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Crossveinless d is a vitellogenin-like lipoprotein that binds BMPs and HSPGs, and is required for normal BMP signaling in the *Drosophila* wing

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SUMMARY

The sensitivity of the posterior crossvein in the pupal wing of *Drosophila* to reductions in the levels and range of BMP signaling has been used to isolate and characterize novel regulators of this pathway. We show here that *crossveinless d* (*cv-d*) mutations, which disrupt BMP signaling during the development of the posterior crossvein, mutate a lipoprotein that is similar to the vitellogenins that comprise the major constituents of yolk in animal embryos. Cv-d is made in the liver-like fat body and other tissues, and can diffuse into the pupal wing via the hemolymph. Cv-d binds to the BMPs Dpp and Gbb through its Vg domain, and to heparan sulfate proteoglycans, which are well-known for their role in BMP movement and accumulation in the wing. Cv-d acts over a long range in vivo, and does not have BMP co-receptor-like activity in vitro. We suggest that, instead, it affects the range of BMP movement in the pupal wing, probably as part of a lipid-BMP-lipoprotein complex, similar to the role proposed for the apolipophorin lipid transport proteins in Hedgehog and Wnt movement.

KEY WORDS: BMP, Dpp, Gbb, Lipoprotein, Vitellogenin, Wing venation

INTRODUCTION

The localized BMP signaling that initiates the development of the posterior cross vein (PCV) from the epithelia of the *Drosophila melanogaster* pupal wing depends in large part on the BMPs Decapentaplegic (Dpp) and Glass bottom boat (Gbb), which are secreted from the earlier arising longitudinal veins (LVs) (Conley et al., 2000; Ray and Wharton, 2001; Ralston and Blair, 2005). Although it is not known why signaling is heightened specifically in the PCV region, the sensitivity of PCV development to reductions in the range or levels of BMP signaling has allowed the isolation and characterization of several regulators of BMP signaling. Loss of the secreted BMP-binding proteins Short gastrulation (Sog), Crossveinless-Twisted gastrulation 2 (Cv-Tsg2), Tolloid-related (Tlr; Tok – FlyBase) or Crossveinless 2 (Cv-2, also known as BMPER in vertebrates) reduces BMP signaling in the developing PCV: Sog and Cv-Tsg2 are thought to increase the movement of BMPs from the LVs to the PCV, Tlr to release BMPs from Sog, and Cv-2 to increase the transfer of BMPs to their receptors (Conley et al., 2000; Serpe et al., 2005; Shimmi et al., 2005a; Vilmos et al., 2005; Serpe et al., 2008). The LVs are less sensitive to these factors largely because they are specified much earlier in development by other pathways.

We here use the sensitivity of the PCV to identify and characterize the gene mutated by *crossveinless d* (*cv-d*), first isolated by Bridges (Bridges, 1935). We found the *cv-d* encodes a vitellogenin-like lipoprotein, similar to the vitellogenins that comprise the major constituents of yolk in animal embryos. Cv-d binds BMPs and heparan sulfate proteoglycans (HSPGs) and increases BMP signaling in the PCV, probably by increasing the range of BMP signaling.

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MATERIALS AND METHODS

Stocks

Stocks were from the Bloomington *Drosophila* Stock Center, except *UAS-cv-d-RNAi* (from NIG-Fly and VDRC), *hml-gal4* (from Utpal Banerjee, UCLA, USA), and FRT^{42D} $botv^{103}$/*CyO* (Han et al., 2004b) and FRT^{2A} $dally^{80}$ dlp^{A187}/*TM6,Tb* (Han et al., 2004a) from Xinhua Lin (Cincinnati Children's Hospital, OH, USA). New *cv-d* alleles were generated using EMS and screening over $cv\text{-}d^{1}$. Clones were generated using FRTs and either *hs-Flpase*, *en-gal4 UAS-flpase* or *hh-gal4 UAS-flpase*.

Constructs

Cv-d constructs, which are tagged at the C-termini with V5, were generated from RE08719 and RE25382 from the Bloomington *Drosophila* Genome Project. RT-PCR used primers TTATGCAACTACAACTACATCGGAGG and TCCTCCGCTCCGACAAGATCTCGC.

Histology

In situ hybridization and antibody staining were as previously described (Ralston and Blair, 2005; Shimmi et al., 2005a), using rabbit anti-V5 (Bethyl, 1:1000), rabbit anti-Smad3-phospho (Epitomics, 1:2000), mouse anti-DSRF (Active Motif, 1:1000) and rat anti-Cv-d preabsorbed on $cv\text{-}d^{13}$ embryos (1:50-100). Cv-d antiserum was generated by Panigen from a purified, bacterially expressed His tagged fragment (amino acids 43-385).

