Developmental Characterization of Cara Mitad: A Drosophila Nuclear Receptor Co-Regulator

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LOYOLA UNIVERSITY CHICAGO

DEVELOPMENTAL CHARACTERIZATION OF CARA MITAD:
A DROSOPHILA NUCLEAR RECEPTOR CO-REGULATOR

A DISSERTATION SUBMITTED TO
THE FACULTY OF THE GRADUATE SCHOOL
IN CANDIDACY FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

PROGRAM IN MOLECULAR BIOLOGY

BY

CHHAVI CHAUHAN

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“To Ma and Papa”
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<td>20E</td>
<td>20 Hydroxy Ecdysone</td>
</tr>
<tr>
<td>ACT</td>
<td>Actin</td>
</tr>
<tr>
<td>ACV</td>
<td>Anterior Cross Vein</td>
</tr>
<tr>
<td>AF</td>
<td>Activation Function</td>
</tr>
<tr>
<td>ALL</td>
<td>Acute Lymphocytic Leukemia</td>
</tr>
<tr>
<td>ALR</td>
<td>ALL Related</td>
</tr>
<tr>
<td>A/P</td>
<td>Anterior- Posterior</td>
</tr>
<tr>
<td>ASC-2</td>
<td>Activating Signal Cointegrator-2</td>
</tr>
<tr>
<td>ASCOM</td>
<td>ASC-2 Complex</td>
</tr>
<tr>
<td>ASH2</td>
<td>Absent Small Homeotic 2</td>
</tr>
<tr>
<td>B</td>
<td>Bar</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone Morphogenetic Protein</td>
</tr>
<tr>
<td>BR-C</td>
<td>Broad-Complex</td>
</tr>
<tr>
<td>BRM</td>
<td>Brahma</td>
</tr>
<tr>
<td>CMI</td>
<td>Cara Mitad</td>
</tr>
<tr>
<td>CpG</td>
<td>Cytosine Poly Guanine</td>
</tr>
</tbody>
</table>
CV    Cross Vein
CyO   Curly O
Cys   Cysteine
D/ Dsk Disk
DBD   DNA binding domain
Df    Deficiency
DHR38 Drosophila Hormone Receptor 38
DPP   Decapentaplegic
Drev  Dora Reverse Transcriptase
E(z)  Enhancer of Zeste
Ecd   Ecdysoneless
EcR   Ecdysone Receptor
EcRE  Ecdysone Responsive Element
EGFR  Epidermal Growth Factor Receptor
ERα   Estrogen Receptor α
FM    First Multiple
FYR   Phenylalanine and Tyrosine Rich
FYRC  FYR C-terminus
FYRN  FYR N-terminus
Gal4  Galactose 4
Gbb   Glass Bottom Boat
GFP   Green Fluorescent Protein
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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</thead>
<tbody>
<tr>
<td>H</td>
<td>Histone</td>
</tr>
<tr>
<td>H3K4me3</td>
<td>Histone H3 Lysine 4 trimethylated</td>
</tr>
<tr>
<td>HA</td>
<td>Hemagglutinin</td>
</tr>
<tr>
<td>HAT3</td>
<td>Homeobox leucine Zipper Protein 3</td>
</tr>
<tr>
<td>Hh</td>
<td>Hedgehog</td>
</tr>
<tr>
<td>Hh&lt;sup&gt;MRT&lt;/sup&gt;</td>
<td>Hedgehog&lt;sub&gt;Moon Rat&lt;/sub&gt;</td>
</tr>
<tr>
<td>Hin</td>
<td>Happloinsufficiency</td>
</tr>
<tr>
<td>His</td>
<td>Histidine</td>
</tr>
<tr>
<td>HMG</td>
<td>High Mobility Group</td>
</tr>
<tr>
<td>HMTase</td>
<td>Histone Methyl Transferase</td>
</tr>
<tr>
<td>HRP</td>
<td>Horse Radish Peroxidase</td>
</tr>
<tr>
<td>IR</td>
<td>Inverted Repeat</td>
</tr>
<tr>
<td>iro-c</td>
<td>Iroquois Complex</td>
</tr>
<tr>
<td>JH</td>
<td>Juvenile Hormone</td>
</tr>
<tr>
<td>KOH</td>
<td>Potassium hydroxide</td>
</tr>
<tr>
<td>kn</td>
<td>Knot</td>
</tr>
<tr>
<td>L</td>
<td>Longitudinal Vein</td>
</tr>
<tr>
<td>L 1/2/3/4/5</td>
<td>Longitudinal Vein 1/2/3/4/5</td>
</tr>
<tr>
<td>LBD</td>
<td>Ligand Binding Domain</td>
</tr>
<tr>
<td>LXR</td>
<td>Liver X Receptor</td>
</tr>
<tr>
<td>MLL</td>
<td>Mixed Lineage Leukemia</td>
</tr>
<tr>
<td>NIG</td>
<td>National Institute of Genetics</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>NR</td>
<td>Nuclear Receptor</td>
</tr>
<tr>
<td>ORF</td>
<td>Open Reading Frame</td>
</tr>
<tr>
<td>PA1</td>
<td>PTIP Associated 1</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffer Saline</td>
</tr>
<tr>
<td>PBST</td>
<td>PBS Tween</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PCV</td>
<td>Posterior Cross Vein</td>
</tr>
<tr>
<td>PHD</td>
<td>Plant Homeo Domain</td>
</tr>
<tr>
<td>PRIP</td>
<td>Peroxisome Proliferator-Activated Receptor G- Interacting Protein</td>
</tr>
<tr>
<td>PTIP</td>
<td>Pax Transactivation Domain-Interacting Protein</td>
</tr>
<tr>
<td>RAR</td>
<td>Retinoic Acid Receptor</td>
</tr>
<tr>
<td>RBQ3</td>
<td>Retinoblastoma Binding Protein 3</td>
</tr>
<tr>
<td>rp49</td>
<td>Ribosomal Protein 49</td>
</tr>
<tr>
<td>RT</td>
<td>Room Temperature</td>
</tr>
<tr>
<td>RXR</td>
<td>Retinoid X Receptor</td>
</tr>
<tr>
<td>Sax</td>
<td>Saxophone</td>
</tr>
<tr>
<td>Sco</td>
<td>Scutoid</td>
</tr>
<tr>
<td>SET</td>
<td>Suvar 3-9, Enhancer of Zeste, Trithorax</td>
</tr>
<tr>
<td>Sh</td>
<td>Short Hair-pin</td>
</tr>
<tr>
<td>Shv</td>
<td>Shortvein</td>
</tr>
<tr>
<td>SM</td>
<td>Second Multiple</td>
</tr>
<tr>
<td>SNR1</td>
<td>SNF 5 Related 1</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td><strong>Su(var)</strong></td>
<td>Suppressor of Variegation</td>
</tr>
<tr>
<td><strong>SWI/SNF</strong></td>
<td>Switch/ Sucrose Non-fermenting</td>
</tr>
<tr>
<td><strong>Tkv</strong></td>
<td>Thickveins</td>
</tr>
<tr>
<td><strong>TM</strong></td>
<td>Third Multiple</td>
</tr>
<tr>
<td><strong>Trr</strong></td>
<td>Trithorax Related</td>
</tr>
<tr>
<td><strong>Trx</strong></td>
<td>Trithorax</td>
</tr>
<tr>
<td><strong>UAS</strong></td>
<td>Upstream Activating Sequence</td>
</tr>
<tr>
<td><strong>Usp</strong></td>
<td>Ultra Spiracle</td>
</tr>
<tr>
<td><strong>UTX</strong></td>
<td>Ubiquitously Transcribed X Chromosome Tetratricopeptide Repeat Protein</td>
</tr>
<tr>
<td><strong>VDRC</strong></td>
<td>Vienna Drosophila RNAi Center</td>
</tr>
<tr>
<td><strong>WD</strong></td>
<td>Tryptophan (W) Aspartic Acid (D)</td>
</tr>
<tr>
<td><strong>WDR5</strong></td>
<td>WD Repeat Domain 5</td>
</tr>
<tr>
<td><strong>Wds</strong></td>
<td>Will Die Slowly</td>
</tr>
<tr>
<td><strong>Wg</strong></td>
<td>Wingless</td>
</tr>
<tr>
<td><strong>WT</strong></td>
<td>Wild Type</td>
</tr>
<tr>
<td><strong>α</strong></td>
<td>Alpha</td>
</tr>
<tr>
<td><strong>β Gal</strong></td>
<td>β Galctosidase</td>
</tr>
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CHAPTER-1

INTRODUCTION

Cara Mitad (CMI) shows homology to the N-terminal half of mammalian Mixed Lineage Leukemia 2 (MLL2). Here we establish that in Drosophila, CMI plays a critical role in tissue patterning and development. We demonstrate that CMI controls wing vein patterning through the Decapentaplegic (DPP) signaling pathway by using multiple approaches that include genetic epistasis tests, tissue specific knock-down and over-expression of cmi as well as the components of the DPP signaling pathway, and DPPLACZ reporter assays. The loss of function allele, CMI1, enhances loss of DPP function phenotypes in genetic epistasis tests. Wing specific knock-down of CMI results in incomplete veins toward the distal wing margin that are enhanced by the simultaneous knock-down of DPP. In contrast, the over-expression of a tagged full-length HA-CMI transgene results in ectopic veins which are suppressed upon a simultaneous knock-down of DPP. The knock-down and over-expression of CMI, respectively, results in reduced and increased DPP signaling as observed by immuno-staining for phospho-MAD (Mothers against DPP), a DPP downstream effector. The knock-down of CMI suppresses the knock-down of TKV phenotype while the over-expression of HA-CMI enhances TKV knock-down phenotypes. We further show that CMI controls wing vein patterning by regulating DPP transcription through the 3’disk regulatory region at the larval stage and
through the 5’ shortvein (shv) regulatory region at the pupal stage. We suggest that the regulation of *DPP* expression by CMI is a key part of the mechanism that controls wing vein patterning.
CHAPTER-2

LITERATURE REVIEW

2.1 Drosophila as a model organism

*Drosophila melanogaster*, commonly known as the fruit fly, was used as a model system for the first time in the 1900s by Thomas Hunt Morgan to study genetics. Since then, Drosophila has served as a model organism to study almost every aspect of eukaryotic biology that ranges from gene regulation, metabolic/development pathways, embryonic development and behavior to genetic disorders and disease progression. The genome of the fly has been sequenced, leading to the identification of over 13,000 protein-coding genes, thus providing an invaluable resource to fly biologists. Moreover, an unparalleled array of genetic and molecular tools has been generated in Drosophila that make it a unique model organism.

The fact that the genes and the signaling pathways that are present in Drosophila are conserved in other eukaryotes further augments the use of fly to study the complex biology of multicellular organisms. In addition, the life cycle of Drosophila is short, yet, represents various stages of development that are regulated by hormone titers. This dependence of Drosophila development on hormone titers allows us to study the conserved hormone mediated regulation of genes in mammals that is involved not only in development but also in the manifestation of diseases due to hormonal imbalance.
2.2 Hormone dependent regulation of Drosophila Development

The Drosophila development is temperature dependent, being slower at lower temperatures and much faster at higher temperatures, with the life span of about 30 days at 29 °C. The Drosophila life cycle consists of the following five structurally and functionally different stages: the embryo, larva, pre-pupa, pupa and adult (Figure 1). During embryogenesis, insect body plan is created by the maternal and zygotic gene expression, by establishing polarity, segmentation and segment identity, in the order mentioned. After hatching, a series of three larval stages, or instars, occur as the insect feeds and grows, namely, L1, L2 and L3. Transition between each instar is associated with molting when the existing larval cuticle undergoes partial digestion after detaching from the underlying epidermis (apolysis), followed by the shedding of the cuticle (ecdysis).

After ecdysis, a new cuticle is synthesized by the underlying epidermis and secreted. A fully grown L3 larva crawls out of the food, enters a wandering stage, ceases movement in a few hours, becomes immobile and secretes glue proteins from the salivary glands to stick to a dry surface to form a prepupa. At this point, L3 cuticle detaches and a pupal cuticle is secreted, however, the L3 cuticle is retained. This L3 cuticle undergoes tanning and serves as a pupal case that prevents infection and desiccation at this stage. The mature pupa undergoes metamorphosis to give rise to an adult {reviewed in (Riddiford 1993; Berger and Dubrovsky 2005)}. The transition from an embryo to an adult fly takes about 10 days at 25 °C. Every step of this transition is guided by the
fluctuations in hormone titers that allow global gene reprogramming at each developmental step.

Drosophila development is governed by three hormones that include peptide, steroid and sesquiterpenoid lipid-like hormones, namely prothoracicotropic hormone (PTPH), 20 hydroxy ecdysone (20E) (or ecdysone hormone) and juvenile hormone (JH), respectively (Mitchell and Smith 1988; Riddiford 1993; Nijhout 1994; Riddiford 1996; Gilbert, Rybczynski et al. 2002). In the larvae, PTPH triggers the synthesis of ecdysone from the prothoracic gland which is then converted into the active form, 20E, in the mitochondria present in the fat bodies and other tissues (Mitchell and Smith 1988). Levels of 20E and JH then control various developmental transitions. High titers of JH accompanied by peaks of 20E initiate larval molting. At the end of L3, the drop in JH titers and a major peak in 20E initiates puparium formation and the synthesis of pupal cuticle (Figure 1) {reviewed in (Riddiford 1993; Berger and Dubrovsky 2005)}. The onset of adult development requires an increase in 20E titers in the absence of JH (Figure 1) {reviewed in (Riddiford 1993; Berger and Dubrovsky 2005)}. 
Figure 1 Fluctuations in hormone levels drive the progression of development

The developmental stages of Drosophila (bottom) are shown relative to the titers of the steroid hormone ecdysone and the sesquiterpinoid juvenile hormone (JH) (top). The fluctuation of ecdysone titers is shown as the red line, while the JH titer is depicted with a black line. Rising ecdysone levels trigger each of the developmental transitions. Adapted and modified from the review by Tatiana Kozlova and Carl S. Thummel, Trends in Endocrinology and Metabolism, Volume 11, No. 7, 2000.
2.2.1. Role of JH in Drosophila development

JH regulates a range of physiological and developmental processes at all stages of Drosophila development. At the larval stage, high titers of JH ensure that high 20E levels trigger larval molts and prevent metamorphosis. On the other hand, low JH titers at pupal stage prevent the premature differentiation of imaginal discs. In the adult females, JH is required for vitellogenesis and egg production, while, in the adult males, JH is required for normal mating behavior and protein synthesis in male accessory glands {reviewed in (Berger and Dubrovsky 2005)}. METHOPRENE TOLERANT (MET) and ULTRASPITALIRACLE (USP) have been suggested to be possible JH receptors, that can likely mediate some of these functions (Wilson and Fabian 1986; Shemshedini, Lanoue et al. 1990; Riddiford and Ashburner 1991; Jones and Sharp 1997; Pursley, Ashok et al. 2000; Jones, Wozniak et al. 2001; Xu, Fang et al. 2002; Wilson 2004; Miura, Oda et al. 2005).

Although the mechanisms of JH action are not yet understood, JH responsive targets have been identified in various insects. In Drosophila, studies in cultured cells have demonstrated that JH inhibits 20E induction of four small HSP genes and a cluster of micro-RNA encoding genes, MIR-100, MIR-125, and LET-7 (Berger, Goudie et al. 1992; Sempere, Sokol et al. 2003). Also, in the salivary glands, JH has been shown to interfere with the induction of ecdysone puffs in response to 20E (Richards 1978). By administration of exogenous JH before pupa to adult transition, JH has been shown to regulate the 20E-dependent expression of Broad-Complex (BR-C) resulting in re-
expression of *BR-C* and recurrence of the pupal development plan in the Drosophila abdomen (Zhou and Riddiford 2002). Consequently, JH-treated pupae were shown to develop into pupal-adult intermediates that had an adult-like head and thorax, but a pupa-like abdomen (Riddiford and Ashburner 1991). Studies in cultured Drosophila cells have demonstrated that an early ecdysone inducible gene *E75A* can also be induced by JH (Dubrovsky, Dubrovskaya et al. 2004). These authors proposed three functions for *E75A* that included auto down-regulation, promotion of expression of secondary JH-target genes like *JHL-21*, and repression of early ecdysone genes including *BR-C* gene.

Recently, micro-array analysis and Northern blot analysis with RNA isolated from cultured larval organs has allowed the identification of three classes of JH regulated genes. These classes include genes that are induced by JH alone like *E74B*, *PEPCK* and *CG14949*; genes induced by JH in combination with 20E genes like *CG7906*, *CG7924*, *MMNDC*, *BLACK* and *CG11956*; and genes whose JH dependent induction is blocked with 20E like *E74B* and *PEPCK* (Beckstead, Lam et al. 2007). Further experiments need to be carried out to understand the cross-talk between different hormone signaling pathways in Drosophila to regulate complex gene transcription.

A JH response element (JHRE) has been identified in a JH responsive gene, *JHP21* from *Locusta migratoria* and *JH Estersase* from *Choristoneura fumiferana* (Zhang, Saleh et al. 1996; Zhou, Zhang et al. 2002; Kethidi, Li et al. 2006). Microarray analysis in cultured cells from Drosophila and *Apis mellifera* has allowed the identification of 16 JH-induced genes which have been used to screen for JHRE binding proteins using DNA affinity columns (Whitfield, Ben-Shahar et al. 2006; Li, Zhang et al. 2007). Two proteins, FKBP39 and CHD64 have been identified as JHRE binding
proteins that have been shown to interact with each other as well as with ECDYSONE RECEPTOR (ECR), USP and MET, using both yeast two hybrid assays and GST pull-down experiments (Li, Zhang et al. 2007). Current models suggest that ECR and USP can heterodimerize with FKBP (Alnemri, Fernandes-Alnemri et al. 1994) and other cofactors, bind to ecdysone responsive elements (ECREs) of ecdysone responsive genes and regulate their transcription during metamorphosis. During molting, on the other hand, high titers of both JH and 20E might result in a competition and consequently, the expression of JH responsive genes through their regulation from JHREs. The identification of proteins that bind to JH as well as multi-protein complexes that bind to JHRE, will further our understanding of JH action.

2.2.2. Role of 20E in Drosophila development

Changes in the titer of 20E coordinate gene expression during the development of Drosophila. The molecular mechanism by which 20E controls metamorphosis has been elucidated using studies based on the puffing patterns of the giant larval salivary gland polytene chromosomes (Clever 1964). Experiments examining changes in the puffing patterns of the larval polytene chromosomes upon 20E stimulation have proposed a hierarchical model whereby 20E promotes the appearance of the early puffs (Ashburner 1974; Ashburner, Chihara et al. 1974; Ashburner and Richards 1976; Thummel 1996). This hierarchical model has been confirmed by molecular characterization of the early puff genes.

The 2B5, 74EF and 75B early puffs contain the BR-C, E74 and E75 genes, respectively, that encode families of transcription factors (Burtis, Thummel et al. 1990;
Segraves and Hogness 1990; DiBello, Withers et al. 1991) that are required for developmental responses to ecdysone during metamorphosis (Kiss, Beaton et al. 1988; Restifo and White 1991) and directly regulate gene transcription (Urness and Thummel 1995; Crossgrove, Bayer et al. 1996).

