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Splits ends is a tissue/promoter specific regulator of Wingless signaling

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SUMMARY

Wingless directs many developmental processes in *Drosophila* by regulating expression of specific target genes through a conserved signaling pathway. Although many nuclear factors have been implicated in mediating Wingless-induced transcription, the mechanism of how Wingless regulates different targets in different tissues remains poorly understood. We report here that the *split ends* gene is required for Wingless signaling in the eye, wing and leg imaginal discs. Expression of a dominant-negative version of *split ends* resulted in more dramatic reductions in Wingless signaling than *split ends*-null alleles, suggesting that it may have a redundant partner. However, removal of *split ends* or expression of the dominant-negative had no effect on several Wingless signaling readouts in the embryo. The expression pattern of Split ends cannot explain this tissue-specific requirement, as the protein is predominantly nuclear and present throughout embryogenesis and larval tissues. Consistent with its nuclear location, the *split ends* dominant-negative acts downstream of Armadillo stabilization. Our data indicate that Split ends is an important positive regulator of Wingless signaling in larval tissues. However, it has no detectable role in the embryonic Wingless pathway, suggesting that it is a tissue or promoter-specific factor.

Key words: *Drosophila*, *wingless*, *split ends*, SHARP, MINT

INTRODUCTION

The *Drosophila* Wingless (Wg) protein is a founding member of the Wnt family, which consists of secreted glycoproteins that are conserved throughout the animal kingdom. Wnts play vital roles in a wide range of events during development in worms, flies, amphibians and mice (Cadigan and Nusse, 1997). Inappropriate activation of the Wnt signaling pathway has also been implicated in several forms of human cancers (Polakis, 2000).

Cells respond to Wg via a highly conserved signaling cascade that centers on Armadillo (Arm). In unstimulated cells, Arm is constitutively expressed but the cytosolic pool is phosphorylated by a degradation complex containing two kinases, Shaggy/Zeste white 3 (Sgg/Zw3) and Casein Kinase Iα (CKIα). Phosphorylated Arm is then rapidly degraded through the ubiquitination/proteosome pathway (Yanagawa et al., 2002). Wg signaling, through a membrane receptor complex, activates the cytoplasmic protein Disheveled, which in turn inhibits the function of the degradation complex, resulting in the stabilization and accumulation of Arm (Cadigan and Nusse, 1997; Polakis, 2000). The consensus view of downstream events is that the stabilized Arm translocates to the nucleus where it forms a complex with the DNA-binding protein TCF (Pangolin – FlyBase) and two other proteins, Legless and Pygopus (Pygo) (Belenkaya et al., 2002; Kramps et al., 2002; Parker et al., 2002; Thompson et al., 2002). How this nuclear complex mediates the regulation of Wg transcriptional targets is not understood, though several other factors have also been implicated in the process (for a review, see Hurlstone and Clevers, 2002).

In the absence of Arm, TCF is thought to transcriptionally repress Wg target genes by interacting with the transcriptional co-repressor Groucho (Cavallo et al., 1998). In addition, the ARID domain protein Osa has been shown to repress Wg target genes by acting in a chromatin remodeling complex that contains the bromodomain protein Brahma (Collins et al., 1999; Collins and Treisman, 2000). Binding of Arm to TCF somehow blocks the functions of these factors and converts TCF into an activator (Hurlstone and Clevers, 2002).

The Wg signaling pathway is used repeatedly throughout fly development where it exerts differential regulation on many genes in various tissues and cell types (Klingensmith and Nusse, 1994; Zecca et al., 1996). The molecular basis for this specificity is not well understood. Some of these differential responses are due to combinatorial inputs of multiple signaling cascades (Campbell et al., 1993; Lockwood and Bodmer, 2002). In other instances, there is evidence suggesting that other co-factors may be involved in regulating the activity of Arm/TCF in specific tissues or stages. Such examples include the zinc-finger protein Teashirt (Tsh) and transcriptional repressor Brinker in the embryonic ventral epidermis and midgut (Gallet et al., 1998; Waltzer et al., 2001; Saller et al., 2002) and the nuclear protein Lines (Lin) in the dorsal epidermis of the embryo (Hatini et al., 2000).

This report describes the role of the *split ends* (*spen*) gene

in Wg signaling. Spen is a predominantly nuclear protein containing three RNA recognition motifs (RRM) and a SPOC domain at the C terminus (Kuang et al., 2000; Rebay et al., 2000; Wiellette et al., 1999). Spen has previously been implicated in neuronal cell fate, survival and axonal guidance (Chen and Rebay, 2000; Kuang et al., 2000), cell cycle regulation (Lane et al., 2000) and repression of head identity in the embryonic trunk (Wiellette et al., 1999). At the genetic level, *spen* has been suggested to act with the Hox gene Deformed (Wiellette et al., 1999) and the EGF/Ras signaling pathway (Chen and Rebay, 2000; Rebay et al., 2000). The human homolog SHARP has been shown to act as a transcriptional co-repressor for steroid hormone receptors (Shi et al., 2001) and RBP-Jκ, which mediates Notch signaling (Oswald et al., 2002). The mouse homolog MINT has also been shown to bind to specific DNA sequences through its RRM domains (Newberry et al., 1999). We demonstrate that Spen is a positive regulator of Wg signaling in the larval eye, wing and leg imaginal discs. Consistent with its nuclear location, Spen acts downstream of stabilized Arm. Interestingly, we could find no requirement for *spen* in embryonic Wg signaling, indicating that it is a tissue or target promoter specific regulator of the pathway.

MATERIALS AND METHODS

Drosophila strains

The P[*sev-wg^{ts}*] transgene has been described previously (Cadigan et al., 2002); the P[*sev-wg*] stock was from Konrad Basler (Brunner et al., 1997); the P[*GMR-arm**]^{F36} stock (Freeman and Bienz, 2001) was kindly provided by Mariann Bienz; P[*GMR-hid*]^{10} and P[*GMR-rpr*] were from Bruce Hay; P[*GMR-hid*]^{IM} was from from John Abrams and Antony Rodriguez; P[*UAS-EGFR^{DN}*] was from Matt Freeman (Freeman, 1996); and P[*UAS-lacZ*] was from the Bloomington stock center, as were the Gal4 drivers P[*GMR-Gal4*], P[*armadillo-Gal4*] (*arm-Gal4*) and P[*paired-Gal4*] (*prd-Gal4*). P[*engrailed-Gal4*] (*en-Gal4*) and P[*patched-Gal4*] (*ptc-Gal4*) were from Ron Johnson; P[*daughterless-Gal4*] (*da-Gal4*) was from Andreas Wodarz; P[*twist-Gal4*] (*twi-Gal4*) and P[*twi-Gal4*]; P[*24B-Gal4*] were from Rolf Bodmer; P[*UAS-TCF^{DN}*] was from M. Piefer; P[*dpp-lacZ*] (Blackman et al., 1991) was from Laurel Raftery; and *dpp^{blk}* was from Jessica Treisman. The *spen^{k07721}* and *spen^{k13624}* lines were obtained from the Berkeley *Drosophila* Genome Project (http://www.fruitfly.org). *spen^{3}*, P[*FRT, hs-neo*]^{40A} (Kuang et al., 2000) and *spen^{XIE1796}*, P[*FRT, hs-neo*]^{40A} (P. Kolodziej, personal communication) were provided by Peter Kolodziej. *spen^{3}* is a point mutation that results in a truncation after the RRMs (at amino acids 964) (Kuang et al., 2000). *spen^{XIE1796}* is a 10 bp deletion causing a frameshift that affects the C-terminal 99 residues of Spen, removing more than half of the SPOC domain (P. Kolodziej, personal communication).

