

Feedback regulation of *Drosophila* BMP signaling by the novel extracellular protein Larval Translucida

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SUMMARY

The cellular response to the *Drosophila* BMP 2/4-like ligand Decapentaplegic (DPP) serves as one of the best-studied models for understanding the long-range control of tissue growth and pattern formation during animal development. Nevertheless, fundamental questions remain unanswered regarding extracellular regulation of the ligand itself, as well as the nature of the downstream transcriptional response to BMP pathway activation. Here, we report the identification of *larval translucida* (*ltl*), a novel target of BMP activity in *Drosophila*. Both gain- and loss-of-function analyses implicate LTL, a leucine-rich repeat protein, in the regulation of wing growth and vein patterning. At the molecular level, we demonstrate that LTL is a secreted protein that antagonizes BMP-dependent MAD phosphorylation, indicating that it regulates DPP/BMP signaling at or above the level of ligand-receptor interactions. Furthermore, based on genetic interactions with the DPP-binding protein Crossveinless 2 and biochemical interactions with the glypican Dally-like, we propose that LTL acts in the extracellular space where it completes a novel auto-regulatory loop that modulates BMP activity.

KEY WORDS: BMP signaling, DPP, LTL, Feedback, Imaginal disc, Morphogen

INTRODUCTION

Throughout multicellular animals, the long-range coordination of patterning and growth requires the action of secreted signaling molecules from a small number of highly conserved gene families. This relatively limited palette of signaling systems generates a diversity of cell- and tissue-specific responses by eliciting context-specific transcriptional outputs [e.g. BMPs (Hogan, 1996), WNTs (Croce and McClay, 2008), Hedgehog (Jiang and Hui, 2008), FGFs (Itoh, 2007; Ornitz and Itoh, 2001) and Notch (Hansson et al., 2004)]. However, beside the tissue-specific transcriptional response, both intra- and extracellular modulation of signaling activity also play a crucial role in determining the response to most, if not all, secreted signals. Indeed, while core components of signaling systems are highly conserved throughout evolution (Erwin, 2009), a broad spectrum of regulators can shape context-specific activity. *Drosophila* BMPs, for example, perform multiple functions throughout development, and are subjected to context- and developmental stage-specific extracellular regulation (Umulis et al., 2009).

Drosophila imaginal discs originate as small invaginations of the embryonic ectoderm and proliferate to form epithelial sacs that differentiate adult cuticular structures during pupal metamorphosis (Cohen, 1993). In the wing imaginal disc, the BMP2/4-like ligand DPP is expressed in a precisely patterned stripe at the anterior-posterior margin (Padgett et al., 1987; Posakony et al., 1990; Zecca et al., 1995). DPP/BMP protein is secreted from these source cells and is thought to form a morphogen gradient that controls both patterning and growth of the wing disc (Nellen et al., 1996), as evidenced by a well-characterized gradient response to pathway

activity (Entchev et al., 2000; Lecuit et al., 1996; Teleman and Cohen, 2000). Still, precisely how DPP/BMP moves to elicit this graded response remains unclear, with evidence supporting active transport by planar transcytosis (Entchev et al., 2000; Kicheva et al., 2007), diffusion in the apical extracellular space through the disc lumen (Gibson et al., 2002), facilitated extracellular diffusion through the action of heparan sulfate proteoglycans (HSPGs) (Akiyama et al., 2008; Belenkaya et al., 2004) or some combination of the above (Affolter and Basler, 2007).

Mechanisms of transport aside, secreted DPP/BMP binds to a heterodimeric receptor complex consisting of the type II receptor Punt and the type I receptor Thickveins (TKV), which in turn phosphorylate the cytosolic transducer Mothers Against DPP (MAD) (Brummel et al., 1994; Letsou et al., 1995; Sekelsky et al., 1995). Phosphorylated MAD (PMAD) interacts with Medea and is accumulated in the nucleus, where the complex binds to regulatory elements in DPP/BMP target genes (Gao et al., 2005). The PMAD/Medea complex primarily acts via repression of Brinker (BRK), a transcriptional repressor that inactivates DPP targets (Kirkpatrick et al., 2001; Affolter and Basler, 2007). The DPP/BMP transcriptional response is thus formed through a combination of de-repression of BRK target genes and direct activation. The known targets of this cascade in the wing disc are surprisingly limited, including repression of BRK and the novel extracellular protein encoded by *pentagone* (*pent*) (Vuilleumier et al., 2010), and activation of the transcription factors Optomotor Blind (OMB) (Grimm and Pflugfelder, 1996; Nellen et al., 1996) and Spalt (Kuhnlein et al., 1994; Nellen et al., 1996), as well as the inhibitory SMAD6 homolog Daughters Against DPP (DAD) (Tsuneizumi et al., 1997). Beyond this initial transcriptional response, however, relatively little is known about downstream effectors of the pathway that ultimately control growth or effect morphogenesis.

During wing development, BMP signaling has multiple roles where it not only determines final wing size by promoting growth and epithelial morphogenesis during larval stages (Gibson and

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Perrimon, 2005; Schwank et al., 2008; Shen and Dahmann, 2005), but it also plays a crucial role in patterning the longitudinal veins (LV) and the anterior and posterior crossveins (ACV and PCV) during pupal development. Although LV formation requires the cooperation of several different signaling pathways (de Celis et al., 1996; Sotillos and De Celis, 2005), the development of CVs relies almost entirely on BMP signaling, and has thus served as a sensitive readout for functional assays of proteins that modulate pathway activity. *dpp* mRNA is expressed throughout the LVs during pupal development, but not in the developing CVs. Hence, additional mechanisms are required to facilitate transport of the ligand to target cells in the presumptive CV region (Ralston and Blair, 2005). Currently, transport is thought to be accomplished by a complex of the extracellular proteins Short gastrulation (SOG) and Crossveinless (CV), which binds to BMPs, inhibiting the signal while mediating transport perpendicular to the LVs (Ralston and Blair, 2005; Serpe et al., 2005; Shimmi et al., 2005; Vilmos et al., 2005). Within the CV territory, the metalloprotease Tolloid-related (TLR) cleaves SOG, enabling the transported ligand to signal to receptors (Marques et al., 1997; Serpe et al., 2005; Umlis et al., 2009). The sensitivity of CV development to perturbations in DPP/BMP signaling has allowed the identification of numerous additional DPP-modulators, including the BMP binding protein Crossveinless 2 (CV-2) (Conley et al., 2000; Serpe et al., 2008), the transmembrane protein Kekkon5 (Evans et al., 2009) and the LEM-domain protein MAN1 (Wagner et al., 2009).

In the present study, we employ transcriptional profiling of *Drosophila* wing disc cell clones lacking BMP activity to identify and characterize *larval translucida* (*ltl*, CG32372), a novel BMP target gene. Both sequence analysis and functional experiments demonstrate that *ltl* is a target of BMP signal transduction. To elucidate the function of LTL, we generate a series of sequenced alleles exhibiting larval and adult phenotypes. We show that elimination of LTL results in reduced wing growth and vein patterning defects associated with ectopic DPP/BMP activity. Intriguingly, we find that overexpression of LTL inhibits wing growth, while simultaneously abolishing DPP/BMP signaling in the presumptive PCV. These complex phenotypes suggest an intricate concentration-dependent BMP-modulatory function for LTL, not unlike that recently described for CV-2 (Serpe et al., 2008). Consistent with this view, we show that LTL is a secreted protein that genetically interacts with *cv-2* and physically interacts with the glypican Dally-like (DLP).