**BR-C** encodes a family of related transcription factors that share a common core region and contain one of four possible pairs of zinc finger domains (DiBello, Withers et al. 1991; Bayer, Holley et al. 1996). *E74* consists of two overlapping ecdysone-inducible transcription units, *E74A* and *E74B*. These transcripts encode two isoforms, E74A and E74B, respectively, which contain distinct N-terminal domains and a common C-terminal domain with an ETS DNA-binding motif (Burtis, Thummel et al. 1990). The *E74B* transcript is induced first, at ecdysone levels 25-fold lower than *E74A* (Karim and Thummel 1991).

*E75* uses nested promoters and encodes three isoforms, E75A, E75B and E75C, that are orphan nuclear receptors in the nuclear hormone receptor superfamily (Feigl, Gram et al. 1989; Segraves and Hogness 1990). Analysis of expression pattern and the effects of mutations in these early genes has provided evidence that these transcription factors mediate 20E response in different tissues and developmental stages by controlling parallel genetic pathways (Thummel 1996). The early genes encode for transcription factors that trigger the transcription of secondary response genes known as the late genes that include transcription factors as well as genes important for various developmental events. It is the regulation of the early and late genes by ecdysone that pushes Drosophila cells through the different stages of development (Riddiford 1993; Huet, Ruiz et al. 1995; Thummel 1996; Henrich, Rybczynski et al. 1999).
Ecdysone activates transcription of early response genes by binding to heterodimeric receptor complex composed of ECR and USP (Yao, Segraves et al. 1992; Yao, Forman et al. 1993). Genetic and molecular analysis of both of these genes suggests that these molecules are essential for normal development and act as predicted at the top of the ecdysone response hierarchy (Oro, McKeown et al. 1992; Bender, Imam et al. 1997). However, subtle phenotypic differences between USP and ECR mutants suggest the possibility that receptors other than the ECR-USP heterodimer could be involved in mediating a response to ecdysteroids (Buszczak and Segraves 1998). These possibilities are discussed in greater details in the following sections.

2.3 An over-view of the nuclear receptor superfamily

Nuclear receptors (NR) are highly conserved transcription factors that are activated upon ligand binding (Evans 1988; Olefsky 2001). 48 NRs have been identified in the human genome that can be subdivided into six subfamilies based on amino acid sequence identity (Figure 2) (Laudet 1997; 1999; Zhang, Burch et al. 2004; King-Jones and Thummel 2005). The structure of NRs can be divided into six domains, an N-terminal A/B regulatory domain that contains ligand-independent transactivation function (AF1); a highly conserved DNA binding domain (DBD) called the C domain that contains two zinc fingers which bind to specific hormone response elements (HREs); a hinge region called the D domain which connects the DBD and the ligand binding domain (LBD) and has transactivation (AF2) activity; a LBD called the E domain which can also bind to coactivators and corepressors; and a variable C-terminal F domain (Kumar and Thompson 1999; Klinge 2000; Warnmark, Treuter et al. 2003).
Biochemical and structural analysis has shown that upon ligand binding, the NRs undergo a structural change (Ribeiro, Kushnerr et al. 1995). The LBD is recognized by short leucine rich hydrophobic motifs (consensus LXXLL/ NR box) present within each steroid receptor coactivator (SRC) that is necessary and sufficient for the interaction (Heery, Kalkhoven et al. 1997; Torchia, Rose et al. 1997; Smith and O'Malley 2004). Biochemical data suggests that the sequences flanking the NR box impart specificity to the NRs (Darimont, Wagner et al. 1998; McInerney, Rose et al. 1998). This data is supported by the data from crystal structure analysis of various mammalian receptors in the presence of coactivators (Darimont, Wagner et al. 1998; Nolte, Wisely et al. 1998; Shiau, Barstad et al. 1998). Coactivators have multiple NR specific LXXLL motifs that can possibly bind different NRs in response to various hormones (McInerney, Rose et al. 1998; Zhou, Cummings et al. 1998; Shao, Heyman et al. 2000).

Unlike the coactivators, corepressors like the nuclear receptor corepressor (nCoR) and the silencing mediator of retinoic acid and thyroid hormone receptor (SMRT) interact with unliganded nuclear receptors through a consensus sequence (LXX I/H IXXX I/L) also called the CoR/ Corr-box whose interaction site maps to the same hydrophobic groove as the coactivators (Horlein, Naar et al. 1995; Nagy, Kao et al. 1997; Hu and Lazar 1999; Perissi, Dasen et al. 1999; Webb, Anderson et al. 2000).

The 48 vertebrates NR genes are all represented in Drosophila through its 18 NR encoding genes (Figure 2) (King-Jones and Thummel 2005).
Figure 2 The evolution and genetics of vertebrate and Drosophila NRs

The figure above summarizes the genes in the six NR superfamilies from Drosophila and mammals. The numbers on the right represent the various NR superfamilies (1-6). The
mammalian NR genes are highlighted in blue while the Drosophila genes are indicated by red arrows. The figure is adapted from the review by Kirst King-Jones and Carl S. Thummel, Nature Reviews Genetics, Volume 6, 2005.
2.3.1. Drosophila nuclear receptors

The Drosophila ecdysteroid receptor is a heterodimer of ECR (NR1H1), a member of the nuclear hormone receptor superfamily (Koelle, Talbot et al. 1991) and USP (NR2B4), the only fly ortholog of mammalian RETINOIC X RECEPTOR (RXR) (Oro, McKeown et al. 1990; Koelle, Talbot et al. 1991; Christianson, King et al. 1992; Yao, Segraves et al. 1992; Thomas, Stunnenberg et al. 1993; Yao, Forman et al. 1993). ECR functions like a ligand-dependent transcription factor that along with USP, binds to a palindromic ECRE present in the promoter of many 20E inducible genes (Mestril, Schiller et al. 1986; Riddihough and Pelham 1986; Riddihough and Pelham 1987; Cherbas, Lee et al. 1991; Rudolph, Morganelli et al. 1991; Mangelsdorf and Evans 1995). Similar to the vertebrate thyroid hormone receptor, the ECR/USP receptor can associate with the HREs to repress basal expression of neighboring genes in the absence of ligand (Cherbas, Lee et al. 1991; Dobens, Rudolph et al. 1991). The presence of 20E triggers ECR/USP dimerization, converting the repressor into a transcriptional activator (Yao, Forman et al. 1993).

In Drosophila, both ECR and USP are present as multiple isoforms (Henrich, Sliter et al. 1990; Oro, McKeown et al. 1990; Shea, King et al. 1990; Koelle, Talbot et al. 1991; Talbot, Swyryd et al. 1993; Henrich, Szekely et al. 1994). The ECR gene encodes three protein isoforms, ECR A, ECR B1 and ECR B2 (Koelle, Talbot et al. 1991; Talbot, Swyryd et al. 1993). The A and B isoforms are generated using alternative promoters, whereas the B1 and B2 isoforms result from alternative mRNA splicing. All isoforms
have identical DNA and ligand binding domains but unique amino terminal domains (Talbot, Swyryd et al. 1993). Using isoform specific antibodies as well as specific mutations, the roles of each isoform and their expression pattern have been identified (Robinow, Talbot et al. 1993; Truman, Talbot et al. 1994; Bender, Imam et al. 1997; Cherbas, Hu et al. 2003). All three ECR isoforms can interact with USP with equal affinity (Talbot, Swyryd et al. 1993; Thomas, Stunnenberg et al. 1993; Mangelsdorf and Evans 1995; Bender, Imam et al. 1997; Buszczak and Segraves 1998; Hall and Thummel 1998).

USP encodes for a single polypeptide that has two isoforms of 54 and 56 kDa corresponding to unphosphorylated and phosphorylated variants (Song, Sun et al. 2003). The functional significance of USP phosphorylation is not known yet. USP is widely expressed temporally and spatially (Henrich, Sliter et al. 1990; Oro, McKeown et al. 1990; Shea, King et al. 1990; Henrich, Szekely et al. 1994).

ECR functions upon heterodimerizing with USP, while USP can function both as a homodimer or a heterodimer with mammalian nuclear receptors, such as RETINOIC ACID RECEPTOR, THYROID HORMONE RECEPTOR, and VITAMIN D RECEPTOR (Christianson, King et al. 1992; Yao, Segraves et al. 1992; Mangelsdorf and Evans 1995). In Drosophila, USP can heterodimerize with ECR as well as DROSOPHILA HORMONE RECEPTOR 38 (DHR38), a member of the orphan nuclear receptor superfamily (Yao, Segraves et al. 1992; Sutherland, Kozlova et al. 1995). Biochemical and genetic analysis have demonstrated that DHR38/ USP complexes enhance ligand-dependent transcription in response to ecdysteroids through an atypical ecdysteroid signaling pathway which is independent of direct ligand binding (Baker,
Shewchuk et al. 2003). Baker et. al. also proposed that the native USP could be activated by an unknown ligand, allowing for the formation of a DHR38/ USP complex that incorporates activated USP (Baker, Shewchuk et al. 2003). This hypothesis is supported by the data from X-ray crystallography studies that led to the identification of a large hydrophobic pocket in USP which can potentially be occupied by a lipophilic ligand (Billas, Moulinier et al. 2001; Clayton, Peak-Chew et al. 2001). DHR38 seems to lack a functional ligand binding domain, suggesting that unlike the ECR/ USP complex in which ECR is activated upon ligand binding, in DHR38/ USP complex, a yet unknown ligand might activate USP.

ECR/ USP can interact with other cellular factors to regulate target gene expression. A co-repressor, SMRTER (SMR/ vertebrate SMRT ortholog) is one such factor (Tsai, Kao et al. 1999). In vitro studies have shown that SMR can bind to ECR, while immunostaining studies have shown that SMR an USP can co-localize on specific bands and interbands on the polytene chromosomes as well as to dSIN3A, a protein associated with the nucleosome/ histone modifying protein RPD3/ HDAC (Tsai, Kao et al. 1999). These authors have proposed a model in which in the absence of 20E, the ECR/ USP/ SMR complex loosely associates with a target gene ECRE and recruits histone deacetylase complex which results in transcriptional repression. In contrast, upon ligand binding, the ECR/ USP complex recruits unidentified co-activators upon replacing SMR which results in gene transcription.

Mutations affecting the different ECR isoforms and USP have been characterized (Henrich, Rybczynski et al. 1999). Genetic studies have shown that lesions in both the DBD and the LBD of ECR result in embryonic lethality (Kozlova and Thummel 2000;
Kozlova and Thummel 2003). The analysis of N-terminal lesions in ECR-B1 demonstrates a disruption in puffing pattern in the salivary gland polytene chromosomes of L3 larvae followed by death around puparium formation (Bender, Imam et al. 1997). Genetic analysis has demonstrated that the mutations in DBD of USP result in lethality during the L1 to L2 molt as against embryonic lethality observed upon the maternal loss of *USP* (Perrimon, Engstrom et al. 1985; Oro, McKeown et al. 1990; Henrich, Szekely et al. 1994). Also, the loss of zygotic functions of *USP* results in partial larval lethality at the L2 stage. The larvae that do survive the L2 stage show extra posterior spiracles, suggesting a defect in the L1 cuticle (Perrimon, Engstrom et al. 1985; Oro, McKeown et al. 1992). It is likely that in the absence of zygotic USP, maternally derived USP supports survival till the L1 stage. However, it is not clear why mutations in *ECR* result in lethality earlier than mutations in *USP*. It is possible that at the embryonic stage, ECR partners with a yet unidentified binding partner such as *Drosophila Hormone Receptor 3* (*DHR3*) which has been shown to bind to ECR in vitro (White, Hurban et al. 1997). This hypothesis is supported by the fact that mutations in *DHR3* result in embryonic lethality (Carney, Wade et al. 1997). Alternatively, USP might partner with DHR38 to signal independent of ECR, as *DHR38* mutants exhibit cuticle defects similar to those in *USP* mutants (Hall and Thummel 1998; Kozlova, Pokholkova et al. 1998; Baker, Shewchuk et al. 2003).

In addition, the mutations in *ECR* and *USP* also affect the ecdysone signaling hierarchy. The loss of *ECR* function leads to a decrease in the transcription of early genes like *BR-C*, *E74*, and *E75* at the onset of metamorphosis (Hall and Thummel 1998; Li and Bender 2000). The loss of function of *USP* leads to precocious differentiation as a result
of the inability to activate early genes, such as DH3, ECR and E75B; and precocious expression of late targets like βFTZ-F1 and the Z1 isoform of BR-C (BRC-Z1) (Schubiger and Truman 2000).

As mentioned earlier, NRs can interact with numerous co-regulatory proteins that include coactivators and corepressors. In Drosophila, Trithorax-related (TRR) is one such ecdysone receptor coactivator and is discussed in more detail in the next section.

2.3.2. TRR as an ecdysone receptor coactivator

In Drosophila, the TRITHORAX RELATED (TRR) gene encodes the SET [SU(VAR) 3-9, E(Z), TRITHORAX] domain containing protein TRR, which is closely related to the well studied HOX gene activator TRITHORAX (TRX) (Sedkov, Benes et al. 1999). Biochemical evidence has demonstrated that TRR is a histone methyltransferase which can trimethylate lysine 4 of histone H3 (H3-K4) (Sedkov, Cho et al. 2003).

Biochemical analysis has revealed that the nuclear receptor binding motifs of TRR can bind ECR and its hetero-dimeric partner USP in a hormone dependent manner (Sedkov, Benes et al. 1999; Sedkov, Cho et al. 2003). Genetic and biochemical analysis has demonstrated that TRR functions as an ecdysone receptor coactivator to regulate the transcription of a principal developmental-factor gene, HEDGEHOG (HH) in an ecdysone dependent manner. This regulation of HH by TRR has been demonstrated in both ecdysone inducible embryonic S2 cells as well as the eye imaginal disc, where it is required for the HH-dependent progression of the morphogenetic furrow (Sedkov, Benes et al. 1999; Sedkov, Cho et al. 2003).
TRR contains several conserved protein domains that are found in transcriptional regulators and shows homology to the C-terminus of ALL-RELATED (ALR/MLL2) (Figure 4). These domains, their functions and the necessary components that are required for coactivator function are discussed in the following sections.

(Note: There is a lot of confusion in the literature regarding MLL nomenclature as some of the MLL protein designations have been changed. For the sake of simplicity, in this document, ALR/MLL2 will be called ALR unless otherwise stated. But, note that in some publications ALR/MLL2 has been called MLL4).

Figure 3 Comparison of various SET domain containing proteins

The schematic diagram above shows the various SET domain containing proteins from different species. On the left is the name of each protein with the species name at the bottom. The following are the abbreviations used in the figure: MLL-MIXED LINEAGE LEUKEMIA, TRX-TRITHORAX, CMI-CARA MITAD and TRR-TRITHORAX
RELATED. The size of each protein is listed on the right. The key at the bottom of the figure depicts the color scheme for various domains. Note: MLL2 in the figure above is ALR.
2.4 Gene transcription is regulated through histone modifications

The genetic information in eukaryotes is stored in a DNA-protein complex called chromatin. Chromatin consists of DNA and protein complexes called nucleosomes. The fundamental repeating unit of chromatin, the nucleosome core particle, consists of ~146 bp of DNA wrapped around a histone octamer consisting of two copies of each of the core histones-H2A, H2B, H3 and H4 (Kornberg and Lorch 1999). The DNA between two nucleosomes, called the spacer DNA, associates with linker histone, H1 and other proteins {reviewed in (Bartova, Krejci et al. 2008)}. Each core histone has a structured domain; N-terminal unstructured tails of 25-40 amino acids; and shorter C terminal tails. These histone tails undergo epigenetic modifications such as acetylation, methylation, phosphorylation, etc. {reviewed in (Grant 2001)}, serving as carriers of the epigenetic information through cell generations. These epigenetic marks are maintained and modified by various proteins such as Histone acetyltransferase (HATs) like GCN5 and PCAF; histone deacetylases (HDACs) like RPD3 and HDAC1; histone methyltransferases (HMTases) like MLL and TRX and histone demethylases like UTX {reviewed in (Grant 2001)}.

2.4.1. Histone methyltransferases activate gene transcription

HMTases are enzymes that catalyze the transfer of one to three methyl groups to lysine and arginine residues of histone proteins. Some HMTases have a characteristic domain called the SET domain that has been shown to have H3K4 specific HMTase
activity required for transcriptional activation while H3K9, H3K79 and H3K27 are required for transcriptional repression in a number of eukaryotic species (Santos-Rosa, Schneider et al. 2002; Goo, Sohn et al. 2003; Schneider, Bannister et al. 2004; Schubeler, MacAlpine et al. 2004; Bernstein, Kamal et al. 2005; Dillon, Zhang et al. 2005; Martin and Zhang 2005; Pokholok, Harbison et al. 2005; Dehe and Geli 2006; Ruthenburg, Allis et al. 2007). The SET domain is a 130 amino acid long domain that was first characterized in SU(VAR)3-9 (Tschiersch, Hofmann et al. 1994).

In yeast, SET1 protein is the only enzyme to methylate H3K4 (Jenuwein, Laible et al. 1998; Briggs, Bryk et al. 2001; Roguev, Schaft et al. 2001). Unlike yeast, humans and Drosophila have multiple proteins with H3K4 methyltransferase activity, each of which has unique roles. The mammalian SET domain containing proteins include SET1 proteins that are related to yeast SET1 and MIXED LINEAGE LEUKEMIA (MLL) proteins, MLL1-5. Some of the Drosophila SET domain containing proteins include SUPPRESSOR OF VARIEGATION 3-9 (SU(VAR)3-9), ENHANCER OF ZESTE 4 (EZ4) as well as the Drosophila MLL orthologs, TRX and TRR (Figure 3). (Tschiersch, Hofmann et al. 1994; Stassen, Bailey et al. 1995; Sedkov, Benes et al. 1999).

2.4.2. SET domain containing proteins are part of different SET1-like complexes

The SET domain containing proteins associate with other proteins to form multi-subunit coactivator complexes like the yeast SET1 complex. Table 1 lists the components of the yeast SET1 complex and those in various mammalian SET1-like complexes (Ruthenburg, Allis et al. 2007). Despite the fact that these complexes share a number of common sub-units, they differ in other sub-units, presumably required for
impacting specificity to each complex. In particular, various MLL proteins form part of
different SET1-like complexes, suggesting MLL dependent target specificity.
Table 1 Comparison of the components of the SET1 like complexes in yeast and human

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<th>Yeast</th>
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|        | Set1 A &B  | Ash2 Ash2 Ash2 |
|        | Bre2       | Ash2 Ash2 Ash2 |
|        | Spp1       | CGBP CGBP CGBP |
|        | SWd1       | Rbbp5 Rbbp5 Rbbp5 |
|        | Swd2       | Swd2            |
|        | Swd3       | WDR5 WDR5 WDR5 |
|        | Sdc1       | Dpy30 Dpy30 Dpy30 |
|        | HCF1       | HCF1            |
|        |            | ERα ERα ERα    |
|        |            | ASC-2 ASC-2 ASC-2 |
|        |            | MOF             |
|        |            | α-tubulin       |
|        |            | β-tubulin       |
|        |            | PTIP            |
|        |            | UTX             |
|        |            | PA1             |
Comparison of the components of the yeast SET1 complex with various mammalian SET1-like complexes. The components in red are conserved among all human SET1 like complexes and form the core subunits of these complexes. MLL5 containing SET1 like complex has not yet been identified. MLL2 in the above table refers to ALR.
2.5 Description of MLL proteins and their domains

There are five known MLL (MLL1-5) genes that encode five MLL proteins (MLL1-5) (Figure 3). These proteins have a number of similar domains which suggests that the MLL genes might have undergone duplication over time. MLL genes are essential as the deletion or truncations in MLL1, ALR and MLL4 genes in mice result in embryonic lethality. However, the MLL proteins are not functionally redundant as the mutations in different MLLs show independent phenotypes (Yu, Hess et al. 1995; Glaser, Schaft et al. 2006; Lee, Lee et al. 2006). In particular, the rearrangements of the MLL1 gene in humans are associated with a variety of aggressive human leukemias, including acute lymphoblastic leukemia (ALL) and acute myelogenous leukemia (AML) (Akao, Mizoguchi et al. 1998; Miller, Krogan et al. 2001; Orphanides and Reinberg 2002; Hess 2004; Sims, Mandal et al. 2004). The knock-down of ALR in cervical cancer HeLa cells results in defects in cell growth, migration, adhesion and spreading (Issaeva, Zonis et al. 2007). The mammalian MLL1 and MLL4 maintain HOX gene expression during development (Yu, Hess et al. 1995; Glaser, Schaft et al. 2006; Tan, Sindhu et al. 2008). In addition, the deletion of MLL1 SET domain in mice leads to a homeotic phenotype, suggesting that the SET methyltransferase domain might contribute to the regulation of HOX genes by MLL1 (Terranova, Agherbi et al. 2006). MLL1-4 have been shown to have intrinsic H3K4 methyltransferase activity (Milne, Briggs et al. 2002; Goo, Sohn et al. 2003; Wysocka, Myers et al. 2003; Hughes, Rozenblatt-Rosen et al. 2004; Yokoyama, Wang et al. 2004).
The various domains present in different MLL proteins include the Plant Homeodomain (PHD) zinc fingers, Phenylalanine and Tyrosine rich (FYR) region which is split into two parts FYR N-terminus (FYRN) and FYR C-terminus (FYRC), NR binding motifs, proteolytic cleavage site, bromodomain, CxxC domain, DNA-binding High Mobility Group (HMG) box, AT-hooks and an evolutionarily conserved C-terminal SET domain (Figure 3).

