disc, similar to the buffering seen with $L13^{M3}$ (Fig. 7E). Likewise, the $L13^{M7/8/9}$ reporter was also repressed medially in regions of high Dpp signaling, although significant derepression was seen laterally (Fig.

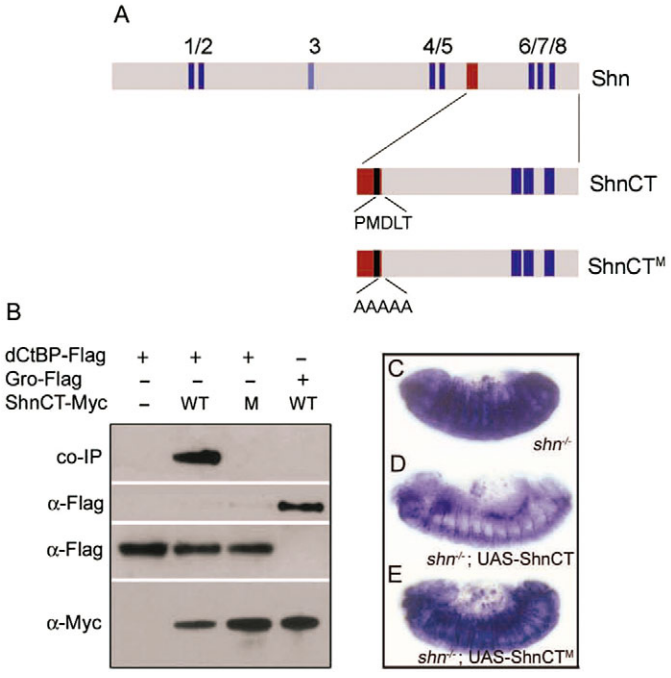

Fig. 5. Shn interacts with the short-range co-repressor dCtBP. (**A**) Full-length Shn and ShnCT, a polypeptide sufficient for Dpp-dependent repression of *brk*, are shown. Zinc-finger domains are marked in blue. A ~100 residue domain required for repression (red bar) includes a CtBP interaction motif PMDLT, which was mutated in $ShnCT^{M}$ as shown. (**B**) ShnCT interacts with dCtBP but not with Gro. Extracts from S2 cells transfected as indicated, were immunoprecipitated with anti-Myc and probed with anti-Flag. Expression levels were monitored by probing separate blots with anti-Flag or anti-Myc. Wild-type ShnCT bound dCtBP but not Gro, while $ShnCT^{M}$ failed to interact with dCtBP. (C-E) The CtBP interaction motif contributes to repression in vivo. (**C**) The brk^{X47} reporter is expressed ubiquitously in *shn*⁻ embryos. (**D**) In *shn*⁻ embryo, *Hsp70-Gal4*-driven expression of ShnCT restores *brk-lacZ* repression in the dorsolateral ectoderm and rescues dorsal closure defects. (**E**) $ShnCT^{M}$ is unable either to repress *brk-lacZ* or to rescue the *shn*⁻ morphology.