For mosaics, *spen^{9C7}* and *spen^{14C2}* were recombined onto a P[*FRT, hs-neo*]^{40A} chromosome as described previously (Xu and Rubin, 1993). The P[*FRT, hs-neo*]^{82B}, *pygo^{10}* stock was described previously (Parker et al., 2002). Clonal markers were P[*arm-lacZ*]^{3R} (from D. J. Pan) and a P[*Ubi-GFP^{nls}*] on 2L (Davis et al., 1995). Mitotic clones were induced with P[*hs-flp*]^{1} (Golic and Lindquist, 1989) in the wing (60-90 minute heatshock 48-72 hours after egg laying), and P[*eyeless-flp*]^{T11} (*eye-flp*) or P[*eye-flp*]^{T12} (Newsome et al., 2000) in the eye. Embryonic balancer chromosome markers were CyO P[*larB208*] (Grossniklaus et al., 1992) and TM3 *eve-lacZ*.

P[*UAS-spen^{DN}*] lines were constructed by PCR amplifying the C-terminal 2.8 kb of the *spen*-coding region (amino acids 4540-5476) using the oligos 5′GGAAGATCTATGCCGAAGAAGAAGCGCAAGGTGGTTGCCGCCAGTCATTTGGCACC3′ and 5′CCGCTCGAGTTAGACAGTAGCGATGACAATCAG3′, digesting with *Bgl*II and *Xho*I, ligating into pUAST and injecting into *w^{1118}* embryos to obtain transgenics. The first primer contains a nuclear localization signal (PKKKRKV). Two lines were used, P[*UAS-spen^{DN}*]^{II} and P[*UAS-spen^{DN}*]^{III}, the latter of which gave significantly stronger phenotypes. These transgenes cause midline glia defects similar to those previously observed in *spen* mutants (Chen and Rebay, 2000). In addition, the severity of this phenotype is enhanced in *spen* heterozygotes and a *spen^{DN}* rough eye phenotype is suppressed in animals carrying a duplication of the *spen* locus (D.B.D. and I.R., unpublished). These results suggest that the Spen^{DN} protein is acting to antagonize endogenous *spen* activity.

Excisions were generated from *spen^{k07721}* and *spen^{k13624}* using the *Δ2-3, Sb* chromosome (Robertson et al., 1988); homozygous viable revertant lines were isolated.

Fly crosses were maintained at 25°C unless otherwise noted.

Isolation of new *spen* alleles

The *spen* alleles were generated using the mutagen ethyl methane sulfonate, and identified in a screen for modifiers of the P[*sev-wg^{ts}*] interommatidial bristle phenotype. The screen was performed at 17.6°C as described previously (Cadigan et al., 2002). Two suppressors belonged to a single lethal complementation group and were subsequently found to be allelic to *spen* (see Results). The molecular nature of these alleles was not determined.

Whole-mount staining and microscopy

Immunostaining was as described previously (Cadigan and Nusse, 1996). Rat anti-Spen (1:1000) was from P. Kolodziej, affinity-purified rabbit anti-Wg antisera (1:50) and mouse anti-Dfz2 (1:50) were from R. Nusse, mouse monoclonal anti-Ac (1:20) was from the Developmental Hybridoma Bank, rat anti-Elav (1:100) was from G. Rubin, and guinea pig anti-Slp1 (1:100) was from S. Small. Guinea pig anti-Sens (1:1000) was from H. Bellen, rabbit anti-Eve (1:100) was from Z. Han and R. Bodmer, mouse monoclonal anti-En supernatant (1:2) was from the University of Iowa Hybridoma Bank, mouse and rabbit anti-β-galactosidase (1:500) were from Sigma and Cappel, respectively. Cy3- and Cy5-conjugated secondary antibodies were from Jackson Immunochemicals and Alexa Flour 488-conjugated secondaries were from Molecular Probes. All fluorescent pictures were obtained with a Zeiss Axiophot coupled to a Zeiss LSM510 confocal apparatus. All images were processed as Adobe Photoshop files. Cuticles were prepared and photographed as previously described (Bhanot et al., 1999). Flies were prepared for scanning electron microscopy (SEM) as described (Cadigan et al., 2002). The samples were viewed with a scanning electron microscope and photographed using Polapan 400 film (Kodak).

RESULTS

Mutations in *spen* dominantly suppress a sensitized Wg phenotype in the eye

Misexpression of *wg* in the eye via the *sevenless* promoter (P[*sev-wg*]) blocks interommatidial bristle formation, owing to Wg-mediated repression of proneural gene expression (Cadigan et al., 2002; Cadigan and Nusse, 1996). P[*sev-wg^{ts}*] flies express a temperature-sensitive form of Wg (Wg^{ts}). At 17.6°C, the Wg^{ts} protein is partially active, and these animals have 150-200 bristles/eye (compared to 600/eye in wild type) (Cadigan et al., 2002) (Fig. 1A). This temperature was chosen as a sensitized background in which a screen for dominant modifiers was performed in order to identify genes that interact with *wg*.

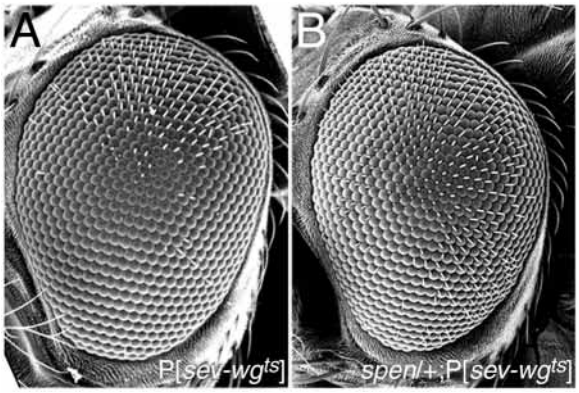

Fig. 1. *spen* dominantly suppresses a P[*sev-wg*^{ts}] eye bristle phenotype. Micrographs are SEMs of adult fly heads. (A,B) Flies contain the P[*sev-wg*^{ts}] transgene and are heterozygous for either the parental chromosome *iso*^{5A1} (A) or *spen*^{9C7} (B), both reared at 17.6°C. The *spen* heterozygotes display significant suppression of the P[*sev-wg*^{ts}] partial loss of interommatidial bristles. A similar suppression was also seen with *spen*^{14C2} (data not shown).

Two suppressors of the P[*sev-wg*^{ts}] phenotype increase the number of bristles to ~400/eye (Fig. 1B, data not shown). They form one lethal complementation group, which was mapped to the tip of chromosome 2L between 21B4-B6. Several lines of evidence demonstrate that these suppressors are alleles of *spen*, and they will hereby be referred to as *spen*^{9C7} and *spen*^{14C2}. First, both *spen*^{9C7} and *spen*^{14C2} fail to complement alleles of *spen*. Second, immunostaining using Spen-specific antiserum (Kuang et al., 2000) showed that Spen protein is dramatically reduced in clones of *spen*^{14C2} (Fig. 2, clones marked by lack of GFP), and is present at higher than wild-type levels in *spen*^{9C7} clones (data not shown). Third, *spen*^{9C7} and *spen*^{14C2} germline clone embryos have head sclerite defects similar to those seen with other *spen* alleles (data not shown) (Wiellette et al., 1999). Finally, identical effects on several Wg readouts in the eye and the wing were observed in clones of the suppressors and known alleles of *spen* (see below).

We found Spen protein to be predominantly nuclear in eye imaginal discs (Fig. 2), consistent with previous reports in the embryo (Kuang et al., 2000; Wiellette et al., 1999). Spen is ubiquitously expressed throughout embryogenesis as well as in the larval eye, wing and leg imaginal discs (data not shown).