In vitro analyses

Co-immunoprecipitation from S2 cell supernatant was as described previously (Serpe et al., 2008) using anti-FLAG (Sigma) or anti-V5 (Bethyl) agarose beads. BMP signaling experiments in S2 cells (Kirkpatrick et al., 2001; Gao et al., 2005) were performed with the *p3xUbx-lacZ* reporter, which contains three repeats of TCTTTCT*GGACTGGCG*TCA*GCGCCGGCGC*TCT (binding sites for *Mad* in italics and *Mad/Medea* underlined) 5′ of *lacZ*. A batch of S2 cells was subdivided equally into non-coated 24 well plates at ~10^6 cells in 0.5 ml per well, and 200 ng total of DNA for each condition (Table 1) was transfected using DDAB; transfections were performed in duplicate or

Table 1. Transfected DNA for in vitro signaling assays

Transfected DNA	Amount (ng) used (Fig. 5) 5A,C	5D	5B	5E
p3xUbx-lacZ	30	30	30	30
pPAc-Luciferase	5	5	3.3^x or 5^y	5
pAc-mad-flag	5	5	3.3^x or 5^y	5
pAwgal4	25	25	25	25
pUAS-cv-d-V5 (full or Δ) or *pUAST*	60	60	20+40,40+20 or 60+0	60
pAc-tkvQD or *pPAc*	30	–	30	30
pUAST	35	65	48.3^x or 45^y	45
pRM-sog or *pUAST*	10	10		

triplicate. Two days after transfection, chemiluminescent β-galactosidase (Galacto-Star, Applied Biosystems) and Luciferase (Steady-Glo, Promega) assays were performed, normalizing the β-galactosidase activity to luciferase activity to correct for transfection efficiency. For experiments A, C and D, all cells were treated with $CuSO_4$ after transfection because *pRM-sog* uses a metallothionein promoter. For experiment C, control cells from A were exposed 48 hours after transfection to Sog-conditioned or Cv-d-conditioned medium (taken 48 hours after transfection from the *cv-d*-expressing or *sog*-expressing cells used for assay in A) for 4 hours. For experiment D, cells were exposed 24 hours after transfection to 4 nM recombinant Dpp (R&D Systems) for an additional 24 hours. For experiments B and E, data from different batches of cells were combined by first normalizing values to the average negative control value for that batch. For experiment B, two batches of cells were run under slightly different conditions (x and y in Table 1). *pAc-mad-flag* was used to improve the response to BMP signaling; in experiments lacking this, the *3xUbx-lacZ* reporter was also inhibited by co-transfected *cv-d* (data not shown).

RESULTS

cv-d^1 mutates *CG31150*

We mapped *cv-d*^{1} (Bridges, 1935) using deletions, followed by meiotic recombination relative to known P-element insertion sites, to place *cv-d*^{1} within a region containing five predicted genes (Fig. 1A,B). Sequencing *cv-d*^{1} DNA and mRNA revealed a genomic deletion and an mRNA splicing error in one candidate, *CG31150* (Fig. 1E). We used EMS to generate new *cv-d* alleles, 11 of which had mis-sense or nonsense mutations in the CG31150 coding region (Fig. 1E; supplementary material Fig. S1A). Expression of either of the two available *UAS-CG31150-RNAi* stocks using the strong, ubiquitously expressed *tubulin* promoter (*tub*)-*gal4* driver generated PCV-less adults (Fig. 1C; supplementary material Fig. S1C), and *tub-gal4*-driven expression of a UAS construct containing a V5-tagged version of CG31150 (*UAS-cv-d-V5*) rescued crossvein development in *cv-d* mutants (Fig. 1D; supplementary material Fig. S1D).