The PHD finger was first identified in a homeodomain protein in *Arabidopsis thaliana*, Homeobox Leucine Zipper Protein 3 (HAT3) (Millar, Scott et al. 1993). PHD fingers are Cys4-His-Cys3 motifs that are required for protein-protein interaction and may be present as a single finger or in clusters in the MLL proteins (Figure 3). The PHD fingers can also recognize either methylated or unmodified lysine residues in histone tails {reviewed in (Baker, Allis et al. 2008)}. Internal deletion of exon 8 of *MLL1* that results in exclusion of critical cysteine residues of the first PHD finger without affecting the reading frame is a normal splice variant which has also been observed in rare cases of acute lymphoblastic T-cell leukemia (Lochner, Siegler et al. 1996; Ayton and Cleary 2001). How this internal deletion alters the function of MLL and whether it is sufficient to promote leukemogenesis have yet to be determined. In addition, point mutations, deletions or chromosomal translocations that target PHD fingers encoded by many genes (such as *RAG2, ING, NSD1, ATRX*) have been associated with wide range of human pathologies including immunological disorders, cancers and neurological diseases {reviewed in (Baker, Allis et al. 2008)}.

The SET domain is preceded by a pre-SET domain and a FYR region split into two parts FYRN and FYRC. The FYRN and FYRC domains are separated by a
proteolytic cleavage site in the MLL family proteins (MLL1, MLL4 and TRX). Upon proteolytic cleavage, the FYRN and FYRC become part of two different halves (MLL\textsuperscript{N} and MLL\textsuperscript{C}) of the MLL1 and MLL4 proteins. FYRN and FYRC domains mediate MLL\textsuperscript{N} and MLL\textsuperscript{C} fragment heterodimerization after TASPASE1 proteolytic cleavage (Hsieh, Cheng et al. 2003; Takeda, Chen et al. 2006; Liu, Takeda et al. 2008). However, these domains are present adjacent to one another in the ALR and MLL3 proteins in which a proteolytic cleavage site has not been identified. The fact that FYRN and FYRC are located close to each other in ALR and MLL3 suggests that these proteins might not have to undergo proteolytic cleavage to perform their functions; ALR and MLL3 seem to be the result of gene duplication.


The AT hooks are short motifs that are thought to target MLL to the DNA by allowing specific binding of MLL to the minor groove of AT-rich or nucleosomal or bent DNA. AT hooks are thought to stabilize protein-DNA interactions by inducing conformational changes like DNA bending which can affect the recruitment of various factors and thereby affect gene transcription (Macrini, Pombo-de-Oliveira et al. 2003).
The CxxC (C= cysteine; X = any amino acid) domain of MLL can bind to unmethylated CpG DNA dinucleotides, is contained within a potential repression domain, and recruits histone deacetylases, thereby modulating MLL function as an activator/repressor (Zeleznik-Le, Harden et al. 1994; Birke, Schreiner et al. 2002; Xia, Anderson et al. 2003).

The HMG box was originally identified as the domain of the HMG-box (HMGB) family proteins that mediate their binding to DNA (Baxevanis and Landsman 1995). HMG proteins can also mediate sequence specific DNA binding as in the case of proteins from HMG-nucleosome (HMGN) family (Bustin 2001; Thomas and Travers 2001; Agresti and Bianchi 2003). Loss of yeast HMGB-type proteins results in genomic instability, shortened life span and hypersensitivity to DNA-damaging agents (Giavara, Kosmidou et al. 2005). Primary mouse embryonic fibroblasts (MEFs) derived from HMGB1-/- mice also exhibit chromosomal instability that results in high levels of aneuploidy and spontaneous chromosomal aberrations (Giavara, Kosmidou et al. 2005; Stros, Launholt et al. 2007).

The NR binding motifs of ALR as well as Menin have been shown to interact with estrogen receptor (ER-α) and regulate estrogen responsive genes (Lee, Lee et al. 2006; Lee, Lee et al. 2008). siRNA mediated knock-down of ALR in HeLa cells has been shown to impair ER-α mediated transactivation of CATHEPSIN D and pS2 (Mo, Rao et al. 2006; Issaeva, Zonis et al. 2007).
2.5.1. Mammalian ALR regulates target genes in response to hormone signaling

Mammalian ALR is a component of two independent co-activator complexes, the ALR complex that regulates gene transcription in response to ERα signaling (Mo, Rao et al. 2006) and the ASCOM complex that regulates target gene transcription through the RXR as well as the LIVER X RECEPTOR (LXR) signaling (Goo, Sohn et al. 2003). It is not clear what determines the recruitment of ALR to either ASCOM or ALR complex. Also, it is not known if the ALR and ASCOM complexes have exclusive targets that respond to ERα, RXR and LXR signaling, respectively. Further studies are needed to rule out the possibility that the ALR and ASCOM complexes represent the same complex that recruits different subunits at different times or in response to different hormone signals.

The MLL2 and ASCOM complexes share common core proteins that include ABESENT SMALL HOMEOTIC 2 (ASH2), RETINOBLASTOMA BINDING PROTEIN (RBBP3), WD REPEAT DOMAIN 5 (WDR5), hDPY30 and CpG BINDING PROTEIN (CGBP) (Wysocka, Swigut et al. 2005; Mo, Rao et al. 2006). The various subunits that have so far been identified in each complex are listed in Table 1.

ASH2 is the mammalian homolog of Drosophila ASH2, a TRX protein that is required for the regulation of HOX genes (LaJeunesse and Shearn 1995; Adamson and Shearn 1996). Biochemical studies in HeLa cells have shown that ASH2 is required for trimethylation of MLL1 target gene promoters (Steward, Lee et al. 2006).

RBBP5 is a member of the highly conserved subfamily of WD-repeat proteins. The WD domain is found in retinoblastoma (RB) binding proteins.
WDR5 is a highly conserved WD40 repeat protein. Peptide pull-down assays in HEK293 nuclear extracts have demonstrated that WDR5 preferentially binds dimethylated H3K4 and is essential for H3K4 trimethylation. Knock-down studies in HEK293 cells and in X. laevis have shown that ASH2, RBBP5 and WDR5 regulate the expression of the MLL1 target genes HOXA9 and HOXC8 (Wysocka, Swigut et al. 2005).

hDPY30 is the mammalian ortholog of the DPY30 protein which is an essential component of the dosage compensation complex that balances X-linked gene expression in C. elegans. hDPY30 is a conserved member of some HMT complexes where it can directly interact with ASH2L (Cho, Hong et al. 2007; Wang, Lou et al. 2009).

CGBP is a CpG binding protein. Knock-down studies in HEK293 cells have demonstrated that CGBP is required for both the H3K4 trimethylation of the promoter as well as the expression of the MLL1 target gene, HOXA7. It has also been demonstrated that the knock-down of CGBP prevents the recruitment of MLL1 into HOXA7 promoter (Ansari, Mishra et al. 2008).

Analysis of ALR expression in mouse embryo and adult human tissues indicates multiple functions for ALR during development (Prasad, Zhadanov et al. 1997). The knock-down of ALR in HeLa cells results in defects in cell spreading, migration, adhesion and growth. Further, in vivo experiments where ALR-deficient HeLa cells were introduced in athymic nude mice with tumors demonstrated tumor shrinkage with delayed tumor development kinetics compared to ALR positive HeLa cells. Also, in-vitro knock-down of ALR in HeLa cells led to the identification of a number of ALR targets that include genes with roles in development, growth and adhesion. MOTHERS AGAINST DECAPENTAPLEGIC HOMOLOG 6 (MADH6)/ SMAD6 is one such gene that
was identified. MADH6 is involved in Transforming Growth Factor β (TGF β)/ Bone Morphogenetic Protein (BMP) signaling (Issaeva, Zonis et al. 2007).

Unlike MLL-1 and TRX which regulate HOX gene expression, ALR has not been shown to directly regulate HOX gene expression. However, recently, ALR was shown to be recruited to the HOXC8 locus by ACTIVATING PROTEIN 2δ (AP2δ) along with ASH2L, suggesting a possibility that ALR might control HOX gene transcription (Tan, Sindhu et al. 2008).

2.5.2. ALR is represented by two independent proteins in Drosophila

In Drosophila, ALR is represented by two independently encoded and transcribed proteins: TRR and CARA MITAD (CMI) (Figure 4). TRR is homologous to the C-terminal half of mammalian MLL2 and contains three nuclear receptor binding motifs and a SET domain. Like ALR, TRR was shown to have H3K4 specific trimethylation activity (Sedkov, Cho et al. 2003).

Despite the fact that TRR functions as a conserved nuclear receptor co-activator in response to ecdysone signaling, some of the key features present in ALR which are responsible for tethering a coactivator (complex) to the chromatin are missing from TRR. In order to determine whether the functions of the N-terminal half of ALR could be performed by another protein in Drosophila, we performed a BLAST analysis to determine if there was a protein in Drosophila that contains the missing ALR domains. Based on our BLAST search, we identified a novel gene that we named CARA MITAD (CMI) (meaning “my dear half”, in Spanish). CMI encodes a putative protein which shows homology to the N-terminal half of mammalian ALR (Figure 4). CMI has the
missing PHD fingers, HMG box, and hormone receptor binding motifs found in the N-terminal portion of ALR. The fact that Drosophila ALR is split into two proteins provides us with a unique opportunity to study the roles of the domains found in the N-terminal portion of ALR, independent of the HMTase function of the SET domain.

This study focuses on understanding the role of CMI as a NR coactivator, identifying CMI target genes, determining if the function of CMI is dependent on TRR, and to study the role of CMI in Drosophila development. In order to study the role of CMI in adult development and patterning, we have used Drosophila wing as a model system.
Figure 4 Drosophila CMI and TRR are homologous to mammalian ALR protein

A comparison between the mammalian ALR protein with Drosophila CMI and TRR proteins. Various domains in the proteins are indicated. The chromosomal location of the genes encoding for each protein is listed in the brackets below each protein.
2.6  **Drosophila wing as model for tissue patterning**

The Drosophila wing serves as an excellent model system for studying various signaling pathways and the cross-talk between them because all major conserved signaling pathways involved in tissue growth and patterning are required for wing development. In addition, the roles of each of these pathways have been well documented in wing patterning (Blair 2007). Wing is used as a classical model to place a novel protein in a specific signaling pathway. This is done by looking at the ability of the mutations in the novel protein or the mis-expression of the protein either to enhance or suppress pathway specific phenotypes.

2.6.1.  **Structure of the Drosophila wing**

The adult fly wing has five main longitudinal veins (LVs), LV 1-5 that are formed from the proximal to the distal end of the wing and two main cross-veins (CV) that connect the LVs (Figure 5). The anterior cross-vein (ACV) connects L3 and L4 and the posterior cross-vein (PCV) connects L4 and L5 (Blair 2007).

The adult Drosophila wing develops from ~15-20 cells (at embryogenesis) to ~50,000 cells during the late third instar larval stage in the wing imaginal disc. The imaginal discs are epidermal sheets that form the adult external structures like the wings, legs and body wall in flies. These imaginal discs are specified as groups of precursor cells during embryogenesis that proliferate during the larval stage to form mature discs (Williams, Paddock et al. 1993; Goto and Hayashi 1997). Vein formation can be
separated into two phases: establishment of a six to seven cell wide prospective vein (pro-vein) domain in the third instar larva and the refinement of this domain to two to three rows of vein cells by restriction of vein cell differentiation at the pupal stage (reviewed in De Celis 1998; De Celis and Diaz-Benjumea 2003). The wing disc evaginates at the pupal stage to give rise to the adult wing. The cells along the veins are the only living cells in the mature wing as the intervein cells are lost soon after the flies emerge from the pupal cases (Kiger, Natzle et al. 2007).
Figure 5 The structure of a Drosophila wing

A wild type *Oregon R* wing with veins and cross-veins. L1, L2, L3, L4 and L5 refer to the longitudinal veins 1-5, ACV- Anterior Cross vein, PCV- Posterior Cross Vein.
There are five major signaling pathways that determine the positioning of the veins and the interveins in the Drosophila wing. These pathways include HH, Drosophila EPIDERMAL GROWTH FACTOR RECEPTOR (EGFR), BMP mediated by DECAPENTAPLEGIC (DPP), WNT/ WINGLESS (WG) and NOTCH (N) signaling pathways {reviewed in (Blair 2007)}. The roles of each pathway in wing patterning, both at the larval and the pupal stages, are discussed in the following sections.

2.6.2. Development of the Drosophila wing imaginal disc

The wing disc cells are programmed for sensory structure formation (Campuzano and Modolell 1992) or for vein development (Sturtevant and Bier 1995) by the differential expression of genes at the larval stage. This differential gene expression programs the imaginal primordium to establish anterior-posterior (A/P), proximal-distal (P/D) and dorsal-ventral (D/V) polarity at embryonic, larval and pupal stages (Garcia-Bellido, Ripoll et al. 1973).

The precursors of the LVs run along the P/D axis of the wing and first appear at the larval stage in the wing discs. However, the precursors for the ACV and PCV, that bridge the LVs, do not appear until the early stages of pupal development (Conley, Silburn et al. 2000). The following sections discuss the roles of each signaling pathway in the development of the wing imaginal disc.
2.6.2.1. **HH signaling is required for A/P axis determination in the wing imaginal disc**

The A/P axis is determined by the expression of ENGRAILED (EN) and HH in the posterior compartment (Kornberg, Siden et al. 1985). The secreted morphogen HH, spreads from the posterior (HH producing domain) to the anterior (HH receiving domain) compartment, activating the expression of its targets in a concentration dependent manner (Eaton and Kornberg 1990; Dominguez, Brunner et al. 1996; Ruiz i Altaba 1997; Ingham and McMahon 2001). This canonical HH signaling is mediated by the GLI-like transcription factor, CUBITUS INTERRUPTUS (CI) which is expressed only in the anterior compartment (Blair 1995; Irvine and Rauskolb 2001; Blair 2003; Blair 2003). Cells on the anterior side of the A/P compartment boundary respond by expressing a number of genes, including the gene encoding for BMP-4-like signaling protein DPP (Padgett, St Johnston et al. 1987; Basler and Struhl 1994; Tabata and Kornberg 1994; Felsenfeld and Kennison 1995; Sanicola, Sekelsky et al. 1995; De Celis 1998).

HH signaling is required for the development of the L3 and L4 proveins and for the maintenance of the distance between them. The L3 provein is formed in the anterior cells receiving low levels of HH signal, while the L4 provein is formed just to the posterior of the A/P boundary in cells that cannot receive HH signal (reviewed in (Blair 2007)). The placement and spacing between L3 and L4 veins is thus controlled by the domains where HH is received. The gain of HH signaling leads to an increase in the distance between L3 and L4, while a loss of HH signaling decreases the distance (Mullor,
Calleja et al. 1997; Nestoras, Lee et al. 1997; Strigini and Cohen 1997; Biehs, Sturtevant et al. 1998). In addition, the cells that receive high levels of HH specify intervein cells, whereas the cells that receive no or low levels of HH signal form proveins (reviewed in Blair 2007).

The short-range HH activity is mediated by the activation of a number of transcription factors. These transcription factors include COLLIER or KNOT (COL or KN) in the A/P organizer cells which are immediately anterior to the A/P boundary and receive high doses of HH (Vervoort, Crozatier et al. 1999; Bier 2000; Mohler, Seecoomar et al. 2000); IROQUIOS GENE COMPLEX (IRO-C) transcription factors in the disc (Gomez-Skarmeta, Diez del Corral et al. 1996; Gomez-Skarmeta and Modolell 1996); and VEIN (VN) and EN in the anterior wing compartment (de Celis and Ruiz-Gomez 1995; Guillen, Mullor et al. 1995; Simcox, Grumbling et al. 1996; Mullor, Calleja et al. 1997).

KN acts in a cell-autonomous manner to up-regulate intervein-specific, DROSOPHILA SERUM RESPONSE FACTOR (DSRF) and down-regulate EGFR in these cells, thereby imposing an intervein fate, which results in the formation of the L3-L4 intervein region. KN also acts through a non cell-autonomous mechanism to promote formation of the L4 vein by up-regulating the expression of a gene encoding for EGFR ligand, VN (Crozatier, Glise et al. 2003). VN in turn diffuses and activates the EGFR pathway in cells posterior to the A/P boundary and refines the L4 pro-vein territory (Simcox, Grumbling et al. 1996; Crozatier, Glise et al. 2003).
2.6.2.2. DPP signaling is required for the pre-patterning of LVs in the wing imaginal disc

DPP is a member of the transforming growth factor beta (TGFβ) family of secreted signaling molecules and the fly ortholog of the vertebrate BMPs, BMP2/BMP4 that are required for bone formation and adult limb regeneration (Padgett, St Johnston et al. 1987). DPP often signals with the BMP-7-like protein GLASS BOTTOM BOAT (GBB/60A) which is expressed uniformly in the wing (Doctor, Jackson et al. 1992; Chen and Struhl 1998; Khalsa, Yoon et al. 1998).

The DPP signaling pathway is utilized at multiple points during development, with varying targets in each tissue and stage. In the wing imaginal disc, DPP is expressed in a stripe down the midline of the wing disc and forms a long range BMP signaling gradient that positions the LVs along the A/P axis of the wing disc (De Celis 1998). In particular, the formation of L3 and L4 proveins requires heightened BMP signaling, while, the initiation of L2 and L5 proveins requires low levels of BMP signaling (Tanimoto, Itoh et al. 2000).

