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The transcription factor Schnurri plays a dual role in mediating Dpp signaling during embryogenesis

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SUMMARY

Decapentaplegic (Dpp), a homolog of vertebrate bone morphogenic protein 2/4, is crucial for embryonic patterning and cell fate specification in *Drosophila*. Dpp signaling triggers nuclear accumulation of the Smads Mad and Medea, which affect gene expression through two distinct mechanisms: direct activation of target genes and relief of repression by the nuclear protein Brinker (Brk). The zinc-finger transcription factor Schnurri (Shn) has been implicated as a co-factor for Mad, based on its DNA-binding ability and evidence of signaling dependent interactions between the two proteins. A key question is whether Shn contributes to both repression of *brk* as well as to activation of target genes. We find that during embryogenesis, *brk* expression is derepressed in *shn* mutants. However, while *Mad* is essential for Dpp-mediated repression of *brk*, the requirement for *shn* is stage specific. Analysis of *brk*; *shn* double mutants reveals that upregulation of *brk* does not account for all aspects of the *shn* mutant phenotype. Several Dpp target genes are expressed at intermediate levels in double mutant embryos, demonstrating that *shn* also provides a *brk*-independent positive input to gene activation. We find that Shn-mediated relief of *brk* repression establishes broad domains of gene activation, while the *brk*-independent input from Shn is crucial for defining the precise limits and levels of Dpp target gene expression in the embryo.

Key words: *Drosophila*, BMP, TGFβ, Smad co-factor, Transcriptional regulation

INTRODUCTION

Positional information along the dorsoventral (D/V) axis of the Drosophila embryo is assigned by the activity of two signaling pathways, one maternal and the other zygotic. The maternal pathway culminates in a nuclear gradient of the morphogen Dorsal (Dl) that activates target gene expression in distinct domains in the ventral half of the embryo. In addition, Dl directly represses expression of genes such as *decapentaplegic* (*dpp*) and *zerknüllt*, restricting their transcription to the dorsal region of the pre-cellular blastoderm (reviewed in Rusch and Levine, 1996). Dpp, a secreted growth factor related to vertebrate bone morphogenetic protein (BMP) 2/4, is a key component of a zygotic activity gradient of BMP signaling that patterns the dorsal side of the embryo (Ferguson and Anderson, 1992; Wharton et al., 1993). During early development a second BMP-related ligand Screw (Scw) acts with Dpp to achieve peak levels of signaling. The gradient of Dpp/Scw activity results in the establishment of distinct thresholds of gene activation that are required for the differentiation of dorsal tissues such as the amnioserosa and the dorsal ectoderm (Nguyen et al., 1998; Neul and Ferguson, 1998). Dpp is also essential for cell fate specification later in embryogenesis, and in the larval imaginal discs, where it plays an important role in growth and patterning (reviewed in Neumann and Cohen, 1997). Like other ligands of the BMP family, Dpp signals through a complex of type I and type II transmembrane serine-threonine kinases that trigger the translocation of a receptor-specific Smad/co-Smad complex from the cytoplasm to the nucleus (Hoodless et al., 1996; Newfeld et al., 1997; Das et al., 1998; Hudson et al., 1998; Wizotskey et al., 1998). Smads can directly activate transcription of downstream target genes but bind DNA with weak affinity. Furthermore, the consensus Smad-binding site is of low complexity and is predicted to occur approximately once every kilobase. As a result, it is widely believed that association of Smads with other DNA-binding proteins is essential for pathway- and promoter-specific gene activation (reviewed in Massagué and Wotton, 2000).

Dpp signal transduction was initially thought to consist of a linear pathway culminating in the direct activation of gene expression by the BMP-specific Smad *Mothers against dpp* (*Mad*) and the co-Smad *Medea* (*Med*) (reviewed in Raftery and Sutherland, 1999). However, Dpp signaling also negatively regulates transcription of the repressor Brinker (Brk), which inhibits Dpp target gene expression. Analysis of imaginal disc clones in which inputs from both Dpp and Brk were simultaneously removed has indicated that Dpp-responsive

genes can be grouped into three categories (Campbell and Tomlinson, 1999; Jazwinska et al., 1999a; Minami et al., 1999). The first class of genes depends entirely on Mad-mediated relief of *brk* repression. Promoters for the second class of targets incorporate both positive inputs from Mad as well as negative inputs from Brk. Finally, genes belonging to the third class are directly activated in response to Mad but are not repressed by Brk. Recent studies have highlighted a similar differential requirement for *brk* and *Mad* in activating Dpp target genes in early embryonic patterning (Jazwinska et al., 1999b; Ashe et al., 2000). Thus, the transcriptional response to Dpp depends on the balance between signaling-mediated activation and antagonism of target gene expression.

A number of transcription factors are known to lend specificity to activin and TGFβ signaling by interacting with Smads. However, the only DNA-binding partners to be identified in BMP signaling are the zinc-finger proteins OAZ in vertebrates and Schnurri (Shn) in *Drosophila* (Dai et al., 2000; Hata et al., 2000; Udagawa et al., 2000). Another *Drosophila* protein, Tinman, has been shown to potentiate Mad/Medea activity, but it is not known if this involves direct protein-protein interactions (Xu et al., 1998). Several lines of evidence implicate Shn in the nuclear response to Dpp. Mutations in *shn* affect the expression of a wide range of Dpp-inducible genes in the embryo as well as in the wing imaginal disc, indicating that it is crucial for responsiveness to Dpp (Arora et al., 1995; Grieder et al., 1995; Staehling-Hampton et al., 1995; Torres-Vazquez et al., 2000). Shn is localized to the nucleus and associates directly with Mad in a signaling-dependent fashion (Dai et al., 2000; Udagawa et al., 2000). Furthermore, we have identified Shn-binding sites in a Dpp-responsive enhancer from the *Ultrabithorax* (*Ubx*) gene that mediates weak activation in vivo. Co-expression of Shn and Mad synergistically stimulates transcription of this reporter in cell culture assays (Dai et al., 2000). Taken together these results argue that Shn is involved in the regulation of Dpp target genes and acts as a DNA-binding co-factor for Mad. In light of these findings, it is striking that absence of *shn* activity during embryogenesis has markedly less severe consequences than loss of *dpp*. Embryos homozygous for strong loss-of-function alleles of *shn* are defective in dorsal closure and are weakly ventralized, but differentiate a normal amnioserosa (Arora et al., 1995; Grieder et al., 1995; Staehling-Hampton et al., 1995). In contrast, embryos that lack *dpp*, *Mad*, *Med* or the type I receptor *thickveins* (*tkv*), do not differentiate any dorsally derived structures and are strongly ventralized (Irish and Gelbart, 1987; Brummel et al., 1994; Nellen et al., 1994; Das et al., 1998; Hudson et al., 1998; Wizotskey et al., 1998). Formally, three potential mechanisms could explain the milder phenotype of *shn* mutant embryos. One possibility is that Shn acts with Mad exclusively in gene activation and is therefore only required for one aspect of Dpp function. A second model is that Shn is dedicated to Mad-mediated repression of *brk* and thus makes an indirect contribution to the activation of a subset of *dpp* target genes. A third alternative is that Shn is required for both activation of target genes as well as *brk* repression, but makes a lesser contribution compared with Mad.

We describe results showing that *shn* plays a dual role in mediating Dpp signaling during embryogenesis. Thus, our data favor the third model in which Shn affects target gene expression through *brk*-dependent and *brk*-independent mechanisms. We find that *shn* is essential both for repression of *brk* as well as activation of specific Dpp target genes during late stages of embryogenesis. Our results indicate that Shn may regulate transcription by promoting interactions between Mad and DNA and/or the transcriptional machinery. These findings provide new insights into the role of Smad-interacting factors and suggest that *shn* activity is crucial in establishing biologically relevant thresholds of gene activation.

MATERIALS AND METHODS

Mutant stocks

The mutant stocks employed in this study, shn^{P4738}/CyO; brk^{XH}, *FRT 101/FM7*; $brk^{38\text{-}20}$/FM7; brk^{M68}/FM7; tkv^{7}/CyO; tkv^{8}/CyO; and dpp^{H48}/CyO, P{*dpp-P23*} are all null or severe loss-of-function alleles (Nüsslein-Volhard et al., 1984; Padgett et al., 1993; Nellen et al., 1994; Arora et al., 1995; Campbell and Tomlinson, 1999). The P-element insert in $brk^{38\text{-}20}$ drives *lacZ* expression (Minami et al., 1999). Homozygous mutant embryos were distinguished from their heterozygous siblings by using marked balancers (*FM7*, *B*, *sn*, *ftz-lacZ*; CyO, *wg-lacZ*; *CyO*, *hb-lacZ*). The Mad^{12} null allele was used for generating germline clones (Das et al., 1998).

In situ hybridization and visualization of *lacZ* reporters

Antisense riboprobes were derived from genomic DNA fragments or cDNA clones for the following genes: *brk*, *pnr*, *ush*, *dpp*, *sna* and *lacZ*. RNA in situ hybridization was carried out with modifications, as described (Manoukian and Krause, 1992). The *lacZ* reporters used were $brk^{38\text{-}20}$, brk^{X47}, a viable enhancer trap (Campbell and Tomlinson, 1999), and Dad^{P1883}, a recessive-lethal enhancer trap line (Tsuneizumi et al., 1997). For *Scr* we used the midgut reporter *P{HZR+0.8X/H}* located on the *TM6B* balancer (Newfeld et al., 1997).

Immunohistochemistry

Antibody staining was carried out as previously described (Torres-Vazquez et al., 2000), using rat anti-dCreb-A (1:15,000; Andrew et al., 1997), monoclonal FP3.38 anti-Ubx (1:20; White and Wilcox, 1984) and monoclonal anti-β-gal (1:2000; Promega). Primary antibodies were detected using the appropriate alkaline phosphatase-conjugated secondary antibody (1:5000; Promega) or Vectastain kit (Vector Laboratories).