MATERIALS AND METHODS

RNA extraction for microarrays

Homozygous *tkv^{xtr}* clones (Gibson and Perrimon, 2005) were generated using the MARCM technique (Lee and Luo, 1999). To prepare RNA for array probe synthesis, extruding homozygous clones (GFP+) and neighboring wild-type tissue (GFP-) were isolated surgically. Pooled clones were lysed in TRIzol (Invitrogen). Affymetrix array experiments were performed at the Stowers Institute Microarray Facility.

Mutagenesis and molecular characterization of *ltl* alleles

To create deletions covering *ltl*, 2-day-old *w; PBac{WH}f03786/PBac{WH}f03786* males were irradiated at 3500 Rads and mated to *w; Ly/TM3,Sb* females. Progeny exhibiting loss of eye pigmentation due to disruption of *PBac{WH}f03786* were analyzed by PCR. The smallest deletion characterized was *w; Df(D26)*. To generate point mutations, 25 mM EMS was used to mutagenize 2-day-old isogenized *CantonS* males (a gift from Scott Hawley, Stowers Institute for Medical Research, Kansas City, MO, USA). To identify putative *ltl* alleles, mutants were tested for

failure to complement *w; Df(D26)/TM3, Gal4-twi, P{UAS-2xEGFP}*, organized into complementation groups and sequenced according to standard protocols.

P{UAS-ltl} and *P{UAS-ltl::GFP}C5* misexpression constructs

To generate *P{UAS-ltl}*, *ltl* cDNA was amplified from RE09158 (Berkeley Drosophila Genome Project) and cloned into *P{UAS-T}* (Brand and Perrimon, 1993). Transformant lines were generated using standard methods (Genetic Services, Cambridge MA). For LTL::GFP, we generated *P{UAS-NotI-ltl-XhoI-STOP-AgeI}C5* by amplifying the *ltl* ORF (minus the stop codon) from RE09158 using PCR primers adapted with *NotI* (5') and *XhoI*-TGA(STOP)-*AgeI* (3') sequences. This *NotI-ltl-XhoI-STOP-AgeI* cassette was subcloned into *P{UAS-C5}* (Le et al., 2007). To generate *P{UAS-N-gfp::ltl}*, the GFP ORF excluding its stop codon was amplified from pGFP (Clontech) with *KpnI*- and *NotI*-adapted primers and then subcloned, in frame, into the endogenous *KpnI* and engineered *NotI* sites of *P{UAS-NotI-ltl-XhoI-STOP-AgeI}C5*. To create *P{UAS-ltl::gfp-C}*, a *gfp-stop* PCR product was inserted into the engineered *XhoI* and *AgeI* sites in *P{UAS-NotI-ltl-XhoI-STOP-AgeI}C5*. To create the C-terminal fusion *P{UAS-ltl^{HA-2XFLAG}}*, *ltl* cDNA was digested from *P{UAS-ltl}* and ligated into *XhoI/NotI* digested *P{UAS-HA-2XFLAG}* vector (a gift from Jerry Workman, Stowers Institute for Medical Research, Kansas City, MO, USA).

Drosophila genetics

Animals were maintained on standard molasses-based diet at 25°C.

Drosophila stocks used in this study were:

w; Df(D26)/TM3,Sb,GAL4-twi,P{UAS-2xEGFP},
w; ltl¹/TM3,Sb,GAL4-twi,P{UAS-2xEGFP},
w; ltl²/TM3,Sb,GAL4-twi,P{UAS-2xEGFP},
w; ltl³/TM3,Sb,GAL4-twi,P{UAS-2xEGFP},
w; ltl⁴/TM3,Sb,GAL4-twi,P{UAS-2xEGFP},
w; ltl^N/TM3,Sb,GAL4-twi,P{UAS-2xEGFP},
w; ltl² FRT80B/TM3,Sb, GAL4-twi,P{UAS-2xEGFP},
w; P{UAS-ltl}¹ (II),
w; P{UAS-ltl}⁵ (III),
w; P{UAS-ltl^{HA-2XFLAG}} (III),
w; P{UAS-N-gfp::ltl}/CyO,
w; P{UAS-ltl::gfp-C} (III),
cv-2¹ (Bloomington Drosophila Stock Center),
w; engrailed-Gal4, P{UAS-cv-2}^{gmc18} (a gift from Seth Blair, University of Wisconsin, Madison, WI, USA) and
w; UAS-dlp-HA#5 (a gift from Hiroshi Nakato, University of Minnesota, Minneapolis, MN, USA).

Polyclonal antibody production

To purify LTL protein, *ltl* cDNA was cloned into pDONR221 using the BP clone kit (Invitrogen). *ltl* cDNA was then recombined into the pVCH6 expression vector (Min et al., 2004) with the LR clone kit (Invitrogen). Rosetta DE3 pLysS cells (Novagen) were transformed with the resulting pVCH6-*ltl* construct. LTL was induced by addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Recombinant His-tagged LTL was purified using HiTrap HP columns (GE Healthcare, Piscataway, NJ). Two rabbits were immunized to generate anti-LTL (Yenzym, South San Francisco, CA, USA).

Immunocytochemistry, confocal microscopy and image analysis

Imaginal discs were fixed and processed according to standard protocols. For pupal wing staining, we modified the protocol of Ralston and Blair (Ralston and Blair, 2005). Briefly, pupae were partially dissected and fixed in 4% paraformaldehyde in PBS at 4°C overnight. Wings were then isolated by dissection and fixed for an additional 30 minutes using 4% paraformaldehyde in PBS with 1.5% NP40. Samples were washed four times for 20 minutes with 0.3% Triton X-100 in PBS (0.3% PBT). Primary antibody incubation was overnight at 4°C, followed by four 20-minute washes with 0.3% PBT. Secondary antibody incubation was carried out for 2 hours at room temperature followed by four 20-minute washes with 0.3% PBT. To quantify PMAD activity, pixel intensities within a fixed area of the dorsal compartment of 20 discs were obtained using the plot profile

function of ImageJ. To minimize variation, we chose 20 comparable discs from both genotypes, matched the optical sections, and imaged under identical parameter settings. Rabbit anti-LTL was used at 1:3000, rabbit anti-pMad at 1:2000 (a gift from Ed Laufer, Columbia University, NY, USA) and Phalloidin-546 at 1:250 (Invitrogen). All samples were imaged on a Leica SP5 AOBs confocal microscope system.

Western blotting and in situ hybridization

For western blots, total protein was extracted from 200 embryos using lysis buffer [RIPA buffer (Pierce, Rockford, IL), one protease inhibitor cocktail tablet and 0.2 mM phenylmethylsulfonyl fluoride]. Extracts from controls and mutant embryos selected for lack of a GFP-marked balancer chromosome were mixed with LDS loading buffer (Invitrogen) and loaded onto a polyacrylamide gel. Membranes were probed with anti-LTL (1:4000). For loading controls, membranes were re-probed with mouse anti- α -Tubulin (1:1000, Sigma).

In situ hybridizations on wing discs were performed using standard protocols (Sturtevant et al., 1993). DIG-labeled antisense *ltl* RNA probe was generated from BDGP cDNA RE09158 and used at a 1:50 dilution. In situ hybridizations on pupal wings were carried out as described (M. Sturtevant and E. Bier, personal communication; <http://superfly.ucsd.edu/bierlab/research/protocols/imagdisc.html>).