Reductions in BMP signaling can either shift the position of the LVs or lead to gaps, while heightened/ectopic BMP signaling is sufficient to induce ectopic venation (de Celis 1997). L2 and L5 are positioned by the gradient of DPP signaling at set distances from the source of DPP along the A/P boundary. Reducing DPP-mediated BMP signaling often reduces the distance between L2 and L5 without affecting the distance between L3 and L4, in severe cases L2 fuses with L3 and L5 with L4 (Spencer, Hoffmann et al. 1982; Segal and Gelbart 1985). 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 (Spencer, Hoffmann et al. 1982; Segal and Gelbart 1985). A partial loss of DPP and GBB function can also block the formation of the L4 provein (Bangi and Wharton 2006).

DPP determines growth and patterning in the wing disc by dose sensitive activation of transcription factors like **SPALT (SAL)**, **SPALT-RELATED (SALR)** that are centered around the A/P boundary just posterior to the L2 provein; **IRO-C** that is expressed posterior to the SAL/ SALR boundary; and **OPTOMOTER BLIND (OMB)** that is expressed in the L5 provein region in response to low levels of BMP signaling (de Celis, Barrio et al. 1996; Gomez-Skarmeta, Diez del Corral et al. 1996; Grimm and Pflugfelder 1996; Lecuit, Brook et al. 1996; Nellen, Burke et al. 1996; Cook, Biehs et al. 2004).

Most of the DPP downstream effects are mediated by the SAL gene complex that is formed by two adjacent genes: **SAL** (Doctor, Jackson et al. 1992) and **SALR**, which encode for zinc finger containing transcription factors (Kuhnlein, Frommer et al. 1994; de Celis, Barrio et al. 1996; Barrio, de Celis et al. 1999; de Celis and Barrio 2000). Interactions between the transcription factors encoded by **SAL** and **SALR** further confine the expression of **KNIRPS-COMPLEX (KNI-C)** that includes **KNI** and **KNIRPS-REALTED/ KNRL** and **IRO-C** to the veins L2 and L5, respectively (Spencer, Hoffmann et al. 1982; Segal and Gelbart 1985; de Celis, Barrio et al. 1996; Gomez-Skarmeta, Diez del Corral et al. 1996; Biehs, Sturtevant et al. 1998; Lunde, Biehs et al. 1998; de Celis and Barrio 2000; Entchev, Schwagedissen et al. 2000; Teleman and Cohen 2000; Lunde, Trimble et al. 2003). Further, KNI/ KNRL appear to link the positional information provided by BMP signaling to the activation of the EGFR signaling along L2 by
stimulating the expression of RHOmboïd (RHO) at the late third instar (Lunde, Biehs et al. 1998).

In addition to the activation of SAL/ SALR and IRO-C genes in the wing disc, DPP also activates VESTIGIAL (VG) in all wing blade cells (Kim, Sebring et al. 1996; Kim, Johnson et al. 1997).

DPP signaling in the wing disc is regulated by the expression of its receptor THICKVEINS (TKV) that limits DPP diffusion (Lecuit and Cohen 1998) and its antagonist BRINKER (BRK) that binds to DPP response elements (Kirkpatrick, Johnson et al. 2001). The binding of DPP to TKV not only triggers DPP signaling but also limits DPP diffusion (Lecuit and Cohen 1998). Therefore, the localized repression of TKV results in peaks of DPP activity on either side of the A/P axis. This regulation of DPP signaling is further complicated by its regulation by HH signaling which induces the expression of putative transcription factor MASTER OF THICKVEINS (MTV/SCRIBBLER/ BRAKELESS) between L3 and L4, which in turn represses expression of the BMP receptor TKV (Funakoshi, Minami et al. 2001).

DPP and BRK form an inverse gradient in the wing disc, such that DPP signaling represses BRK, which in turn represses targets such as SAL and OMB (Campbell and Tomlinson 1999; Jazwinska, Kirov et al. 1999; Minami, Kinoshita et al. 1999). Thus, HH signaling as well as the DPP receptor, TKV and DPP antagonist BRK refine DPP signaling domains to pre-pattern LV proveins. This pre-patterning of the proveins by BMP signaling in turn serves as a prerequisite for a localized EGFR signaling {reviewed in (Blair 2007)}.
2.6.2.3. **EGFR signaling is required for the development of LV proveins in the wing imaginal disc**

EGFR is a receptor tyrosine kinase (RTK) that functions in a wide spectrum of developmental processes in Drosophila (Diaz-Benjumea and Hafen 1994; Schweitzer and Shilo 1997). In the wing imaginal disc, EGFR signaling is required for the formation of LV proveins as there is heightened EGFR signaling within the LV proveins. Early provein cells express targets of EGFR signaling such as RHO and STAR (S) that enhance EGFR signaling via. EGF-like ligands SPITZ (SPI), KEREN (KRN), and ARGOS that inhibits EGFR signaling as part of a negative feedback loop (Rutledge, Zhang et al. 1992; Sturtevant, Roark et al. 1993; Sawamoto, Okano et al. 1994; Schweitzer, Howes et al. 1995; Sturtevant and Bier 1995; Golembio, Schweitzer et al. 1996; Guichard, Biehs et al. 1999; Reich and Shilo 2002). EGFR signaling in the veins leads to the accumulation of phosphorylated protein kinase (MAPK), a downstream effector (Gabay, Seger et al. 1997).

EGFR can also signal in a RHO and S independent manner through a NEUREGULIN-like protein, VN (Schnepp, Grumbling et al. 1996). VN is strongly expressed between the L3 and L4 proveins and likely plays an instructive role in their induction as well as the formation of other LVs as a combination of hypomorphic VN and RHO alleles almost completely block the formation of adult veins (Garcia-Bellido and de Celis 1992; Simcox, Grumbling et al. 1996).

Further, EGFR signaling controls vein-intervein cell fate as EGFR signaling is both necessary and sufficient to suppress intervein specific factor, DSRF in the wing disc. This suppression of DSRF promotes provein formation (Roch, Baonza et al. 1998;
Ralston and Blair 2005). *DSRF* mutations induce ectopic venation and *RHO* expression during the pupal stages (Fristrom, Gotwals et al. 1994; Sturtevant and Bier 1995; Roch, Baonza et al. 1998). Removal of *DSRF* induces ectopic vein formation even within hypomorphic *EGFR* clones, suggesting that the down-regulation of *DSRF* in vein cells caused by EGFR activity is a critical step in vein specification and likely mediates vein-intervein choice (Roch, Baonza et al. 1998).

Further, EGFR signaling is necessary and sufficient for vein formation from the mid third instar through the first day of pupal development as reducing EGFR activity in the wing disrupts provein markers in the mid to late third instar discs (Gabay, Seger et al. 1997; Roch, Baonza et al. 1998; Guichard, Biehs et al. 1999; Martin-Blanco, Roch et al. 1999; Ralston and Blair 2005) and blocks vein formation in adults (Diaz-Benjumea and Garcia-Bellido 1990; Diaz-Benjumea and Hafen 1994; Sturtevant and Bier 1995; Guichard, Biehs et al. 1999). Moreover, loss of function mutations in EGFR pathway cause loss of veins as against the gain-of-function alleles and/or over-expression that results in ectopic veins (Diaz-Benjumea and Hafen 1994).

Further, EGFR plays a late role in promoting intervein development through HH signaling mediated *KN* expression in the L3-L4 intervein, which represses EGFR targets in the center of L3-L4 intervein region (Nestoras, Lee et al. 1997; Guichard, Biehs et al. 1999; Martin-Blanco, Roch et al. 1999; Vervoort, Crozatier et al. 1999; Wessells, Grumbling et al. 1999; Mohler, Seecoomar et al. 2000).
2.6.2.4. **N signaling is required for D/V axis determination in the wing imaginal disc**

The D/V axis in the wing imaginal disc is determined by the local activation of a membrane receptor protein-**NOTCH** (N) along the D/V boundary {reviewed in (Blair 2007). N in turn directs the expression of **VG** and **WINGLESS (WG)** which are required for wing growth (Blair 1995; De Celis 1998). In addition to N signaling, the D/V axis is also determined by the expression of **APTEROUS (AP)** in the dorsal compartment at the larval stage (Cohen, McGuffin et al. 1992).

N-ligands, **DELTA** (DL) and **SERRATE** (SER) are preferentially expressed in the center of the provein and activate N signaling in the adjacent cells. N signaling leads to the expression of the N-downstream gene **ENHANCER OF SPLIT mβ** \{E(spl) mβ\} in the vein-intervein boundary cells that prevents vein formation (Kooh, Fehon et al. 1993; de Celis 1997; de Celis, Bray et al. 1997; Huppert, Jacobsen et al. 1997). Decrease in N signaling leads to a characteristic phenotype in which the veins are fairly uniformly thick with deltas at the tip of the LVs {reviewed in (De Celis 2003)}.

In the wing disc, the interactions between N and EGFR divide each vein into two complementary domains, a central domain where EGFR is active and two boundary domains where N is active (de Celis, Bray et al. 1997; Crozatier, Glise et al. 2003). Notch and EGFR signaling are mutually dependent at this stage as EGFR activity regulates **DL** expression in the veins, while N signaling represses **RHO** in the adjacent cells (Sturtevant and Bier 1995; de Celis 1997; Huppert, Jacobsen et al. 1997). Therefore, N signaling is antagonistic to the EGFR signaling in the formation of LVs as N signaling prevents vein formation in the wing imaginal discs while EGFR signaling promotes vein formation.
In addition to its requirement for EGFR signaling, wild type levels of N signaling in early third larval instar disc are also required to antagonize HH activation of KN expression, to define precisely the over-lapping A/P positions to the L3 vein and associated sensory organs (Crozatier, Glise et al. 2003). However, the mechanism that contributes to transforming the HH gradient into a sharp limit of KN expression has not been determined yet.

**2.6.2.5. WNT/ WG signaling is required for D/V axis determination in the wing imaginal disc**

In the early third instar wing imaginal disc, N signaling between dorsal and ventral cells results in the expression of WG in a narrow stripe of edge cells in the D/V compartment boundary of the prospective wing blade (reviewed in (Irvine and Rauskolb 2001)). WG then induces distal (marginal) development in the neighboring cells (Phillips and Whittle 1993; Blair 1994; Couso, Bishop et al. 1994; Irvine and Rauskolb 2001). Also, WG signaling is both necessary and sufficient for the formation of the bristle precursors (Phillips and Whittle 1993; Blair 1994; Couso, Bishop et al. 1994).

The loss of WG signaling leads to death of both vein and intervein tissue along the wing margin. On the other hand, mis-expression of WG can induce ectopic venation in cells that are not generating ectopic sensory organs (Lunde, Trimble et al. 2003).

**2.6.3. Development of the Drosophila pupal wing**

At the pupal stage, the positioned and initiated LV proveins are further refined from broad regions into narrower veins, through the cross-talk between the same signaling pathways.
The LV proveins are formed separately on the prospective dorsal and ventral surfaces of the wing imaginal disc and are aligned at the pupal stage to form lacunae (hollow tubes) of the mature veins. When the wing surfaces align, transcription factors pass from one surface of the wing to the other, often more strongly from the dorsal to the ventral surface (Milan, Baonza et al. 1997; Guichard, Biehs et al. 1999).

2.6.3.1. **HH and N signaling determines L3 and L4 positioning in the pupal wing**

HH signaling sets up the stage for the position of and spacing between L3 and L4 veins as described in section 2.6.2.1. In addition to the HH signaling pathway, the N signaling pathway is required for the maintenance of width between L3 and L4.

At the pupal stage, N signaling plays a short-range role in refining the boundary between vein and intervein cells as the removal of N signaling during the pupal stage results in broad veins in adults (Shellenbarger and Mohler 1975; de Celis, Mari-Beffa et al. 1991; Sturtevant and Bier 1995; Huppert, Jacobsen et al. 1997; Crozatier, Glise et al. 2003) while gain in N signaling results in loss of adult veins (de Celis and Garcia-Bellido 1994; Lunde, Trimble et al. 2003).

2.6.3.2. **DPP signaling is required for the development of distal LVs**

At the transition from the larval to the pupal stage, there is a change in the expression pattern of *DPP*. DPP is lost from the midline stripe and appears in all of the LVs, leading to heightened BMP signaling in the LVs (Yu, Sturtevant et al. 1996; de Celis 1997; Conley, Silburn et al. 2000; Ralston and Blair 2005). The mechanisms of this switch in expression of *DPP* from the A/P boundary to the veins are not known.
At the pupal stage, DPP acts locally to maintain the previously specified LV fate. This vein-specific expression of \textit{DPP} is disrupted in \textit{DPP}\textsuperscript{shortvein} homozygotes and leads to the loss of all but the most proximal portions of the LVs (Segal and Gelbart 1985; Posakony, Raftery et al. 1990; de Celis 1997; Ray and Wharton 2001; Sotillos and de Celis 2006). Thus, DPP signaling at the pupal stage is required to maintain the development of at least distal vein cells. Once \textit{DPP} is expressed in the veins, it contributes to maintaining the expression of both \textit{DL} and \textit{RHO} in these cells to activate N and EGFR signaling, respectively (de Celis 1997).

During pupal stages, BMP signaling leads to a decrease in \textit{TKV} expression within the veins and a resulting heightened expression at vein boundaries, likely due to the repression of \textit{DPP} expression in the boundary cells (de Celis 1997). Thus, reducing \textit{TKV} activity broadens the region of \textit{DPP} expression which in turn broadens the veins, either through DPP signaling by the residual TKV or via another DPP receptor, SAXOPHONE (SAX) (de Celis 1997). Another proposed mechanism suggests that TKV reduces the range of signaling by binding and sequestering DPP produced in the veins (Lecuit and Cohen 1998; Tanimoto, Itoh et al. 2000).

In addition to DPP, GBB also plays a role in specific vein regions, such as the distal tips of the LVs (Burke and Basler 1996; Singer, Penton et al. 1997; Ray and Wharton 2001; Bangi and Wharton 2006; Bangi and Wharton 2006). Misexpression of DPP, GBB, or an activated form of TKV can induce ectopic venation (de Celis 1997; Bangi and Wharton 2006; Sotillos and de Celis 2006).
2.6.3.3. DPP and EGFR signaling is required for the development of ACV and PCV

At the pupal stage, CVs are precisely positioned along the P/D axis of the wing in response to BMP signaling. The earliest marker to appear in the CVs is locally heightened BMP signaling (from 18 h to 22 hr after pupariation [AP]) (Conley, Silburn et al. 2000). EGFR signaling is eventually activated along the CVs, likely by the activation of \( RHO \) and \( S \) in response to BMP signaling. The EGFR signaling is required for the maintenance of CVs into adult stages. Nonetheless, the exclusive role for BMP signaling at early stages makes the CVs especially sensitive to reductions in BMP signaling (reviewed in (O'Connor, Umulis et al. 2006)).

Signaling in the ACV is prefigured by the expression of \( DPP \) in a stripe that intersects the ACV (Ralston and Blair 2005). However, in the PCV region, there is no expression of \( DPP \) initially (reviewed in (O'Connor, Umulis et al. 2006)). Rather, \( DPP \) is expressed in the adjacent LVs and moves from the LVs into the adjacent PCV region (Ralston and Blair 2005). The initial development of PCV depends on heightened BMP signaling or an EGFR independent decrease in \( DSRF \) expression (Ralston and Blair 2005).

BMP signaling in the PCV requires both DPP and GBB and their transport into the PCV domain via a complex containing two extracellular BMP binding molecules, \textsc{Short Gastrulation (SOG)}\textsc{, the Drosophila CHORDIN homolog, and Crossveinless (CV)}\textsc{, a member of the twisted gastrulation family (Conley, Silburn et al. 2000; Ray and Wharton 2001; Ralston and Blair 2005; Serpe, Ralston et al. 2005; Vilmos, Sousa-Neves et al. 2005).}
Removing endogenous SOG results in a loss of signaling in the PCV and therefore, a crossveinless phenotype (incomplete/missing PCV) (Serpe, Ralston et al. 2005; Shimmi, Ralston et al. 2005). On the other hand, strong over-expression of SOG leads to loss of PCV (Yu, Sturtevant et al. 1996). While high levels of SOG can inhibit signaling in the wing, low levels of over-expression stimulate signaling distant from the site of mis-expression (Shimmi, Ralston et al. 2005). Loss of CV also leads to loss of BMP signaling in the PCV and hence a crossveinless phenotype (Shimmi, Ralston et al. 2005; Vilmos, Sousa-Neves et al. 2005).

2.6.3.4. **EGFR signaling is required for the maintenance of CVs**

As mentioned in section 2.6.2.3, BMP signaling is required for the initiation of CVs while EGFR signaling is required for their maintenance. The EGFR signaling switches from veins to interveins between 24 h and 30 h AP, pMAPK is lost from the veins and becomes heightened in interveins (Guichard, Biehs et al. 1999; Martin-Blanco, Roch et al. 1999). However, by 33 h AP, pMAPK expression switches back to the veins (Marenda, Vrailas et al. 2006). Little is known about the cause or result of this switch.

In addition to the maintenance of CVs, high doses of EGFR signaling are required for DPP activation in vein cells during pupal development, where DPP signaling acts locally to promote vein differentiation (de Celis 1997; Ray and Wharton 2001), as mentioned in section 2.6.3.2.
2.7 Structure and regulation of DPP

Proper spatial and temporal regulation of DPP expression is necessary for normal patterning during embryonic and imaginal disc development. The developmental complexity of DPP expression is reflected in the structure of the gene that extends over 55kb of genomic DNA. The DPP gene has been divided into three genetically distinct regions that include two huge cis-regulatory regions: a ~ 31 kb 5' shortvein (shv) region and a ~ 25 kb 3' imaginal disk specific (disk/d) region; and an exon-coding haploinsufficient (Hin) region (Spencer, Hoffmann et al. 1982; Segal and Gelbart 1985; St Johnston, Hoffmann et al. 1990) (Figure 6). There are five promoters for DPP in the shv and Hin regions. Despite the fact that the transcripts are restricted to both shv and Hin regions, each of the RNAs contains the same coding information that is located in the Hin region. Thus, DPP encodes for a single polypeptide (St Johnston, Hoffmann et al. 1990). The expression of DPP in various tissues is spatially and temporally regulated through the cis-regulatory elements (St Johnston, Hoffmann et al. 1990).

There have been numerous studies involving classical mutant analysis and germ line transformation that have allowed specific functions to be ascribed to the various regions of DPP during development. Hin region is the protein coding region that is specifically required for dorsal-ventral patterning of the embryo (Hoffmann and Goodman 1987; Irish and Gelbart 1987; St Johnston, Hoffmann et al. 1990). The mutations in the Hin region lead to a loss of all DPP function and exhibit haploinsufficiency (Hin/+ animals are embryonic lethal) (Irish and Gelbart 1987).
Figure 6 A schematic showing the structure of the DPP genomic region

A schematic representation of the *DPP* genomic region. The *DPP* regulatory region consists of two regulatory regions: ~31 kb 5’ shortvein (shv) region and ~25 kb 3’ disk region. p, lc and lnc represent three categories of mutant alleles in the shv region while V, III and II represent the three classes of mutant alleles in the disk region. The phenotypes of the mutations that are closest to the haploinsufficient (Hin) region (shown in red) are most severe while those that are farthest from the Hin region (shown in blue) are the mildest. Mutations in the central Hin region lead to embryonic lethality. The locations of various promoters are shown in the bottom schematic.
The disk region controls HH dependent larval expression of DPP and is required for the elaboration of P/D adult appendage development (Spencer, Hoffmann et al. 1982; Segal and Gelbart 1985). Mutations in the disk region result in defects in the imaginal discs and the adult derivatives of the imaginal discs (Spencer, Hoffmann et al. 1982; Bryant 1988; Masucci, Miltenberger et al. 1990; Blackman, Sanicola et al. 1991). Most of the DPP disk mutations involve gross chromosomal rearrangements that include inversions, translocations or deletions within the disk region that extend into adjacent loci (St Johnston, Hoffmann et al. 1990). Based on their genetic location and the severity of the phenotypes, the DPP disk alleles can be sorted into three phenotypic groups (Spencer, Hoffmann et al. 1982) (Figure 6). The most severe disk V alleles have breakpoints in an 8 kb region proximal to the shv-Hin transcription unit. Animals homozygous for disk V mutations exhibit extensive loss of imaginal discs, resulting in death at pupariation. The milder disk III mutations are distal to the Hin region and have breaks within a 10 kb region. The animals homozygous for disk III mutations exhibit a loss of distal material from all the adult appendages. Finally, the mildest disk II mutations interrupt a 2 kb region most distal to the shv-Hin region. The animals homozygous for disk II mutations exhibit defects in the wing, haltere and male genitalia (St Johnston, Hoffmann et al. 1990).