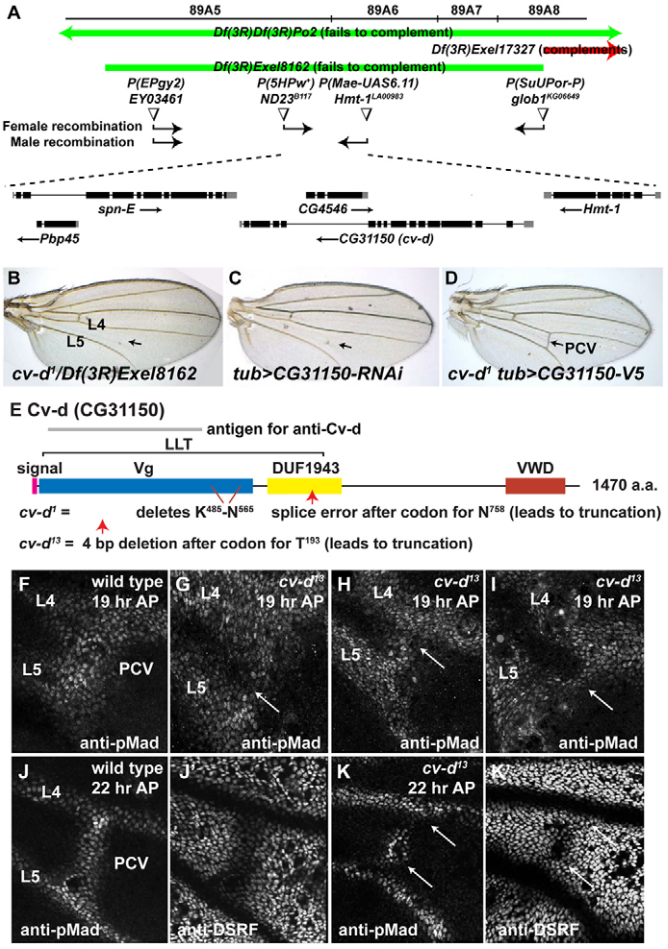

Fig. 1. *cv-d* mutants disrupt *CG31150* and reduce BMP signaling in the developing PCV. (**A**) Mapping of *cv-d*^{1} to five candidate genes, using molecularly defined Exel deletions (Parks et al., 2004), recombination relative to *w*$^+$ P element insertions in *w*; *cv-d*^{1} *Pr/P element* females and transposase-mediated recombination at P element insertions (Chen et al., 1998) in Δ2-3; *Gl cv-d*^{1} *Pr/P element* males. (**B**) Disruption of PCV (arrow) in *cv-d*^{1}*/Df(3R)Exel8162* adult wing, normally found between longitudinal veins 4 and 5 (L4 and L5). (**C**) Disruption of PCV (arrow) in *tub-gal4 UAS-CG31150-RNAi* (VDRC #3975) adult wing. (**D**) Rescue of PCV (arrow) in *cv-d*^{1} homozygote by *tub-gal4 UAS-CG31150-V5*. (**E**) Structure of Cv-d, and predicted alterations in *cv-d*^{1} and *cv-d*^{13} mutants (supplementary material Fig. S1A). (**F-K′**) Comparison of BMP signaling in PCV regions of 19 hour AP (F-I) and 22 hour AP (J-K′) wild type (F,J,J′) and *cv-d*^{13} (G-I,K,K′) wings. Anti-pMad staining is reduced and anti-DSRF staining heightened in regions of the *cv-d*^{13} PCV (arrows).

Cv-d is a vitellogenin-like protein

Cv-d is vitellogenin (Vtg)-like lipoprotein, similar to the Vtgs, which are major constituents of yolk in many animals (e.g. *C. elegans* Vit-6, BLAST score 3e-16; chick Vtg2, BLAST score 3e-16). Like other Vtg family members (Smolenaars et al., 2007a), Cv-d has an N-terminal Large Lipid Transfer (LLT) domain, containing the Vitellogenin N (Vg) and DUF1943 Pfam motifs, and, near the C terminus, a partial von Willebrand Factor D (VWD) domain (Fig. 1E).

This was especially surprising as insect yolk Vtgs are produced only in adult sexually mature females (Ziegler and Van Antwerpen, 2006), whereas Cv-d affects BMP signaling at pupal stages (see below) and in both sexes. However, Cv-d is not a direct homolog of the Vtgs that have been isolated from the yolks of insects. *Drosophila* lacks such a homolog; oocytes take up lipase-like yolk proteins using the *Drosophila* Vtg receptor Yolkless (Barnett et al., 1980; Schonbaum et al., 1995). Sequence comparisons indicate that Cv-d is instead part of a distinct family of Vtg-like lipoproteins that is conserved even in insects that also have yolk Vtgs (Smolenaars et al., 2007a) (supplementary material Fig. S1E). The only other members of this family characterized to date are the 160 kDa Melanization Enabling Protein of the beetle *Tenebrio molitor* (160MEP, also known as Melanization-related protein), which is found in the hemolymph of both sexes and enhances melanization reactions (Lee et al., 2000), and the 160 kDa Very High Density

