Development 126, 3323-3334 (1999)
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DEV5329

The role of *brinker* in mediating the graded response to Dpp in early *Drosophila* embryos

Anna Jaźwińska[1], Christine Rushlow[2,*] and Siegfried Roth[1,*]

[1]Institut fuer Entwicklungsbiologie, Universitaet zu Koeln, Gyrhofstr. 17, 50923 Koeln, Germany
[2]Department of Biology, New York University, 100 Washington Square East, New York, NY 10003, USA
*Authors for correspondence (e-mail: chris.rushlow@nyu.edu; sroth@biolan.uni-koeln.de)

Accepted 13 May; published on WWW 5 July 1999

SUMMARY

Brinker (Brk), a novel protein with features of a transcriptional repressor, regulates the graded response to Decapentaplegic (Dpp) in appendage primordia of *Drosophila*. Here, we show that in the embryo *brk* also has differential effects on Dpp target genes, depending on the level of Dpp activity required for their activation. Low-level target genes, like *dpp* itself, *tolloid* and early *zerknüllt*, show strong ectopic expression in ventrolateral regions of *brk* mutant embryos; intermediate-level target genes like *pannier* show weak ectopic expression, while high-level target genes like *u-shaped* and *rhomboid* are not affected. Ectopic target gene activation in the absence of *brk* is independent of Dpp, Tkv and Medea, indicating that Dpp signaling normally antagonizes *brk's* repression of these target genes. *brk* is expressed like *short gastrulation* (*sog*) in ventrolateral regions of the embryo abutting the *dpp* domain. Here, both *brk* and *sog* antagonize the antineurogenic activity of Dpp so that only in *brk sog* double mutants is the neuroectoderm completely deleted.

Key words: *brinker*, Dpp target genes, Transcriptional repression, Neuroectoderm, *sog*, *Drosophila melanogaster*

INTRODUCTION

In both early insect and early vertebrate embryos, gradients of BMP activity are established that are responsible for most aspects of patterning along the embryonic dorsoventral axis (Ferguson, 1996). In *Drosophila*, the BMP2/4 homolog Decapentaplegic (Dpp) is an important component of a BMP activity gradient, which has its highest levels along the dorsal midline of cellular blastoderm embryos and declines towards more lateral regions. The high levels determine the cell fate of the extraembryonic amnioserosa while lower levels specify the dorsal epidermis. The absence of Dpp activity in ventral regions is required for the formation of the neurogenic ectoderm, which gives rise to both the ventral epidermis and the central nervous system. Thus, as in vertebrates, BMP in flies promotes the formation of epidermis and suppresses that of neural tissues (for reviews see Rushlow and Roth, 1996; Biehs et al., 1996; Wilson et al., 1997).

The importance of BMP activity gradients in DV patterning poses two questions. How are these gradients formed in early embryos and how is the graded distribution transformed into distinct cellular responses? Both in vertebrates and in insects gradient formation depends in part on secreted inhibitors that are expressed complementary to the BMPs (for a review see Cho and Blitz, 1998). One of these inhibitors is chordin in vertebrates and its homolog *short gastrulation* (*sog*) in *Drosophila* (Sasai et al., 1994; François et al., 1994; Holley et al., 1995; Schmidt et al., 1995; Biehs et al., 1996). In vertebrates the regulatory mechanisms that lead to the expression of *chordin* and *BMP* in complementary domains are not well understood. However, in *Drosophila*, *sog* and *dpp* are target genes of the maternal Dorsal (Dl) protein gradient, which has peak levels in ventral nuclei and declining levels laterally. Dl activates *sog* and represses *dpp* and thus confines *dpp* to the dorsal side of the embryo and *sog* to a ventrolateral stripe abutting the *dpp* domain. In ventralmost regions Dl activates the expression of the genes *twist* and *snail*, which repress *sog* and specify the mesodermal anlagen, but themselves are not affected by Dpp signaling (for a review see Rusch and Levine, 1996).

Chordin and *sog* are BMP-binding proteins which compete with ligand binding to the receptor and thus prevent signaling. They are cleaved by metalloproteases (*tolloid* (*tld*) in *Drosophila* and its homologs in vertebrates) expressed in the same domain as the BMPs (Piccolo et al., 1997; Marqués et al., 1997). Cleavage of the ligand/inhibitor complex results in the release of the active ligand. Thus, the activity gradient is not formed by diffusion of the ligand itself, but rather by diffusion of its inhibitor and of ligand/inhibitor complexes. Moreover, the actual gradients might result from the combined activity of several ligands interacting with different receptors and being differentially susceptible to inhibition, as has been recently shown for *Drosophila*. Here, the Dpp activity gradient results from an intricate interplay of Dpp with a second BMP-like ligand, Screw (Scw; Neul and Ferguson, 1998; Nguyen et al., 1998). Dpp and Scw each preferentially signal through

different type I receptors. While Dpp signals through Thick veins (Tkv) and is required for all aspects of the DV pattern, Scw signals through Saxaphone (Sax) and is required only to induce dorsalmost cell fates. To explain this observation a synergistic interaction between Tkv activated by Dpp and Sax activated by Scw has been proposed, which potentiates Dpp signaling in dorsalmost positions.

How BMP gradients are transformed into a series of different cellular responses depends on the way target genes are regulated in an activity-dependent manner. Here, the SMAD proteins, the cytoplasmic signal transducers of all TGF-β family members, play a crucial role since they directly mediate between receptor activation and transcriptional response (for reviews see Heldin et al., 1997; Whitman, 1998). Receptor activation leads to phosphorylation of Class I SMADs, which then associate with Class II SMADs and translocate into the nucleus to directly bind to DNA recognition sites in target gene promoters (for a review see Derynck et al., 1998). In vertebrates dose-dependent responses to BMP can be mimicked by injection of different amounts of Class I SMAD proteins (Suzuki et al., 1997; Wilson et al., 1997). Therefore, it was suggested that graded BMP signaling leads to a gradient of nuclear concentrations of kinase-activated SMAD proteins, which in turn bind to target promoter sites with different affinities, thereby eliciting concentration-dependent threshold responses (Wilson et al., 1997). However, the situation now appears to be more complex. Firstly, BMP signaling also activates negatively acting SMADs, which do not enter the nucleus but compete with Class I and Class II SMADs for receptor binding or complex formation, respectively. This results in a non-linear relationship between receptor activation and nuclear SMAD concentrations (for a review see Whitman, 1998). Secondly, the actual transcriptional response to SMAD activation appears to be strongly dependent on the simultaneous binding and probable complex formation with other transcriptional regulators such as the transcription factors FAST-1, FAST-2, or Jun/Fos in vertebrates (for a review see Derynck et al., 1998). The spatial distribution of such regulators themselves could be important for gradient interpretation.

So far attention has been paid mainly to proteins which, together with the SMADs, act as activators of transcription. However, one case is known from *C. elegans* where activated SMADs counteract the specific repression exerted by a DNA-binding inhibitory SMAD (Patterson et al., 1997; Thatcher et al., 1999). In addition, recent evidence was provided from *Drosophila* that one purpose of SMAD signaling is to antagonize transcriptional repression of Dpp target genes (Campbell and Tomlinson, 1999; Jaźwińska et al., 1999; Minami et al., 1999). In the wing, the Brinker (Brk) protein acts as a repressor of low and intermediate level Dpp target genes. These genes are expressed upon loss of *brk* even in the absence of SMAD signaling, suggesting that Brk acts as a direct transcriptional repressor. Since *brk* expression itself is negatively regulated by Dpp, target gene activation at least in part results from downregulation of *brk*.

Here, we show that in the early embryo *brk* plays a role comparable to that in the wing disc, with some differences. In contrast to the wing disc, loss of *brk* in the embryo leads to ectopic expression of *dpp*. However, by injection of activated Dpp receptor RNA we confirm that in the embryo *dpp* is its own target, and thus *dpp* repression by *brk* is a special case of target gene regulation. Moreover, most genes that are repressed by *brk* in the embryo are also targets of repression by maternal Dl, suggesting that *brk* could act as a corepressor of Dl. However, using double mutant analysis and ectopic expression of *brk* we show that *brk* represses Dpp target genes independently of Dl. Consistent with *brk's* role in the wing disc, embryonic target gene activation in the absence of *brk* is independent of SMAD activity. Thus, in both contexts, *brk* acts parallel or downstream to SMADs as a specific repressor of low and intermediate level Dpp target genes. *brk* is expressed like *short gastrulation* (*sog*) in ventrolateral regions of the embryo abutting the dorsal *dpp* domain, and in *brk* mutants *dpp* expression expands to cover the entire ectoderm. In this situation *sog* is largely responsible for Dpp gradient formation since *brk sog* double mutant embryos have almost no polarity information in the ectoderm. The double mutants consist mainly of mesoderm and unstructured dorsal epidermis. Thus, *brk* and *sog* together specify the neuroectoderm of *Drosophila* embryos.

