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The *Drosophila* Gene *brinker* Reveals a Novel Mechanism of Dpp Target Gene Regulation

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Summary

***decapentaplegic* (*dpp*), a *Drosophila* member of the TGFβ family of secreted molecules, functions as a long-range morphogen in patterning of the embryo and the adult appendages. Dpp signals via the SMAD proteins Mad and Medea. Here we show that in the absence of *brinker* (*brk*), Mad is not required for the activation of Dpp target genes that depend on low levels of Dpp. *brk* encodes a novel protein with features of a transcriptional repressor. *brk* itself is negatively regulated by Dpp. Dpp signaling might relieve *brk*'s repression of low-level target genes either by transcriptional repression of *brk* or by antagonizing a repressor function of *brk* at the target gene promoters.**

Introduction

Members of the transforming growth factor β (TGFβ) superfamily are secreted peptides that regulate a vast array of cellular processes in organisms as diverse as *Caenorhabditis*, *Drosophila*, and mouse (for review, see Hoodless and Wrana, 1998). All members of the TGFβ superfamily signal through a heteromeric receptor complex consisting of distinct type I and type II transmembrane serine/threonine kinase receptors. The activated type I receptor phosphorylates members of the SMAD class of proteins that translocate to the nucleus and function directly as transcriptional regulators (for review, see Massagué, 1998; Whitman, 1998). The simplicity of this pathway may allow tight coupling of external ligand concentration with transcriptional responses, making the pathway particularly useful in developmental contexts where the activating ligands fulfill morphogen functions. Indeed, members of two subgroups of the TGFβ superfamily, activins and bone morphogenetic proteins (BMPs), have been shown to act as morphogens in patterning of early vertebrate embryos (Green and Smith, 1990; Gurdon et al., 1994; Dosch et al., 1997; Wilson et al., 1997). Likewise, the best characterized *Drosophila* representative of the BMPs, *decapentaplegic* (*dpp*), forms long-range morphogen gradients that control dorsal–ventral (D–V) patterning in the embryo and anterior–posterior (A–P) patterning of the adult appendages (Ferguson and Anderson, 1992a; Wharton et al., 1993; Zecca et al., 1995).

For studying morphogen function at a molecular level, the *Drosophila* wing primordium has emerged as an excellent model system. Here, a Dpp gradient with peak levels in the center of the primordium provides positional information along the A–P axis of the later wing. In recent years, considerable knowledge has been gained on the formation of the gradient, the relation to its target genes, and on the way these target genes specify pattern elements of the adult wing. Gradient formation occurs by spreading of Dpp protein from a narrow stripe of *dpp* transcription that extends along the A–P compartment boundary (Lecuit et al., 1996; Nellen et al., 1996). Two target genes, *optomotor blind* (*omb*; Grimm and Pflugfelder, 1996) and *spalt* (*sal*; de Celis et al., 1996), are known that are activated in nested domains by distinct concentration thresholds of Dpp. In addition, the Dpp type I receptor *thick veins* (*tkv*) is not uniformly distributed along the A–P axis but has higher levels in lateral regions of the primordium (Haerry et al., 1998; Lecuit and Cohen, 1998). These high receptor levels both sensitize signal reception and limit the spreading of the ligand. The expression of the receptor itself is negatively regulated by Dpp, suggesting that a complex interplay between ligand and receptor levels shapes the gradient and affects target gene expression.

The function of the SMAD proteins that mediate between receptor activation and transcriptional response has also been studied in the wing primordium. On the basis of structural and biochemical studies mainly done on vertebrates, three classes of SMAD proteins can be distinguished (for review, see Heldin et al., 1997). At least one member of each of these three classes is also involved in Dpp signaling in the wing. *Mad* belongs to class I, comprising the receptor-regulated SMADs that are direct substrates of the activated type I receptor kinase. *Mad* is essential for all aspects of Dpp signaling, and consequently, *Mad* mutant clones completely abolish target gene expression in the wing disc (Raftery et al., 1995; Sekelsky et al., 1995; Lecuit et al., 1996; Das et al., 1998). Upon phosphorylation, the receptor-regulated SMADs form complexes with the co-SMADs (class II) and then translocate to the nucleus. The *Drosophila* representative of this class is *Medea* (Raftery et al., 1995; Das et al., 1998; Hudson et al., 1998; Wisotzkey et al., 1998). In the wing, *Medea* seems to be differentially required to potentiate Dpp responses, with regions more distant from the Dpp source being more sensitive to a loss of *Medea* (Wisotzkey et al., 1998). Finally, class III includes inhibitory SMADs that interfere with or antagonize the function of class I and II SMADs. They may act at the level of receptor interaction or at the level of complex formation with the co-SMADs (Hayashi et al., 1997; Nakao et al., 1997; Hata et al., 1998). *Dad* is such an inhibitory SMAD that downregulates Dpp signaling in the wing primordium (Tsuneizumi et al., 1997). *Dad* is

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induced by Dpp, so its inhibitory function is probably highest in regions of high Dpp activity. This negative feedback loop might modulate the duration and intensity of the signal.

So far little is known about how Mad–Medea complexes in the nucleus regulate *omb* and *sal*, the major target genes of Dpp in the wing. In other contexts, it has been well established that SMAD proteins directly bind to DNA recognition sites in the target gene promoters (Kim et al., 1997; Yingling et al., 1997; Dennler et al., 1998; Shi et al., 1998; Xu et al., 1998; Zawel et al., 1998). Dorsal mesoderm induction in *Drosophila* currently provides the best understood example. Here, the Dpp target gene *tinman* has binding sites for Mad and Medea in its enhancer region. However, as shown by a functional dissection of this enhancer region, correct *tinman* induction by Dpp requires the binding of both activators and repressors in the vicinity of SMAD proteins (Xu et al., 1998). A picture emerges in which the actual transcriptional response to SMAD activation is strongly dependent on the simultaneous binding and probable complex formation with other transcriptional regulators like the transcription factor Fast1 (Chen et al., 1996; Liu et al., 1997). In the wing disc, so far only one other such regulator has been identified that, like the SMADs, is required for target gene activation, namely the putative zinc finger transcription factor Schnurri (Arora et al., 1995; Grieder et al., 1995; Burke and Basler, 1996).

In this paper we present evidence that *omb* and *sal* expression in the wing disc occurs by antagonizing the repressive function of a new protein encoded by the *brinker* (*brk*) gene. *brk* is also required for D–V patterning in the embryo and may therefore play a more general role in Dpp target gene regulation. In the wing disc, *brk* is expressed in lateral regions distant from the A–P compartment boundary where it prevents the ectopic activation of *omb* and *sal*. However, while *omb* expression can be entirely explained by *dpp* antagonizing *brk*, *sal* needs additional Dpp inputs to attain its highest expression levels. Ectopic expression of target genes in *brk* mutant clones does not require functional Mad, indicating that *brk* is not just a modulator of Dpp signaling strength like the inhibitory SMAD proteins. We propose that high-level target genes are regulated in a different manner by Dpp than low-level target genes. The latter are not subject to direct activation by the pathway but rather are regulated by the relief of repression. We discuss why this mechanism could be important in the context of morphogen function.