Cuticle preparations

Appropriately aged embryos were dechorionated and cleared in a Hoyers-lactic acid 1:1 mixture at 65°C, and photographed using dark-field optics.

RNA injections

Precellular blastoderm stage embryos carrying the brk^{X47} reporter were injected under halocarbon oil using a Narishige IM300 microinjector (60-70 pL/embryo). A modified *tkv* cDNA corresponding to a constitutively activated form of Tkv (TkvA) was used for in vitro transcription reactions (Nguyen et al., 1998). Injected embryos were maintained at 25°C until the required stage, recovered from the halocarbon oil, fixed and processed to visualize *lacZ* expression.

Heat-shock experiments

To induce expression of P{HS-*dpp*.BP} 0-15 hour egglays of the appropriate genotype were subjected to two 1 hour heat shocks at 37°C separated by a 1 hour recovery at 25°C (Twombly et al., 1996). Following this, the embryos were aged 3 hours and stained to visualize either *brk-lacZ* or *sna* expression. The brk^{X47}, brk^{X47}; shn^{P4738}, and brk^{XH}; shn^{P4738} stocks carried one copy of P{HS-*dpp*.BP}.

RESULTS

shn is required for repression of *brk* in late embryogenesis

In the embryo, *brk* transcription is restricted to a broad stripe of ventrolateral cells on either side of the prospective mesoderm by two distinct regulatory mechanisms. Initially *brk* is activated in a localized manner by the maternal morphogen Dl, while in a later zygotic phase it is ubiquitously activated but confined to ventrolateral cells as a result of Dpp-mediated repression in dorsal cells. Thus, in *dpp*-null embryos, *brk* expression is initiated correctly but becomes derepressed throughout the dorsal region by stage 8 (Fig. 1A,B; staging according to Campos-Ortega and Hartenstein, 1985; Jazwinska et al., 1999b). At cellular blastoderm, *shn* is transcribed in the dorsal half of the embryo in a pattern similar to that of *dpp* and complementary to that of *brk* (Arora et al., 1995). To test whether *shn* activity is required for *brk* repression, we examined the distribution of *brk* mRNA in *shn* mutant embryos. We found that the upregulation of *brk* expression in *shn* mutants occurs only at late stage 10/stage 11 (Fig. 1C,D). Ectopic *brk* transcript is first detected in a subset of dorsal cells in a segmental repeat pattern, in addition to its normal expression in the ventral neurogenic ectoderm. By late stage 11 this periodicity is obscured and *brk* mRNA is uniformly distributed throughout the mutant embryo in both dorsal and ventral ectodermal cells (Fig. 1E,F). The delay in *brk* derepression in *shn* mutants compared with *dpp* nulls indicates that although *shn* is required for Dpp-dependent downregulation of *brk*, it may not be essential early in development.

We next examined the requirement for *shn* activity in restricting *brk* transcription in the developing gut. In germband-retracted embryos, *brk* expression can be detected in three distinct sites in the gut visceral mesoderm and endoderm, that are broadly complementary to sites of *dpp* expression in parasegment 3 (ps3) and ps7 (Fig. 1G; Jazwinska et al., 1999b). At this stage, *shn* mRNA is distributed throughout the gut but is enriched in regions that show high levels of *dpp* transcript (Arora et al., 1995). Analysis of homozygous *shn*-null embryos revealed uniform *brk* expression along the entire length of the visceral mesoderm and endoderm, indicating that Shn is also involved in negative regulation of *brk* transcription in the midgut (Fig. 1H).

Phenotypic analysis indicates a role for *shn* in the absence of *brk* activity

We have previously shown that Shn can directly stimulate transcription of a Dpp target gene (Dai et al., 2000). The derepression of *brk* in *shn* mutants indicates that another mechanism by which Shn could induce target gene expression is by eliminating Brk. To test whether *shn* provides a significant positive input to *dpp* signaling through a *brk*-independent mechanism we examined the phenotype of embryos lacking both *shn* and *brk* function. If *shn* acts solely by inhibiting *brk*, the *brk*; *shn* double mutants would be expected to resemble *brk* mutants, i.e., loss of *shn* activity should have no additional consequences in a brk^- background. Alternatively, if Shn affects patterning independently of *brk*, the double mutants would be predicted to have an intermediate phenotype.

The larval cuticle provides excellent markers for cell fate along the D/V axis that are sensitive to alterations in Dpp activity. Peak levels of Dpp signaling result in specification of the amnioserosa, while intermediate levels promote development of the dorsal ectoderm and suppress differentiation of the ventral neurogenic ectoderm. In the larval cuticle the dorsal ectoderm can be distinguished by the presence of fine dorsal hairs, while the ventral ectoderm is characterized by heavily pigmented denticles (Fig. 2A). The greater efficacy of Dpp signaling in the absence of the repressor *brk* results in an expansion of dorsal fates and a concomitant loss of ventral ectoderm that is reflected in the reduced width of the ventral denticle belts (Fig. 2B; Jazwinska et al., 1999b; Lammel et al., 2000). In contrast, abolition of *shn* activity results in loss of dorsally derived ectoderm and a modest expansion of ventral cell fates. The mis-specification of the dorsalmost epidermal cells in *shn* mutants causes a failure of dorsal closure and a 'dorsal open' phenotype in which the internal organs are often extruded (Fig. 2C; Nüsslein-Volhard et al., 1984; Arora et al., 1995; Grieder et al., 1995; Staehling-Hampton et al., 1995). A striking aspect of the *brk*; *shn* double mutant phenotype is that the dorsal closure defect is ameliorated and the dorsal epidermis is contiguous (Fig. 2D). Based on this criterion, *brk* function appears epistatic to *shn*. However, a detailed analysis reveals that *shn* is epistatic in other aspects of the cuticle phenotype. We find that in *brk*; *shn* double mutants the dorsal epidermis is reduced and the ventral denticle belts are wider compared to *brk* null embryos (Fig. 2B,D). Thus, the double mutants are more ventralized than brk^- embryos, but less ventralized than *shn* mutants. This intermediate phenotype is remarkably similar to that caused by the partial loss-of-function shn^{IM56} allele at 18°C (Arora et al., 1995). We used the location of the bilaterally symmetric Keilin's organs (KO) and the ventral black dots (VBDs), sensory organs that differentiate at specific positions on either side of the ventral midline, to estimate changes in the size of the ventrolateral ectoderm (Fig. 2E-G; Campos-Ortega and Hartenstein, 1985). Consistent with the loss of ventral fates in *brk* mutants, the paired KOs and the VBDs are approximately 50% closer in brk^- larvae compared with wild type (Fig. 2E,F). The ventralization of *brk*; *shn* double mutants is apparent in the increased distance between the KOs, relative to both *brk* mutant and wild-type larvae (Fig. 2F,G). The expansion of ventral fates in the double mutants is associated with a coordinate reduction in the dorsal ectoderm compared with *brk*-null embryos. In addition we observe a partial loss of the dorsolaterally derived Filzkörper in the double mutant, a defect that also occurs in shn^- but not brk^- embryos (Fig. 2B-D). Thus, our data indicate that specification of dorsal cell fates requires a contribution from *shn* that is independent of *brk* function.

shn does not regulate *dpp* target gene expression in early embryogenesis

The intermediate phenotype of the double mutants suggested that *brk* and *shn* do not act in a linear pathway and that *shn* is involved in induction of Dpp target genes independently of its role in mediating *brk* repression. In order to address this issue at the level of gene regulation, we examined the transcription of several Dpp-responsive genes in the ectoderm at different stages of embryogenesis. We first assayed the expression of

two genes, *u-shaped* (*ush*) and *pannier* (*pnr*), which are differentially regulated by *dpp* signaling in the early blastoderm (Winick et al., 1993; Cubadda et al., 1997). *ush* mRNA is restricted to a small group of cells straddling the dorsal midline, where it is activated in response to peak levels of *dpp* and is insensitive to loss or overexpression of Brk. In contrast, the wider domain of *pnr* expression requires both positive input from Dpp signaling as well as relief of repression by *brk* (Jazwinska et al., 1999b; Ashe et al., 2000). We find that loss of *shn* does not affect expression of either *ush* or *pnr* at the blastoderm stage. In *brk*; *shn* double mutants, the expression of these genes is essentially similar to that seen in *brk*$^{-}$ animals, i.e. *ush* is unaltered and *pnr* is expanded ventrally relative to wild type (Fig. 3A-C; and data not shown). These results indicate that *shn* may not contribute to Dpp-mediated specification of dorsal cell fates prior to gastrulation, and are consistent with the fact that *shn* mutant embryos do not display defects in early D/V patterning.

shn and brk provide differential inputs to Dpp-responsive gene expression during germband extension

At later stages of embryogenesis Dpp signaling is required for the maintenance of dorsal ectodermal fates as well as for patterning larval and adult precursors. Mutations in *shn* affect the expression of several Dpp target genes during this period and result in mis-specification of dorsolateral cell fates, similar to that caused by zygotic loss of the Dpp receptors *tkv* and *punt* (*put*) (Affolter et al., 1994; Brummel et al., 1994; Arora et al., 1995; Grieder et al., 1995; Letsou et al., 1995; Ruberte et al., 1995; Staehling-Hampton et al., 1995). We therefore assayed the requirement for *shn* and *brk* activity in these Dpp-mediated events.

