

Matching catalytic activity to developmental function: Tolloid-related processes Sog in order to help specify the posterior crossvein in the *Drosophila* wing

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Summary

The *Drosophila tolloid* (*tld*) and *tolloid related* (*tlr*) gene products belong to a family of developmentally important proteases that includes Bone Morphogenetic Protein 1 (Bmp1). Tld is required early in *Drosophila* development for proper patterning of dorsal embryonic structures, whereas Tlr is required later during larval and pupal stages of development. The major function of Tld is to augment the activity of Decapentaplegic (Dpp) and Screw (Scw), two members of the Bmp subgroup of the Tgf β superfamily, by cleaving the Bmp inhibitor Short gastrulation (Sog). In this study, we provide evidence that Tlr also contributes to Sog processing. Tlr cleaves Sog *in vitro* in a Bmp-dependent manner at the same three major sites as does Tld. However, Tlr shows different site selection preferences and cleaves Sog with slower kinetics. To test whether these differences are important *in vivo*, we investigated the role of Tlr and Tld during development of the posterior crossvein (PCV)

in the pupal wing. We show that *tlr* mutants lack the PCV as a result of too little Bmp signaling. This is probably caused by excess Sog activity, as the phenotype can be suppressed by lowering Sog levels. However, Tld cannot substitute for Tlr in the PCV; in fact, misexpressed Tld can cause loss of the PCV. Reducing levels of Sog can also cause loss of the PCV, indicating that Sog has not only an inhibitory but also a positive effect on signaling in the PCV. We propose that the specific catalytic properties of Tlr and Tld have evolved to achieve the proper balance between the inhibitory and positive activities of Sog in the PCV and early embryo, respectively. We further suggest that, as in the embryo, the positive effect of Sog upon Bmp signaling probably stems from its role in a ligand transport process.

Key words: Tolloid, Bmp, Sog, Crossvein *Drosophila*, 18w

Introduction

Extracellular proteases are important regulators of a wide variety of biological processes, including developmental patterning. Bone Morphogenetic Protein 1 (Bmp1), first identified in osteogenic extracts of bone (Wozney et al., 1988), is the prototype of a structurally conserved set of metalloproteases known as the Bmp1/Tld family. These proteases are implicated in regulating several important developmental events in a broad range of species (Suzuki et al., 1996; Marqués et al., 1997; Picolo et al., 1997; Blader et al., 1997; Connors et al., 1999; Clark et al., 1999; Wolfman et al., 2003). Each family member has a highly similar structure containing an astacin-like protease domain followed by CUB protein-protein interaction domains that are interspersed with EGF-like motifs (Bond and Beynon, 1995). These proteases are synthesized as pro-enzymes that are activated upon removal of an N-terminal propeptide by dibasic endopeptidases (Barr, 1991).

The Bmp1/Tld-like proteases process a number of different extracellular matrix (ECM) components, including type I to III procollagen (Kessler et al., 1996), laminin 5 (Amano et al.,

2000), biglycan (Scott et al., 2000), lysyl oxidase (Uzel et al., 2001) and some members of the Tgf β family (Wolfman et al., 2003). However, the best understood developmental patterning of Bmp1/Tld-like proteins is in the early *Drosophila* embryo, in which Tolloid (Tld) is required for specification of dorsal structures (Shimell et al., 1991). Tld acts by cleaving the secreted Bmp antagonist Sog to release the Bmp2/4 homolog Dpp from an inhibitory complex (Marqués et al., 1997). In *Drosophila*, graded Dpp activity in dorsal cells is achieved by diffusion of Sog from its ventrolateral site of synthesis into the dorsal domain (Ferguson and Anderson, 1992; François et al., 1994; François and Bier, 1995; Marqués et al., 1997; Srinivasan et al., 2002). The diffusion of Sog from ventrolateral cells serves two purposes. First, it inhibits Dpp activity in dorsolateral regions and second, it helps transport Dpp (Wang and Ferguson, 2005; Eldar et al., 2002; Shimmi et al., 2005a) towards the dorsal side in a complex with Twisted gastrulation (Tsg), a second extracellular protein that modulates patterning within dorsal cells (Mason et al., 1994; Ross et al., 2001). Dorsally expressed Tld proteolytically cleaves Sog and liberates Dpp from the inhibitory complex (Marqués et al., 1997; Shimmi and O'Connor, 2003). Thus, mutations in *tld*

result in a partially ventralized phenotype that is similar to, but less severe than *dpp* mutants (Arora and Nüsslein-Volhard, 1992; Jürgens et al., 1984).

In vertebrates, Bmp1/Tld-like proteases regulate the activity of Chordin, a Sog homolog, similar to the way Tld regulates Sog activity in *Drosophila* embryos. Bmp1 family members can process Chordin (Piccolo et al., 1997; Blader et al., 1997) and co-overexpression of vertebrate Tld proteases with Chordin can reverse the dorsalizing effects of Chordin upon early *Xenopus* development (Piccolo et al., 1997; Scott et al., 1999a). Mutations in the zebrafish *mini-fin* gene, which encodes a Tld-related enzyme that is able to cleave Chordin in vitro (Blader et al., 1997), exhibit dorsoventral patterning defects (Connors et al., 1999).

The *Drosophila* genome encodes a second Tld-like protein that maps immediately upstream of *tld*. This gene has been referred to as *tolloid-related 1* (*tlr*; *18w* – FlyBase) or *tolkin* (*tok*) (Nguyen et al., 1994; Finelli et al., 1995). At the blastoderm stage, *tlr* expression overlaps that of *tld*. However, after embryonic stage 15, *tld* expression is no longer seen in any embryonic tissue, while *tlr* is heavily expressed in muscles, a subset of cells within the CNS, and in the corpus allatum of the ring gland (Nguyen et al., 1994; Finelli et al., 1995). During larval stages *tlr* and *tld* show identical expression patterns within the imaginal discs; however, the *tlr* transcript is much more abundant.

Despite several studies, the biological function(s) of Tlr remain ill defined. Embryos mutant for *tlr* show no significant defects in dorsal patterning, although it has recently been demonstrated that the level of Sog protein present in the dorsal region of *tlr tld* double mutant embryos is higher than in *tld* single mutants (Srinivasan et al., 2002). This led to the speculation that, like Tld, Tlr might recognize Sog as a substrate. However, the biological relevance of such processing was not clear.

Null alleles of *tlr* are largely lethal during larval and pupal stages but result in a small number of escaper flies in which the posterior crossvein (PCV) of the adult wing is absent (Nguyen et al., 1994; Finelli et al., 1995). This is intriguing, as the *dpp* and *sog* gene products are reused in pupal stages of development to help initiate and maintain wing veins (Posakony et al., 1990; Yu et al., 1996; de Celis, 1997; Conley et al., 2000; Ray and Wharton, 2001; Ralston and Blair, 2005). During pupal stages, *dpp* is expressed in longitudinal vein primordia and helps maintain their fate (Posakony et al., 1990; de Celis, 1997), while *sog* is expressed in complementary intervein cells and refines vein formation (Yu et al., 1996), probably by blocking an auto-activating Dpp feedback loop. Two smaller veins, called crossveins, develop late in this process, from tissue that is initially specified to be intervein material. PCV development is initiated by high levels of Bmp signaling. Therefore, the PCV is especially sensitive to reductions in such signaling (Conley et al., 2000; Ralston and Blair, 2005). The localized, high level of Bmp signaling that initiates PCV development is probably supplied from ligands produced in the longitudinal veins (Ralston and Blair, 2005). The molecular mechanism responsible for localizing high levels of Bmp signaling activity to the crossvein primordia is not clear. The *tlr* mutant phenotype suggests that Tlr may aid PCV development by cleaving Sog and thus regulating its activity during crossvein development.

In this report, we provide biochemical evidence that Tlr, like Tld, can cleave Sog, albeit with different kinetics and site selection specificities. In addition, we further characterize the *tlr* loss-of-function phenotype and find that Tlr activity is required to ensure localized, high level Bmp signaling during crossvein specification. Moreover, we find that Tlr activity during crossvein specification is required to cleave Sog, and that Sog exhibits both agonist and antagonistic activities towards Bmp signaling during pupal wing patterning. Taken together with recent data indicating that the source of Bmp ligand for crossvein specification is likely to be longitudinal vein cells (Ralston and Blair, 2005), we formulate a model whereby Sog and Tlr recapitulate the embryonic roles of Sog and Tld in transporting and releasing Bmp ligands to achieve spatially restricted patterns of signaling. As Tld can not substitute for Tlr during PCV formation and Tlr can not substitute for Tld in the embryo, we further speculate that the different catalytic properties of Tlr and Tld towards a common substrate (Sog) have been matched to their respective developmental processes: rapid processing for the quickly developing embryo and slow processing for the more slowly developing PCV.