MATERIALS AND METHODS

Crosses and stocks used to analyze *brk* function in the embryo

To distinguish hemizygous or homozygous mutant embryos from their heterozygous siblings, balancers were used which carried early expressing lacZ transgenes (*FM7c ftz-lacZ*; *CyO hb-lacZ*; *TM3 hb-lacZ*) whenever possible. In all other cases the genetically expected number of embryos with a given phenotype was confirmed.

brk deficiencies:
Df(1) ct^{J4}, In (1)dl^{49}, f / FM7c ftz-lacZ
Df(1) Sxl-ra/Y y^+ct^+ Sxl^+
Df(1) ct^{4b1}/ FM7
Df(1) RF19 / FM7

brk: *yw brk ^{M68}/ FM7c ftz-lacZ*

brk germline clones: germline clones were induced in *brk ^{M68} FRT 101 / ovo^{DI} FRT101* second or third instar larvae by the Flp-FRT technique (Chou and Perrimon, 1996).

sog: *sog^{YS06} / FM7c ftz-lacZ*

brk sog: *yw brk ^{M68} sog^{YS06} / FM7c ftz-lacZ*

sog plus four copies of *dpp^+: sog^{YS06} / FM7c ftz-lacZ; Dp (2; 2) DTD48 /CyO23, P[dpp^+]*

brk dpp: *yw brk ^{M68}/ FM7c ftz-lacZ; dpp^{H46} sna^{IIG} twi^{S60} / CyO23, P[dpp^+]*. *dpp^{H46}* is a null allele (Wharton et al., 1993). *dpp* mutant embryos were identified by absence of ventral furrow formation, which is eliminated by loss of *sna* and *twi*.

brk tkv: *yw brk ^{M68}/ FM7c ftz-lacZ; tkv^{O1} /tkv^{Sz3}* crossed to *FM7c ftz-lacZ; tkv^{O1} / CyO hb-lacZ*. *tkv^{O1}* is an antimorphic allele, *tkv^{Sz3}* is a strong hypomorphic allele (Terracol and Lengyel, 1994; Nellen et al., 1994). Eggs from transheterozygous *tkv^{O1} /tkv^{Sz3}* females fertilized with *tkv^{O1}* sperm develop ventralized embryos that are comparable to those of *dpp* null.

brk Medea: *yw brk ^{M68}/ FM7c ftz-lacZ; hs-flp 38/+; Med^1 e FRT82B / ovo^{DI} FRT82B* females crossed to *FM7c ftz-lacZ;; Med^1 e FRT82B / TM3 hb-lacZ*. *Med^1* is a null allele (Das et al., 1998). *Med^1* germline clones were induced in the second or third instar larvae by the Flp-FRT technique (Chou and Perrimon, 1996).

brk Tl^{9Q}: *yw brk ^{M68}/ +;; Tl^{9Q}/+* were crossed to wild-type males. *Tl^{9Q}* causes a dominant maternal ventralization (Anderson et al., 1985).

dpp dl: *dpp^{H46} dl^1/ CyO23, P[dpp^+]* were crossed to *Dp (2; 2) DTD48 dl^1/ CyO* to generate *dpp^{H46} dl^1 /Dp (2; 2) DTD48 dl^1* females,

which were crossed to dpp^{H46} / *CyO23, P[*dpp^{+}*]* males (Wharton et al., 1993).

UAS brk: Jaźwińska et al. (1999).

tub GAL4: this stock carries a transgene in which the DNA binding domain of GAL4 fused to the VP16 transcriptional activation domain is expressed from the α4-tubulin promoter (gift of D. St Johnston).

Preparation of embryonic cuticle

For the analysis of embryonic cuticle, non-hatched larvae were dechorionated and mounted in a mixture of Hoyer's medium and lactic acid 2:1 (Roberts, 1998).

Immunochemistry and in situ hybridization

Immunostaining of embryos with rabbit anti-Krüppel was done as described previously (Roth et al., 1989). Detection of transcripts in situ was performed as outlined in Tautz and Pfeifle (1989) and double-label in situ hybridization according to O'Neill and Bier (1994) and Hauptman and Gerster (1996). The embryos were mounted in Durcupan-ACM and sectioned as described in Roth et al. (1989). *LacZ* expression was detected using rabbit anti-βGal (Cappel) antibodies and by in situ hybridization with an antisense *LacZ* probe.

RNA injections

Capped *Tkv** mRNA (Hoodless et al., 1996) and *brk* mRNA were made from pCS2-Tkv* or pCS2-brk (Rupp et al., 1994), respectively, linearized with *Not*I and transcribed using SP6 mRNA polymerase using the Message Machine kit (Ambion). mRNA was injected into preblastoderm embryos as previously described (Ferguson and Anderson, 1992a). For both *Tkv** and *brk*, the injected mRNAs had concentrations of approximately 100 ng/ µl.