In stage 11 embryos, the expression patterns of *dpp* and its target genes, *pnr* and *daughters against dpp* (*Dad)*, define three overlapping domains of increasing width in the dorsal ectoderm (Fig. 4A,E,I). *dpp* is transcribed in a single row of cells at the border between the epidermis and the amnioserosa providing a cell fate marker for the 'leading edge' of the dorsal ectoderm during germband extension. In *shn*$^{-}$ mutants the level of *dpp* mRNA in these cells is severely reduced (Fig. 4B). Likewise, *pnr* expression is correctly initiated and refined, but the transcripts are lost prematurely (see Fig. 3B; Fig. 4F; Grieder et al., 1995). In addition, *Dad-lacZ* expression in the dorsal ectoderm is seen in fewer cells compared with wild-type embryos (Fig. 4I,J). Absence of *brk* activity affected transcription of two of the three marker genes. In *brk* null mutants the dorsal stripe of *dpp* expression appears unaltered, but the *pnr* and *Dad* expression domains are wider relative to wild type (Fig. 4C,G,K). Analysis of embryos lacking both *brk* and *shn* function revealed a differential requirement for *shn* activity in the regulation of these markers. We observed that *dpp* transcription in the 'leading edge' cells is restored to near wild-type levels in the double mutant (Fig. 4D). This is striking given the premature loss of *dpp* mRNA in these cells in *shn*$^{-}$ embryos (Fig. 4B), and suggests that *dpp* transcription at this location is primarily dependent on repression of *brk*. Expression of *pnr* and *Dad* in the dorsal ectoderm was also recovered in the double mutants, although the domains are narrower in *brk*; *shn* embryos relative to *brk* mutants (Fig. 4H,L; compare with Fig. 4G,K). This effect is readily apparent in ventral views of stage 13 embryos. In *shn* mutants, the two bands of *Dad*-expressing cells that flank the ventral neurogenic ectoderm are located further apart compared with wild type, consistent with their partially ventralized phenotype (Fig. 4M,N; Arora et al., 1995). Conversely, in embryos that lack *brk* activity, the ventral ectoderm is significantly reduced owing to expansion of dorsal cell fates (Fig. 4O). It is evident that in *brk*; *shn* double mutants, the distance between the two domains of *Dad* expression is midway between that observed in either single mutant, and equivalent to wild-type embryos (Fig. 4P). Thus, the alterations in gene expression in dorsal cells are correlated with corresponding changes in the size of the ventral region. Collectively, these data show a differential requirement for *shn* and *brk* function in the regulation of three Dpp-responsive genes in the dorsal ectoderm. While the absence of *brk* activity is sufficient for *dpp* expression in the 'leading edge',

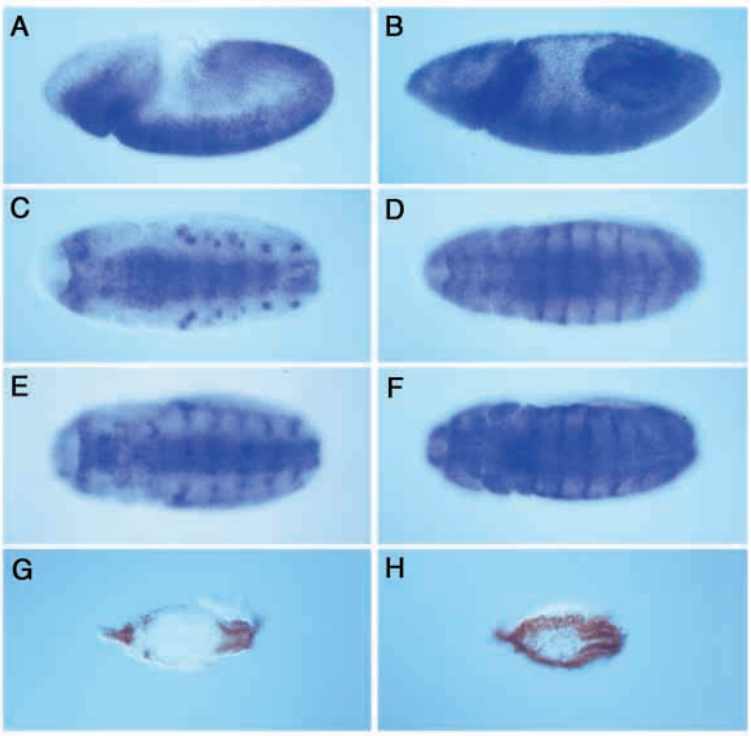

Fig. 1. *shn* is required for repression of *brk* transcription. Distribution of *brk* mRNA in wild type (A,C,E), *dpp*^{H48} (B) and *shn*^{P4738} (D,F) mutants. All embryos are oriented with their anteriors towards the left. (A,B) At stage 8, *brk* expression is restricted to the ventrolateral region in wild-type (A) embryos, while in *dpp* mutants (B) *brk* transcripts are distributed throughout the ectoderm. Ventral views of wild-type embryos at (C) early and (E) late stage 11, showing *brk* expression in the ventral neuroectoderm and in a segmentally reiterated pattern corresponding to cells surrounding the tracheal pits. (D) *shn* mutant embryos at early stage 11 initially show *brk* derepression in a small subset of dorsal cells in each segment, and (F) eventually express *brk* throughout the embryo by late stage 11. (G,H) *brk-lacZ* expression in the dissected gut. (G,H) In wild type (G), three distinct domains of expression can be detected in the gut visceral mesoderm and endoderm, while in *shn* mutants (H), *brk-lacZ* expression is contiguous along the length of the gut.

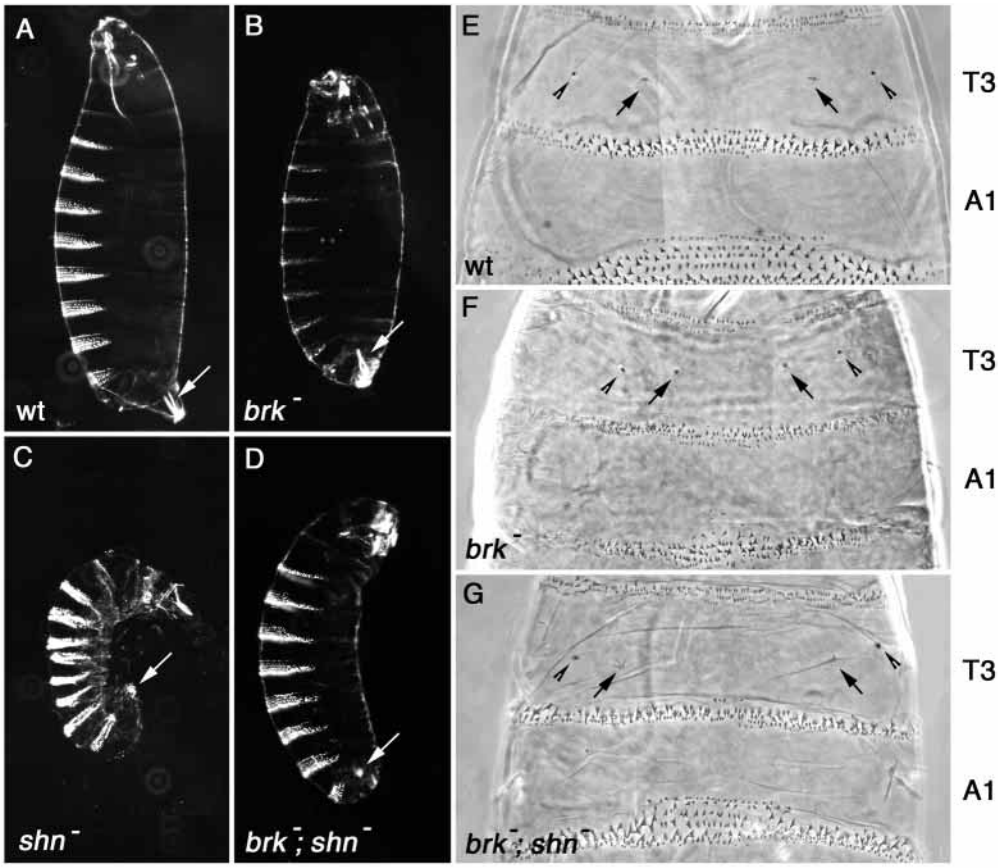

Fig. 2. *shn* and *brk* make distinct contributions to patterning the ectoderm. (A-D) Dark field images showing lateral views of larval cuticles oriented with their anteriors towards the top and ventral towards the left. (A) Wild-type larva showing strongly pigmented denticle belts that derive from the ventral ectoderm, while the dorsal ectoderm is characterized by fine dorsal hairs. The dorsolaterally derived Filzkörper are marked with white arrows. (B) In brk^{XH} mutants the dorsal ectoderm is expanded and there is a corresponding loss of ventral epidermis that is apparent in the reduced width of the denticle belts. Filzkörper differentiate normally. (C) shn^{P4738} mutants display a 'dorsal open' phenotype, owing to loss of dorsal cell fates and are partially ventralized. (D) brk^{XH}; shn^{P4738} double mutant embryos have an intermediate phenotype. The dorsal hole typical of *shn* mutants is rescued, but the dorsal ectoderm is significantly reduced compared with *brk*-null animals and the cuticle is relatively more ventralized. Both in *shn* as well as in *brk*; *shn* larvae the Filzkörper are reduced. (E-G) High magnification views using phase contrast optics to detect changes in size of the ventral ectoderm in wild type (E), brk^{XH} (F) and brk^{XH}; shn^{P4738} (G) larvae. The third thoracic segment (T3) and the first abdominal segment (A1) are in view. The black arrows mark the paired Kielin's organs, while the arrowheads point to the ventral black dots (VBDs) that are located anterior to T3, but are absent from the abdominal segments (E). In *brk* mutants (F), the distance between these bilaterally symmetrical sense organs indicates the loss of ventral cell fates due to expansion of the dorsal epidermis. (G) By comparison, the *brk*; *shn* double mutant larva is relatively ventralized. In addition, the first row of anterior facing denticles in A1 (typically absent in *brk* mutants) is recovered in the double mutants.

expression of *pnr* and *Dad* reveals an additional positive input from *shn*. These results support our analysis of the cuticle phenotype of the double mutant and suggest that the *brk*-independent contribution from *shn* may be crucial for achieving the threshold response in cells that receive low levels of *dpp* signaling, rather than for maximal levels of expression (see Discussion).

