The *Drosophila* COMPASS-like Cmi-Trr coactivator complex regulates *dpp*/*BMP* signaling in pattern formation

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ABSTRACT

Drosophila Cara Mitad (Cmi, also known as Lpt) is the N-terminal homolog of mammalian Mixed Lineage Leukemia 2 (MLL2/ALR), a core component of COMPASS-like nuclear receptor coactivator complexes. Cmi is required for the activation of ecdysone hormone targets and plays a critical role in development and tissue patterning. Using multiple approaches that include genetic interaction tests and tissue specific knockdown and overexpression of *cmi*, we demonstrate that Cmi has important functions in controlling wing vein patterning through regulation of the conserved Decapentaplegic (Dpp) signaling pathway. The loss of function allele, *cmi1*, enhances loss of *dpp* function phenotypes in genetic epistasis tests. Wing specific knockdown of *cmi* results in incomplete veins towards the distal wing margin that are enhanced by the simultaneous knockdown of *dpp*. In contrast, the overexpression of a tagged full-length *HA-cmi* transgene results in ectopic veins that are sensitive to Dpp levels. The knockdown and overexpression of *cmi* result in reduced and increased Dpp signaling as observed by immunostaining for phospho-MAD (Mother against DPP), a downstream effector of Dpp function. shRNAi depletion of *cmi* suppresses a *tkv* reduced function phenotype while the overexpression of *HA-cmi* enhances *tkv* RNAi phenotypes. We further show by enhancer reporter assays and chromatin immunoprecipitation that Cmi controls wing vein patterning by regulating *dpp* transcription directly or indirectly through the 3′ disc regulatory region at the larval stage and through the 5′ shortvein (shv) regulatory region at the pupal stage. Our data reveals that Cmi is a key part of the mechanism that controls wing vein patterning through nuclear receptor regulation of the Dpp signaling pathway.

Introduction

Gene transcription is tightly regulated by the crosstalk between nuclear receptors, co-repressors, co-activators and chromatin remodeling complexes. In the absence of hormones, the corepressor complexes mask the hormone receptor binding sites. However, in the presence of hormones, the nuclear receptors form heterodimers, displace the corepressors and recruit coactivators to allow for gene transcription (reviewed in Rosenfeld et al., 2006).

Nuclear receptors regulate target gene expression through associations with cofactors that include the Set1 family of histone H3K4 methyltransferases (HMTase) embedded in large multimeric complexes referred to as Set1/COMPASS-like complexes (Eissenberg and Shilatifard, 2010; Shilatifard, 2012). In mammals, these methyltransferases are members of the Mixed Lineage Leukemia (MLL) family

of proteins that contain both the HMTase domain, as well as clustered chromatin binding PHD (plant homeodomain) zinc-finger domains. One MLL subfamily appears to interact directly with several nuclear receptors through a conserved LXXLL motif. Members of this group include the homologous and perhaps functionally redundant MLL2/ALR (HGNC 7133; NCBI NM003482.3) and MLL3/HALR (HGNC 13726) proteins (Lee et al., 2009). MLL2/ALR is a component of several COMPASS-like nuclear receptor co-activator complexes, including the MLL2 and ASCOM complexes. As there has been considerable confusion regarding the nomenclature of the MLL2/ALR protein (variously referred to synonymously as MLL2, MLL4 and ALR), for simplicity we will refer to the mammalian protein as MLL2 throughout this report. The MLL2 complex regulates gene transcription in response to estrogen receptor α (ERα) signaling (Mo et al., 2006) and the ASCOM complex regulates target gene transcription through the retinoic acid receptor (RAR) as well as the liver X receptor (LXR) (Goo et al., 2003; Lee et al., 2008). This transcriptional regulation is dependent on the covalent histone lysine methylation activity of MLL2 (Issaeva et al., 2007). Moreover, knockdown of *MLL2* in HeLa cells results in defects in cell adhesion and migration and was associated with reduced

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tumorigenicity in mice. Issaeva et al. (2007) also identified a number of MLL2 target genes involved in cell adhesion, proliferation and developmental signaling, including MADH6 [MAD (mothers against decapentaplegic) *Drosophila* Homolog 6] that is involved in Transforming Growth Factor β (Tgfβ)/Bone Morphogenetic Protein (BMP) signaling and *DKK1* involved in Wnt/Wg receptor signaling. MLL2 has also been shown to regulate transcription of the homeobox gene *Hoxc8* by its recruitment through Activating protein 2δ (Ap2δ) along with ASH2L, another component of the MLL2 complex (Tan et al., 2008). Similarly, *HOXB9* expression is regulated in part through collaboration between MLL3 and estrogen receptors (Ansari et al., 2011).

Recent reports have revealed important functions for COMPASS-like complexes in both development and human disease. A COMPASS-like complex is present in *Caenorhabditis elegans* and is involved in epigenetic regulation in the germ line and attenuation of RAS-signaling during development (Fisher et al., 2010; Li and Kelly, 2011). Several studies have demonstrated links between Kabuki Syndrome (OMIM 147920) and mutations in the *MLL2* gene (Hannibal et al., 2011; Li et al., 2011; Ng et al., 2010; Paulussen et al., 2011). Kabuki Syndrome is characterized by facial dysmorphia, skeletal and cardiac abnormalities and developmental delay. Mutations in *MLL2* and *MLL3* have also been associated with a variety of cancers, including breast (Wang et al., 2011), renal (Dalgliesh et al., 2010) and bladder (Gui et al., 2011) cancers, childhood medulloblastoma (Parsons et al., 2011), and nearly one-third of all newly diagnosed cases of non-Hodgkin lymphoma (Morin et al., 2011). The human MLL2 protein is also implicated in several key cancer signaling pathways, including the p53 and cAMP pathways that involve the function of the retinoic acid receptor (Guo et al., 2012; Lee et al., 2009). Therefore, MLL2 and MLL3 provide important functional links between epigenetic gene regulation, animal development and disease.

In *Drosophila*, both MLL2 and MLL3 are represented by a single orthologous set of proteins, Cmi (also known as Lpt or Lost PHD fingers of Trr) and Trr, that are both present in the same COMPASS-like complex (Chauhan et al., 2012; Mohan et al., 2011; Sedkov et al., 2003). The 163 kDa Cara Mitad (Cmi/CG5591/Lpt) contains two conserved clusters of PHD fingers (PHD1–4, PHD 5–7), an HMG domain and several LXXLL nuclear receptor binding motifs (Chauhan et al., 2012). The 267 kDa Trithorax-related (Trr) protein carries the HMTase domain, conserved FY-rich regions and several additional LXXLL motifs (Sedkov et al., 2003). Cmi and Trr appear to represent the N- and C-terminal *Drosophila* counterparts to both MLL2 and MLL3, respectively, and both are capable of direct contact with the *Drosophila* Ecdysone Receptor/Ultraspiracle (EcR/Usp) nuclear receptor heterodimer (Chauhan et al., 2012; Sedkov et al., 2003). Mutations in both the *cmi* and *trr* genes have revealed important common functions in regulating ecdysone hormone targets during development, suggesting that the conserved PHD and HMTase features are both essential for hormone-dependent gene regulation. Trr has an essential role in regulating the ecdysone dependent transcription of *hedgehog* (*hh*) and *Broad-complex* (*BR-C*) both in S2 cells and the eye imaginal disc (Johnston et al., 2011; Sedkov et al., 2003). Mutations in *trr* have also been shown to affect Dpp signaling in the eye imaginal disc downstream of Hh signaling, presumably through *trr* functions in regulating *hh* expression (Sedkov et al., 2003). Cmi functions as nuclear receptor coactivator to positively regulate ecdysone dependent transcription in vivo from an Ecdysone Receptor response element (ECRE) as well as the early ecdysone pathway genes *E74A* and *E74B* (Chauhan et al., 2012) that are produced from the *Eip74EF* locus. Null alleles of *cmi* die as larvae with cuticle and locomotion defects while in vivo knockdown in embryos is lethal (Chauhan et al., 2012). We have observed broad tissue patterning defects upon *cmi* misregulation (overexpression and knockdown) that include disrupted eye development, ectopic sex combs in male prothoracic legs and wing pattern defects. Knockdown of *cmi* in the wing results in the appearance of incomplete longitudinal veins, while overexpression of *cmi* results in ectopic vein development. These phenotypes are highly penetrant and completely opposite, suggesting a common regulatory target (s) whose expression is sensitive to Cmi levels. Moreover, the ectopic wing vein phenotype associated with *cmi* overexpression is suppressed by the simultaneous loss of *ultraspiracle* function (encoding the *Drosophila* ortholog of mammalian RXR) revealing a potentially novel link between the *cmi*-dependent vein patterning pathway and nuclear receptor activity.

These data support the hypothesis that mammalian MLL2 and *Drosophila* Cmi/Trr COMPASS-like complexes are essential for the proper regulation of developmentally important hormone-dependent genes. One possible target is the *dpp* gene, as misregulation of *dpp* in the developing fly wing results in opposite overexpression and loss of function phenotypes (de Celis, 2003; Martin-Castellanos and Edgar, 2002) that are remarkably similar to those associated with misregulation of *cmi*. Dpp is a member of the conserved Tgfβ family of secreted signaling molecules and the fly ortholog of the vertebrate BMP proteins BMP2/BMP4 that are required for bone formation and adult limb regeneration (Padgett et al., 1987). In mammals, misregulation of the tumor suppressive Tgfβ pathway has been linked to oncogenesis through modulation of tumor microenvironments, cellular differentiation and invasion (Massague, 2008). The Dpp signaling pathway is utilized at multiple points during *Drosophila* development, with varying targets in each tissue/stage. During larval development, Hh is expressed in posterior compartment wing cells and diffuses into the anterior compartment to up-regulate Dpp signaling (Sturtevant and Bier, 1995). Transcription of *dpp* is spatially and temporally regulated by the 3′ disc regulatory region during the larval development in a Hh-dependent manner and through the 5′ shortvein (shv) regulatory region in a Hh-independent manner during the pupal stages (St Johnston et al., 1990). However, despite evidence for the regulation of mammalian MADH6 by MLL2, and Hh by Trr in the *Drosophila* eye imaginal disc, there is no evidence for the direct in vivo regulation of either the Hh or Dpp signaling pathways in the developing wing by Cmi or Trr, nor is there any evidence for direct regulation of the mammalian Tgfβ/BMP pathway by nuclear hormone receptors or the MLL2 coactivator complex.

