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schnurri Is Required for *dpp*-Dependent Patterning of the Drosophila Wing

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The BMP-related ligand Decapentaplegic (Dpp) has a well-characterized role in pattern formation during Drosophila embryogenesis and in larval development. Previous work has shown that transcription of Dpp-responsive genes requires the activity of the BMP-specific Smad, Mothers against dpp (Mad). In this study we investigated the role of the zinc finger transcription factor Schnurri (Shn) in mediating the nuclear response to Dpp during adult patterning. Using clonal analysis, we show that wing imaginal disc cells mutant for *shn* fail to transcribe the genes *spalt, optomotor blind, vestigial,* and *Dad,* that are known to be induced by *dpp* signaling. *shn* clones also ectopically express *brinker,* a gene that is downregulated in response to *dpp,* thus implicating Shn in both activation and repression of Dpp target genes. We demonstrate that loss of *shn* activity affects anterior-posterior patterning and cell proliferation in the wing blade, in a manner that reflects the graded requirement for Dpp in these processes. Furthermore, we find that *shn* is expressed in the pupal wing and plays a distinct role in mediating *dpp*-dependent vein differentiation at this stage. The absence of *shn* activity results in defects that are similar in nature and severity to those caused by elimination of Mad, suggesting that Shn has an essential role in *dpp* signal transduction in the developing wing. Our data are consistent with a model in which Shn acts as a cofactor for Mad. **© 2000 Academic Press**

Key Words: **Schnurri; BMP signaling; *dpp;* Smad; transcription factor; wing imaginal disc; Drosophila.**

INTRODUCTION

The BMPs (bone morphogenetic proteins) are structurally related secreted growth factors that are conserved across the phylogenetic spectrum and have been implicated in a variety of developmental process in both vertebrates and invertebrates. These include establishment of embryonic axes, development of the heart, limbs, and neural tissue, and control of the cell cycle (reviewed in Massagué, 1998; Raftery and Sutherland, 1999). So far three BMP ligands, *decapentaplegic* (*dpp*), *screw* (*scw*), and *glass bottom boat* (*gbb*), have been identified in Drosophila (Arora *et al.*, 1994; Doctor *et al.*, 1992; Padgett *et al.*, 1987; Wharton *et al.*, 1991). Among these, the role of Dpp has been most extensively characterized. Mutations in *dpp* affect multiple patterning events during embryogenesis as well as in development of the larval imaginal discs (Posakony *et al.*, 1991; Spencer *et al.*, 1982). Like most members of the TGF-β superfamily, Dpp binds to a heteromeric complex of type I and type II receptors with intrinsic serine/threonine kinase activity (reviewed in Derynck and Feng, 1997; Heldin *et al.*, 1997; Massagué and Chen, 2000). The Dpp type II receptor Punt (Put) activates the type I receptor Thick veins (Tkv), that in turn propagates the signal by phosphorylating Mothers against dpp (Mad), a founding member of the family of intracellular signal transducers collectively known as Smads (reviewed in Podos and Ferguson, 1999; Raftery and Sutherland, 1999). In both the embryo and in imaginal discs, Dpp signaling is potentiated by other BMP ligands such as Scw and Gbb, that act through an independent type I receptor Saxophone (Sax; Haerry *et al.*, 1998; Neul and Ferguson, 1998; Nguyen *et al.*, 1998).

Phosphorylation of receptor-specific Smads, such as Mad, triggers the formation of a hetero-oligomeric complex with a co-Smad (Smad4) that lacks a phosphorylation domain. In Drosophila, the Smad4 homolog Medea (Med), functions as a co-Smad (Das *et al.*, 1998; Hudson *et al.*, 1998; Wisotzkey *et al.*, 1998). The Smad complex translocates into the nucleus and regulates transcription of downstream target

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genes (reviewed in Massagué and Chen, 2000). Smads can bind DNA sequences of low complexity, but do so with relatively weak affinity (Shi *et al.*, 1998; Zawel *et al.*, 1998). There is accumulating evidence that *in vivo* Smads interact with other transcription factors that increase the specificity and/or the range of the cellular responses to the signal. The first such cofactor to be identified was the winged helix transcription factor FAST-1, that forms tripartite complexes with Smad2/4 as well as Smad3/4 to regulate expression of several activin-responsive genes in vertebrates (Chen *et al.*, 1996). Activin- and TGF-β-responsive Smads have also been shown to interact with additional DNA-binding transcription factors including AP1 (Fos-Jun), TFE3, the homeodomain proteins Mixer and Milk, and members of the AML family (reviewed in Massagué and Wotton, 2000). In contrast only one transcription factor, the vertebrate zinc finger protein OAZ, is known to complex with BMP-specific Smads and mediate transcription (Hata *et al.*, 2000).

In Drosophila, Mad and Medea have been shown to bind to enhancer sequences in the Dpp target genes *tinman* (*tin*), *Ultrabithorax* (*Ubx*), and *vg* (Kim *et al.*, 1997; Kim *et al.*, 1996; Waltzer and Bienz, 1999; Xu *et al.*, 1998), but no Smad-interacting DNA-binding factors have been identified. Although the homeobox protein Tin cooperates with Mad/Medea in regulating its own expression, it does not appear to physically interact with Mad or Medea (Xu *et al.*, 1998). Several lines of evidence indicate that the nuclear protein Schnurri (Shn) may act as a transcriptional cofactor for Mad. *shn* encodes a multiple zinc finger protein that is homologous to the mammalian major histocompatibility enhancer binding proteins, MBP1 and MBP2 (Arora *et al.*, 1995; Grieder *et al.*, 1995; Staehling-Hampton *et al.*, 1995). Mutations in *shn* are embryonic lethal and strongly enhance partial loss-of-function *dpp* alleles. Previous studies have established that in *shn* mutants the expression of Dpp target genes during embryogenesis is either attenuated or abolished. It has recently been shown that Shn and Mad can interact directly (Udagawa *et al.*, 2000; Dai *et al.*, 2000). Furthermore, Shn binds to sites in a Dpp-responsive enhancer element and acts synergistically with Mad to induce transcription of Dpp target genes in a cell culture assay. Based on these results, we have proposed that Shn acts as a cofactor with Mad to regulate gene expression during embryogenesis (Dai *et al.*, 2000).

Mutations in *shn* do not affect all *dpp* target genes in the embryo, raising the possibility that its participation in Dpp signal transduction may be tissue and/or stage specific (Grieder *et al.*, 1995; J. Torres-Vazquez and K. Arora, unpublished data). Therefore we wished to determine whether there is an obligatory requirement for Shn in mediating Dpp function during imaginal disc development. The contribution of *shn* to adult patterning has not been analyzed, although there is some evidence suggesting that Shn is required postembryonically for cell proliferation in discs (Burke and Basler, 1996; Penton *et al.*, 1997). Here we focus on examining the role of *shn* in the wing imaginal disc, where *dpp* is expressed in a narrow stripe of cells at the anterior-posterior (A/P) compartment boundary. It is believed that diffusion of Dpp from its site of expression results in a gradient of signaling along the A/P axis that patterns the wing epithelium in a concentration-dependent manner. Support for the role of *dpp* as a morphogen comes from the observation that genes such as *spalt* (*sal*), *optomotor blind* (*omb*), *vestigial* (*vg*), and *Daughters against dpp* (*Dad*) respond to different levels of Dpp signaling and are activated in nested domains of expression centered on the A/P boundary (reviewed in Neumann and Cohen, 1997; Podos and Ferguson, 1999). Dpp signaling also represses transcription of the nuclear protein Brinker (Brk), that in turn negatively regulates Dpp target gene expression. Thus *brk* is expressed in an inverse pattern with respect to the predicted gradient of *dpp* signaling, and serves to sharpen the threshold response to Dpp (Campbell and Tomlinson, 1999; Jazwinska *et al.*, 1999a,b; Minami *et al.*, 1999). Subsequently, during pupal development *dpp* plays a role in specification of the veins that mark the surface of the adult wing. *dpp* is expressed in narrow stripes of cells that correspond to the future wing veins and acts in concert with the EGFR and Notch (N) pathways to promote their differentiation (de Celis, 1997; Posakony *et al.*, 1991; Segal and Gelbart, 1985; Yu *et al.*, 1996).