At late stages of embryogenesis, *dpp* is involved in establishing the anlagen of structures such as the salivary glands and the imaginal discs. Dpp signaling restricts the salivary gland primordia to a small group of ventrolateral ectodermal cells in ps2, and loss of *dpp* or *shn* activity results in dorsal expansion of genes such as *dCreb-A* (CrebA – FlyBase) that mark the salivary glands (Fig. 5A,B; Henderson et al., 1999). Conversely, loss of *brk* activity leads to a reduction in the size of this domain (Fig. 5C; Lammel et al., 2000). *brk*; *shn* double mutant embryos display a phenotype essentially similar to that of *brk* mutants (Fig. 5D), indicating that the correct spatial restriction of *dCreb-A* does not require a *brk*-independent input from *shn*. Next we examined the requirement for *shn* and *brk* in specification of the wing and haltere disc primordia. *dpp* expression in a cluster of lateral cells in the second and third thoracic segments (T2 and T3) is crucial for allocation of these imaginal discs. The highest levels of Dpp signaling in the dorsal most cells within these domains result in induction of the disc primordia that are marked by expression of the transcription factor *snail* (*sna*) in T2 and T3, respectively (Fig. 5E; Goto and Hayashi, 1997). Disruption of *dpp* signaling at late stages, such as in zygotic *tkv* mutants, results in loss of *sna* expression and the failure to specify imaginal disc cells (Goto and Hayashi, 1997). In situ hybridization experiments showed that these domains of *sna* expression are absent in mutants lacking *shn* activity, but are enlarged in brk^{-} embryos (Fig. 5F,G). Strikingly, in *brk*; *shn* double null embryos *sna* expression is essentially abolished in the imaginal disc primordia, similar to shn^{-} embryos (Fig. 5H). These data provide evidence that *shn* contributes a *brk*-independent input to activation of *sna* transcription and is necessary for specification of the wing and haltere discs, even in the absence of repression by Brk.

Dpp-responsive gene expression in the visceral mesoderm incorporates *brk*-dependent and *brk*-independent inputs from *shn*

In addition to its role in specification of ectodermal cell fates, *dpp* is involved in patterning both the visceral mesoderm and the underlying endoderm of the embryonic midgut. Dpp signaling in these tissues is implicated in regulation of its own

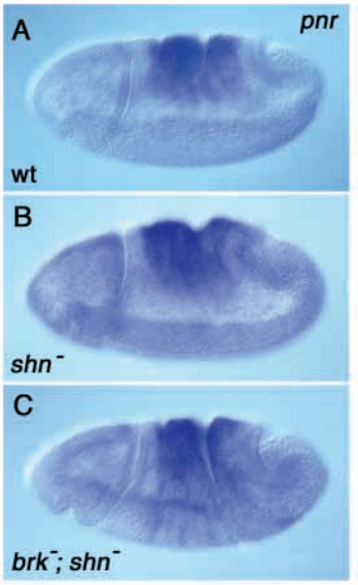

Fig. 3. Dpp-responsive gene activation in early embryogenesis. Distribution of *pnr* mRNA in stage 7 embryos arranged with anterior towards the left and dorsal side upwards. (A) In wild-type embryos, Dpp signaling initiates *pnr* transcription in the dorsal blastoderm cells. (B) In shn^{P4738} mutants, *pnr* expression is unaltered, while in brk^{-} embryos (not shown), and in brk^{XH}; shn^{P4738} double mutants (C), *pnr* is upregulated in ventrolateral cells.

expression as well as that of genes such as *Ubx* and *Sex combs reduced* (*Scr*) (reviewed by Bienz, 1997). The upregulation of *brk* throughout the visceral mesoderm in shn^{-} embryos (Fig. 1H), raises the possibility that the loss of Dpp-responsive gene expression in the midgut (Arora et al., 1995; Grieder et al., 1995: Staehling-Hampton et al., 1995) could be at least partially due to repression by Brk. We therefore assayed the relative contribution of *brk* and *shn* to regulation of gut-specific target genes.

In germband retracted embryos, *dpp* is expressed at several sites in the gut including the visceral mesoderm of ps3 and ps7 where its transcription is subject to autoregulation (Fig. 6A;

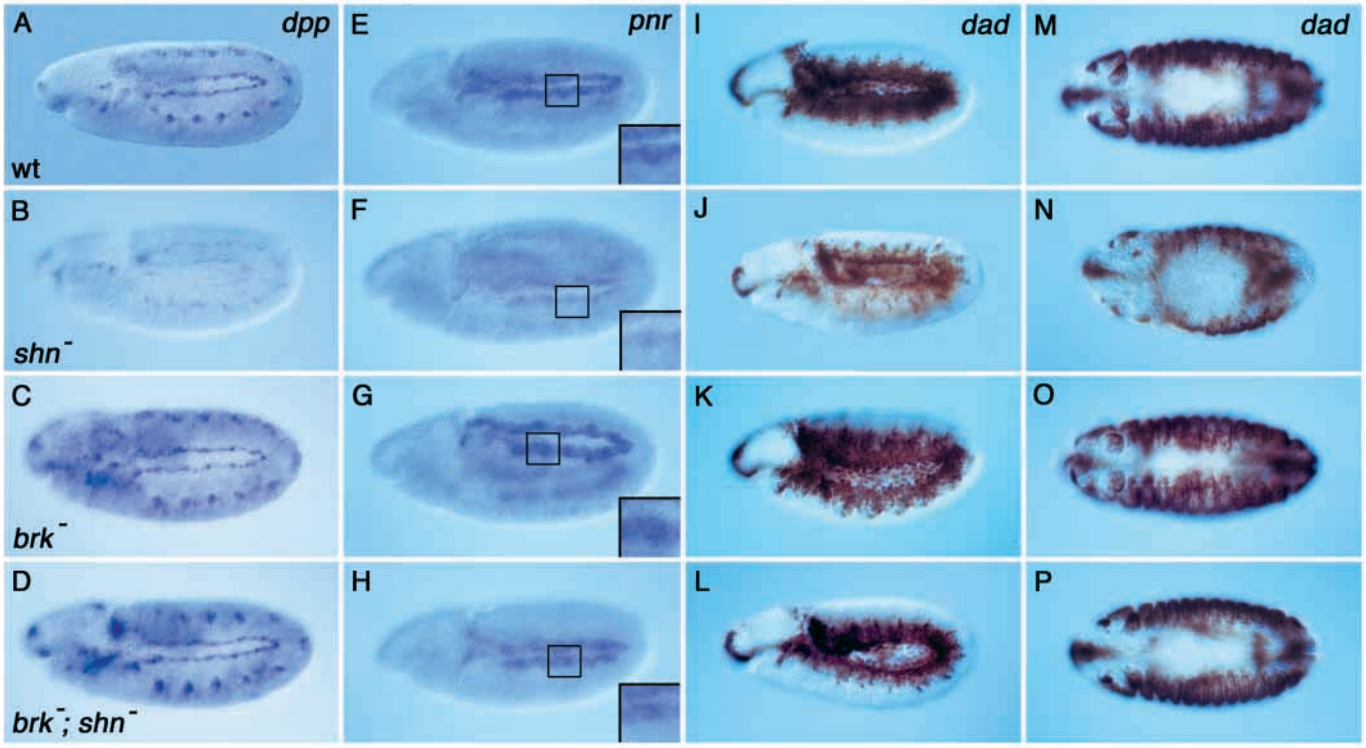

Fig. 4. Expression of ectodermal markers in embryos lacking *shn* and *brk* activity. (A-L) Lateral views of germband extended embryos at stage 11 and (M-P) ventral views of stage 13 embryos after germband retraction. The genotype of the embryos is indicated in the left bottom corner of each row and the top right corner in each column indicates the marker assayed. Boxed areas are shown at high magnification in the inset. (A) In wild-type embryos, *dpp* transcripts can be seen in a single row of cells at the 'leading edge' of the dorsal ectoderm, as well as in a reiterated pattern in lateral cells. *dpp* expression in the 'leading edge' is not maintained in shn^{P4738} mutant embryos (B), but is unaltered in brk^{XH} embryos (C). brk^{XH}; shn^{P4738} double mutants (D) show wild-type levels of *dpp* expression at the 'leading edge'. (E) *pnr* mRNA is present at high levels in the dorsal epidermis in wild-type animals. (F) In *shn* mutants, *pnr* is initiated correctly but is eventually lost at germband extension. (G) In *brk* mutants, the domain of *pnr* expression is expanded relative to wild-type animals, while (H) in *brk*; *shn* double mutants *pnr* is localized to a narrow region when compared with *brk* mutants. In wild-type embryos, expression of *Dad-lacZ* can be detected throughout the dorsal ectoderm and amnioserosa at germband extension (I), as well as after germband retraction (M). In *shn* mutant embryos at comparable stages (J,N), *Dad* expression in the dorsal ectoderm is severely reduced (staining in the amnioserosa persists). In embryos lacking *brk* (K,O), the dorsal ectodermal domain is expanded relative to wild type. This expansion is not maintained in double mutant animals (L,P), as is evident in ventral views of embryos at stage 13. The bilateral domains of *Dad-lacZ* expressing cells are expanded (and hence closer together) in brk^{-} animals (O), compared with *brk*; *shn* double mutants (P).

Hursh et al., 1993). Embryos mutant for *shn* lack *dpp* mRNA at both these locations (Fig. 6B). In contrast, *brk* mutant embryos show expanded *dpp* expression in ps7 (Fig. 6C). We did not observe a comparable expansion in ps3, although the small size of this domain may preclude detection of a modest increase in the number of cells that express *dpp*. In *brk*; *shn* embryos, *dpp* expression in both domains is affected. This is most striking in ps3, where *dpp* transcription is essentially lost in the double mutant. Expression in ps7 is significantly reduced, not only compared with *brk* mutants but also relative to the levels encountered in wild-type animals (compare Fig. 6A with Fig. 6D). The changes in *dpp* expression in ps7 were quantified using a micrometer reticule. Analysis of at least 30 embryos of each genotype revealed that in *brk*$^{-}$ animals expression in ps7 was increased by 15%, while in the double mutants the domain was reduced by 22% compared with wild type. The compromised *dpp* expression in the double mutant demonstrates that *shn* activity is required for wild-type levels of *dpp* transcription, even in the absence of Brk. It is well documented that *dpp* expression in ps7 promotes the transcription of the DNA-binding protein Ubx (Fig. 6E), that in turn stimulates transcription of *dpp* (Bienz, 1997). In embryos mutant for *shn*, Ubx fails to accumulate in ps7 (Fig. 6F; Grieder et al., 1995; Dai et al., 2000). Analysis of embryos that lack both *brk* and *shn* activity revealed lower levels of Ubx expression compared with *brk* mutant and wild-type embryos (Fig. 6G,H), further implicating Shn in the direct induction of *Ubx* by Dpp signaling.