In this study we report genetic and reporter gene analyses that suggest a novel role for the nuclear receptor coactivator Cmi in wing patterning by spatial and temporal regulation of *dpp* transcription both in the wing imaginal disc and the pupal wing, in a Hh independent manner. We further demonstrate that this requirement for Cmi is necessary but not sufficient for *dpp* transcription. Our results suggest important and unanticipated functions of conserved COMPASS-like nuclear receptor coactivator complexes in the developmental regulation of essential cell signaling pathways.

Materials and methods

Drosophila culture

All stocks and genetic crosses were maintained on standard cornmeal/dextrose medium. Transgenic *Drosophila* shRNAi lines were obtained from the Vienna Drosophila RNAi Center (VDRC), National Institute of Genetics (NIG-Fly) or were generated as described (Chauhan et al., 2012). The hemagglutinin (HA) epitope-tagged full-length *cmi* rescue and *cmi-IR* knockdown transgenes were expressed under UAS_{GAL4} control (Chauhan et al., 2012). All other fly strains and GAL4 drivers used in this study were obtained

from the Bloomington *Drosophila* Stock Center and are described in Flybase (http://flybase.bio.indiana.edu).

Examination of imaginal discs, pupal and adult wings

The regulation of *dpp* transcription enhancers was assayed using *lacZ* reporter genes as described (*dpp^{shv}-lacZ.RD2* (Hursh et al., 1993) and *dpp-lacZ^{dpp.BS3.0}RA* (Blackman et al., 1991)). Dissected larval imaginal discs and pupal wings were examined for *lacZ* expression by histochemical staining for β-galactosidase activity (Johannes and Preiss, 2002; Marenda et al., 2004). Third instar larvae were staged on blue food (0.05% bromophenol blue). Imaginal discs and early pupae (25–46 h APF) were dissected in ice cold PBS. Whole pupae were removed from their pupal cases but left intact. Larval tissues and dissected pupae were fixed in 1.5% glutaraldehyde for 15 min at room temperature. The fixing solution was removed and replaced by pre-warmed staining solution [10 mM $NaH_2PO_4 \cdot H_2O/Na_2\ HPO_4 \cdot 2H_2O$ (pH 7.2.), 150 mM NaCl, 1 mM $MgCl_2$, 3.1 mM $K_4[FeIII(CN)_6]$, 3.1 mM $K_3[FeII(CN)_6]$, 0.3% Triton X-100] with X-gal (25 μl of 8% X-gal in DMSO). Reactions were incubated 1 h to over-night at 37 °C until optimal color development was achieved followed by rinsing with PBS. Larval imaginal discs were mounted in 80% glycerol and photographed at 100X magnification using an Olympus BX41 microscope. Pupal wings were dissected away from the remaining tissues and re-stained for 1 h at 37 °C followed by an additional hour at room temperature, then mounted in 80% glycerol and photographed. Wings from adult female flies were dissected in 100% isopropanol, placed in DPX mounting fluid (Fluka Chemika), and examined on a Leica MZ16 stereo dissecting microscope at 63X magnification.

Immunostaining of imaginal discs and pupal wings

Dissected wing imaginal discs from third instar larvae and pupal wings (25–46 h APF) were fixed in 4% formaldehyde and stained as described in (de Celis, 1997). The activation of Dpp downstream effector phospho-Mad (pMad) was assayed by immunostaining with phospho Smad3 (pS423/425) rabbit monoclonal antibody (Epitomics, Inc.) used at 1:200 dilution. Horseradish peroxidase-conjugated Donkey anti-rabbit secondary antibodies (Jackson Immunoresearch) were used at a 1:500 dilution. Imaginal discs and pupal wings were mounted and photographed as described above.

Chromatin immunoprecipitation

Bulk chromatin was isolated from wild type *OregonR* embryos (0–8 h after egg laying) or wandering third instar larvae as described (Negre et al., 2010). Chromatin immunoprecipitation (ChIP) was carried out using polyclonal antibodies recognizing Cmi (Lpt) (Chauhan et al., 2012) and DNA samples prepared for Illumina sequencing (Oh et al., 2013). Cmi genomic enrichments are expressed as conservative fold changes plotted on a log2 scale following background IgG subtraction. Data for EcR, H3K4me1, H3K27Ac and RNA Pol II enrichments were obtained from the modENCODE project (http://www.modencode.org/).

RNA analysis

Expression of the *UAS_{GAL4}:cmi-IR* transgene (Chauhan et al., 2012) was controlled using the widely expressed *P(GawB)69B-GAL4* driver with crosses performed at 29 °C. Animals were staged according to standard guidelines using morphological landmarks (Bainbridge and Bownes, 1981). Total RNA from 100 early pupae was prepared using the RNAqueous extraction system (Applied

Biosystems, Foster City, CA). Transcript levels of *Eip74EF* (*E74A*) and *hh* were analyzed by SYBR Green quantitative real time PCR on reverse transcriptase reactions (qRT-PCR) performed in triplicate using GoTaq qPCR Master mix (Promega). Levels of mRNA were analyzed by comparative *Ct* method. qRT-PCR primers were used that spanned at least one intron when appropriate corresponding to *Eip74EF* common region isoforms, as well as the *hh* gene. The ribosomal protein gene *rp49* was used as a reference standard. All primer sequences are available upon request.

Results

cmi genetically interacts with dpp

We recently found that *trr* cooperates with *cmi* (CG5591/FBgn0263667, also known as *Lpt*) in hormone regulated gene transcription as part of a *Drosophila* COMPASS-related nuclear receptor coactivator complex, and *cmi* has important functions in tissue patterning (Chauhan et al., 2012). Using a combination of loss of function mutations and GAL4-directed expression of shRNAi (*cmi-IR*) transgenes as well as ectopic overexpression (*HA-cmi*) in *Drosophila*, we identified highly penetrant and reproducible wing pattern defects associated with reduced *cmi* that include shortened and incomplete veins, while gain of *cmi* function results in ectopic veins and reduced overall wing size.

The *cmi* loss and gain of function wing phenotypes are strongly consistent with defective Dpp signaling (de Celis, 2003; Martin-Castellanos and Edgar, 2002). To clarify the role of Cmi in wing patterning, we first looked for regulation of the Dpp pathway by testing for dose-sensitive genetic interactions between the *cmi^1* null allele and mutations that affect genes required for Dpp signaling.

The expression of *dpp* during development is controlled through both the 5′ and the 3′ regulatory regions (St Johnston et al., 1990) (Fig. S1). The *dpp^{d-ho}* allele is a recessive mutation in the *dpp* 3′ disc regulatory region that produces flies displaying held-out wings when homozygous and in trans-heterozygous combinations with the *dpp^{s1}* mutation that affects the 5′ short vein (shv) region (Gelbart, 1982; Irish and Gelbart, 1987). While *dpp^{s1} dpp^{d-ho}* homozygotes have highly penetrant held-out wing and shortened vein phenotypes as a consequence of reduced Dpp signaling, heterozygous flies appear normal. Another mutation affecting the 3′ disc region (*dpp^{d14}*) is fully recessive (Fig. 1A), while flies carrying transheterozygous combinations with *dpp^{s1} dpp^{d-ho}* exhibit a held-out wing phenotype (Fig. 1B). Flies heterozygous for *dpp^{d14}* and the *cmi^1* null mutation display a completely penetrant held out wing phenotype (Fig. 1C) consistent with reduced Dpp function.

We next looked for interactions using the shortened vein phenotype of reduced function *dpp* mutants. Homozygous *dpp^{s1}* shv mutant flies display a shortened L4 vein phenotype (Fig. 1D). While heterozygous *dpp^{s1}/cmi^1* flies show a shortened crossvein (Fig. 1E), both L4 and posterior crossvein (PCV) shortening is enhanced when *dpp^{s1}* is homozygous (Fig. 1F; Table 1). The L4 longitudinal vein is further shortened in homozygous *dpp^{s1} dpp^{d-ho}* flies (Fig. 1G), while heterozygotes appear normal. In *cmi^1/dpp^{s1} dpp^{d-ho}* double heterozygotes the L4 vein is significantly shortened (Fig. 1H) and the incomplete vein phenotype is strongly enhanced when the *dpp* alleles are homozygous in the presence of *cmi^1* (Fig. 1I). These results support the view that *cmi* regulates the development of wing veins through interactions with the Dpp signaling pathway.