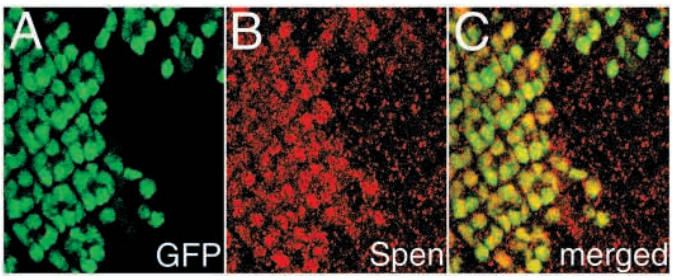

Fig. 2. Spen protein is severely reduced in *spen*^{14C2} clones. (A-C) Confocal images of a third instar eye imaginal disc containing *spen*^{14C2} clones generated by *ey-FLP*. Clones were marked by the absence of nuclear GFP (A) and stained for Spen (B); the merged image is shown (C). Spen signal is predominantly nuclear (C) and is greatly reduced in *spen*^{14C2} clones.

Spen potentiates Wg signaling in the eye

The suppression of the P[*sev-wg*^{ts}] phenotype suggests that Spen is a positive effector of Wg signaling in the eye. To

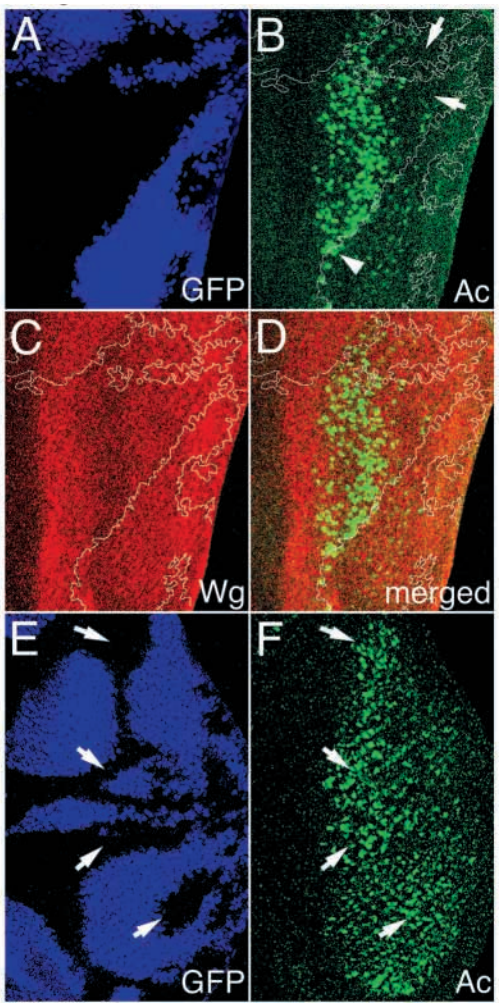

Fig. 3. *spen* is required for maximal Wg-dependent repression of proneural genes in the eye. (A-D) Confocal images of a P[*sev-wg*] pupal eye (3-6 hours after pupal formation – APF) containing *spen*^{3} clones generated by *ey-FLP*. Clones of *spen*^{3} were marked by the absence of GFP (A; clonal boundaries shown by the white lines in B-D) and were stained for Ac (B) and Wg (C); the merged image is shown (D). P[*sev-wg*] eyes have high levels of Wg behind the morphogenetic furrow (C) and low levels of Ac (B) outside the *spen*^{3} clones. Ac is derepressed in much of the clone (B; note that cells in and ahead of the MF are not competent to express Ac) but significant Ac repression still occurs inside the clone (arrows in B). Transgenic Wg levels are unaffected in *spen*^{3} clones (C). Similar Ac derepression and P[*sev-wg*] expression are also seen in *spen*^{14C2} clones (data not shown). Occasional non-autonomous derepression of Ac is observed adjacent to *spen* clones (B, arrowhead). (E,F) Confocal images of a pupal eye (3-6 hours APF) containing *spen*^{14C2} clones generated by *ey-FLP*. Clones of *spen*^{14C2} were marked by the absence of GFP (E) and were stained for Ac (F). Ac expression is elevated in *spen*^{14C2} clones (arrows in F; note that the laser intensity is lower than in B).

examine this in more detail, we used clonal analysis with *spen* mutant alleles and *spen* hypomorphic combinations to explore its requirement on Wg-dependent inhibition of bristle formation and morphogenetic furrow (MF) initiation.

Ectopic Wg in P[*sev-wg*] flies represses the expression of a proneural protein, Acheate (Ac) (Cadigan and Nusse, 1996). In clones of *spen* in a P[*sev-wg*] background, Ac is significantly derepressed (Fig. 3B), while the expression of Wg is not affected (Fig. 3C). This strongly suggests that *spen* alleles do not suppress the P[*sev-wg*] phenotypes by reducing Wg expression. Ac is not derepressed in all areas of the *spen* clones (Fig. 3B, arrows), indicating that a significant level of Wg-dependent Ac repression still occurs in the absence of *spen*. Ac derepression is occasionally seen in cells adjacent to the *spen* clones (Fig. 3B, arrowhead), which may be caused by defects in Ras-dependent activation of Delta (Dl) expression (see Discussion).

The conclusion that *spen* is partially required for Wg-dependent bristle inhibition is complicated by the fact that loss of *spen* causes an increase in Ac expression in wild-type eyes as well (Fig. 3E,F). As Wg signaling is thought to play no physiological role in proneural gene expression in the interior of the eye (Cadigan et al., 2002; Cadigan and Nusse, 1996), these data indicate that removal of *spen* is affecting a Wg-independent process that could account for the effects of *spen* on P[*sev-wg*]-mediated Ac inhibition.

To determine if *spen* was required for other Wg readouts in the eye, we examined its effect on Wg-mediated inhibition of the MF. The MF is a coordinated wave of apical constriction of the columnar epithelial cells that triggers differentiation of the fly eye. The MF starts at the early third larval instar and sweeps across the eye imaginal disc, from the posterior to the anterior (Wolff and Ready, 1993). Clusters of photoreceptors develop behind the MF (Fig. 4G,J, marked by the neuronal protein Elav). Wg is expressed at the dorsal and ventral edges of the eye disc (Fig. 4D,J), where it inhibits MF initiation (Ma and Moses, 1995; Treisman and Rubin, 1995). Decapentaplegic (Dpp) signaling at the posterior edge represses Wg expression. In the eye-specific dpp^{blk} mutant, Wg expression in early third instar discs expands posteriorly (Royet and Finkelstein, 1997) (compare Fig. 4J with 4K), causing a partial inhibition of the MF, reduced photoreceptor differentiation (Fig. 4H) and resulting in an adult small eye phenotype (Fig. 4B) (Chanut and Heberlein, 1997). Posterior expansion and upregulation of Wg is also observed in late third instar dpp^{blk} eyes (Fig. 4E).