Results

The Embryonic Phenotype of *brk* Indicates an Involvement in Dpp Signaling

Dpp in flies and BMP2/4 in vertebrates pattern the D–V axis of early embryos via an evolutionarily conserved mechanism (for review, see Ferguson, 1996). They promote the formation of nonneurogenic ectoderm and suppress that of the neurogenic ectoderm. In *Drosophila*, the nonneurogenic ectoderm gives rise to the dorsal epidermis of the larvae (DE in Figure 1A). By screening a collection of 3200 X-linked lethal mutations, one mutant was identified that showed a strong expansion of the dorsal epidermis and concomitant reduction of the ventral epidermis, indicating a defect in Dpp signaling (Figure 1B). This mutant was called *brk*. The *brk* phenotype is different from that of all other known *Drosophila* D–V patterning mutants (Arora and Nüsslein-Volhard, 1992). However, it resembles the phenotypes caused by ectopic expression of *dpp* or an activated version of the Dpp receptor *thick veins* (*tkv**) (Ferguson and Anderson, 1992b; Biehs et al., 1996; Nellen et al., 1996; Rushlow and Roth, 1996). This indicates that *brk* negatively affects either the distribution of Dpp, the reception of the Dpp signal, or the activation of Dpp target genes. Since the wing imaginal disc offers the simpler model system for studying Dpp function in flies, we addressed the question of *brk*'s role in the Dpp pathway by inducing homozygous *brk* mutant clones in the wing disc.

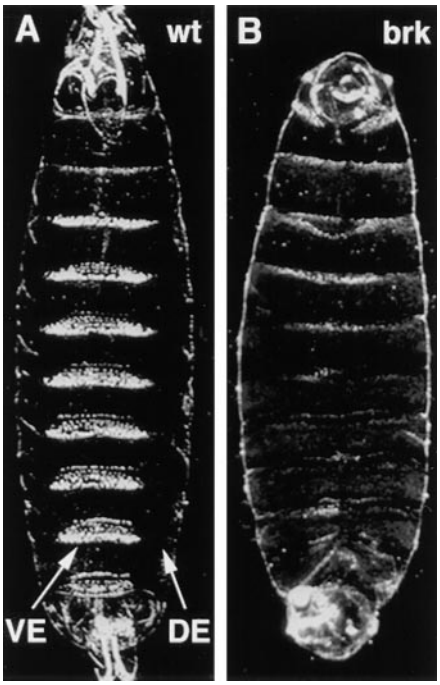

Figure 1. The Cuticle Secreted by *brk* Mutant Larvae

(A) Wild type (wt). (B) brk^{M68}/Y. Dark-field photographs showing ventral views of larval cuticle. Anterior is up. (A) wt with the strongly pigmented ventral denticle belts of the ventral epidermis (VE). The dorsal epidermis (DE) carries weakly pigmented dorsal hairs not visible at this magnification. (B) *brk* with expanded dorsal epidermis at the expense of ventral epidermis as can be seen by the almost complete absence of ventral denticle belts.

brk Acts as a *dpp* Antagonist in Wing Patterning

dpp is required for both cell proliferation and A–P patterning in wing imaginal discs (Burke and Basler, 1996; Lecuit et al., 1996; Nellen et al., 1996). Ectopic expression of *dpp* leads to outgrowths and pattern duplications in both the anterior and posterior compartments of the wing (Capdevila and Guerrero, 1994; Ingham and Fietz, 1995). These outgrowths and duplications are highly nonautonomous, that is, small patches of *dpp*-expressing cells have long-range organizing effects on the surrounding tissue (Zecca et al., 1995), reflecting the fact that Dpp spreads from the local source of its production into neighboring regions. *brk* mutant clones also cause

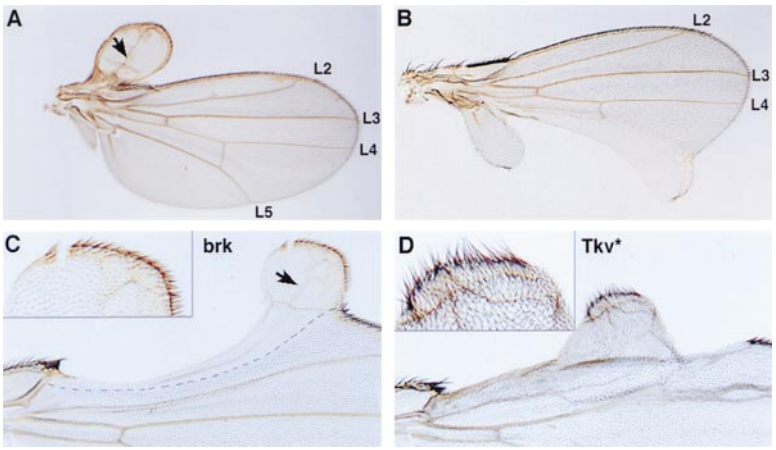

Figure 2. *brinker* Mutant Clones Affect Wing Morphology

(A) A *brk* clone in the proximal anterior part of the wing blade promotes an outgrowth composed exclusively of mutant cells marked with *yellow* and *forked*. It harbors anterior-most structures like the vein L2 (arrow) and a triple row of bristles at the margin. The numbering of longitudinal veins 2–5 is indicated along the wing margin.

(B) A *brk* clone in the posterior part of the wing blade causing both outgrowth and notching of the posterior wing margin. Formation of vein L5 is suppressed.

(C and D) Comparison of defects caused by *brk* clones (C) and clones expressing activated *tkv* (*Tkv**) (D). Notches and outgrowths are observed in both cases. Note the presence of an ectopic vein L2 inside the *brk* clone (arrow) that is absent in the *Tkv** flp-out clone. The insets show that the *brk* mutant clone carries a triple row of margin bristles, whereas the *Tkv** clone is surrounded by double row–type bristles.

outgrowths and pattern duplications in anterior and posterior regions of the wing, indicating that removal of *brk* leads to ectopic activation of the Dpp pathway (Figures 2A and 2B). However, the outgrowths are entirely cell autonomous, meaning that all cells belonging to the outgrowth are mutant for *brk*. This suggests that *brk* is not a negative regulator of *dpp* expression. Accordingly, *dpp* expression is not changed in third instar larval discs containing *brk* clones irrespective of their position within the disc (Figure 3A).