A prediction of this model is that *dpp* loss of function wing vein phenotypes would be sensitive to Cmi levels. We used a wing-specific GAL4 driver (*C765-GAL4*) and shRNAi (IR) transgenes to
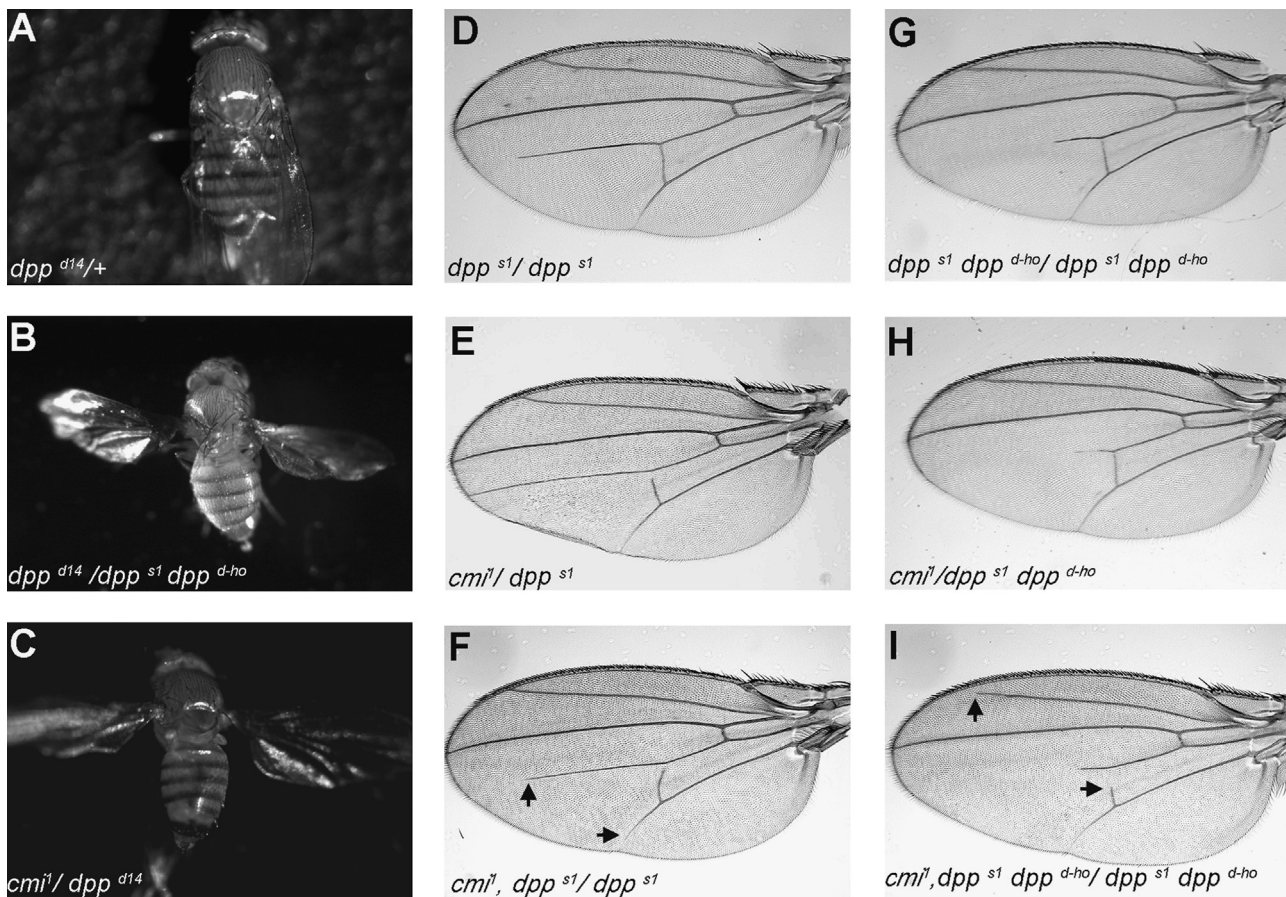
Fig. 1. Loss of *cmi* function enhances *dpp* mutant phenotypes. (A) Heterozygous *dpp^d14^/+* flies show normal wings. (B) *dpp^d14^/dpp^s1^ dpp^d-ho^* transheterozygous flies have held out wings. (C) *cmi^1^/dpp^d14^* display a similar held out wing phenotype. (D) *dpp^s1^/dpp^s1^* flies display an incomplete L4 vein towards the distal wing margin. (E) *cmi^1^/dpp^s1^* transheterozygous flies show incomplete PCV. (F) *cmi^1^ dpp^s1^/dpp^s1^* flies show an enhancement of the incomplete L4 and PCV phenotypes observed in (D) and (E) as indicated by the arrows. (G) *dpp^s1^ dpp^d-ho^/dpp^s1^ dpp^d-ho^* homozygotes show incomplete L4 towards the distal wing margin. (H) *cmi^1^/dpp^s1^ dpp^d-ho^* transheterozygous flies show an enhancement of the incomplete L4 observed in (G). (I) *cmi^1^, dpp^s1^ dpp^d-ho^/dpp^s1^ dpp^d-ho^* flies display enhancement of the incomplete L2, L4 and PCV phenotypes as indicated by the arrows. All pictures were taken at the same magnification and all crosses were carried out at 25 °C.

Table 1
Genetic interaction between mutant alleles of *cmi* and *dpp*.

Genotype	Percent showing phenotype			
	Inc. L2	Inc. L3	Inc. L4	Inc. PCV
*dpp^s1^/dpp^s1^*	–	–	94	35
*cmi^1^/dpp^s1^*	–	–	–	4
*cmi^1^, dpp^s1^/dpp^s1^*	6[a]	–	100[b]	71[b]
*dpp^s1^dpp^d-ho^/dpp^s1^dpp^d-ho^*	65	–	100	10
*cmi^1^/dpp^s1^ dpp^d-ho^*	–	–	–	6
*cmi^1^, dpp^s1^ dpp^d-ho^/dpp^s1^ dpp^d-ho^*	93[c]	–	100	43[c]

[a] New genetic interaction.
[b] Enhancement of *dpp^s1^* phenotype.
[c] Enhancement of the *dpp^s1^ dpp^d-ho^* phenotype.

knockdown both *cmi* and *dpp* simultaneously. Modest expression of either the *dpp-IR* or the *cmi-IR* in the wing disc resulted in shortened wing veins, as expected (Fig. 2A–C). Simultaneous knockdown of both *dpp* and *cmi* resulted in further shortening of the wing veins (Fig. 2D and Table 2). *HA-cmi* overexpression in the wing disc results in both ectopic vein formation and a disrupted proximal L2 vein (Fig. 2E). Simultaneous overexpression of *HA-cmi* and *dpp-IR* results in suppression of both the incomplete vein phenotypes observed upon *dpp* knockdown as well as the ectopic vein phenotypes observed upon *cmi* overexpression (Fig. 2F, Table 2).

One possible interpretation of these results is that *cmi* acts upstream of *dpp*, perhaps through positive regulation of *dpp* transcription in presumptive wing vein cells; however, these data may also be explained by *cmi* function downstream through regulation of Dpp-responsive target genes. To address this question, we ectopically expressed *dpp* under GAL4 control, bypassing any possible regulation by *cmi*. Ectopic expression of *UAS-dpp* in the wing results in severe wing defects that include blisters, ectopic veins and tissue overgrowth (Capdevila and Guerrero, 1994; Staehling-Hampton et al., 1994). Neither increasing nor decreasing Cmi levels, through expression of *HA-cmi* or *cmi-IR*, in combination with *UAS-dpp* had any significant effect on the *UAS-dpp* wing phenotype (our unpublished results). This suggests that *cmi* most likely functions upstream of Dpp, rather than on downstream targets of Dpp signaling in the presumptive wing vein cells during pupal development.

cmi genetically interacts with Dpp receptors

Dpp acts as a morphogen to direct development of cells both locally, as well as at a distance from its normal expression domain through diffusion. In order for cells to respond to the Dpp signal, receiving cells must express receptors (Type I and Type II receptor kinases) that are required to transmit the signal that activates gene expression (Affolter and Basler, 2007). The local action of Dpp is mediated through restriction of the signal to a narrow strip of cells by the Type I receptors, Thickveins (Tkv) and Saxophone (Sax), with Tkv assuming a more potent and possibly direct role (Affolter
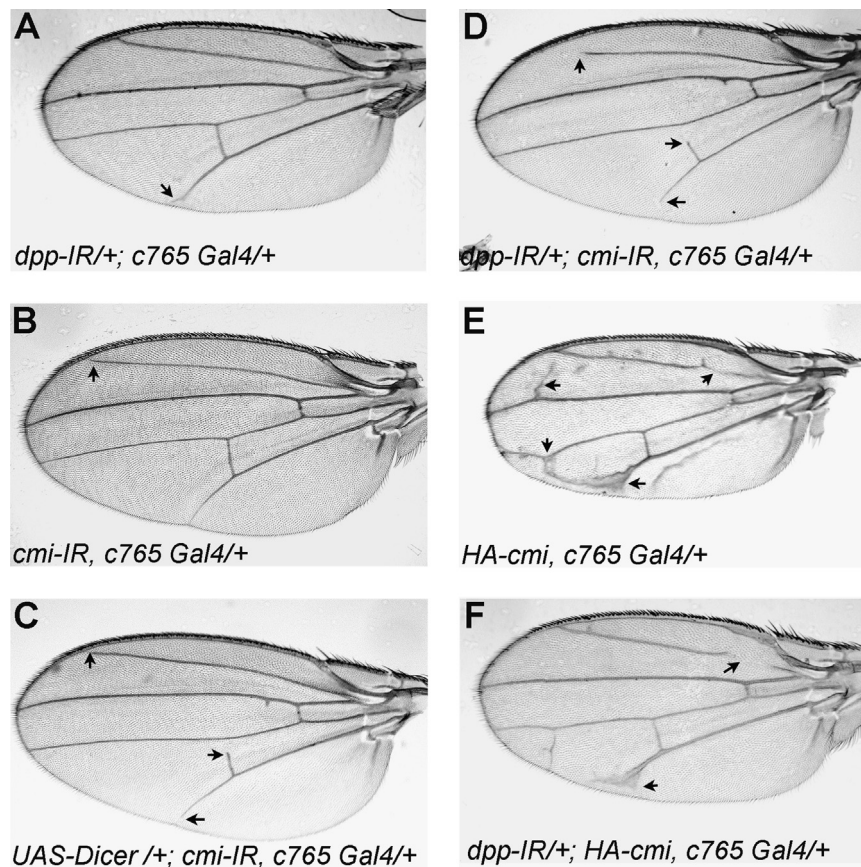
Fig. 2. Reduced *dpp* modifies *cmi* loss- and gain-of-function phenotypes. Short hairpin RNAi (shRNAi) was used to reduce *dpp* function (*dpp-IR*) in combination with reduced *cmi (cmi-IR)* or ectopic *cmi (HA-cmi)* in the developing wing using a specific GAL4 driver. (A) Wings from *dpp-IR/+; C765 GAL4/+* flies shows incomplete L5 as indicated by the arrow. (B) *cmi-IR, C765 GAL4/+* flies display an incomplete L2 vein. (C) *UAS-Dicer/X; cmi-IR, C765 GAL4/+* flies exhibit incomplete L2, L5 and PCV. (D) *dpp-IR/+; cmi-IR, C765 GAL4/+* wing. Note the enhancement of the incomplete vein phenotypes observed in (A) and (B) as indicated by the arrows. (E) *HA-cmi, C765 GAL4/+* flies show ectopic LVs towards the distal wing margin, indicated by the arrows. (F) *dpp-IR/+; HA-cmi, C765 GAL4/+* flies show a suppression of the *HA-cmi, C765 GAL4/+* phenotype observed in (D). All pictures were taken at the same magnification.

Table 2
Summary of the *cmi* and *dpp* misexpression phenotypes.

Phenotype	*dpp-IR/+; C765-GAL4*	*C765-GAL4, HA-cmi/+*	*C765-GAL4, HA-cmi/+; dpp-IR/+*	*C765-GAL4, cmi-IR/+*	*C765-GAL4, cmi-IR/+; dpp-IR/+*
Ectopic L2	–	100	55[a]	–	–
Ectopic L3	–	100	25[a]	–	–
Ectopic L4	–	100	32[a]	–	–
Ectopic L5	–	100	87[a]	1	–
Incomplete ACV	–	14	–	–	–
Proximal L2 incomplete	–	47	20[a]	–	–
Distal L2 incomplete	2	–	–	10	82[b]
Distal L5 incomplete	2	–	–	1	38[b]

Numbers represent the percentage of flies showing a given phenotype.