The dpp^{blk} small eye phenotype is greatly suppressed by removal of *wg* activity (Treisman and Rubin, 1995). To examine the effect of *spen* on this Wg readout, we reduced *spen* dosage in the dpp^{blk} background using transheterozygotes of $spen^{k07721}$ and $spen^{k13624}$, two P-element insertions in the *spen* region. These alleles fail to complement $spen^{9C7}$, $spen^{14C2}$ and several other *spen* alleles, and they have reduced viability when transheterozygous (Wielette et al., 1999) (data not shown). Reduction of *spen* gene activity significantly rescued photoreceptor formation and the adult small eye phenotype of

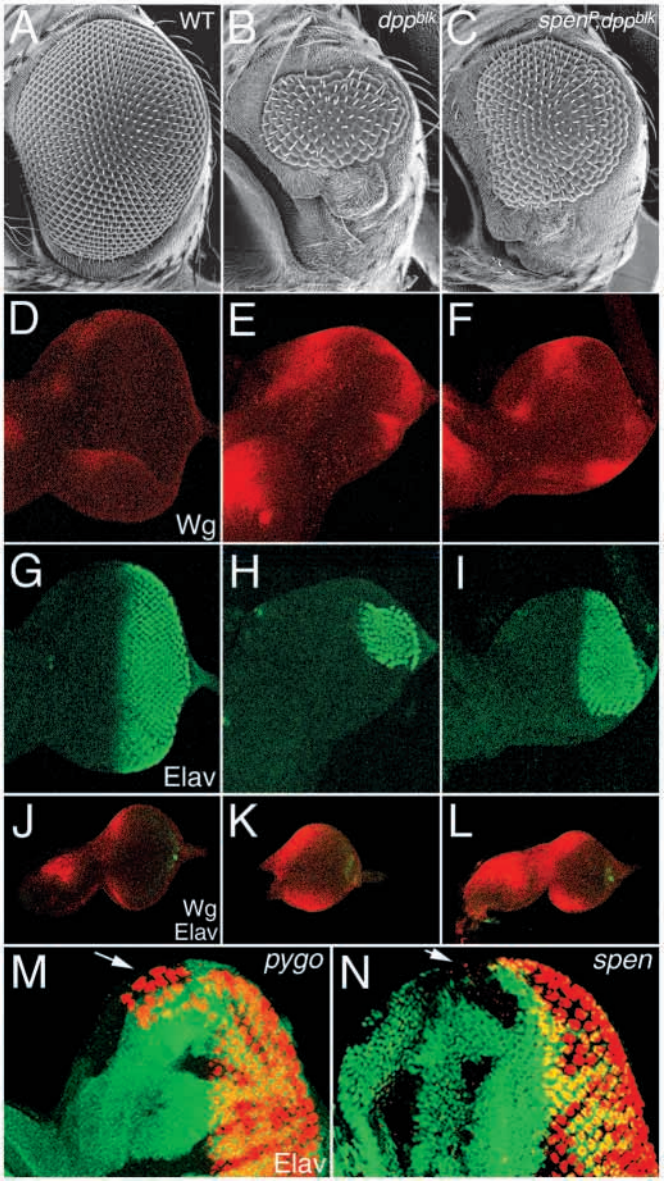

Fig. 4. *spen* potentiates Wg-dependent repression of the morphogenetic furrow. (A-C) SEM images of adult eyes of wild-type (A), dpp^{blk} (B) and $spen^{k07721}$, $dpp^{blk}/spen^{k13624}$, dpp^{blk} (C) flies. (D-L) Confocal images of eye imaginal discs with the following genotypes: $spen^{k07721\text{-}rev}/spen^{k13624\text{-}rev}$ (D,G,J), $spen^{k07721\text{-}rev}$, $dpp^{blk}/spen^{k13624\text{-}rev}$, dpp^{blk} (E,H,K) and $spen^{k07721}$, $dpp^{blk}/spen^{k13624}$, dpp^{blk} (F,I,L). The *k07721-rev* and *k13624-rev* alleles are homozygous viable revertants of the *spen* P-element alleles *k07721* and *k13624* resulting from precise excisions. Late third instar eyes (D-I) and early third instar eyes (J-L) are stained for Wg (D-F,J-L) and Elav (G-L). Ectopic Wg in dpp^{blk} eyes inhibits the furrow, thereby reducing Elav-positive photoreceptors (compare G with H) and resulting in a small eye phenotype (compare A with B). Reduced *spen* dosage in transheterozygotes of hypomorphic alleles increases the number of photoreceptors (I) and the adult eye size (C); the higher than normal Wg level in dpp^{blk} eyes (compare D with E and J with K) is unaffected in the dpp^{blk}, *spen* mutants at early third instar (L) and slightly reduced at late third instar (F). (M,N) Confocal images of third instar eye imaginal discs showing Elav (red) and either *lacZ* (M, green) or GFP (N, green). Clones of $pygo^{10}$ (M) and $spen^{14C2}$ (N) (marked by the absence of *lacZ* or GFP) were generated by *ey-FLP*. *pygo* clones at the edge of the eye block Wg-dependent furrow inhibition and produce ectopic photoreceptors (arrow in M). By contrast, ectopic photoreceptors do not form in *spen* clones at similar positions (arrow in N; $spen^{14C2}$: n=16; $spen^{3}$: n=11).

dpp^{blk} eyes (compare Fig. 4I with 4H and 4C with 4B). We could detect no change in Wg expression in early third instar *dpp*^{blk} eyes with *spen* reduction (compare Fig. 4L with 4K). *spen* reduction does lead to a decrease in posterior Wg expression at a later stage (compare Fig. 4F with 4E), yet the dorsal and ventral Wg expression remains higher than wild type (Fig. 4D). We believe this late reduction in Wg is unlikely to influence the initiation and progression of the MF, although we cannot rule out this possibility. With this caveat, the data are consistent with *spen* being required for Wg signaling downstream of Wg in MF inhibition.

In an otherwise wild-type eye, loss of Wg at the lateral edges allows ectopic MF initiation and inward progression (Ma and Moses, 1995; Treisman and Rubin, 1995). Therefore, removal of Wg signaling components at the lateral edge of the eye should result in an ectopic MF. This is indeed observed in mutant clones of *pygo* (Fig. 4M, arrow), in which Wg signaling is blocked (Belenkaya et al., 2002; Kramps et al., 2002; Parker et al., 2002; Thompson et al., 2002). To establish the role of *spen* in Wg-mediated MF inhibition in the wild-type situation, we looked at *spen* clones at similar positions. In contrast to *pygo*, clones of *spen* never give rise to ectopic photoreceptors (Fig. 4N, arrow), suggesting that *spen* is not essential for Wg signaling in this context.

spen is required for Wg signaling in the wing and leg

To determine whether *spen* plays a role in Wg signaling in other tissues, we examined its effects on the developing wing. In the third instar wing imaginal disc, *wg* is expressed in a narrow stripe along the dorsoventral (DV) border from which it emanates to form a morphogen gradient that regulates the expression of many genes (Neumann and Cohen, 1997; Zecca et al., 1996). The zinc-finger nuclear protein Senseless (Sens) is activated by Wg signaling in the proneural clusters on either side of the DV border, immediately adjacent to the *wg*-expressing stripe (Nolo et al., 2000; Parker et al., 2002) (Fig. 5A). In addition, Wg signaling refines the distribution of Wg protein by auto-repression of *wg* expression (Rulifson et al., 1996) and downregulation of the Wg receptor Fz2 (Cadigan et al., 1998). Thus, loss of Wg signaling in this tissue would lead to loss of Sens expression, expansion of the Wg stripe, and derepression of Fz2.

Loss of *spen* function in clones of several *spen* alleles leads to reduction or complete blockage of Sens expression (Fig. 5A) with incomplete penetrance. Wg levels are either normal or occasionally slightly derepressed (Fig. 5B); thus, the loss of Sens is not due to reduced Wg expression. *spen* clones are typically significantly smaller than their wild-type twin spots, suggesting that *spen* is required for optimal growth. The small size of the clones raises the possibility that trace amounts of Spen protein may remain in each cell, resulting in the incomplete penetrance of Wg defects. We used earlier heat shocks to induce *hs-FLP* to try to obtain larger *spen* clones at the DV boundary, with no success. Immunostaining showed that Spen protein levels were not detectable inside the *spen*^{3} clones in the wing (data not shown). Thus, to the best of our knowledge, *spen* is needed for maximal Wg signaling in the wing disc, but is not essential.