In this study, we generated clones lacking *shn* function and analyzed their effect on Dpp target gene expression in the wing disc, as well as the phenotypic consequences in adult wing patterning. We report that loss of *shn* activity affects all three aspects of wing development that are known to be Dpp dependent. In addition to the known requirement for *shn* in cell survival, we demonstrate for the first time that *shn* is required for specification of cell fates along the A/P axis. Consistent with this, we show that the expression of Dpp target genes involved in mediating A/P patterning is altered in clones lacking *shn* activity. We also find that *shn* is expressed in pupal wings, and is involved in the process of vein differentiation at this stage. The defects seen in *shn* clones reflect a cell autonomous loss in Dpp signaling and are strikingly similar in severity to those caused by loss of the receptor Tkv, or the signal transducer Mad. Our analysis demonstrates an essential role for *shn* in regulating Dpp target gene expression in the wing, and support the idea that Shn functions as a cofactor for Mad during adult patterning.

MATERIALS AND METHODS

Drosophila Strains

The mutant stock dpp^{s1} has been described previously (Segal and Gelbart, 1985). The shn^{IB09} and shn^{IM56} alleles are strong hypomorphs that show essentially similar phenotypes *in trans* to a deficiency for the region (Nüsslein-Volhard *et al.*, 1984). For the temperature-sensitive allele shn^{IM56}, this holds true at the restrictive temperature (≥25°C). Both alleles have been molecularly characterized and encode truncated proteins (Arora *et al.*, 1995).

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$shn^{3.3J}$ is also a strong hypomorph that was identified in an independent screen (Matunis *et al.*, 1997). *CyO, S* was obtained from the Umea stock center.

Adult Clonal Analysis

For clonal analysis, the alleles shn^{IM56} and shn^{IB09} were recombined on to an FRT42D chromosome. Somatic loss-of-function *shn* clones were generated using the FLP/FRT system (Golic and Lindquist, 1989; Xu and Rubin, 1993). The parental strains were transferred successively to fresh vials, to collect 12-h egg lays. Mitotic recombination was induced at different time points by submerging the vials in a 37°C water bath for 1.5 h. The timing of induction was either at 30, 54, 78, or 90 (±6) h after egg laying (AEL), that corresponds to first, second, early, and late third instar larval stages, respectively. After heat treatment the progeny were maintained at 25°C, unless otherwise noted.

The following recessive markers were used for *shn* clonal analysis in the adult wing. The mutation *yellow* (y^1) was used to identify clones at the margin by their lack of pigmentation. Clones in the wing blade were marked with *prickle* (pk^1) which alters the polarity of trichome hairs, and the corresponding wild-type twin spots were marked with *shavenoid* (sha^{koj}) that differentiates stunted trichome hairs. For clones induced in a *Minute* background, *shn* mutant cells were identified using the trichome hair marker *forked* (f^{36a}). For the generation of clones, flies of the genotype *y, [hsFLP 122]; FRT42D,* shn^{allele}*/TSTL* or *CyO, S* were crossed to either *yw/Y;* $P[ry^+$*, neo FRT] 42D,* $P[y^+, ry^+]$ *44B* (for *shn* clones marked with y^1) or $P[ry^+$*, hsFLP]* f^{36a}*; FRT42D,* w^+*,* f^{+72}*, M(2R)/CyO* females (for *shn* clones marked with f^{36a}, these clones were scored only in male progeny). To generate *shn* clones with marked twin-spots *y, [hsFLP 122]; FRT42D,* sha^{koj}*/CyO, S* females were crossed with *+/Y; FRT42D,* shn^{allele}*,* pk^1*/CyO* or *CyO, S* males. Progeny in which *shn* clones were induced were identified by the absence of the balancer chromosome. Experiments to determine the temporal requirement for *shn* activity as well as the mutant phenotype in adult wings were also carried out with a $shn^{3.3J}$*, FRT43D* stock using the appropriate marker chromosomes. Similar results were obtained with all three alleles, with the exceptions noted in the text for shn^{IM56} at the permissive temperature (18°C).

Analysis of Clones in Imaginal Discs

To assess the recovery of *shn* clones induced at different larval instars *y, [hsFLP 122]; FRT42D, arm > lacZ* females were mated with *y, [hsFLP 122]; FRT42D,* shn^{allele}*/TSTL* or *CyO, S* males. Twelve-hour egg lays were collected and heat-shocked at 54, 66, or 78 h AEL, respectively, and analyzed at 114 h AEL. Mutant clones were identified by the absence of *lacZ* expression using antibody staining. To analyze *dpp* target gene expression in *shn* mutant cells *yw, [hsFLP 122]; FRT42D, hsp70-CD2* flies were crossed with *y, [hsFLP 122]; FRT42D,* shn^{allele}*/TSTL* flies. Clones were induced at 78 h AEL (±6 h) and identified by lack of expression of a *hsp70-CD2* transgene in shn^+ cells. Expression of CD2 was induced at 114 h AEL (±6 h) by heat shocking animals in a 37°C water bath for 70 min (Jiang and Struhl, 1995). Following a 60-min recovery period, the discs were processed for immunofluorescence. The following β-Gal reporter lines were used to follow *dpp* target gene expression: omb^{X35}, a viable enhancer trap line (Sun *et al.*, 1995); Dad^{P1883}, a recessive lethal enhancer trap line (Tsuneizumi *et al.*, 1997); brk^{X-47}, a viable enhancer trap (Campbell and Tomlinson, 1999); and vg^{Qh12}, the *vg* quadrant enhancer fused to *lacZ* (Kim *et al.*, 1996). Expression of *sal* was detected with a rabbit anti-Sal antibody (kindly provided by Rosa Barrio and Jose de Celis). Mutant clones were not independently marked in wing discs in which *sal* expression was analyzed.

Wing imaginal discs were fixed in 4% formaldehyde, 0.5% Tween 20, 1X PBS for 30 min. The following antibodies were used: rabbit anti-β-Gal (USB), Cy3-conjugated donkey anti-Rabbit (Jackson Immunological Labs), FITC-conjugated mouse anti-Rat CD2 (OX34, Serotec), and Cy2-conjugated donkey anti-mouse (Jackson Immunological Labs). After staining the discs were washed in PBT (1% Tween 20, 1X PBS), mounted in 5% *n*-propylgallate, 0.1 M Tris, pH 7.0, 80% glycerol, and examined using a Bio-Rad MRC 1024 UV confocal microscope.

In Situ Hybridization and Clonal Analysis in Pupal Wings

For analysis of *tkv* expression in pupal wing clones *y, [hsFLP 122]; FRT42D,* sha^{koj}*/CyO, S* females were mated with *+/Y; FRT42D,* shn^{IM56}*/CyO, S* males. Eggs were harvested every 12 h and clones were induced in late third instar wandering larvae or white prepupae. After the heat-shock treatment, animals were shifted to 29°C for 24 h and subsequently processed for RNA *in situ* hybridization. For analysis of *Dad* expression in *shn* mosaic wings *y, [hsFLP 122]; FRT42D, hsp70-CD2,* y^+*;* Dad^{P1883}*/TM6B* females were mated with *y, [hsFLP 122]; FRT42D,* shn^{allele}*/TSTL* males. Both shn^{IB09} and shn^{IM56} alleles were used. Clones were induced successively at 114 and 138 h AEL (±6 h), and the animals were allowed to continue development at 25°C or 29°C (for shn^{IM56}). Animals in which mutant clones had been induced were identified by absence of *Tubby* (*Tb*), a dominant marker present in both *TSTL* and *TM6B* balancers.

Pupal wings were dissected and processed for immunostaining or *in situ* hybridization as described in Sturtevant *et al.* (1993), with the modification that the hybridization was carried out at 55°C. Digoxigenin-labeled *tkv, dpp,* and *shn* antisense riboprobes were used for hybridization and visualized using an anti-digoxigenin antibody (Boehringer Mannheim). *Dad-lacZ* expression in pupal wings was visualized using an anti-β-Gal monoclonal antibody and an alkaline phosphatase-conjugated secondary. After staining, pupal wings were progressively dehydrated in ethanol and rehydrated in PBT. Wings were mounted in 70% glycerol-PBS and analyzed using Nomarski optics.

Heat Shock Rescue of dpp^{s1}

dpp^{s1} animals as well as a dpp^{s1} stock carrying three copies of the *hsp70-shn* transgene were heat-shocked using a protocol modified from Yu *et al.* (1996). One-hour egg lays from both strains were collected in vials containing standard cornmeal-agar food and allowed to develop at 25°C, until 18 h after puparium formation (APF). The pupae were heat-shocked for 30 min at 37°C in a water bath and then allowed to recover at room temperature for 30 min. This treatment was repeated five times, and then the animals were allowed to develop until eclosion at 25°C. In control experiments, 1-h egg lays from both strains were reared at 25°C without heat shock treatment and analyzed. Wings were mounted in methyl salicylate/Canada balsam, 1/1, and photographed using bright-field optics.