The shv region controls proper gut morphogenesis in the larva, HH independent expression of DPP in the pupal wing vein and head capsule formation in the adult (Segal and Gelbart 1985; Immergluck, Lawrence et al. 1990). Mutations in the shv region result in the shortening or loss of the longitudinal veins towards the distal wing margin (Segal
and Gelbart 1985; St Johnston, Hoffmann et al. 1990; de Celis, Bray et al. 1997; Sotillos and de Celis 2006). Based on their phenotypes and their location with respect to the Hin region, the shv mutations can be grouped into three classes. The weakest shv-p (pupariation) mutations are located most distal to the Hin region and lead to lethality at late pupariation when heterozygous with any other shv mutation (Segal and Gelbart 1985). Mild shv-lc (larval lethal, complement disk mutations) mutations are more proximal to the Hin region and animals that are homozygous for these mutations undergo larval lethality, while the animals trans-heterozygous for these mutations and the disk mutations survive to adulthood as shv-lc mutations complement the disk mutations. The strongest shv-lnc (larval lethal, non-complementing) mutations are most proximal to the Hin region and the animals homozygous for the mutations in this class exhibit larval lethality, while the trans-heterozygous animals for these mutations and disk mutations fail to reach adulthood as the mutations in shv-lnc class fail to complement disk mutations (St Johnston, Hoffmann et al. 1990).

The genetic studies mentioned above and regulatory region fusions used in reporter based assays have provided evidence for the temporal regulation of DPP transcription through the cis-regulatory regions (Masucci, Miltenberger et al. 1990; Blackman, Sanicola et al. 1991; Hursh, Padgett et al. 1993). However, not much is known about the positive and negative signals that might direct DPP transcription during various stages of development. In the embryos, DPP transcription is known to be controlled by the interplay between multiple enhancer and silencer elements (Huang, Schwyter et al. 1993). In the wing, genetic and in vitro binding studies have identified several vein-specific transcription factors like ARAUCAN (ARA), KNI, VVL and DPP pathway
effectors like MOTHERS AGAINST DPP (MAD), and MEDEA that can bind to the conserved residues in these regulatory regions (Johnson, Bergman et al. 2003; Sotillos and de Celis 2006). However, there is no supporting bio-chemical evidence in the literature yet that demonstrates how these regulatory regions functionally control DPP expression.

2.8 An overview of the DPP signaling pathway in the Drosophila wing

The TGFβ signaling pathway elicits responses using a simple core signaling pathway in conserved vertebrates, insects and nematodes. This basic signaling is comprised of two receptor serine/threonine protein kinases (receptor types I and II) that bind the ligand. Upon ligand binding, the constitutively active type II receptor kinase activates the type I receptor which in turn phosphorylates a specific member of cytoplasmic transducers, so called receptor regulated Smads (R-Smads). The phosphorylated Smads translocate into the nucleus and assemble into multi-subunit complexes that regulate target gene transcription (Figure 7) {reviewed in (Heldin, Miyazono et al. 1997; Massague 1998; Raftery and Sutherland 1999)}.

Drosophila TGFβ signaling pathway components include three ligands {DPP, SCREW (SCW), and GBB}; three type I receptors {TKV, SAX and BABOON (BABO)}; three type II receptors {PUNT (PUT), WISHFUL THINKING (WIT) and STK-D}; and one Smad protein {MAD} (Brummel, Twombly et al. 1994; Nellen, Affolter et al. 1994; Penton, Chen et al. 1994; Terracol and Lengyel 1994; Xie, Finelli et al. 1994; Letsou, Arora et al. 1995; Ruberte, Marty et al. 1995; Twombly, Blackman et al. 1996; Simin, Bates et al. 1998; Brummel, Abdollah et al. 1999).
Figure 7 DPP/TGFβ signaling pathway

(A) Schematic drawing showing the steps in the TGFβ signaling pathway in which the ligand triggers the assembly of a receptor complex that phosphorylate Smads, which in turn assemble transcriptional complexes to regulate target gene transcription. The type I
receptors are activated by the type II receptors which in turn phosphorylate Smads. The Phosphorylated Smads along with co-Smads assemble transcriptional complexes that bind to DNA to regulate target gene expression. This figure was adapted and modified from (Massague and Chen 2000). (B) A schematic of the DPP signal transduction pathway that lists the various steps involved in DPP signaling. DPP is received by Type I and type II receptor complex, which activates MAD. MAD and MEDEA then form a complex to allow tissue specific target gene expression. The genes that are activated in response to DPP signaling in various tissues are listed below the tissue type. This figure has been adapted and modified from (Raftery and Sutherland 1999).
Of the above mentioned components, DPP, GBB, TKV, SAX, PUT and MAD are involved in DPP mediated BMP signaling in the Drosophila wing. DPP and GBB create gradients of BMP signaling along the A-P axis (Tanimoto, Itoh et al. 2000). The DPP homodimer or the DPP-GBB hetero-dimer is received by either TKV-PUT hetero-dimer or SAX-PUT hetero-dimer (Doctor, Jackson et al. 1992; Chen and Struhl 1998; Khalsa, Yoon et al. 1998; Shimmi, Umulis et al. 2005).

The local action of DPP is mediated through restriction of the signal to a narrow stripe of cells by the Type I receptors, TKV and SAX, with TKV assuming a more potent and possibly direct role (O'Connor, Umulis et al. 2006; Affolter and Basler 2007). SAX demonstrates low affinity binding to GBB and has been proposed to boost the intracellular level of DPP signal transmitted by TKV (Brummel, Twombly et al. 1994; Nellen, Affolter et al. 1994; Singer, Penton et al. 1997). The DPP receptors themselves are targets of BMP signaling and are down-regulated in response to BMP signaling (de Celis 1997; Lecuit and Cohen 1998; Tanimoto, Itoh et al. 2000; Ralston and Blair 2005).

Mis-expression of either DPP, GBB or an activated form of TKV results in ectopic venation as a result of spreading of the DPP signal (de Celis, Bray et al. 1997; Bangi and Wharton 2006; Sotillos and de Celis 2006). Mutations that remove the kinase domain of both type I and II receptors show dominant negative activity (Brand, MacLellan et al. 1993; Haerry, Khalsa et al. 1998).

The BMP receptors phosphorylate MAD that mediates all of the DPP signaling. Hence, the phosphorylation state of MAD (p-MAD) can be used as an intracellular marker to monitor DPP morphogen activity (Raftery, Twombly et al. 1995; Sekelsky, Newfeld et al. 1995; Newfeld, Chartoff et al. 1996; Wiersdorff, Lecuit et al. 1996; Kim,
Johnson et al. 1997; Newfeld, Mehra et al. 1997; Raftery and Sutherland 1999; Tanimoto, Itoh et al. 2000). p-MAD is detected over a broad area near the DPP source at the A/P compartment border in the wing imaginal disc (Tanimoto, Itoh et al. 2000).

MAD loss of function phenotypes are similar to DPP loss of function phenotypes. Also, partial reduction in MAD activity exacerbates phenotypes associated with specific DPP mutant genotypes (Raftery, Twombly et al. 1995; Sekelsky, Newfeld et al. 1995; Newfeld, Mehra et al. 1997). In addition, the gain of function phenotypes observed in TKVQ199D mutants (constitutively active TKV) can be suppressed by MAD mutations (Hoodless, Haerry et al. 1996).

MEDEA, a co-Smad, functions downstream of both DPP and TKV in responding cells (Newfeld, Mehra et al. 1997; Das, Maduzia et al. 1998). Gene expression studies using genetically mosaic wing imaginal discs have revealed differences in the requirements for the R-Smad, MAD and the co-Smad, MEDEA. Reducing MAD function results in the reduction in expression of SAL and OMB (Lecuit, Brook et al. 1996). MEDEA, on the other hand, is essential for OMB expression only in the cells that receive low levels of DPP signal (Wisotzkey, Mehra et al. 1998).

In addition to their role in vein development, DPP and GBB are both required for CV formation as loss of either DPP or GBB blocks signaling in the PCV (Ray and Wharton 2001; Ralston and Blair 2005). While the expression of SAX is not required for the formation of PCV, the expression of TKV is reduced in the PCV as a result of heightened BMP signaling (Ray and Wharton 2001; Ralston and Blair 2005).
2.9 The role of ECR and USP in wing patterning

Although critically important in the steroid hormone response of larval tissues, the genes in the ecdysone signaling cascade appear to have less impact in some imaginal tissues (Kozlova and Thummel 2002). Somatic clonal analysis in the wing imaginal disc using USP null clones has demonstrated that the loss of function of ecdysone receptor complex results in a failure of activation of early genes as well as precocious (premature) differentiation of sensory neurons in the margin (Schubiger and Truman 2000), suggesting a role for USP in repressing sensory neuron development and 20E in removing this repression. Further, studies using loss of function alleles of USP and ECR have demonstrated that both the components of the ecdysone receptor are needed to repress differentiation of the sensory organs in the wing disc through the de-repression of 20E target gene, BROAD (BR) (Schubiger, Carre et al. 2005). Schubiger et. al. also demonstrated a BR-independent repression of campaniform sensilla (sensory neurons) on the third vein by ECR/USP, suggesting a temporal regulation of sensory neurons in the wing, by 20E (Schubiger, Carre et al. 2005).

Genetic studies using mutations in ECR, CROOKED LEGS (CROL), an ecdysone inducible gene that encodes a family of zinc finger proteins; and genes encoding for integrin sub-units have demonstrated that the loss of ECR and CROL function results in defects in wing morphogenesis and cell adhesion through the regulation of INTEGRIN expression during metamorphosis (D'Avino and Thummel 2000). In particular, adult escapers that are transheterozygous for mutations in CROL and ECR show wing blisters as well as misshapen wings and legs. Also, flies homozygous for a semi-lethal
hypomorphic mutation, $CROL^{lex15}$ often have malformed wings or abnormal venation. Similar wing defects are also observed in some escaper flies that are homozygous for hypomorphic $ECR^{K06210}$ mutation which results from a $P$-element insertion that disrupts the ECR-A coding region and causes a reduction in ECR-B mRNA (D'Avino and Thummel 2000). In addition, flies transheterozygous for hypomorphic $ECR^{A483T}$ mutation and $ECR$ null mutations demonstrate wing defects that include ectopic CVs (Bender, Imam et al. 1997; Tsai, Kao et al. 1999). The additional CVs associated with $ECR$ mutations have not been observed in $CROL$ mutant wings suggesting that $CROL$ and $ECR$ have overlapping as well as unique functions during wing development. Understanding this 20E dependent regulation of wing development becomes even more difficult due to the fact that $CROL$ and $ECR$ cross-regulate each other, while $CROL$ is required for maximal $ECR$ expression during prepupal development, $CROL$ transcription itself is induced by 20E (D'Avino and Thummel 1998).

Flies transheterozygous for either $ECR^{M554fs}$, a small deletion in the LBD or $ECR^{C300Y}$, a missense mutation in the DBD show allele specific interactions with mutations in the genes involved in integrin family of cell receptors like $MYS^{n42}$ and $IF^{3}$ that result in defects in wing patterning (Wilcox, DiAntonio et al. 1989).

In vivo studies have indicated that a few escaper flies transheterozygous for $ECR^{A483T}$ and $ECR^{261st}$, an allele that removes both the DBD and the LBD domains of ECR, display significant delays in development, blistered wings, and defective tergites, indicating a role for ECR in the development of these tissues (Tsai, Kao et al. 1999). However, there is no evidence for the regulation of any wing vein specific genes by either 20E or ECR/ USP so far.
In addition to the role of ECR and USP in the wing development, organ culture experiments as well whole animal studies have revealed a requirement for 20E, ECR and USP in the morphogenetic furrow progression in the developing Drosophila eye disc (Li and Meinertzhagen 1995; Zelhof, Ghbeish et al. 1997; Brennan, Ashburner et al. 1998; Champlin and Truman 1998).
CHAPTER-3

MATERIALS AND METHODS

3.1 Culture of Drosophila and Fly Stocks

All stocks and genetic crosses were maintained at 25°C and 60-80% humidity on standard Drosophila yeast-cornmeal-dextrose medium unless otherwise indicated. Transgenic Drosophila shRNAi lines were obtained from the Vienna Drosophila RNAi Center (VDRC), National Institute of Genetics (NIG) (Mishima, Japan). The HA epitope-tagged full-length CMI transgene was designed to be expressed under UASGAL4 control {unpublished (Chauhan, Zraly et.al)}. 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). Expression of shRNAi and HA-epitope tagged full-length CMI constructs was carried out by crosses with various GAL4 driver strains at 18°C, 25°C or 29°C as indicated.

3.2 Fly stocks and genetic manipulations

Mutations in the CMI gene were generated by ∆2-3 transposase mediated excision of a P-element (EPgy2{EY06424}) residing approximately 380bp 5’ to the CMI transcript initiation site. Females from w; wg^{sp-1}/CyO; P{∆2-3}99B, Sb/TM6B flies were crossed to Df (2R)or^{BR11} cn^{l} bw^{l} sp^{l}/SM6a sp^{2} males and w; Df (2R)or^{BR11} cn^{l} bw^{l}
sp1/CyO; P{Δ2-3}99B, Sb/+ male progeny were next crossed to w; P{EPgy2}EY06424 females. Male progeny (w; Df (2R)orBR11 cn1 bw1 sp1/ P{EPgy2}EY06424; P{Δ2-3}99B, Sb/+ ) were next crossed to y1 w*; P{en2.4-GAL4}e22c/SM5 cn2 sp2 females. Individual curly wing non-speck male progeny (w-; AP{EPgy2}EY06424/SM5 cn2 sp2) were next crossed to Df (2R)orBR11 cn1 bw1 sp1/SM6a sp2 females and curly non-complementing (non-speck) flies were self-crossed to establish potential CMI mutant stocks. The presence of chromosomal deletions was first assessed by PCR using genomic DNA templates and deletion endpoints were determined by sequencing the PCR products. Both the CMI1 and CMI2 mutants were found to harbor small chromosomal deletions that remove the CMI promoter and first 61bp and 124bp of the CMI ORF, respectively. Reverse-transcriptase coupled PCR (RT-PCR) was performed as described (Zraly, Marenda et al. 2004) using RNA prepared from homozygous CMI mutant embryos balanced with a green fluorescent protein (GFP) reporter. PCR primers were used that spanned an intron of CMI (Forward 5’ GGTCTTCTGCACGGAACATT 3’; Reverse 5’ TCGATTGTCTGTGTTGGGATGA 3’).

3.3 Lethal phase analysis

Embryos from the OregonR, CMI1/ CyO ACTIN GFP, CMI2/ CyO ACTIN GFP, Sco/ CyO ACTIN GFP stocks and from crosses between CMI1/ CyO ACTIN GFP X CMI2/ CyO ACTIN GFP flies were collected on molasses containing caps and stored in the dark at room temperature (RT), for a period of 24 hrs. Lethal phase analysis of the CMI mutants was carried out as described (Marenda, Zraly et al. 2003). 100 embryos and
100 larvae from each instar were observed from every genotype for lethal phase determination.

3.4 Ecdysone feeding experiment

Embryos from various experimental crosses (\textit{CMI}^I/\textit{CyO ACTIN GFP}, \textit{CMI}^2/\textit{CyO ACTIN GFP} and \textit{CMI}^I/\textit{CyO ACTIN GFP} X \textit{CMI}^2/\textit{CyO ACTIN GFP}) as well as control stocks (\textit{OregonR} and \textit{Sco/ CyO ACTIN GFP}) were collected for 3 to 4 hours. Embryos were maintained at 25°C on molasses containing caps and allowed to hatch. Larvae homozygous for \textit{CMI} null alleles were then selected by their lack of GFP expression. The experimental and control larvae were analyzed for their ability to feed on 20E and their ability to undergo pupariation as described earlier (Gates, Lam et al. 2004). 100 larvae from each instar from every genotype were fed ecdysone.

3.5 Locomotion assay

The third-instar foraging stage larvae for the given genotypes were briefly washed with distilled water and moved to the center of a fresh experimental molasses containing plate. The larvae were then observed and auto-photographed for 2 minutes using Leica MZ16 stereo dissecting microscope. The photographs were then linked to form a movie using Leica software to study the path morphology, locomotion and the distance travelled by each larva. 10 larvae of every genotype were scored for this analysis.
3.6 **Olfaction assay**

The experimental and control larvae (obtained as described in section 3.4) were all washed with distilled water to remove any residual food. The larvae were then lined up on one end of an agar containing petri-plate with yeast paste at the other end of the plate. The larvae were then observed for their ability to smell the yeast paste and reach the other end of the plate within 2 minutes. 10 larvae from each genotype were scored for this analysis.

3.7 **Touch Sensitivity Assay**

The third-instar foraging stage experimental and control larvae (obtained as described in section 3.4) were used to perform touch sensitivity assay at 25 °C. The larvae were briefly washed with distilled water to remove any remaining food and transferred to the center of a fresh experimental plate with a soft paintbrush. The sensitivity was tested by touching each larva gently with an eyelash. The sensitivity was scored on a point system as described earlier (Caldwell, Miller et al. 2003). 10 larvae from each genotype were scored for this analysis.

3.8 **Darth Vader Assay**

We used a slightly modified version of the Darth Vader assays (Dettman, Turner et al. 2001; Caldwell, Miller et al. 2003). An agarose plate was illuminated from beneath with a white light transilluminator. One half of the plate was covered with aluminum foil to prevent the light from entering (“the dark side”) while the other half was lit. Single
larvae were placed in the middle of the test plate and observed for 90 s or till the larva reached the edge of the plate. 10 larvae from each genotype were scored for this analysis.

3.9 Generation of CMI shRNAi transgenic flies

Transgenic fly strains harboring CMI knockdown (CMI-IR) constructs were generated by injecting the appropriate engineered constructs into $w^{118}/\gamma^{1}w^{*}$ embryos (Bestgene, Inc., Chino Hills, CA). Knockdown of CMI in vivo was carried out using shRNAi constructs under GAL4-inducible control (Lam and Thummel 2000; Dietzl, Chen et al. 2007). A ~500bp N-terminal portion of the CMI ORF was cloned as an inverted repeat (antisense-sense) separated by a 74bp region of the WHITE gene intron into the pWIZ vector (Lee and Carthew 2003). Primers used for cloning the CMI sequences: Forward 5’ AAGCTCTAGAGCGGGCAAGGTG TGCTGTTTAT 3’; Reverse 5’ AAGCTCTAGAGCTCAATGTCCGTCGGAG 3’. Several independent transgene insertion lines were obtained for both constructs.