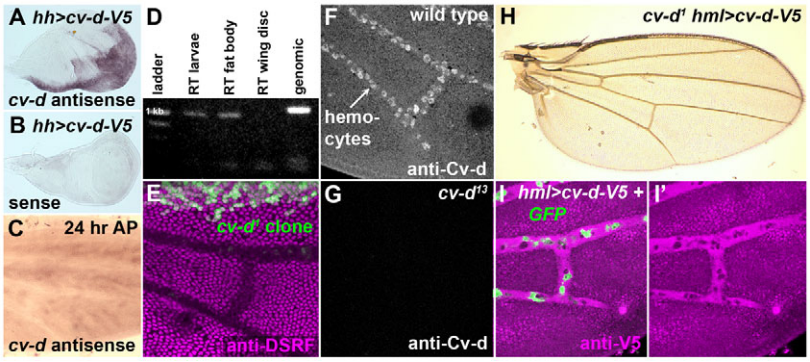

Fig. 2. Cv-d is made in fat body and can promote PCV development from outside the wing. (**A**,**B**) In situ hybridization using *cv-d* antisense (A) or sense (B) probes in wing imaginal discs overexpressing *UAS-cv-d-V5* in the posterior half with *hh-gal4*. Anterior staining with antisense probe (A) is as faint as staining with sense probe (B). (**C**) Overstained in situ using *cv-d* antisense probe in 24 hour AP pupal wing. (**D**) RT-PCR showing expression of *cv-d* mRNA in whole larvae and dissected fat body, but not in dissected wing imaginal discs. The PCR product from genomic DNA is approximately 100 bp larger. (**E**) Normal suppression of anti-DSRF staining (purple) in PCV of pupal wing with large posterior homozygous $cv\text{-}d^{1}$ clone (identified by the absence of green GFP). On the other surface of the wing (not shown) the clone encompassed the entire frame of the photograph. (**F**,**G**) Anti-Cv-d staining in wild type (F) and $cv\text{-}d^{13}$ (G) 24 hour AP wings. In wild type, the staining is ubiquitous and is higher in the hemocytes found in wing vein hemolymph; in $cv\text{-}d^{13}$, staining is absent. (**H**) Rescue of PCV in adult $cv\text{-}d^{1}$ wings after expression of *UAS-cv-d-V5* with *hml-gal4*. (**I**,**I'**) Spread of Cv-d-V5 staining (purple) from hemocytes (green) into pupal wing after *hml-gal4*-driven expression of *UAS-cv-d-V5* and *UAS-GFP*.

Lipoprotein (VHDL) found in the larval hemolymph of the honeybee *Apis mellifera* (Shipman et al., 1987). Unlike insect yolk Vtgs (Tufail and Takeda, 2008), members of the Cv-d/160MEP family are not cleaved into smaller fragments prior to secretion, either in vivo (Shipman et al., 1987; Lee et al., 2000) or in vitro (supplementary material Fig. S1B). The Cv-d/160MEP family is also distinct from insect apolipophorin and apolipophorin-related lipid transport proteins, despite sharing some domain structures (Smolenaars et al., 2007a) (supplementary material Fig. S1E).

cv-d mutants disrupt BMP signaling in the posterior crossvein

Although adult *cv-d* homozygotes are rare in balanced stocks, all of our *cv-d* alleles are homozygous viable, as are all of the *gal4 UAS-cv-d-RNAi* combinations we have tried, and produce fertile adults whose only visible external defects are disruption of the PCV and, occasionally, the distal tip of vein L5, and a slight variable reduction in wing size (0-9% linear reduction, average 7%) (supplementary material Fig. S2A-C). This is true even of our strongest allele, $cv\text{-}d^{13}$, which is predicted to remove all of the DUF1943 and VWD domains and most of the Vg domain (Fig. 1E), making it a possible null. With weaker alleles, the vein phenotype varies from complete loss of the PCV to wild type, while in $cv\text{-}d^{13}$ adults, the PCV is either partially (16%) or wholly (84%) absent (supplementary material Fig. S1D).

cv-d mutants disrupt BMP signaling in the pupal PCV (Fig. 1I-N′). Although PCV development is also sensitive to signaling mediated by the only EGF receptor (EGFR) in *Drosophila*, BMP signaling in the PCV precedes EGFR signaling, and the EGFR can be eliminated prior to 24 hours after pupariation (AP) without disrupting PCV-specific BMP signaling (Ralston and Blair, 2005). $cv\text{-}d^{1}$ and $cv\text{-}d^{13}$ could reduce BMP signaling from pupal PCVs as early as 17-19 hours AP, detected with an antiserum specific for the C-terminal-phosphorylated form of the receptor-activated Smad Mothers against dpp (anti-pMad); the stained region was abnormally thin and weak near the center of the PCV, distant from the LVs (Fig. 1J-L). By 22-24 hours AP, wider gaps appeared in anti-pMad staining, accompanied by failures in the repression of *Drosophila Serum Response Factor* expression (*DSRF*, also known as *blistered*) (Fig. 1M′), which is normally mediated by BMP signaling at this stage (Conley et al., 2000; Ralston and Blair, 2005). Therefore, Cv-d can affect the initial stages of BMP signaling in the PCV, and is unlikely to affect EGFR signaling alone. The more extreme PCV defects observed at later stages indicate that Cv-d is also important for maintaining signaling.

Cv-d is, however, permissive for BMP signaling in veins, rather than instructive. Overexpression of *UAS-cv-d-V5* did not induce the ectopic veins typical of gains in BMP signaling (Fig. 1D; supplementary material Fig. S3A). When we drove *UAS-cv-d-V5* with strong wing-blade drivers such as *nub-gal4* or *hh-gal4*, the only external defect was scalloping of the wing margin (supplementary material Fig. S3A); this may be caused by the reduced Wingless levels along the prospective wing margin that we observed in some late third instar discs (supplementary material Fig. S3B).

$cv\text{-}d^{13}$ did not cause any difference in the BMP signaling in late third instar wing discs that results from the earlier expression of Dpp along the anterior-posterior compartment boundary (supplementary material Fig. S2D,E), but not along veins.

Cv-d can be transported into the wing by hemolymph

Both the yolk Vtgs and apolipophorins of insects are produced by the fat body and circulated through the body via the hemolymph (Rodenburg and Van der Horst, 2005; Ziegler and Van Antwerpen, 2006). The same is probably true for Cv-d. We detected *cv-d* message by RT-PCR from dissected larval fat body (Fig. 2D), and by in situ hybridization in embryos (not shown), similar to results reported by the FlyAtlas Organ/Tissue Expression database (flybase.org) (Chintapalli et al., 2007).