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RESULTS

shn Is Required for Dpp-Dependent Cell Fates in the Wing

We have previously shown that *shn* is broadly expressed in the wing imaginal disc, suggesting that it could be involved in the transcriptional regulation of Dpp target genes during larval development (Arora *et al.*, 1995). In order to determine the contribution of *shn* to Dpp signaling, we generated clones of homozygous mutant cells that lack *shn* activity and examined their effect on adult wing patterning. To control for variability in genetic background the clonal analysis was carried out using three different alleles, shn^{1B09}, shn^{1M56} and $shn^{3.3J}$, that were identified in two separate screens (Matunis *et al.*, 1997; Nüsslein-Volhard *et al.*, 1984). For all three alleles clones were recovered at a significantly higher frequency when somatic recombination was induced during mid–late third larval instar, suggesting that *shn* clones induced earlier in development experience a growth disadvantage or do not survive to adulthood (see Materials and Methods). This is consistent with reports using the shn^{1B09} allele that implicate *shn* in cell proliferation (Burke and Basler, 1996; Penton *et al.*, 1997).

We first analyzed the effect of loss of *shn* activity in the wing margin using mutations in the *yellow* (*y*) gene to identify clones lacking *shn* function. The anterior wing margin differentiates three unique types of bristle that form excellent markers for anterior-posterior patterning. Each bristle type is found in a specific domain along the wing margin that corresponds to a distinct level of Dpp signaling. The anteriormost position is occupied by the distal costa bristles that are followed by the triple row bristles, and finally the double row bristles. The latter are closest to the A/P boundary and require the highest levels of Dpp signaling for specification (Figs. 1A, B; Singer *et al.*, 1997). Although clones of *shn* induced at 90 h after egg lay can be recovered throughout the anterior wing margin, they were observed at a much higher frequency in the region containing the triple row bristles compared to the double row bristle domain (Table 1). In addition, clones recovered in the triple row bristle domain tend to be larger than clones in the double row domain, as judged by the percentage of clones that differentiated at least three or more bristles within each region (Table 1, and Figs. 1C, D). We encountered anterior shifts in cell fate in *shn* clones located in the double row bristle domain. Within these clones, margin cells differentiated the thicker triple row bristles instead of the finer bristles typical of the double row (Fig. 1C). The bristle transformations occurred in a cell-autonomous manner and affected all bristles within the clone. Thus clones lacking *shn* function behave like cells further away from the source of Dpp. The phenotypes of these clones indicate a clear requirement for *shn* in anterior-posterior patterning of the wing. Mutant *shn* clones located in the region containing triple row bristles showed no phenotypic consequences (Fig. 1D). In summary, the data demonstrate that both in cell proliferation and in A/P patterning, Shn activity is more stringently required in cells that are closer to the source of Dpp and thus exposed to higher levels of signaling.

The requirement for *shn* in cell proliferation is apparent in the underrepresentation of mutant cells with respect to wild-type cells in the wing blade as well. As shown in Fig. 1E, shn^{-} clones marked with *prickle* (pk^{1}) are smaller than the corresponding twin spots marked with *shavenoid* (sha^{koj}). In addition, in several instances sha^{koj} clones in the wing were not accompanied by a corresponding pk^{1} clone (data not shown). In agreement with the idea that cells lacking *shn* experience a growth disadvantage (Burke and Basler, 1996), larger clones of *shn* mutant cells were recovered when they were generated in the presence of a *Minute* mutation on the shn^{+} chromosome. In these animals, we also observed a loss of wing blade tissue and margin structures, phenotypes that are likely to result from cell death or a failure of cell proliferation (Fig. 1F). In a number of cases small patches of *shn* mutant cells were recovered immediately adjacent to the missing portion of the wing, suggesting that the deleted region comprised cells lacking *shn* function that did not survive to adulthood (see clone marked with *forked* in Fig. 1F).

A third class of phenotypic defects was observed in *shn* mutant clones that abut or bisect a vein. Mutant cells within such clones do not differentiate vein tissue (Figs. 1G–I). As has been reported previously, *shn* clones on the dominant (protruding) surface of the vein are associated with interruptions in the vein tissue (Figs. 1G, H; Burke and Basler, 1996). Clones on the nonprotruding surface do not appear to disrupt the vein, although it is possible that the loss of vein tissue is obscured by the stronger pigmentation on the dominant surface. The loss of venation is almost exclusively cell autonomous. However, in a few rare instances we have observed clones on one surface that apparently impact vein differentiation on both sides of the wing (see area highlighted in blue in Fig. 1H). This is intriguing since there is no evidence to suggest that *dpp* is involved in communication between the dorsal and the ventral wing surfaces. A possible explanation could be that loss of vein fates impacts the expression of other signals that are involved in interactions between the two wing surfaces (reviewed in Garcia-Bellido and de Celis, 1992). The failure of *shn* clones to adopt vein cell fate is observed throughout the wing and thus is not specific to any particular vein position or compartment. In addition, we have observed an interesting non-cell-autonomous effect associated with small *shn* clones that impinge on veins. In these situations vein differentiation is disrupted within the mutant clone, but wild-type cells surrounding the clone differentiate ectopic veins. Such clones can result in branching and disruption of endogenous veins, as well as bifurcations that encapsulate the mutant tissue (Figs. 1H, I). In all such instances the ectopic vein comprises wild-type cells entirely (see Figs.

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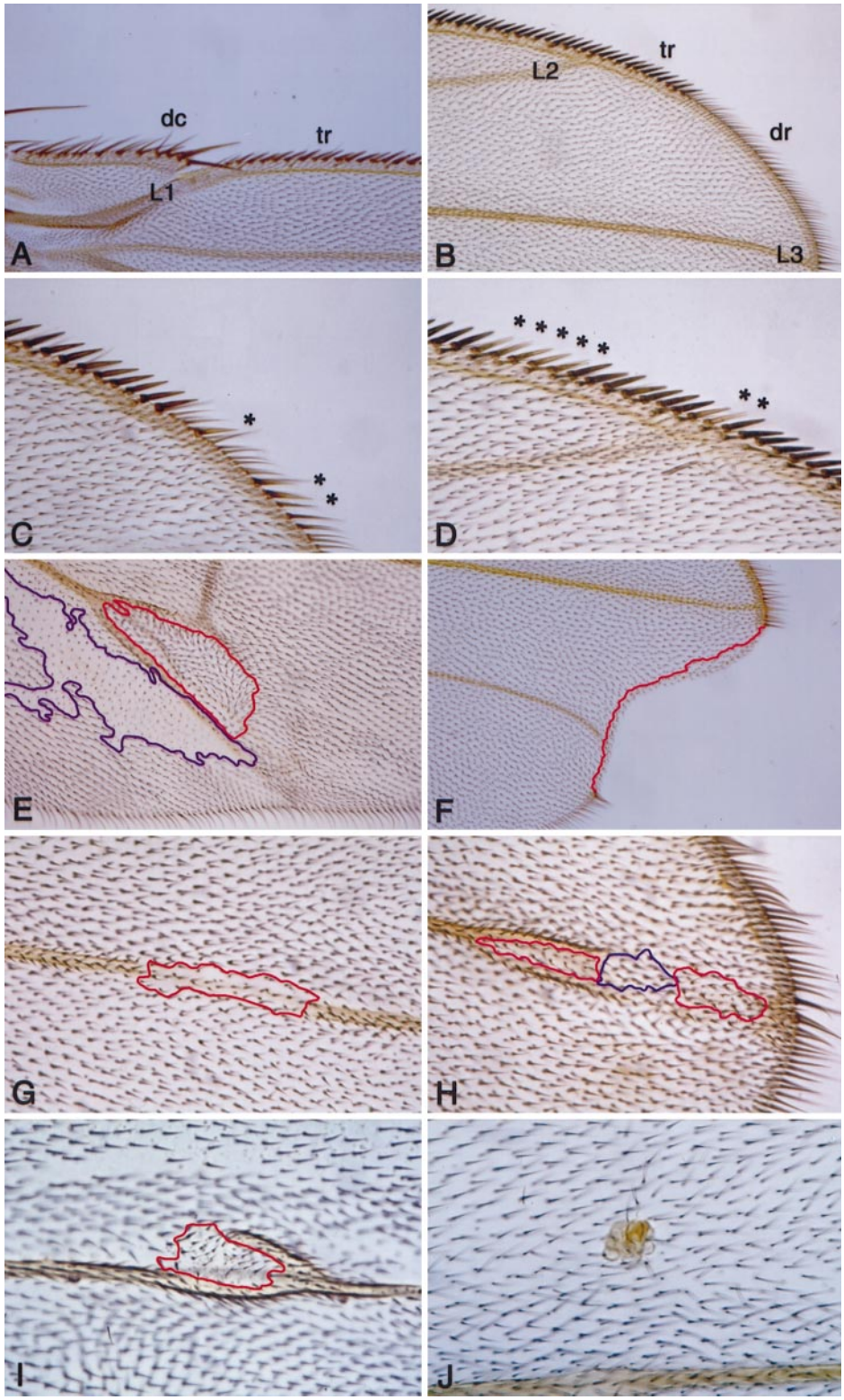