We also assayed expression of *Scr* in the gut visceral mesoderm, where it is restricted to ps4 owing to negative regulation by Dpp signaling in ps3 (Fig. 6I; Newfeld et al., 1997). In *Mad* mutants and *dpp* alleles that specifically affect gut development, *Scr* expression expands anteriorly to include cells of ps3. Conversely ectopic activation of the Dpp pathway eliminates *Scr* expression in its endogenous domain in ps4 (Newfeld et al., 1997). Using a β-gal reporter we find that in embryos lacking *brk* activity, *Scr* expression in ps4 is severely reduced (Fig. 6K). Interestingly, in *shn* as well as *brk*; *shn* double mutants, *Scr* is expressed in a larger domain that includes ps3 and ps4, indicating that Shn function is epistatic with respect to this Dpp target gene as well (Fig. 6J,L). As it is not known whether *Scr* is directly repressed by *dpp* signaling, our data could reflect an essential requirement for *shn* in activation of a Dpp target gene in ps3 that in turn downregulates *Scr* transcription. In summary, analysis of Dpp-responsive genes at different embryonic stages and in different tissues provides compelling evidence that *shn* contributes a *brk*-independent input to gene regulation in a stage and promoter-specific manner.

tkv makes a greater contribution to gene activation relative to *shn*

We wished to assess the input to dorsal patterning mediated by *shn* relative to the total contribution from Dpp signaling at comparable stages of development. This is not straightforward, as the requirement for *dpp* in early D/V patterning prevents the use of null alleles to analyze its role in late embryogenesis. One way to circumvent this problem is by examining the consequences of zygotic loss of *tkv* function that result in a mutant phenotype very similar to that of *shn* null embryos (Fig. 7A). In situ hybridization experiments revealed that *brk* transcription was upregulated in *tkv* mutants at stage 11 comparable with *shn*-null animals (data not shown). We also analyzed the phenotype resulting from simultaneous loss of *brk* and *tkv*, both at the level of the differentiated cuticle and gene activation. Interestingly, we find that *brk*; *tkv* larvae resemble *brk*; *shn* animals, in that the 'dorsal open' phenotype typical of zygotic *tkv* mutants is rescued, the Filzkörper are reduced, and the ventral ectoderm is expanded compared with *brk* mutants (Fig. 7B; see Fig. 2D). The restoration of dorsal closure and the differentiation of a contiguous dorsal ectoderm in the *brk*; *tkv* double mutants is notable because it suggests that in this aspect of *dpp* function *brk* is epistatic not only with respect to *shn*, but also to *tkv*. This was confirmed by examining *dpp* transcription in the

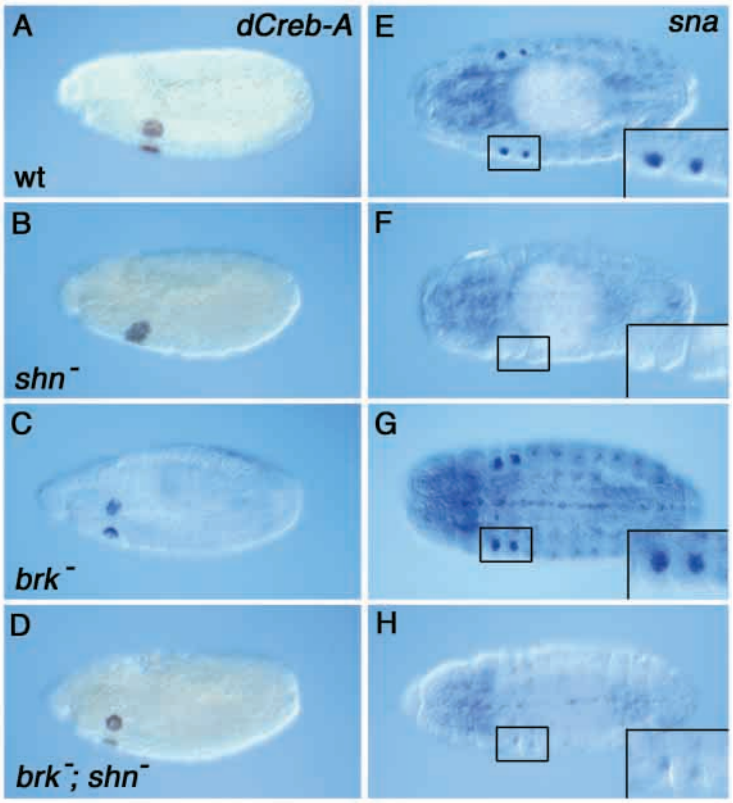

Fig. 5. Analysis of *dCreb-A* (*CrebA* – FlyBase) and *sna* expression in *shn* and *brk* mutant animals. (A-D) Lateral views of stage 11 embryos stained with antibody against dCreb-A, and (E-H) ventral views of stage 13 embryos hybridized with probe for *sna* mRNA. The genotype is indicated in the bottom left-hand corner of each row and the top right-hand corner in each column indicates the marker assayed. Boxed areas are shown at high magnification in the inset. (A) Expression of *dCreb-A* in the ventrolateral region marks the salivary gland primordia in wild-type embryos. (B,C) *dCreb-A* expression domain is enlarged (B) in shn^{P4738} mutants and reduced (C) in brk^{XH} embryos. (D) In brk^{XH}; shn^{P4738} embryos, *dCreb-A* expression is essentially similar to that in *brk* mutants. (E) In wild-type embryos *sna*-expressing cells in the second and third thoracic segments mark the wing and haltere disc primordia, respectively. (F) Embryos that lack *shn* activity fail to express *sna* at these locations, while (G) *brk* mutants show an increase in the size of these domains. (H) *sna* is expressed at extremely low levels in double mutant embryos that lack both *brk* and *shn* activity.

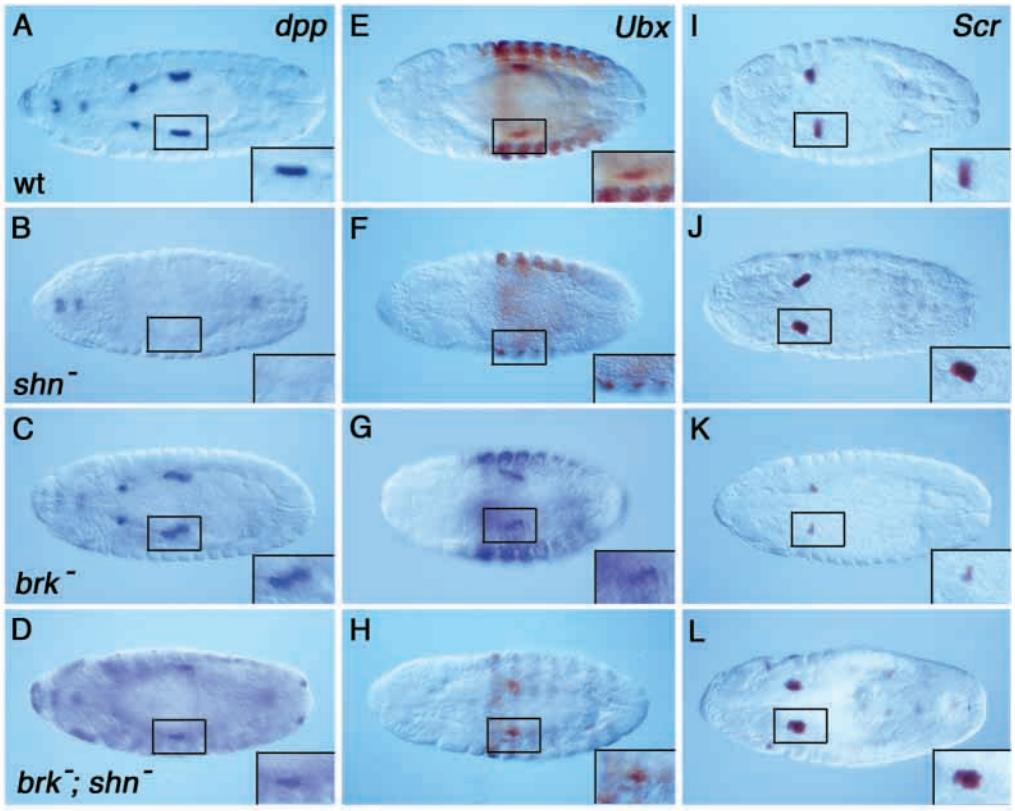

Fig. 6. Dpp target genes in the midgut incorporate *brk*-dependent and *brk*-independent inputs from *shn*. (A-L) Dorsal views of germband retracted embryos at stage 13 displaying Dpp-responsive gene expression in the visceral mesoderm. The genotype of the embryos is indicated in the bottom left-hand corner of each row, and the top right-hand corner in each column indicates the marker assayed. Boxed areas are shown at high magnification in the inset. (A-D) In situ hybridization to visualize *dpp* mRNA. (A) In wild-type embryos, *dpp* transcription in ps3 and ps7 of the midgut is maintained through an indirect autoregulatory loop, and (B) expression at both sites is lost in shn^{P4738} embryos. (C) In brk^{XH} animals the ps7 domain is expanded, while expression in ps3 is essentially unaffected. (D) In embryos that lack *brk* and *shn* activity, *dpp* transcription in ps7 is detected in a smaller domain and at lower levels compare with wild type, while expression in ps3 is invariably reduced and sometimes undetectable. (E) Ubx protein is present in the visceral mesoderm of ps7 as well as in the ectoderm in wild-type embryos. Expression is lost in *shn* mutants (F), marginally increased in brk^{XH} embryos (G), and can be detected at intermediate levels in the double mutant (H). (I-L) Anti-β-gal staining to visualize expression of an *Scr* reporter in the midgut. (I) In wild-type animals, *Scr-lacZ* expression is restricted to ps4 of the midgut, owing due to downregulation by Dpp signaling in ps3. (J) *Scr* expression is expanded anteriorly in the absence of *shn*. (K) In *brk* mutant animals, the *Scr* domain is reduced, but brk^{XH}; shn^{P4738} double mutants (L) express *Scr-lacZ* in a broader region, as seen in *shn* mutants.