FlyAtlas also detected moderate *cv-d* expression in other tissues, such as larval and adult brain, and adult heart. Our in situ hybridization experiments, however, suggest that *cv-d* mRNA is either absent or low in wing imaginal discs, as staining levels appeared equivalent with antisense and sense probes (Fig. 2A,B),

and we could not detect any message by RT-PCR from dissected discs (Fig. 2D). It is difficult to isolate pupal wings for RT-PCR without fat body contamination, but in situ staining of pupal wings was faint, even after overstaining (Fig. 2C). PCV development was also not disrupted by very large *cv-d*^{1} clones in the wing (Fig. 2E), or by combining *UAS-cv-d-RNAi* with strong wing drivers such as *nub-gal4* or *hh-gal4* (data not shown).

By contrast, we found Cv-d protein in many tissues, including the wing (Fig. 2F), using an antiserum to a region of the Cv-d Vg domain (Fig. 1E); staining was absent in *cv-d*^{13} homozygotes (Fig. 2G). Staining was particularly high in the hemocytes that circulate through hemolymph in embryos and through the lumen and veins of pupal wings. As hemocytes can act as scavengers, this may represent uptake from the hemolymph.

Our results strongly suggest that most of the Cv-d in the wing is being supplied via the hemolymph. As it is difficult to remove simultaneously the non-wing sources of Cv-d, we tested the converse: whether Cv-d supplied through the hemolymph was sufficient for normal function. We rescued *cv-d*^{1} mutants by driving *UAS-cv-d-V5* expression with several different drivers, including the hemocyte driver *hml-gal4* (Goto et al., 2003) (Fig. 2H); Cv-d-V5 spreads well outside the *hml-gal4*-expressing hemocytes in pupal wings (Fig. 2I,I′). Thus, Cv-d function in the wing can be supplied by tissues other than the wing epithelia.

The Cv-d Vg domain interacts with BMPs and is required for Cv-d activity

How might Cv-d promote BMP signaling in the wing? While Cv-d could help lipid delivery into the wing, like other lipoproteins, such delivery is likely to be minimal, and there is no indication that other changes in lipid delivery can affect *Drosophila* BMP signaling (see Discussion).

Instead, we found that we found that the FLAG-tagged BMPs Dpp and Gbb both bound to Cv-d-V5 in S2 cell supernatants, suggesting a direct link between Cv-d and the regulation of extracellular BMPs. When we mixed Dpp-FLAG or Gbb-FLAG supernatant with Cv-d-V5 supernatant, anti-FLAG beads co-precipitated Cv-d-V5 (Fig. 3A); conversely, anti-V5 beads co-precipitated Gbb-FLAG (Fig. 3B). Deletion (Δ) constructs (Fig. 3C) showed that the Cv-d Vg domain is sufficient for, and likely to be necessary for, binding BMPs. Cv-d-V5Δ(DUF1943+VWD) co-precipitated with both Dpp-FLAG and Gbb-FLAG (Fig. 3D). This occurs despite the likely reduction in lipid binding with this construct; the DUF1943 domain contains two amphipathic β-sheets (βA and βB) that make up a large part of the lipid-binding cavity (Smolenaars et al., 2007a), and similar deletions from apolipophorin largely eliminate lipid binding (Smolenaars et al., 2007b). Cv-d-V5Δ(VWD) also co-precipitated with Dpp-FLAG but not with Gbb-FLAG (Fig. 3E), suggesting that presence of the DUF1943 domain reduced binding. We did not detect any co-precipitation of Cv-d-V5Δ(Vg+DUF1943) or Cv-d-V5Δ(Vg) with Dpp-FLAG or Gbb-FLAG (data not shown).

Although PCV development in *cv-d*^{13} mutants was rescued by Cv-d-V5, removal of the BMP-binding Vg domain [Cv-d-V5Δ(Vg)] blocked this rescue, even though all of the constructs were secreted and diffused in wing discs at roughly equivalent levels (data not shown). However, although the BMP-binding Vg domain is necessary, it is not sufficient for Cv-d activity, as deletion constructs that retained the Vg domain also failed to rescue *cv-d*^{13}. Reduced activity after deletion of DUF1943 suggests that lipid binding is also essential for Cv-d function; the function of the VWD domain is unknown.