[a] Suppression of the *HA-cmi* phenotype.

[b] Enhancement of the *cmi-IR* phenotype.

and Basler, 2007; O'Connor et al., 2006). A prediction based on the above genetic tests of *cmi* function, would be that a reduction in the expression of the Dpp receptors Tkv and Sax would affect the *cmi* loss and gain of function phenotypes if Dpp signaling was a primary target.

In order to test our hypothesis, we first performed epistasis tests using our *cmi¹* null allele combined with mutations in the two receptor genes that result in vein thickening and ectopic veins (abnormal branching) as a consequence of the spread of Dpp signaling

beyond its normal domain. *sax^KG05725* is a hypomorphic allele that results from a P-element insertion upstream of *sax* (Dworkin and Gibson, 2006) and *sax⁴* is an amorphic allele (Singer et al., 1997); while *tkv¹* is a cold temperature sensitive hypomorphic mutation (Diaz-Benjumea and Garcia-Bellido, 1990; Terracol and Lengyel, 1994). Recombinants were generated between *cmi¹* and *tkv¹*, *cmi¹* and *sax⁴*, and between *cmi¹* and *sax^KG05725*, as all mutant alleles are recessive. Recombinants were crossed to parental flies to generate homozygous or transheterozygous *tkv* and *sax* alleles along with the *cmi¹*
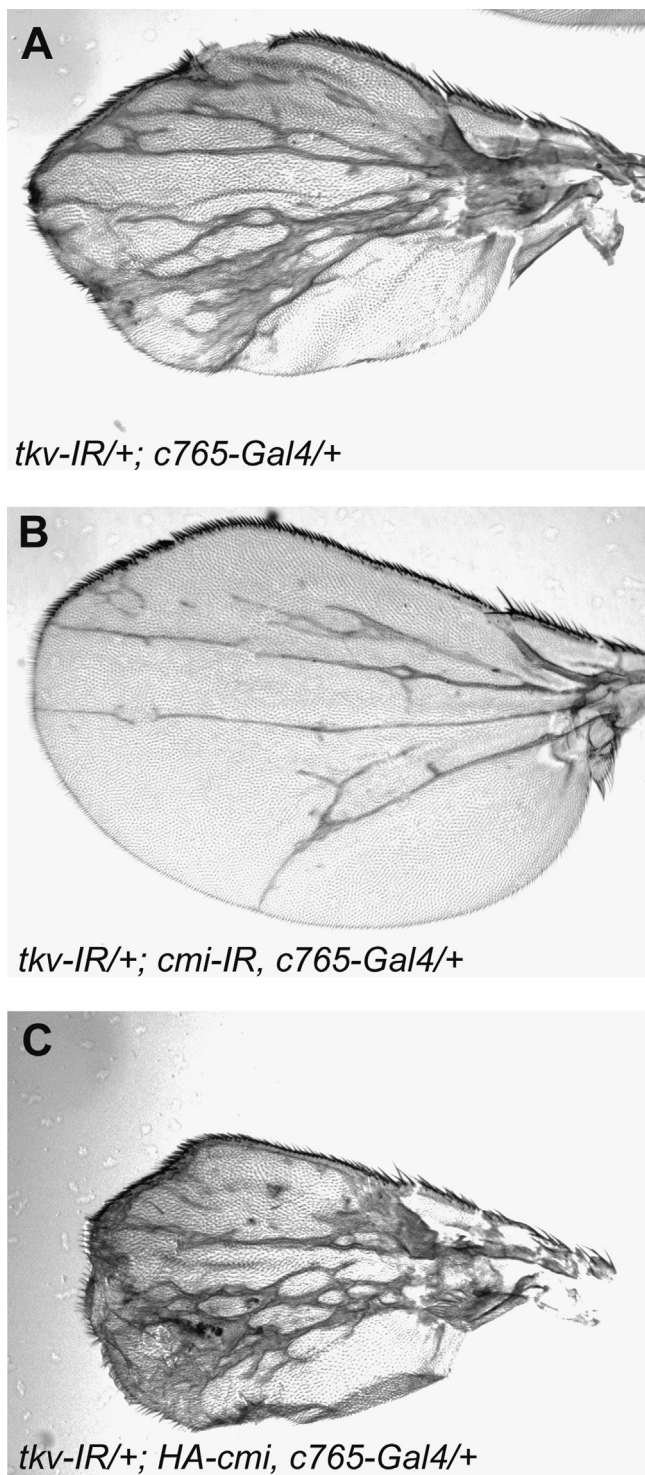
Fig. 3. Interactions between *cmi* and *tkv*. Wing-specific knockdown or overexpression of *cmi* was carried out in combination with knockdown of *tkv*. (A) *tkv-IR/+*; *C765 GAL4/+*. Note the rounded wings with extensive ectopic wing veins. (B) *tkv-IR/+*; *cmi-IR, C765 GAL4/+* flies show enlargement of the wings and a suppression of the ectopic vein phenotype observed in (A). (C) *tkv-IR/+*; *HA-cmi, C765 GAL4/+* flies exhibit a decrease in the overall wing size and an enhancement of the ectopic vein phenotype observed in (A). All pictures were taken at the same magnification and all crosses were carried out at 25 °C.

heterozygote. The results of these crosses are summarized in Table S1. In these genetic tests, reduced *cmi* function exhibited a modest suppression of the thickened vein phenotype associated with reduced *tkv* and *sax* function (our unpublished results) consistent with models in which *cmi* positively regulates Dpp signaling.

The *cmi¹* null mutant is fully recessive and thus likely to have only a slight reduction in Dpp levels. Therefore, we verified our epistasis tests using in vivo knockdown and overexpression of *cmi* together with simultaneous knockdown of *tkv* using a *tkv-IR* transgene. Wing defects apparent upon knockdown of *tkv* in the imaginal disc are similar to overexpression of *dpp*, including enlargement of wing size in addition to ectopic veins (Fig. 3A). As expected, the simultaneous knockdown of *cmi* and *tkv* together results in a suppression of *tkv* knockdown phenotypes, presumably as a consequence of reducing Dpp signaling (Fig. 3B). In contrast, the overexpression of *HA-cmi* results in an enhancement of the *tkv* knockdown phenotypes, as would be expected if Dpp signaling was elevated (Fig. 3C).

We next assayed for the activation of Dpp downstream effector Mad (Mothers against Dpp) by performing immunostaining with phospho-Mad antibodies (anti-pMad). Mad (mammalian R-Smad) is downstream of both Dpp and Tkv and is responsible for the activation of Dpp target genes (Kim et al., 1997; Newfeld et al., 1996; Wiersdorff et al., 1996). In the third instar wing imaginal disc, pMad staining is observed along the A-P boundary (Fig. 4A) (Newfeld et al., 1996). As expected, the knockdown of *cmi* results in a decrease in the amount of pMad staining (Fig. 4B), presumably as a result of reduced Dpp signaling. In contrast, the overexpression of *HA-cmi* results in diffused pMad staining, likely due to the spreading of the Dpp signal (Fig. 4C). Within the pupal wings pMad is localized to the developing wing veins (Fig. 4D). The knockdown of *cmi* results in decreased pMad staining (Fig. 4E) and the overexpression of *HA-cmi* results in ectopic pMad staining towards the distal wing margin (Fig. 4F). Since the phosphorylation of Mad depends on Dpp signaling, our results suggest that Cmi regulates Mad phosphorylation indirectly through regulation of Dpp.

cmi regulates dpp transcription through the 3′disc regulatory region during larval development