FIG. 1. Adult phenotypes caused by *shn* mutant clones. Details of *shn* mutant clones in adult wings. Clones depicted were induced at 90 h AEL in all cases except in F (114 hours AEL). All wings are oriented with proximal to the left. (A–D) Cells along the anterior wing margin that lack *shn* activity display shifts in anterior cell fates. Sections of a wild-type wing showing the proximal (A) and distal (B) region of the

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TABLE 1
Recovery of *shh* Mutant Clones in the Anterior Wing Margin

Allele	Temperature	No. of wings recovered	No. of clones recovered	Clones in the triple row bristle region containing		Clones in the double row bristle region containing	
				1–2 bristles	3 or more bristles	1–2 bristles	3 or more bristles
shn^{IM56}	18°C	50	82	48 (58.6%)	30 (36.6%)	4 (4.9%)	0 (0%)
shn^{IM56}	25°C	51	82	60 (73.2%)	16 (19.5%)	5 (6.1%)	1 (1.2%)
shn^{IB09}	25°C	45	73	37 (50.7%)	27 (37.0%)	7 (9.6%)	2 (2.7%)

Note. Clones lacking *shn* function were induced at 90 h AEL marked with y^1. Larvae were raised at 25°C prior to clone induction and maintained at the temperatures listed above after clone induction; 25°C is the restrictive temperature for shn^{IM56}. The clone size was estimated by counting the number of y^1 marked large mechanosensory bristles found within clones in the anterior wing margin. These margin elements are typical of the triple row bristle region and are absent from the double row bristle region. Differentiation of the large mechanosensory bristles in the later domain represents a cell fate transformation due to loss of Shn activity. Note that the percentage of clones containing three or more bristles is significantly reduced in shn^{IM56} clones at the restrictive temperature.

1E, H, I; and Discussion). In rare cases we have observed ectopic veins formed by wild-type cells that do not appear to be in the vicinity of a mutant clone (data not shown). Presumably in these cases, the *shn* clone associated with the ectopic vein did not survive to adult stages. Taken together, our results demonstrate that *shn* activity is required for Dpp responsiveness in multiple aspects of wing development: A/P patterning of the imaginal disc, cell survival, and vein differentiation in pupal wings.

Finally, wings in which *shn* clones are induced frequently differentiate small circular arrays of cells, which are pigmented like vein cells but do not possess a compact linear form (Fig. 1J). We were unable to determine the genotype of these cells since they lack trichome hairs and thus were not labeled by any of the clonal markers we used. The clusters appear more frequently in the anterior compartment and were encountered both adjacent to endogenous veins and in the intervein region.

Expression of Dpp Target Genes in the Wing Disc Requires Shn Activity

In order to determine whether the A/P patterning defects observed in adult wings lacking *shn* function correlate with loss of Dpp signaling, we analyzed the expression of several Dpp-responsive genes in *shn* mutant clones (see Materials and Methods). The size and distribution of *shn* clones in the larval wing disc follow the same general pattern as those described above for adult clones. Induction of mitotic recombination at 30 and 54 h AEL resulted in infrequent recovery of *shn* clones. By contrast, clones induced at early third instar were consistently recovered throughout the wing disc. We therefore induced clones at 78 h AEL in order to assay target gene expression. Consistent with the requirement for *dpp* in cell proliferation/survival, *shn* mutant clones were smaller than their wild-type twin spots, and clones in the wing pouch located close to the A/P boundary tended to be smaller in size than those further away from the source of Dpp.

anterior wing margin. The distal costa (dc), triple row (tr), and double row (dr) bristles occur at stereotypic positions and serve as landmarks along the A/P axis. The longitudinal veins L1–L3 are indicated. The transition from dc to tr occurs at L1, and from tr to dr between L2 and L3. shn^{IM56} clones located in the dr region (C) differentiate tr bristles, while clones in the tr domain (D) maintain their cell fate. Mutant bristles were identified by their yellow color and are marked with asterisks. (E, F) *shn* mutant clones suffer a growth disadvantage compared to wild-type cells. (E) shn^{IM56} clone marked with pk^1 located near vein L5 (outlined in red) is smaller than its corresponding wild-type twin spot marked with sha^{koj} (outlined in blue). (F) In a shn^{IM56} clone induced in a Minute background, loss of wing blade tissue in the distal portion of the wing results in a notch between veins L3 and L5. *shn* mutant cells marked with f^{36a} are present at the border of the deleted tissue in the wing blade. (G–I) Effects of *shn* clones on wing venation. (G) A shn^{IM56} clone (marked with f^{36a} on the dorsal surface of L3) prevents differentiation of vein tissue on the dominant side, but does not affect the opposite surface as evidenced by the faint pigmentation. In (H, I) shn^{IM56} clones (outlined in red) prevent vein differentiation autonomously. In the clone on the left, wild-type cells adjacent to the clone adopt vein cell fate. The boundary of the ectopic vein is not sharp adjacent to the mutant clone, but is well defined on the side farther away from the clone. This can also be seen in the mutant clone in (E). The clone marked with blue in (H) is located on the ventral surface, away from the viewer, and is a rare example of a clone on one surface causing loss of venation on the nondominant surface as well. (J) Wings containing *shn* clones differentiate clusters of pigmented tissue that protrude from the plane of the wing.

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The gradient of Dpp signaling in the wing imaginal disc results in activation of different target genes at distinct thresholds. As a consequence the transcription factors Sal, Omb, and Vg are expressed in nested domains and provide positional information for patterning the A/P axis of the wing (Lecuit *et al.*, 1996; Nellen *et al.*, 1996). Dpp activity also triggers expression of Dad, a Smad-related protein that modulates the response to Dpp by competing with Mad for receptor binding (Tsuneizumi *et al.*, 1997). We observed that *shn* mutant clones in the wing disc do not express the Dpp-inducible genes *omb*, *Dad* and *vg* (Figs. 2A–C). This is true for all clones in the anterior and the posterior compartments, as well as for clones that straddle the A/P boundary. Thus the requirement for *shn* in target gene expression is not restricted to cells that express *dpp*, but extends to all cells that respond to Dpp signaling in the wing pouch. As seen in Figs. 2A–C, target gene expression is uniformly lost from all cells within a clone and is not dependent on the distance of the clone from the source of the Dpp signal. These results indicate that mutations in *shn* result in the absence of a detectable response to Dpp rather than simply attenuating the signal. In a separate set of experiments, we found that expression of the Dpp target *sal* was lost in a similar manner in *shn* mosaic wing discs (data not shown). Thus, Shn is required for the activation of genes that respond to both low and high thresholds of Dpp signaling.

In addition to induction of target gene expression, it has recently been shown that *dpp* signaling represses transcription of Brk, a negative regulator of Dpp-dependent gene expression (Campbell and Tomlinson, 1999; Jazwinska *et al.*, 1999a; Minami *et al.*, 1999). Analysis of *brk* in *shn* mutant clones revealed that *brk* transcription is upregulated in the central region of the wing disc, where it is normally repressed by high levels of *dpp* signaling (Fig. 2D). Thus loss of Shn activity in the wing disc affects the expression of genes that are downregulated by Dpp signaling, as well as genes that are activated by Dpp. In all cases the transcription of Dpp-responsive genes is affected in a cell-autonomous manner. Based on these results we infer that Shn is required for the transcriptional response to Dpp in adult patterning. It is significant that the effects of loss of *shn* activity on target gene expression are as strong as those encountered in clones lacking Mad function (Kim *et al.*, 1997; Lecuit *et al.*, 1996; Minami *et al.*, 1999). These observations indicate that both transcription factors are critical for mediating Dpp signaling, consistent with the idea that Shn and Mad act together to regulate Dpp target gene expression.