LTL and DLP co-immunoprecipitation

Approximately 1000 wing imaginal discs were isolated from w^{1118} animals in ice-cold PBS with 1×Roche Complete Protease Inhibitor (PI) and PMSF. Discs were sonicated in 200 μ l of NP40 Lysis Buffer (Invitrogen). For input control, 10 μ l of lysate was set aside. The remaining 190 μ l was divided into two aliquots and 250 μ l of NP40 Lysis Buffer with PI was added to each tube. For LTL pulldown experiments at 4°C, 10 μ l of rabbit-anti-LTL was added to one tube overnight and then both tubes were incubated with 60 μ l of A/G Plus Beads (Santa Cruz Biotechnology) for 4 hours. The beads were washed and then eluted with 30 μ l of 4× SDS loading buffer at 70°C for 5 minutes. Western blotting was carried out according to standard methods with mouse anti-DLP 13G8-c antibody (1:200; Developmental Studies Hybridoma Bank). For pulldown with DLP, sonication and immunoprecipitation were carried out in ice-cold PBS with PI and PMSF instead of NP40. Immunoprecipitation was performed with approximately 6 μ g of mouse anti-DLP and the blot was probed with rabbit-anti-LTL (1:3000).

RESULTS

Transcriptional profiling of the BMP response in the wing disc

To identify novel transcriptional targets of BMP signaling in the developing wing imaginal disc, we employed two distinct expression-profiling strategies. In the first, we used *Omb-Gal4*, *UAS-GFP* wing discs to surgically isolate GFP positive BMP-responding cells, and then compared their transcriptional profile to GFP negative cells from the same discs. In the second approach, we took advantage of the fact that mutant cell clones lacking the DPP/BMP receptor TKV form extruded epithelial cysts (Gibson and Perrimon, 2005; Shen and Dahmann, 2005). *tkv^{xt}* mutant cell clones induced by the MARCM technique (Lee and Luo, 1999) were surgically isolated from third-instar wing discs based on GFP expression (Fig. 1A). Two or three individual clone lysates were then pooled together to recover sufficient RNA for comparison with adjacent GFP-negative control tissue (see Fig. S1 in the supplementary material).

Both array experiments were performed in triplicate and data were analyzed for highly enriched genes showing upregulation in the DPP/BMP-responsive OMB domain and concomitant downregulation in mutant cell clones. Hierarchical clustering identified a number of known DPP/BMP-responsive genes, including *omb* (Grimm and Pflugfelder, 1996), *spalt* (Kuhnlein et al., 1994), *doc1*, *doc2* and *doc3* (Hamaguchi et al., 2004), and the

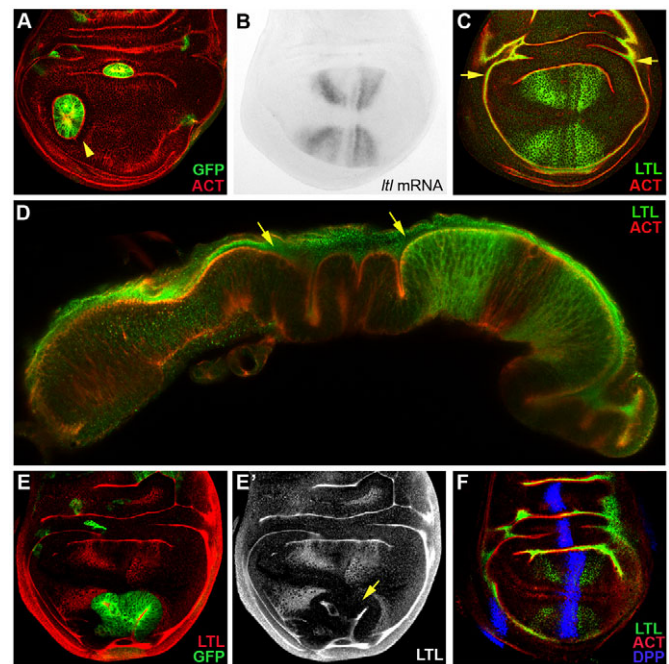


Fig. 1. DPP signaling is required for LTL expression in the wing disc. (A) *tkv^{xt}* homozygous mutant clones labeled with GFP (yellow arrowhead) were isolated from third instar discs for expression profile analysis. (B) Expression pattern of *ltl* mRNA. (C) LTL protein (green) shows an identical intracellular distribution to the *ltl* mRNA, with extracellular protein visible in the apical lumen (yellow arrows). Phalloidin counterstaining of F-Actin (ACT) is red. (D) An xz section through the wing disc reveals abundant extracellular LTL (green) throughout the lumen (yellow arrows), apical to F-Actin accumulation at the adherens junctions (red). (E, E') GFP+, *tkv^{xt}* mutant clones lacking BMP activity (green) exhibit cell-autonomous elimination of LTL expression (yellow arrow in E'). (F) The LTL expression domain (green) is precisely centered on the *dpp* expression domain (blue), as revealed by a *dpp-LacZ* reporter. Intracellularly, LTL protein is primarily apical, hence the basal optical sections here and in E' have significantly weaker signal than more apical sections (as in C).

inhibitory SMAD encoded by *dad* (Tsuneizumi et al., 1997). Interestingly, the most highly BMP-repressed gene in the OMB comparison was PENT, a newly described BMP feedback regulator (Vuilleumier et al., 2010). Highly ranked among the known DPP/BMP-responsive transcripts in both arrays was *CG32372*, an uncharacterized leucine-rich repeat (LRR) protein. Based on subsequent phenotypic analysis, *CG32372* was re-named *larval translucida* (*ltl*).

larval translucida is a target of BMP signaling

As expected for a BMP-responsive target in the wing imaginal disc, in situ hybridization revealed *ltl* mRNA expression in an asymmetric gradient domain, centered on the row of DPP-secreting cells along the AP compartment boundary of the blade primordium (Fig. 1B). Although BMP signaling is essential for development of all imaginal discs, spatially localized *ltl* gene expression was not detected in leg or eye discs (data not shown), indicating that patterned expression of *ltl* is subject to wing-specific control. Confirming these results, antibodies raised against LTL showed a similar distribution of intracellular LTL protein centered on the AP compartment boundary of the wing disc, together with a broad apical extracellular signal in the disc lumen (Fig. 1C,D).

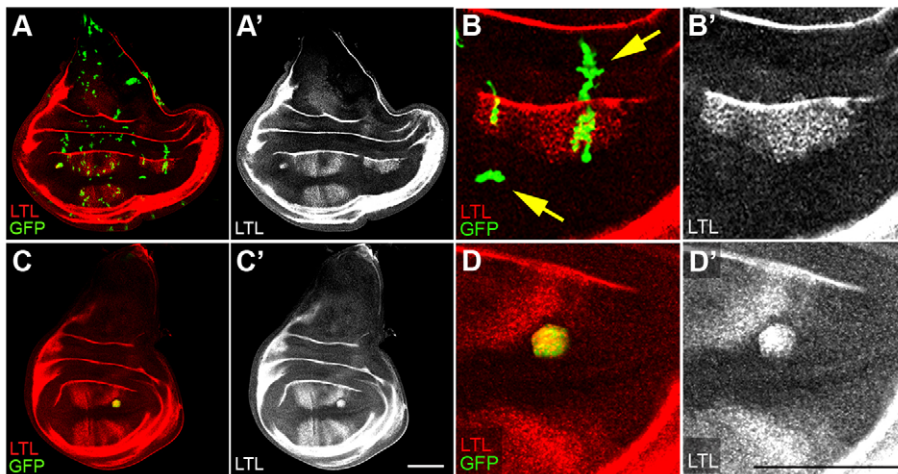


Fig. 2. Non-autonomous regulation of *ltl* expression by DPP. (A,A') Clones expressing *UAS-dpp* and GFP (green) cause imaginal disc overgrowth and activate LTL nonautonomously. (B,B') Detail of the disc in A. DPP does not induce LTL outside the wing pouch or in the wing margin territory (yellow arrows). (C,C') GFP-marked clones expressing *UAS-tkv^{QD}* exhibit cell autonomous LTL activation. (D,D') Detail of the disc in C. Scale bars: 50 μ m.