The effect of a putative dominant-negative *spen* transgene (*spen*^{DN}) on Wg targets in the presumptive wing provides evidence that the clonal analysis of *spen* underestimates the contribution of *spen* to Wg signaling. The *spen*^{DN} construct contains the C-terminal 936 amino acids of *spen*, including a nuclear localization signal and the conserved SPOC domain. *en-Gal4* was used to express *spen*^{DN} throughout the posterior compartment of the wing disc, while the anterior compartment remains wild type. With this Gal4 driver, *spen*^{DN} caused reduced (Fig. 5D) or complete absence (Fig. 5F) of Sens expression. The penetrance of loss of Sens expression was higher than seen in *spen* clones. More strikingly, a significant Wg depression was always observed, even when Sens is only moderately affected (Fig. 5E). By contrast, no effect on Wg-dependent Fz2 inhibition was observed (Fig. 5G). Thus, the *spen*^{DN} construct indicates an absolute requirement for *spen* in Wg stripe refinement and an important role in Wg-mediated

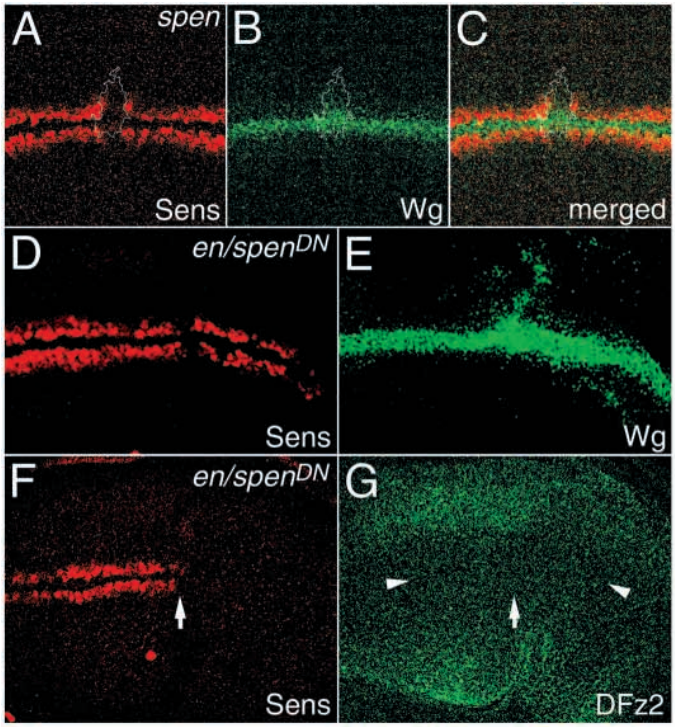

Fig. 5. *spen* is required for Wg signaling in the wing imaginal disc. (A-C) Confocal images of third instar wing imaginal discs containing randomly generated *spen*^{XIE1796} clones (marked by the absence of GFP, boundary shown by white lines). Samples were stained for Sens (A) and Wg (B); the merged image is shown (C). Reduction or total lack of Sens (A) was observed in some *spen* clones (*spen*^{9C7}: 23% and 26%, respectively, n=31; *spen*^{14C2}: 50% and 6.7%, n=30; *spen*^{3}: 27% and 0%, n=30; *spen*^{XIE1796}: 30% and 8.6%, n=70), and Wg level is unaffected or slightly derepressed (B), consistent with attenuated Wg signaling in cells that lack *spen*. (D-G) Confocal images of third instar wing imaginal discs containing the transgenes P[*en-Gal4*] and P[*UAS-spen*^{DN}]^{II} stained for Sens (D,F), Wg (E) and Fz2 (DFz2 in figure) (G). Flies were reared at 18°C. Expression from P[*UAS-spen*^{DN}] in the posterior compartment of the wing reduces (D) or eliminates (F) Sens expression (the posterior compartment is towards the right of the arrows in F and G; 44% are similar to D, 27% are more severe but some Sens remains and 29% are as in F, n=68) and derepresses Wg (E, 97%, n=31). By contrast, expression of Fz2 is either unaffected (compare arrowheads; G, 73%, n=45) or reduced throughout the posteior compartment (data not shown, 27%).

regulation of Sens, but it has no discernable role in Wg-dependent Fz2 repression.

We believe that the *spen*^{DN} experiments may also underestimate the importance of *spen* in Wg signaling. Gal4 is known to be cold sensitive in flies and we observe a strict temperature dependence in our *en-Gal4*/*spen*^{DN} experiments. The discs shown in Fig. 5 were reared at 18°C. At higher temperatures (e.g. 20°C or 25°C) where Gal4 is more active, we observe either gross deformities of the wing disc or organismal lethality before the 3rd instar larval stage (data not shown). Thus, we cannot assay the effect of *spen*^{DN} on Wg signaling when expressed at higher levels than the ones shown in Fig. 5. However, at those levels, *spen*^{DN} expression results in morphologically normal wing discs with strong Wg signaling defects.

In the leg imaginal discs, we see a similar situation as has just been described in the developing wing (Fig. 6). In the leg, Wg signaling inhibits *dpp* expression in the ventral portion of the discs (Brook and Cohen, 1996; Heslip et al., 1997; Jiang and Struhl, 1996) (Fig. 6A-C). Expression of *spen*^{DN} with *ptc-Gal4*, which is active in a stripe overlapping both the *dpp* and *wg* expression domains, causes a complete breakdown of disc morphology (data not shown) at 25°C. At lower temperatures (18-22°C), small leg discs are observed with either normal restriction of *dpp-lacZ* expression to the dorsal half (Fig. 6D-F) or derepression in the ventral region (Fig. 6G-I). This derepression is consistent with a block in Wg signaling.