The interactions between *cmi* and *tkv* suggested that *cmi* was affecting Dpp signaling by regulating the expression of the *dpp* gene that is expressed in a narrow region near the A–P boundary in the third larval instar imaginal wing disc. Dpp then diffuses into both the anterior and posterior wing regions to regulate growth and to position the longitudinal veins (LV) along the A–P axis (O'Connor et al., 2006). Since we observed a genetic interaction between *cmi* and a mutation in the *dpp* 3′ disc enhancer region, we tested a *dpp* enhancer fusion to the *lacZ* gene (*dppBS3.0-lacZ*) (Blackman et al., 1991) to determine whether *cmi* could regulate *dpp* through the 3′ region (Fig. S1). The *dppBS3.0-lacZ* transgene construct contains 10 kb of disc regulatory sequences linked to a β-galactosidase reporter whose expression recapitulates much of the normal *dpp* expression pattern on the anterior side of the A–P compartment boundary (Blackman et al., 1991; Raftery et al., 1991; Sanicola et al., 1995) (Fig. 5A). Recombinants were generated between the *dppBS3.0-lacZ* reporter and *HA-cmi* as well as the *cmi-IR* to determine if *cmi* had a regulatory influence on *dpp* transcription. We carried out the overexpression and knockdown of *cmi* using an imaginal disc driver, P(GawB)69B-GAL4 at 25 °C. Developmentally staged late L3 larval wing discs were examined using histochemical X-Gal staining to measure *lacZ* expression. Knockdown of *cmi* resulted in strongly reduced *dppBS3.0-lacZ* expression in approximately 70% of the wing imaginal discs examined (Fig. 5B). Depletion of *trr* using the wing-specific *C765-GAL4* driver resulted in strongly reduced *dppBS3.0-lacZ* expression in approximately 50% of the wings examined, consistent with cooperation between *cmi* and *trr* regulating *dpp* transcription (Fig. S2). Conversely, overexpression of *HA-cmi* resulted in a strong increase in *dppBS3.0-lacZ* expression (Fig. 5C). While the overall level of *dppBS3.0-lacZ* expression was modulated in response to increasing or decreasing *cmi*, the pattern was
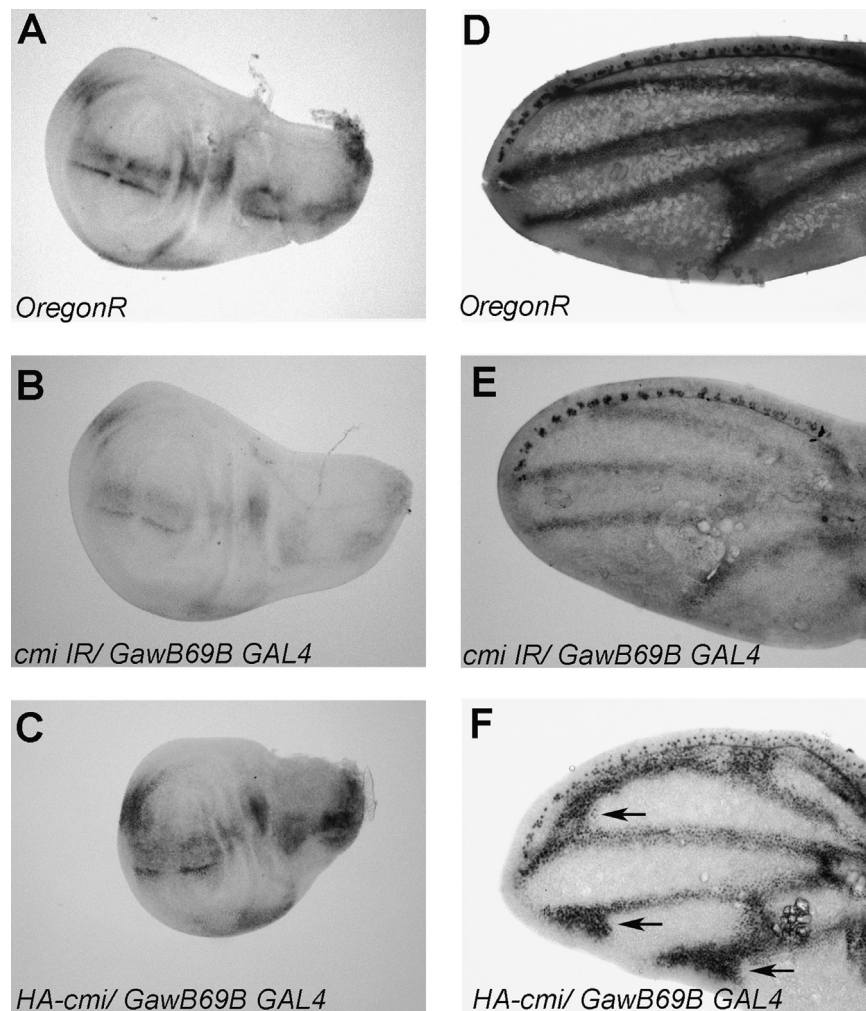
Fig. 4. Phosphorylated Mad levels are sensitive to Cmi. Larval wing discs and pupal wings were immunostained using antibodies against phosphorylated Mad (pMad), a downstream effector of Dpp signaling. (A–C) Wing imaginal discs from third instar larvae. (D–F) Wings from 25–46 h old pupae. (A) Control discs from wild type *OregonR* larvae show pMad immunostaining along the anterior–posterior compartment boundary. (B) *cmi-IR/P[GawB]69B-GAL4* wing discs show a reduction in pMad immunostaining. (C) *HA-cmi/P[GawB]69B-GAL4* wing discs showing diffused or expanded pMad along the A–P boundary. (D) Wild type *OregonR* pupal wings show pMad localized in the wing veins and the anterior wing margin. (E) *cmi-IR/P[GawB]69B-GAL4* pupal wings display a marked decrease in the pMad within the wing veins, although pMAD levels are unchanged in cells along the anterior margin. (F) Pupal wings from *HA-cmi/P[GawB]69B-GAL4* exhibit ectopic pMad in cells near the distal wing margin, as indicated by the arrows. All pictures were taken at the same magnification.

unchanged despite ectopic expression of *cmi* in the *HA-cmi* background. This result suggests that *cmi* is necessary though not sufficient for *dpp* transcription in the larval wing disc to modulate expression within its normal domain.

cmi regulates dpp transcription through the 5′ shv regulatory region during pupal development

Dpp expression is restricted to the presumptive wing veins and margin during pupal development where it acts within its local environment to maintain longitudinal vein (LV) fate as well as at a distance to specify the positions of the crossveins (CV) (de Celis, 1997; Posakony et al., 1990). These events are controlled by Dpp signaling during the pupal stage by the regulation of *dpp* transcription through the 5′ shv regulatory region (Figs. 5D, S1) (Christoforou et al., 2008). The knockdown and overexpression of *cmi* results in wing patterning defects in the distal end of the longitudinal veins and the crossveins. We therefore hypothesized that *cmi* also regulates *dpp* transcription during the pupal stage through the 5′ shv regulatory region. To test this hypothesis, we made use of *dpp^shv-lacZ.RD2* reporter transgene that contains an 8.9 kb region of the *dpp* 5′ region adjacent to the coding exons, linked to a *lacZ* gene (Hursh

et al., 1993). The expression of *lacZ* in this construct mimics normal *dpp* transcription in the pupal wing veins (Fig. 5D) (Christoforou et al., 2008). Recombinants carrying the *dpp^shv-lacZ.RD2* and *HA-cmi* or *cmi-IR* were crossed to *P(GawB)69B-GAL4* at 25 °C. Pupal wings were dissected and *lacZ* expression determined. Expression of the *cmi-IR* resulted in strongly reduced lacZ staining in the wing veins and loss of lacZ staining in the wing margin (Fig. 5E). Moreover, the wing veins appeared to be shortened, consistent with the adult *cmi-IR* phenotype. Similar results were obtained when *dicer* was expressed in the *cmi-IR* background (our unpublished results). In contrast, ectopic overexpression of *HA-cmi* resulted in an elevated level of lacZ staining throughout the wing veins and margin (Fig. 5F). In addition we observed lacZ staining in regions of the pupal wing where ectopic veins appear when *cmi* is overexpressed. Our lacZ staining results confirm that *dpp* is a target of *cmi* regulation either directly or indirectly through the 5′ transcript regulatory region at the pupal stage.

cmi regulates wing pattern development independent of hh

During larval development, Hh is expressed in posterior compartment wing cells and diffuses into the anterior compartment to up-regulate Dpp signaling (Sturtevant and Bier, 1995).
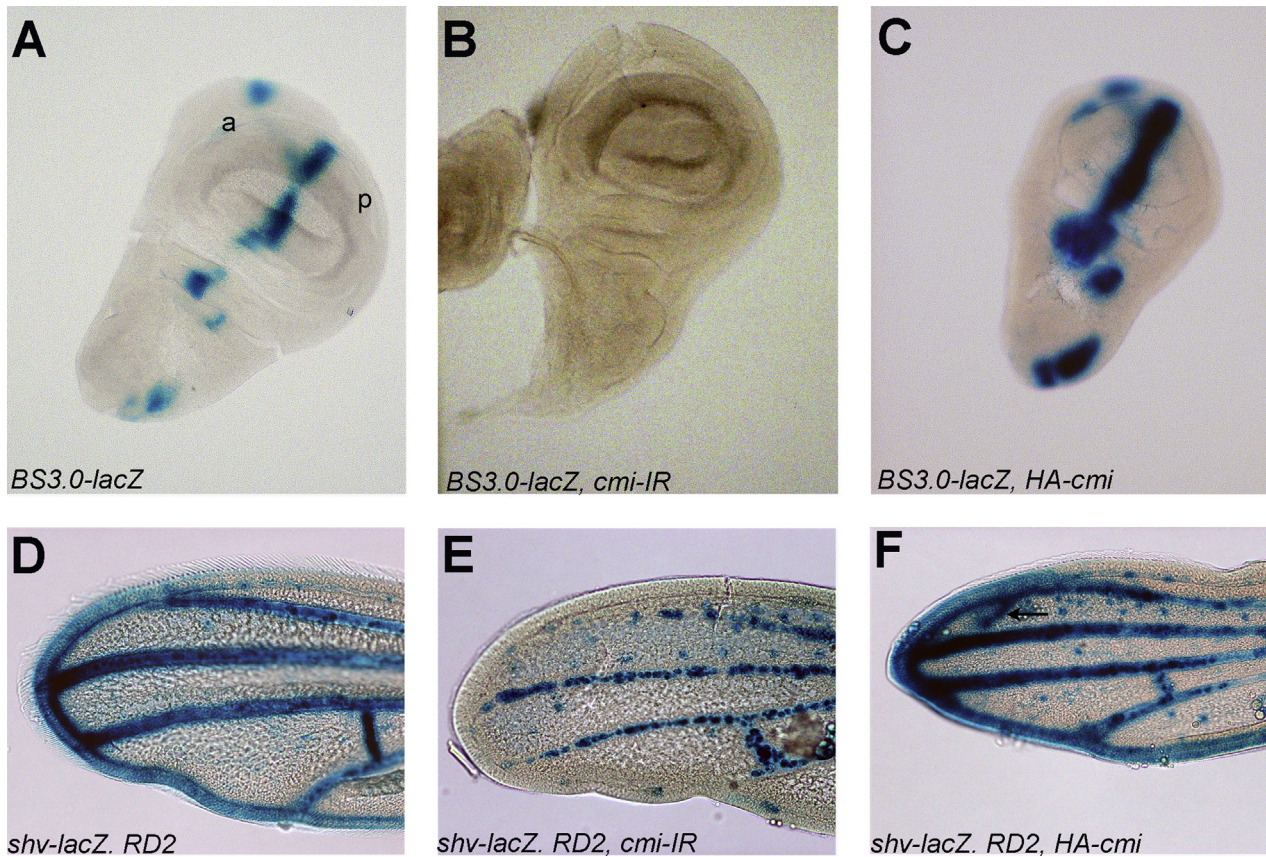
Fig. 5. Cmi positively regulates transcription from the *dpp* 3′ disc enhancer during larval development and the 5′ shv enhancer during pupal development. Larval wing discs and pupal wings were examined for *dpp-lacZ* reporter gene activity using a β-galactosidase activity stain upon knockdown or overexpression of *cmi* with the *P*[GawB]69B-GAL4 driver. (A–C) Wing imaginal discs from third instar larvae were examined using the *dppBS3.0-lacZ* 3′ disc enhancer reporter. (D–F) Wings from 25–46 h old pupae were examined using the *dpp*^{shv}-*lacZ.RD2* 5′ shortvein enhancer. (A) Control wild type *OregonR* wing discs show the expression of the *lacZ* reporter throughout the imaginal disc, primarily along the A–P axis. (B) *dppBS3.0-lacZ, cmi-IR/+* discs show a widespread reduction in *lacZ* expression. (C) *dppBS3.0-lacZ, HA-cmi/+* discs display an increase in lacZ staining within the normal *dppBS3.0* 3′ disc enhancer expression domain. Note that the size of the wing imaginal disc in (B) is slightly larger than the wild type disc in (A), while the disc in (C) is significantly smaller. All pictures were taken at the same magnification. Discs are oriented with anterior to the left. (D) Wild type *OregonR* pupal wings display *dpp*^{shv}-*lacZ.RD2* expression in all the longitudinal veins (L2–L5), the PCV and the wing margin. (E) *dpp*^{shv}-*lacZ.RD2, cmi-IR* pupal wings show incomplete veins along with reduced lacZ staining in the longitudinal veins and wing margin. (F) *dpp*^{shv}-*lacZ.RD2, HA-cmi* wings show elevated lacZ staining as well as ectopic veins (L2, L3 and L4) near the distal wing margin. All pupal wing pictures were taken at the same magnification and all wings were stained in parallel.