We next tested the requirement for BMP signaling in LTL activation. Extruded *tkv^{vir}* mutant clones, lacking BMP activity, exhibited a clear cell-autonomous reduction in LTL protein and mRNA levels (Fig. 1E,E'; in situ data not shown). These experiments indicate that in the wing, BMP signaling is essential for *ltl* expression, which could reflect either indirect or direct transcriptional activation. Consistent with the latter, the spatial pattern of LTL was precisely centered on the expression domain of DPP itself (Fig. 1F). Further, phylogenetic analysis of related *Drosophila* species indicates that the *ltl* region contains numerous highly conserved consensus binding sites for the cytosolic transducer MAD as well as the DPP target-repressor BRK (see Fig. S2 in the supplementary material). Among these was one highly conserved promoter sequence matching the recently reported DPP activation element motif (AE) (Weiss et al., 2010) (data not shown). Indeed, we confirmed that the *ltl* expression patterns in *D. erecta*, *D. simulans*, *D. willistoni* and *D. yakuba* wing discs were nearly identical to that observed in *D. melanogaster* (see Fig. S3 in the supplementary material).

We next tested whether DPP was sufficient to induce *ltl*. Small GFP-marked cell clones expressing *UAS-dpp* were generated using the *flp-out* technique (Pignoni and Zipursky, 1997; Struhl and Basler, 1993). Along with the expected overgrowth, ectopic DPP induced large patches of LTL expression over a distance of several cell diameters from the source clone (Fig. 2A,B). Arguing against indirect induction, clones expressing a constitutively active TKV receptor (*UAS-tkv^{QD}*) (Nellen et al., 1996) showed strictly cell-autonomous LTL expression (Fig. 2C,D). Notably, the capacity of either *UAS-dpp* or *UAS-tkv^{QD}* to induce LTL expression was limited to specific regions of the wing primordium. DPP-expressing clones in the hinge and margin territories, for example, did not activate *ltl*, revealing a complex regulation of the LTL spatial pattern by multiple patterning inputs (Fig. 2B,B'). Even broad overexpression of DPP in the dorsal compartment (with *Ap-Gal4 > UAS-dpp*) induced LTL throughout the wing blade, but not in the hinge, margin or AP boundary regions (see Fig. S4 in the supplementary material). In sum, these experiments demonstrate that BMP signaling is both necessary and sufficient to induce *ltl* expression in the blade primordium of the developing wing disc.

Generation and molecular characterization of *ltl* alleles

The only identifiable protein domains within LTL are 13 LRRs (Fig. 3A). Despite its extracellular localization, we did not identify a predicted signal peptide. To analyze LTL function, we performed an

EMS mutagenesis and isolated a complementation group of four alleles that also failed to complement a deficiency covering *ltl* (Fig. 3B). Two early larval lethal alleles encoded premature stop codons (*ltl¹*, *G15** and *ltl²*, *G455**), while two weaker alleles carried predicted missense mutations (*ltl³*, *L72F* and *ltl⁴*, *D704N*; Fig. 3C). Western blots showed elimination of LTL protein in the putative null alleles and reduction of LTL levels in the presumptive hypomorphs (Fig. 3D). Additional alleles were obtained for which we could not define the lesion, including a putative protein null, *ltl^N* (Fig. 3D). The most striking phenotype, observed in transheterozygous allele combinations (such as *ltl²/ltl³*), was a pronounced bloating of mutant larvae, resulting in pupal lethality (Fig. 3E; for phenotypes of all allele combinations, see Table S1 in the supplementary material). Furthermore, when raised under optimal conditions, ~36% of *ltl¹* and *ltl²* homozygotes survived to the third instar and exhibited the same phenotype (data not shown). The translucent, edemic appearance of mutant animals was not due to overgrowth, as it was readily rescued by allowing the hemolymph to drain through a small incision in the larval cuticle (see Movie 1 in the supplementary material). As this was the first phenotype to emerge from our screen, we re-named *CG32372 larval translucida*.

LTL controls both wing size and vein patterning

Consistent with a role for *ltl* in wing development, *ltl¹/ltl⁴* or *ltl²/ltl⁴* transheterozygotes survived to adulthood and exhibited wing phenotypes. These defects included subtle modifications of wing size and shape, along with ectopic vein material associated with the second longitudinal vein (L2) and the ACV and PCV structures (Fig. 3F,G). Intriguingly, both wing growth and vein patterning are BMP-dependent processes (Affolter and Basler, 2007; De Celis, 2003), suggesting that *ltl* could encode a feedback regulator of BMP signaling.

To circumvent larval lethality of the null alleles and eliminate *ltl* function in the wing, we recombined *ltl²* onto the FRT80B chromosome and induced mutant cell clones using the *flp/FRT* system (Lee and Luo, 1999; Xu and Rubin, 1993). To overcome the possibility of non-autonomous rescue of mutant clones by neighboring wild-type cells, we generated wing discs devoid of *ltl* expression using the Minute technique (Morata and Ripoll, 1975). Following induction of GFP-positive mutant cell clones in a slow-growing Minute background, the majority of wing discs exhibited complete or nearly complete elimination of heterozygous cells, as well as LTL expression (see Fig. S5 in the supplementary material). The resulting wings showed consistent reduction in size, variable