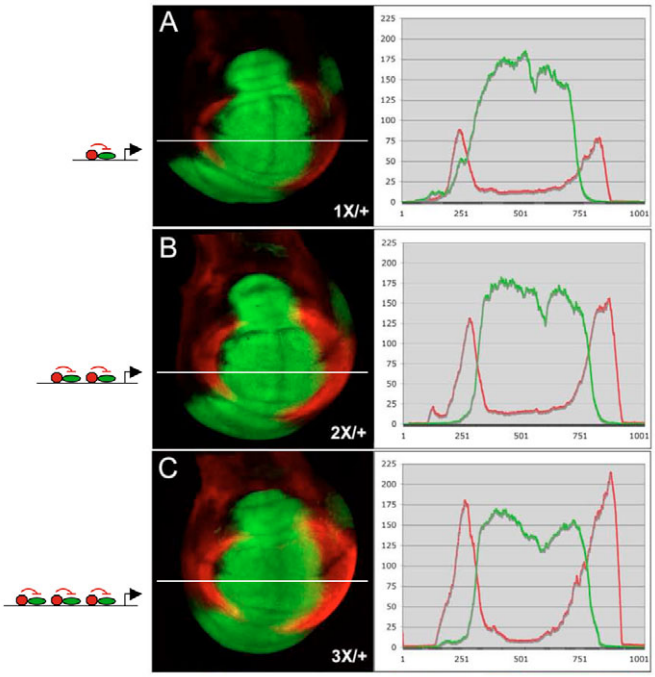

Fig. 6. The *brk* promoter integrates inputs from multiple modules. Confocal images of wing discs (dorsal upwards, anterior leftwards) showing reporter-*lacZ* (red) and *omb-Gal4>UAS-eGFP* expression (green). The module number and organization (green oval represents activator, red the SMM complex) is depicted schematically. Discs in A-C were stained in parallel and visualized using identical settings. Graphs show signal intensity in the red and green channels, measured at the white bar. (**A**) A 1×/+ reporter containing a single copy of module-3 (L12) was expressed in the wing pouch region lateral to *omb>GFP*. (**B**) The 2×/+ and (**C**) 3×/+ reporter drive increasingly higher levels of expression, but showed no significant overlap with *omb>GFP*.

7G). $L13^{M7/8/9}$, which lacked three SMM sites, showed more pronounced derepression compared with $L13^{M3}$ with a single mutant SMM site. We also assayed reporters in which more than one module was disrupted ($L13^{M3+M7/8/9}$). Remarkably, despite stronger derepression in lateral regions, expression of this transgene was still not detected near the AP boundary, indicating that the remaining Dpp-sensitive modules retained the ability to override the effects of the mutant modules, albeit in a narrow central domain (Fig. 7H).

Thus, although each module is capable of generating an expression pattern independently, the endogenous pattern does not result from mere superimposition of modular inputs. Instead, in the context of a multi-modular promoter, wild-type repressed modules appear to override the contribution of individual 'derepressed' modules thus buffering their effect in regions of high Dpp signaling.

DISCUSSION

The *brk* promoter contains multiple enhancer modules

The *brk* gene is unique in that eleven SMM sites are present in its regulatory region: no other locus in the genome has more than three sites. These sites are widely dispersed over 16 kb and separated from each other by 0.35 to 5.5 kb, with the exception of sites 7/8/9, which are clustered in a 183 bp region (see Fig. 4). We have shown that for seven of the eleven SMM sites (3, 4, 5, 7/8/9 and 10), sequences that mediate transcriptional activation are located within ~380 bp of the SMM sites. These SMM sites and linked activator sequences can independently generate *brk*-like expression patterns, suggesting that they function as autonomous modules. The fact that the L1 transgene, which contains a single SMM site (#1), also drives a *brk*-

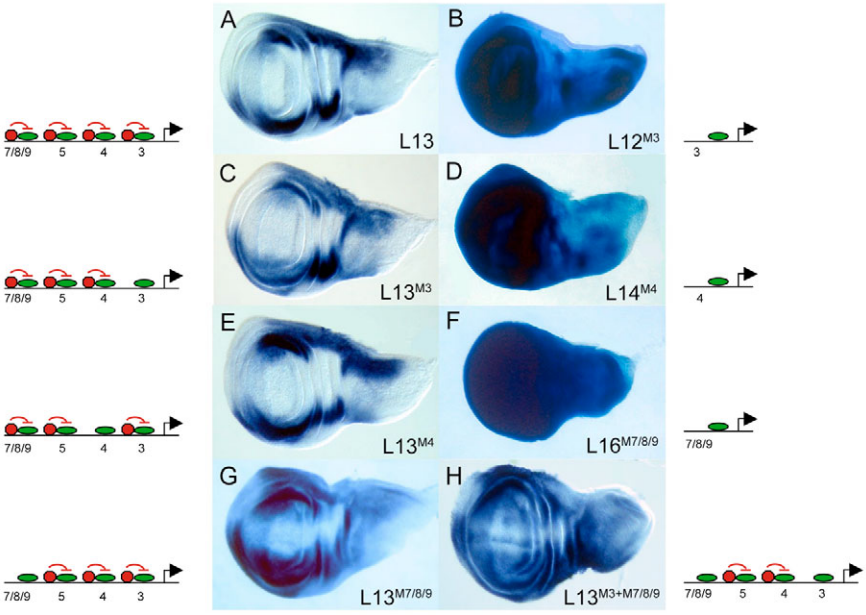

Fig. 7. The impact of Dpp-insensitive mutant modules is ameliorated in multimodular *brk* reporters. Wing discs stained for *lacZ* to visualize reporter expression. The module number and organization are depicted schematically. (**A**) Wild-type L13 reporter. (**B**,**D**,**F**) In fragments containing individual modules (see Fig. 4 for nomenclature; mutant modules indicated in superscript), disruption of SMM sites results in expression throughout the wing pouch. (**C**,**E**,**G**) By contrast, multimodular L13 reporters containing the same lesions do not show derepression in the medial region (compare with B,D,F, respectively). (**H**) $L13^{M3+M7/8/9}$, which contains two mutant modules shows significant expansion of lateral expression but continues to be repressed medially, where Dpp activity is highest. The narrow central stripe of expression corresponds to the AP boundary where pMad levels are lower (Tanimoto et al., 2000).