Materials and methods

Fly stocks

sog^{Y126} and *sog^{P1}* are homozygous lethal hypomorphic alleles (Ferguson and Anderson, 1992; François et al., 1994) and were obtained from Eric Wieschaus and Ethan Bier. *sog^{P129D}* is weak homozygous viable allele and was obtained from Chip Ferguson. *tld^{B4}* is a null allele (Childs and O'Connor, 1994). *tlr^{ex[2-4]1}* is a null allele (Nguyen et al., 1994), while *tlr^{E1}* is a strong allele and *tld^{P1a}* is a small deletion that removes both *tld* and *tlr* (Finelli et al., 1995). The *da-Gal4* line was obtained from Armin Manoukian and expresses *Gal4* ubiquitously. The *A9-Gal4* driver expresses *Gal4* throughout the wing pouch and has been described previously (Haerry et al., 1998). The UAS-*sog*-HA and UAS-*tsG*-HA flies have been described previously (Yu et al., 2000). UAS-*tld* was obtained from Mike Hoffmann. UAS-*tlr* constructs were generated by insertion of a complete *tlr* cDNA in the pUAST vector (Brand and Perrimon, 1993). Germline transformation of these constructs was performed according to standard protocols.

Molecular constructs

To make the *tlr*-HA constructs, a *NotI* site was introduced by site-directed mutagenesis (QuickChange, Stratagene) downstream of bp 4729 in the *tlr* cDNA (GenBank sequence U12634) and was used to insert a triple HA epitope at the C-terminus of *tlr* ORF. A processing site mutant (pmTlr, R516A and R519A) and catalytically dead (cdTlr, S669F) forms were engineered by site-directed mutagenesis. Astacin domain swapping was accomplished in two steps. For example for Tld^{astTlr}, the sequence coding for the Tlr astacin domain was PCR amplified (Expand High Fidelity PCR System, Boehringer Mannheim) using primers that had overhangs homologous to the flanking sequences in *tld* cDNA. Then, the PCR product was used as a pair of matching primers in an amino acid switch step, using QuickChange site-directed mutagenesis kit (Stratagene) and a *tld*-based template. All junctions were verified by sequencing. Tld^{astTlr} has the following composition: Tld amino acids M1-R126, Tlr A520-K719 and then Tld C327-end. Tlr^{astTld} has Tlr amino acids M1-R519, Tld A127-K326 and then Tlr C720-end. Additional details are available upon request. All tagged proteins were placed in pRmHaI (Bunch et al., 1988) for transfections into S2 cells, in pUAST (Brand and Perrimon, 1993) to generate transgenic lines or in Bluescript II SK(+) (Stratagene) for mRNA synthesis.

Protein production and purification

Drosophila S2 cells were used for producing recombinant proteins as described previously (Shimmi and O'Connor, 2003). The cleared supernatant (conditioned medium) was used directly for in vitro cleavage assays and western blotting. Cell pellets were disrupted by boiling in SDS-loading buffer, followed by centrifugation and the clarified supernatant analyzed by western blotting as the soluble cell pellet fraction.

For in vitro cleavage assays, mixtures of proteins were incubated for the indicated times at 25°C in the presence of 1× reaction buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2.5 mM CaCl₂, 1 μM ZnCl₂ and Complete™, EDTA-free protease inhibitor cocktail, Roche).

Immunoprecipitation and immunoblotting

Protein samples were separated by SDS-PAGE on 4-12% NuPAGE gels (Invitrogen) and transferred onto PVDF membranes (Millipore). Primary antibodies were used at the following dilutions: anti-HA 12CA5 (Roche) 1:2000; anti-Myc A14 (Santa Cruz) 1:1000; rat anti-Gbb (raised against the C-terminus of Gbb, kindly provided by Mike Hoffmann and Grace Boekhoff-Falk) 1:500. Immune complexes were visualized using HRP-conjugated secondary antibodies (Jackson Immuno Research Laboratories) and ECL Super Signal (Pierce). Alternatively, for simultaneous multiple detection, anti-rat Alexa Fluor 680 and anti-mouse IRDye 800 secondary antibodies were used at 1:5000 dilution, followed by scanning with Odyssey Infrared Imaging System (Li-cor Biosciences).

For Sog-Tld/Tlr co-immunoprecipitation, equivalent amounts of wild-type and variant Tld-HA and Tlr-HA proteins in conditioned media were mixed with Sog-Myc for 3 hours at room temperature in a final volume of 250 μl. The mixtures were diluted with an equal volume of 0.4% BSA in mock conditioned media, followed by immunoprecipitation at 4°C overnight with Affinity Matrix Mono HA.11 (Covance). The samples were washed five times in ice-cold PBS and analyzed as above. For Dpp-Gbb heterodimer analysis, equivalent amounts of individually expressed and co-expressed ligands were preincubated for 2 hours at room temperature in a final volume of 1 ml. The mixtures were divided for immunoprecipitation with Affinity Matrix Mono HA.11 (Covance) and with anti-Flag M2-Agarose (Sigma) at 4°C overnight. The samples were washed with ice-cold PBS and eluted with non-reducing SDS-loading buffer to prevent the dissociation of antibodies from matrix. Prior to electrophoresis and analysis as described above, β-mercaptoethanol was added to the samples.

RNA localization and immunohistochemistry

In situ hybridization to pupal wings was performed with digoxigenin-labeled RNA probes and visualized with alkaline phosphatase precipitates as previously described (Conley et al., 2000). Specimens were stained using anti-pMad (gift from P. ten Dijke) at 1:1000 dilution followed by anti-rabbit HRP (Jackson Immuno Research Laboratories) at 1:2000 and visualized using the fluorescein TSA kit (NEB) or as described by Ralston and Blair (Ralston and Blair, 2005).

Secondary axis assay

Xenopus embryos were obtained by in vitro fertilization of eggs with testes homogenates. Embryos were dejellied in 2% cysteine and staged. For secondary axis induction assays, synthetic capped mRNAs were injected into one of the two adjacent ventral blastomeres of four-cell stage embryos in the equatorial region. The injection volume was up to 5 nl at the concentrations indicated. mRNAs for injections were transcribed using either T7 or SP6 Message Machine kits (Ambion).

Results

Tlr is a secreted protease that processes Sog in vitro

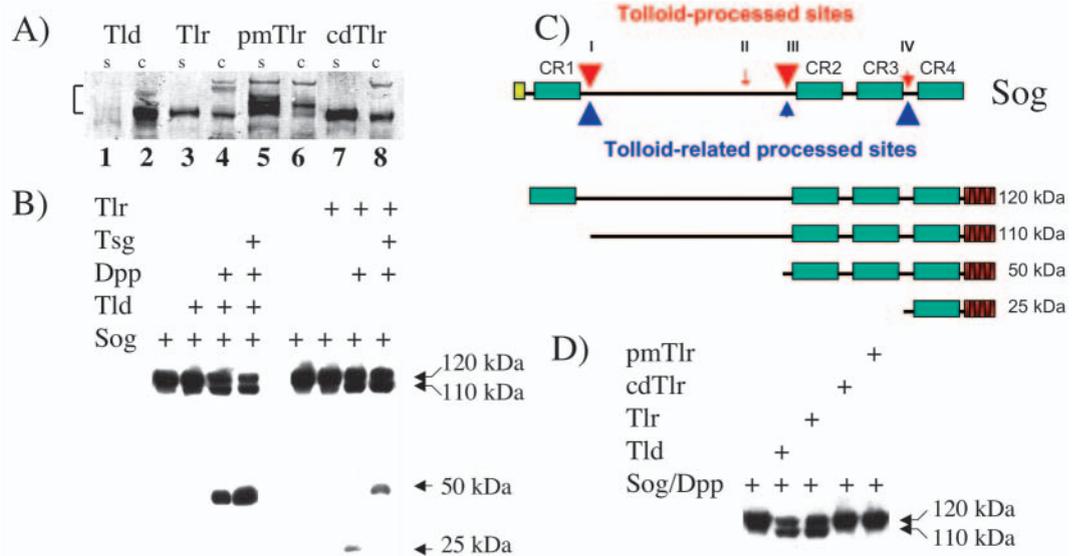
The observation that *tlr-tlr* double mutant embryos show