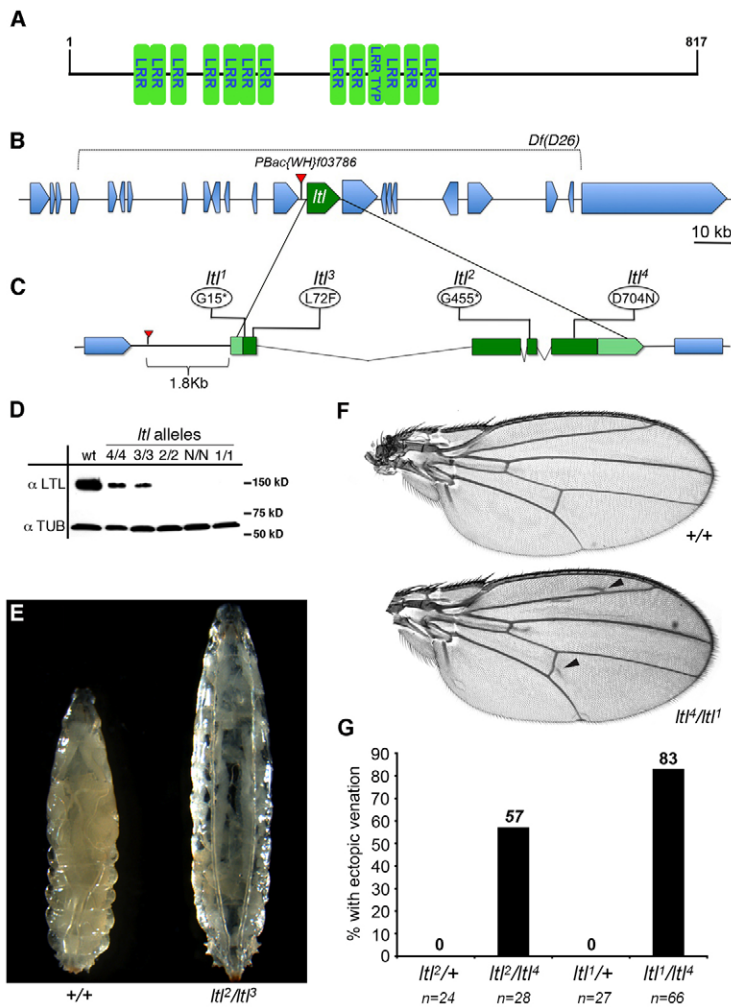


Fig. 3. Molecular and phenotypic characterization of *Itl* alleles. (A) Thirteen LRR repeats are located along the LTL protein sequence. (B) Map of the *Itl* genomic region showing the *PBac(WH)103786* insertion and the approximate breakpoints of *Df(D26)*. (C) Detailed map of sequenced mutations in the *Itl*-coding region, along with predicted amino acid changes. (D) Western blot showing reduction of LTL protein levels in homozygous embryos of the hypomorphic alleles *Itl3* and *Itl4*, and elimination of LTL protein in embryos homozygous for *Itl1*, *Itl2* and *ItlN*. (E) The inflated larval *translucida* phenotype of *Itl2/Itl3* (right) compared with a wild-type control animal (left). (F) Transheterozygous combinations of *Itl4* and *Itl1* or *Itl2* results in ectopic vein formation adjacent to LV2, the ACV and the PCV (arrowheads). (G) The ectopic venation phenotype is highly penetrant in wings from animals with different transheterozygous allele combinations (*Itl2/Itl4* and *Itl1/Itl4*).

effects on shape, and highly penetrant defects in the patterning of LV2 and the PCV (Fig. 4A,B). We quantified the growth defects by measuring wing area in the *FRT80B Itl2* mutants compared with a *FRT80B* control, and observed variable size reductions averaging 13% of total wing area, with extreme cases showing up to 25% reduction (Fig. 4C). Similar effects were observed for the independent alleles *Itl1* and *ItlN* (data not shown). Together, these experiments indicate that elimination of LTL produces patterning defects in the PCV and LV2, as well as variable defects in wing size regulation.

Ectopic LTL antagonizes both wing growth and crossvein patterning

Although the experiments above suggest a role for *Itl* in promoting wing growth, the ectopic venation phenotypes could suggest a more specific function in the inhibition of BMP activity. Because removing a DPP/BMP inhibitor would be predicted to increase growth, these paradoxical results raise two possibilities: (1) that the growth-promoting and PCV-patterning functions of LTL are molecularly distinct activities; or (2) that LTL could both promote and inhibit DPP/BMP signaling in a stage-, context- or concentration-dependent manner.

To probe the effects of ectopic LTL on wing development, we generated transgenic flies carrying a Gal4-inducible *UAS-Itl* transgene, *P{UAS-Itl}5*. Under control of the ubiquitous wing blade driver *A9-Gal4*, ectopic *Itl* expression resulted in an ~20%

reduction in wing size compared with controls. Extreme cases exhibited wing area reductions of up to 32% (Fig. 4D-F). This effect on wing size was coupled with total elimination of the PCV, and consistent elimination or disruption of the ACV. These findings indicate that LTL antagonizes growth and crossvein patterning. In order to test whether LTL inhibits wing growth by modulating BMP signaling, we quantified PMAD signal intensity in *A9-Gal4>UAS-Itl5* discs compared with controls (Fig. 4G,H). In the presence of ectopic LTL, PMAD signal intensity was reduced by an average of 12% (Fig. 4I). Although we cannot rule out simultaneous effects of LTL on other pathways, these findings suggest that ectopic LTL flattens peak BMP signaling levels without affecting the overall shape of the activity gradient.

LTL antagonizes BMP signaling during PCV development

During pupal development, patterning of the PCV requires precise extracellular control over BMP ligand movement and is therefore highly sensitive to perturbations in signaling activity (Ralston and Blair, 2005). We took advantage of this to test the effect of LTL on BMP signal transduction. In pupal wings, the BMP ligands DPP and GBB are expressed in the presumptive vein territories (Wharton et al., 1999; Yu et al., 2000), where high levels of PMAD are also observed (Fig. 5A). Consistent with transcriptional activation of *Itl* by DPP/BMP signaling during pupal wing development, we observed expression both *Itl* mRNA and protein

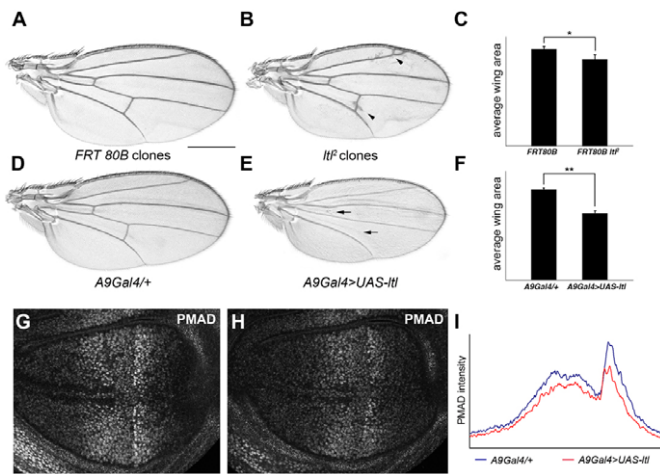


Fig. 4. LTL functions in both wing growth and vein patterning. (A) MARCM/Minute wing with FRT80B control clones. (B) Elimination of LTL in *Itl²* clones reduced wing size and caused ectopic vein formation at LV2 and the PCV (arrowheads). (C) The mean size reduction in wings bearing large *Itl²/Itl²* mutant clones was variable but averaged 13% (* $P < 0.0005$, $n = 20$ wings). (D, E) Overexpression of LTL in the wing disc using *A9Gal4* results in adult wing size reduction and elimination of both crossveins (arrows) compared with controls (*A9Gal4/+*). (F) Quantification of the 20% size reduction in *A9Gal4>UAS-Itl* compared with controls (** $P < 0.0001$, $n = 20$ wings). Results are mean \pm s.d. (G) PMAD staining in an *A9Gal4/+* control disc. (H) PMAD staining in a wing disc from *A9Gal4>UAS-Itl* imaged under identical conditions. (I) PMAD intensity profile plots show reduced BMP/DPP activity in the LTL overexpressing discs ($n = 20$ discs). Scale bar: 400 μ m.

centered on the developing LV and CV territories (Fig. 5B,C). Furthermore, LTL protein exhibits what appears to be a broad extracellular distribution throughout the interior of the pupal wing blade (Fig. 5C).