RESULTS

brk affects the shape of the Dpp activity gradient in early *Drosophila* embryos

The Dpp activity gradient that is established in the dorsal half

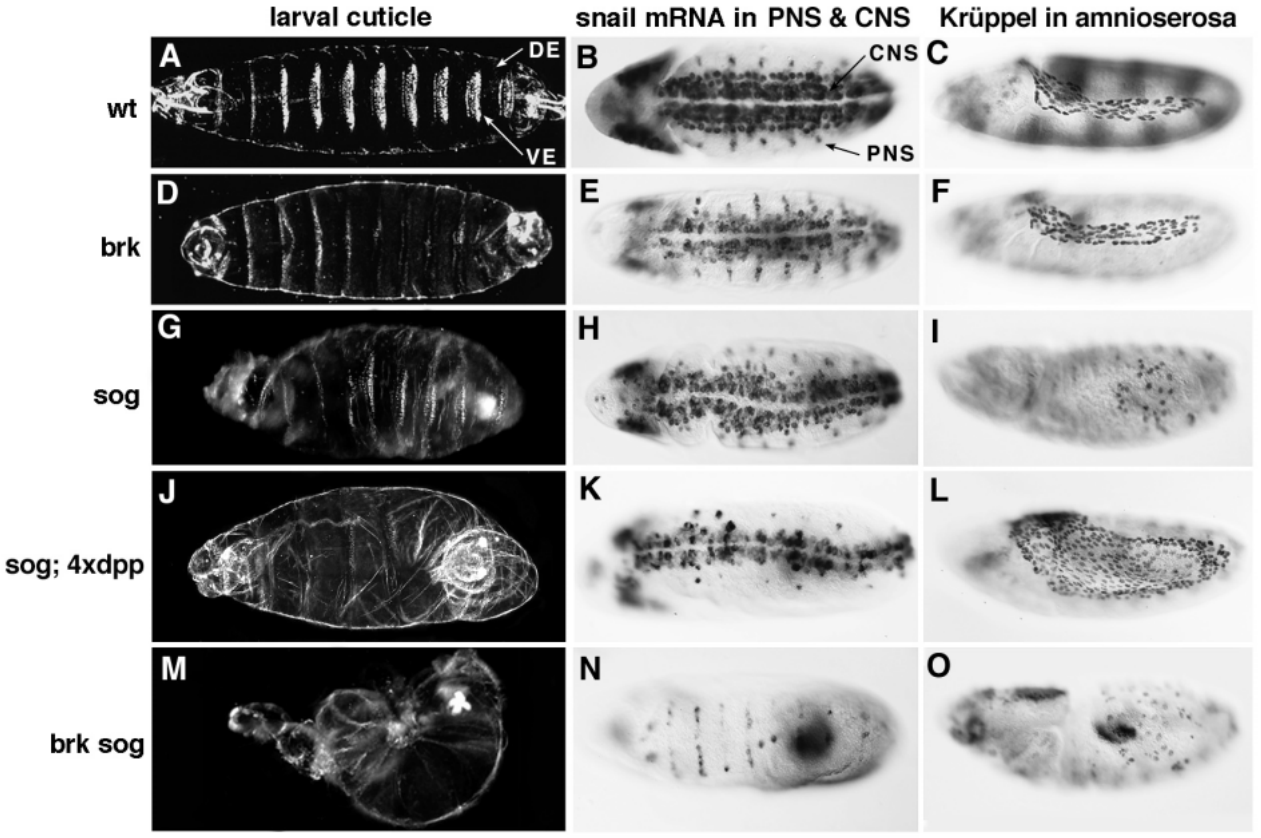

Fig. 1. *brk* and *sog* together specify the ventral ectoderm of early *Drosophila* embryos. (A-C) Wild type, (D-F) brk^{M68}/Y, (G-I) sog^{SYO6}/Y, (J-L) sog^{SYO6}/Y*; DTD 48/ DTD48*, (M-O)$brk^{M68}sog^{YSO6}/Y$. (A,D,G,J,M) Dark-field photographs showing the ventral side of the larval cuticle. Anterior is to the left. (A) Wild type with strongly pigmented denticle belts of the ventral epidermis (VE). The dorsal epidermis (DE) carries weakly pigmented dorsal hairs. (D) In *brk* mutants the ventral denticle belts have largely disappeared due to an expansion of dorsal epidermis. (G) *sog* mutants have abnormal shapes due to a failure in germ band extension. The reduction in the size of the ventral denticle belts is less severe than in *brk* mutants. (J) *sog* with four copies of dpp^{+} shows a cuticle phenotype resembling that of *brk*. (M) *brk sog* mutants have only dorsal epidermis. (B,E,H,K,N) Ventral view of embryos during germ band extension. The neuroblast pattern is visualized using *snail* (*sna*) mRNA expression as a marker. (B) Wild-type embryos showing three rows of neuroblasts on each side of the ventral midline (CNS). The small cells expressing *sna* in more lateral positions are peripheral nervous system (PNS) precursors. (E) In *brk* mutant embryos the number of neuroblasts in the two outer rows is severely reduced. (H) In *sog* mutant embryos only neuroblasts in the first outer row are affected. (K) In *sog* with four copies of dpp^{+} in average only one row of neuroblasts is left. (N) In double mutant embryos the neuroblasts are completely deleted and only PNS precursors can be found at the ventral side. (C,F,I,L,O) Krüppel protein expression is used as a marker for amnioserosa cells. Wild-type (C) and *brk* mutant embryos (F) with normal numbers of amnioserosa cells. (I) Dorsal view of a *sog* mutant embryo shows only a small number of scattered amnioserosa cells. Germ band extension is abnormal. (L) *sog* with four copies of dpp^{+} has strongly enlarged amnioserosa. (O) Lateral view of double mutant embryo shows scattered amnioserosa cells. Germ band extension is abnormal.

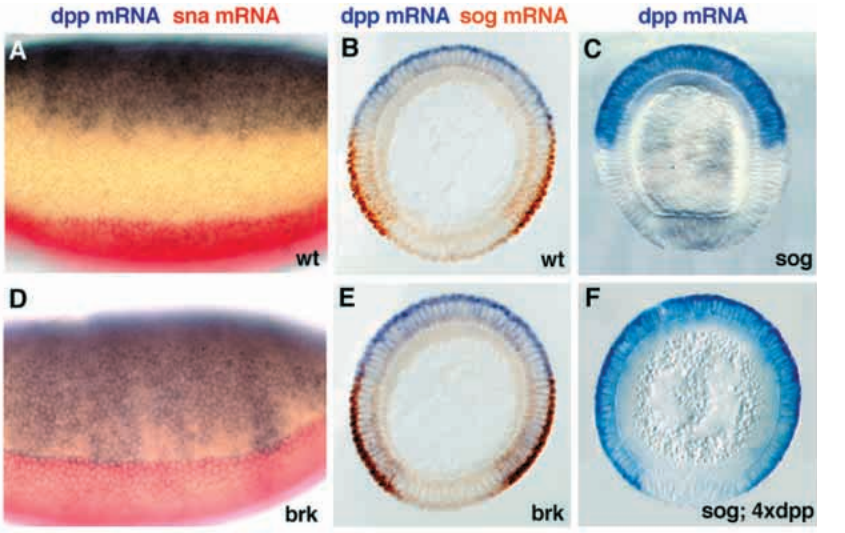

Fig. 2. In contrast to *sog*, *brk* mutant embryos show expanded *dpp* expression. (A,D) Lateral view of blastoderm embryos. Anterior is to the left and dorsal up. *dpp* expression is shown in dark blue and mesodermal *snail* expression in red. (A) In wild type (wt) *dpp* is repressed in ventrolateral regions (yellow). (D) In a *brk* mutant embryo *dpp* expression expands to the border of the *snail* domain. (B,E,C,F) Cross sections through cellular blastoderm or early gastrulating embryos showing *dpp* (blue) expression and *sog* (brown) expression (B,E). (B) Wild type. *sog* expression abuts *dpp* expression. (E) *brk* mutant embryo. *sog* expression is normal despite the dramatic expansion of *dpp*. (C) sog^{SYO6}/Y. *dpp* is only slightly expanded. (F) sog^{SYO6}/Y; *Dp(2;2) DTD 48/ Dp(2;2)DTD48 dpp* expands to a similar degree as in *brk* mutant embryos.

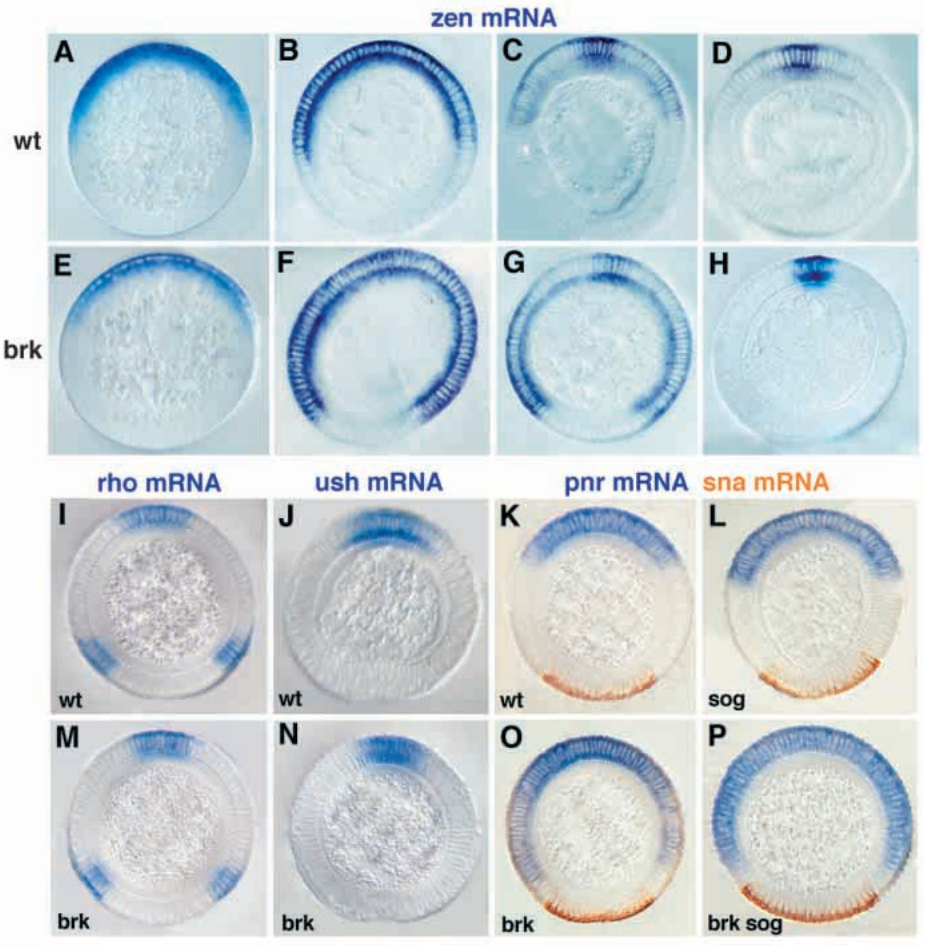

Fig. 3. The expression of Dpp target genes in *brk* mutant embryos. Cross sections through embryos at approximately 50% egg length. (A-D, I-K) wild type; (E-H, M-O) brk^{M68}/Y; (L) sog^{SYO6}/Y; (P) $brk^{M68}sog^{YSO6}/Y$. (A,E) Early syncytial blastoderm (stage 4): *zen* is repressed in ventral and lateral regions of wt and *brk* mutant embryos. (B,F) Early cellular blastoderm (stage 5a): different from wt, the *zen* domain expands to the border of the mesoderm in *brk* mutant embryos. (C,G) Cellular blastoderm slightly older than B,F: *zen* expression starts refinement in both wt and *brk* mutant embryos. (E,J) Late cellular blastoderm shortly before gastrulation. *zen* is expressed in a narrow 5- to 6-cell-wide domain in wt and *brk* mutant emrbyos. (I,M) The dorsal stripe of *rho* expression is approximately 12 cells wide in both wt and *brk* mutant embryos at the cellular blastoderm stage. (J,N) The dorsal stripe of *ush* expression is approximately 14 cells wide in both wt and *brk* mutant embryos at early gastrulation. (K-P) *pnr* (blue) and *sna* (brown) expression. (K) In wt the *pnr* domain is approximately 32 cells wide (n=9). (L) In sog mutants *pnr* expands, comprising about 45 cells (n=10). (O) In *brk* mutants low levels of *pnr* expand ventrally and stop five cells short of the *sna* domain. (P) In *brk sog* mutants high levels of *pnr* expand ventrally and also stop five cells short of the *sna* domain.