The effects of *brk* mutant clones are similar to those of an activated version of the Dpp receptor Thick veins (Tkv*; Figure 2D). Both are cell autonomous, and in both cases outgrowths are often accompanied by notches. This similarity indicates that loss of *brk* results in locally restricted pathway activation. However, a detailed comparison of the cuticular markers present in outgrowths induced by *brk*, as compared to those induced by Tkv*, suggests that loss of *brk* leads to a lower level of pathway activation than Tkv*. Some structural elements of the adult wing, including wing veins and the bristle types along the margin, can be correlated with certain levels of Dpp activity. In the anterior compartment, vein L2 and the triple row bristles of the margin depend on low levels of Dpp, while higher levels are required for double row margin bristles (de Celis, 1996; Singer et al., 1997). *brk* mutant clones close to the A–P compartment boundary in regions of peak levels of Dpp (posterior to vein L2 and anterior to the L4/L5 intervein region) have no phenotypic effects. Outgrowths, venation defects, and notches are observed if *brk* clones are located in regions more distant from the A–P boundary, corresponding to low Dpp levels (Figures 2A–2C). Anterior outgrowths frequently harbor a vein L2 recognized by corrugation at the ventral side of the wing, and their margins carry triple row bristles (Figures 2A and 2C). Thus, they are composed of structures dependent on low levels of Dpp. In contrast, outgrowths induced by ectopic Tkv* do not carry a vein L2, and their margins are occupied by an irregular array of double row bristles characteristic of high levels of Dpp activity (Figure 2D). This comparison indicates that removal of *brk* leads to the formation of structures corresponding to low or intermediate Dpp levels.

To investigate the developmental origin of these pattern rearrangements, we analyzed the expression of the Dpp target genes *omb* and *sal* in third instar larval imaginal discs that contained *brk* mutant clones. In wild-type discs, *omb* and *sal* are expressed in nested domains centered around the stripe of *dpp* expression; *omb* being activated by low levels of Dpp has the broader expression domain, and *sal* requiring higher levels has the narrower domain (Lecuit et al., 1996; Nellen et al., 1996; Figure 6I). *brk* clones located in the endogenous *omb* and *sal* regions had no visible effect on *omb* and *sal* expression, respectively. However, all clones outside the respective domains but within the wing pouch primordium showed strong ectopic *omb* and weak *sal* expression, both in a strictly cell-autonomous manner (Figures 3B, 3C, and 3E). If *brk* mutant clones started at the endogenous *sal* domain and extended laterally, *sal* expression within the clone was often observed to decline continuously with increasing distance from the A–P compartment boundary (Figure 3E). In contrast, patches of cells expressing Tkv* always showed high levels of *sal* expression (Figure 3D). This again indicates that loss of *brk* does not result in maximal activation of the Dpp pathway. In summary, loss of *brk* in the wing disc leads to an activation of target genes dependent on low or intermediate levels of Dpp signaling and to an expansion of corresponding fates. However, fates depending on highest Dpp levels are not affected. Thus, *brk* function is most important in regions where the Dpp gradient has diminishing levels or where a further spreading of Dpp signaling has to be prevented.

omb and *sal* Expression in *brk* Clones Is Independent of *dpp*, *tkv*, and *Mad*

The cell autonomy of *brk* clones suggests that *brk* acts as an intracellular negative regulator of the pathway. As such, *brk* could be a negative modulator of signaling strength. In that case, the *brk* phenotype would result

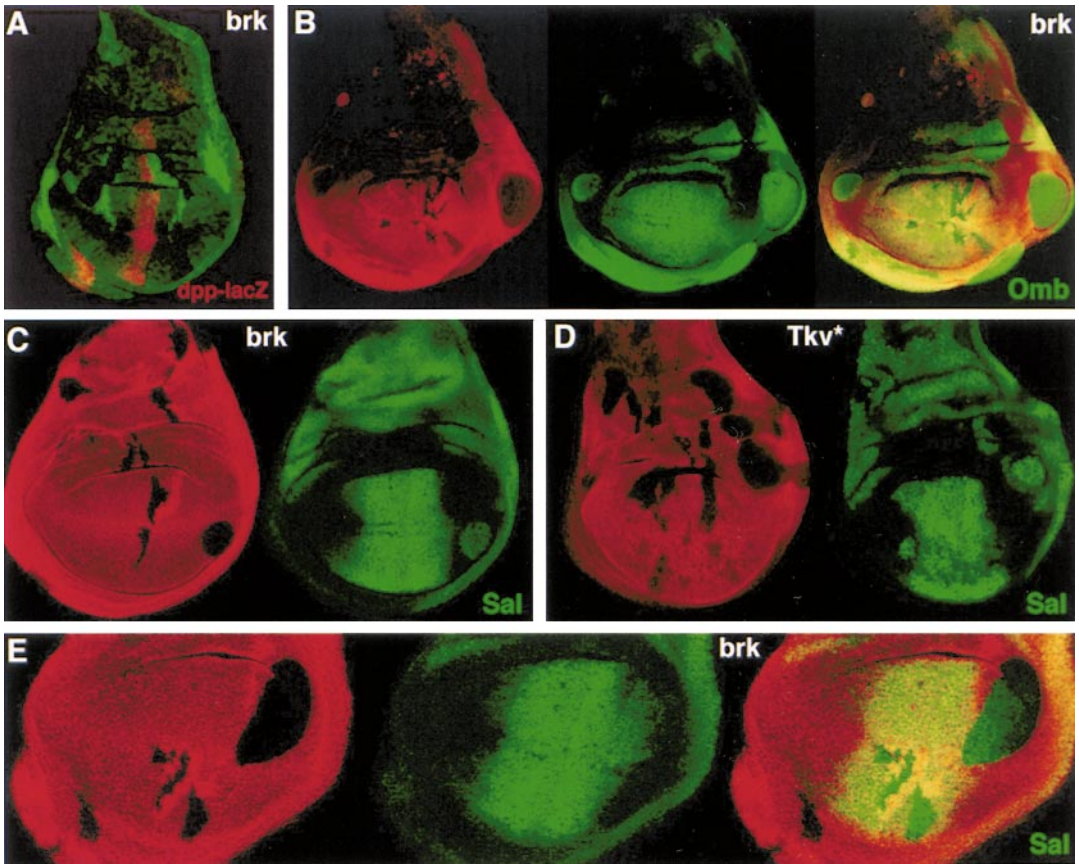

Figure 3. *brk* Acts as a Dpp Antagonist

(A) *brk* clones in a wing disc marked by the absence of N-Myc (green). *dpp* expression shown by staining for *dpp-lacZ* (red) is not affected. (B–E) Expression of Dpp target genes in *brk* clones or *Tkv** flp-out clones marked by the absence of *arm-lacZ* (red). Omb protein (B) and Sal protein (C and D) are shown in green. *omb* and *sal* are ectopically induced in a cell-autonomous manner in *brk* clones (B, C, and E) or in Tkv*-expressing cells (D). However, note the weak level of *sal* expression in *brk* mutant clones compared to *sal* expression in its endogenous domain and in *Tkv** flp-out clones. (E) *sal* expression in a large *brk* clone that starts at the endogenous *sal* domain and extends posteriorly. While the neighboring wild-type cells have a sharp border of *sal* expression, the *brk* mutant cells show a continuous decline of *sal* expression from the endogenous domain toward the edge of the wing pouch.

from amplification of residual levels of pathway activity. Alternatively, *brk* might act completely independently of pathway activation. In this case, the phenotype would be expressed even in the absence of pathway components. Furthermore, *brk* could act as an antagonist either at the receptor level, at the level of the SMAD proteins, or directly at the level of target gene promoters. To genetically address these questions, we constructed double mutants of *brk* with mutations in pathway components and assayed their effects on the expression of the Dpp targets *omb* and *sal*.