Shn Is Transcribed in the Pupal Wing and Is Required for Responsiveness to Dpp

The adult wing contains five longitudinal veins (L1 to L5) and two cross veins that are located at stereotypic positions along the wing blade (Fig. 3A). The positioning and differentiation of these veins occur in two distinct stages, both of which require input from *dpp*. Initially, Dpp signaling in the imaginal disc is required to specify the position of individual veins. This aspect of *dpp* function is likely to be mediated by activation of transcription factors such as *sal* and *spalt-related* (*salr*), since the position of the L2 and L5 veins is specified at the boundary of Sal/Salr expression (de Celis and Barrio, 2000; de Celis *et al.*, 1996; Sturtevant *et al.*, 1997). Subsequently, during pupal development *dpp* is expressed in all prospective veins and is critical for their differentiation (Fig. 3B; de Celis, 1997; Posakony *et al.*, 1991; Segal and Gelbart, 1985; Yu *et al.*, 1996). Thus the requirement for *shn* in wing venation could stem from its early role in mediating Dpp signaling during A/P patterning and/or from a later requirement in vein differentiation. As a first step in determining whether *shn* could play a role in late stages of vein differentiation, we examined its expression in the pupal wing. We found that *shn* is expressed throughout the wing blade and its transcription is modulated in the presumptive vein territory (Fig. 3C). *shn* mRNA is present at high levels at the vein border cells and at lower levels within the developing veins. This pattern is reminiscent of *tkv* expression at a comparable stage with the exception that significant levels of *shn* transcript are also observed in the intervein region (Fig. 3D) (de Celis, 1997).

To determine whether the process of vein differentiation is sensitive to *shn* at pupal stages, we examined the effect of altering Shn dosage in a dpp^{s1} background. This allele is a mutation in the *cis*-regulatory region of the *dpp* promoter that specifically reduces expression in the pupal wing in the distal L2 and L4 vein domains, but does not affect transcription at other stages (de Celis, 1997; St. Johnston *et al.*, 1990). As a consequence wings from dpp^{s1} adults are characterized by incomplete longitudinal veins L2 and L4 that fail to reach the wing margin (Fig. 4A; compare with wild-type wing in Fig. 3A). Heterozygosity for *shn* in a dpp^{s1} background has been shown to result in severe truncation of L2 and L4 veins as well as reduction of the posterior cross vein (Fig. 4B; Staehling-Hampton *et al.*, 1995), consistent with a loss of responsiveness to Dpp. Therefore we tested whether increasing the level of Shn in a stage-specific manner could compensate for the reduced level of signaling in a dpp^{s1} homozygous mutant background. Transgenic flies were generated carrying the Shn coding sequence under the control of a heat shock promoter (*hsp70-shn*). Ubiquitous Shn expression was induced 18–23 h after puparium formation (APF) in the period during which ectopic Dpp is known to impact vein differentiation (Yu *et al.*, 1996). We observed that excess Shn results in considerable rescue of the dpp^{s1} phenotype (Fig. 4C). In 65% of the wings (81/124) the L2 vein was rescued and reached the wing margin, and 94% of the wings (116/124) displayed a significant increase in the length of L4 distally, such that it spanned more than three-quarters of the wing blade. In control dpp^{s1} animals that were heat-shocked but did not carry a transgene, only 2% of the wings (4/199) contained a complete L2 and 0.5% (1/198) showed L4 veins of a length comparable to the rescued wings. Weaker phenotypic rescue (32% for L2 and 79% for L4) was observed in

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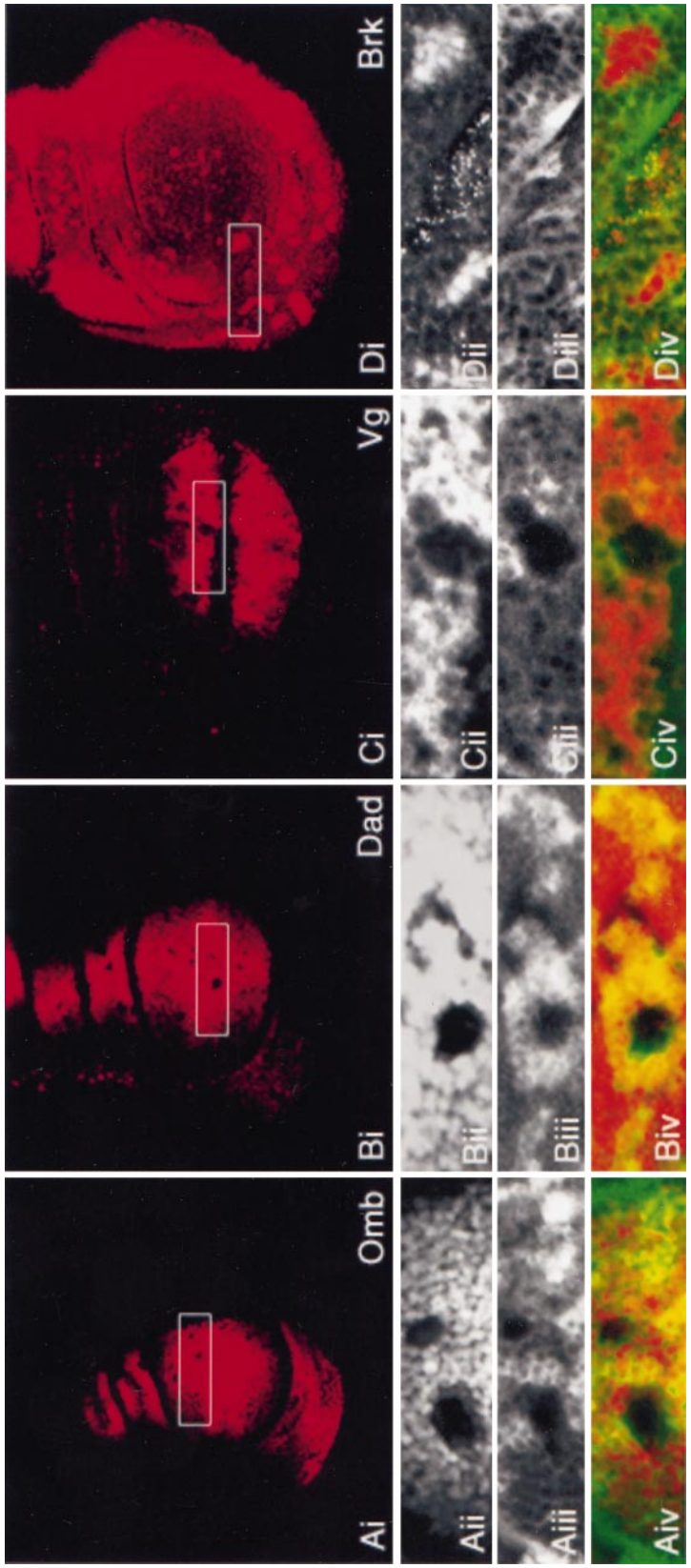

FIG. 2. Loss of *shn* affects the expression of Dpp-responsive genes in wing discs. Confocal photomicrographs of wing imaginal discs in which shn^{IM56} clones were induced at 78 h AEL and visualized at late third instar. The expression of Dpp target genes was monitored using the nuclear β-Gal reporter lines omb^{X35} in (A), Dad^{P1883} in (B), the *vg* quadrant enhancer vg^{Q} in (C), and brk^{X47} in (D). Anterior is left, dorsal is up. Mutant clones were identified by lack of labeling for CD2 (in green). (Ai–Di) Imaginal discs displaying reporter gene expression in red. Triple inset panels contain enlarged views to demonstrate the consequence of loss of *shn*. (Aii–Dii) Expression of *dpp*-responsive genes. (Aiii–Diii) Expression of clonal marker CD2. Wild-type twin spots containing two copies of the marker transgene appear brighter. (Aiv–Div) Merged image with shn^{+} cells in green and target gene expression in red. In general, *shn* mutant clones are smaller and are recovered less frequently compared to wild-type twin spots. Cells in *shn* mutant clones fail to express *omb*, *Dad*, and *vg*, but ectopically express *brk*. In all cases loss of *shn* has a cell-autonomous effect on target gene expression and includes cells far away from the *dpp*-expressing cells at the A/P boundary.