of cellular blastoderm embryos subdivides the ectoderm into three major territories that can be identified by the cell types they will comprise and/or by the cuticular structures they secrete (Fig. 1A-C; Ferguson and Anderson, 1992a,b; Wharton et al., 1993; Biehs et al., 1996). High levels of Dpp activity specify the dorsalmost region, which differentiates into large amnioserosa cells that express Krüppel (Kr) protein (Fig. 1C; Gaul et al., 1987). These cells form extraembryonic tissue that does not produce cuticle. Lower Dpp levels in the dorsolateral region specify the non-neurogenic ectoderm harboring peripheral nervous system precursors that can be identified as small segmentally repeated *snail* (*sna*)-expressing cells (Alberga et al., 1991; PNS in Fig. 1B). This region gives rise to cuticle with weakly pigmented dorsal hairs (DE, dorsal epidermis in Fig. 1A). Finally, the ventral (neurogenic) ectoderm requires very low levels or absence of Dpp to form

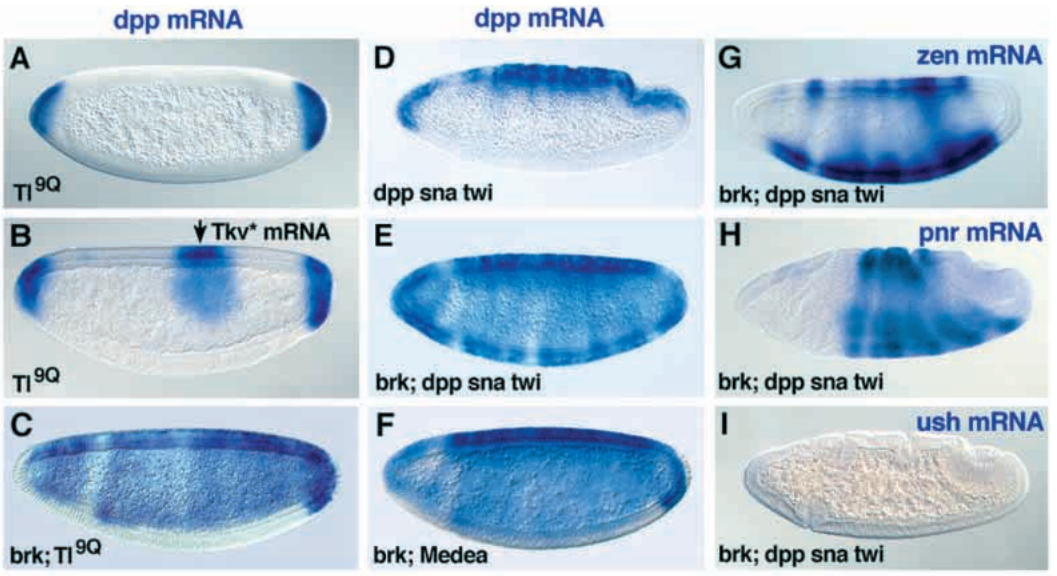

Fig. 4. Target gene activation in *brk* mutant embryos in the absence of dpp. (A-F) Lateral views of embryos at cellular blastoderm stage or early gastrulation showing *dpp* expression. Dorsal is up and anterior to the left. (A) Embryos derived from Tl^{9Q}/+ females are ventralized. *dpp* expression is restricted to the termini. (B) Injection of mRNA encoding a constitutively activated form of the Dpp receptor Tkv (Tkv*) into maternally ventralized embryos induces *dpp* expression at the site of the injection (arrow). (C) Loss of zygotic *brk* function in a Tl^{9Q}/+ maternal background leads to derepression of *dpp*. (D) In dpp^{H46} sna^{IIG} twi^{S60} triple mutant embryos, which can be identified by the absence of the ventral furrow, *dpp* mRNA remains detectable in a dorsal domain like in wild type. (E) Loss of *brk* in this background leads to *dpp* expression around the entire embryonic circumference. (F) brk^{M68}/*Y;; Med*^{1} / *Med*^{1} embryos derived from females carrying brk^{M68}/*FM7 ftz-LacZ; Med*^{1} / *Med*^{1} germline clones crossed to *FM7 ftz-LacZ; Med*^{1} / *TM3 hb-LacZ* males. Double in situ hybridization with *dpp* and *LacZ* probes allowed the identification of double mutant embryos, which show expanded *dpp* expression. (G-I) brk^{M68}/*Y;* dpp^{H46} sna^{IIG} twi^{S60} / dpp^{H46} sna^{IIG} twi^{S60} showing ectopic *zen* (G) and *pnr* (H), but no *ush* (I) expression.

the neuroblasts of the central nervous system, which also express *sna* (Alberga et al., 1991; CNS in Fig. 1B). This region secretes cuticle with heavily pigmented ventral denticles (VE, ventral epidermis in Fig. 1A).

The cuticle of *brk* mutant embryos has an enlarged region carrying dorsal hairs and a smaller region carrying ventral denticles (Fig. 1D). The number of *sna*-expressing neuroblasts in the ventral neurogenic region is reduced (Fig. 1E). This indicates that *brk* mutations lead to an expansion of dorsolateral fates and a reduction of ventrolateral fates. However, despite these lateral fate shifts, the number of Kr-expressing amnioserosa cells is not different from wild type (Fig. 1F). Thus, *brk* specifically affects cell fates depending on low or intermediate levels of Dpp signaling, while those that require peak levels are not altered.

To identify the underlying causes of the visible changes in cell fate, we examined the effect of *brk* on the expression of two groups of DV patterning genes. The first group consists of *dpp*, *zen* and *tld*, whose expression is initiated very early in syncytial blastoderm stages. Since they are ventrally repressed by Dl protein their expression domains are confined to the dorsal 40% of the egg circumference (Figs 2A, 3A; Rushlow et al., 1987; Ray et al., 1991; Shimell et al., 1991; data not shown for *tld*). In *brk* mutant embryos *dpp*, *zen* and *tld* expression is initiated normally as shown for *zen* in Fig. 3E. However, in contrast to wild type their expression domains expand ventrally during mid-cellularization (Figs 2A,D, 3B,F). Double in situ hybridization shows that *dpp* expression abuts, and sometimes overlaps, the first cell row of the *sna* domain whose blastoderm expression marks the mesodermal anlagen (Fig. 2D). These data demonstrate that *brk* is not required for the early ventrolateral repression of these genes, but is essential to prevent their lateral expansion during cellularization. Since at these stages Dl protein is still present in the lateral nuclei, *brk* might function to augment the Dl-mediated repression of *dpp*, *zen* and *tld*.

The second group of DV patterning genes includes *rhomboid* (*rho*, dorsal expression domain; Bier et al., 1990), *u-shaped* (*ush*; Haenlin et al., 1995; Cubadda et al., 1997) and *pannier* (*pnr*; Winick et al., 1993; Heitzler et al., 1996), which are not direct targets of repression by maternal Dl. The initiation of their expression during cellularization requires prior formation of the Dpp activity gradient (*rho*, Bier et al., 1990; *ush*, C. R., unpublished results; *pnr*, Winick et al., 1993). Therefore, they are candidates for being direct targets of Dpp signaling in the embryo. They are expressed in domains straddling the dorsal midline that are 12 (*rho*), 14 (*ush*) and 32 (*pnr*) cells wide at cellular blastoderm (cell counts at approx. 50% egg length; Fig. 3I-K). The two narrowly expressed genes *rho* and *ush* are not changed in *brk* mutant embryos (Fig. 3M,N). This is also true for late *zen* expression, which in *brk* mutant embryos, as in wild type, refines to a narrow 5- to 6-cell-wide stripe along the dorsal midline despite the prior expansion (Fig. 3C,D,G,H). However, *pnr* expression expands in *brk* mutant embryos and low ectopic *pnr* levels can be seen in a broad lateral domain that stops about five cells short of mesodermal *sna* expression (Fig. 3O). Thus, *brk* does not affect Dpp target genes that are expressed in dorsalmost regions and supposedly depend on highest Dpp levels. However, a target gene that is expressed in a wider domain, and is therefore presumably activated by intermediate levels of Dpp, is expanded.

The described alterations in DV patterning appear to result from complete loss of *brk* function, and deficiencies uncovering *brk* produce phenotypes identical to the original point mutation brk^{M68} (see Materials and methods). This mutation contains a stop codon causing an early truncation of the protein (Jaźwińska et al., 1999). The phenotypes are also

not enhanced in embryos derived from females in which germline clones homozygous for *brk*^{M68} had been induced (see Materials and methods), indicating that the zygotic phenotype is not ameliorated by maternal expression.