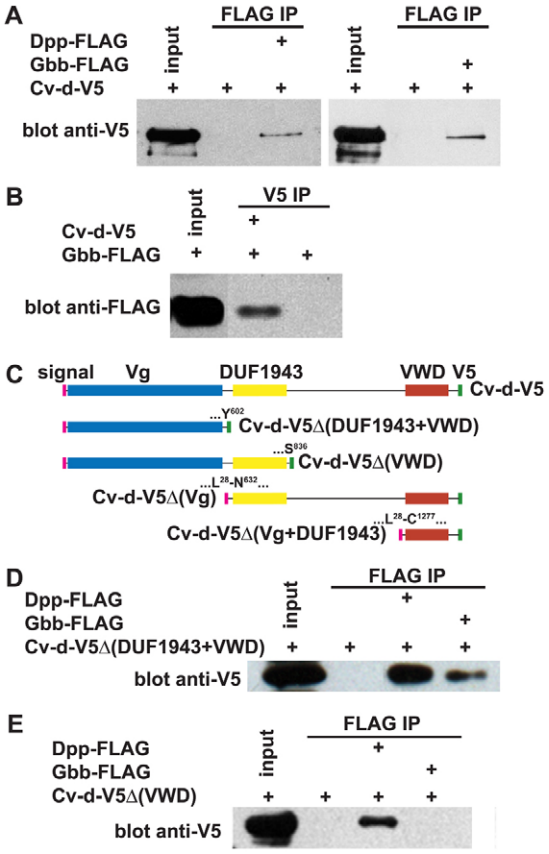

Fig. 3. Cv-d interacts with BMPs. (**A**,**B**,**D**,**E**) Immunoprecipitation assays using S2 cell supernatants containing the indicated tagged proteins. (**C**) Structures of the full-length and deleted V5-tagged Cv-d constructs.

Cv-d accumulation and PCV development requires HSPGs

Cv-d also interacts with HSPGs. In clones that lack Brother-of-tout-velu (Botv), an EXT polymerase that is required for the synthesis of the heparan sulfate side chains that are linked to proteoglycan core proteins (Han et al., 2004b; Takei et al., 2004), the levels of extracellular Cv-d-V5 accumulation were greatly reduced (Fig. 4A).

In wing imaginal discs, cell-bound glypican HSPGs increase the movement of Dpp through the disc epithelium and the accumulation of Dpp distant from its site of synthesis (Fujise et al., 2003; Belenkaya et al., 2004; Bornemann et al., 2004; Takei et al., 2004). The glypicans are not, however, obligate BMP co-receptors, either in wing discs or pupal wings. Mitotic recombinant clones lacking *dally* and *dlp* disrupted anti-pMad staining or DSRF suppression, often resulting in incomplete or fragmented PCVs, but this effect was not cell-autonomous, and anti-pMad staining or DSRF suppression was maintained in mutant PCV cells two to three cell diameters from wild-type cells, consistent with an effect on the range of BMP movement (Fig. 4B-D).

Cv-d does not act like a co-receptor in vitro

Secreted BMP-binding proteins are thought to promote signaling in the developing crossveins either by acting over a long range to increase BMP movement (Sog, Cv-Tsg2), as has been demonstrated in embryos (Shimmi et al., 2005b; Wang and

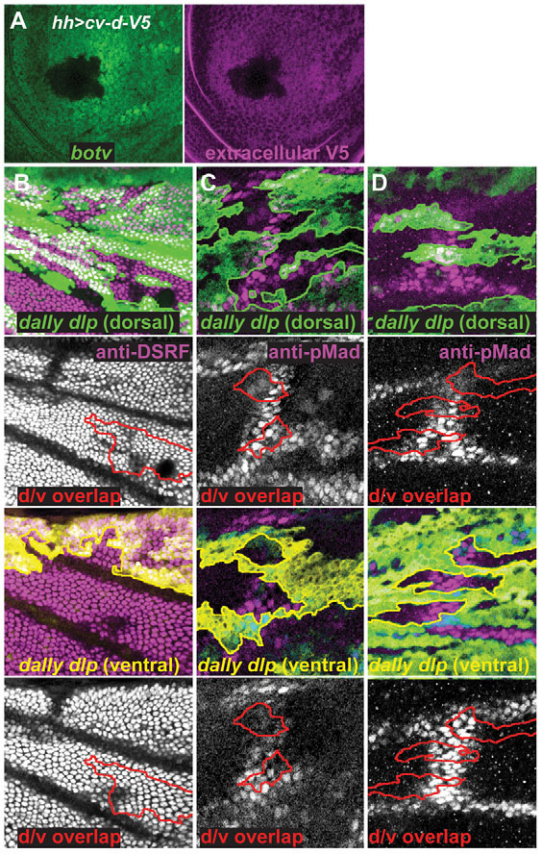

Fig. 4. HSPGs interact with Cv-d and affect PCV development. (**A**) Extracellular Cv-d-V5 (purple) in wing disc is reduced in *botv* clone (absence of green GFP). (**B-D**) Dorsal and ventral epithelia around pupal PCV with clones lacking *dally* and *dlp* (absence of green or yellow GFP). Green and yellow outlines show dorsal and ventral clones, respectively; red outlines show overlapping regions missing *dally* and *dlp* on both surfaces (d/v overlap). PCV, which is marked by the suppression of DSRF (G; purple and white) or the presence of pMad (H,I; purple and white), forms in cells two to three cell diameters distant from wild-type cells in the same or the opposite epithelium.

Ferguson, 2005), or by acting over a short range as non-obligate co-receptors or exchange proteins (Cv-2) (Serpe et al., 2008; Umulis et al., 2009). Cv-d does not affect BMP signaling in embryos or wing discs where Dpp movement can be easily assessed, and it is technically difficult to monitor BMP movement in pupal wings. However, there is another line of evidence, beyond the range of action of a protein, that has been used to argue for a particular role: the activity of the protein in vitro. When cells in vitro are bathed in BMPs, and thus BMP movement through tissues is not an issue, putative co-receptors such as Cv-2 can still increase signaling (Kamimura et al., 2004; Serpe et al., 2008; Kelley et al., 2009), whereas molecules like Sog that aid movement in vivo bind BMPs in vitro and only inhibit signaling (Shimmi and O'Connor, 2003).