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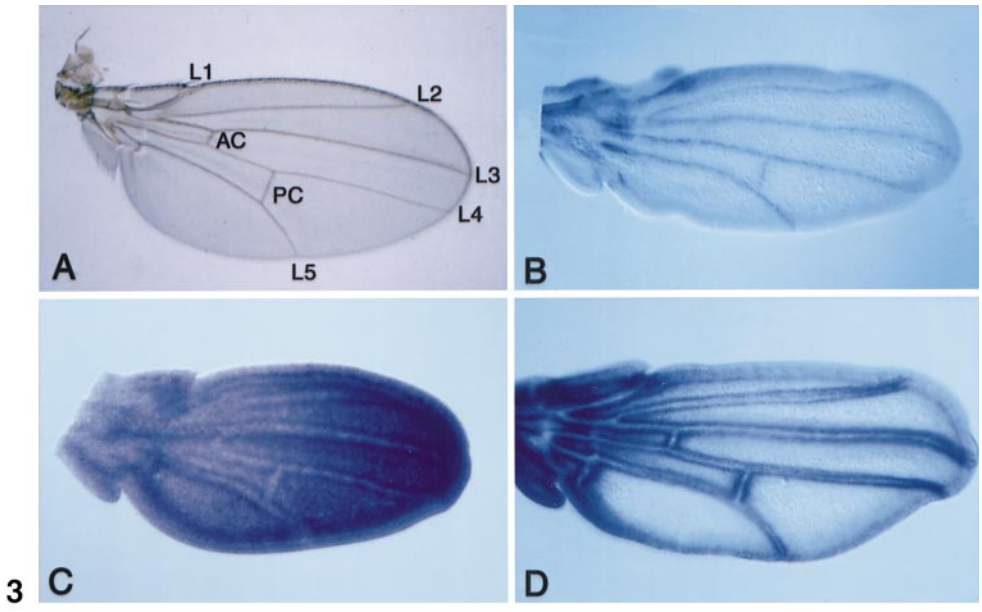

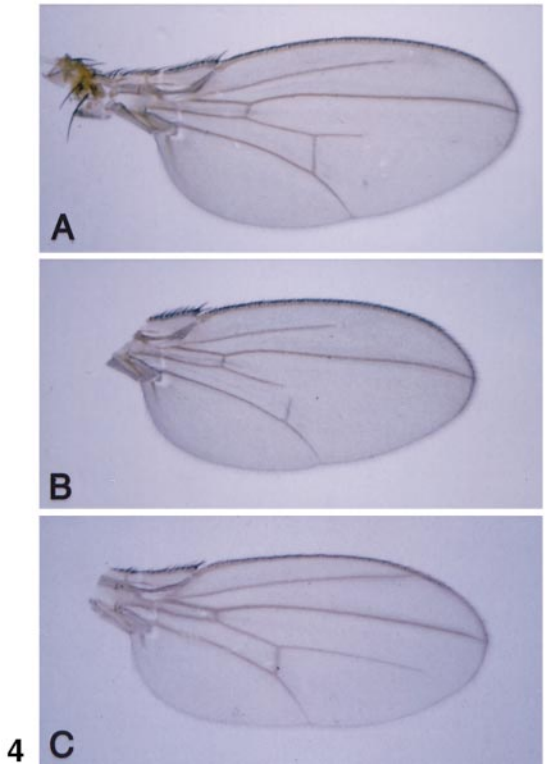

FIG. 3. Expression of *shn* in pupal wings. (A) Wild-type wing showing longitudinal veins (L1-L5) as well as the anterior (AC) and posterior (PC) cross veins. (B–D) Wild-type pupal wings 24–30 h APF hybridized with digoxigenin-labeled antisense riboprobes to detect transcription of *dpp* (B), *shn* (C), and *tkv* (D). (B) *dpp* is expressed in the prospective vein cells of L1–L5. (C) *shn* mRNA can be detected throughout the wing blade, but is present at higher levels flanking each vein. (D) Transcription of the receptor *tkv* is also modulated in vein territories. Maximal levels of mRNA are detected in the border cells on either side of the presumptive vein.

FIG. 4. The dpp^{s1} phenotype is sensitive to dosage of *shn*. (A) Adult wings from dpp^{s1} homozygous animals exhibit distal truncation of the L2 and L4 veins. (B) In dpp^{s1}, $+/dpp^{s1}$, shn^{IB09} wings, the truncation of both L2 and L4 veins is exacerbated and the posterior cross vein is reduced in size. (C) Elevated levels of Shn provided by a *hsp70-shn* transgene in a dpp^{s1} background rescues the loss of longitudinal vein L2 and significantly increases the length of L4 distally.

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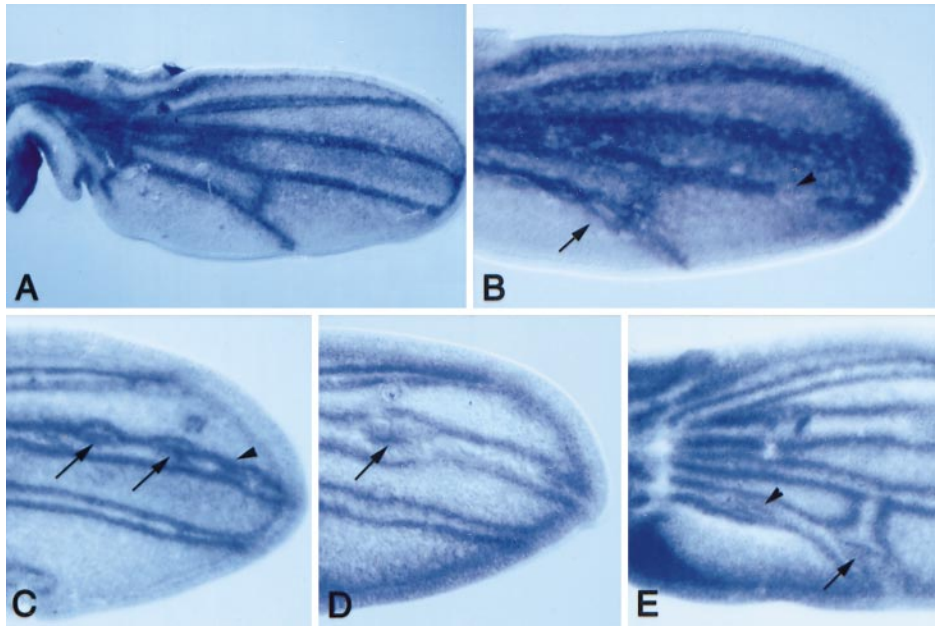

FIG. 5. *shn* affects *Dad* and *tkv* expression in pupal wings. (A) In wild-type pupal wings *Dad* is expressed in the prevein territory in a pattern somewhat wider than that of *dpp* (compare with Fig. 3B). Low level graded expression is also observed in the intervein regions near the A/P boundary (between veins L3 and L4). This may be due to perdurance of β-Gal from earlier expression in the imaginal disc during the third larval instar. *Dad* expression was visualized using the Dad^{P1883} reporter. (B) *Dad* expression in pupal wings containing shn^{IM56} clones. Expression is abolished in a section of the L4 vein marked with an arrowhead. In some cases loss of *Dad* expression is associated with ectopic transcription in adjacent cells (arrow in L5). Reporter gene expression is also lost in patches of cells throughout the wing blade in intervein regions. (C–E) Alterations in pattern of *tkv* expression in shn^{IM56} mosaic wings, visualized using antisense riboprobe. Ectopic domains of *tkv* expression are attributed to loss of *shn* function. In several instances the flanking domains of *tkv* are unaltered (marked with an arrowhead in C, E). In other examples flanking tracks of *tkv* expression diverge around ectopic *tkv* (marked with arrows in C–E). The pupal wings depicted in this figure are 24–30 h APF.

dpp^{s1}animals carrying the *hsp70-shn* transgene even in the absence of heat shock. This effect is most likely a result of low level read-through from the *hsp70* promoter at 25°C, the temperature at which the fly stocks were maintained. Overall, our results suggest that *shn* is required for Dpp responsiveness during pupal development and enables cells to trigger the process of vein differentiation.