To test whether ectopic expression of *omb* and *sal* in *brk* clones requires Dpp ligands, we used a *trans*-heterozygous combination of two hypomorphic *dpp* alleles that leads to rudimentary wings (Figure 4A) and elimination of *omb* expression in the wing blade primordium (Figure 4C). Induction of *brk* clones in this background leads to *omb* (Figure 4D) and *sal* expression (data not shown) and to small outgrowths composed of wing blade material (Figure 4B), demonstrating that target gene activation in *brk* mutant cells occurs in the absence of normal Dpp levels.

tkv and *Mad* have been demonstrated to be required in a cell-autonomous manner for the expression of *omb* and *sal* (Burke and Basler, 1996; Lecuit et al., 1996; Nellen et al., 1996). To test whether this requirement is maintained in a *brk* mutant background, double mutant clones were induced (see Experimental Procedures). Both *brk*; *tkv* and *brk*; *Mad* double mutant clones express high levels of *omb* (data not shown) and low levels of *sal*, similar to that observed in *brk* single mutant clones (Figures 4E and 4F). In the case of *sal*, the expression level in double mutant clones is low even if the clone is located in the endogenous *sal* domain (see inset in Figure 4E). Thus, removal of *brk* leads only to a certain level of *sal* expression, and the higher levels normally seen in the *sal* domain must reflect some additional signaling input from the Dpp pathway. These data indicate that in the absence of *brk*, neither *tkv* nor *Mad* are required for *omb* and low-level *sal* expression. Since for both *tkv* and *Mad* we used null alleles (tkv^{a12}: Nellen et al., 1994; Terracol and Lengyel, 1994; Mad^{12}: Raftery et al., 1995; Das et al., 1998), we conclude that removal of *brk* leads to Dpp target gene activation by a mechanism independent of pathway activity. The fact that *brk* acts downstream or in parallel to Mad suggests that *brk* itself

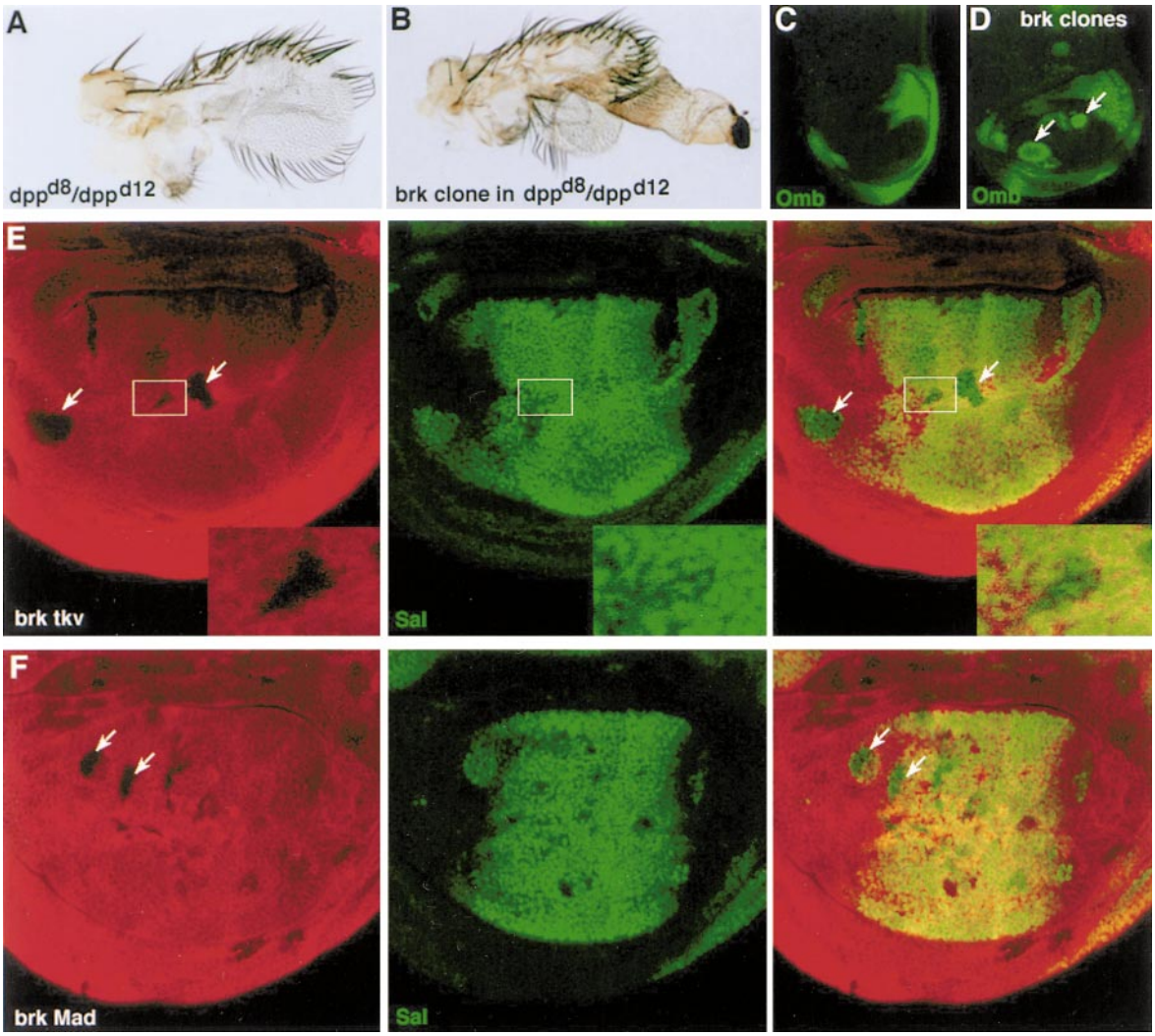

Figure 4. *brk* Is Epistatic to Components of the Dpp Signaling Pathway

(A) Rudimentary adult wing from dpp^{d8}/dpp^{d12} flies. In the strongly reduced wing discs of this genotype (C), *omb* is absent in the wing pouch primordium. (B and D) *brk* mutant clones induced in this background lead to outgrowth of wing material (B) and ectopic *omb* expression (D, arrows). (E and F) Double labeling for Sal (green) and *arm-lacZ* (red). The discs carry single and double mutant clones; however, only double mutant clones completely lack *arm-lacZ*. See Experimental Procedures for genotypes. (E) *brk*; *tkv* double mutant clones using an amorphic *tkv* allele (tkv^{a12}; arrows) express reduced levels of *sal* irrespective of whether they are outside or within the normal *sal* domain (see inset). (F) *brk*; *Mad* double mutant clones (arrows) using an amorphic *Mad* allele (Mad^{12}) also show reduced levels of *sal* expression.