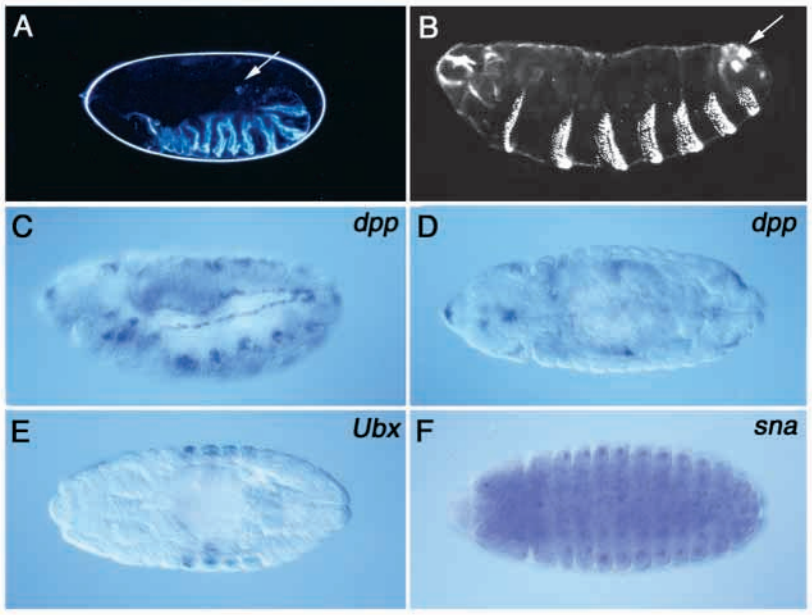

Fig. 7. Elimination of *brk* function partially compensates for the loss of *tkv* activity. (A-B) Dark field images of larval cuticles from tkv^{8} and brk^{XH}; tkv^{8} double mutant animals. Anterior is towards the left and dorsal is upwards. (A) The loss of zygotic *tkv* activity results in a 'dorsal open' cuticle phenotype that closely resembles that of *shn* null animals (see Fig. 2C). Failure of dorsal closure results in a prominent dorsal hole, which is rescued in *brk*; *tkv* double mutants (B). Double mutant larvae differentiate a contiguous dorsal epidermis, but display reduced Filzkörper (arrow) and are more ventralized than *brk* null animals (see Fig. 2B). (C-F) Expression of Dpp-responsive genes in *brk*; *tkv* double mutants. The marker assayed is indicated in the top right corner in each column. (C) *dpp* transcription is restored to near wild-type levels in cells at the leading edge of the dorsal ectoderm in brk^{M68}; tkv^{7} mutants. (D) In contrast, *dpp* mRNA in ps3 and ps7 and (E) Ubx expression in ps7 of the gut visceral mesoderm is at residual levels in the double mutants. (F) Likewise, *sna* transcription is essentially abolished in the second and third thoracic segments of double mutant embryos. For expression in wild-type and *brk* mutant controls refer to Figs 4-6.

'leading edge' of the dorsal ectoderm. Although *dpp* expression at this site is abolished in *tkv* mutants (Affolter et al., 1994), it is restored to wild-type levels in *brk*; *tkv* animals, as observed in *brk*; *shn* mutants (Fig. 7C; see Fig. 4D). We next analyzed expression of three genes that require a *brk*-independent positive input from *shn*. It has been previously

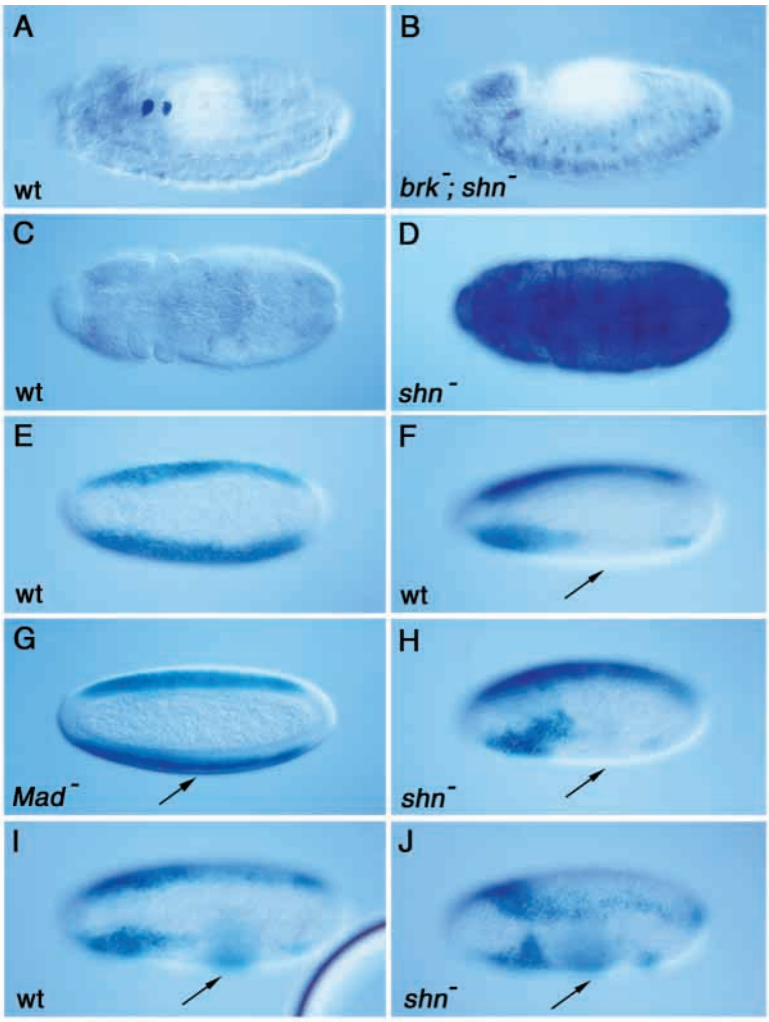

Fig. 8. Regulation of *brk* by ectopic Dpp signaling. (A,B) Ubiquitous Dpp cannot induce *sna* expression in the absence of *shn* activity. Embryos are oriented anterior to the left and viewed ventrally. Genotypes are as marked. Induction of Dpp results in expansion of *sna* expression in the imaginal disc primordia in T2 and T3 in a stage 13 wild-type embryo (A), but is ineffective in inducing *sna* expression in a *brk*; *shn* double mutants (B). (C,D) Downregulation of *brk* in response to ectopic Dpp signaling in extended germband embryos at stage 11. Heat-shock induction of Dpp results in almost complete loss of *brk-lacZ* reporter gene expression in wild-type animals (C), but not in shn^{P4738} mutants (D). (E-H) Response of *brk* to ectopic Dpp signaling in stage 5/6 embryos. Embryos are oriented anterior to the left, ventral up. (E) Expression of a *brk-lacZ* reporter can be detected in two ventrolateral stripes of cells that flank the presumptive ventral mesoderm (not stained) in a control animal. Injection of constitutively activated Tkv receptor (TkvA) mRNA at 10 ng/μl is equally effective in repressing *brk-lacZ* expression in wild-type (F) and shn^{-} (H), but not in *Mad*-null embryos (G). Embryos were always injected laterally on the same side (arrow marks site of injection), thus the opposite uninjected side serves as an internal control. Interestingly, both in wild type (I) and *shn* mutants (J), injection of higher concentrations of TkvA mRNA (50 ng/μl) results in localized *brk* activation at the site of injection (below the plane of focus). In both backgrounds, *brk-lacZ* expression in its endogenous domain (in the plane of focus) continues to be repressed.

shown that tkv^{-} embryos lack expression of *dpp* and *Ubx* expression in the gut (Affolter et al., 1994). In the *brk*; *tkv* double mutants, only residual expression of these genes can be detected (Fig. 7D,E). Likewise, expression of *sna* in the primordia of the wing/haltere imaginal discs is lost in *tkv* mutants, and is only marginally recovered in embryos that lack *brk* and *tkv* (Fig. 7F; Goto and Hayashi, 1997). Although these Dpp-inducible genes were expressed at low levels in *brk*; *shn* double mutants as well, the loss is more severe in *brk*; *tkv* animals, indicating a greater contribution from *tkv* relative to *shn* in gene activation. This is in contrast to *dpp* expression in the 'leading edge' cells, where *shn* and *tkv* appear to contribute equally to relief of *brk* repression.

Shn is essential for Dpp-mediated activation of *sna* and repression of *brk*

We have shown that Shn contributes to gene expression through both *brk* repression and direct activation. However, an important question is whether Shn is essential in either of the mechanisms by which Dpp-responsive genes are induced. To assess the requirement for *shn* in gene activation, we examined expression of *sna*, a gene that shows severely reduced transcription in *brk; shn* double mutants compared with wild-type embryos (see Fig. 5). Ubiquitous expression of Dpp using a heat-shock promoter results in expansion of *sna* expression in T2 and T3 in wild-type embryos (Fig. 8A). In contrast, excess Dpp was unable to induce *sna* transcription in double mutant embryos that lacked both *shn* and the repressor *brk* (Fig. 8B). This result demonstrates that *shn* is obligately required for activation of *sna* by Dpp.