Shn Affects Gene Expression in the Pupal Wing

Among the Dpp-responsive genes in the wing imaginal disc (*sal, vg, omb,* and *Dad*), only *Dad* is known to affect vein differentiation during pupal development (K. Tsuneizumi, personal communication). To determine whether *shn* activity is required for target gene expression in pupal wings, we induced *shn* clones during prepupal stages and assayed their effect on transcription of *Dad*. Although in these experiments the clones were not labeled, the changes in gene expression are consistent with, and appear to anticipate, the vein phenotypes characteristic of *shn* clones in the adult wing (see Materials and Methods). We first examined expression of the Dpp target gene *Dad* in wild-type pupal wings, using the *P1883*-LacZ enhancer trap that is inserted upstream of the Dad transcription unit (Tsuneizumi *et al.*, 1997). Strong expression of Dad was detected in the presumptive vein cells in a domain somewhat broader than that of *dpp* transcription (Fig. 5A; compare with Fig. 3B). A weak signal was seen in intervein cells close to the A/P boundary that falls between the L3 and L4 veins. This could result from perdurance of *Dad*-LacZ expression in the wing disc during larval development. Wings from dpp^{s1} animals carrying a copy of the transgene showed reduced expression of *Dad* in L2 and L4 confirming that it is sensitive to changes in the level of Dpp signaling (data not shown). In pupal wings mosaic for *shn,* we frequently observed loss of *Dad*-LacZ in vein cells and occasional large gaps in expression consistent with the loss of vein tissue in adults (see arrowhead in Fig. 5B; compare with Fig. 1G). Hence we infer that lack of Shn activity results in loss of *dpp*-responsive gene expression in pupal wings. Interestingly, we also detected ectopic *Dad* expression adjacent to vein cells that showed loss of *Dad*. This is reminiscent of the non-cell-autonomous effect associated with *shn* clones in the adult wing, i.e., the differentiation of ectopic veins by wild-type cells surrounding the mutant clone (see arrow in Fig. 5B; compare with Figs. 1E, H). The ectopic expression of

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Dad in pupal wings mosaic for *shn* suggests that the differentiation of vein tissue adjacent to *shn* clones may be mediated by Dpp (see Discussion).

Although *tkv* is not considered a canonical *dpp* target gene, it serves as a valuable marker since its transcription is keenly sensitive to levels of Dpp signaling. In the pupal wing *dpp* and *tkv* have overlapping but complementary expression patterns (Figs. 3B, D; de Celis, 1997; Yu *et al.*, 1996). The receptor is expressed at low levels in the vein cells that transcribe *dpp* and at higher levels on either side. These patterns result from a signaling-dependent feedback mechanism which ensures that *tkv* transcription is down-regulated in vein cells that are exposed to high levels of Dpp signaling, while *dpp* transcription is reduced in border cells that express high levels of *tkv* (de Celis, 1997). Thus in *shn* clones located within the vein domain, loss of *dpp* responsiveness would be expected to result in ectopic expression of *tkv*. In agreement with this, we found that *shn* mosaic wings frequently showed derepression of *tkv* within the presumptive vein region, while the flanking domains of *tkv* remained unaffected (see arrowhead in Figs. 5C, E). Despite the high levels of *tkv* expression, these cells would be defective in signaling and should fail to differentiate veins. Such examples are likely to correlate with adult clones of *shn* mutant cells that show loss of vein differentiation (see Fig. 1G). In other instances, small domains of ectopic *tkv* are bordered by tracks of *tkv* expression that loop around their normal positions (see arrows in Figs. 5C–E). These perturbations in the *tkv* pattern are suggestive of *shn* clones in the adult wing that do not differentiate vein tissue but are surrounded by ectopic veins on both sides (see Figs. 1E, I). In conclusion, our data demonstrate that *shn* activity is required for the expression of *dpp*-responsive genes during pupal vein differentiation, in addition to its earlier role in patterning in the wing disc.

DISCUSSION

Cell-Autonomous Requirement for shn in Expression of Dpp Target Gene and Specification of Positional Information

During larval development *dpp* is known to act as a morphogen in the wing imaginal disc. Transcription of *dpp* in a narrow stripe of cells along the A/P compartment boundary results in a diffusion gradient of the ligand that elicits concentration-dependent responses in cells of the entire wing pouch. Different levels of Dpp activity trigger the expression of transcription factors such as *sal, omb,* and *vg,* in nested domains that subdivide the disc and mediate aspects of wing patterning (Lecuit *et al.*, 1996; Nellen *et al.*, 1996). The results presented here demonstrate that *shn* activity is required in adult wings for interpreting the Dpp gradient in order to specify correct positional information (Fig. 1). Support for this view comes from the fact that *shn* loss-of-function clones fail to express *sal, omb, Dad,* and *vg* (Figs. 2A–C). In all cases, the transcription of the *dpp*-inducible target gene was unaffected in *shn* mutant clones located outside the wild-type domain of expression for each gene. It is important to note that the loss of gene expression in clones mutant for *shn* occurs in a cell-autonomous manner and affects cells at a distance from the source of the ligand Dpp. Therefore these phenotypes cannot be attributed to a requirement for *shn* in regulating *dpp* expression at the A/P boundary. Rather, our data indicate that *shn* function is required in the cells responding to the Dpp signal. Strikingly similar loss of expression of *sal, omb,* and *Dad,* as well as the *vg* quadrant enhancer, is encountered in mutants for components of the Dpp pathway, such as *tkv* and *Mad,* that eliminate *dpp* signaling (Kim *et al.*, 1997; Lecuit *et al.*, 1996; Nellen *et al.*, 1996; Tsuneizumi *et al.*, 1997). In contrast, the requirement for *Med* appears to be position dependent, since cells within the clone that are closer to the A/P boundary show residual *omb* expression (Wisotzkey *et al.*, 1998). A similar graded effect of *sax* null clones on *sal* expression is consistent with the idea that Sax is only required for interpreting peak levels of Dpp signaling (Singer *et al.*, 1997). Therefore the loss of target gene expression in *shn* mutant clones is significant and suggests that like Mad, Shn activity is essential for the transcriptional response to Dpp.

In the adult wing, the Dpp gradient is manifest in the specification of morphologically distinct sense organs and bristles in stereotypic positions along the anterior wing margin. Alterations in the level of signaling by ectopic activation of the *dpp* pathway or loss of the type I receptors *tkv* and *sax* have been shown to result in corresponding transformations in bristle pattern (Haerry *et al.*, 1998; Jazwinska *et al.*, 1999a; Minami *et al.*, 1999; Singer *et al.*, 1997; Sturtevant *et al.*, 1997). The absence of *shn* activity affects the ability of cells to perceive the *dpp* signal since clones in the wing margin show anterior shifts in cell fate typical of cells that are further away from the source of Dpp (Fig. 1). Interestingly, clones lacking *sax* retain residual A/P polarity and show a graded shift in cell fates, consistent with a partial loss of responsiveness. In contrast, absence of *shn* function affects all cells within the clone in a manner similar to loss of *tkv* signaling (Fig. 1C; Singer *et al.*, 1997). The bristle transformations observed along the wing margin in *shn* mutant clones are likely to result from the loss of *sal* expression, since of the downstream *dpp* target genes, only mutations in *sal* are known to cause similar anterior shifts in cell fate (Sturtevant *et al.*, 1997). This view is substantiated by the fact that both *tkv* and *sax* mutant clones that are characterized by loss or attenuation of *sal* expression also show defects in bristle pattern (Singer *et al.*, 1997). Despite the apparent overlap in the mutant phenotypes of *shn* and *sal* clones, a clear distinction can be made between the requirement for these two genes. Clones deficient for *sal* display a vein mispositioning phenotype in a location-specific manner. The boundary of *sal*/*salr*-expressing and -nonexpressing cells in the imaginal disc is critical for specifying the position of the future L2 and L5 veins. Clones lacking *sal* located between L2 and L3 frequently cause

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posterior displacement of L2, while clones in the region between L4 and L5 result in anterior displacement of L5. It has been postulated that loss of *sal* in these domains results in a novel sal^{+}/sal^{-} boundary and differentiation of an ectopic vein closer to the source of Dpp (de Celis and Barrio, 2000; de Celis *et al.*, 1996; Sturtevant *et al.*, 1997). By contrast, although *shn* clones fail to express *sal*, we do not observe differentiation of ectopic veins within the mutant tissue. *tkv* loss-of-function clones are similar to *shn* in this respect (Singer *et al.*, 1997). One explanation for this apparent paradox is that *sal* function is only required during disc development to specify vein location, while *shn* and *tkv* have an additional (essential) role in vein differentiation in pupal stages (see below).