enhanced levels of Sog protein accumulation in the dorsal domain (Srinivasan et al., 2002) suggested that Tlr might process Sog. To examine this possibility, we first produced various tagged forms of the Tlr protein in cell culture. Previously, we found that insertion of an HA epitope at the C terminus of the Tld protein did not interfere with its enzymatic activity (Marqués et al., 1997). Therefore, we constructed a similar C-terminally HA-tagged version of Tlr. When transiently expressed in S2 cells under the control of a metallothionein-inducible promoter, this construct produces a secreted protein of 122 kDa detectable in the conditioned media (Fig. 1A, lane 3). The predicted mass of intact Tlr is 161 kDa. However, like Tld, Tlr has a putative -1,-4 dibasic pro-protein convertase site found just upstream of the astacin domain (Barr, 1991), suggesting that removal of an N-terminal pro-domain might be responsible for the size difference. Consistent with this view, we observe both the processed and intact pro-enzyme in the cell pellet (Fig. 1A, lane 4). To determine if processing occurs at the putative pro-protein convertase site, we mutated the -1,-4 dibasic residues in Tlr to alanine. The resultant mutant form of the enzyme (pmTlr) is secreted into the conditioned media as a mixture of the full-length form in which the pro-peptide is not removed and few smaller randomly processed species (Fig. 1A, lane 5). Last, we created a putative dominant-negative allele by changing S669 to F in the astacin domain (cdTlr-catalytically dead) (Fig. 1A, lanes 7 and 8). A similar mutation in the *tlr*^{10E95} allele has been shown to be catalytically inactive and to have antimorphic properties (Childs et al., 1994; Marqués et al., 1997).

To examine the abilities of these proteins to process Sog, we used an in vitro assay system that was previously developed to characterize Sog cleavage by Tld (Marqués et al., 1997; Shimmi and O'Connor, 2003). In these studies, it was determined that, in the presence of Dpp, Tld processes Sog at one minor (II) and three major sites (I, III and IV, Fig. 1C). Processing occurs preferentially at sites I and III, although the selection of cleavage site can be influenced by the presence of Tsg, another small secreted protein that makes a tripartite complex with Dpp and Sog (Ross et al., 2001; Shimmi and O'Connor, 2003). We find that Sog is also an in vitro substrate for Tlr, and that Tlr processes Sog in a ligand-dependent reaction (Fig. 1B). Analysis of fragment sizes (Fig. 1B,C) and microsequencing (M.S. and M.B.O., unpublished) reveals that Tlr processes Sog at the same three major sites as does Tld. However, Tlr preferentially cleaves Sog at sites I and IV in the absence of Tsg, and Tsg promotes cleavage at site III (Fig. 1B). We also find that the pmTld and cdTlr variant proteins were unable to process Sog (Fig. 1D, lanes 4 and 5), indicating that, like Tld, Tlr activity requires removal of the pro-domain as well as an intact catalytic domain.

To further compare the catalytic activities of Tld and Tlr for Sog processing, we performed time course processing assays. Protease levels were quantified by western analysis of the similarly C-terminal triple HA-tagged enzymes (Fig. 2A, insert). As shown in Fig. 2A, Tlr processed Sog less efficiently at all time points compared with Tld, despite the fact that there is fivefold more Tlr in the reaction. Reactions were carried out in the presence of 3×10⁻¹⁰ M Dpp (R&D Systems), a concentration shown previously to stimulate approximately half maximal levels of processing (Shimmi and O'Connor, 2003). Using the bacterial recombinant Dpp conferred the

Fig. 1. Biochemical characterization of the Tlr protein. (A) Western blotting analysis of the Tld-HA, Tlr-HA proteins and various modified forms. Shown are supernatants (s) and soluble cell fraction (c) from transfected S2 cells probed with anti-HA 12CA5 antibody. The bracket on the left indicates position of unprocessed and partially processed forms. The amount of secreted Tld is always low and the band is often smeared due to glycosylation (Marqués et al., 1997). (B) Comparison of Sog processing by Tld and Tlr, in the absence or presence of Dpp and Tsg. The indicated combinations of proteins were incubated for 16 hours at 25°C and Sog fragments were visualized with anti-Myc A14 antibody. Under these conditions, processing is incomplete, enabling the different fragments to be seen. The molecular weight of full-length Sog is 120 kDa and the C-terminally Myc tagged products shown are of 110 kDa, 50 kDa and 25 kDa. The 25 kDa band in the Tld lane is not visible with this level of exposure. (C) Schematic representation of the Tld and Tlr cleavage sites. The position of cleavage site II is indicated but this site is very weak for Tld (Shimmi et al., 2003) and undetectable for Tlr. Sog processing by Tlr requires an active, enzymatically intact astacin domain. (D) Equivalent amounts of wild-type and modified Tlr proteins were tested for their Sog processing activity by incubation for 40 hours at 25°C in the combinations indicated. Processing at site I only (full-length Sog versus the 110 kDa fragment) is shown. pmTlr, processed mutant Tlr; cdTlr, catalytically dead Tlr.



advantage of allowing accurate quantification of the ligand introduced in reactions; however, with this recombinant ligand, Sog processing is limited to sites I and III. When quantifying Sog processing by Tlr and Tld, the time taken for the disappearance of the full-length Sog (120 kDa band) and the appearance of the 50 kDa processing product appeared approximately doubled in the case of Tlr (Fig. 2A, lower panels). Coupled with the five-fold excess of Tlr relative to Tld in these reactions, the results suggest that under these *in vitro* conditions, Tlr processes Sog approximately one order of magnitude slower than Tld. Addition of even higher amounts of Tlr (10-fold and 25-fold more than Tld) to the Sog processing reaction slightly increased the disappearance of the full-length Sog (120 kDa band) and the appearance of the 110 kDa product (Fig. 2B), but had no effect upon the production of the 50 kDa product (not shown). To address the possibility that the Dpp requirements for the two enzymes might be different, we carried out the Sog processing in the presence of three times more Dpp (1×10^{-9} M). This increased Dpp concentration did not significantly alter the rate of Sog processing by Tlr (Fig. 2C, compare with 2B), indicating that the Dpp concentration was not limiting under these conditions. Therefore, the slow kinetics of Sog processing by Tlr appears to stem from a slow proteolytic activity and not from inefficient use of the required co-substrate. Even in co-transfection experiments, where Sog, Dpp, and Tld or Tlr were expressed in the same cells, Tlr was a less effective enzyme than Tld (not shown).

The reduced processing rate of Tlr compared with Tld could be the result of slow enzyme-substrate association, increased enzyme-substrate dissociation and/or an intrinsic reduction in the protease catalytic activity. To determine if the slow processing rate was the result of an intrinsic difference in the

catalytic activity of the astacin protease domain, we swapped the astacin catalytic domain in each protein with that of its paralog. When these chimeric proteins were assayed for processing *in vitro*, we found that Tld containing the Tlr astacin domain was a fast processing enzyme, while Tlr containing the Tld astacin domain was still a slow processing enzyme. Thus, the activity seen correlated with the CUB and EGFs and not with the astacin domain (Fig. 2D). This suggests that it is likely that the initial binding or presentation of the Sog substrate differs between these enzymes. Consistent with this view, we found that a complex consisting of Sog and wild-type Tlr was stable enough to be co-immunoprecipitated (Fig. 2E, lane 3). By contrast, Tld-Sog complexes do not co-immunoprecipitate under the same experimental conditions and are seen only if enzymatically inactive forms of Tld are used (Fig. 2E, compare lane 2 with lanes 1, 4 and 5). Taken together these results indicate that Tlr is not able to process Sog as efficiently as Tld probably because the substrate is not as efficiently presented to the active site.