In summary, *brk* mutations affect the Dpp activity gradient in the embryo by expanding the domains of expression of *dpp* and one of its activators (*tld*) into ventrolateral regions. Despite the uniform expression of *dpp* in the entire ectoderm, Dpp activity levels appear to be only mildly increased in the ventrolateral region since only low-level (*zen*) or intermediate-level (*pnr*) target genes are ectopically expressed, causing a reduction in the size of the nervous system and ventral epidermis accompanied by an expansion of dorsal epidermis. Peak levels of Dpp in dorsalmost positions appear to be normal, judging from both target gene expression and cell type differentiation.

brk and *sog* act independently to specify the ventral neurogenic ectoderm

The question arises as to why uniform expression of *dpp* in the ectoderm is compatible with the substantial degree of DV polarity exhibited by *brk* mutant embryos. In wild type, Dpp activity is polarized by *sog* expression in the ventrolateral region of the embryo (Fig. 2B) such that ventrolateral Dpp activities are reduced and a peak of activity is established centered on the dorsal midline (Zusman et al., 1988; François et al., 1994; Biehs et al., 1996). Embryos mutant for *sog* show a reduction of ventrolateral fates, albeit to a weaker degree than *brk* embryos (Fig. 1G,H; see legend for details). In contrast to *brk* embryos, they differentiate only a small number of scattered amnioserosa cells (Fig. 1I,). The lateral fate shift is not accompanied by strong expansion of *dpp* transcription as seen in *brk* embryos (Fig. 2C), and *pnr* expression is not as greatly expanded as in *brk* embryos (Fig. 3L).

In *brk* embryos, *sog* is still expressed in the ventrolateral domain of the ectoderm (Fig. 2E). To test whether this expression accounts for the DV polarity in the ectoderm of *brk* embryos, we constructed *brk* embryos that were also mutant for *sog*. In contrast to either single mutant, the ectoderm of *brk sog* embryos forms only dorsal-type cuticle hairs and completely lacks ventral denticles (Fig. 1M). During germ band extension, neuroblast expression of *sna* cannot be detected; instead *sna* expression in the peripheral nervous system (PNS) precursors, normally restricted to dorsolateral regions, expands into the ventral region of the embryo (Fig. 1N). This suggests a complete deletion of the ventral neurogenic region. We confirmed this observation using a number of different molecular markers for the neuroectoderm including *msh* (D'Alessio and Frasch, 1996), the *achaete-scute* transcript *l'sc* (Cabrera et al., 1987) and the *Enhancer of split* transcript *m5* (Knust et al., 1992). In all cases the early neuroectodermal expression of these genes was eliminated in double mutant embryos, although expression was only slightly affected in each of the single mutants (data not shown). This indicates that Dpp activity is elevated in the ventrolateral regions of *brk sog* double mutants to reach levels that completely suppress neurogenesis. Despite this, there is no corresponding increase in Dpp activity at the dorsal side of double mutant embryos. This can be seen by comparing *sog* plus extra copies of *dpp*$^{+}$ (Ferguson and Anderson, 1992b; Biehs et al., 1996) with *brk sog* embryos. In the former case Dpp levels are increased along the entire embryonic circumference, which leads not only to a strong reduction of the ventral ectoderm (Fig. 1J,K), but also to the rescue of the amnioserosa defect of *sog*. Moreover, this rescue is accompanied by a large increase in the number of amnioserosa cells compared to wild type (Fig. 1L). In contrast, in *brk sog* double mutant embryos the amnioserosa defect of *sog* is not rescued (Fig. 1O). This demonstrates that *brk* action is confined to the ventrolateral region and that despite ectopic expression of *dpp* in *brk* mutants there is no detectable increase in Dpp activity at the dorsal side even in a situation where Dpp activity levels are limiting.

Taken together, *brk* and *sog* have both overlapping and distinct roles in shaping the Dpp activity gradient of the *Drosophila* embryo. While *sog* has an important function in providing peak levels of the gradient necessary for amnioserosa development, *brk* and *sog* together are essential to limit the ventral extension of the anti-neurogenic activity of *dpp*. Interestingly, *brk sog* double mutants do not completely eliminate all polarity of the ectoderm. This can be seen from the expression of *pnr*, which in the double mutant has uniformly high ectodermal levels except in a 5-cell wide stripe bordering the mesoderm (Fig. 3P). Thus, in addition to *brk* and *sog*, other ventrally localized factors provide patterning information for the ectoderm. Candidates for this function are members of the *spitz* group of genes (Schweitzer et al., 1995; Golembo et al., 1998).

Ectopic target gene expression in *brk* mutant embryos does not require Dpp, Tkv or Medea

In contrast to the embryo, in wing imaginal discs *brk* mutant cells ectopically express Dpp target genes without affecting *dpp* expression itself. Furthermore, this ectopic target gene expression does not require the components of the Dpp signaling pathway. This indicates that *brk* is a negative transcriptional regulator of Dpp target genes in wing imaginal discs that is counteracted by Dpp signaling (Campbell and Tomlinson, 1999; Jaźwińska et al., 1999; Minami et al., 1999). Embryonic and imaginal disc data can be reconciled if *dpp* in the embryo is a target gene of Dpp signaling, directly or indirectly, as has been suggested earlier (Rushlow and Roth, 1996; Biehs et al., 1996). To test this possibility rigorously, we injected mRNA encoding an activated version of the Dpp type I receptor Thick veins (Tkv*, Hoodless et al., 1996) into embryos from *Toll*^{9Q} heterozygous mothers. *Toll*^{9Q} is a dominant ventralizing mutation which causes high nuclear levels of Dl protein to be uniformly present in the ectodermal region. This results in repression of *dpp* in the entire trunk region of the embryo (Fig. 4A; Anderson et al., 1985; Ray et al., 1991). At the site of Tkv* mRNA injection, expression of *dpp* was observed (Fig. 4B), confirming that activation of the Dpp signaling pathway can induce expression of *dpp*. We then asked whether the same effect can be produced by removal of *brk* in a *Toll*^{9Q} background. This was the case; *brk* embryos derived from *Toll*^{9Q} mothers show uniform expression of *dpp* throughout their entire ectoderm (Fig. 4C). This suggests that ectopic *dpp* expression in *brk* mutants does not require prior expression of the Dpp ligand. This conclusion was confirmed by double mutant analysis using an amorphic *dpp* allele (*dpp*^{H46}), which in early embryos produces normal amounts of *dpp* transcripts (Fig. 4D; Wharton et al., 1993). To identify the

dpp mutant embryos we used a triple mutant chromosome which, in addition to *dpp*, carries *sna* and *twi* mutations. *dpp sna twi* triple mutant embryos can be recognized by the lack of ventral furrow formation (Fig. 4D). *brk; dpp sna twi* quadruple mutant embryos (see Materials and methods) show expanded *dpp* and *zen* expression (Fig. 4E,G) and secrete cuticles exhibiting a partial transformation of ventral into dorsal epidermis (data not shown). Thus, the *brk* phenotype in the embryo, as in imaginal discs, does not depend on endogenous *dpp* activity.

We further confirmed that neither receptor activity nor SMAD function are required for target gene expression in the absence of *brk*. To that end, we constructed double mutants of *brk* with mutations in the Dpp receptor *tkv* (Terracol and Lengyel, 1994; Nellen et al., 1994; Brummel et al., 1994; Penton et al., 1994) or in the cytoplasmic signal transducer *Medea* (Hudson et al., 1998; Das et al., 1998; Wisotzkey et al., 1998). Both *brk*; *tkv* and *brk*; *Medea* double mutant embryos (see Materials and methods) showed expanded *dpp* expression (Fig. 4F; data not shown for *tkv*). These observations are most significant for *Medea* since the allele we used ($Medea^{1}$) is predicted to cause a truncation that completely eliminates *Medea* function (Das et al., 1998). Thus, *brk* could function as a specific transcriptional repressor of Dpp target genes, as previously suggested for imaginal discs. However, the experiments presented so far do not rule out the possibility that in the embryo *brk* acts as a corepressor of Dl, since the target genes studied here, *dpp*, *tld* and *zen*, are all targets of Dl repression, and since the expansion of *pnr* in *brk* embryos (Fig. 3O) might be a secondary consequence of the expansion of *dpp*. Therefore, we tested whether *pnr* expression, which is absent in *dpp* mutants (Winick et al., 1993), is restored in *brk; dpp sna twi* quadruple mutant embryos. Indeed, the quadruple mutants show uniform expression of *pnr* along the DV axis at levels corresponding to those found in lateral region of *brk* embryos (Fig. 4H). These data strongly support the view that *brk* acts as specific repressor of low and intermediate-level Dpp target genes. High level target genes are not affected by *brk* and are consequently not expressed in *brk; dpp sna twi* quadruple mutant embryos as shown for *ush* (Fig. 4I).

brk expression indicates an involvement in many aspects of Dpp signaling

In imaginal discs *brk* is expressed complementary to regions of Dpp signaling. The same principle applies for its expression during embryonic development. In late syncytial and in cellular blastoderm embryos *brk* is expressed in a ventrolateral stripe, which is initially 9-10 nuclei wide and expands to encompass 18 nuclei shortly before gastrulation (Fig. 5A,B). A narrow gap separates the initial stripe dorsally from the expression domain of *dpp* (data not shown). At the ventral side expression stops one cell row short of the *snail* domain (Fig. 5J). Thus, the *brk* domain is largely overlapping *sog* expression, in agreement with our finding that *brk* and *sog* together are required for the establishment of the ventral neurogenic ectoderm (Fig. 5K). Furthermore, the domain also corresponds to the region of the embryo showing ectopic *dpp*, *zen* and *tolloid* misexpression in the absence of *brk*. This expression pattern is thus consistent with the view that *brk* may directly repress the transcription of these genes.