may act at the transcriptional level. Target gene activation by Dpp would then be accomplished by inhibiting *brk*'s repressor function.

brk Encodes a Novel Protein

We began the cloning of the *brk* region of the X chromosome by isolating P element–induced lethal mutations of *brk* (see Experimental Procedures). Plasmid excision and subsequent isolation and sequencing of cDNAs identified a single open reading frame of 704 amino acids (schematized in Figure 5A and listed in 5B). The predicted protein sequence contains several regions of repeated amino acids. There are three glutamine-rich regions, an alanine- and histidine-rich region, a serine-rich region, and one run of eight histidines. Blast database searches that filtered out these repeats failed to identify significant homology to any protein in the data banks. However, searching specifically with the N-terminal 100 amino acids of Brk revealed a weak homology between amino acids 44–99 (yellow box in Figure 5A) and the homeobox domains from several proteins (listed in Figure 5C). The predicted secondary structure of this region of Brk contains two alpha helices, one from residues 10–16 and another from residues 31–56, and a less structured region in between them. Noticeably, the conserved amino acids (R-43, Q-44, W-48, Q-50), which are part of the DNA recognition helix in the homeodomain (Gehring et al., 1994), are also present in the C-terminal alpha helix in this region of Brk. Although speculative, this suggests that Brk may bind to DNA.

The sequence PMDLSLG at 377–383 is similar to a motif (P-DLS-K) present in several proteins known to act as transcriptional repressors that were shown to interact with the corepressor CtBP (Nibu et al., 1998).

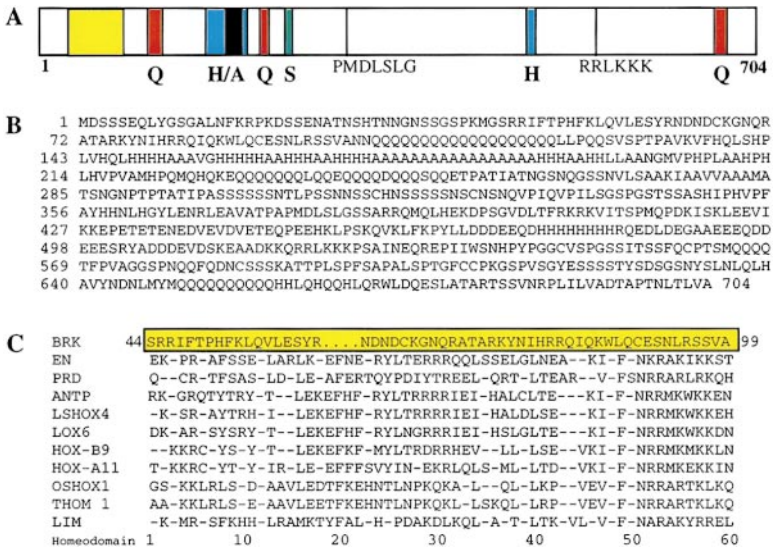

Figure 5. *brk* Encodes a Novel Protein

(A) Schematic representation of the Brk protein, highlighting several regions of repetitive amino acids (aa). NLS indicates the position of the putative nuclear localization signal (aa 517–525), and PMDLSLG marks a putative repression domain (aa 377–383). The specific type of repeat is listed below the colored boxes. The yellow box in the N-terminal region represents a region that shows weak homology to the homeodomain (see below).

(B) The predicted 704 aa sequence of Brk.

(C) The top line (in yellow) represents a 56-amino acid stretch (aa 44–99 of Brk-yellow box in A) that shows weak homology to the homeodomains of several proteins. Below Brk is listed the Antennapedia (ANTP) homeodomain, which did not come through in the search (15% identity), and the homeodomains from seven proteins (21%–23% identities).

In addition, there is a short stretch of basic amino acids (KKQRRLKKK) at position 517–525 that is reminiscent of the nuclear localization signal of SV40 large T antigen (Kalderon et al., 1984). The presence of these basic residues and the putative DNA-binding domain and transcriptional repression domain, together with the genetic data that Brk inhibits the pathway downstream of the SMADs, suggests that Brk functions as a repressor of Dpp target genes.

DNA sequencing of the brk^{M68} EMS-induced null allele (Experimental Procedures) identified the lesion to be a G-to-A transition that changes the 87th codon (TGG=W) to a stop codon (TAG). Thus, the brk^{M68} truncated protein contains only about 10% of the normal Brk amino acid sequence. This observation, along with the fact that the brk^{M12} P-induced mutation can be reverted to wild type (see Experimental Procedures), provides evidence that we have indeed cloned the *brk* gene.

brk Expression Is Negatively Controlled by Dpp

Interestingly, *brk* expression in the imaginal discs is not uniform but shows complementarity to regions of Dpp signaling. In wing discs, *brk* is highly expressed in lateral regions that are distant from the Dpp source in the center of the disc (Figure 6A). In leg discs, *brk* expression is lowest in the dorsal compartment, which is specified by high levels of Dpp signaling (Lecuit and Cohen, 1997; Figure 6B). Double stainings for *brk-lacZ* and Omb protein demonstrate the complementarity between high levels of *brk* transcription and the expression of a low-threshold target gene of Dpp in wing and leg imaginal discs (Figures 6C and 6D). They also reveal a narrow zone of overlap between low *brk* levels and *omb* expression in the wing pouch (yellow region in Figure 6C), suggesting that *brk* expression extends into regions of low-level Dpp signaling. In this region of overlap between Omb and *brk*, *brk* levels are declining in a graded fashion and become undetectable at positions where Sal expression starts (Figure 6E). The complementarity between *brk* expression and regions of Dpp signaling may reflect a negative regulation of *brk* by Dpp. Consistent with this view, clones of mutant cells missing the Dpp receptor Tkv express high levels of *brk* irrespective of their location within the wing pouch (Figures 6E and 6F). Thus, *brk* expression would occur evenly throughout the wing pouch in the absence of a Dpp gradient emanating from the center of the disc. An important function of Dpp signaling in the wing disc might be to generate the asymmetric distribution of a repressor (such as *brk*) of its target genes.