Bmp heterodimers are the preferred catalyst for Tld and Tlr processing of Sog

The catalytic role of Bmps in stimulating Sog processing is thought to be caused by a Bmp-induced conformational change within Sog that better presents the substrate to the Tld protease domain. In the embryo, both Dpp and another Bmp family ligand, Screw (Scw), are required for proper dorsal patterning. In imaginal and pupal tissues, Dpp and the Bmp family ligand Glass bottom boat (Gbb) are required for proper patterning (Khalsa et al., 1998; Wharton et al., 1999; Ray and Wharton, 2001). It has recently been determined that the primary patterning ligand in the early embryo is likely to be a heterodimer of Dpp and Scw formed by intermolecular

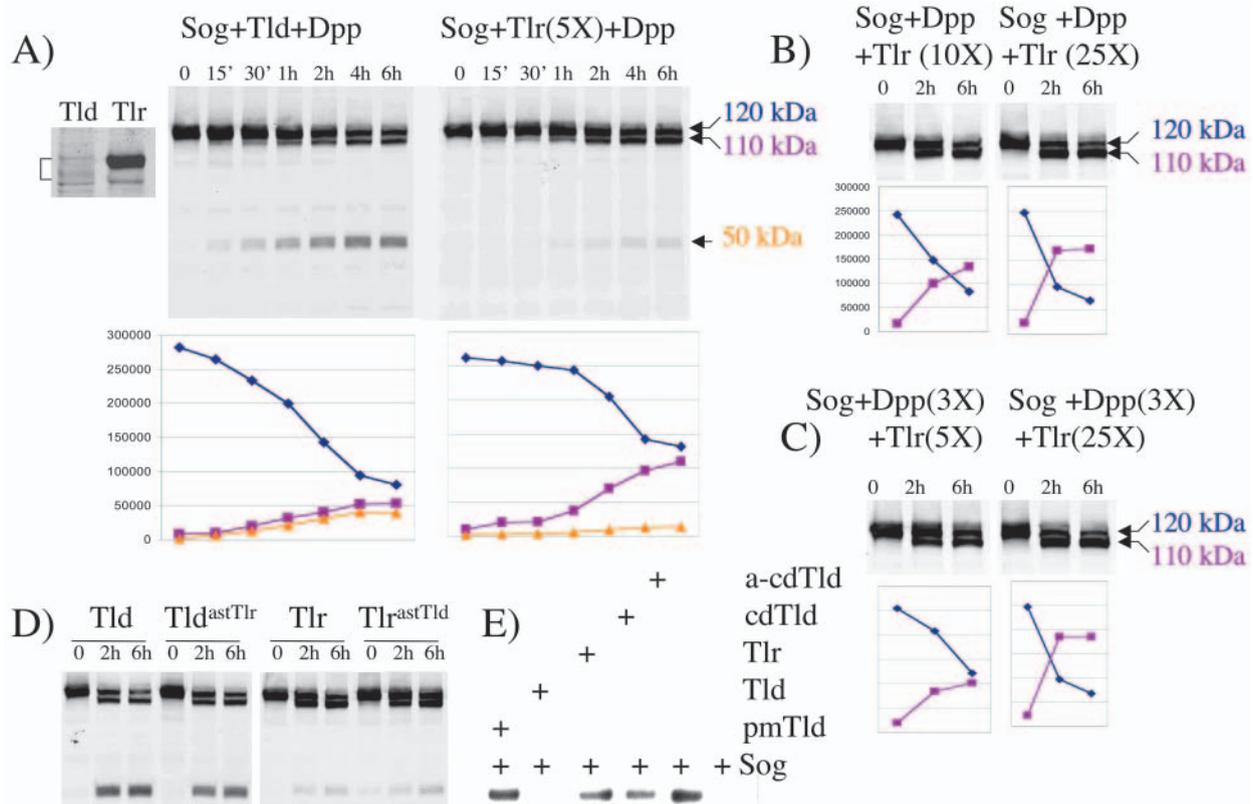


Fig. 2. Tlr exhibits slow Sog processing kinetics compared with Tld. (A) Time course of Sog processing by Tlr and Tld. The amount of Tlr in the reactions was fivefold more than the amount of Tld, as estimated by quantitative fluorescence measurements of the anti-HA immunoreactivity of the equivalently C-tagged Tld-HA and Tlr-HA contained in the bracketed area. The Dpp (R&D Systems) concentration was 3×10^{-10} M. The processing reactions were incubated at 25°C. Aliquots were removed at the indicated times, and were analyzed by immunoblotting with anti-Myc A14 antibody. The processing products were quantified using Odyssey Infrared Imaging System. The graphs below indicate the quantitation of the indicated bands at each time point. (B) Time course processing of Sog with increasing amounts of Tlr protein as indicated (10- and 25-fold excess when compared with Tld). (C) Time course of Sog processing in the presence of 1×10^{-9} M Dpp and variable amounts of Tlr protein as indicated. (D) Time course assays in which the Tld and Tlr protease domains have been swapped. In Tld^{astTlr} the Tld astacin domain has been replaced with the Tlr astacin domain, while Tlr^{astTld} is the converse construct. (E) Sog co-immunoprecipitates with Tlr and catalytically inactive Tld variants but not with active Tld. Equivalent amounts of wild-type and modified Tld-HA and Tlr-HA proteins were mixed with Sog-Myc for 3 hours at room temperature followed by immunoprecipitation with the anti-HA 12CA5 antibody. Sog was detected with anti-Myc A14 antibody.

disulfide bridge during ligand processing and secretion (Shimmi et al., 2005a). In these studies, Dpp/Scw heterodimers were found to stimulate Sog processing better than either homodimer. In imaginal and pupal tissues Dpp/Gbb heterodimers have been suggested to play important patterning roles (Ray and Wharton, 2001). We therefore tested the relative abilities of Bmp homodimers and heterodimers to stimulate processing of Sog by Tlr. We generated homodimers by transfection of individual expression plasmids in *Drosophila* S2 cell cultures. Heterodimers were generated by co-transfection of two Bmp-ligand expression plasmids. This produces a significant proportion of heterodimers together with individual homodimers (Fig. 3A) (Shimmi et al., 2005a).

Among the homodimers, Dpp and Gbb stimulate processing of Sog by either Tld or Tlr to an equivalent degree (Fig. 3C, lanes 3,4,8,9). By contrast, Sog processing was enhanced in the presence of heterodimers of Dpp/Gbb (Fig. 3C, lanes 5 and 10). Our comparison is a qualitative one, because in this study a rigorous measurement of the homodimer/heterodimer ratio was not determined; however, the same total amount of ligand was

used in each case. The enhancement we observed cannot be explained simply by an additive effect of two homodimers, as the activity of heterodimers exceeded the processing activity of equivalent amounts of the two homodimers added together to the reaction mix (Fig. 3C, compare lane 5 with 6 and lane 10 with 11).

Tlr suppresses Sog gain-of-function phenotypes in vivo

As Tlr can process Sog in vitro, we tested whether it could also function as a modulator of Sog activity in vivo. In *Xenopus* embryos, injecting *sog* mRNA into the ventral marginal zone blocks Bmp signaling and produces a secondary dorsoventral axis (Holley et al., 1995; Piccolo et al., 1997). We used this assay to determine if Tlr can reverse the effects of Sog. As shown in Fig. 4A, ventral injection of 2 ng of *sog* mRNA induces secondary axes in ~61% ($n=83$) of the resulting tadpoles. However, when *sog* mRNA was co-injected together with an equimolar amount of *tld* or *tlr* mRNA, secondary axis induction was reduced to 11% ($n=120$) and 16% ($n=149$),