During germ band extension *brk* continues to be expressed in the ventral ectoderm where it appears to be restricted to neuroectodermal cells during neuroblast segregation (Fig. 5C,D,L). New sites of expression appear in the ventral mesoderm at stage 10 (Fig. 5E,L), when Dpp-signaling from the ectoderm induces dorsal mesoderm (Frasch, 1995). During stage 11 some ectodermal cells surrounding the tracheal pits express *brk* (Fig. 5F). The tracheal pits lie in between the dorsolateral and the ventral stripe of *dpp* expression, and patterning of the invaginating tracheal cells has been shown to be influenced by *dpp* (Wappner et al., 1997; Vincent et al., 1997). During germ band retraction the developing midgut broadly expresses *brk* (Fig. 5G). In stage 13, three localized domains of expression can be seen in the visceral mesoderm and endoderm of the developing gut: a domain slightly anterior to the region of the developing gastric caeca, a domain approximately at the location of parasegment 5 and a posterior domain at the border between hindgut and midgut (Fig. 5H). These regions lie between domains of *dpp* expression in the visceral mesoderm where *dpp* mediates inductive processes between visceral mesoderm and endoderm (for a review see Bienz, 1996). Finally, *brk* is expressed during dorsal closure in a broad stripe of ectodermal cells localized ventrally to the leading edge cells in which *dpp* expression controls morphogenesis (Fig. 5I; for a review see Noselli, 1998). In summary, these expression patterns suggest a potential role of *brk* in all major aspects of Dpp signaling in the embryo.

brk is activated by maternal Dorsal and repressed by Dpp

The ventrolateral expression of *brk* in early embryos suggests that *brk*, like *sog* and *rho*, is a target gene of the maternal Dl protein gradient (Ip et al., 1992; François et al., 1994). In support of this notion, we found that *brk* expression is completely abolished in maternally dorsalized embryos (Fig. 6A). Conversely, in maternally ventralized embryos derived from the $Toll^{9Q}$ heterozygous mothers (used above in Fig. 4), *brk* expression is initiated along the entire embryonic circumference except in the presumptive mesoderm (Fig. 6B). In *sna twi* mutant embryos (Fig. 6D) and in *sna* single mutants (data not shown) *brk* expression is uniform at the ventral side. Thus, as shown for *rho* (Ip et al., 1992), *sna* might be a ventral repressor of *brk* transcription.

The complementarity between *brk* expression and regions of Dpp signaling in the embryo might arise if *brk* is itself negatively regulated by Dpp, as previously shown in imaginal discs (Campbell and Tomlinson, 1999; Jaźwińska et al., 1999; Minami et al., 1999). To test this idea, we examined *brk* expression in *dpp* mutant embryos. Here, *brk* expression is normal before the onset of gastrulation, but subsequently expands towards the dorsal side of the embryo so that *brk* becomes uniformly expressed in the entire ectoderm (Fig. 6E). The opposite phenotype results if *dpp* expression expands into the ventrolateral region, as in a *sog* mutant embryo with extra wild-type copies of *dpp* (Fig. 2N; Frasch, 1995; Rushlow and Roth, 1996; Biehs et al., 1996). These embryos exhibit a strong repression of *brk* transcription in the ventrolateral region although a small domain of *brk* expression is maintained close to the border of the mesoderm (Fig. 6F). This residual expression might be responsible for the narrow stripe of neuroblasts which still forms in *sog* embryos with four copies of dpp^{+} (Fig. 1K). We wondered whether the expansion of *brk*

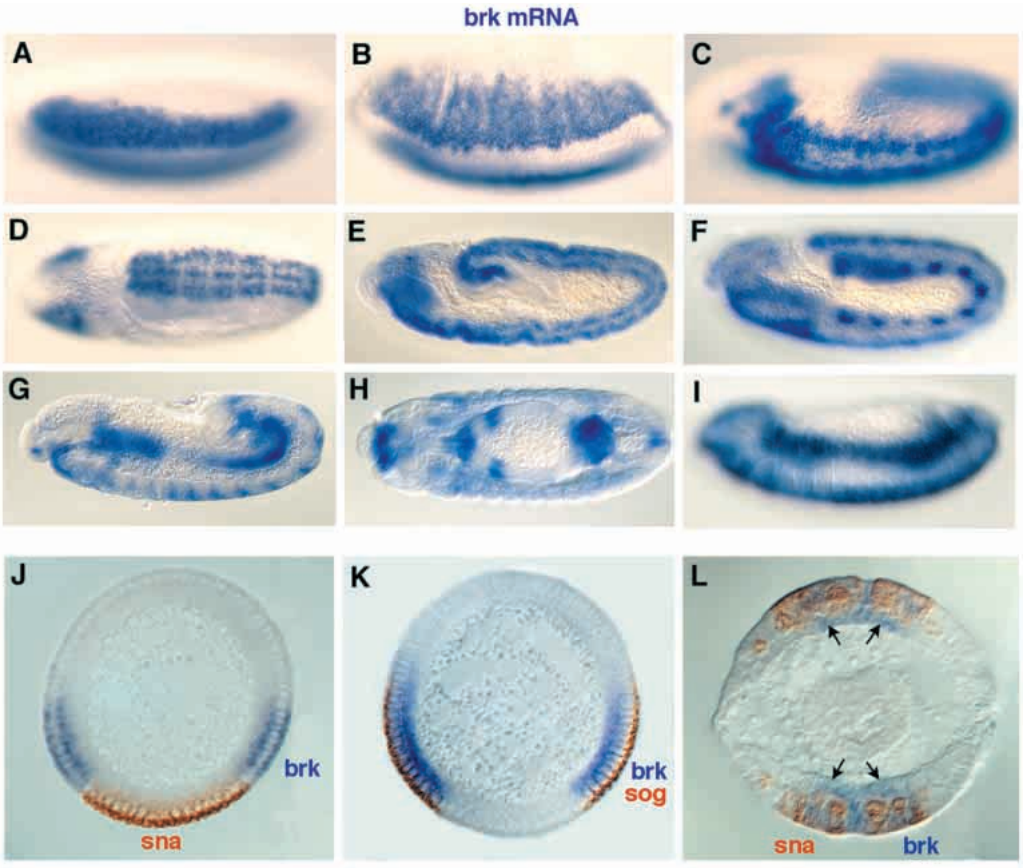

Fig. 5. Expression of *brk* during embryonic development. (A-I) *brk* mRNA distribution during successive stages of embryonic development. (A) Lateral surface view of syncytial blastoderm embryo. *brk* expression is initiated in a 9- to 10-cell-wide ventrolateral stripe. (B) Lateral surface view of embryo at beginning of gastrulation. *brk* expression has expanded to encompass an 18 cell-wide stripe. (C) During germ band extension *brk* is confined to the cells of the neurogenic ectoderm. (D) Dorsal view of extending germ band embryo shows *brk* in a pattern comparable to that of E(spl)m5 in neuroctodermal cells being excluded from cells delaminating as neuroblasts (L). (E) Optical midsection through stage-10 embryo. *brk* is expressed in the ventral mesoderm and in neuroectodermal cells; compare to transverse section shown in (L). (F) Lateral surface view of stage-11 embryo. Some ectodermal cells surrounding the tracheal pits express *brk*. (G) Optical midsection through stage-12 embryo shows srong *brk* expression in developing gut. (H) Stage 13. *brk* is expressed in three distinct domains of the gut, which encompass cells of the visceral mesoderm and endoderm. (I) During dorsal closure *brk* is expressed in a broad stripe of ectodermal cells localized ventrally to the leading edge cells. (J-L) Cross sections through embryos. (J) Syncytial blastoderm embryo. *brk* expression (blue) stops one cell short of the *snail* domain (brown). (K) Cellular blastoderm. *brk* RNA (blue) is present in the same cells as *sog* RNA (brown). While *brk* RNA is localized basally, *sog* RNA is concentrated apically. (L) Stage 9. *brk* expression (blue) in the ectoderm is restricted to neuroectodermal cells and excluded from delaminating neuroblasts expressing *snail* (yellow). *brk* is also expressed in the ventral mesoderm (arrows).

expression in *dpp* mutants requires the previous Dl-dependent activation of *brk* transcription. In *dl dpp* double mutant embryos, *brk* is initially not expressed (data not shown); nevertheless, uniform *brk* expression is initiated during gastrulation (Fig. 6C). Thus, absence of *dpp* leads to derepression of *brk* irrespective of whether Dl is present, indicating that other mechanisms of transcriptional activation of *brk* exist that are normally counteracted by Dpp signaling.