like pattern, strongly argues for a sixth module in addition to the five we have demonstrated (see Figs 1, 4). Thus, the 11 SMM sites in the *brk* regulatory region probably correspond to a total of 9 or 10 distinct modules, depending on whether the 7/8/9 cluster represents one or more modules. The evolutionary conservation of this unusual promoter organization provides additional support for its functional importance. Analysis of *brk* flanking regions in *D. pseudoobscura* and *D. virilis*, which are 30 and 40 million years distant from *D. melanogaster*, identified 12 and 11 SMM sites, respectively, arranged with a similar spacing relative to the basal promoter. Furthermore, 11 sites are found upstream of the *brk*-coding region in the mosquito *Anopheles gambiae*, which is separated from *Drosophila* by ~200 million years (L.Y. and R.W., unpublished).

Functional consequences of a modular promoter organization

How does the *brk* promoter read the pMad gradient and generate a complementary graded expression pattern, and what benefit could the presence of multiple modular enhancers confer in generating the Brk gradient? Our work, as well as earlier studies (Muller et al., 2003; Pyrowolakis et al., 2004), indicate that SMM sites act as sensors for Dpp signaling by binding a repressor complex that antagonizes broadly expressed activators in a dose-dependent manner. Our data that SMM-mediated repression has a limited range suggests that each module can autonomously generate an output representing the balance between activation and signaling-dependent repression within that module. The patterns produced by individual modules probably reflect variation in SMM site sequence and affinity, the distance between SMM and activator sites, activator site sequence and number, as well as whether sites for additional transcription factors are present.

The endogenous *brk* pattern does not appear to reflect the activity of a single 'dominant' module, but rather is a composite pattern resulting from integration of multiple modular inputs. This can be inferred from the fact that large promoter fragments containing more than one module (e.g. L2 and L6) drive patterns that resemble, but are not identical to, those of their constituent modules. Furthermore, the additive effect of module multimerization on expression levels in regions of low Dpp activity is also consistent with integration across modules (see Fig. 6). Finally, strong support for this idea comes from the buffering capacity of multimodular promoter fragments (see below).

A significant feature of the *brk* promoter is the remarkable ability of intact modules to override medial activation by mutant modules that are Dpp insensitive. This is apparent from our data that activators uncoupled from Dpp-dependent repression drive strikingly different expression patterns in isolation than they do in the context of larger fragments containing additional wild-type modules (see Fig. 7). Thus, disruption of the SMM sites in module 3 ($L12^{M3}$), module 4 ($L14^{M4}$) and module 7/8/9 ($L15^{M7/8/9}$) caused derepression throughout the center of the wing disc. However, the same mutations in a larger fragment containing several additional modules ($L13^{M3}$, $L13^{M4}$ and $L13^{M7/8/9}$) resulted in no derepression in the center of the disc. These results are inconsistent with a simple model in which only modules unbound by SMM complexes contribute to the transcriptional output of the promoter. If this were the case, in cells at the AP boundary, high levels of Dpp signaling would repress all intact modules in the $L13^{M}$ variants, leaving the constitutively active mutant module(s) free to interact with the transcriptional machinery. As a consequence, $L13^{M}$ variants would be expected to upregulate expression throughout the medial region of the disc. One potential explanation for the ability of wild-type modules to dampen expression from mutant modules could be that activators from SMM-repressed modules may compete disproportionately with activators from unrepressed modules for access to the transcriptional machinery, thus diluting the effect of the mutant modules. Alternatively, the SMM repressor complexes bound at multiple modules could act cooperatively (perhaps by modifying chromatin structure), thus reducing the output from adjacent mutant modules. In both cases, the absence of any expression in the medial region even with two Dpp-insensitive modules present (see Fig. 7H), argues that repressed modules make a significant contribution to the transcriptional output compared with the derepressed modules. Such an integrative mechanism also provides a framework for understanding how poorly resolved patterns like those generated by module 4 (see Fig. 4D), could be refined to generate the wild-type *brk* pattern. An important consequence of this promoter logic is that although individual SMM repression complexes act locally, modules in aggregate can, nevertheless, exert a long-range/global effect on promoter activity.