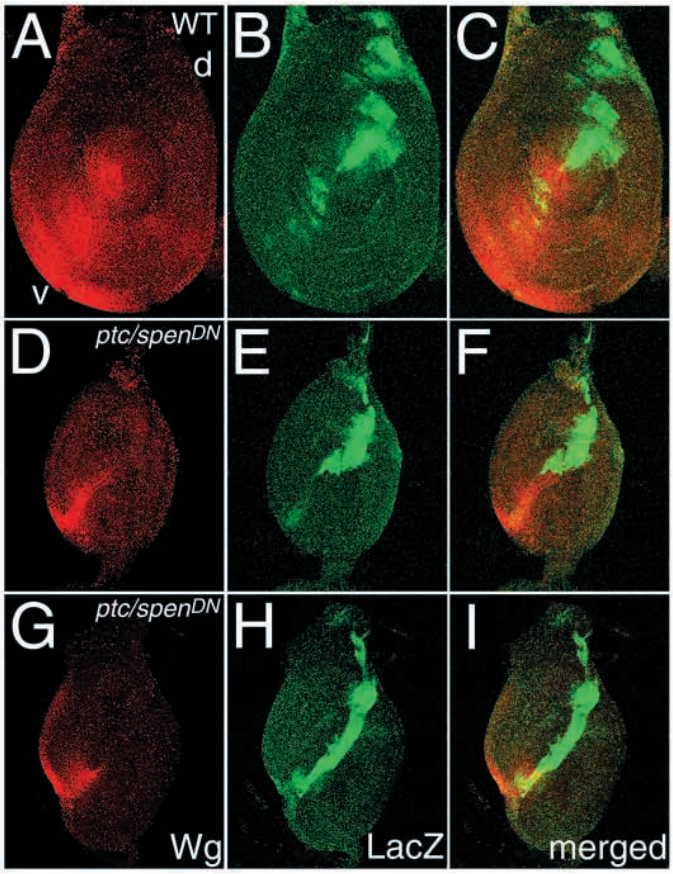

Fig. 6. *spen* is required for Wg signaling in the leg imaginal disc. (A-I) Confocal images of third instar leg imaginal discs of wild type (A-C) and animals containing transgenes P[*ptc-Gal4*], P[*dpp-lacZ*] and P[*UAS-spen*^{DN}]^{II} (D-I) reared at 21°C. Samples were stained for Wg (A,D,G) and *lacZ* (B,E,H); merged images are shown (C,F,I). All panels are shown with the same magnification. Ventral (v) expression of Wg (A) restricts *lacZ* expression to the dorsal (d) half (B) in the wild type. Expression from P[*UAS-spen*^{DN}] along a stripe overlapping both the *wg* and *dpp* domains either has no effect on the restriction of *lacZ* expression (D-F) or leads to derepression of *lacZ* in the ventral region (G-I; note the overlap of Wg and *lacZ* in I; 9% are similar to I, 45% have significant but incomplete derepression of *lacZ*, and 46% are similar to F, *n*=35). This derepression is consistent with a block in Wg signaling.

spen acts downstream of Arm stabilization

To address the question of where *spen* acts in the Wg signaling pathway, we carried out epistasis analysis using *spen*^{DN}. In the absence of Wnt signaling, Arm is phosphorylated at serine and threonine residues at its N terminus by CKIα and Sgg/Zw3, and then degraded (Peifer et al., 1994; Yanagawa et al., 2002). Mutations in these residues render Arm resistant to degradation (Freeman and Bienz, 2001; Pai et al., 1997). Expression of these Arm mutants (Arm*) in flies activates Wg signaling independent of upstream components (Pai et al., 1997). When expressed under the control of the eye-specific *GMR* enhancer, Arm* reduces eye size dramatically (Freeman and Bienz, 2001) (Fig. 7A). Co-expression of *spen*^{DN} using the *GMR-Gal4* driver severely suppresses this phenotype (Fig. 7B), indicating that *spen* potentiates Wg signaling downstream of Arm stabilization. Expression of *spen*^{DN} could also suppress a small eye phenotype caused by *GMR*-driven expression of a dominant-negative version of the EGF receptor (*EGFR*^{DN}) but not of the pro-apoptotic factors *head involution defective* or *reaper* (data not shown). This specificity indicates that *spen*^{DN} does not affect *GMR* enhancer-mediated expression.

spen is not required for Wg signaling in the embryo

Embryos completely lacking *spen* gene activity have head defects and ectopic sclerite formation (Wiellette et al., 1999), as well as axonal path-finding and midline glial cell defects (Chen and Rebay, 2000; Kuang et al., 2000). No defects in Wg-dependent developmental decisions were observed. We have

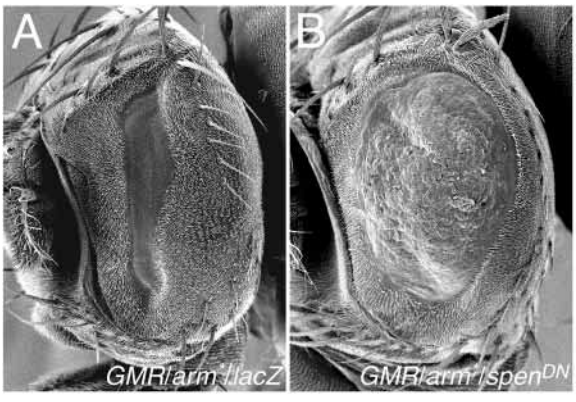

Fig. 7. *spen*^{DN} acts downstream of Arm stabilization. Micrographs are SEMs of adult fly heads from flies containing P[*GMR-Gal4*], P[*GMR-arm**]^{F36} and either P[*UAS-lacZ*] (A) or P[*UAS-spen*^{DN}]^{II} (B) reared at 25°C. Expression of an activated form of Arm activates Wg signaling and produces an eye that is severely reduced in size and is suppressed by P[*UAS-spen*^{DN}].

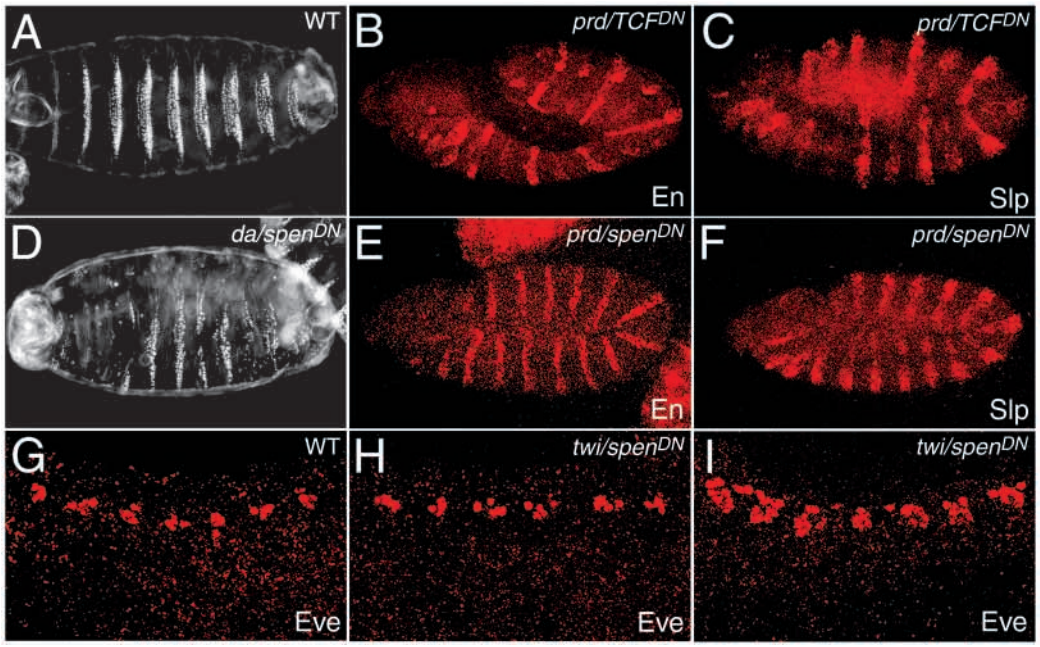

Fig. 8. *spen* is not required for Wg signaling in the embryo. (A,D) Micrographs of cuticles of wild type (A) and embryos containing transgenes P[*da-Gal4*] and P[*UAS-spen^{DN}*]^{III} (D) reared at 25°C. Expression from P[*UAS-spen^{DN}*] has variable effects on cuticle patterning, ranging from wild type (P[*UAS-spen^{DN}*]^{II} at 25°C, data not shown), moderate reduction of denticles (D) to complete disruption of cuticle formation (P[*da-Gal4*], P[*UAS-spen^{DN}*]^{III} containing embryos reared at 29°C, data not shown). (B,C,E,F) Confocal images of stage 11 embryos containing P[*prd-Gal4*] and either P[*UAS-TCF^{DN}*] (25°C; B,C) or P[*UAS-spen^{DN}*]^{III} (29°C; E,F). Samples were stained for En (B,E) or Slp1 (C,F). En and Slp1 stripes remain wild type in *spen^{DN}*-expressing embryos. (G-I) Confocal images of stage 13 wild-type embryos (G) and embryos containing transgenes P[*twi-Gal4*] and P[*UAS-spen^{DN}*]^{II} (H,I) reared at 25°C. Expression from P[*UAS-spen^{DN}*] has variable effects on the Eve pericardial expression, ranging from wild type (H) to an increase in Eve-positive cells (I). Some embryos containing transgenes P[*twi-Gal4*], P[*24B-Gal4*] and P[*UAS-spen^{DN}*]^{II} reared at 25°C or 29°C also display disorganization of Eve-expressing cells or occasional gaps missing Eve expression, in addition to an overall increase in Eve-positive cells (data not shown). These effects are qualitatively different from a blockage of Wg signaling [ectopic denticles, loss of En (B) and Slp-1 (C) stripes and loss of Eve in pericardial cells].

confirmed this by examining *spen* germline clones for several Wg readouts (described in detail below), all of which were normal (data not shown). As the *spen^{DN}* construct gave stronger Wg defects in the imaginal discs than loss of *spen*, we also examined the effects of *spen^{DN}* expression on Wg targets in the embryo.