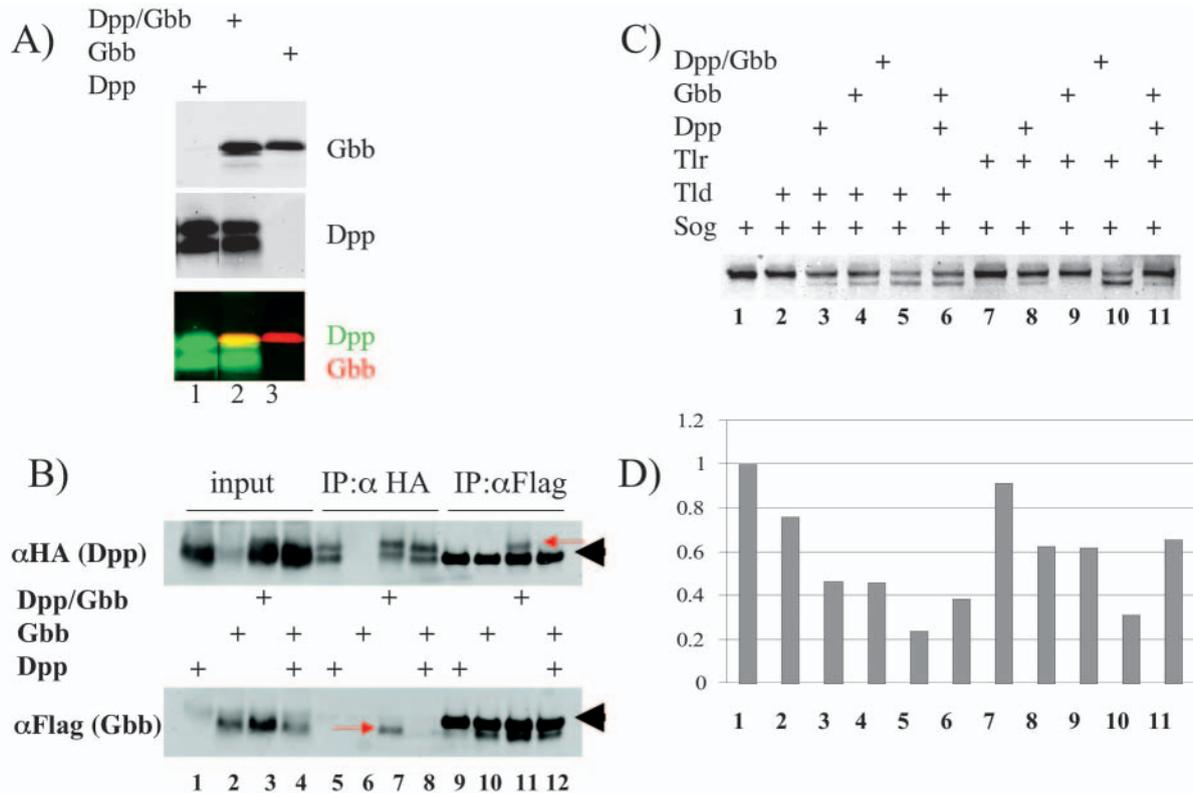


Fig. 3. Sog processing is enhanced by a Dpp/Gbb heterodimer. (A) Western blot showing a representative sample of ligand produced by cells expressing either Dpp-HA alone (lane 1), Gbb (lane 3) or co-transfected Dpp-HA and Gbb (lane 2). The immunoblots were simultaneously probed for Dpp-HA with anti-HA 12CA5 antibody and Gbb with rat anti-Gbb C terminus antibody. The blots were scanned with Odyssey Infrared Imaging System. (B) Co-expression of Dpp-HA and Gbb-Flag leads to heterodimer formation. The top panel is probed for the presence of Dpp-HA, while the bottom panel is probed for Gbb-Flag using the anti Flag M2 antibody. Lanes 1-4 show the input levels of ligands present in the starting samples. Lanes 5-8 are immunoprecipitations with the anti-HA antibody. Lanes 9-12 show immunoprecipitated material using the anti-Flag antibody. The red arrows indicate co-immunoprecipitation of Dpp and Gbb which is only seen in the samples (lanes 7 and 11) where the two ligands were co-transfected into the same S2 cells and not in samples where homodimers were mixed together (lanes 8 and 12). The 25 kDa band (black arrowhead) present in all anti-Flag M2 immunoreactions is due to the mouse IgG light chain detached from the beads under our experimental conditions. (C) Sog processing by Tld and Tlr (16 hours at 25°C) in the presence of Dpp or Gbb homodimers was compared with the processing in the presence of Dpp and Gbb heterodimers (Dpp/Gbb). Equivalent amounts of Bmp ligands estimated by their immunoreactivity (A) were included in the processing reactions as indicated. (D) Percentage of remaining full-length Sog relative to the no-enzyme containing processing reaction (lane 1) was quantified using the Odyssey Infrared Imaging System (see Materials and methods).

respectively. These results suggest that like Tld, Tlr is able to process Sog in *Xenopus* embryos.

In contrast to the case in *Xenopus*, overexpression of Sog during *Drosophila* development produces very mild phenotypes (Yu et al., 1996). However, stronger phenotypes can be generated by the combined overexpression of Sog and Tsg, which together make a more potent Bmp inhibitory complex (Yu et al., 2000; Ross et al., 2001). In the wing imaginal disc, for example, overexpression of Sog and Tsg inhibits Bmp signaling, producing a smaller wing with altered patterns of venation (Fig. 4B) (Shimmi and O'Connor, 2003). As shown in Fig. 4B, co-overexpression of Sog and Tsg with Tlr is able to reverse the small wing phenotype and restore normal patterning. These results suggest that Tlr is able to process Sog in *Drosophila* under in vivo conditions.

Tlr is required for Bmp signaling during the pupal crossvein formation

Previous analysis of *tlr* mutants found that these animals die

mostly in the larval and pupal stages of development. However, rare escapers exhibit loss of the crossveins (Nguyen et al., 1994) (Fig. 5B). Crossvein formation is initiated by high levels of Bmp signaling beginning 19 hours after pupariation (AP) (Conley et al., 2000; Ralston and Blair, 2005). We therefore examined the expression of *tlr* transcript during pupal wing development. For comparative purposes, we also examined *tld* expression in pupal wings. As illustrated in Fig. 5A we found that *tlr* is expressed in the intervein regions, while *tld* was not detectable above background. The absence of detectable *tld* expression suggests that Tlr is the major contributor of Tld-like activity to the venation process during pupal development.

To determine directly whether *tlr* is required for Bmp signaling in the developing crossveins, we assayed for accumulation of the phosphorylated form of Mad (pMad) in the crossvein region of *tlr^{ex[2-41]}/tlr^{E1}* mutants during pupal wing development. In wild-type wings dissected at 19 hours AP, pMad signal is evident in a broad region around the PCV (Fig. 5B); by 24 hours AP this region starts to narrow and by

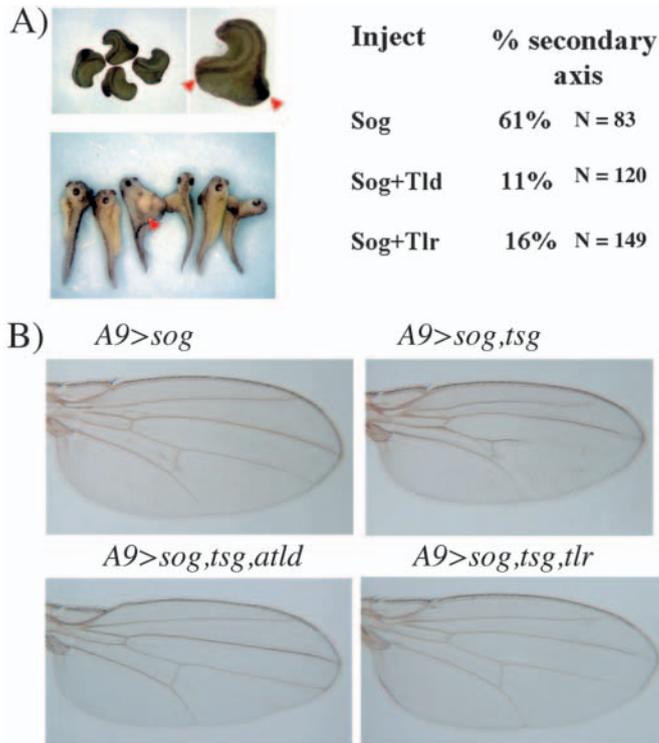


Fig. 4. Tlr suppresses Sog gain-of-function activities in vivo. (A) Injections of *sog* mRNA in one of the ventral four-cell stage blastomeres of *Xenopus* embryos induces secondary axis formation (red arrowheads) in tadpoles shown at development stages 19 and 28. Co-injection of *tld* and *tlr* inhibit Sog-mediated secondary axis formation. To ensure equimolar amounts of mRNA, we used 2 ng capped mRNA for *sog* and *tld* and 4 ng for *tlr*, which had a transcript approximately twice as long as *tld* because of the longer pro-peptide and UTR sequences. (B) Wing phenotypes produced by ectopic expression of Sog and Tsg are suppressed by addition of activated Tld (*atld*) (*UAS-tld27*) or wild-type Tlr (*tlr*) (*UAS-tlr3*).

30 hours AP a narrow stripe forms corresponding to the PCV (Conley et al., 2000). In *tlr^{ex[2-41]}/tlr^{E1}* mutant wings, pMad is severely reduced at 19 hours AP (Fig. 5B). As an internal reference, we note that pMad accumulation in the longitudinal veins and in the axons and nuclei of the sensory neurons along the anterior margin is not affected in *tlr^{ex[2-41]}/tlr^{E1}* mutants. By 24 hours AP, pMad nuclear staining was completely lost in the PCV region of almost all *tlr^{ex[2-41]}/tlr^{E1}* mutant wings (20/22 wings).