We used a similar approach to determine whether Shn was essential for repression of *brk*. Overexpression of Dpp after gastrulation is sufficient to repress endogenous *brk* in a wild-type background (Fig. 8C). However, in *shn* mutants, *brk* continues to be expressed widely even after induction of Dpp (Fig. 8D). From this experiment we conclude that *shn* is strictly required for Dpp-mediated repression of *brk* during late embryogenesis. In light of the above data, the fact that *brk* derepression occurs at an earlier stage in *dpp*-null embryos (stage 8) compared with *shn* mutants (stage 11), was puzzling (see Fig. 1B,D). To test whether *shn* is redundant for Dpp-mediated repression of *brk* during early development, we injected mRNA encoding an activated form of the Dpp type I receptor Tkv (TkvA) into precellular blastoderm stage embryos (Nguyen et al., 1998), and analyzed *brk-lacZ* expression at stage 5/6 (Fig. 8E-H). Embryos were always injected ventrolaterally on one side, allowing reporter gene expression on the opposite side to serve as an internal control. As seen in Fig. 8F, ectopic activation of the Dpp pathway in wild-type embryos results in inhibition of *brk* expression

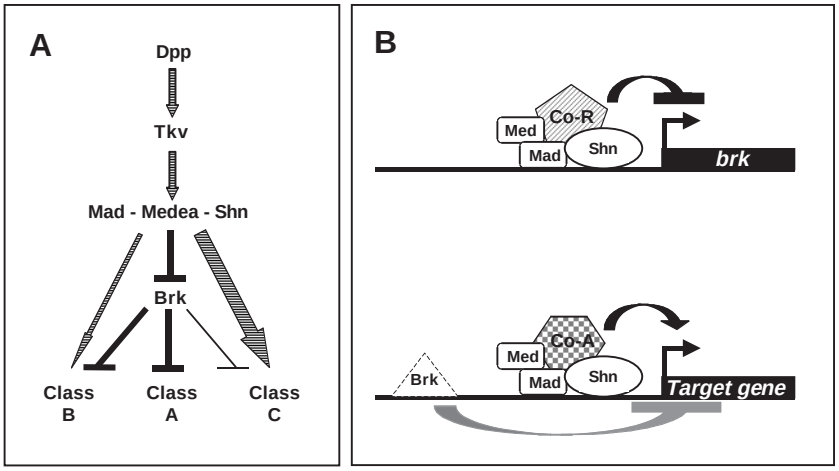

Fig. 9. Shn affects Dpp target genes through a dual mechanism. (A) Binding of Dpp to Tkv triggers Mad phosphorylation and accumulation of Mad/Med in the nucleus where they interact with Shn. The tripartite complex is involved both in repression of *brk* transcription as well as in activation of Dpp target genes. Downstream responses can be assigned to three classes based on their requirement for *shn* and *brk* activity. *shn* contributes indirectly to the expression of class A genes, through relief of *brk* repression. Promoters belonging to class B and C incorporate inputs from Brk as well as Shn/Mad for activation, but can be distinguished based on the level of contribution from Shn. In *brk*; *shn* double mutants, the expression of class B genes is comparable with that in wild-type animals, indicating that in the absence of both factors, Mad-mediated signaling is sufficient for expression within the normal domain. However the wider domain of expression encountered in *brk* mutants is not sustained by Mad in the double mutants, suggesting that Shn activity may be crucial in cells that are exposed to minimal levels of Dpp signaling. In contrast, Class C targets display a pronounced reduction both in the size of the domain and levels of gene expression in the double mutant, not only relative to brk^- but also compared with wild-type animals. Thus, Shn makes a significant *brk*-independent positive input to regulation of these genes in the endogenous domain. (B) Shn may facilitate Mad activity, perhaps by enhancing interaction of Mad with DNA and/or components of the transcriptional machinery, such as co-activators (Co-A) and co-repressors (Co-R) in a promoter-specific manner. Additional DNA-binding proteins may be involved in recruiting the Co-R.

unilaterally from the side into which TkvA was injected. We wished to ascertain whether repression of *brk* by TkvA requires Mad activity. As in the case of *dpp* mutants, *brk* expression in embryos lacking both maternal and zygotic *Mad* is initiated correctly but is upregulated at stage 8 (data not shown). Significantly, TkvA was ineffective in repressing *brk-lacZ* expression in *Mad* null embryos, indicating that Mad is essential for repression of *brk* (Fig. 8G). In contrast, in *shn* mutant animals TkvA was able to inhibit *brk-lacZ* expression in a localized manner (Fig. 8H). The incidence of inhibition at 10 ng/μl of TkvA was similar in embryos lacking *shn* activity and in wild-type controls, underscoring the fact that Mad can repress *brk* in the absence of Shn. These results suggest that Shn may play a stage- or enhancer-specific role in *brk* repression, rather than having a mechanistically essential function.

Another intriguing result from the mRNA injection experiments was that under some circumstances TkvA was capable of inducing low level activation of *brk-lacZ*. This occurred both in wild-type and in *shn* mutant embryos, although the effect was more pronounced in the absence of Shn. In all instances *brk-lacZ* activation was limited to a small group of cells in the center of a domain that lacked reporter gene expression, suggesting that activation is associated with higher levels of Dpp signaling at the site of injection, while comparatively lower levels of TkvA at the periphery are sufficient to repress *brk* expression in its endogenous domain. Supporting this view, the percentage of embryos showing ectopic *brk-lacZ* expression increased at higher levels of TkvA both in wild type and *shn* mutants (Table 1). This expression appears to represent de novo activation rather than lack of repression, as it can occur even in the lateral region of the embryo in cells that do not normally express *brk* (Fig. 8I,J). Strikingly, we never observed *brk* activation (or repression) in *Mad*-null embryos in response to excess TkvA (see Table 1). It is possible that the dose-dependent induction of *brk-lacZ* in wild-type and shn^- embryos is a consequence of 'squelching' by Mad at high levels of signaling, and its enhancement in *shn* mutants reflects a role for Shn in increasing the efficiency of Mad interactions with transcriptional complexes (see Discussion).

Table 1. Response of *brk* to ectopic Dpp signaling in different genetic backgrounds

Genotype	TkvA dose (ng/μl)	Response of *brk-lacZ* reporter		
		No effect	Repression	Activation*
Wild type	10	12%	69%	19%
shn^{P4738}	10	9%	57%	34%
Mad^{12}	10	100%	–	–
Wild type	50	–	49%	51%
shn^{P4738}	50	–	14%	86%
Mad^{12}	50	100%	–	–

*Note that all embryos that display ectopic activation of reporter at the site of injection, also show repression of *brk-lacZ* in the endogenous domain of expression. For each set of experiments, more than 50 embryos of the relevant genotype were injected and scored.

DISCUSSION

Shn is likely to function as a transcriptional co-factor for Mad in the nuclear response to Dpp based on its DNA-binding ability and the demonstration of signaling-dependent interactions between the two proteins (Dai et al., 2000; Udagawa et al., 2000). In this study we have shown that Shn (like Mad) contributes to regulation of Dpp target genes through two distinct mechanisms: by direct activation and by restricting expression of the repressor Brk. Our results indicate that Shn acts as a facilitator of Mad activity and enhances its interaction with DNA or components of the transcriptional machinery (summarized in Fig. 9).

Shn is not a dedicated repressor of *brk* transcription

Genetic evidence implicates both Shn and Mad in *dpp*-dependent repression of *brk*. Cells that lack *Mad* or *shn* ectopically express *brk* and fail to activate the Dpp-responsive genes *optomotor-blind* (*bifid* – FlyBase) *vestigial*, *spalt* and *Dad* in the wing disc (Campbell and Tomlinson, 1999; Jazwinska et al., 1999a; Minami et al., 1999; Marty et al., 2000; Torres-Vazquez et al., 2000). We have found that abolition of *shn* or *Mad* activity results in upregulation of *brk* in the embryo and that in the absence of *shn* ectopic Dpp cannot suppress *brk* expression (Figs 1, 8D; data not shown). As Shn and Mad interact directly, an attractive hypothesis is that a Shn/Mad complex is involved in the Dpp-dependent repression of *brk*. It has recently been suggested that Dpp signaling bifurcates downstream of Mad/Med into a Shn-dependent pathway, leading to *brk* repression and a Shn-independent pathway that triggers gene activation (Marty et al., 2000). According to this model, Shn acts primarily as a dedicated repressor that switches Mad from a transcriptional activator to a transcriptional repressor on the *brk* promoter (Marty et al., 2000). However several lines of evidence from our study are incompatible with such an interpretation.

A strong argument that *shn* has additional roles beyond *brk* repression comes from the fact that simultaneous loss of *brk* and *shn* activity results in a phenotype that is distinct from that of *brk*-null animals. If the sole function of *shn* is to mediate *brk* repression, then *shn* activity should be redundant in a *brk* mutant background. However, both at the overt phenotypic level as well as in the regulation of individual target genes, *brk*; *shn* double mutants display defects consistent with lower levels of Dpp signaling compared with embryos that lack *brk* alone (Figs 2, 4-6). These results indicate that *shn* participates in gene activation through a *brk*-independent mechanisms as well (see below). The finding that Shn is not obligately required to suppress *brk* transcription prior to germband elongation, while Mad is essential in this process, also argues against an exclusive role for Shn as a Mad co-repressor. In *dpp*- and *Mad*-null embryos *brk* is upregulated at stage 8, while in embryos lacking *shn* function, derepression occurs approximately 3 hours later than the transition of *brk* regulation from maternal to zygotic control (Fig. 1B,D,F; and data not shown). Thus, *brk* transcription is insensitive to the absence of *shn* function at a time when it is responsive to Dpp and Mad. This idea is reinforced by the fact that ectopic Dpp signaling (through TkvA) can repress *brk* transcription at stage 5/6 in both wild-type and *shn*$^{-}$ animals, but not in *Mad*-null embryos (Fig. 8F-H; Table 1). Collectively these data provide compelling evidence against a model in which all aspects of the *shn* mutant phenotype result from derepression of *brk* transcription.