The specialized architecture of the *brk* promoter may provide a mechanism to respond to Dpp signaling in a uniquely precise and robust fashion. Multiple modules allow simultaneous parallel reads of the pMad gradient, thus increasing the precision with which the *brk* promoter detects Dpp morphogen levels. Integration would also be predicted to increase the fidelity of the *brk* promoter response by making it less sensitive to fluctuations at any individual module. This fidelity would be further enhanced by a disproportionate contribution from repressed as opposed to active modules. This buffering ability of the *brk* promoter is likely to be important in preventing stochastic fluctuations or transcriptional noise in wild-type animals (Arias and Hayward, 2006; Blake et al., 2003; Kaern et al., 2005), as well as in rendering *brk* transcription more resistant to mutational insults.

The *brk* promoter organization is distinct from other modular promoters

Several developmentally important genes have modular promoters consisting of multiple non-overlapping enhancers that function autonomously to generate a composite expression pattern. The segmentation gene *eve* provides an archetypal example, with five enhancers that drive expression in seven discrete stripes in the embryonic blastoderm (Fujioka et al., 1999; Goto et al., 1989; Harding et al., 1989). Although *brk* resembles *eve* in its modular promoter organization and the ability of individual modules to function independently, three key differences make *brk* unique. First, individual *eve* elements are bound by different combinations of activators and repressors, and thus drive expression in distinct stripes in the embryo. By contrast, individual *brk* modules respond to a common set of repressive cues and drive expression in largely overlapping domains. Second, in any given region of the embryo, the *eve* pattern represents the output of a single enhancer. By contrast, multiple *brk* modules are active in each cell and contribute collectively to the final expression pattern. A final crucial difference is that in *eve* short-range repression prevents crosstalk between enhancers that drive expression in different stripes, while in *brk* the outputs of modules that appear to respond autonomously to the Dpp gradient are integrated. Why do *brk* and *eve* cis-regulatory elements display different properties, even though both use the CtBP co-repressor? One potential explanation arises from the fact that CtBP functions as part of a complex that includes histone deacetylases, histone methylase/demethylases and SUMO E2/E3 ligases (Chinnadurai, 2007). CtBP complexed with SMM on the *brk* promoter may recruit a different subset of activities from a CtBP-gap gene complex on *eve* enhancers. In addition, the SMM complex

itself may recruit unique activities to the *brk* promoter. Furthermore, as the activators that mediate *brk* and *eve* expression are likely to be distinct, they may be affected by CtBP differentially.

Shn is likely to interact with additional co-repressors and co-activators

Two lines of evidence argue that SMM activity is distance dependent: Shn interacts directly with the co-repressor dCtBP, and there is a functional requirement for close linkage of SMM sites and activator sequences. Short-range repression appears to be a property of the SMM complex in other contexts as well, as an SMM site located ~89 bp from a germ cell-specific enhancer in the *bag of marbles* (*bam*) gene fails to mediate repression when this spacing is increased (Chen and McKearin, 2003). Furthermore, an SMM site and activator sequences are closely linked in a compact 514 bp Dpp-dependent enhancer in the *gooseberry* (*gsb*) promoter (Pyrowolakis et al., 2004). Loss of dCtBP binding strongly reduces repression by $ShnCT^{M}$, demonstrating that this interaction is relevant in vivo. However, $ShnCT^{M}$ still retains residual ability to repress *brk*-LacZ, and *brk* is not ectopically expressed in *dCtBP* clones in the wing disc (Hasson et al., 2001) (D. Bornemann and R.W., unpublished). This could indicate that the dCtBP interaction motif actually has a different function in vivo. Alternatively, Shn may employ redundant repression strategies, consistent with the current view that Shn proteins act as scaffolds for co-repressors, and indeed co-activators and other modulators, enabling the Smad complex to elicit different transcriptional responses dependent on cellular context (Jin et al., 2006; Yao et al., 2006).

The identity of the activator(s) targeted by the SMM repression complex remain to be determined, as do the precise sequences to which it binds. It is possible that different *brk* modules incorporate inputs from distinct activators, and that some of these activators are spatially or temporally restricted. In addition to inputs from the SMM complex and the activator, there is genetic evidence that *brk* negatively autoregulates its own expression, most prominently in the mediolateral regions of the wing disc (Hasson et al., 2001; Moser and Campbell, 2005). Consistent with this, the *brk* promoter contains multiple sites that match the Brk consensus (L.-C.Y. and R.W., unpublished) and may mediate autoregulation.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/135/12/2183/DC1

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