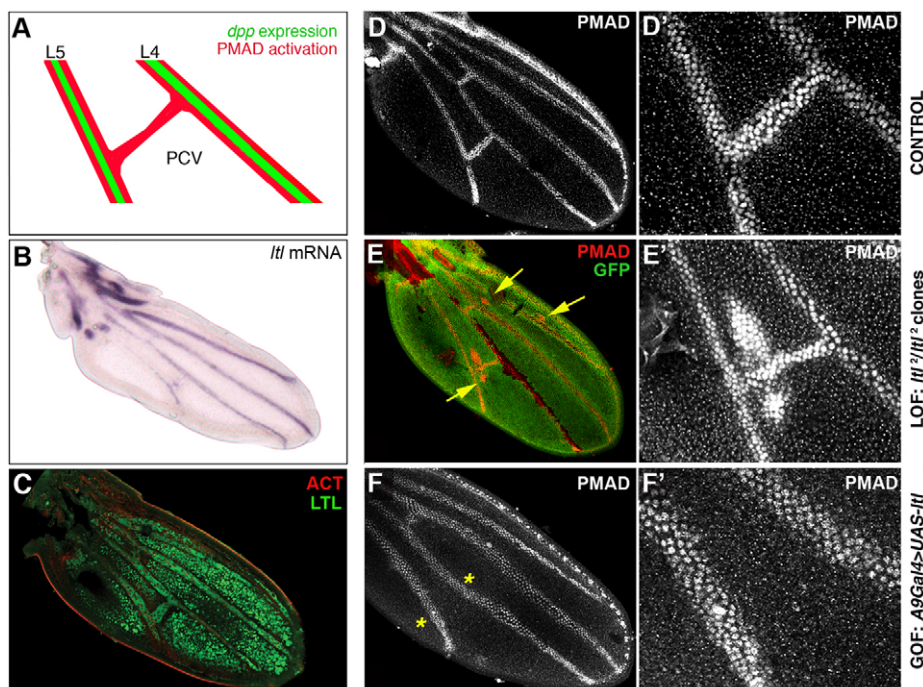


Fig. 5. LTL modulates BMP signaling at or above the level of MAD phosphorylation. (A) During pupal wing development, *dpp* is expressed along the longitudinal veins (green); DPP ligand is then transported into the PCV region where it activates PMAD expression (red). (B) *Itl* mRNA is expressed in the developing pupal vein territories, with weaker expression visible in the presumptive crossveins. (C) LTL protein is intracellularly enriched in the presumptive vein territories, but is also distributed throughout the extracellular space between the basal surfaces of the wing epithelium. (D) Control PMAD staining on wild-type pupal wing marks the forming longitudinal and crossveins. (D') Detail of PMAD staining in the presumptive PCV of a control wing. (E, E') Elimination of LTL in large homozygous *Itl²* MARCM clones (GFP+) induces ectopic PMAD staining along LV2, LV3 and the PCV (yellow arrows). (E') PMAD staining in the presumptive PCV territory. (F, F') Expression of UAS-*Itl* with *A9Gal4* abolishes PMAD signal in the presumptive PCV territory (**).

Because loss of LTL caused excessive PCV formation (Fig. 4B) and ectopic LTL eliminated both crossvein structures (Fig. 4E), we next asked whether perturbations of *Itl* activity directly modulate PMAD levels in the crossvein primordia. We eliminated *Itl* by generating large GFP-labeled homozygous *Itl²* mutant clones in a Minute background. Under these conditions, little or no LTL protein was detected (data not shown), and strong ectopic PMAD staining was consistently observed associated with the PCV, along with sporadic ectopic PMAD in the region the ACV and LV2 (Fig. 5D-E'). This demonstrates that *Itl* normally restricts BMP signaling in these regions. Conversely, ectopic expression of *Itl* under the control of *A9Gal4* eliminated PMAD activation in the presumptive PCV (Fig. 5F,F'). Taken together, these results indicate that in the pupal wing, LTL antagonizes BMP signaling at or above the level of MAD phosphorylation. This would suggest either an intracellular function at the level of receptor/MAD interaction, or an extracellular function at the level of ligand/receptor interaction, not unlike several of the known DPP/BMP modulators (Umulis et al., 2009).

Extracellular localization of LTL

To discern autonomous versus non-autonomous function of LTL, we used *ptc-Gal4* to drive ectopic LTL in a narrow stripe of anterior cells at the A/P boundary of the wing disc. Under these conditions, overall wing area was reduced. Furthermore, the PCV was non-autonomously eliminated in all progeny (see Fig. S6 in the supplementary material). Similar effects on overall wing size were obtained with other spatially restricted drivers such as *Ap-* and *hh-Gal4* (data not shown). These results are most consistent with a non-autonomous activity of LTL, and we therefore examined the tissue-level distribution of LTL protein in greater detail.

Immunostaining of fixed wing discs revealed an intense extracellular distribution of LTL in the apical lumen between the apposed peripodial and columnar epithelia (Fig. 1D). This luminal signal was widely dispersed beyond sites of *Itl* expression. Consistent with specificity of the antibody for LTL protein, luminal staining was eliminated in *Itl²* homozygous mutant discs

(see Fig. S5 in the supplementary material). In confocal XZ sections through the entire wing blade primordium, LTL showed a punctate distribution within cell bodies of the columnar epithelium. However, the most intense signal was detected in the apical lumen (see Fig. S7 in the supplementary material). Intriguingly, one of the few proteins proposed to localize to the wing disc lumen is DPP itself (Gibson et al., 2002), although experiments that specifically address whether luminal DPP can signal to the columnar epithelial cells are lacking. Still, as extracellular LTL was also observed in the pupal wing (Fig. 5C), we conclude that LTL is secreted and most probably functions in the extracellular environment, either at the cell surface or in the extracellular space.

Secreted LTL::GFP disrupts PCV development

To confirm the immunolocalization of LTL, we also examined the distribution of LTL::GFP fusion proteins. Transgenic lines carrying Gal4/UAS-inducible C- and N-terminal fusions were generated and crossed to *hh-Gal4*, driving either *UAS-ltl::gfp-C* or *UAS-N-gfp::ltl* expression solely in the disc posterior compartment. Wing discs from *w; hh-Gal4/UAS-ltl::gfp-C* larvae exhibited an extracellular distribution of LTL::GFP-C indistinguishable from endogenous LTL, with the intensity of luminal fluorescence exceeding that of the intracellular fraction (Fig. 6A). By contrast, the N-terminal fusion product (N-GFP::LTL) was expressed but was not detectably secreted (Fig. 6B). Intracellularly, the C-terminal fusion (LTL::GFP-C) exhibited punctate cytoplasmic localization in the disc posterior compartment, much like endogenous LTL (Fig. 6C). By contrast, the N-GFP::LTL fluorescence showed a diffuse cytoplasmic pattern (Fig. 6D), indicating that the N-terminal fusion protein was translated but either improperly trafficked or misfolded and thus never secreted. Under the control of *hh-Gal4*, secreted LTL::GFP-C eliminated PCV formation and inhibited wing growth in a manner similar to unmodified LTL (Fig. 6E), but non-secreted N-GFP::LTL had no visible effect on wing development (Fig. 6F). These experiments not only confirm the localization of LTL to the wing disc lumen, but also strongly suggest that proper processing and secretion of LTL is essential for its ability to regulate BMP signaling.