3.10 Single Fly PCR

To isolate DNA using a single fly, a single male fly of the required genotype was squished in 50 ul of Squishing Buffer (1M Tris, pH 8.2, 0.5M EDTA, pH 8.0; 5M NaCl and 20mg/ml proteinase K). This solution was then subjected to PCR with one cycle of 35 degrees for 30 minutes followed by 95 degrees for 5 minutes to deactivate the proteinase K. The DNA isolated was collected as the supernatant of this sample spun down and stored at -20 °C for later use.
3.11 Mounting abdomens

We used a slightly modified version of mounting Drosophila abdomens (Jeong, Rokas et al. 2006). The abdomens from control and experimental flies were treated with 10% potassium hydroxide (KOH) for 30 minutes to dissolve the guts. The abdomen was then cut into half; washed with 30% ethanol followed by subsequent washes with 70% and 100% ethanol; and mounted on slides using DPX mountant. Mounted slides were then analyzed under a light microscope.

3.12 Cuticle preparation

Mutant chromosomes were outcrossed to eliminate balancer chromosomes from the stocks before mutant cuticles were collected. Embryos were collected for approximately 12 hours and aged for more than 24 hours before preparing cuticles by standard techniques (Sucena and Stern 2000). Hatched larvae were collected and, after washing in water, treated with 1:4 glycerol: lactic acid and mounted in 80% glycerol. Mounted slides were then analyzed under a light microscope.

3.13 DNA Sequencing

All constructs were sequenced at the Loyola University Medical Center Sequencing Facility. Sequencing primers were provided by the sequencing facility. Sequences were compared to the available sequences deposited in the GenBank on the website of the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov).
3.14 CMI antibodies and immunostaining

A peptide corresponding to CMI amino acids 749-762 was used to prepare rabbit polyclonal antiserum (GenScript, Inc., Piscataway, NJ). Immunostaining of imaginal discs and pupal wings was performed as described (Zraly, Marenda et al. 2003) using CMI antibodies at 1:500 dilution and anti-rabbit HRP secondary antibodies at 1:500 (Jackson Immunoresearch).

Dissected wing imaginal discs from third instar larvae and pupal wings (25-46h APF) were fixed in 4% formaldehyde and stained as described in (de Celis 1997). The activation of DPP downstream effector MAD (P-Mad) was assayed by performing immuno-staining 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 1:500 dilution. Imaginal wings were mounted and photographed as described in the following section. The experimental and control samples were stained in parallel in all cases.

3.15 Examination of fly wings

A minimum of 100 fly wings were examined for each cross unless otherwise stated. Drosophila wings were dissected in isopropanol then mounted in DPX (Fluka) and photographed using a Leica MZ16 stereo dissecting microscope at 63X. All wings were dissected from female flies, unless otherwise stated.
3.16 X-gal staining of imaginal discs and pupal wings

The regulation of DPP transcription enhancers was assayed using LACZ reporter genes as described (DPPshv-LACZ.RD2) (Hursh, Padgett et al. 1993) and DPP-LACZ$^{dpp.RS3.0}$ (Blackman, Sanicola 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, Zraly et al. 2004). Third instar larvae were staged on blue food (0.05% bromophenol blue added to standard culture medium). Imaginal discs and early pupae (25-46h 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 15min at room temperature. The fixing solution was removed and replaced by pre-warmed staining solution [10mM NaH$_2$PO$_4$.H$_2$O/ Na$_2$HPO$_4$.2H$_2$O (pH 7.2.), 150 mM NaCl, 1mM 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 ul of 8% X-gal in DMSO). Reactions were incubated 1h 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 one hour at 37°C followed by an additional hour at room temperature, then mounted in 80% glycerol and photographed. The experimental and control samples were stained in parallel in all cases.
3.17 Generation of recombinants

Various recombinants were generated as described in the Appendix.
### Table 2 Primers used to detect the extent of deletion upon P-element excision

<table>
<thead>
<tr>
<th>Forward Primers</th>
<th>Primers (5’—3’)</th>
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<tbody>
<tr>
<td>HA CMI F1</td>
<td>ACA CCT AGG CGG TAC CAC TG</td>
</tr>
<tr>
<td>CMI P Sequencing F’</td>
<td>CG GAA TTC CG TTG TGC CAT CAT TTT TCC AG</td>
</tr>
<tr>
<td>CMI Genomic F3</td>
<td>CAG CTG AAT GCG ATT GGT AA</td>
</tr>
<tr>
<td>CMI GST Sequencing P’</td>
<td>ATA GCA TGG CCT TTG CAG</td>
</tr>
<tr>
<td>CMI Genomic F2</td>
<td>CTC CAG CAG CTC CAT AAA CC</td>
</tr>
<tr>
<td>CMI Sequencing Primer</td>
<td>GGA CTC CAA CAT GCT CGA GT</td>
</tr>
<tr>
<td>CMI GST Sequencing P’</td>
<td>ATA GCA TGG CCT TTG CAG</td>
</tr>
<tr>
<td>CMI FL Sequencing P’</td>
<td>TCG ATT GTC TGT TGG GAT GA</td>
</tr>
<tr>
<td>CMI RNAi F’</td>
<td>AAG CTC TAG AGC GGG CAA GGT GTG CTG TTT AT</td>
</tr>
<tr>
<td>Drev RTPCR F</td>
<td>TGT TAG CAT GGC TGT GTA TCG</td>
</tr>
<tr>
<td>CMI F2 P Sequencing</td>
<td>CGG AAT TCC GCT CCA GCA GCT CCA TAA ACC</td>
</tr>
<tr>
<td>CMI F4 P Sequencing</td>
<td>CGG AAT TCC GCC AGA GAG TTG TCG TTG</td>
</tr>
<tr>
<td>CMI F4</td>
<td>CCA GAG AGT TGT CGT TGT GG</td>
</tr>
<tr>
<td>CMI P Genomic F’</td>
<td>CTT CAG GAA CCC GTT GAT GT</td>
</tr>
</tbody>
</table>

### Reverse Primers

| HA CMI R1       | CTC CTC CAT GCT CAC AAC AA |
| CMI P Sequencing R’ | CG GGA TCC CG TTT TAC CAA TCG CAT TCA GC |
| CMI Genomic R2   | TGC GTT TCT TGT TGA TCT GC |
| Cmi Genomic R3   | ACT GGA AAA ATG ATG GCA CA |
| CMI RNAi R’      | AAG CTC TAG AGC TCA AAT GTT CCG TGC AGA AG |
| Drev RTPCR R’    | AGC TCG GAA ACG GTG TTG |
| CZR’             | CTC CTC CAT GCT CAC AAC AA |
The table above lists the various primers that were used to screen the extent of deletion in the mutants generated from the $P$-element excision mutagenesis screen.
CHAPTER-4

RESULTS

4.1 Drosophila CMI is the conserved counterpart to the N-terminus of the ALR family of nuclear receptor coactivators

Key structural features of the ALR coactivator family include a histone methyltransferase domain (SET domain) responsible for trimethylation of histone H3 lysine 4, PHD finger domains that are coordinated by zinc atoms and are thought to bind to trimethylated lysine 4 of histone H3, nuclear receptor (NR) binding motifs, FY-rich regions and an HMG domain implicated in DNA binding. A Drosophila ortholog, known as TRR (TRITHORAX RELATED), is related to the carboxyl terminal region of the ALR family, including the FY-rich and SET domains (Sedkov, Cho et al. 2003). Similar to the ALR family in mammals, TRR is required for hormone dependent gene expression. TRR lacks the N-terminal PHD fingers and HMG domain that may contribute to chromatin binding or possibly corepressor function in the absence of ligand. The PHD finger domains are highly conserved and are found among a diverse set of proteins involved in chromatin recognition and regulation (Aasland, Gibson et al. 1995; Bienz 2006; Mellor 2006). These domains mediate associations with repressor proteins, such as histone demethylases and deacetylases (Xia, Anderson et al. 2003; Shi, Matson et al. 2005; Soliman and Riabowol 2007). The Type 3 PHD finger domain recognizes and binds
trimethylated lysine 4 on histone H3 (Martin, Cao et al. 2006; Shi, Hong et al. 2006; Wysocka, Swigut et al. 2006). Therefore, we searched for other Drosophila genes that might encode these features and identified a single homolog we named CMI (CARA MITAD; CG5591). The CMI gene potentially encodes for a 163kDa protein with similar overall structure to the N-terminus of the ALR family, including two PHD finger clusters, an HMG domain and three potential NR binding sequences (Figure 8). Based on the phylogenetic analysis using the conserved PHD Type 3 finger, CMI appears to be equally related to both the ALR/MLL2 and HALR/MLL3 proteins, suggesting a common ancestral origin and possible duplication of the mammalian orthologs (Figure 3 and Figure 8).

**Figure 8** A rooted dendrogram showing clustering of the PHD fingers from various SET domain containing proteins and Drosophila CMI
The figure above shows a rooted dendrogram of the conserved third PHD finger of the second cluster from various SET domain containing proteins of different species. The PHD finger from MLL1 and 4 cluster together while the PHD finger from MLL2 and 3 clusters in a separate branch in the tree. The following abbreviations are used in the figure: MLL- MIXED LINEAGE LEUKEMIA, TRX- TRITHORAX, ALR- MLL2/ MIXED LINEAGE LEUKEMIA2, and TRR- TRITHORAX RELATED. The key for the species is as follows: Dya- Drosophila yakuba, Dsi- Drosophila simulans, Dan- Drosophila ananassae, Der- Drosophila erecta, Dwi- Drosophila willinstoni, Dse- Drosophila sechellia, Dps- Drosophila pseudoobscura, Dvi- Drosophila virilis, Dm- Drosophila mojavensis, Cp- Culex pipiens, Aa- Aedes aegypti, Ag- Anopheles gambiae, Am- Apis mellifera, Nav- Nasonia vitripennis, Is- Ixodes scapularis, Bm- Bombyx mori, Dv- Drosophila virilis, Dp- Daphnia pulex, Ph- Pediculus humanus, Rp- Rhodnius prolixus, Ap- Acyrthosiphon pisum, Dm- Drosophila melanogaster, and Tc- Tribolium castaneum. http://align.genome.jp/ program was used to generate this phylogenetic tree.
It is interesting to note that a single ALR ortholog exists in the nematode *C. elegans*, in the primitive metazoan *Nematostella*, in *Tribolium*, an insect of the order Coleoptera and even in *Anopheles; Aedes* and *Culex* (Diptera and Nematocera). However, these orthologs appear to be part of a single unit that includes the SET domain as well, implying that CMI and TRR ‘split’ at some point in the evolution of Diptera. A phylogenetic analysis of the conserved SET domain of TRR supports our hypothesis for the presence of a common ancestral origin and possible duplication of the mammalian orthologs (Figure 9).

Surprisingly, the *CMI* gene is located at cytological position 60A9 near the right end of the second chromosome, whereas the *TRR* gene is located on the X chromosome at 2B14. Thus, it is likely that the two genes split and acquired the abilities to be independently regulated.
Figure 9 A rooted dendrogram showing the clustering of the SET domains from various SET domain containing proteins from different species.
The figure above shows a rooted dendrogram for the SET domain from SET1 and MLL proteins from different species. The SET domain from the SET1 proteins from various species cluster together. The SET domain from MLL1 and 4 cluster together in one branch while the SET domain from MLL2 and 3 cluster in a separate branch. The various abbreviations used are as follows: MLL- MIXED LINEAGE LEUKEMIA, TRX-TRITHORAX, ALR- MLL2/ MIXED LINEAGE LEUKEMIA2, and TRR-TRITHORAX RELATED. The key for the species is as follows: Dv- Drosophila virilis, Cp- Culex pipiens, Aa- Aedes aegypti, Ag- Anopheles gambiae, Am- Apis mellifera, Nav- Nasonia vitripennis, Is- Ixodes scapularis, Bm- Bombyx mori, Ph- Pediculus humanus, Rp- Rhodnius prolixus, Ap- Acyrthosiphon pisum, Dm- Drosophila melanogaster, Tc- Tibolium castaneum. Hs- Homo sapiens, Gm- Glossina morsitans, Nvi- Nasonia vitripennis, Xt- Xenopus tropicalis, Xl- Xenopus laevis, Tr- Takifugu rubripes, Anc- Anolis carolinensis, and Gg- Gallus gallus. http://align.genome.jp/ program was used to generate this phylogenetic tree.
4.2 CMI excision mutants are amorphs

The generation of mutations in the CMI gene using Δ2-3 transposase mediated excision of a P-element (P\(\{\text{EPgy2}\}\text{EY06424}\)), resulted in eight excision mutants that failed to complement two independent overlapping deficiencies that remove CMI (Table 3). The inability of these mutants to complement the deficiencies suggests that these eight excisions remove an essential gene residing in the deficiencies, near the site of the P-element insertion. These mutants were numbered 93, 97, 169, 174, 175, 187, 198 and 207, in the order they were generated. These eight mutant lines were tested for their ability to complement each other as well as two independent deficiencies that remove regions 3’ and 5’ of CMI, respectively. The non-complementation analysis suggests that these eight mutations fall within two complementation groups (A and B), each containing four independent alleles (Table 4). To determine the extent of deletion upon P-element excision in these mutants, a PCR based screen was performed using multiple sets of primers, extending on either side of the P-element insertion site. The PCR analysis using primers within a neighboring gene transcribed from the opposite strand, DORA REVERSE TRANSCRIPTASE (DREV) and CMI suggests that Group B is most likely to have affected CMI. Two of the Group B mutants, CMI\(^1\) (187) and CMI\(^2\) (207) were cloned and sequenced. Sequencing results show that CMI\(^1\) is a unidirectional deletion of 485 base pairs and removes 61 base pairs (20 amino acids) of the CMI open reading frame (ORF). CMI\(^2\) is the result of a bidirectional deletion of 1074 base pairs that removes 124 amino acids of the DREV ORF and 68 amino acids of the CMI ORF.
Excision mutants 174 and 198 were not used in further analysis as they resulted in larger bi-directional deletions, extending into the \textit{DREV ORF}.

Homozygous \textit{CMI}^1 and \textit{CMI}^2 flies do not reach adulthood, suggesting that \textit{cmi} is an essential gene and the homozygous loss of \textit{CMI} is lethal. RT-PCR analysis on extracts from the homozygous \textit{CMI}^1 and \textit{CMI}^2 larvae confirmed a lack of production of \textit{CMI} transcript in \textit{CMI}^1 and \textit{CMI}^2 homozygous animals; a decreased production of neighboring \textit{DREV} transcript in \textit{CMI}^1 homozygous animals; and a loss of \textit{DREV} transcript in \textit{CMI}^2 homozygous animals (Figure 10). The \textit{CMI} transcript is detected in both \textit{OregonR} and the original \textit{P}-element carrying lines that served as controls (Figure 10). Our RT-PCR data confirms that both \textit{CMI}^1 and \textit{CMI}^2 mutations are amorphs. Since, the \textit{CMI}^1 mutant results from a uni-directional deletion into \textit{CMI}, we focused on \textit{CMI}^1 mutants for the rest of our analysis.
<table>
<thead>
<tr>
<th>Mutant #</th>
<th>93</th>
<th>97</th>
<th>169</th>
<th>174</th>
<th>175</th>
<th>187</th>
<th>198</th>
<th>207</th>
<th>Df(^1)</th>
<th>#16887(^2)</th>
<th>#18387(^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>93</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>C</td>
<td>-</td>
<td>C</td>
<td>C</td>
<td>-</td>
<td>C</td>
<td>-</td>
<td>C</td>
</tr>
<tr>
<td>97</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>C</td>
<td>-</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>-</td>
<td>C</td>
<td>C</td>
</tr>
<tr>
<td>169</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>C</td>
<td>-</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>-</td>
<td>C</td>
<td>C</td>
</tr>
<tr>
<td>174</td>
<td>C(^5)</td>
<td>C</td>
<td>C</td>
<td>-</td>
<td>C</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>C</td>
<td>C</td>
</tr>
<tr>
<td>175</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>C</td>
<td>-</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>-</td>
<td>C</td>
<td>C</td>
</tr>
<tr>
<td>187</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>-</td>
<td>C</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>C</td>
<td>C</td>
</tr>
<tr>
<td>198</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>-</td>
<td>C</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>C</td>
<td>C</td>
</tr>
<tr>
<td>207</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>-</td>
<td>C</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>C</td>
<td>C</td>
</tr>
<tr>
<td>Df</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

The first column and the first row list the excision mutant number. Each mutant was crossed to the remaining mutants; to a large deficiency \([\text{Df} \text{(or-BR 11)}/ \text{CyO}]\) that removes \textit{CMI}; and two smaller deficiencies \#16887 and \# 18387 that remove regions 3’ and 5’ of \textit{CMI}, respectively. This analysis allows testing for the ability of the mutants to complement each other and the deficiencies. The mutants highlighted in red failed to complement other mutants in red while the ones in green failed to complement other mutants in green.