Ectopic Expression of *brk* Blocks Normal Dpp Function

To test whether the spatial regulation of *brk* is indeed important for normal Dpp function, we ectopically expressed *brk* in the center of the wing pouch. To this end, we used the GAL4/UAS system (Brand and Perrimon, 1993) to express *brk* under the control of the *omb* promoter (Lecuit et al., 1996). We confirmed that this misexpression did not interfere with the transcription of endogenous *dpp* (date not shown). Despite this, *brk* expression in the *omb* domain caused a strong reduction of wing size (Figures 6G and 6H); the corresponding third instar larval discs do not have the normal folded morphology and contain fewer cells than wild-type discs. *omb* and *sal* expression are strongly reduced in the wing pouch region of such discs and can be seen only in some centrally located scattered groups of cells (Figures 6I and 6J). Although we cannot explain the stochastic aspect of the *omb* and *sal* pattern in these discs, ectopic *brk* expression clearly leads to the repression of both Dpp target genes in most of the cells in which they would normally be expressed. Interestingly, *brk* affects *omb* and *sal* expression even in regions of high Dpp signaling close to the compartment boundary. These data suggest that *brk* expression is a powerful antagonist of Dpp signaling and must be tightly controlled to avoid interference with normal Dpp function.

Discussion

The experiments described in this paper and the accompanying paper by Campbell and Tomlinson (1999; this issue of *Cell*) provide evidence for a new regulatory

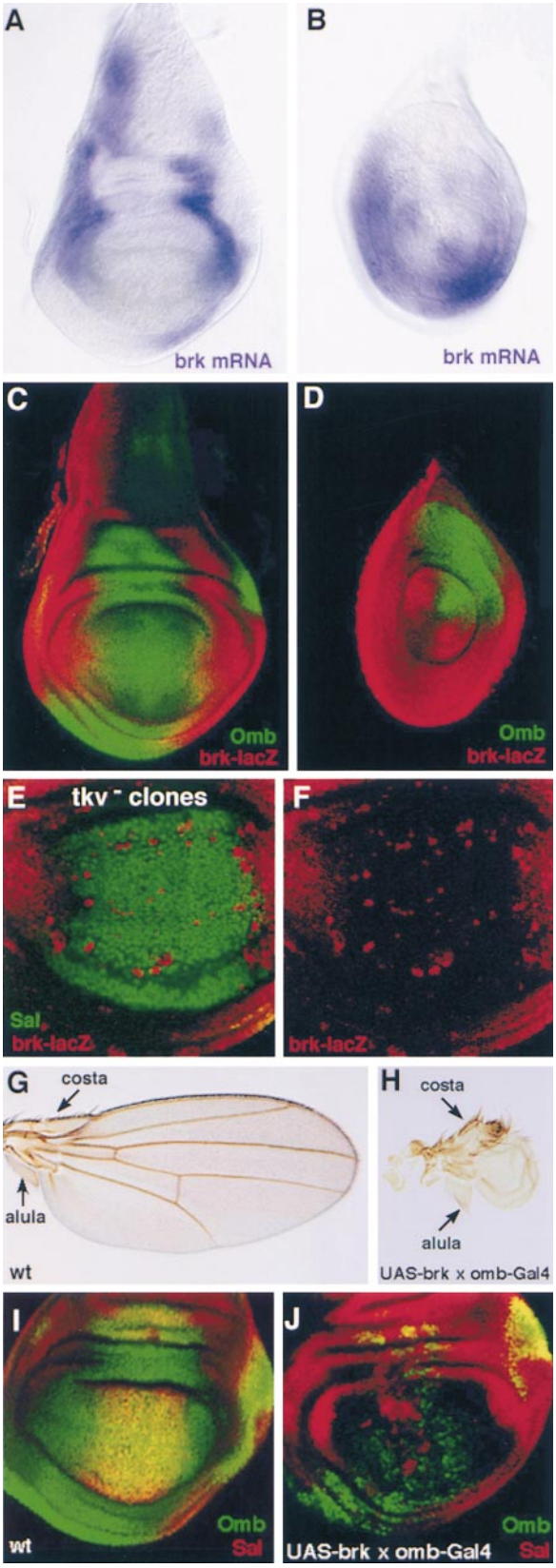

Figure 6. Normal and Altered *brk* Expression in Imaginal Discs

(A and B) *brk* mRNA distribution. (C and D) Double staining showing *brk-lacZ* (red) and Omb protein (green) distribution. (A and C) In wing imaginal discs, the expression of *brk* is highest in lateral regions and declines toward the center of the disc in the wing pouch. Only a slight overlap of *brk* and *omb* expression is visible in the wing pouch region (yellow). (B and D) In leg imaginal discs, *brk* is expressed complementary to regions of Dpp signaling. Expression is lowest in a broad domain of the dorsal compartment in which *omb* is expressed. (E and F) *tkv* mutant clones marked by the absence of *sal* expression (green) lead to ectopic induction of *brk-lacZ* (red). The spatial relation between endogenous *brk* and *sal* expression can be seen. The *sal* expression starts where the *brk* expression has declined to nondetectable levels. (G–J) Effects of ectopic expression of *brk* in the center of the wing disc. (G) Wild-type wing. (H) Severe reduction of wing blade if *brk* is expressed under the control of *omb*-Gal4. (I and J) Omb (green) and Sal (red) proteins in wild-type wing disc (I) and in a wing disc in which *brk* is expressed under the control of *omb*-Gal4 (J). Expression of *brk* in the *omb* domain leads to a severe reduction of *omb* and *sal* expression in the wing pouch region.

mechanism by which Dpp activates target genes that depend on low or intermediate levels of signaling activity. To activate these genes, Dpp has to antagonize their repression by Brinker, a novel protein with features of a putative transcriptional repressor. Since this mechanism is also involved in dorsal–ventral patterning of the *Drosophila* embryo, which is evolutionarily conserved, we expect that it will operate in vertebrates too.