The unexpected result that at high levels TkvA mediates activation of *brk* promoter, while at low levels it causes repression reveals a possible mechanism by which Shn contributes to Mad activity (Fig. 8I,J; Table 1). One explanation for these concentration-dependent effects of TkvA could be that the default mode of Mad action is transcriptional activation, and interaction with a co-repressor (perhaps present in limiting amounts) is crucial to bring about repression. Cells that receive very high levels of signaling could experience 'squelching', owing to excess nuclear Mad that binds to the *brk* promoter without recruitment of the co-repressor, thus promoting activation rather than repression. Supporting this idea, injection of TkvA into embryos that lack Mad did not induce either *brk* activation or repression (Fig. 8G; Table 1). The increased frequency of ectopic *brk* expression in *shn*$^{-}$ embryos could indicate that Shn stabilizes a Mad/co-repressor complex on the *brk* promoter. It is worth bearing in mind that even in *shn*$^{-}$ embryos, ectopic activation did not occur independent of *brk* repression in the peripheral cells (Fig. 8J). Thus, it appears that Shn does not determine whether Mad acts as an activator or as a repressor, but may promote its interaction with other factors that determine the polarity of Mad transcriptional activity (see below and Fig. 9).

Shn is essential for *brk*-independent gene activation

Analysis of Dpp-responsive gene expression in *brk*; *shn* double mutants has allowed us to assess the *brk*-independent input from *shn* to gene activation at different developmental stages in a range of tissues. Although it has not been demonstrated that each of these markers is a direct target of Dpp signaling, we can distinguish three categories of responses based on these studies (Fig. 9A). In the first group (class A), exemplified by *dpp* in the leading edge of the dorsal ectoderm, expression in the double mutant is indistinguishable from that in *brk*$^{-}$ embryos (Fig. 4). Thus, *shn* contributes to class A gene expression primarily by relief of *brk* repression. Promoters belonging to class B include *Dad* and *pnr* in the dorsal ectoderm during germband extension. Expression of class B genes is downregulated in the double mutant compared with *brk*$^{-}$ embryos, but is equivalent to wild-type levels. We infer from this result that in the absence of Brk and Shn, Mad-mediated activation may be sufficient for expression within the normal domain, but cannot sustain the lateral expansion encountered in *brk* mutants. A third category of responses (class C) includes *dpp* and *Ubx* in the midgut, and *sna* in the primordia of the wing/haltere imaginal discs. Genes in this class show significantly reduced levels of expression in the double mutant, not only relative to *brk*$^{-}$ but also compared with wild-type animals. Class C promoters incorporate a *brk*-independent positive input from *shn* that is necessary for wild-type levels of expression. The inability of ectopic Dpp to induce *sna* expression in *shn* mutants demonstrates the essential nature of the requirement for Shn in activation of class C genes (Fig. 8A,B).

It is evident that repression of *brk* is crucial for expression of all three classes of genes described, and as such accounts for a significant part of the positive input from *shn* to gene activation. In addition, our data suggest that Mad and Shn contribute equally to repression of *brk* and regulation of class A genes (Fig. 7). However, the fact that *brk* activity is only partially epistatic with respect to class B and C promoters, indicates that the majority of genes examined in this study integrate positive inputs from *shn*, as well as negative inputs from *brk*. The near wild-type expression of class B genes in double mutant embryos suggests that the *brk*-independent input from *shn* may be crucial at the margins of the expression domains and may be less significant in regions of the embryo that receive moderate to high levels of Dpp signaling. In contrast, the positive input from *shn* to class C targets appears to be important throughout the domain of expression. The observation that genes such as *dCreb-A* and *Scr* that are repressed by *dpp* signaling are also sensitive to loss of *brk*, raises the possibility that Dpp regulates their expression indirectly. In this event, the *dpp* target genes

that mediate repression of *dCreb-A* and *Scr* would belong to classes A and C, respectively.

The partial restoration of *dpp* target gene expression in the double mutants relative to *shn*$^{-}$ embryos provides a basis for interpreting the cuticle phenotype. Homozygous *brk*; *shn* animals as well as *brk*; *tkv* mutants have an intermediate phenotype in that they show rescue of the dorsal closure defect observed in *shn* and *tkv* mutants, but also display a reduced dorsal epidermis compared with *brk*-null embryos (Figs 2B-D, 7B). Both *dpp* and *pnr* have been implicated in dorsal closure, which results from movement of the epidermal cells over the amnioserosa and their suturing at the midline (reviewed in Noselli and Agnes, 1999). In light of this, the recovery of their expression in the dorsalmost ectodermal cells in the double mutants correlates well with the restoration of dorsal closure (Figs 4, 7). Likewise, the compromised expression of dorsal ectodermal markers such as *Dad* and *pnr* in *brk*; *shn* embryos relative to *brk* null animals, provides molecular correlates for the ventralization observed in the double mutants.

Mechanistic basis of Shn function

The data presented in this study indicate that Shn can mediate both gene activation and *brk* repression in response to Dpp signaling. An important question is whether Shn has a Mad-independent role in activation. Shn contains a potential activation domain, and the human ortholog of Shn (PRDII-BF1) can elicit a 10-fold increase in gene expression in transfection assays (Seeler et al., 1994). However, a Shn-Gal4 fusion protein does not activate transcription in yeast, and Shn is only marginally effective in stimulating a Dpp-responsive reporter in the absence of Mad in cell culture assays (Dai et al., 2000). Taken together these results suggest that Shn acts by promoting Mad binding to DNA and/or its interactions with the transcriptional machinery (Fig. 9B). There is ample precedent for such a mechanism, as several vertebrate DNA-binding Smad partners such as FAST1, OAZ, Mixer and Milk, do not have an innate ability to stimulate transcription, but potentiate gene activation by Smads in a pathway specific manner (reviewed in Massagué and Wotton, 2000). A prediction from our data is that promoters of class B and class C genes are likely to contain binding sites for Shn as well as Mad, and that Shn increases Mad specificity by recruiting it to a subset of promoters that contain binding sites for both proteins. Analysis of gene expression in *brk*; *tkv* mutants demonstrates that for class B and class C genes *Mad* provides a greater *brk*-independent input compared with *shn*, consistent with the idea that Mad plays a primary role in Dpp-dependent gene activation and that *shn* facilitates *Mad* activity. Further support comes from the observation that deletion of Mad sites in the Ubx midgut enhancer had a more profound effect than abolition of Shn binding (Szuts et al., 1998; Dai et al., 2000). It has been shown that Mad interacts with Nejire (Nej), the *Drosophila* homolog of the co-activator p300/CREB binding protein (CBP) (Akimura et al., 1997; Waltzer and Bienz, 1998). Reduction in *nej* activity affects the expression of *ush*, *pnr* and *Ubx*, and disrupts events that are *dpp* and *shn*-dependent, like tracheal migration and imaginal disc patterning (Waltzer and Bienz, 1999; Ashe et al., 2000). It is interesting to speculate that Shn may interact directly with Nej and stabilize complex formation between Mad/Medea and Nej.

The requirement for Shn and Mad in both aspects of Dpp signaling suggests that Shn does not confer the ability to activate or repress transcription. It appears more likely that the activity of the Mad/Shn complex is modulated in a promoter specific fashion analogous to the mechanisms that convert Dl from an activator to a repressor (Mannervik et al., 1999; Chen and Courey, 2000). Similarly, the presence of binding sites for factors that bring co-repressors into proximity with Mad/Shn could permit inhibition of transcription at the *brk* promoter while target genes that lack these sites could be activated in the same cells (Fig. 9B). It has been shown that Smad4 interacts with the co-repressor TGIF and the co-activator CBP in a mutually exclusive manner (Wotton et al., 1999). Thus, the ability to recruit co-activators as opposed to Smad co-repressors (such as cSki and SnoN), or more general transcriptional repressors like Groucho or CtBP, would be crucial to determining whether Dpp stimulation resulted in activation or repression of the target gene.

It is conceivable that in addition to repressing *brk* transcription, Shn and Mad could prevent residual Brk protein in the nucleus from binding to target gene promoters through steric hindrance or direct competition for common binding sites. Related anti-repression mechanisms have been postulated for Smad1 and Smad2 that interact with the transcriptional repressors Hoxc-8 and SIP1, respectively, triggering their dissociation from the osteopontin and X-Bra promoters (Verschueren et al., 1999; Yang et al., 2000). Although such a mechanism could potentially enhance the efficiency with which Shn and Mad antagonize *brk* activity, it does not account for the *brk*-independent input from *shn* observed in *brk*; *shn* embryos, as there is no Brk protein in the double mutants.

Shn activity is functionally redundant during early embryonic patterning

Despite the fact that *shn* transcripts are present from precellular blastoderm stage onwards, loss of *shn* activity does not affect either *brk* repression or the expression of Dpp target genes until germband extension. Germline clonal analysis and ds-RNAi experiments (S. P. and K. A., unpublished data) indicate that the insensitivity of Dpp target gene expression to loss of *shn* during early embryogenesis is unlikely to result from perdurance of maternal message. Thus, the ‘weakness’ of the *shn* mutant phenotype may reflect a limited temporal requirement for *shn* in *dpp* signaling, rather than a lesser requirement for *shn* activity throughout development. The functional redundancy of *shn* during early patterning could be due to the presence of another protein that contributes a Shn-like activity to Dpp signal transduction. Alternatively, Mad activity alone could be sufficient for induction of early D/V patterning genes if they contain promoter elements that are more sensitive to Mad. It is also possible that the higher levels of nuclear Mad resulting from the synergy between Scw and Dpp in early embryogenesis renders the potentiation of Mad by Shn unnecessary (Nguyen et al., 1998; Neul and Ferguson, 1998). Finally, given the conserved nature of the BMP signal transduction pathway and the identification of Shn homologs in humans, frogs and worms, it is possible that Shn-like proteins in other systems potentiate Smad activity in an analogous manner.

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