We therefore examined the effects of Cv-d on S2 cells transfected with the transcriptional reporter *3xUbx-lacZ*, which contains three repeats of a short sequence that binds the Smads Mad and Medea, and drives *lacZ* expression in response to BMP signaling (Kirkpatrick et al., 2001). S2 cells produce endogenous *dpp* and *gbb* mRNA (data not shown), and thus have a basal level of BMP signaling that is sensitive to the extracellular milieu; reporter gene expression was reduced after reducing the levels of free BMPs with Sog, and was increased by an activated form of the BMP receptor Thickveins (TkvQD) (Fig. 5A) or the addition of Dpp (Fig. 5C).

Transfection with *cv-d* did not increase reporter gene expression. Instead, Cv-d caused a modest but significant decrease in reporter gene expression (Fig. 5A); the decrease was stronger as levels of transfected *cv-d* DNA increased (Fig. 5B). Cv-d-containing supernatant did not affect signaling (Fig. 5C), and transfected *cv-d* did not significantly weaken the effects of added Dpp (Fig. 5D), suggesting that the inhibition is quite weak and requires a high ratio of Cv-d to BMP. The Cv-d deletion constructs also failed to increase BMP signaling, although their ability to inhibit signaling was significantly reduced compared with full-length Cv-d-V5 (Fig. 5E). These results argue that Cv-d does not have co-receptor-like activity.

DISCUSSION

Our evidence indicates that Cv-d, a member of the Cv-d/160MEP family of Vtg-like lipoproteins, acts over a long range to promote BMP signaling in the developing PCV of the *Drosophila* wing, probably after having been delivered to the wing via the hemolymph. Cv-d binds both BMPs and HSPGs, and Cv-d activity in vivo requires the presence of its BMP-binding Vg domain. As Cv-d does not promote signaling in vitro, in vivo it more likely acts by increasing the movement of Dpp and Gbb from the LVs or their accumulation in the PCV region. This is consistent with the initial BMP signaling defects found near the center of *cv-d* mutant PCVs (Fig. 1G-I). The later appearance of defects near the LVs may be caused by the LV-specific expression of vein-inhibiting signals such as Delta and Argos (Conley et al., 2001; Christoforou et al., 2008). $cv\text{-}d^{1}$ and $cv\text{-}d^{13}$ also reduce the number of adult jump muscle fibers (R. M. Cripps, personal communication), the development of which is sensitive to changes in BMP signaling and loss of Cv-Tsg2 (Jaramillo et al., 2009). Thus, Cv-d probably regulates BMP signaling in at least two different contexts.

The activity of Cv-d in BMP signaling has interesting parallels to the signaling activities of the *Drosophila* Apolipophorins, which have a similar domain structure to Cv-d/160MEP proteins. Apolipophorins shuttle lipids from the fat body and digestive tract to other tissues via the hemolymph (van der Horst et al., 2002; Rodenburg and Van der Horst, 2005; Smolenaars et al., 2007a), but their role is not limited to lipid delivery. Apolipophorins can increase the range over which signaling proteins move in the wing disc, an effect thought to be mediated by diffusion of an extracellular complex that contains lipids, Apolipophorins and lipid-linked signaling proteins such as Hedgehog, Wnts and HSPGs (Panakova et al., 2005; Eugster et al., 2007). Although BMPs are not lipid-linked proteins, Cv-d activity also requires both BMP and lipid binding motifs, consistent with BMP movement via a lipid-lipoprotein complex.

The loss of Apolipophorins can also affect signaling by loss of lipid delivery, and thus lipid-dependent intracellular signaling, such as the lipid-mediated signal transduction triggered when Hedgehog binds to its receptor Patched (Khaliullina et al., 2009). However, the evidence does not support a similar role for Cv-d-mediated lipid delivery in BMP signaling. Lipids have not been linked to the transduction of canonical BMP signaling, and reducing lipid delivery to wings through lipid starvation or reduction in *Drosophila* Apolipophorin expression greatly shrinks the size of the wing but does not alter venation or BMP signaling (Panakova