Ectopic expression of *brk* blocks Dpp expression even in the absence of Dorsal

Ectopic *pnr* expression in *brk; dpp* double mutant embryos shows that *brk* does not entirely function as a corepressor of Dl. However, the latter still could apply to *brk* function in regulating *dpp*, *tld* and early *zen*. To test whether *brk* can act as repressor in the absence of Dl we first expressed UAS-*brk* under the control of a maternally expressed GAL4 driver to achieve uniformly high-level expression of *brk* in early wild-type embryos. This completely suppresses the formation of the dorsal *dpp* domain (Fig. 7E,G). It also abolishes terminal expression of *dpp*, which is not subject to regulation by Dl (see *dpp* expression in the termini of Tl^{9Q} embryos in Fig. 4A). The resulting embryos secrete only cuticle with ventral denticle belts and resemble *dpp* mutant embryos (Fig. 7A,C). Thus, early uniform expression of *brk* can completely block all *dpp* expression. We also injected *brk* mRNA into *dl* mutant embryos that uniformly express *dpp* (Fig. 7F). At the site of injection *dpp* is repressed (Fig. 7H) and the injected embryos form cuticle with ventral denticles that resembles the cuticle from *dl dpp* double mutants (Wharton et al., 1993; Fig. 7B,D). However, since *dpp* is not uniformly repressed cuticles often show a transition from ventral epidermis to dorsal epidermis with increasing distance from the site of injection. In the region of transition dorsolateral structures are formed, such as Filzkörper (FK in Fig. 7D). This indicates that different amounts of repression by *brk* might lead to different ectodermal cell fates.

In summary, the ectopic expression data show that *brk* repression of *dpp* is independent of prior repression by Dl and therefore *brk* functions in early embryos, like in imaginal discs, as a regulatory component of the Dpp signaling pathway.

DISCUSSION

In *Drosophila* Dpp acts as a morphogen in two completely different developmental contexts: in DV patterning of the early embryo and in AP patterning of the appendage primordia

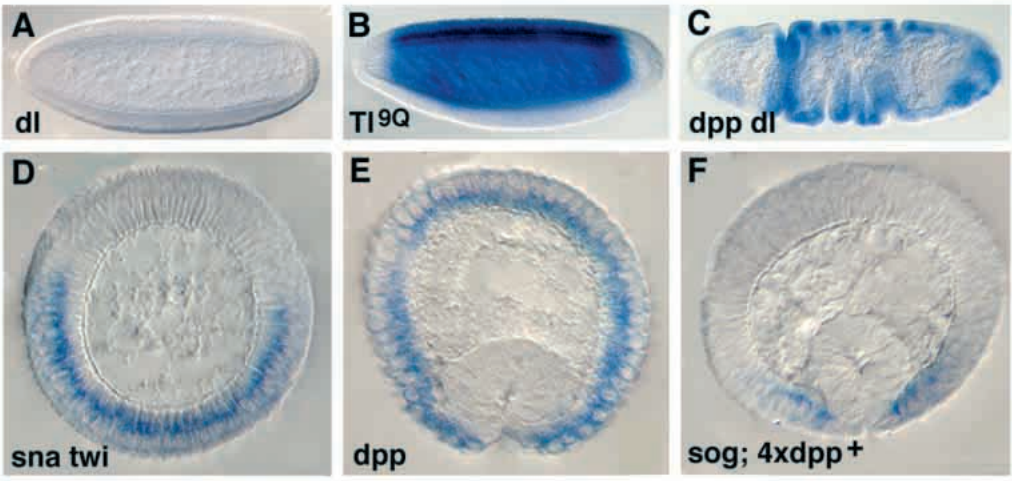

Fig. 6. Regulation of *brk* expression by maternal Dorsal and *dpp*. (A) No *brk* expression can be detected in completely dorsalized embryos derived from *dorsal* mutant females. (B) In ventralized embryos derived from Tl^{9Q}/+ females early *brk* expression is uniform in the entire ectoderm. (C) If in the *dorsal* mutant background, *dpp* is removed, *brk* expression is initiated during gastrulation although it was absent at earlier stages. (D-F) Cross sections through gastrulating embryos. (D) In sna^{IIG} twi^{S60} double mutant embryos mesodermal anlagen are not established and consequently ventral furrow formation is abolished. *brk* is expressed uniformly at the ventral side. (E) dpp^{H46} mutant embryo shows uniform *brk* expression in ectoderm during germ band extension. (F) sog^{SYO6}/Y; *DTD 48/ DTD48* shows strongly reduced *brk* expression. Only a small ventral domain remains.

(Ferguson and Anderson, 1992a,b; Wharton et al., 1993; Nellen et al., 1996; Lecuit et al., 1996). In the first case, gradient formation is a fast process and occurs in an epithelium in which no cell divisions take place; in the second case gradient formation is a slow process and occurs in an epithelium that constantly grows by cell division. In each context the gradients are established by completely different molecular mechanisms: in the embryo mainly by diffusion of an inhibitor (Sog) into a region of uniform expression of *dpp* (François et al., 1994; Holley et al., 1996), and in the imaginal discs mainly by the spreading of Dpp protein from a local source of *dpp* transcription in the center of the primordium (Lecuit and Cohen, 1998). In addition, in each context Dpp signaling affects a completely different set of target genes. Despite these differences there are some remarkable similarities, which might identify mechanisms important for gradient formation and morphogen function. In both cases two different BMP-type ligands each activate a different type I receptor, which results in synergistic effects important for correct establishment of the activity gradients (Nguyen et al., 1998; Neul and Ferguson, 1998; Haerry et al., 1998; Khalsa et al., 1998). In both cases receptor expression itself is influenced

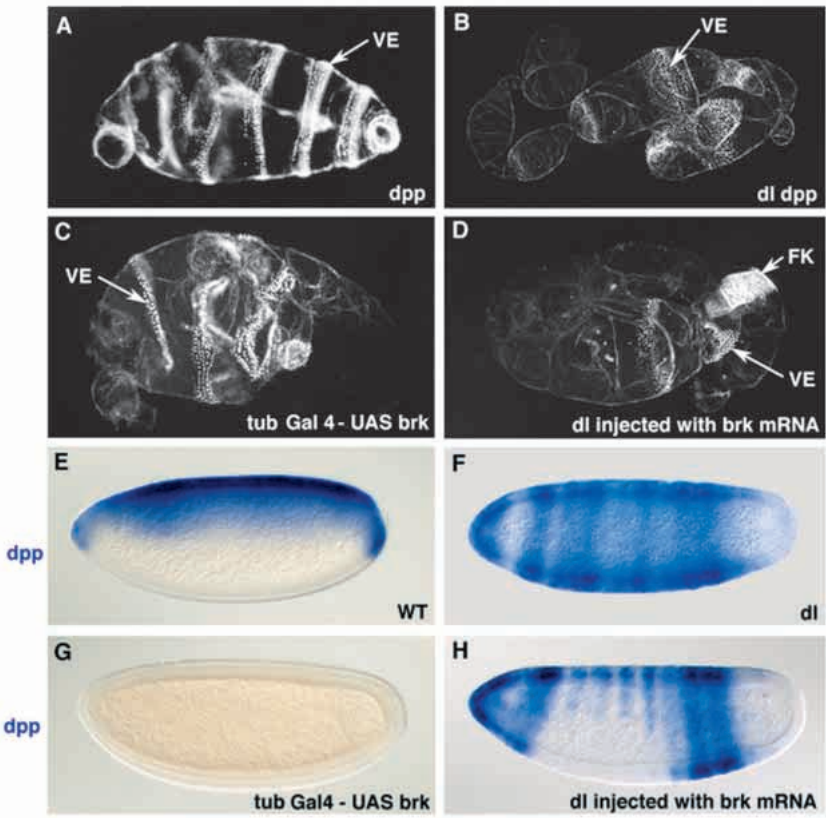

Fig. 7. Ectopic expression of *brk* ventralizes Drosophila embryos. (A-D) Dark-field photographs of the larval cuticle. (A) dpp^{H46}/dpp^{H46}. Cuticle consists only of ventral epidermis (VE). (B) dpp^{H46} mutant embryos derived from *dl* mutant females (see Materials and methods). Cuticle consists only of ventral epidermis (VE). (C) UAS *brk* expressed uniformly in early embryos using tub VP16 GAL4 as driver leads to completely ventralized cuticle. (D) Injection of *brk* mRNA into completely dorsalized embryos derived from *dl* mutant females leads to production of ventral epidermis (VE) at the site of injection and to the formation of Filzkörper (FK) at a certain distance from the site of injection. (E-H) Blastoderm embryos after in situ hybridization to detect *dpp* mRNA. (E) Dorsal and terminal *dpp* domains in wild type. (F) Uniform expression of *dpp* in *dl* mutant embryo. (G) Uniform *brk* expression controlled by tub VP16 GAL4 in an otherwise wild-type background completely abolishes *dpp* transcription. (H) Injection of *brk* mRNA into dorsalized embryo leads to repression of *dpp* at the site of injection.