LTL interacts with Cv-2 and the glypican DLP

To better define the mechanism by which LTL modulates BMP signaling, we used both gain- and loss-of-function experiments to screen for interactions between *ltl* and known pathway components. This approach identified a synergistic genetic interaction between LTL and Cv-2, a BMP-binding protein with a central role in crossvein patterning (Ralston and Blair, 2005; Serpe et al., 2008). Surprisingly, weak ectopic LTL was sufficient to rescue the phenotype of *cv-2* mutants. Animals homozygous for *cv-2¹*, a hypomorphic allele, were 100% posterior crossveinless under standard conditions (Fig. 7A). However, in flies carrying two copies of the leaky transgenic construct *P{UAS-ltl}⁵*, 45% of wings showed a fully restored PCV (Fig. 7B). This rescue was dose-dependent: one copy of the *UAS-ltl⁵* transgene fully restored the PCV in only 6% of wings (Fig. 7C,D). As additional evidence for genetic interactions, the loss of *cv-2* enhanced viable *ltl* transheterozygotes to pupal lethality (*cv-2¹/cv-2¹; ltl²/ltl⁴*; data not shown). This indicates that *ltl* and *cv-2* may be partially redundant in some developmental processes. Consistent with the possibility that LTL and Cv-2 perform similar functions, overexpression of each protein alone (under the control of *en-Gal4*) resulted in reduction of total wing area and loss of the PCV (Fig. 7E). Co-

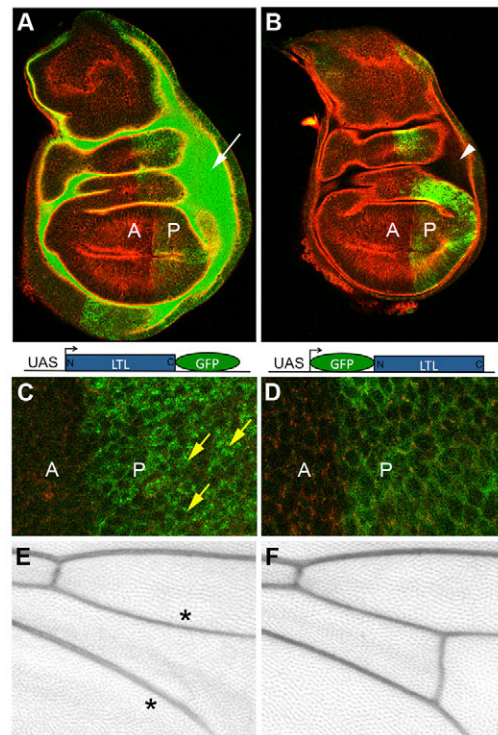


Fig. 6. Secretion-dependent activity of LTL::GFP fusion proteins. (A) *hh-Gal4*-mediated expression of *UAS-ltl::gfp-C* in the wing disc posterior compartment results in broad GFP signal in the lumen (arrow) throughout the wing disc (anterior, A; posterior, P). (B) By contrast, N-terminally tagged LTL is not secreted (arrowhead). (C) Intracellularly, LTL::GFP-C is localized in granular, punctate structures (yellow arrows). (D) By contrast, non-secreted N-GFP::LTL is diffusely distributed throughout the cytoplasm. (E) Adult wing from *hh-Gal4>UAS-ltl::gfp-C*, showing that the fusion protein retains biological activity inhibits PCV formation (**). (F) *hh-Gal4>UAS-N-gfp::ltl* adult wing. The non-secreted N-GFP::LTL fusion has no detectable biological activity.

overexpression of both proteins led to additive effects on wing size reduction and synergistic effects on patterning, including deletions of LV4 in the proximity of the presumptive PCV (Fig. 7E-G).

During pupal wing development, Cv-2 is proposed to bind both DPP and the glypican Dally (Serpe et al., 2008). Several other morphogens are known to interact with Dally and the related protein DLP in the extracellular space, including Wingless (Yan et al., 2009; Lin and Perrimon, 1999) and Hedgehog (Desbordes and Sanson, 2003; Gallet et al., 2008). We therefore used co-overexpression assays to test for functional interactions between LTL and DLP. Driving weak ectopic LTL with *A9Gal4>UAS-ltl^{HA-2XFLAG}* resulted in an ~14% reduction of wing size (Fig. 8A), whereas overexpression of DLP alone using *A9Gal4>UAS-dlp* (Giraldez et al., 2002) caused a slight increase of size (Fig. 8B). By contrast, simultaneous expression of both proteins disrupted wing morphogenesis and strongly enhanced the size reduction to ~42% of controls (Fig. 8C,D). These findings suggest that LTL could modulate BMP signaling through interactions with both Cv-2 and DLP. Consistent with this, we performed co-immunoprecipitation experiments and identified a reciprocal association between endogenous LTL and endogenous DLP in vivo (Fig. 8E; see Fig. S8 in the supplementary material). We could not detect similar interactions between LTL and Cv-2 or DPP itself (data not shown).

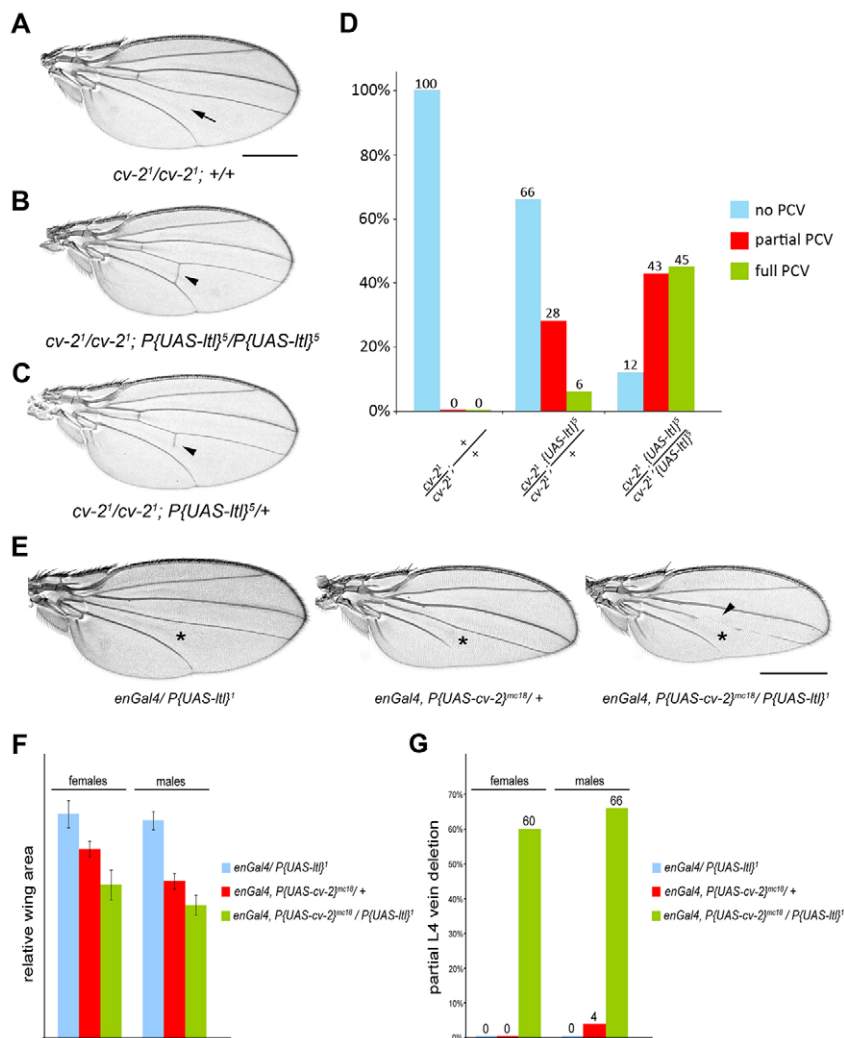


Fig. 7. *ltl* genetically interacts with *cv-2*. (A) *cv-2¹* homozygous wings lack the PCV (arrow). Scale bar: 400 μ m. (B) Introducing two copies of a leaky *UAS-*ltl*⁵* transgene fully rescued the PCV in 45% of *cv-2¹* homozygotes and partially in 43% (arrowhead). (C) A single copy of *UAS-*ltl*⁵* fully rescued the PCV in 6% of *cv-2¹* homozygotes, and partial rescue was detected in 28% (arrowhead). (D) Quantification of PCV rescue indicating the mean results ($n=100$ male wings in all cases). (E) Overexpression of either *UAS-*ltl*¹* or *UAS-*cv-2** using *enGal4* results in wing size reduction and loss of the PCV (asterisks). Co-overexpression of both proteins results in stronger wing size reduction, loss of the PCV and deletions in LV4, mostly in the proximity of the PCV territory (arrowhead). Scale bar: 500 μ m. (F) Quantification of the size reduction of each genotype in both sexes. Data represent the mean relative wing area; error bars indicate s.d. ($n=50$ wings). (G) Quantification of the phenotypes described in E, from both sexes for each genotype ($n=50$ wings).