1 \(\text{Df(or BR-11)/ CyO}\) and removes 59F6-59F8; 60A12-60A16
2 \(y^{w67c23}; \text{P(EPgy2)}CG5594EY08304/CyO; 3’ of cmi\) and removes 60 A8
3 \(w^{118}; \text{PBac}[WH]CG5602f00902/CyO; 5’ of cmi\) and removes 60 A9-11
4 Does not complement
5 Complements
Table 4 The P-element excision mutants fall into two complementation groups

<table>
<thead>
<tr>
<th>Group 1</th>
<th>Group 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>93</td>
<td>174</td>
</tr>
<tr>
<td>97</td>
<td>187 ( (CMI^1) )</td>
</tr>
<tr>
<td>169</td>
<td>198</td>
</tr>
<tr>
<td>175</td>
<td>207 ( (CMI^2) )</td>
</tr>
</tbody>
</table>

The table above lists the two groups of mutations that were obtained from \( P\{EPgy2\}/EY06424 \) excision mutagenesis screen. The mutants in Group 1 fail to complement other Group 1 mutants but complement Group 2 mutants and vice-versa. \( CMI^1 \) and \( CMI^2 \) mutants both fall in Group 2.
Figure 10 *CMI* transcript is absent in *CMI¹* and *CMI²* mutants

RT-PCR analyses of the late second instar (L2) homozygous larvae. The genotype of the larvae that were used to generate extracts is listed at the top. The names of the transcripts tested using RT-PCR is listed on the left-hand side of the gel pictures. The analysis shows that no *CMI* transcript is made in *CMI¹* and *CMI²*. However, *DREV* transcript is made in *CMI¹* homozygous mutant larvae, although at a reduced level. On the other hand, in *CMI²* homozygous mutant larvae, no *DREV* transcript is observed. Primers to detect *RIBOSOMAL PROTEIN49* (*RP49*) are used as a control for the RT-PCR.
4.3 Loss of CMI results in lethality

Since the homozygous CMI null animals do not make it to adulthood, we performed a lethal phase analysis on the homozygous mutant embryos. The lethal phase analysis revealed that all embryos homozygous for the CMI null mutations (CMI<sup>1</sup> and CMI<sup>2</sup>) hatch at rates similar to the wild type control (Table 5), suggesting that they either die at a later stage or the maternal contribution of CMI masks an essential requirement for CMI during the embryonic stage. To address if CMI was critically required at the larval stage, we performed a lethal phase analysis on the homozygous mutant larvae. Larvae from OregonR and Sco/ CyOActGFP flies were used as negative controls. Non-fluorescent larvae from the crosses between Df(2R)orBR-11/ CyOActin GFP or CMI<sup>1</sup>/CyO Act GFP to Df(2R) or BR-11/ CyO Actin GFP flies were used as positive controls. The number of animals that died at each larval stage was determined as shown in Table 5. Our data demonstrates that a majority of the homozygous mutant animals die during the late larval stage, predominantly at late L2 and less frequently during L3 (Table 5), suggesting a critical requirement for CMI during larval development. However, RNA in situ analysis has revealed a maternal contribution of CMI in the embryos (www.FlyBase.net), which possibly allows the homozygous null CMI mutants to reach the larval stages. Moreover, our observations suggest that the loss of CMI prompts larvae to stay in the larval stage for a prolonged period of time before they die as well as a failure to proceed into the pupal stages. We further carried out a more detailed phenotypic analysis of these mutants and observed that the CMI null larvae show various
phenotypic defects such as loss of body segmentation, presence of necrotic patches, histolysis, premature tanning and locomotion defects (these larvae are more sluggish than the WT controls) (data not shown). The lethality associated with the homozygous null CMI larvae, the inability of these mutants to undergo pupariation and the presence of necrotic patches, as well as histolysis and premature tanning in these larvae suggests a defective hormone signaling in CMI null mutants, further supporting our hypothesis that like ALR and TRR, CMI is a hormone dependent nuclear receptor coactivator.
Table 5  *CMI* mutants die during larval development

<table>
<thead>
<tr>
<th>Genotype</th>
<th>% fail to hatch</th>
<th>N (embryo)</th>
<th>1&lt;sup&gt;st&lt;/sup&gt; instar</th>
<th>2&lt;sup&gt;nd&lt;/sup&gt; instar</th>
<th>3&lt;sup&gt;rd&lt;/sup&gt; instar</th>
<th>Pre-pupa</th>
<th>N (larvae)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/+</td>
<td>1</td>
<td>100</td>
<td>5%</td>
<td>0%</td>
<td>3%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>cmi&lt;sup&gt;1&lt;/sup&gt;/+</td>
<td>3</td>
<td>100</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>cmi&lt;sup&gt;2&lt;/sup&gt;/+</td>
<td>8</td>
<td>100</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>cmi&lt;sup&gt;1&lt;/sup&gt;/cmi&lt;sup&gt;1&lt;/sup&gt;</td>
<td>n.a.</td>
<td>n.a.</td>
<td>11%</td>
<td>57%</td>
<td>24%</td>
<td>8%</td>
<td>0%</td>
</tr>
<tr>
<td>cmi&lt;sup&gt;2&lt;/sup&gt;/cmi&lt;sup&gt;2&lt;/sup&gt;</td>
<td>n.a.</td>
<td>n.a.</td>
<td>15%</td>
<td>72%</td>
<td>8%</td>
<td>5%</td>
<td>0%</td>
</tr>
<tr>
<td>Df(2R) or BR11/ CyOActGFP</td>
<td>n.a.</td>
<td>n.a.</td>
<td>12%</td>
<td>24%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>cmi&lt;sup&gt;1&lt;/sup&gt;/Df(2R)or-BR11</td>
<td>n.a.</td>
<td>n.a.</td>
<td>22%</td>
<td>50%</td>
<td>12%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>Sco/ CyOActGFP</td>
<td>n.a.</td>
<td>n.a.</td>
<td>3%</td>
<td>16%</td>
<td>1%</td>
<td>1%</td>
<td>0%</td>
</tr>
</tbody>
</table>

The percentage of *CMI* mutants that die at various stages in development are listed above. 100 embryos of the given genotype were collected to study the embryonic lethal phase. n refers to the total number of eggs and larvae collected for each study. To determine the lethal phase in *CMI* mutants at later stages of development, mutant first instar larvae were collected and monitored. +/+ (+ = WT) and Sco/CyOActGFP were used as controls (n.a. = not applicable).
4.4 The lethality observed in the CMI excision mutants is rescued by expressing a tagged full-length CMI in vivo

We hypothesized that if the larval lethality observed in the CMI excision mutants is due to the loss of CMI alone (not DREV), then the expression of a full-length CMI should rescue the lethality. Using the Gateway Cloning System, we established nine independent transgenic fly lines on the 2nd and 3rd chromosomes bearing a 3X FLAG and 3X Hemagglutinin (HA) epitope fused to the N-terminus of full-length CMI. These transgenic lines were tested to determine any lethality or phenotypes associated with the insertions. Table 6 summarizes the characterization of these HA-tagged full-length CMI transgenic lines.

The transgenic lines were further tested by inducing CMI transgene expression using GaWB69B GAL4 driver at 25 °C and performing a Western Blot analysis on the pupal extracts from this cross, using HA antibodies (Chauhan et. al., unpublished results). GAL4 is 881 amino acid long yeast protein that was identified in the yeast Saccharomyces cerevisiae as a regulator of galactose inducible genes such as GAL10 and GAL1. The GAL4 system has been modified to activate the transcription of target transgenes by making use of a GAL4 inducible upstream activating sequence (UAS) in the transgenes (Figure 11).
Table 6 Characterization of HA CMI transgenic lines

<table>
<thead>
<tr>
<th>Line No.</th>
<th>Sex of G1</th>
<th>Chromosome</th>
<th>Adult Location</th>
<th>Insertion homozygous viable</th>
<th>Homozygous flies fertile</th>
<th>Phenotype associated with insertion</th>
<th>Phenotype with OregonR Both @ 25 and 29°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>3</td>
<td>Yes</td>
<td>Yes</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>2</td>
<td>Yes</td>
<td>Yes</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>2</td>
<td>Yes</td>
<td>Yes</td>
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</tr>
<tr>
<td>4</td>
<td>M</td>
<td>3</td>
<td>Yes</td>
<td>Yes</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>2</td>
<td>Yes</td>
<td>Yes</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>6</td>
<td>M</td>
<td>2</td>
<td>Yes</td>
<td>Yes</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>7</td>
<td>M</td>
<td>3</td>
<td>Yes</td>
<td>Yes</td>
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<td>None</td>
</tr>
<tr>
<td>8</td>
<td>M</td>
<td>2</td>
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<td>None</td>
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<tr>
<td>9</td>
<td>M</td>
<td>2</td>
<td>Yes</td>
<td>Yes</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
</tbody>
</table>

The table above shows a summary of the characterization of the various HA CMI lines. The table lists the chromosomes in which the transgene was inserted. In all the cases, the insertion was homozygous viable suggesting that the insertion of the transgene did not lead to mutations in any other essential genes. The flies were all homozygous fertile and did not have any obvious phenotypes associated with the insertion itself. The last column shows the result from a cross between each transgenic line and WT flies at 29°C, further showing that the transgenic flies did not have any phenotypes associated with the insertion.
GAL4-UAS system can be used to induce any transgene by crossing the GAL4 containing flies (driver) to the fly containing the *UAS-transgene* (responder). In the progeny, the GAL4 protein is being made that binds the UAS and in turn leads to the transcription of the transgene. Various drivers are available that activate transgenes in a tissue and time dependent manner. Adapted from the review by Daniel ST Johnston, Nature reviews, Genetics, March 2002, Volume 2.
Table 7 Summary of CMI rescue data

<table>
<thead>
<tr>
<th>Maternal Genotype</th>
<th>Paternal genotype</th>
<th>Zygotic genotype</th>
<th>Temp.</th>
<th>Viability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GawB69B GAL4/ GawB69B</td>
<td>HA cmi/ CyO</td>
<td>HA cmi/ +; GawB69B</td>
<td>29</td>
<td>0</td>
</tr>
<tr>
<td>GAL4</td>
<td></td>
<td>GAL4/+</td>
<td>25</td>
<td>10</td>
</tr>
<tr>
<td>Df(or BR-11)/CyO; GawB69B</td>
<td>Df(or BR-11)/CyO;</td>
<td>cmi1/Df(or BR-11);</td>
<td>29</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>GawB69B GAL4/</td>
<td>GawB69B GAL4/+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Df(or BR-11)/CyO; GawB69B</td>
<td>HA cmi/ CyO</td>
<td>HA cmi/ Df(or BR-11);</td>
<td>29</td>
<td>0</td>
</tr>
<tr>
<td>Df(or BR-11)/CyO</td>
<td>cmi1, HA cmi/ CyO</td>
<td>cmi1, HA cmi/ Df(or BR-11);</td>
<td>29</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>25</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>22</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GawB69B GAL4/ GawB69B</td>
<td>cmi1, HA cmi/ CyO</td>
<td>cmi1, HA cmi/ +;</td>
<td>29</td>
<td>0</td>
</tr>
<tr>
<td>GAL4</td>
<td></td>
<td>GawB69B GAL4/+</td>
<td>25</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Df(or BR-11)/CyO; GawB69B</td>
<td>cmi1, HA cmi/ CyO</td>
<td>cmi1, HA cmi/ Df(or BR-11);</td>
<td>29</td>
<td>0</td>
</tr>
<tr>
<td>GAL4/ GawB69B GAL4</td>
<td></td>
<td>25</td>
<td>17*</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>43R</td>
<td></td>
</tr>
<tr>
<td>Df(or BR-11)/CyO; GawB69B</td>
<td>Df(or BR-11)/CyO</td>
<td>Df(or BR-11)/Df(or BR-11);</td>
<td>29</td>
<td>0</td>
</tr>
<tr>
<td>GAL4/ GawB69B GAL4</td>
<td></td>
<td>25</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>22</td>
<td>0</td>
</tr>
</tbody>
</table>
The table above summarizes the data from the rescue experiment. The first two columns list the maternal and the paternal genotypes of the flies used for the crosses, respectively. The following columns list the relevant zygotic phenotype followed by the temperature at which the crosses were carried out. The last column gives the percentage of the flies of the given zygotic genotype that made it to adulthood as compared to their sibling controls.

*- indicates rescue of lethality

R- progeny for the reciprocal cross
4.5 *CMI* null mutants exhibit developmental defects

We hypothesized that if *CMI* is critically required for the hormone dependent biological processes in the development of *Drosophila*, then the loss of *CMI* should give rise to phenotypic defects associated with development. We performed a series of tests that demonstrated various developmental defects in larvae homozygous for *CMI* null mutation, as discussed below.

The third instar foraging larvae show a preference for dark areas (Sawin-McCormack, Sokolowski et al. 1995). Results from the Darth Vader light sensitivity assay to test if these mutants were light insensitive, like some other mutants in the ecdysone signaling pathway, revealed that *CMI* null mutants do not show a difference in phototaxis as compared to the WT control animals (data not shown). These results suggest that *CMI* is not required for the regulation of genes in the ecdysone signaling pathway that are involved in mediating phototaxis response.

We further analyzed *CMI* mutants to test their sluggish movement by observing their locomotion behavior. Our data indicates that *CMI* homozygous mutants exhibit a slower rate of movement and traverse a smaller area as compared to the WT control animals (video not shown). Further, to test if these locomotion defects were due to muscle malfunction, we performed a touch sensitivity test on the *CMI* homozygous mutants. However, a statistical analysis of the data reveals that *CMI* mutants are not touch insensitive (data not shown), suggesting that the locomotion defects observed in the *CMI* mutant larvae are not due to muscle dysfunction.
4.6 **CMI null mutants are not defective in ecdysone bio-synthesis**

To determine if the inability of the *CMI* mutants to make it to adulthood was either due to a defect in ecdysone bio-synthesis or due to a defect in hormone signaling, 20E was administered to the mutant animals. Our observations revealed that feeding 20E did not rescue the lethality of the *CMI* 

mutants (data not shown), suggesting that the molting defects that are observed are not due to a defect in ecdysone bio-synthesis but could be due to a defect in hormone signaling. To further rule out the possibility that the *CMI* null mutants have an olfactory defect that could result in the larvae not feeding and hence the observed phenotypes, we performed an olfactory assay. Our data showed that both WT and mutant larvae were able to smell the food, reach it and also feed on it at similar rates (data not shown), suggesting that the phenotypes observed in these animals are neither due to an olfactory defect nor due to malnutrition.

4.7 **CMI null animals are defective in ecdysone signaling**

The developmental transition from the larval to the pupal stage is carried out under a tight regulation of genes by JH and 20E. The fact that *CMI* mutant larvae exhibited pre-mature tanning and prolonged larval stages, suggested a mis-regulation of hormone dependent events in these animals. We, therefore, examined if these animals exhibited any molting defects like the duplication of mouth-hooks that are associated with abnormal hormone signaling. The WT second instar larvae have small mouth-hooks with a few large teeth (Figure 12A), as against the WT third instar larvae that have large mouth-hooks with many small teeth (Figure 12B). The third instar *CMI* 

homozygous
larvae show improper mouth-parts in the second and the third instar larvae (Figure 12C and D) as well as retention of both second instar and third instar mouth-hooks (Figure 12E), phenotypes observed in mutations in genes like *ECR, USP, ECDYSONELESS (ECD)* and *RIGOR MORTIS (RIG)* that are components of the hormone signaling pathway (Perrimon, Engstrom et al. 1985; Oro, McKeown et al. 1992; Hall and Thummel 1998; Schubiger, Wade et al. 1998; Li and Bender 2000; Carney, Robertson et al. 2004; Gates, Lam et al. 2004; Davis, Carney et al. 2005). Our results suggest that an improper hormone signaling could be responsible for these molting defects in the *CMI* null mutants.
Figure 12 Molting defects in *CMI* null homozygous mutants

The figure above shows the mouth-hooks from WT and *CMI* null homozygous larvae. (A) WT L2 have a few large teeth (B) WT L3 have numerous small teeth. Homozygous null *CMI* mutants die as L2/L3 larvae with defects in molting (shedding of the larval cuticle) and malformed L2 mouth hooks (C), L3 mouth hooks (D), as well as retained L2 and malformed L3 (E) mouth hooks in mutant L3 larvae.
4.8 *CMI*\(^I\) genetically interacts with *ECR*

To further determine if *CMI* is involved in the ecdysone signaling pathway, we looked for a genetic interaction between *CMI* and *ECR* by looking for dominant synthetic phenotypes upon crossing *CMI*\(^I\) null allele to various loss of function alleles of *ECR*. While neither heterozygous *CMI*\(^I\) nor *ECR*\(^{A483T}\) (an EMS mutation in the LBD of the ECR-B1 ORF) flies show any wing specific phenotypic defects, we observed incomplete PCV (a late patterning event) in 50% of flies that are transheterozygous for *CMI*\(^I\) and *ECR*\(^{A483T}\) (Figure 13A), suggesting an *ECR* dependent role for *CMI* in wing patterning. However, we did not observe any wing patterning defects in flies transheterozygous for *CMI*\(^I\) and other *ECR* loss of function alleles, which means this interaction is allele specific. In addition, the wing specific knock-down of *ECR* did not result in any wing patterning defects, further suggesting that the role of *CMI* in the early wing development is not entirely *ECR* dependent (data not shown).

4.9 *CMI*\(^I\) genetically interacts with *TRR*

TRR is known to play a role in eye patterning by regulating HH in response to ecdysone signaling (Sedkov, Cho et al. 2003). Based on the structural similarity between *CMI* and ALR and the fact that ALR is split into CMI and TRR in Drosophila, we hypothesized that CMI and TRR co-operate in regulating their targets and hence in tissue patterning. We, therefore, tested if TRR could genetically interact with CMI to give rise
to tissue patterning defects. We performed a genetic epistasis test between CMI1 null allele and TRR4 hypomorphic alleles, neither of which exhibit wing patterning defects when heterozygous with a WT allele. However, we observed incomplete ACV, L4 and L5 veins in 50% of the escaper male flies, as well as shortening of the wing size in flies that are transheterzygous for CMI1 and TRR4 (Figure 13B). Our genetic epistasis results suggest that CMI and TRR might synergistically co-operate to control wing vein patterning, possibly through an event regulated by the ecdysone signaling pathway.
Figure 13 *CMI* genetically interacts with *ECR* and *TRR*

Wing patterning defects observed in flies transheterozygous for mutations in *CMI* and *ECR* and *TRR*. (A) *CMI<sup>1</sup>/ECR<sup>A483T</sup>* wing shows incomplete PCV while a *TRR<sup>4</sup>/Y; CMI<sup>1</sup>/+* wing shows incomplete L4, L5 and ACV as indicated by the arrows.
4.10  The over-expression of CMI results in developmental defects

To determine the role of CMI in Drosophila development, we over-expressed CMI by crossing the HA-CMI transgenic lines to various ubiquitous GAL4 driver lines. A summary of driver-specific phenotypes observed upon the over-expression of CMI are listed in Table 8.
Table 8 Summary of CMI over-expression phenotypes

<table>
<thead>
<tr>
<th>No.</th>
<th>GAL4 driver used</th>
<th>Temp. in °C</th>
<th>Phenotype</th>
<th>% with phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Actin 5c GAL4(^E)</td>
<td>29</td>
<td>Embryonic lethal</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>25</td>
<td>Embryonic lethal</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>Sevenless GAL4</td>
<td>25</td>
<td>Rough eye</td>
<td>68</td>
</tr>
<tr>
<td>3</td>
<td>A9 GAL4</td>
<td>29</td>
<td>Pupal lethal (males)</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>25</td>
<td>Severe wing defects</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Severe wing blistering /</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Wings reduced in size</td>
<td>100</td>
</tr>
<tr>
<td>4</td>
<td>e22cGAL4(^E)</td>
<td>29</td>
<td>Embryonic lethal</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>25</td>
<td>Embryonic lethal</td>
<td>100</td>
</tr>
<tr>
<td>5</td>
<td>Distalless GAL4</td>
<td>29</td>
<td>Extra humoral bristle</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td></td>
<td>25</td>
<td>Extra humoral bristle</td>
<td>10</td>
</tr>
<tr>
<td>6</td>
<td>GawB69B GAL4</td>
<td>29</td>
<td>Pupal lethal</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>25</td>
<td>Wing patterning defects</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ectopic sex combs</td>
<td></td>
</tr>
</tbody>
</table>

The table above lists the various phenotypes that are observed upon over-expressing CMI using different drivers (as listed on the left) at different temperatures. On the right is the percentage of animals that showed the phenotype.

\(^U\) = Ubiquitously expressed

\(^E\) = Embryonically expressed
Consistent with the role of \textit{CMI} as a global regulator, we observed a range of phenotypes upon over-expression of \textit{CMI} using various tissue and stage specific \textit{GAL4} driver lines that range from defects in the wings, abdomen and legs to lethality (Table 8, Figure 14 and Figure 15). The phenotypes associated with the over-expression of the \textit{CMI} tagged transgene were further studied by altering the severity of the over-expression by performing this analysis at various different temperatures (\textit{GAL4} is a yeast protein that is most active as a transcription factor at 29 °C, thus potentially leading to more severe phenotypes at higher temperatures as compared to the lower temperatures). The over-expression phenotypes indicate that it is critical to maintain appropriate levels of \textit{CMI} for the proper development of Drosophila.