Hormone-dependent transcription of the *hedgehog* (*hh*) gene in cultured *Drosophila* S2 cells is regulated by the HMTase activity of Trr (Johnston et al., 2011) and genetic studies have revealed that *trr* regulates *hh* function in the developing *Drosophila* eye (Sedkov et al., 2003). Mutations in *trr* also affect Dpp morphogen signaling in the eye imaginal disc downstream of Hh signaling, presumably through *trr* functions in regulating *hh* expression. Thus, we sought to determine if gain or loss of *cmi* function in the *hh* expressing region could affect patterning in the anterior portion of the wing, consistent with a role in regulating *hh* during larval wing development.

We first addressed this possibility using genetic tests. The Cmi protein is broadly expressed throughout the larval and pupal wing tissues at essentially uniform levels (Fig. S3) and null mutations in *cmi* are completely recessive; however, wing phenotypes associated with strong loss of *cmi* function observed with RNAi (Chauhan et al., 2012) are not similar to *hh* mutant somatic clone phenotypes in the wing (Burke et al., 1999; Kojima et al., 1994; Lee et al., 1992). Heterozygous combinations of a *cmi¹* null allele and the *hh*^{AC} amorphic mutant did not display any obvious interaction phenotype. The *hh*^{moonrat} (*hh*^{MRT}) allele is a dominant gain of function mutation that results in the ectopic expression of *hh* in the anterior compartment, leaving the posterior compartment unaffected (Felsenfeld and Kennison, 1995; Tabata and Kornberg, 1994). The *hh*^{MRT} mutation results in overgrowth with vein pattern

defects near the distal wing margin partly reminiscent of *cmi* overexpression phenotypes; however, increased (*HA-cmi*) or reduced (*cmi-IR*) *cmi* expression had no significant effect on the *hh*^{MRT} phenotype (our unpublished results).

Next, we expressed both *cmi-IR* and the epitope-tagged full length cDNA (*HA-cmi*) in the posterior wing compartment using *P*[en2.4-GAL4]e16E driver that produces GAL4 protein in a pattern that mimics the transcription pattern of the *engrailed* gene (Fig. 6B). Expression of the *cmi-IR* in the posterior wing compartment results in the formation of incomplete veins only in the posterior region of the wing (Fig. 6C). Co-expression of *Dicer-2* to increase the efficiency of *cmi* knockdown (Dietzl et al., 2007) led to a strong wing blistering (Fig. 6D). While reduced *hh* function results in a decrease in the spacing between the L3 and L4 longitudinal veins at the A/P boundary, reduced *cmi* in the posterior compartment had no effect on the L3/L4 spacing, suggesting that *hh* was not affected. Overexpression of the *HA-cmi* using the same *en-GAL4* driver resulted in lethality prior to the emergence of adult flies, possibly due to high level expression in embryos (Chauhan et al., 2012). We therefore carried out a weaker overexpression of *cmi* using *P*[EPgy2]EY06424, a *P*-element insertion that contains a GAL4-responsive enhancer 320 base pair upstream of the 5′ region of *cmi*. We observed that modest overexpression of *cmi* using *en-GAL4* driver resulted in the formation of ectopic veins only in the posterior wing compartment (Fig. 6E).
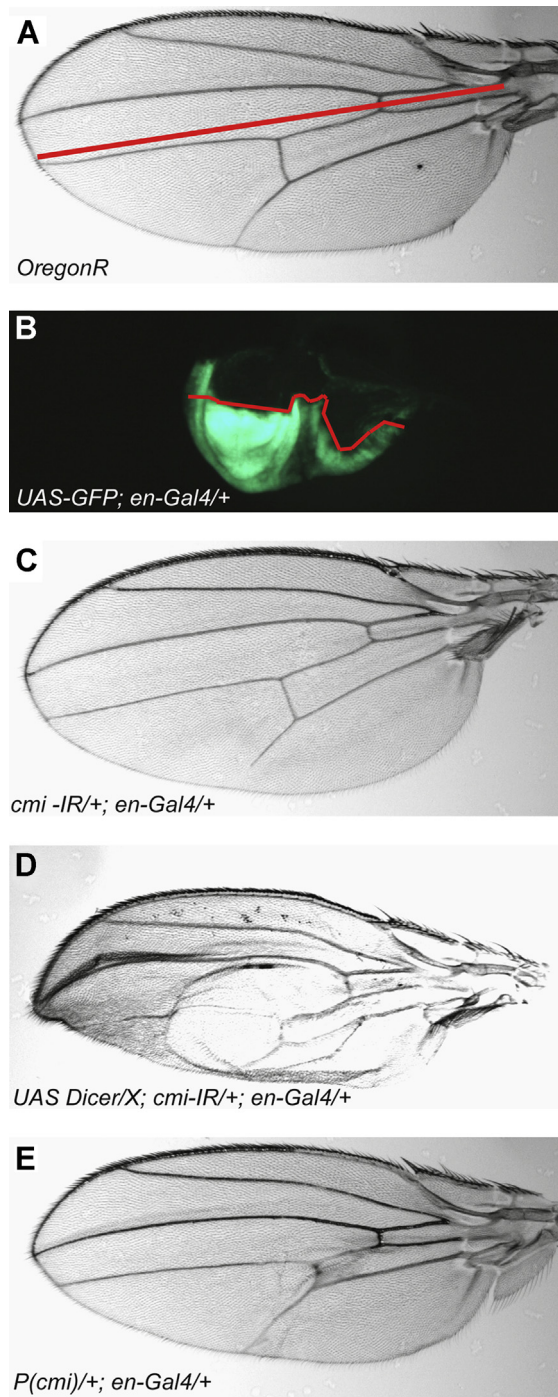
Fig. 6. Targeted depletion and overexpression of *cmi* leads to compartment-specific wing patterning defects. (A) Adult wing from a wild type *OregonR* fly. The position of the *A–P* axis is indicated by the horizontal line. (B) GFP expression is restricted to the posterior compartment in a third instar larval wing imaginal disc of the genotype *P[en2.4-GAL4]e16E/+*; *UAS-GFP/+*. The line marks the approximate boundary between the anterior and the posterior wing compartments. (C) *cmi-IR/+*; *P[en2.4-GAL4]e16E/+* flies exhibit an incomplete L5 vein. (D) *UAS-Dicer/X*; *cmi-IR/+*; *P[en2.4-GAL4]e16E/+* flies display wing blistering in the posterior wing compartment and defects in the L4 and L5 veins. (E) *P(cmi)/+*; *P[en2.4-GAL4]e16E/+* flies show wing blistering in the posterior compartment. *P(cmi)=P[Epgy2] EY06424*. All wing pictures (A, C–E) were taken at the same magnification and all crosses were carried out at 29 °C. In all panels, anterior is at the top.

Since phenotypes associated with *cmi* loss- and gain-of-function were restricted to the posterior compartment in this functional assay, we conclude that Cmi is unlikely to affect *hh* expression levels in the larval wing disc. To address this question
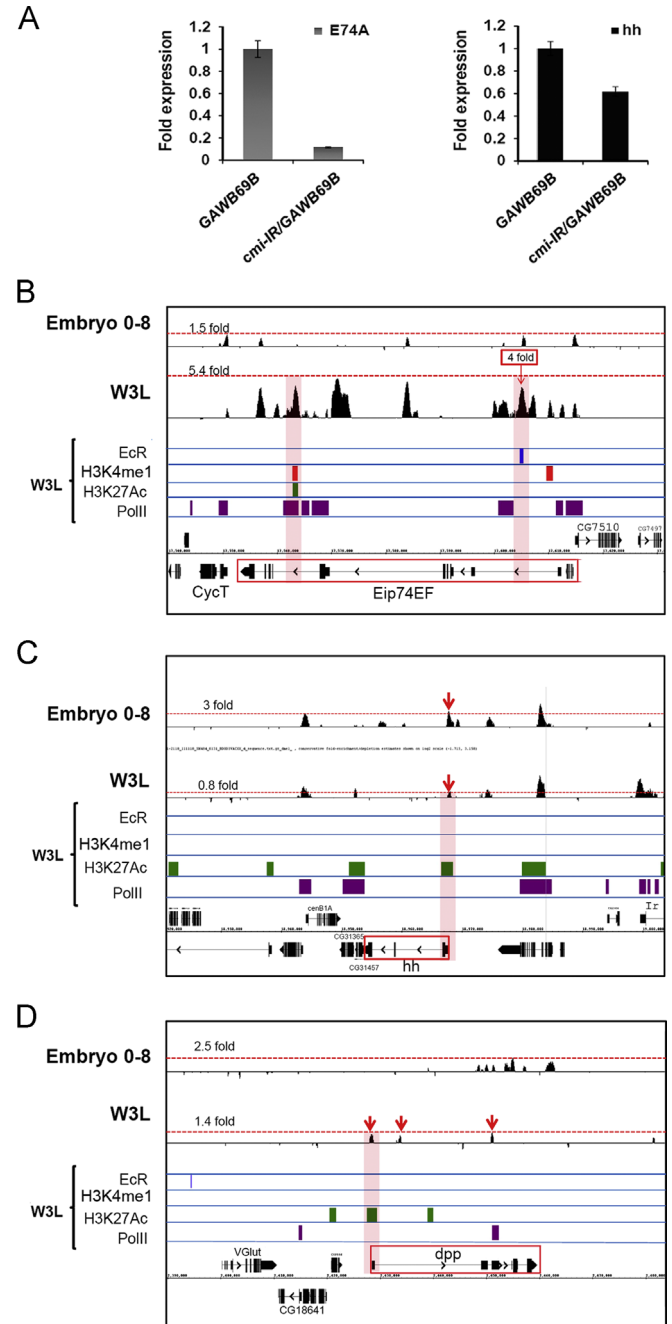
Fig. 7. Cmi directly regulates the early ecdysone-inducible *Eip74Ef* (*E74*) gene and is moderately enriched within the *dpp*, but not the *hh* locus. (A) Quantitative (qPCR) measurements of *Eip74EF* (*E74A*) and *hh* transcripts following widespread knockdown of *cmi* in vivo using *P[GawB]69B-GAL4* driven expression of *cmi-IR*. (B–D) Enrichments of Cmi, EcR, H3K4me1, H3K27Ac and RNA Polymerase II at specific genomic locations. ChIP-seq analyses were carried out using chromatin prepared from embryos (0–8 h after egg laying) and wandering third instar larvae (W3L). Fold enrichments for Cmi are indicated at genomic loci for (B) *Eip74EF*, (C) *hh*, (D) *dpp*, with the maximal enrichment of Cmi at each developmental stage shown by the dashed lines. In panel B, the shaded regions highlight Cmi accumulation that coincides with EcR occupancy and dual H3K4me1 and H3K27Ac marks. In panels C and D, the highest enrichments at the W3L stage are highlighted.