Based on these findings, we propose that LTL is secreted into the extracellular space where it physically associates with glypicans and acts to modulate BMP signaling through functional interactions with Cv-2 and perhaps additional factors. Importantly, the physical association of LTL with DLP also suggests an avenue by which LTL could modulate additional signaling pathways.

DISCUSSION

Regulation of DPP/BMP signaling can occur at the level of ligand processing and activation (Kunnapuu et al., 2009), ligand homo- and heterodimerization (Aono et al., 1995; Bangi and Wharton, 2006), spatial control of receptor expression levels (Funakoshi et al., 2001) and modulation of intracellular signal transduction (Tsuneizumi et al., 1997). An additional level of regulation plays out in the extracellular space and is mediated by secreted proteins that promote or inhibit ligand movement or sequester BMPs from their receptors (Vuilleumier et al., 2010; Umulis et al., 2009). In this study, we used microarray analysis of mutant cell clones to assess the transcriptional response to BMP signaling in the wing imaginal disc. This approach identified the novel DPP/BMP target *ltl*, which encodes an LRR protein we propose to be an extracellular feedback regulator of BMP signaling. In the larval wing blade primordium, DPP/BMP signaling is both necessary and sufficient for *ltl* expression (Figs 1 and 2). In the pupal wing, *ltl*

expression also closely mirrors the pattern of DPP/BMP activity. Elimination of LTL from developing wing primordia caused a reduction of overall wing size and resulted in ectopic venation attributable to localized increases of DPP/BMP activity at the level of MAD phosphorylation (Figs 3-5). Overexpression of LTL caused a reduction of peak PMAD intensity in the larval disc and a reduction of wing size in adults. During pupal stages, LTL overexpression caused a complete loss of PMAD activity in cells of the presumptive PCV (Fig. 5F). Combined, our gain- and loss-of-function results suggest that LTL can modulate BMP activity in a stage-, concentration- or context-dependent manner.

An important, but still enigmatic, aspect of this study was the intense localization of both LTL protein and LTL::GFP-C to the apical lumen of wing imaginal discs (Fig. 1D; Fig. 6A). Despite a report of nearly identical DPP localization (Gibson et al., 2002), the protein composition of the lumen and the role of apical/luminal proteins in imaginal disc development remain unclear. Studies by Belenkaya et al. (Belenkaya et al., 2004) and Han et al. (Han et al., 2004) advocate models of facilitated extracellular DPP and HH morphogen movement by the glypicans Dally and DLP. Precisely where this movement occurs along the apical-basal axis of the epithelium is less clear, but recent evidence indicates that apical secretion is a crucial component of DLP function, and that DLP may govern the uptake of both WG and HH ligands at the apical

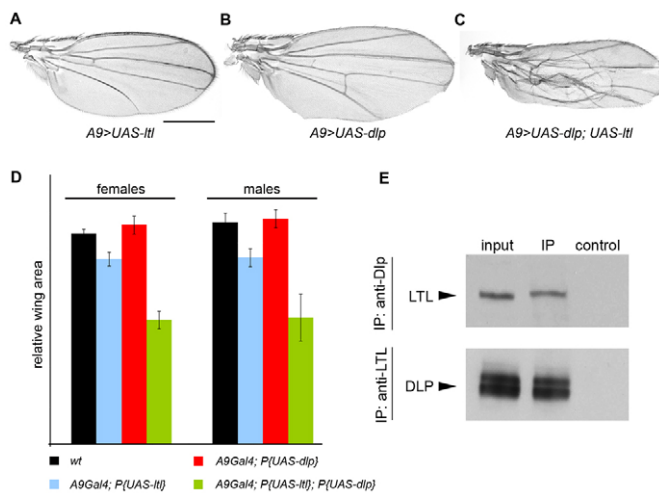


Fig. 8. Genetic and biochemical interactions between LTL and DLP. (A) Overexpression of *UAS-Itl*^{HA-2XFLAG} using *A9Gal4* results in ~14% wing size reduction and loss of the PCV. (B) Overexpression of DLP using the same driver results in a slight increase of wing size along with chipping of the wing margin. (C) Co-overexpression of LTL and DLP results in a strong size reduction of 42% on average. Scale bar: 400 μ m. (D) Quantification of the size effects of the different genotypes in both sexes. Data represent the mean relative wing area; error bars indicate s.d. ($n=20$ wings for each). (E) Endogenous LTL co-immunoprecipitates with endogenous DLP from wing disc protein extracts. The reciprocal interaction was also detected, although neither protein bound AVG plus beads non-specifically in controls.

epithelial surface (Gallet et al., 2008). The findings presented here suggest that LTL activity requires secretion (Fig. 6), and provide evidence that LTL forms a complex with DLP in vivo (Fig. 8E). Interestingly, two vertebrate chondroitin sulfate LRR proteins, Biglycan (Moreno et al., 2005) and Tsukushi (Ohta et al., 2004), form an extracellular inhibitory complex with BMP4 and Chordin, the vertebrate homologs of DPP and SOG. The general function of extracellular LRR proteins thus remains an important avenue for future experiments.

Although we have been able to define a function for LTL in BMP antagonism in the presumptive crossveins, LTL exhibits a paradoxical role with respect to growth. Here, both increasing and decreasing LTL levels reduced the size of the wing (Fig. 4). It is not yet clear whether these effects result from the role of LTL in BMP modulation, or perhaps effects on other growth-regulatory processes. Indeed, if LTL interacts with HSPGs, it is conceivable that multiple signaling pathways are affected. In the case of BMP signaling, one possibility is that gain and loss of *Itl* both flatten the activity gradient in the larval disc. However, we did not detect obvious effects on the PMAD activity gradient in wing discs from *Itl*² homozygous escapers (data not shown). A second formal possibility is that the growth defects in *Itl* mutant wings result from a disruption of pupal development, when BMPs are not generally thought to regulate growth. A third and final alternative is suggested by recent work on the biphasic DPP modulator Cv-2. Extracellular Cv-2 binds to DPP and facilitates receptor-ligand interaction, and elimination of Cv-2 inhibits signaling. At the same time, overexpression of Cv-2 protein is proposed to bind DPP in excess, resulting in elimination of the PCV (Serpe et al., 2008), and also causing wing size reduction (Fig. 7E). As Cv-2 and LTL have at least partially redundant functionality (Fig. 7), endogenous levels

of LTL could similarly facilitate movement or activity of BMPs, whereas excessive levels of LTL could oversaturate the system and cause an inhibitory effect. In this sense, both gain- and loss-of-function manipulations would produce the same result: a smaller wing. Future experiments should distinguish between these possibilities.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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