The Tlr effect on crossvein specification is mediated through Sog

As Sog is expressed in the intervein regions during pupal wing development and overexpression of Sog can block PCV formation and pMad accumulation (Yu et al., 1996; Ralston and Blair, 2005), the *tlr* mutant phenotype may be caused by excess accumulation of unprocessed Sog. If this model is correct, then reducing the level of Sog by using *sog* heterozygotes or weak *sog* heteroallelic combinations in a *tlr* mutant background might partially restore PCV formation. As illustrated in Fig. 6A, we found that *sog^{Y126/+}; tlr^{ex[2-41]}/tlr^{E1}* escaper animals exhibit partial PCVs, with significantly more

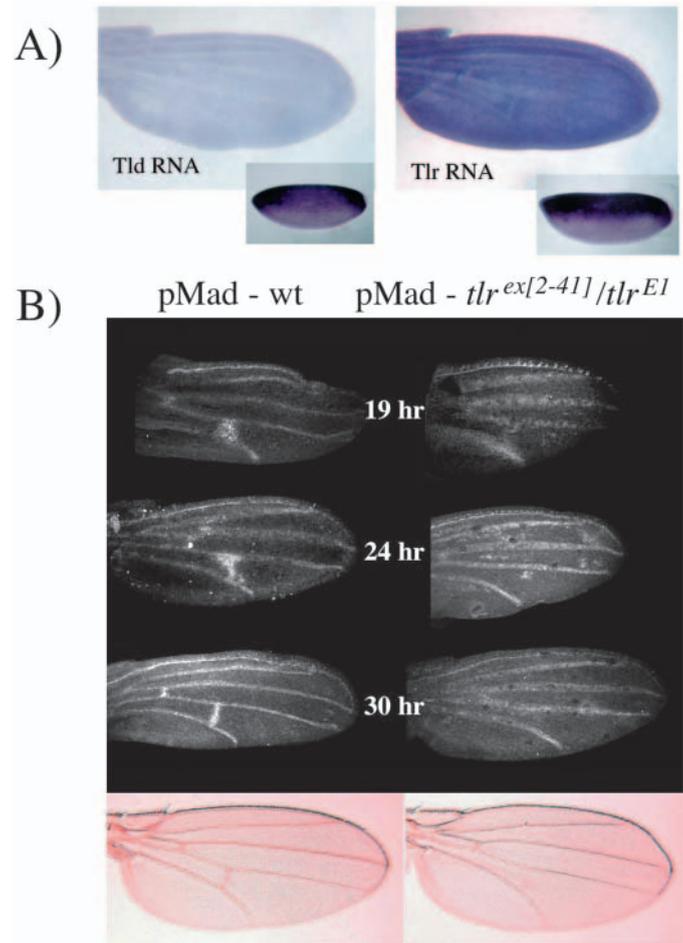


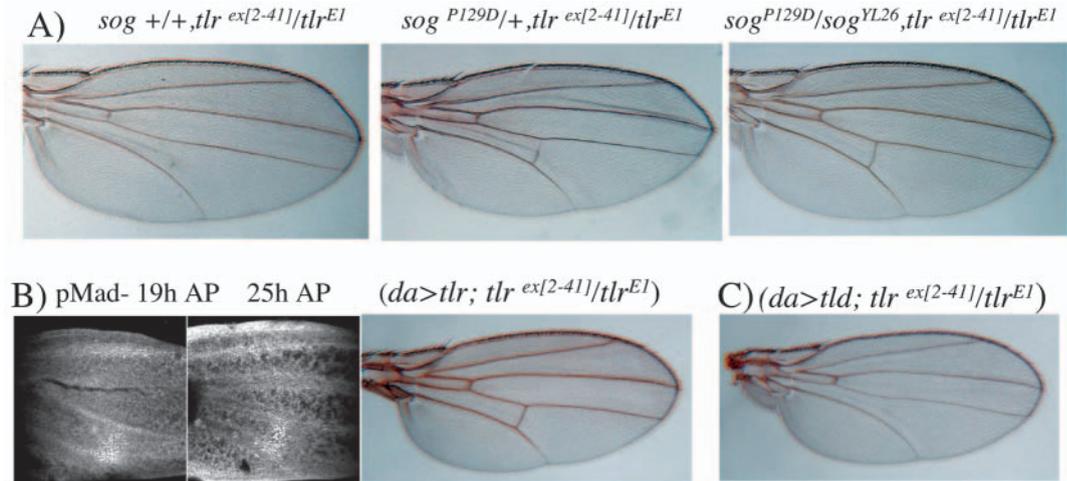
Fig. 5. *tlr* is expressed in the pupal wings and is required for the Bmp signaling in the posterior crossvein. (A) Expression of *tld* and *tlr* in 30 hours AP pupal wings is compared by in situ hybridization with antisense *tld* and *tlr* DIG-labeled probes for the same length of time. Inserts show control hybridization of embryos at the early cellularization stages using the same probes. (B) Accumulation of pMad during the pupal wing development in wild-type and *tlr* mutant animals 19 hours after pupariation (AP), 25 hours AP and 30 hours AP. The bottom panel shows the corresponding adult wings, with the *tlr* mutant wing missing the posterior crossvein.

crossvein material than seen in *tlr⁻* escapers. Moreover, the crossveins were almost completely restored in animals in which Sog was further reduced by introducing an additional weak *sog^{P129D}* allele (*sog^{Y126}/sog^{P129D}; tlr^{ex[2-41]}/tlr^{E1}*). Thus, we concluded that the loss of Bmp signaling in the posterior crossvein in the *tlr* mutants is probably due to accumulation of excess unprocessed Sog.

Tld cannot substitute for Tlr during PCV development

Our biochemical analyses indicate that Tld and Tlr have similar, but not identical, effects on Sog processing. In the embryo, *tlr* and *tld* are expressed in a similar patterns but *tlr* overexpression is unable to rescue the loss of *tld* (Nguyen et al., 1994), suggesting that the lower cleavage activity or altered site specificity of Tlr render it unable to effectively substitute

Fig. 6. The loss of PCV in *tlr* mutants is rescued by reducing endogenous Sog levels or by overexpressing Tlr, but not by overexpressing Tld. (A) *sog* heteroallelic combinations of the indicated genotype restores the PCV structure in *tlr* mutant animals. (B) Overexpression of *UAS-tlr(2)* with *da>Gal4* driver rescues Bmp signaling in the PCV of developing *tlr* mutant pupal wings, as well as the PCV in the adult wings. (C) Overexpression of *UAS-tld(16)* with *da>Gal4* driver does not rescue the PCV in the *tlr* mutant escapers.



for Tld. In the pupal wing, *tld* is not detectably expressed above background (Fig. 5A) and it is not evident whether it could replace *tlr* during PCV development. Therefore, we attempted to rescue the *tlr* mutant phenotype by overexpressing *tlr* or *tld* transgenes ubiquitously in the wing with the *da-Gal4* and the *A9-Gal4* drivers. We found that the PCVs of the adult flies, as well as the Bmp signaling in the PCV region, were largely restored by ubiquitous expression of *tlr* (pMad at 21 hours AP was fully rescued in 7/9 wings, partially rescued in 1/9 wings; at 24 hours AP pMad was fully rescued in 6/8 wings, not rescued in 2/8 wings with *da-Gal4* driver; Fig. 6B). However, we were unable to obtain similar rescue of *tlr* mutant phenotypes by driving expression of a *tld* transgene with *A9-Gal4* or *da-Gal4*; adult PCVs were still missing from such flies (Fig. 6C). Thus, although similar, Tld and Tlr are probably adapted for different developmental contexts: the former active in the embryo and the latter during later developmental stages.

Sog plays a positive role in Bmp signaling during PCV formation

An unexpected result of our misexpression experiments was that misexpression of wild-type *tld* often disrupted the PCV (Fig. 7A). Expression of a constitutively active form of Tld (Marqués et al., 1997) in the posterior of the wing, using *en-Gal4*, caused a range of phenotypes. Thirty-one out of 52 adult wings had expanded PCVs, as might be expected with excess Bmp signaling. However, in 18/52 adult wings, the PCV was completely or partially missing, a result expected from reduced Bmp signaling (Fig. 7B).

One way of explaining this result is to posit that Sog has both an inhibitory and positive effect on Bmp signaling during PCV development. There is precedent for this in the early embryo: Sog is required to specify amnioserosa, the dorsal-most tissue that requires high levels of Bmp signal. It has been proposed that this positive function arises because Sog aids in the transport of ligand so that it is concentrated at the dorsal midline (Holley et al., 1995; Marqués et al., 1997; Eldar et al., 2002; Shimmi et al., 2005a). Recent results indicate that signaling in the PCV depends on Dpp produced in adjacent longitudinal veins (Ralston and Blair, 2005), and thus a similar transport role for Sog might be required in the pupal wing.

To determine if Sog is also required to achieve peak levels of Bmp signaling in the PCV, we generated *sog* heteroallelic

combinations with reduced Sog protein level and monitored PCV formation. We find that the survival rate of the *sog*^{P129D}/*sog*^{YL26} animals was reduced to 31% (20 progeny enclosed compared with 64 of the control genotype *sog*^{P129D}/*FM7*); of these, 15% (*n*=40 wings) exhibited partial loss of the PCV (Fig. 7C). The rest of the escapers (85%) exhibited more subtle alterations in which the PCV appeared as a fragmented line instead of a continuous refined structure. For the stronger allelic combination *sog*^{P129D}/*sog*^{P1} genotype, the survival rate was further reduced to 12% (15 adults compared with 124 *sog*^{P129D}/*FM7* animals) and the phenotype was stronger: 46% of the surviving progeny (*n*=30 wings) exhibited various degrees of loss of the PCV (Fig. 7D,D'). These results indicate that, as in the embryo, Sog is required to establish peak levels of Bmp signaling during PCV specification.