Wild-type embryos have a distinctive denticle patterning on their ventral cuticles with trapezoidal arrays of denticle belts intermittent with naked cuticles (Fig. 8A). Wg signaling is required for naked cuticle formation; and *wg* mutants form ectopic denticles in place of naked cuticle (Nusslein-Volhard and Wieschaus, 1980). The cuticles of embryos carrying *UAS-spen^{DN}* and the ubiquitous driver *da-Gal4* were examined. At 25°C, these embryos show cuticle phenotypes ranging from wild type (data not shown) to moderate reduction of denticle formation (Fig. 8D), inconsistent with reduced Wg signaling. At 29°C, *spen^{DN}* expression causes complete disruption of cuticle formation (data not shown).

At moderate expression levels, *spen^{DN}* caused cuticular defects, similar to those reported for *spen* mutants (Wiellette et al., 1999), including defects in head structures (e.g. reduced or missing dorsal bridge) and apparent ectopic sclerites (data not shown). At higher expression levels, there was an almost complete block of head involution and head cuticle formation. Thus, the lack of Wg signaling defects in the cuticle is unlikely to be due to insufficient expression of *spen^{DN}*. Additionally, *prd-Gal4*, which is expressed in alternating segments throughout the embryo, was also used to express *spen^{DN}*, and no ventral cuticle defects were observed, even at 29°C.

The effects of *spen^{DN}* expression were further characterized using molecular markers. En is normally expressed in epidermal stripes of single segment periodicity. Wg is required for the maintenance of En expression (DiNardo et al., 1988); expression of a dominant-negative *TCF* (*TCF^{DN}*), which blocks Wg signaling in the nucleus, causes En to fade from the epidermis by full germband extension (Fig. 8B). Expression of *spen^{DN}* using *prd-Gal4* (Fig. 8E), or the ubiquitous drivers *arm-Gal4* and *da-Gal4* (data not shown) does not affect En expression at full germband extension, indicating that *spen* is not needed for En maintenance. Similarly, expression of another Wg target in the embryonic ectoderm, Sloppy-paired 1 (Slp1) (Lee and Frasch, 2000), is markedly reduced by *TCF^{DN}* expression (Fig. 8C), but is not affected by *spen^{DN}* expression under the control of *prdGal4* (Fig. 8F), *armGal4* or *daGal4* (data not shown).

Wg signaling in the mesoderm is required for the expression of Even-skipped (Eve) in a subset of pericardial cells (Wu et al., 1995) (Fig. 8G). At 25°C or 29°C, Eve expression in embryos expressing *spen^{DN}* throughout the mesoderm using *twi-Gal4* ranges from wild-type (Fig. 8H) to an increased number of Eve-positive cells (Fig. 8I). Some embryos expressing *spen^{DN}* via two mesodermal drivers (*twi-Gal4* and *24B-Gal4*) simultaneously exhibit general disorganization of Eve-positive pericardial cells, with occasional segmental gaps (one or two per embryo) missing Eve expression, and an overall increased number of Eve-positive cells (data not shown). As Eve expression is always present in these embryos, and segments missing Eve are always concurrent with those with more Eve expression in the same embryo, we conclude that *spen* is not required for Wg signaling in this readout, and that functions of *spen* in other pathways or the non-specificity of *spen^{DN}* may be the culprit for the defects in Eve expression.

DISCUSSION

Spen is required for normal Wg signaling in imaginal discs

In this study, a total of seven distinct readouts of Wg signaling were examined in imaginal discs. They are: inhibition of interommatidial bristle formation (Figs 1, 3); MF initiation/progression (Fig. 4); repression of Wg and DFz2, and activation of Sens expression at the presumptive wing margin (Fig. 5); inhibition of *dpp* expression in the dorsal leg (Fig. 6); and reduction of eye size (Fig. 7). Wg regulation of six of these readouts is significantly blocked by partial or complete removal of *spen* and/or the expression of $spen^{DN}$. These results provide a strong genetic argument that *spen* is required for Wg signaling in these tissues.

Interpreting *spen* phenotypes is complicated by the fact that *spen* has been implicated in several other pathways. Can these functions explain the apparent loss of Wg signaling phenotypes we observed? *spen* has been found to act with *Deformed* to suppress head identity in the embryonic trunk (Wiellette et al., 1999) and *spen* genetically interacts with cell cycle mutants (Lane et al., 2000). We think it unlikely that these *spen* functions can account for the phenotypes observed. However, Spen has also been shown to be involved with the Ras and Notch signaling pathways, which do affect the readouts we employed for studying Wg signaling. Therefore, it is possible that some of the *spen* phenotypes we have documented are due to disruption of these signaling cascades, though we argue below that this is unlikely.

spen mutations affect some Ras targets in a way that suggests it acts positively in Ras signaling (Chen and Rebay, 2000; Rebay et al., 2000). This may be the explanation for the non-autonomous derepression of Ac expression adjacent to *spen* clones in P[*sev-wg*] eyes (Fig. 3B, arrowhead), as *Dl* expression is activated by the EGF/Ras pathway in the eye (Tsuda et al., 2002). Ras signaling plays a positive role in MF progression (Greenwood and Struhl, 1999; Kumar and Moses, 2001) and elevated Ras signaling can suppress a Wg or Arm induced small eye phenotype (Freeman and Bienz, 2001) (K.M.C., unpublished). Therefore, a reduction in Ras signaling caused by loss of *spen* cannot explain our observations. Ras signaling has no effect on wing margin formation (Diaz-Benjumea and Hafen, 1994; Nagaraj et al., 1999) and acts downstream of Wg/Dpp crossregulation in the leg (Campbell, 2002; Galindo et al., 2002), again arguing that the role of Spen in Ras signaling cannot account for the apparent Wg signaling defects we observed.

Expression of Suppressor of Hairless [Su(H)], a transcription factor required for Notch signaling, is significantly reduced in *spen* mutant embryos (Kuang et al., 2000). Can a reduction of Notch signaling explain our results? Notch signaling is required for interommatidial bristle inhibition (Cagan and Ready, 1989) so this could explain the requirement of *spen* for Wg-dependent Ac inhibition (Fig. 3). However, Notch is absolutely required for Wg expression at the DV stripe in the wing (Rulifson and Blair, 1995) and plays a positive role in MF progression (Kumar and Moses, 2001). Thus, reducing Notch activity by loss of *spen* or $spen^{DN}$ cannot explain the wider Wg stripe (Fig. 5) and suppression of the dpp^{blk} MF defect (Fig. 4) that we observed.