by signaling which has been shown to be important for spreading of Dpp protein in the wing disc (Biehs et al., 1996; Lecuit and Cohen, 1998; Haerry et al., 1998). Finally, in both cases *brk* acts as negative regulator of low or intermediate level target genes, being itself negatively regulated by signaling.

Comparison of *brk* function in embryo and imaginal disc

In the wing disc *brk* mutant clones have completely cell autonomous effects and lead to cell fate transformations corresponding to low or intermediate levels of Dpp signaling (Campbell and Tomlinson, 1999; Jaźwińska et al., 1999; Minami et al., 1999). Mutant cells autonomously express the Dpp target genes *optomotor blind* (*omb*) and *spalt* (*sal*) without expressing *dpp*. *Omb* and *sal* each represent a target gene of a different class with regard to *brk* regulation. While *omb* shows normal levels of expression in *brk* clones, *sal*, the target gene depending on higher Dpp levels, is only weakly expressed. This indicates that *sal* integrates *brk*-dependent and *brk*-independent mechanisms for its activation. High-level target genes which are not affected by *brk* have not been found in wing discs so far; however, the cell fate transformation caused by ectopic expression of Tkv* indicate that such target genes exist (Jaźwińska et al., 1999). Since in the complete absence of MAD, which is essential for all aspects of Dpp signaling, removal of *brk* leads to the ectopic expression of *omb* and *sal*, we concluded that Dpp signaling acts to relieve *brk* repression. *brk* is expressed in lateral regions of the wing disc in a pattern which might largely result from transcriptional repression by Dpp. This might also be the predominant mechanism by which Dpp signaling relieves Brk's repression of target genes.

The relationship between *dpp* and *brk* is more complex in the embryo than in the wing imaginal disc for several reasons. Firstly, *brk* influences *dpp* expression in the embryo and thus changes the Dpp activity gradient. Therefore, it is more difficult to judge whether target gene mis-expression in *brk* mutants is a direct result of removal of *brk* or of the expansion of Dpp. Secondly, three of the genes, *dpp, zen* and *tld*, whose expression is expanded in *brk*, are also targets of the maternal Dl gradient, and so are subject to both Dl-mediated and Brk-mediated repression in early embryos. Thirdly, *brk* itself is clearly an activated target of Dl when it is first required for repression of *dpp*, *tld* and *zen*; only later during gastrulation does Dpp negatively regulate *brk*. Finally, in the embryo but not in the disc, *brk* acts with *sog* in an intricate way to shape the Dpp gradient.

Despite these complexities *brk's* relationship to the Dpp activity gradient in early embryos and to the Dpp gradient in wing discs is very similar. In both contexts low and intermediate-level targets are misexpressed in Brk mutants independently of Dpp signaling. Using *brk; dpp* double mutant embryos we demonstrated that the ectopic expression of *zen* and *pnr* in *brk* embryos is not a secondary consequence of the activation of *dpp*, but occurs even in the complete absence of Dpp signaling. *pnr* activation in *brk; dpp* double mutant embryos furthermore confirms that repression by *brk* is not dependent on prior repression by maternal Dl since *pnr* is not a direct target gene of Dl repression. *pnr's* regulation by *brk* is strikingly similar to *brk's* regulation of *sal* in the wing disc. *pnr* and *sal* both are intermediate-level Dpp target genes. In *brk* mutant cells they both show weak levels of expression compared with the expression in their normal domains. Thus, in the embryo and in the disc three types of Dpp target genes can be distinguished with regard to regulation by *brk*: those that are fully activated upon loss of *brk*, those that are weakly activated and those that are not affected. The regulation of the first group might occur entirely by antagonizing *brk's* repression; the second group requires both relief of repression by *brk* and positive activation by Dpp; and the third group might only be subject to direct activation by Dpp.

brk-mediated target gene regulation might be important for morphogen function of Dpp

The strongest phenotypic effects of *brk* mutations we have seen so far are in places where Dpp acts as a morphogen. In both the wing disc and embryo *brk* regulates Dpp target genes that are activated at the lower end of the gradient. In these regions special mechanisms of gradient interpretation might be required which are sensitive to small changes in Dpp activity. Target gene regulation by *brk* could provide such a mechanism. The full activation of low-level target genes requires only transcriptional repression of *brk* by Dpp. However, a more non-linear mechanism suited to establish sharp threshold responses would result if target gene promoters had both repressive *brk* and activating SMAD binding sites. Then, Dpp would simultaneously downregulate *brk* transcription and antagonize its function at the target gene promoters. Such a dual mechanism is the more likely as our data demonstrate that it operates for the intermediate targets *sal* and *pnr*. However, experimental support for such a model can only come from a detailed analysis of how *brk* interacts with the target gene promoters.

Brk and Sog: intra- and extracellular Dpp inhibitors establish the neurogenic ectoderm of *Drosophila* embryos

Originally the *brk* mutant phenotype in the embryo attracted our attention because of its similarity to the phenotype caused by *sog* with extra copies of *dpp* (Ferguson and Anderson, 1992b). Since both lead only to a partial deletion of the neurogenic ectoderm we speculated that Brk and Sog had parallel functions and thought that Brk might be another extracellular inhibitor of BMP, like Noggin in vertebrates. Indeed a large number of secreted molecules have been shown to act as BMP inhibitors in early vertebrate development (Cho and Blitz, 1998). However, the epistasis analysis clearly shows that Brk acts in the absence of Dpp and SMAD proteins and thus is likely to be a transcription factor. In accordance with the genetic data Brk protein has a potential DNA binding domain and harbors a motif found in many transcriptional repressors (Campbell and Tomlinson, 1999; Jaźwińska et al., 1999; Minami et al., 1999). Thus, in *Drosophila* the neurogenic region is established by three levels of repression: firstly, maternal Dl represses *dpp* and the Dpp-group genes *tld* and *zen* so that already during syncytial blastoderm stages their expression is confined to the dorsalmost 40% of the egg circumference. Secondly, Dl activates *brk* in ventrolateral regions. During the late cellular blastoderm stage repression by Dl alone becomes insufficient and *brk*, presumably acting also as a transcriptional repressor, is required to prevent the ventral expansion of *dpp*, *zen* and *tld*. Thirdly, Dl activates *sog* which, even in the absence of *brk* when *dpp* is uniformly expressed in

the ectoderm, acts as a sufficiently strong inhibitor of Dpp to allow formation of neurogenic ectoderm. Only when both *brk* and *sog* are removed is the ectoderm almost apolar and early neurogenesis completely suppressed.

Expression of *brk* in Xenopus embryos antagonizes BMP-4 signaling and leads both to a dorsalization of the ventral marginal zone and to neural induction in ectodermal explants (Minami et al., 1999). Thus, a Brk-like activity probably exists in vertebrates that antagonizes BMP and its target genes at the intracellular level providing another means of negative regulation in addition to the large number of extracellular BMP inhibitors.

The authors would like to thank Oliver Karst for excellent technical assistance. We thank Serena Silver for help with the double in situs, Jo Jack and Tom Kline for deficiencies and duplications in the *brk* region and advice about them, Eric Wieschaus for making *brk* germline clones, Manfred Frasch and Rolf Reuter for looking at embryos with us, and Regine Terracol, Rob Ray and Daniel St. Johnston for providing fly stocks. We are grateful to Eric Wieschaus for insightful discussions and critical reading of the manuscript. A. J. is a fellow of the Gottlieb Daimler-und Karl Benz-Stiftung.

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