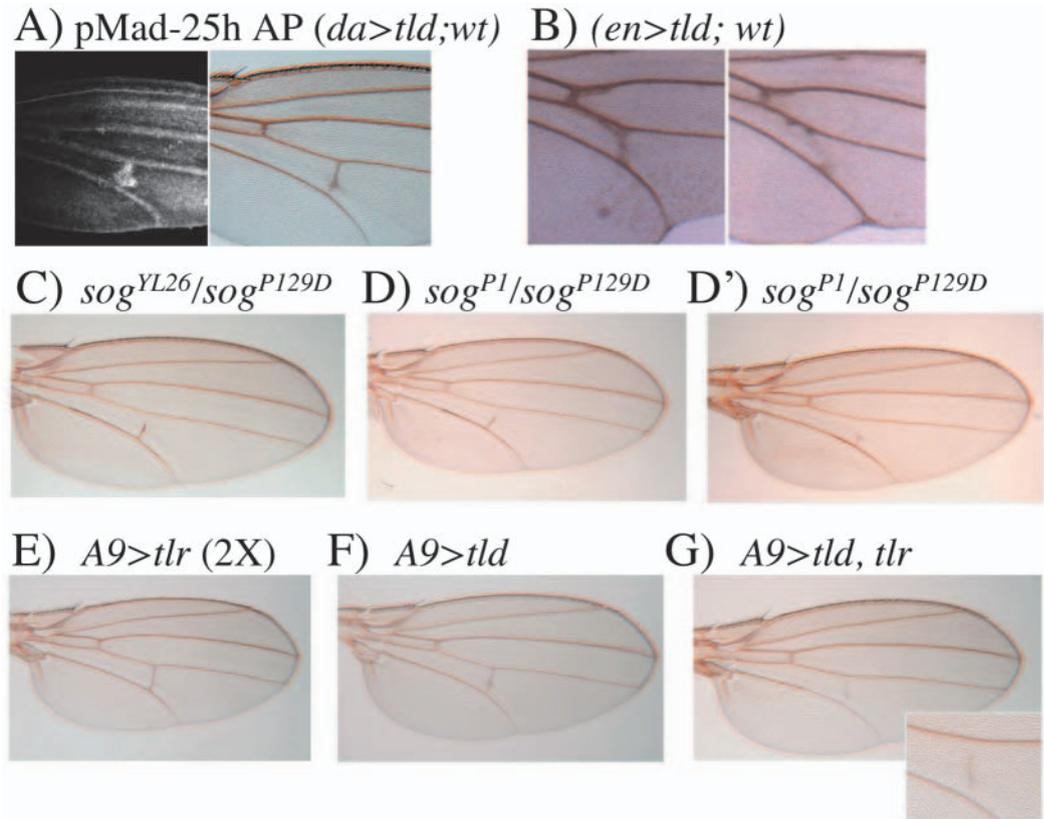
The disruption of the PCV appeared to correlate with the extent of remaining Sog protein, i.e. stronger *sog* allelic combinations or misexpression of stronger/activated *tld* transgenes exhibited stronger loss of PCV structures (this study) (Shimmi and O'Connor, 2003). As a further test of this issue, we used different combinations of *tld* and *tlr* misexpression, each of which on their own did not affect PCV formation but which in combination exhibited loss of the PCV. Thus, as shown in Fig. 7E-G, expression of two copies of a particular *UAS-tlr* line using the *A9-Gal4* driver did not affect PCV formation. Likewise expression of one copy of a particular *UAS-tld* transgene produced only mild effects (a detached PCV in 16% of the wings *n*=102). However, when the *tld* and *tlr* lines were co-overexpressed, the frequency of PCV perturbation was greatly increased to 82% (*n*=192) and the severity of the phenotype was also increased (Fig. 7G). Taken together, these results suggest that an inappropriate increase in total Sog processing activity disrupts the PCV specification most probably by interfering with the positive role of Sog in Bmp signaling within the pupal wing.

Discussion

Kinetic differences in Sog processing by Tld and Tlr probably contribute to the tissue specificity of their actions

In the early embryo, the metalloprotease Tld regulates Bmp

Fig. 7. Reduction of Sog levels perturbs the PCV formation. (A) Overexpression of *UAS-tld(16)* with *da>Gal4* driver causes expanded pMad in the PCV region and expanded PCV material and detachment of PCV from L5. (B) Overexpression of *UAS-tld(16)* with *en>Gal4* in a wild-type background causes widening or absence of PCV. (C) *sog^{YL26}/sog^{P129D}* results in detached PCV. (D,D') In the stronger allelic combination *sog^{P1}/sog^{P129D}*, partial to more complete loss of PCV structures is observed in a larger percentage of animals (see text for numbers). (E) Overexpression of two copies of *UAS-tlr(X)* with *A9>Gal4* does not affect PCV formation. (F) Overexpression of one copy of *UAS-tld(16)* with *A9>Gal4* driver produces detached PCV in 16% of the animals ($n=102$). (G) Overexpression of *UAS-tld(16)* and *UAS-tlr(X)* with *A9>Gal4* driver produces a range of crossvein defects, from detached PCV shown to almost complete loss of PCV structures in 82% ($n=192$) of the adults.



signaling by processing the Bmp antagonist Sog to release the ligand from an inhibitory complex for signaling. In the present study, we provide biochemical and genetic evidence that the Tollloid-related protein Tlr also processes Sog and is required for Bmp signaling. In both cases, Sog is cleaved at three major processing sites. We have recently identified these sites by peptide microsequencing and confirmed that they are the same for Tld and Tlr (M.S. and M.B.O., unpublished). In addition, both enzymes require Bmp ligands to efficiently cleave Sog, something that has so far not been seen for processing of Chordin by any of the vertebrate Tld-like enzymes.

The major distinction between the two *Drosophila* proteases in terms of their Sog processing function is the time and tissue in which they act. Tld activity is primarily confined to the early embryo, while Tlr is required during pupal wing development. To some extent, the functional differences between them can be attributed simply to differences in expression pattern. In the pupal wing we find that Tlr is far more abundantly expressed than Tld, and this alone might account for the lack of redundancy. In the early embryo, however, the situation is more complex. Both enzymes are expressed with similar profiles, but Tlr does not seem to be capable of providing sufficient Sog processing activity, even when several extra copies are provided as transgenes (Nguyen et al., 1994).

Previously, we speculated that this difference in activity might reflect differences in activation of the proteases at the level of pro-peptide removal. Like all the members of the Bmp1 family, Tld and Tlr are secreted as pro-enzymes; the processing of the pro-peptide is necessary for the activation of proteolytic activity, as the N-terminal end of the astacin motif

is buried inside the catalytic domain forming an internal salt bridge (Bode et al., 1992). Mutations at the processing site rendered the enzymes inactive, whereas removal of the pro-peptides produced activated forms of Tld and Tlr. Tlr has a much longer pro-peptide that could either aid or inhibit activation in a tissue-specific manner. However, the inability of Tlr to rescue Tld mutants does not appear to result from an inefficient activation step. We have previously shown that Tld activation, both in the embryo and in S2 cells, is very inefficient (Marqués et al., 1997) with most of the protein found in the pro-enzyme state. By contrast, we show here that pro-peptide removal from Tlr is very efficient in S2 cells, and the same is true when Tlr is ectopically expressed in the embryo (M.S. and M.B.O., unpublished).

Instead, it seems likely that the difference in kinetics of Sog processing by Tlr is the reason behind the inability of Tlr to rescue *tld* mutants. We show here that Tlr is much less efficient at cleaving Sog in vitro than Tld. Given the rapid developmental time of early embryogenesis, where patterning by Bmps during cellularization occurs within approximately a 30 minute time window, the slower kinetics of Sog processing by Tlr may not support proper patterning. Indeed, our recent computational work has shown that a three to fourfold reduction in kinetic properties of Tld will completely disrupt the patterning process (D. Umulis, H. Othmar and M.B.C., unpublished).