Loss of *brk* Mimics Low or Intermediate Levels of Dpp Signaling

brk mutant clones in the wing primordium show effects only in boundary regions of the Dpp gradient, that is, in lateral regions of the wing pouch where the transition between low-level signaling and absence of signaling presumably takes place. *brk* expression is restricted to these regions. In these regions, *brk* clones produce cell fate transformations in a cell-autonomous manner that resemble an activation of the Dpp pathway. However, *brk* is not a block in the pathway that if removed leads to maximal pathway activation. In that respect, it is clearly different from other well-studied negative regulators like protein kinase A in the Hedgehog pathway (for review, see Kalderon, 1995) or *shaggy/zest-white 3* in the Wingless pathway (for review, see Klingensmith and Nusse, 1994). Mutations in these genes lead to high-level pathway activation. *brk* clones, on the other hand, lead to the formation of structures that correspond to low or intermediate levels of Dpp, a phenotype very different from that observed with the activated Dpp receptor Tkv* (Figures 2C and 2D). Accordingly, target genes are activated in *brk* clones that require only low or intermediate levels of Dpp. These are *omb* and *sal*, respectively (Figure 3). However, *sal* shows only low levels of expression in *brk* clones, indicating that *sal* integrates *brk*-dependent and *brk*-independent modes of regulation (Figure 3C).

brk Does Not Act Like an Inhibitory SMAD

The link of *brk* to low levels of Dpp signaling could indicate that *brk* acts to modulate Dpp signaling strength. In this respect, it could be similar to the negatively acting SMAD6/Dad and SMAD7 (Hayashi et al., 1997; Nakao et al., 1997; Tsuneizumi et al., 1997) that modulate signaling strength by binding to the receptor

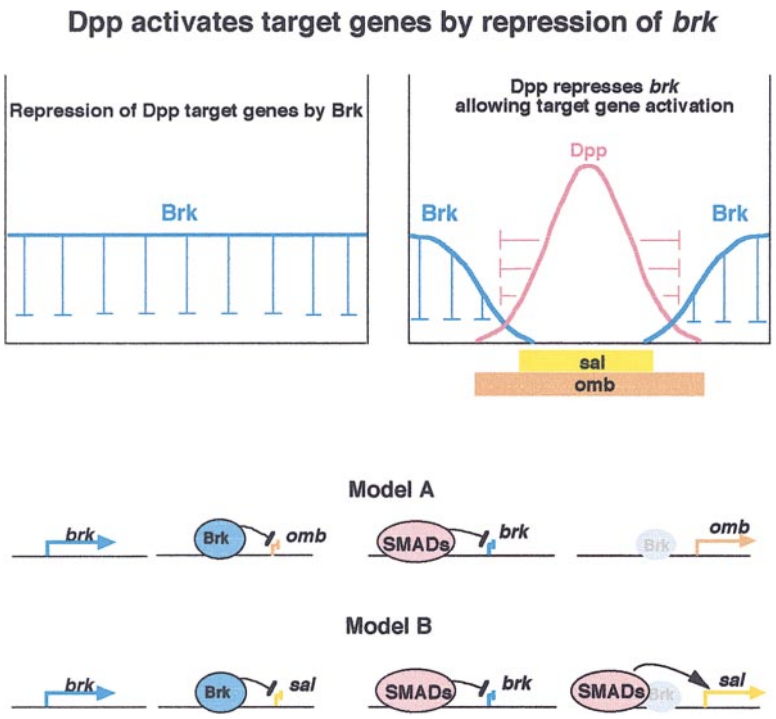

Figure 7. Model of Wing Primordium Patterning by Dpp Antagonizing Brk

In the absence of Dpp signaling (left side), *brk* is uniformly expressed in the wing pouch. This has been shown by inducing receptor null clones that cause strong *brk* expression irrespective of their position (Figures 6E and 6F). Dpp emerging from the center of the wing pouch (right side) has to repress *brk* transcription in order to allow the expression of the target genes *omb* and *sal*. This follows from overexpression studies (Figures 6I and 6J). Two models of target gene regulation can be envisaged. For *omb*, transcriptional repression of *brk* by Dpp signaling (SMADs) is sufficient for expression (model A). Alternatively, transcriptional repression of *brk* is accompanied by SMAD-dependent activation (model B). While both models might apply for *omb*, for high *sal* expression, both transcriptional downregulation of *brk* and Dpp activation are required. At the active promoters, Brk is depicted in light blue to indicate diminishing amounts (model A) and inhibition by SMAD binding (model B).

or the co-SMAD (SMAD4). However, if this was the case, the *brk* phenotype should depend on residual signaling activity and specifically should require the function of Mad, the only receptor-regulated SMAD involved in the Dpp signaling. Our double mutant analysis clearly shows that this is not the case. *omb* and *sal* can be expressed in *brk* clones that are simultaneously lacking Mad function. This demonstrates that relief of *brk* repression alone is sufficient to fully activate *omb* and partially activate *sal*. From the viewpoint of epistasis, a situation similar to that of *brk* has been found in nematodes (Patterson et al., 1997). *daf3* encodes an unusual SMAD protein that antagonizes TGFβ signaling. Double mutants reveal that *daf3* acts independently of the activating SMAD proteins in controlling TGFβ target genes. To explain these epistasis experiments, Daf3 was suggested to constitutively bind to target promoters in the absence of signaling. The kinase-activated SMADs were proposed to bind close to Daf3 to counteract its effect on target gene expression. Important differences between *daf3* and *brk* seem to be that *daf3* transcription is not negatively regulated by signaling and that *brk* seems specifically linked to a context in which a TGFβ molecule fulfills a morphogen function.

Dpp Target Gene Control by *brk*

brk could be a transcription factor based both on its epistatic position in the pathway and on some features of the protein sequence. If *brk* specifically represses only the promoters of low- and intermediate-level target genes of Dpp, then loss of *brk* would lead to the activation of these genes at ectopic positions. At these positions, structures would form that correspond to low or intermediate levels of Dpp signaling, not because signaling has occurred, but instead because a specific subset of target genes had been activated in a signaling-independent way.

If we assume that *brk* is a target gene–specific transcriptional regulator, then two models can be envisaged of how Dpp regulates the target genes controlled by *brk*. In both models, the transcriptional control of *brk* by Dpp plays an important role (Figure 7). Dpp signaling is a potent repressor of *brk* transcription and seems to be required throughout wing development, as the late induction of small *tkv* clones indicates (Figure 6E). As soon as Dpp signaling is abolished, strong *brk* expression can be seen at any position in the wing pouch. If *brk* is ectopically expressed in the center of the wing, then induction of *omb* and *sal* is suppressed even in regions of high Dpp signaling. All these observations suggest that Dpp signaling, at least in part, counteracts *brk* repression by reducing the amount of repressor. The promoter regions responsible for *omb* and low-level *sal* expression might even have only *brk*-binding sites, so that their activation would be completely dependent on downregulation of *brk* expression. Alternatively, these promoters might integrate both the activation by SMAD proteins and repression by *brk*.

brk and the Morphogen Function of Dpp

Interestingly, in both contexts in which Dpp functions as a morphogen, the early embryo and the wing primordium, *brk* is involved in the regulation of target genes that are activated at the low end of the gradient (data not shown for the embryo). In both cases, other target genes exist that show *brk*-dependent and *brk*-independent aspects of their expression, like *sal* in the wing, or that are not regulated by *brk* at all (data not shown). These target genes are always activated by higher levels of Dpp. This might indicate that the regulatory mechanism that is governed by *brk* is specifically linked to the

problem of morphogen function. Boundary regions pose particular problems for the interpretation of morphogen gradients. Close to the source, gradients are likely to have a steep slope that allows discrete thresholds to be easily defined. Morphogen concentrations will be lower at distances far from the morphogen source. The resulting decreased slope will make it more difficult to define sharp expression domains of target genes. This problem can be solved partly by feedback mechanisms that reinforce small differences in morphogen concentration. If Dpp signaling would lead simultaneously to relief of *brk* repression and activation by SMADs, then an enhancement mechanism might result that is very sensitive to small changes in Dpp concentrations and thus could help to define thresholds at the low end of the gradient.