Though no evidence for elevated Notch signaling in *spen* mutants has been reported in *Drosophila*, a recent report has suggested that SHARP, a human Spen homolog, functions as a transcriptional co-repressor for RBP-Jκ/CBL, the ortholog of Su(H) (Oswald et al., 2002). In addition, the fly homolog of human SMRT, which binds to SHARP (Shi et al., 2001), has been shown to act as a negative regulator of Notch signaling (Tsuda et al., 2002). This could mean that loss of *spen* activity in flies results in higher expression of Notch/Su(H) targets, owing to derepression. Although this could conceivably contribute to the MF and wing phenotypes we found, such derepression could not account for the suppression of Wg-dependent reduction of eye size and bristle inhibition (Figs 1, 3) or the derepression of *dpp* expression in the leg (Fig. 6). In summary, the only explanation consistent with all the *spen* (or $spen^{DN}$) imaginal disc phenotypes discussed above is a loss of Wg signaling.

Spen is not required for embryonic Wg signaling

In contrast to the data in the imaginal discs, we could find no evidence for the involvement of *spen* in Wg signaling in the embryo (Fig. 8), either by removing *spen* gene activity or expressing $spen^{DN}$. Thus, it appears that Spen may be a tissue-specific regulator of Wg signaling. Spen is a predominately nuclear protein expressed ubiquitously in embryos and imaginal discs (Kuang et al., 2000; Wiellette et al., 1999) (Fig. 2; data not shown). It could be that a Spen co-factor is not expressed in embryos, or that Spen is post-translationally modified in a tissue-specific way. Alternatively, the specificity could lie in the promoters of the targets that were tested. This appears to be the case in the wing, where Wg and Sens regulation by Wg signaling is *spen* dependent (Fig. 5A-F), while that of Fz2 is not (Fig. 5G).

The negative results we obtained in the embryo cannot be viewed as definitive. Embryos that lack maternal and zygotic *spen* activity could be normal for Wg signaling because of redundancy (see below). Likewise, even though expression of $spen^{DN}$ in the imaginal discs caused strong Wg loss of function phenotypes (Figs 5-7), and caused *spen*-like phenotypes under mild expression conditions in the embryo (data not shown), it is possible that we did not supply adequate amounts of $spen^{DN}$ in our embryonic assays. To address this issue, we used several Gal4 drivers at 29°C (to ensure optimal Gal4 activity; see Results for details). We did observe phenotypes with $spen^{DN}$ not previously reported that are Wg independent. For example, *arm-Gal4*- and *da-Gal4*-driven $spen^{DN}$ expression causes reduced denticle formation to varying degrees in the embryonic ventral cuticle (Fig. 8). A possible explanation is reduced DER/Ras signaling, which promotes the denticle fate by activating *shavenbaby* (Payre et al., 1999). In addition, $spen^{DN}$ expression also causes a variable increase in the number of Eve-expressing cells in the embryonic dorsal mesoderm. This could be explained by a reduction in Su(H) levels, as impairment of Notch signaling causes an increase in Eve-positive pericardial cells (Carmena et al., 2002). Under conditions where $spen^{DN}$ blocked other pathways, we could observe no reduction in Wg signaling.

Spen may have a redundant partner

Our experiments with loss of function *spen* alleles indicate that *spen* is not absolutely required for Wg signaling in the wing and eye. Although reduction of *spen* activity could suppress a dpp^{blk} MF defect (Fig. 4C), which can be explained by a

reduction in Wg signaling, complete removal of *spen* did not cause an ectopic MF (Fig. 4N). Because removal of Wg signaling is known to induce an ectopic MF (Ma and Moses, 1995; Treisman and Rubin, 1995) (Fig. 4M), this indicates that sufficient Wg signaling still occurs in the *spen* clones. In the wing, *spen* clones affect Wg readouts, but with incomplete penetrance (Fig. 5A-C), again indicating a partial reduction in Wg signaling in the absence of *spen*.

Our experiments with $spen^{DN}$ suggest that the partial loss of Wg signaling in *spen* mutants may be due to redundancy. Expressing $spen^{DN}$ causes more severe phenotypes and much higher penetrance in disruption of Sens and expansion of Wg in the wing than complete removal of *spen* (Fig. 5D-F). A likely explanation is that the $Spen^{DN}$ protein also inhibits the function of another gene that has roles in the Wg pathway redundant to *spen*.

Although many genes exist in the fly genome that encode proteins containing RRMs, only one other besides Spen is predicted to encode a protein with both RRMs and a SPOC domain. This factor has been called short Spen-like protein (SSLP or DmSSp) (Kuang et al., 2000; Wiellette et al., 1999) and is referred to as CG2910 in the annotated genome. No genetic or molecular characterization of SSLP has been reported and we are pursuing its possible redundancy with *spen*.

Mechanism of Spen action

Where does Spen act in the Wg pathway? Our epitasis experiments in the eye (Fig. 7) indicate that $Spen^{DN}$ blocks Wg signaling downstream of Arm stabilization. Thus, Spen could act in Arm nuclear import, or in mediating TCF/Arm transcriptional regulation. Consistent with a role in Wg target gene transcription, Spen is predominantly nuclear in imaginal tissues (Fig. 2; data not shown). In addition, the mouse and human homologs of Spen have been implicated as transcription factors (see below).

Studies on the vertebrate homologs of Spen have provided functions for the RRM and SPOC domain that these proteins share with Spen. Spen has three predicted RRMs near its N terminus. The role of RRMs in specific RNA binding is well established (Burd and Dreyfuss, 1994) and the RRM domains in the human Spen homolog SHARP has been shown to bind to the steroid receptor RNA co-activator SRA (Shi et al., 2001). By contrast, the RRM domain of the mouse Spen homolog, MINT, has been shown to bind to specific double-stranded DNA, including the proximal promoter of the osteocalcin gene (Newberry et al., 1999). SHARP also binds to the nuclear receptor co-repressor SMRT and acts as a transcription co-repressor by recruiting histone deacetylases (HDACs) through its SPOC domain (Shi et al., 2001). A similar co-repressor function for SHARP with the DNA-binding protein RBP-Jκ/CBL has also been reported (Oswald et al., 2002). Finally, MINT was also found to interact with Msx2, a known transcriptional repressor (Newberry et al., 1999). These studies on the vertebrate homologs suggest that Spen may bind DNA or RNA at its N terminus, and may regulate the Wg pathway as a transcription corepressor.

Why is *spen* required for only a subset of Wg targets? Based on studies with its vertebrate homologs, could *spen* only regulate the Wg targets that are transcriptionally repressed by TCF/Arm? Wg-dependent transcriptional inhibition through TCF has been shown for the *stripe* gene in the embryo (Piepenburg et al., 2000) and has been suggested for bristle inhibition in the eye (Cadigan et al., 2002). However, no direct targets of Wg signaling in the imaginal discs have been determined and our attempts to determine whether *stripe* repression in the embryo requires *spen* have been inconclusive (H.V.L. and K.M.C., unpublished). It is interesting to note that two embryonic targets tested which were *spen* independant, *eve* and *slp1*, are both directly activated by TCF/Arm (Halfon et al., 2000; Knirr and Frasch, 2001; Lee and Frasch, 2000; Han et al., 2002). Identification of *spen*-dependent direct targets of Wg signaling will be necessary to explore this model.

Two factors have previously been reported that are tissue/promoter-specific regulators of Wg signaling. *tsh* has been shown to be required for Wg-mediated inhibition of denticle formation in the ventral embryonic epidermis (Gallet et al., 1998) and *lin*, which is needed for Wg signaling only in the dorsal epidermis (Hatini et al., 2000). We report a third factor, Spen, which is only needed for imaginal disc regulation of Wg targets. The existence of these specific factors begs the question: what is the difference between the various Wg targets that requires such specificity?

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