directly, we first examined whether increasing or decreasing *cmi* expression would affect *hh* transcription using a *hh-lacZ* reporter line (Emerald and Roy, 1998). There was no significant change in the pattern or expression level of *hh-lacZ* in discs where we reduced or overexpressed *cmi* (our unpublished results). Second, while shRNAi depletion of *cmi* during the late third larval instar stage resulted in strong reduction of *Eip74EF* transcription, there

was only modest lowering of *hh* transcription measured by qRT-PCR (Fig. 7A). We next examined Cmi protein localization within the *Eip74EF*, *hh* and *dpp* genomic regions using ChIP-seq, both in early embryos (0–8 h after egg laying, E08) and wandering third instar larvae (W3L). Coincident with late larval ecdysone-stimulated transcription of *Eip74EF* (Karim and Thummel, 1991), we observed ~5.4 fold enrichment of Cmi to the *Eip74EF* genomic locus, including colocalization with the ecdysone receptor (EcR, blue bar). The Cmi enrichment sites are present downstream of the promoter and at genomic positions within the *Eip74EF* transcribed region associated with RNA PolII and histone marks (H3K4me1 and H3K27Ac) consistent with an active *cis*-acting transcription enhancer site (Bonn et al., 2012) (Fig. 7B). In contrast, there appeared to be no significant enrichment (0.8 fold) of Cmi within the *hh* genomic locus during the W3L stage; although there was enrichment during early embryogenesis, suggesting possible regulation at other developmental stages (Fig. 7C). Cmi was modestly enriched (1.4 fold) within several regions of the *dpp* locus during the W3L stage, including a region also marked by the presence of H3K27Ac near the *dpp* promoter (Fig. 7D). This enrichment varies by developmental stage and we suspect that it varies in a cell-specific manner, as *dpp* is expressed in the wing disc in a small subset of cells and our assays are unable to resolve at that level. In contrast, there was no obvious enrichment of Cmi or histone marks (data not shown) consistent with activation of the *gbb* gene encoding a Tgfβ receptor that may partner with Dpp (Chen et al., 1998). While the genetic and ChIP data suggests that *hh* is not the likely primary target of Cmi regulation in the larval wing, the combination of genetic and in vivo enhancer reporter assays and modest enrichment of Cmi on portions of the *dpp* locus suggests that Cmi functions downstream of Hh or in another signaling pathway to regulate *dpp* expression in the developing wing.

Discussion

In the present study we demonstrate a critical role for Cmi(Lpt), a component of the COMPASS-related nuclear receptor/co-activator complex, in late tissue patterning during *Drosophila* development through regulation of the Dpp signaling pathway. Key findings that support our model include: (1) phenotypes observed upon the gain and loss of *cmi* function in the wing are consistent with misregulated *dpp* expression. Further, *cmi* genetically functions downstream of *hh* in the wing, interacting with both *dpp* and its receptors. (2) Cmi regulates *dpp* transcription through the 3′ disk regulatory region during larval wing development and through the 5′ shv regulatory region during pupal development. (3) The Cmi protein shows modestly enriched binding near the *dpp* promoter during late larval development, consistent with a possible direct role in regulating *dpp* transcription in the wing epithelium. Although one report indicates a possible role for the estrogen receptor (ER) in regulating *BMP-2* transcription (Zhou et al., 2003), to the best of our knowledge this is the first study to have identified a role for nuclear receptor coactivators as key regulators of *dpp* transcription through the 5′ shv regulatory region.

We previously established that Cmi had broad and important functions in tissue patterning throughout development using analyses of null alleles, expression of silencing RNAs and ectopic overexpression in vivo (Chauhan et al., 2012). Although *cmi* is essential, null allele heterozygotes are fully recessive and tissue-specific depletion of *cmi* using targeted shRNAi results in highly reproducible phenotypes. In contrast, overexpression of tagged HA-Cmi results in patterning defects including ectopic sex combs in males, disruption of eye ommatidia, abdomen pigmentation

defects and ectopic wing veins. These results suggest that *cmi* has dose-limiting functions as a global regulator of tissue patterning during *Drosophila* development.

The appearance of *cmi* gain/loss of function phenotypes is largely dependent on other components of the *Drosophila* COMPASS-like nuclear receptor coactivator complex (Chauhan et al., 2012). Mutations in *cmi* and *trr* (*trithorax-related*) genetically interact and both genes are required for hormone-dependent gene transcription (Chauhan et al., 2012; Sedkov et al., 2003). Trr is a histone H3K4 methyltransferase and is important for positive regulation of *hh* transcription both in the eye imaginal disc and in cultured *Drosophila* S2 cells, where *hh* transcription is dependent on ecdysone (Sedkov et al., 2003). While *cmi* mutant phenotypes are observed in the eye (Chauhan et al., 2012) there is no direct evidence for ecdysone dependent *cmi* regulation of *hh* in S2 cells (our unpublished results). Although the gene targets regulated by *cmi* and *trr* in the wing imaginal disc have not been defined, reduced *cmi* and *trr* function in the wing results in aberrant patterning phenotypes that likely result from disruption of one or more developmental signaling pathways.

Cmi is required for proper wing vein patterning through regulation of the Dpp signaling pathway

Wing vein patterning is governed by multiple signaling pathways that include Hh, Egfr, BMP/Dpp, Wnt/Wg and Notch (reviewed in Blair, 2007). In the wing disc, Hh is present at high levels in the posterior wing compartment where it determines the position of and spacing between L3 and L4 by regulating *iroquois complex* (*iro-c*) and *knot* (Farkas and Knopp, 1997) in the L3 provein and the L3/L4 intervein, respectively (Crozatier et al., 2002; Gomez-Skarmeta and Modolell, 1996; Mohler et al., 2000; Mullor et al., 1997; Vervoort et al., 1999). In contrast, the Dpp signaling pathway has been implicated in the development of the L2 and L5 longitudinal veins by regulating the expression of the *spalt*, *knirps*, and *iro* gene complexes (Biehs et al., 1998; de Celis et al., 1996; Entchev et al., 2000; Gomez-Skarmeta and Modolell, 1996; Teleman and Cohen, 2000). Interactions between the transcription factors encoded by these genes confine the expression of Kni-C and Iro-C to the veins L2 and L5, respectively, (de Celis and Barrio, 2000). Misregulation of Dpp signaling results in ectopic veins as a result of a gain of function and incomplete veins due to a loss of function (Segal and Gelbart, 1985; Spencer et al., 1982).

Based on our observations, the wing phenotypes observed upon misregulation of *cmi* could arise as a consequence of Cmi having normal functions in regulating the Hh signaling pathway upstream of Dpp, through control of *dpp* transcription or by influencing Dpp signaling at a downstream step. We did not observe the classical *hh* phenotypes of reduced spacing between the L3 and L4 veins when *cmi* and *trr* functions were reduced. In addition, the data from our genetic interaction and *lacZ* reporter assays did not support a positive regulatory role for *cmi* on *hh* transcription in the wing imaginal discs. While it is possible that Trr and Cmi regulate *hh* in a tissue-specific manner, such as the eye imaginal disc, any regulation of *hh* is most likely dependent on the specific transcription factor(s) required in a particular tissue and developmental stage. For example, although *hh* appears to be potentially regulated by ecdysone in cultured *Drosophila* S2 (late embryonic) cells (Johnston et al., 2011) as well as the eye imaginal disc (Sedkov et al., 2003), it is neither a significant target in *Drosophila* Kc167 cells (mid-embryonic) that differentiate in response to ecdysone, nor a significant target for in vivo regulation during metamorphosis (Gauhar et al., 2009). During revision of this manuscript, Kanda et al. (Kanda et al., 2013) reported that *trr* mutant clones in the larval eye imaginal disc appeared to display increased pMad expression in contrast with our findings using

knockdown and overexpression of *cmi* in the larval and pupal wing discs, raising the possibility that the complex has tissue specific functions in various developmental pathways.

Cmi affects wing patterning by spatial and temporal regulation of dpp transcription

The *dpp* gene is differentially regulated in various tissues through its cis-regulatory elements (St Johnston et al., 1990). The *dpp* genomic locus consists of an exon-coding region (haploinsufficient or Hin) and two major regulatory regions named short-vein (shv) and imaginal disc specific (disc/d) based on mutant phenotypes (Segal and Gelbart, 1985; Spencer et al., 1982) (Fig. S1). The shv region is located 5′ of the coding exons and it controls expression of *dpp* during pupal development. The 3′ disc region is located 3′ of the coding exons and controls larval expression of *dpp*. We have provided evidence for a positive genetic interaction between *cmi¹* and various loss of function mutations both in the 3′ as well as the 5′ regulatory regions of *dpp* and the Dpp type I receptor genes, *tkv* and *sax*.

In the larval wing imaginal disc, *dpp* transcription is regulated through the 3′ regulatory region in a Hh-dependent manner (Hepker et al., 1999; Müller et al., 2003; Parker et al., 2011). Mutations in the 3′ regulatory disc region result in defects in the imaginal discs and the adult derivatives of the imaginal discs (Blackman et al., 1991; Bryant, 1988; Masucci et al., 1990; Spencer et al., 1982). In the wing imaginal disc, Dpp activates *sal* in early L2 development. Low levels of Sal in turn activate *kni* expression that is required for L2 formation. Higher levels of Sal, on the other hand, repress *kni* leading to loss of L2 (de Celis and Barrio, 2000). We have previously observed a loss of proximal L2 upon *cmi* overexpression that may be explained by an increase in *sal* expression as a result of increased Dpp signaling leading to repression of *kni* in wing imaginal disc and hence the loss of proximal L2. Our *lacZ* reporter assays support this view, showing a positive temporal regulation of *dpp* transcription by *cmi* through the 3′ regulatory region at the larval stage.