In addition to its role in A/P patterning, *dpp* is also required non-cell autonomously for the proliferation of cells in the wing imaginal disc (Posakony *et al.*, 1991; Spencer *et al.*, 1982). Alternatively it is possible that Dpp signaling is required to prevent apoptotic cell death. Our clonal analysis reveals a requirement for *shn* in cell proliferation/survival that varies both temporally and spatially in a manner that reflects the graded requirement for Dpp. *shn* mutant clones induced in the first and second larval instar are recovered infrequently. In addition, clones located in extreme anterior or posterior positions further away from the source of *dpp* are larger and recovered more frequently than those located closer to the A/P boundary (Table 1; Figs. 1C and D and Fig. 2). These results are consistent with previous reports supporting the idea that *shn* and *tkv* have linked functions in cell survival and proliferation (Burke and Basler, 1996; Penton *et al.*, 1997). In contrast the requirement for *sax* in cell proliferation is limited. Relatively large clones of *sax* can be recovered even when they are induced in the first larval instar, and these clones are only marginally compromised for growth (Singer *et al.*, 1997). The transcription factors Sal, Omb, and Vg have been implicated in the proliferation of cells in the wing blade. Homozygous *vg* mutant flies have highly reduced wings and clones of cells mutant for *sal* or *omb* exhibit a growth disadvantage (de Celis *et al.*, 1996; Grimm and Pflugfelder, 1996; Lecuit *et al.*, 1996; Simpson *et al.*, 1981; Williams *et al.*, 1991). Thus the reduced viability of cells in the absence of Shn activity may be a consequence of the requirement for *shn* in transcription of these target genes.

shn Is Essential for Wing Vein Differentiation in the Pupal Wing

The positioning and differentiation of wing veins in *Drosophila* is initiated in the imaginal disc and elaborated during pupal wing development. In the imaginal disc, the combined activities of the Dpp and Hedgehog pathways culminate in the restricted expression of genes such as *veinlet* and *Delta* (*Dl*) in longitudinal stripes that represent the future position of individual veins (reviewed in Blair, 1999). These early acting genes are critical for vein differentiation in the pupal wing where they regulate expression of genes such as *dpp*/*tkv* that act later in the process. The vein-competent territories are refined during later stages of pupal development by interactions that require inputs from the Dpp, EGFR, and Dl/N signaling pathways (reviewed in de Celis, 1998; Sturtevant and Bier, 1995). During this stage, the initial broad pattern of *dpp* and *tkv* transcription within the pro-vein domain becomes modulated to reflect the expression patterns depicted in Figs. 3B, D.

Our results provide strong evidence that in addition to its early requirement in patterning the wing disc, there is a later role for Shn in *dpp*-mediated vein differentiation during pupal development. First, heterozygosity for *shn* exacerbates the loss of vein tissue in dpp^{s1} adults, a sensitized genetic background that specifically perturbs wing development at pupal stages (Fig. 4B; Staehling-Hampton *et al.*, 1995). The fact that ectopic expression of Shn immediately preceding pupal wing development partially rescued the dpp^{s1} phenotype lends support to the idea that the phenocritical period for Shn activity in vein differentiation is during pupal stages (Fig. 4C). The spatial distribution of *shn* transcript in pupal wings with maximal levels in the vein border also suggests an important role for *shn* in this tissue. Furthermore, clonal analysis demonstrates that *shn* is essential for differentiation of all longitudinal veins irrespective of their location. Since Dpp signaling in the wing disc determines the position of the L2 and L5 veins (through activation of *sal*), the requirement for *shn* in all veins supports the idea that it is involved in *dpp*-mediated vein differentiation at pupal stages. It is noteworthy that while loss of vein tissue occurs in clones lacking *tkv*, *Mad*, *Med*, and *shn*, none of the downstream *dpp* targets in the disc are known to be essential for vein differentiation. Thus it is likely that Shn may activate other Dpp target genes besides *sal*, *omb*, and *vg*, that mediate vein differentiation.

An intriguing feature of *shn* mutant clones is that although vein differentiation is blocked within the clone in a cell-autonomous manner, in some instances adjacent wild-type tissue is induced to adopt vein cell fate (Figs. 1G–I). This effect is not restricted to mutations in *shn*, and probably results from disruption of Dpp signaling, since *tkv* as well as *Mad* clones also display this behavior (Burke and Basler, 1996; Marquez *et al.*, 2000). The ectopic vein phenotype could result either from upregulation of a diffusible vein-inducing factor in mutant cells defective in Dpp signaling or from downregulation of a signal involved in lateral inhibition of veins. The vein-promoting factor could be *dpp* itself, since it has been shown that in tkv^{1}/tkv^{8} animals with reduced receptor function, *dpp* is expressed ectopically in a wider than normal domain in the pupal wing (de Celis, 1997). Diffusion of excess Dpp would induce adjacent wild-type cells to adopt vein identity (and respond by downregulating receptor expression) while cells farther away express higher levels of *tkv*, thus resulting in the patterns of *tkv* expression observed in Figs. 5C–E and the adult phenotypes in Figs. 1E, H, I. Alternatively, abrogation of Dpp signaling could result in loss of Dl expression in

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mutant cells that include the vein domain and consequently eliminate the inhibition of vein differentiation in adjacent cells via the N pathway. The ectopic veins would express *dpp* that in turn could reorganize *tkv* expression. In this regard it is significant that in adult wings we observe ectopic veins primarily near endogenous vein tissue, suggesting that these interactions may only hold true for clones that occur within the vein-competent domain. A similar explanation may account for the ectopic vein differentiation reported for *tkv* and *Mad* clones (Burke and Basler, 1996; Marquez *et al.*, 2000).

Role of shn in Mediating the Nuclear Response to Dpp

Several lines of evidence indicate that *shn* is involved in mediating the nuclear response to Dpp. Shn protein is localized to the nucleus and contains multiple C_2H_2 zinc finger motifs consistent with its role as a DNA-binding factor. Mutations in *shn* abolish expression of several well-characterized *dpp* target genes in the embryo as well as the disc, indicating that its activity is critical for responsiveness to Dpp (this study; Arora *et al.*, 1995; Grieder *et al.*, 1995; Staehling-Hampton *et al.*, 1995). We have recently identified Shn-binding sites in the Dpp-responsive Ubx-B enhancer element that also contains characterized binding sites for Mad. Furthermore, we have shown that Shn and Mad can associate directly and stimulate transcription of a Dpp-responsive reporter in a cooperative manner (Dai *et al.*, 2000). Collectively, these data strongly support a role for Shn as a cofactor for Mad in Dpp signaling.

Dpp signal transduction was initially thought to consist of a linear pathway culminating in the direct activation of target gene expression by Mad and Medea. Recent studies focusing on the role of *Dad* and *brk*, both of which negatively regulate *dpp* target gene expression, have highlighted the fact that the ultimate outcome of Dpp signaling may depend on the balance between activation and antagonism of the pathway. Dad is presumed to act in the cytoplasm by regulating levels of Mad phosphorylation, and clones mutant for *Dad* ectopically transcribe *omb* (Tsuneizumi *et al.*, 1997). We have demonstrated that despite the absence of *Dad* expression, *omb* transcription is not detectable in *shn* clones (Figs. 2A, B). Thus the loss of Dad cannot upregulate Dpp target gene expression in the absence of Shn function.

The transcriptional repressor Brk acts to refine the threshold response to Dpp signaling by inhibiting target gene expression. Mutations in *brk* result in ectopic expression of known *dpp*-responsive genes in the wing disc, and conversely misexpression of *brk* strongly reduces the expression of both *sal* and *omb* (Campbell and Tomlinson, 1999; Jazwinska *et al.*, 1999a; Minami *et al.*, 1999). Analysis of gene expression in cells in which inputs from both Dpp and Brk were simultaneously removed has resulted in the idea 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, exemplified by *omb* in the disc, only requires Dpp-mediated relief from repression by Brk. Thus *omb* is transcribed in *brk; mad* double mutant clones even in the absence of signaling. Promoters for other target genes such as *sal* and *vg* incorporate both positive inputs from Mad and negative inputs from Brk. It has been proposed that a third class of genes is directly activated in response to Dpp signaling and is not repressed by Brk. We have shown that expression of both class I and class II genes are completely abolished in the absence of *shn* activity (Figs. 2A, C; and data not shown). Since no examples of the third class of genes have been identified in the wing disc thus far, it is not possible to evaluate the effect of *shn* on their expression. Based on the current model, the observation that mutations in *shn* affect expression of *omb* (a class I gene) implicates Shn in Dpp-mediated repression of *brk* transcription. In support of this view, loss of *shn* activity in mutant clones results in ectopic *brk* expression (Fig. 2D). The fact that Shn and Mad can interact directly, coupled with the demonstration that both genes are required for regulating *brk* expression, raises the possibility that Shn could mediate Brk repression by acting as a cofactor with Mad. It is striking that in the wing imaginal disc loss of *shn* activity affects expression of all five *dpp* target genes we have assayed. These results suggest that Shn may primarily affect the range of concentrations over which Mad can elicit transcriptional responses, rather than increase its specificity. Genetic epistasis experiments to examine the relative contribution of Shn and Brk, as well as analysis of individual target gene promoters, will be critical in establishing whether Shn acts in concert with Mad in each instance, or has a more limited role in regulating a subset of Dpp responses.

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