Experimental Procedures

Genetic Mosaics in Discs

Loss-of-function clones of genetically marked cells (either by absence of *arm-lacZ* or *hs-N-Myc*) were generated by *hsFlp-FRT* recombination (Xu and Rubin, 1993). Larvae were subjected to a 60–90 min heat shock at 37°C. *brk* mutant clones were induced in larvae 36 ± 12 hr after egg deposition (AEL), which had the following genotypes: *y w brk^{M68} f^{36a} FRT18A/arm-lacZ FRT18A*; *hs-Flp38/+*. *y w brk^{M68} f^{36a} FRT18A/hs-N-Myc FRT18A*; *dpp-lacZ(10638)/+*; *TM3 hsFlp/+*. *brk* clones in a background of *dpp^{d8}/dpp^{d12}*: *y w brk^{M68} f^{36a} FRT18A/hs-N-Myc FRT18A*; *dpp^{d8}/dpp^{d12}*; *TM3 hs-Flp/+*. *dpp^{d8}/dpp^{d12}* is a combination of *dpp^{disc}* alleles (Spencer et al., 1982; Lecuit et al., 1996). *brk* and *tkv* or *brk* and *Mad* double mutant clones were generated in the larvae 72 ± 12 hr AEL: *y w brk^{M68} f^{36a} FRT18A/arm-lacZ FRT18A*; *tkv^{a12}* (or *Mad^{12}*) *FRT40A/arm-lacZ FRT40A*; *TM3 hs-Flp/+*. *tkv^{a12}* and *Mad^{12}* are null alleles (Nellen et al., 1994; Terracol and Lengyel, 1994; Raftery et al., 1995). To generate flies of this genotype, combinations of chromosomes 2 and 3 were balanced over *T* (Δ2,3) *SM6a-TM6b,Tb*. Larvae were selected by the absence of the dominant marker *Tb*. Since each FRT chromosome carrying the mutation was in *trans* to *arm-lacZ*, the complete absence of *arm-lacZ* occurs only in double mutant clones. *tkv* mutant clones were induced in third instar larvae: *y w brk^{M12}-lacZ/+*;*tkv^{a12} FRT40A/hs-N-Myc FRT40A*;*TM3 hs-Flp/+*. *Tkv** flp-out clones were induced in the larvae 36 ± 12 hr AEL. Clones of cells expressing high levels of *Tkv** were made using the flip-out system (Struhl and Basler, 1993) in larvae of the genotype *y w hs-Flp1/+*;*Ubx>lacZ>Tkv*/+* (Lecuit et al., 1996).

Immunochemistry and In Situ Hybridization

Immunostaining with rabbit anti-Krüppel, mouse anti-β-gal (Sigma), rabbit anti-β-gal (Cappel), mouse anti-Omb (kindly provided by G. Pflugfelder), rabbit anti-Sal (by R. Schuh), and anti-myc (1-9E10.2) antibodies was done as described previously (Struhl and Basler, 1993). Detection of transcripts in situ was performed as previously described (Tautz and Pfeifle, 1989). All fluorescent images were obtained with a Leica confocal microscope.

Preparation of Embryonic and Adult Cuticle

For the analysis of embryonic cuticle, nonhatched larvae were dechorionated and mounted in a mixture of Hoyer's medium and lactic acid 2:1. Wings were removed and mounted in Gary's magic mountant medium (Roberts, 1998).

P Element Mutagenesis

We used the "local hop" mutagenesis method (Tower et al., 1993) starting with the enhancer trap line, *P(w+)*38-10 (gift from T. Tabata). This P element is homozygous viable and maps cytologically to 7B1. *brk* maps close to 7B1 about 0.4 map units distal to the *cut* locus at 7B2-3. The Δ2,3 chromosome was used to induce transposition. From 900 independent F1 matings that were performed, 37 X-linked lethal lines were recovered. Of these, four did not complement *brk^{M68}*. Two of these, *brk^{M12}* and *brk^{L9}*, were studied further. A reversion screen was performed using the *brk^{M12}* line. Out of 378 crosses to Δ2,3 to induce mobilization, 25% gave revertant white-eyed males. In addition, we recovered several lethal revertant lines.

Plasmid Excision and cDNA Characterization

Genomic DNA was prepared from female flies carrying the P element, digested with EcoRI, treated with ligase, and transformed to recover plasmids. From the *brk^{L9}* rescued plasmid, a unique restriction fragment was used to screen cDNA libraries made from 0- to 4-hr embryos and imaginal discs (Brown and Kafatos, 1988). One cDNA from each library was sequenced (Biotechnology Center, Utah University). The disc-derived cDNA is 3.2 kb. Primer extension reactions (Promega) were performed using 0- to 8-hr embryonic poly A+ RNA and a primer whose sequence was derived from near the beginning of the cDNA. Extension products were run on a 6% polyacrylamide-urea gel alongside a marker synthesized by dideoxy sequencing of *brk* genomic DNA using the same primer (USB Biochemical Sequenase kit). The extension product migrated to an A residue 129 nucleotides from the first nucleotide of the primer. This A is the first residue of the cDNA and thus the +1 of the *brk* transcription unit, demonstrating that the cDNA is full length at the 5′ end. Comparison of the cDNA and genomic DNA sequences showed no introns in the *brk* transcription unit. The P element insertions map between nucleotides −33 and −32 (*brk^{M12}*) and −40 and −39 (*brk^{L9}*). The cDNA contains an open reading frame of 704 amino acids, beginning at nucleotide +531.

PCR Isolation of *brk^{M68}* Genomic DNA

Brk^{M68} hemizygous mutant embryos were collected by screening 1- to 2-day-old embryos for the *brk* mutant head phenotype. DNA was isolated and PCR was performed (Expand High Fidelity PCR system, Boehringer Mannheim) using primers from the 5′-untranslated leader and the 3′-untranslated trailer of the cDNA. The 2.5 kb PCR fragment was sequenced directly. PCR products were also obtained from genomic DNA of the parental chromosome, *y w FRT 101 w^{+}*. Direct comparison of the two sequences showed only one change, a G-to-A transition at nucleotide 790 of the *brk* transcription unit that leads to a change in amino acid 87, TGG to a TAG stop codon.

Ectopic Expression of brk

The full-length cDNA was cloned into pUAST (Brand and Perrimon, 1993). To analyze the consequences of overexpression of *brk* in imaginal discs, the Omb-Gal4 driver was used (Lecuit et al., 1996).

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