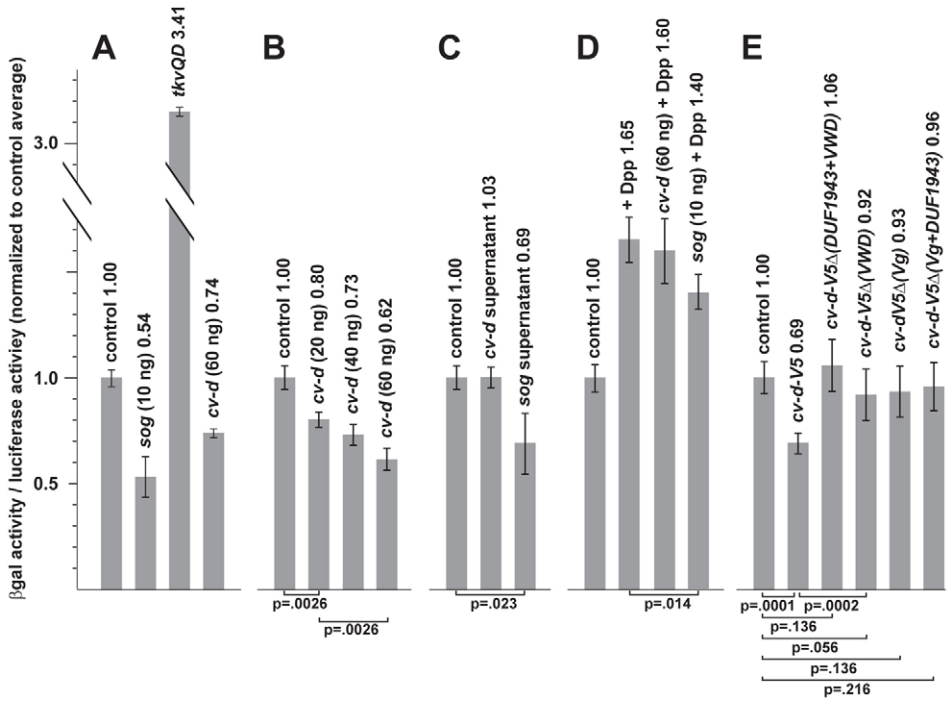

Fig. 5. Cv-d, but not Cv-d deletion constructs, inhibit BMP signaling in vitro. S2 cell signaling assays with the BMP-sensitive *3xUbx-lacZ* construct. The *y*-axis shows the ratio of β-gal activity to control luciferase activity, which is normalized for each experiment to the average control value. The *P* values show comparisons between two selected columns using a single-tailed Mann-Whitney U-test. (**A**) Signaling is inhibited by transfection with *sog* and *cv-d-V5*, and increased by transfection with *tkvQD*. (**B**) Signaling is progressively inhibited by transfection with increasing amounts of *cv-d-V5*. (**C**) Signaling is inhibited by Sog-containing, but not Cv-d-containing, supernatant. (**D**) Signaling that was increased by the addition of recombinant Dpp is inhibited by *sog* but not by *cv-d-V5* transfection. (**E**) Signaling is only reliably reduced by transfection with *cv-d-V5*, but the effects with the deletion constructs were significantly weaker [*cv-d-V5* versus *cv-d-V5Δ(VWD)* comparison shown]. *cv-d-V5Δ(VWD)* may have slightly reduced signaling, but the effect was not significant.

et al., 2005). Cv-d complexes also carry less lipid than apolipophorins. Lipid content in apolipophorin complexes is 30-60% (Ziegler and Van Antwerpen, 2006), but in complexes containing the *Apis* Cv-d homolog VHDL, it is only10% (Shipman et al., 1987). Like VHDL complexes, Cv-d complexes are higher density (lower lipid content) than Apolipophorin complexes (S. Eaton, personal communication).

The LDL-like receptors that mediate lipoprotein uptake do not appear to play a role in PCV development. Loss of the LRP1, LPR1&2, Megalin or the Vtg receptor Yolkless produces viable adults with normal crossveins (Schonbaum et al., 1995; Khaliullina et al., 2009) (S. Eaton, personal communication). The only remaining LDL receptors in *Drosophila* are CG8909/MEGF7, which has been detected in neuronal tissue (FlyAtlas) but not in wing imaginal discs (Khaliullina et al., 2009), and the LRP5/6 homolog Arrow, which is required for Wingless/Wnt signaling during wing disc patterning but has no known role in BMP signaling (He et al., 2004). Thus, it is likely that the effect of Cv-d on BMP signaling is mediated by BMP binding, rather than via lipid delivery alone.

Although Vtgs are best known as the major components of yolk, there is a growing awareness that Vtg-like lipoproteins can have functions outside the yolk, such as immune protection and sperm-egg recognition in vertebrates (Campanella et al., 2011; Zhang et al., 2011), and clotting and melanization in arthropod hemolymph (Hall et al., 1999; Lee et al., 2000). Cv-d demonstrates a new and important function in BMP signaling. Is this function shared by other Vtg family proteins? Intriguingly, *Xenopus* Lipovitellin 1, a Vg domain-containing fragment of VtgA2, was isolated as a BMP4-binding protein, and purified VtgA2 bound to purified BMP4 and ActivinA (but not to TGF-β1) in surface plasmon resonance assays (Iemura et al., 1999). Although the requirement for Vtg in the nutrition of early vertebrate embryos makes it difficult to interpret tests of Vtg function, the evolution of two different Vtg-family proteins in insects, the yolk Vtg and Cv-d/160MEP families, may provide a 'natural experiment' that has allowed us to separate the yolk and non-yolk roles of this protein family, demonstrating for the first time the importance of Vtg-BMP interactions.

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