During the early pupal stage, *dpp* is expressed independent of Hh within all the presumptive veins leading to increased BMP signaling (de Celis, 1997; Ralston and Blair, 2005; Segal and Gelbart, 1985; Yu et al., 1996). The mechanism of this highly specific regulation is not well understood, though it has been reported that mutations in the *dpp* shv region manifest as incomplete veins that fail to reach the wing margin (de Celis, 1997; Segal and Gelbart, 1985; Sotillos and de Celis, 2006; St. Johnston et al., 1990). We found a similar shortened vein phenotype associated with *cmi* knockdown that is consistent with reduced *dpp* expression within presumptive vein cells near the distal wing margin. Our results from genetic epistasis tests using a *cmi¹* null allele are consistent with this view as we observed an enhancement of the *dpp* shv phenotype.

Overexpression of *HA-cmi* in the pupal wing results in ectopic veins that invariably extend from existing veins and frequently appear as 'new' crossveins connecting two longitudinal veins. We suggest that this phenotype reflects ectopic Dpp signaling, as Dpp is required for the proper formation of the crossveins as well as the full extension of the longitudinal veins during pupal development and the ectopic veins express *dpp* through the 5′ shv enhancer region.

Similar to MLL2 and MLL3 in mammals, Cmi functions in concert with nuclear receptors in *Drosophila* to regulate hormone inducible gene expression. It has been reported that the expression of some genes in the BMP/Dpp pathway is regulated in the embryo midgut region through an ecdysone and EcR dependent mechanism (Li and White, 2003). However, there is no evidence for direct regulation of *dpp* transcription in cultured *Drosophila* Kc167 or S2 cells through hormone dependent pathways

(our unpublished observations and (Gauhar et al., 2009)) and the *cmi* wing phenotypes are not substantially modified by reduced EcR function (Chauhan et al., 2012). It is somewhat perplexing why the overexpression of a nuclear receptor cofactor that is usually associated with a large complex, would lead to ectopic expression of *dpp*. In this regard, it is important to note that despite widespread expression of *HA-cmi*, the ectopic veins and *dpp-lacZ* expression are restricted to certain distal regions of the wing, suggesting that Cmi is necessary but not sufficient for *dpp* transcription. Since Cmi and its associated cofactors are presumably recruited to target sites through binding of specific transcription factors, it is likely that the phenotype associated with overexpression of Cmi may be the result of misregulation of an unknown factor. A strong candidate for this is Usp, the *Drosophila* ortholog of the vertebrate RXR receptor, as Cmi directly interacts with Usp and targeted depletion of *usp* in the wing generally suppresses the *HA-cmi* ectopic vein phenotype (Chauhan et al., 2012). If *dpp* is directly regulated by Usp it would likely be EcR independent, as there is no significant binding of EcR/Usp heterodimers in the *dpp* genomic region (Gauhar et al., 2009). In this context, *dpp* regulation might involve Usp partnering with a different receptor and may even be ligand independent. The Cmi/Trr complex may also regulate Dpp signaling via the ETS-domain transcription factor E74, as ecdysone signaling through E74 plays a significant role in BMP signaling in germline stem cell (GSC) maintenance (Ables and Drummond-Barbosa, 2010) and ecdysone signaling via EcR/Usp is required for the development of GSC precursors (Gancz et al., 2011). Our chromatin binding data also leaves open the possibility that the Cmi/Trr complex might directly regulate *dpp* transcription in a subset of cells along the A/P border in the developing wing, likely in collaboration with sequence-specific transcription factors.

Models of Cmi function upstream and downstream of dpp

In the pupal wing, Dpp (BMP2/4) forms a heterodimer with Gbb, a BMP-5/6/7/8 like protein to carry out Tgfβ signaling (Doctor et al., 1992; Khalsa et al., 1998). Dpp and Gbb signal through two Type I Tgfβ receptors, Sax and Tkv and a type II receptor, Punt (Affolter and Basler, 2007). Misexpression of Dpp, Gbb or an activated form of Tkv leads to ectopic venation (Bangi and Wharton, 2006; de Celis, 1997; Sotillos and de Celis, 2006; Terracol and Lengyel, 1994). Similarly, reducing *tkv* function leads to vein thickening, due to spreading of the Dpp signal (de Celis, 1997; Marenda et al., 2004). Our hypothesis that Cmi positively regulates Dpp signaling is further supported by the enhancement of *tkv* knockdown phenotypes upon *cmi* overexpression and the reciprocal suppression of these phenotypes upon *cmi* knockdown.

An alternate scenario is that Cmi contributes to the regulation of downstream components of the Dpp signaling pathway. We previously found that selective GAL4-dependent RNAi depletion of genes encoding *Drosophila* COMPASS complex components, such as *wds*, *ash2* and *utx*, enhanced the *cmi-IR* shortened vein phenotype and suppressed the ectopic veins associated with *HA-cmi* overexpression (Chauhan et al., 2012). Ash2 is known to positively regulate intervein specific genes *net* and *bs*, and negatively regulate the L2 specifying gene *kni* (Angulo et al., 2004). Knockdown of mammalian *MLL2* in HeLa cells results in a decrease in *MADH6* expression, a downstream effector of BMP signaling (Issaeva et al., 2007). In both examples, however, the regulatory effect may be an indirect consequence of decreased Dpp/BMP signaling. Widespread overproduction of Dpp (from ectopic expression) results in tissue overgrowth and is generally lethal, while decreased Dpp is associated with reduced cell division (Affolter and Basler, 2007). In contrast, increasing Cmi levels results
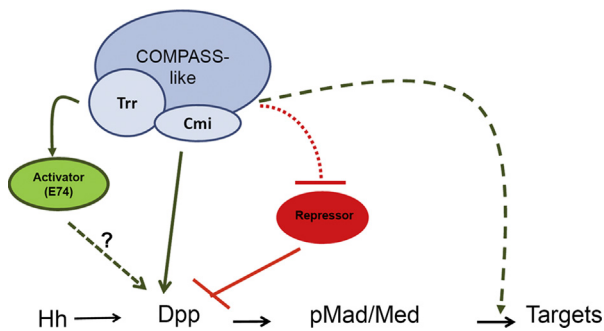
Fig. 8. Model of Cmi/COMPASS-like complex function in regulating the Dpp signaling pathway. Cmi regulates transcription of *dpp* as part of the *Drosophila* COMPASS-like complex that contains the Trr methyltransferase and additional subunits. The Cmi complex can positively regulate Eip74EF (E74) expression that may in turn control transcription of *dpp*. Genetic evidence also suggests that Cmi might directly regulate transcription through enhancers within the *dpp* genomic locus at different stages of development. Cmi may also regulate the expression of repressors of *dpp* transcription or targets of the Dpp signaling pathway through collaborations with additional transcription factors.

in reduced tissue growth while knockdown of Cmi produces larger animals, presumably through increased growth. When Dpp is over-expressed simultaneously with the *cmi-IR* using GAL4, we observed only a few rare escapers that displayed strong ectopic *dpp* phenotypes (our unpublished observations). Overexpression of both Dpp and Cmi together resulted in flies that survived with pattern defects similar to both (ectopic wing veins). Thus, overexpression of Cmi rescues the lethality associated with high level Dpp, suggesting that Cmi might function downstream of Dpp to control Dpp targets involved in cell growth regulation. Although we cannot rule out the possibility that Cmi and the *Drosophila* COMPASS-like complex functions to control downstream Dpp targets, phenotypes associated with GAL4-directed over expression of *dpp* were not suppressed by removing *cmi* function.

We can envisage several ways through which the Cmi/Trr *Drosophila* COMPASS-like complex affects wing patterning via the Dpp signaling pathway based on our findings that Cmi regulates *dpp* transcription (Fig. 8). Although our genetic and protein localization data does not rule out the possibility that the Cmi/Trr complex might directly control Hh expression in a subset of cells, the genetic and chromatin binding data would suggest that the control of Dpp is likely functioning downstream of Hh or through a parallel pathway.

In one scenario the Cmi/Trr COMPASS-like complex regulates the expression of a positive factor required for transcription of *dpp*. One possibility is the hormone responsive E74 transcription factor discussed above; although, it is presently unknown if E74 can directly bind within the *dpp* enhancer regions. Based on the ChIP-seq data, Cmi might also associate with the *dpp* control regions through interaction with enhancer binding transcription factors. Alternatively, the Cmi/Trr complex might negatively regulate an inhibitory factor ('Repressor' in the model) that normally functions to restrict or inhibit *dpp* transcription, such as the Dpp target *brinker* (Blair, 2007; Campbell and Tomlinson, 1999; Cook et al., 2004; Jazwinska et al., 1999; Müller et al., 2003; Ziv et al., 2009). In fact, Brk is a DNA-binding transcriptional repressor with putative binding sites within the *dpp* shv region (Sotillos and de Celis, 2006). Finally, we and others have recently discovered that Cmi (Lpt) and Trr in *Drosophila* (our unpublished data and Herz et al., 2012) and the orthologous human MLL2 complex (Guo et al., 2012) are enriched at many enhancers and gene promoters, opening the possibility that the Cmi/Trr COMPASS-like complex might serve as a coactivator with other unknown transcription factors, some of which might regulate downstream targets of signaling pathways, such as Dpp (our unpublished data).

Conclusions

Our data reveals an important role for Cmi in tissue patterning through its function in a COMPASS-like complex, presumably by mediating the transcriptional regulation of critical target genes. In particular, this likely involves the control of BMP/Tgfβ mediated Dpp signaling during the development of the wing tissue. We conclude that this role is likely through Hh-independent, direct regulation of *dpp,* although the specific transcription factors that Cmi interacts with need to be identified. Furthermore, these results provide a framework for understanding how MLL2 and MLL3 might be involved in regulating cellular adhesion and tumorigenicity, as well as the BMP/Tgfβ signaling pathway in mammalian tissue patterning and cancer (Massague, 2008).

Acknowledgments

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2013.05.018.

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