Although the slow processing kinetics of Tlr towards Sog may prevent it from functioning effectively in early embryonic patterning, this property may be exactly what is required to achieve proper formation of the PCV. Unlike patterning in the

early embryo, formation of the PCV, as assessed by profile of pMad accumulation, occurs over at least a 7 hour time frame (Conley et al., 2000). The slower processing rate of Tlr towards Sog may be required to achieve the appropriate balance of Sog destruction and diffusion (see below) that is necessary for proper patterning to occur. Consistent with this view, we find that overexpression of various UAS-*tld* lines under the control of the *A9-Gal4* driver in a *tlr* mutant background did not rescue PCV formation. In fact, in many cases overexpression of an activated Tld, or co-expression of wild type *tld* and *tlr* together produced loss of the crossvein tissue in a wild-type background. We envision that, under these conditions, the increased level of enzymatic activity results in over digestion of Sog, a situation that would phenocopy *sog* hypomorphs. Consistent with this view, we find that hypomorphic *sog* allelic combinations also result in the loss of the PCV. In addition, it has recently been shown that large *sog* null clones can also result in loss of the PCV (Shimmi et al., 2005b).

In the *Xenopus* assay, we found that Tlr is only slightly less efficient than Tld at reverting secondary axis induction caused by Sog. Although we do not know how well each enzyme is activated in this animal, it should be noted that the developmental time period over which the patterning process functions in *Xenopus* is long compared with early *Drosophila* development. The longer time frame may enable the less efficient protease to produce a similar biological response. Our protease domain swap experiments suggest that the reduced processing rate did not involve evolution of intrinsic differences in the catalytic abilities of the protease domain itself, but rather changes in the way that the Sog substrate initially interacts with the enzyme. In summary, we propose that during evolution there was selection for particular biophysical properties of these two enzymes to properly match the developmental time frame over which the patterning mechanisms operate. We cannot exclude however, that other differences besides kinetic activity might also play a role in providing functional specificity. For example, it is possible that variation in cleavage site selection might also contribute to the different biological activities of Tlr and Tld. It is worth noting in this regard that different fragments of Sog have been shown to have both positive and negative effects when overexpressed in the wing (Yu et al., 2000; Yu et al., 2004). However the *in vivo* roles of endogenous Sog fragments have not been defined.

Recapitulation of the Bmp-mediated dorsal patterning mechanism during specification of the PCV

Our results suggest that a proper balance of Sog and protease activity is necessary to pattern the PCV. Interestingly, the same situation holds true in the early embryo. In this case, Sog plays both positive and negative roles in patterning the dorsal domain (Holley et al., 1995; Ashe and Levine, 1999; Marqués et al., 1997). It is required in dorsolateral regions to block Bmp signaling, but it also acts as an agonist to achieve peak levels of Bmp signals at the dorsal midline. Two types of models have been proposed to account for these dual activities. In one model, the different cleavage fragments of Sog are thought to provide either agonist or antagonist function, but the details of the mechanism are unclear (Ashe and Levine, 1999; Yu et al., 2000). In the other model, both functions are proposed to come about as a result of Sog providing a transport mechanism that

spatially redistributes Bmp ligands from the lateral region to the dorsal most cells (Holley et al., 1995; Ross et al., 2001; Shimmi and O'Connor, 2003; Shimmi et al., 2005a). This transport mechanism also requires the activity of Tsg, a small cysteine-rich secreted protein which has been shown to form a tripartite complex with Sog and Dpp (Ross et al., 2001; Shimmi and O'Connor, 2003). The prevailing view is that as Sog diffuses into the dorsal domain it forms a high affinity complex with Tsg and Dpp. This complex is unable to bind to receptors and is responsible for the antagonistic activity of Sog. At the same time, the complex protects Dpp from degradation and receptor binding allowing it to diffuse and accumulate dorsally where it is released by Tld processing. The ability of Sog to redistribute the Bmp ligands accounts for the agonist function of Sog. Recent computational analyses have provided additional support for this model (Eldar et al., 2002) (D. Umulis, H. Othmar and M.B.C., unpublished).

We propose that the same type of mechanism may be responsible for patterning the PCV. Recent analysis has provided evidence that the longitudinal veins act as the source of Dpp for PCV specification. Dpp is thought to diffuse from these veins into a PCV competent zone (Ralston and Blair, 2005). The exact mechanism by which the competent zone is specified is not clear, but low levels of Sog expression are required (Ralston and Blair, 2005; Shimmi et al., 2005b). *tlr* is expressed within the PCV competent zone during the initial stages of crossvein development (A.R. and S.S.B., unpublished), suggesting the Tlr:Sog ratio will be higher in this region. Furthermore, because processing of the Sog/Dpp complex by Tld-like enzymes is dependent on the Dpp concentration (Shimmi and O'Connor, 2003) (this work), the complex will be most efficiently processed in the center of the competent zone.

According to this model, there is limited processing of Sog and therefore limited release of Dpp from its inhibitor in *tlr* mutants. Conversely, Sog also supplies a positive function for PCV formation, probably by providing a transport mechanism for Dpp, accounting for the partial loss of the PCV in hypomorphic *sog* mutants and complete loss of the PCV in large *sog*-null clones (Shimmi et al., 2005b). The partial reversion of the *tlr* mutant phenotype by introduction of hypomorphic *sog* alleles is also consistent with the view that it is the balance between these two factors that is crucial for proper patterning. Interestingly, this is the way in which Sog was originally identified as an inhibitor of Dpp signaling in the embryo: weak *sog* alleles were isolated as partial suppressors of *tld* mutations (Ferguson and Anderson, 1992). One difference is that, in our case, lowering Sog levels is able to revert a null mutation instead of a hypomorphic condition, as was the case in the embryo. There are at least two possibilities that can explain this suppression effect. First, although these animals may be null for *tlr*, there could be some low level *tld* expression in the pupal wing. If so, then these wings would not be devoid of all Sog-processing activity and therefore lowering Sog levels might enable the limited amount of Tld to provide the proper production-destruction balance. Alternatively, neither Sog nor Tlr may be absolutely required for PCV formation. Instead their functions may be simply to ensure that the patterning occurs reproducibly. Thus, in the absence of both Sog and Tlr, partial PCV formation may occur as a result of some Bmp ligand accumulating in the correct position.

However, under these circumstances, the patterning mechanism would be unreliable and would produce different results on case-by-case basis. To prevent this from occurring, we posit that evolution has selected for supplementary regulatory controls involving Sog and Tlr to ensure that the PCV always forms completely and reliably at the correct position.

Two additional observations make the comparison between formation of the PCV and establishing the high point in embryonic Bmp signaling even more compelling. First, in the embryo, Tsg is required to enable Sog to bind to Dpp and Scw to achieve peak levels of Bmp signaling. Although Tsg and Scw are not transcribed in the pupal wing, we have recently determined that a Tsg-related gene, encoded by the *crossveinless* (*cv*) locus, is expressed in the pupal wing (Shimmi et al., 2005b; Vilmos et al., 2005). As *cv* mutants exhibit a crossveinless phenotype, it seems likely that *Cv* functionally substitutes for Tsg during PCV formation. Second, Gbb, another Bmp-like ligand, may functionally replace Scw as *gbb* hypomorphic mutations lack the PCV and associated Bmp signaling (Wharton et al., 1999; Ralston and Blair, 2005).

A major distinction between embryonic amnioserosa development and PCV formation is that PCV specification also requires the activity of *Cv2*, a protein that contains cysteine-rich repeats similar to those found in Sog, while amnioserosa specification does not, despite the expression of *Cv2* in those cells (Conley et al., 2000) (M.S. and M.B.O., unpublished). Vertebrate homologs of *Cv2* can bind Bmps, and act variously as agonists or antagonists of Bmp signaling in different assays (Kamimura et al., 2004; Coffinier et al., 2002; Binnerts et al., 2004). It is not clear by what mechanism *Cv2* promotes Bmp signaling during PCV formation. It is also not clear why *Cv2* is not required in the early embryo, even though it is expressed in dorsal blastoderm cells (M.S., A.R., S.S.B. and M.B.O., unpublished).

Finally, Tlr plays additional roles during development, besides processing Sog for specification of the PCV. In contrast to *cv* and *cv2* null mutations, which result in homozygous viable and fertile flies (Shimmi et al., 2005b; Vilmos et al., 2005) (M.S. and M.B.O., unpublished), most *tlr* mutant animals die during larval stages (Nguyen et al., 1994; Finelli et al., 1995) when there is no known requirement for Sog. In addition, although reducing Sog levels did suppress the PCV defect observed in the *tlr* mutant escaper flies, it did not increase the frequency of eclosing animals. Therefore Tlr may be required for processing of some other essential component(s) during *Drosophila* development.

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