We further generated recombinants between \textit{e22c \textit{GAL4}}, an embryonic driver and \textit{CMI\textsuperscript{I}} mutants. We observed that the over-expression of \textit{HA-CMI} in the \textit{CMI\textsuperscript{I}} mutant background still results in embryonic lethality (at 25 and 29 °C) (data not shown). These results further confirm that the right levels of \textit{CMI} are very critical at the early embryonic stages of Drosophila development.
Over-expression of *CMI* results in pigmentation defects in adult abdomens. (A) A WT female fly showing the abdominal segments A1-6 with increasing amount of pigmentation with each segment. (B) A WT male fly showing intense pigmentation in A5 and A6. (C) and (D) Over-expression of *CMI* results in diffused and patchy pigmentation in A5 and A6 in the female and male flies respectively.
Figure 15 Over-expression of *CMI* results in tissue patterning defects

Various phenotypic defects are observed upon *CMI* over-expression using tissue specific drivers (A) A WT fly shows normal eye. (B) A *SEVENLESS GAL4/X, HA- CMI/+* fly at 25°C shows an ectopic growth in the eye. (C) The first leg of a WT male fly shows one set of sex combs on the first tarsal segment. (D) A *GawB69B GAL4/ HA-CMI* male shows a duplication of the sex combs at 25°C.
4.11 Knock-down of CMI results in patterning defects

To further determine the role of CMI in adult Drosophila development, we generated shRNAi knock-down lines. A summary for the characterization of the various CMI shRNAi lines is indicated in Table 9.

Consistent with the role of CMI as a global regulator, tissue and stage specific knock-down of CMI using shRNAi construct resulted in a range of phenotypic defects as listed in Table 10. The phenotypes associated with the knock-down of CMI were further studied by altering the severity of the knock-down by performing the analysis at different temperatures. In some cases, the knock-down was carried out in a DICER over-expression background to increase the processing efficiency of the hairpin in generating a silencing RNA (Dietzl, Chen et al. 2007). Consistent with the over-expression phenotypes, the knock-down phenotypes also indicate that it is critical to maintain appropriate levels of CMI for the proper development of Drosophila.
Table 9 Summary of the characterization data for the CMI sh RNAi lines

<table>
<thead>
<tr>
<th>Line No.</th>
<th>Sex of G1</th>
<th>Chromosome Location</th>
<th>Insertion homozygous</th>
<th>Homozygous flies fertile</th>
<th>Phenotype associated with insertion</th>
<th>Phenotype with OregonR</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>2</td>
<td>Yes</td>
<td>Yes</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>2</td>
<td>Yes</td>
<td>Yes</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>3</td>
<td>Yes</td>
<td>Yes</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>X</td>
<td>Yes</td>
<td>Yes</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>2</td>
<td>Yes</td>
<td>Yes</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>6</td>
<td>M</td>
<td>3</td>
<td>Yes</td>
<td>Yes</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>7</td>
<td>M</td>
<td>3</td>
<td>Yes</td>
<td>Yes</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>8</td>
<td>M</td>
<td>2</td>
<td>Yes</td>
<td>Yes</td>
<td>None</td>
<td>None</td>
</tr>
</tbody>
</table>

Summary of the characteristics of the various CMI-IR lines. Listed are the chromosomes in which the transgene was inserted. The last column summarizes the results from a cross between each transgenic line and WT flies at 29°C.
Table 10 Summary of CMI knock-down phenotypes using various GAL4 drivers

<table>
<thead>
<tr>
<th>No.</th>
<th>GAL4 driver</th>
<th>Temp in °C</th>
<th>Phenotype</th>
<th>% showing phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NGT-40\textsuperscript{6} GAL4</td>
<td>29</td>
<td>L2 defective towards wing margin</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Extra humoral bristle</td>
<td>8</td>
</tr>
<tr>
<td>2</td>
<td>A9 GAL4</td>
<td>29</td>
<td>Pupal lethal</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>25</td>
<td>Ectopic ACV with dicer</td>
<td>42</td>
</tr>
<tr>
<td>3</td>
<td>GawB69B GAL4</td>
<td>29</td>
<td>Pupal lethal</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>25</td>
<td>Severely malformed wings with patterning defects</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ectopic eye (eye blistering -small)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Sevenless GAL4</td>
<td>29</td>
<td>patches</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>L2 defective towards wing margin</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td></td>
<td>25</td>
<td>Eye blistering</td>
<td>5</td>
</tr>
<tr>
<td>5</td>
<td>\textsuperscript{e}23\textsuperscript{6} GAL4</td>
<td>29</td>
<td>Embryonic lethal</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>25</td>
<td>Embryonic lethal</td>
<td>100</td>
</tr>
<tr>
<td>6</td>
<td>Actin 5c GAL4\textsuperscript{7}</td>
<td>29</td>
<td>Embryonic lethal</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>25</td>
<td>Embryonic lethal</td>
<td>100</td>
</tr>
<tr>
<td>5</td>
<td>Distalless.GAL4</td>
<td>29</td>
<td>Extra humoral bristle</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td></td>
<td>25</td>
<td>Extra humoral bristle</td>
<td>21</td>
</tr>
</tbody>
</table>

\textsuperscript{6} Embryonically expressed
\textsuperscript{7} ubiquitously expressed
Of the various phenotypes observed upon over-expression and knock-down of $CMI$, the wing vein defects using wing specific drivers ($c765GAL4$ and $GawB69BGAL4$) were the most striking (Figure 16 and Table 10). The knock-down of $CMI$ in the wing resulted in incomplete longitudinal veins and an increase in the wing size. On the other hand, an over-expression of $CMI$ results in ectopic veins and a decrease in the wing size (Figure 16 and Table 13), as discussed in the following sections.

The Drosophila wing is a widely studied model system to understand the various conserved signaling pathways and the cross-talk between them. We, therefore, decided to use the Drosophila wing to understand the function of $CMI$ in adult tissue patterning.
Figure 16 Knock-down and over-expression of *CMI* leads to wing patterning defects

*OregonR/ OregonR*

*HA cmi/c765 Gal4*

*UAS Dicer/+; cmi IR/+; c765Gal4/+*
Wing patterning defects (as indicated by the arrows) that are observed upon CMI knock-down and over-expression. (A) WT OregonR wing (B) HA CMI/+; c765GAL4/+ fly shows ectopic L2, L4 and L5 towards the distal wing margin and an incomplete L2 towards the proximal axis. (C) UAS DICER/+; CMI IR/+; c765GAL4/+ fly shows incomplete L2, L5 and PCV. Note that the knock-down of CMI leads to an increase in the size of the wing whereas an over-expression of CMI results in a decrease in the wing size. All pictures were taken at the same magnification.
4.12 **CMI regulates wing pattern development independent of HH**

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 reproducible and penetrant 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 (Figure 16 and Table 13).

Hormone-dependent transcription of the HH gene in Drosophila S2 tissue culture cells is regulated by the histone methyltransferase activity of TRR and genetic studies have revealed that TRR regulates HH function in the developing Drosophila eye (Sedkov, Cho 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. 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). As discussed in section 4.9, our data demonstrates that TRR cooperates with CMI in hormone regulated gene transcription. 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 (Figure 17).
Figure 17 CMI is present in both vein and inter-vein cells in the pupal wings

(A-B) Wild type OregonR pupal wings that served as (-) antibody controls. (C-D) Wild type OregonR pupal wings immunostained with polyclonal rabbit peptide antibodies against CMI. The CMI protein is present in both vein and intervein cells as shown by the arrows in (C). Note that there is increased expression of CMI along the distal wing margin. (D) CMI is present at similar levels in both vein and intervein cells near the posterior crossvein (PCV).
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 $EN$ gene (Figure 18B). 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 (Figure 18C and Table 11). Co-expression of $DICER$ to increase the efficiency of $CMI$ knock-down (Dietzl, Chen et al. 2007) led to a strong enhancement of the incomplete vein phenotype as well as wing blistering (Figure 18D). In both situations, the incomplete vein phenotypes were restricted to the posterior wing compartment. 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, C. and Zraly, CB., unpublished results). We therefore performed a weaker over-expression 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 $CMI$ (Bellen, Levis et al. 2004). We observed that modest over-expression of $CMI$ using $EN$ GAL4 driver resulted in wing blistering and the formation of ectopic veins only in the posterior wing compartment (Figure 18E).
Figure 18 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 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 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.
Table 11 Summary of phenotypes observed upon misexpression of CMI using an *EN GAL4* driver

<table>
<thead>
<tr>
<th>Genotype</th>
<th>UAS Dicer/X; cmi IR+; enGAL4/+</th>
<th>cmi IR+/+; enGAL4/+</th>
<th>P(cmijy); enGAL4/+</th>
</tr>
</thead>
<tbody>
<tr>
<td>(number of flies scored)</td>
<td>(30)</td>
<td>(56)</td>
<td>(120)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Phenotypes observed</th>
<th>Percent showing phenotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wing blistering in posterior half</td>
<td>87</td>
</tr>
<tr>
<td>Incomplete ACV</td>
<td>3</td>
</tr>
<tr>
<td>Incomplete L5</td>
<td>10</td>
</tr>
<tr>
<td>Incomplete PCV</td>
<td>3</td>
</tr>
<tr>
<td>Ectopic L2</td>
<td>-</td>
</tr>
<tr>
<td>PCV bifurcation</td>
<td>-</td>
</tr>
<tr>
<td>ACV ectopic/thickened</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>-</td>
</tr>
</tbody>
</table>
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 directly, we 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 (data not shown). Thus, HH does not appear to be the primary target of CMI regulation in wing patterning, suggesting that it functions downstream of HH or in another signaling pathway.

4.13 CMI genetically interacts with DPP

The CMI loss and gain of function wing phenotypes are strongly consistent with defective DPP signaling (Martin-Castellanos and Edgar 2002; De Celis 2003). To clarify the role of CMI in wing patterning, we first looked for the regulation of the DPP pathway by testing for dose-sensitive interactions between the CMI\textsuperscript{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, Hoffmann et al. 1990) (Figure 19). The DPP\textsuperscript{d-ho} allele is a recessive mutation in the DPP 3’ disk regulatory region that produces flies with held-out wings when homozygous and in trans-heterozygous combinations with the DPP\textsuperscript{sl} mutation that affects the 5’ short vein (shv) region (Gelbart 1982; Irish and Gelbart 1987). While DPP\textsuperscript{sl} DPP\textsuperscript{d-ho} double 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’ disk region ($DPP^{d14}$) is fully recessive (Figure 20A), while flies carrying transheterozygous combinations with $DPP^{s1} DPP^{d-ho}$ exhibit a held-out wing phenotype (Figure 20B). Flies heterozygous for $DPP^{d14}$ and the $CMI^l$ null mutation display a completely penetrant held out wing phenotype (Figure 20C) 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 (Figure 20D). While heterozygous $DPP^{s1}/CMI^l$ flies show a shortened crossvein (Figure 20E), both L4 and posterior crossvein (PCV) shortening is enhanced when $DPP^{s1}$ is homozygous (Figure 20F and Table 12). The L4 longitudinal vein is further shortened in homozygous $DPP^{s1} DPP^{d-ho}$ flies (Figure 20G), while heterozygotes appear normal. In $CMI^l/DPP^{s1} DPP^{d-ho}$ double heterozygotes the L4 vein is significantly shortened (Figure 20H) and the incomplete vein phenotype is strongly enhanced when the $DPP$ alleles are homozygous in the presence of $CMI^l$ (Figure 20I). These results support the view that $CMI$ regulates the development of wing veins through interactions with the DPP signaling pathway.
Figure 19 Locations of selected *DPP* mutant alleles as well as 5’ and 3’ transcription enhancers within the *DPP* genomic locus.

The *DPP* locus is contained within an approximately 60kb region. Shown are the relative positions and placements of the 5’ shortvein (shv), Haploinsufficiency (Hin) and 3’ regulatory disk regions along with the approximate locations of the mutations used in this study. Also shown are the locations of various *DPP* transcripts, including both coding and non-coding exons. The shaded bars indicate the relative positions of the regions included in the *DPP*<sup>shv</sup>-LACZ.RD2 and *DPPBS3.0*-LACZ reporter constructs.
Figure 20 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^{s1}/DPP^{s1} display a similar held out wing phenotype. (D) DPP^{s1}/DPP^{s1} flies display an incomplete L4 vein toward the distal wing margin. (E) CMI^{s1}/DPP^{s1} transheterozygous flies show incomplete PCV. (F) CMI^{s1}, 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 toward the distal wing margin. (H) CMI^{s1}/DPP^{s1} DPP^{d-ho} transheterozygous flies show an enhancement of the incomplete L4 observed in (G). (I) CMI^{s1}, 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 12 Summary of genetic interaction between loss of function alleles of *CMI* and *DPP*

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Percent showing phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Inc. L2</td>
</tr>
<tr>
<td><em>dpp</em>&lt;sup&gt;1&lt;/sup&gt;/ <em>dpp</em>&lt;sup&gt;1&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td><em>cmi</em>&lt;sup&gt;1&lt;/sup&gt;/ <em>dpp</em>&lt;sup&gt;1&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td><em>cmi</em>&lt;sup&gt;1&lt;/sup&gt;, <em>dpp</em>&lt;sup&gt;1&lt;/sup&gt;/ <em>dpp</em>&lt;sup&gt;1&lt;/sup&gt;</td>
<td>6&lt;sup&gt;8&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>dpp</em>&lt;sup&gt;1&lt;/sup&gt;<em>dpp</em>&lt;sup&gt;d-ho&lt;/sup&gt;/ <em>dpp</em>&lt;sup&gt;1&lt;/sup&gt;<em>dpp</em>&lt;sup&gt;d-ho&lt;/sup&gt;</td>
<td>65</td>
</tr>
<tr>
<td><em>cmi</em>&lt;sup&gt;1&lt;/sup&gt;/ <em>dpp</em>&lt;sup&gt;1&lt;/sup&gt;<em>dpp</em>&lt;sup&gt;d-ho&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td><em>cmi</em>&lt;sup&gt;1&lt;/sup&gt;, <em>dpp</em>&lt;sup&gt;1&lt;/sup&gt;<em>dpp</em>&lt;sup&gt;d-ho&lt;/sup&gt;/ <em>dpp</em>&lt;sup&gt;1&lt;/sup&gt;<em>dpp</em>&lt;sup&gt;d-ho&lt;/sup&gt;</td>
<td>93&lt;sup&gt;10&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

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8 New genetic interaction
9 Enhancement of *dpp*<sup>1</sup> phenotype
10 Enhancement of the *dpp*<sup>1</sup>*dpp*<sup>d-ho</sup> phenotype
11
A prediction of this model is that \textit{DPP} loss of function wing vein phenotypes would be sensitive to CMI levels. We used a wing-specific \textit{GAL4} driver (\textit{c765GAL4}) and shRNAi (IR) transgenes to knock-down both \textit{CMI} and \textit{DPP} simultaneously. Modest expression of either the \textit{DPP-IR} or the \textit{CMI-IR} in the wing disc resulted in shortened wing veins, as expected (Figure 21A, B and C). Simultaneous knockdown of both \textit{DPP} and \textit{CMI} resulted in further shortening of the wing veins (Figure 21D). \textit{HA-CMI} over-expression in the wing disc results in both ectopic vein formation and a disrupted proximal L2 vein (Figure 21E). Simultaneous overexpression of \textit{HA-CMI} and \textit{DPP-IR} results in partial suppression of both the incomplete vein phenotypes observed upon \textit{DPP} knock-down as well as the ectopic vein phenotypes observed upon \textit{CMI} over-expression (Figure 21F and Table 13).
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. Representative pictures are shown from each genotype; however, the severity of the phenotypes varies within a given genotype.
Table 13 Summary of the *CMI* and *DPP* mis-expression phenotypes

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>dpp-IR+/c765/+</th>
<th>c765, HA cmi/+</th>
<th>c765, HA cmi/+; dpp-IR+</th>
<th>c765 Gal4, cmi IR/+</th>
<th>c765, cmi IR/+; dpp-IR/+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ectopic L2</td>
<td></td>
<td>100</td>
<td>55&lt;sup&gt;11&lt;/sup&gt;</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ectopic L3</td>
<td></td>
<td>100</td>
<td>25&lt;sup&gt;12&lt;/sup&gt;</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ectopic L4</td>
<td></td>
<td>100</td>
<td>32&lt;sup&gt;12&lt;/sup&gt;</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ectopic L5</td>
<td></td>
<td>100</td>
<td>87&lt;sup&gt;12&lt;/sup&gt;</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>Inc. ACV/missing</td>
<td></td>
<td>14</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Inc. L2 proximally</td>
<td></td>
<td>47</td>
<td>20&lt;sup&gt;12&lt;/sup&gt;</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Inc. L2 distally</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>10</td>
<td>82&lt;sup&gt;12&lt;/sup&gt;</td>
</tr>
<tr>
<td>Inc. L5 distally</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>38&lt;sup&gt;13&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>11</sup> = suppression of the *HA-cmi* phenotype  
<sup>12</sup> = enhancement of the *cmi-IR* phenotype
One possible interpretation of these results is that \textit{CMI} acts upstream of \textit{DPP}, perhaps through positive regulation of \textit{DPP} transcription in presumptive wing vein cells; however, these data may also be explained by \textit{CMI} function downstream through regulation of DPP-responsive target genes. To address this question, we ectopically expressed \textit{DPP} under GAL4 control, bypassing any possible regulation by \textit{CMI}. Ectopic expression of \textit{UAS-DPP} in the wing results in severe wing defects, including blisters, ectopic veins and tissue overgrowth (Capdevila and Guerrero 1994; Staehling-Hampton and Hoffmann 1994). Neither increasing nor decreasing CMI levels, through expression of \textit{HA-CMI} or \textit{CMI-IR}, in combination with \textit{UAS-DPP} had any significant effect on the \textit{UAS-DPP} wing phenotype (data not shown). This suggests that \textit{CMI} most likely functions upstream of \textit{DPP}, rather than on downstream targets of DPP signaling in the presumptive wing vein cells during pupal development.

\subsection*{4.14 \textit{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 (reviewed in (Affolter and Basler 2007)). The local action of DPP is mediated through restriction of the signal to a narrow stripe of cells by the Type I receptors, THICKVEINS (TKV) and SAXOPHONE (SAX), with TKV assuming a more potent and possibly direct role (O'Connor, Umulis et al. 2006; Affolter and Basler 2007). A prediction based on the above genetic tests of \textit{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 CMI<sup>i</sup> 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<sup>KG05725</sup> is a hypomorphic allele that results from a P-element insertion upstream of SAX (Dworkin and Gibson 2006) and SAX<sup>δ</sup> is an amorphic allele (Singer, Penton et al. 1997); while TKV<sup>i</sup> is a cold temperature sensitive hypomorphic mutation (Diaz-Benjumea and Garcia-Bellido 1990; Terracol and Lengyel 1994). Recombinants were generated between CMI<sup>i</sup> and TKV<sup>i</sup>, CMI<sup>i</sup> and SAX<sup>δ</sup>, and between CMI<sup>i</sup> and SAX<sup>KG05725</sup>, as all mutant alleles are recessive. Recombinants were crossed to parental flies to generate homozygous or transheterozygous TKV and SAX alleles along with the CMI<sup>i</sup> heterozygote. The results of these crosses are summarized in Table 14. In these genetic tests, reduced CMI function exhibited a modest suppression of the thickened vein phenotype associated with reduced TKV and SAX function (data not shown) consistent with models in which CMI positively regulates DPP signaling.

The CMI<sup>i</sup> 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 (which is an inhibitory receptor that prevents the DPP signal from spreading) in the imaginal disc are similar to overexpression of DPP, including enlargement of wing size in addition to
ectopic veins (Figure 22A). As expected, the simultaneous knock-down of $CMI$ and $TKV$ together results in a suppression of $TKV$ knock-down phenotypes, presumably as a consequence of reducing DPP signaling (Figure 22B). In contrast, the over-expression of $HA-CMI$ results in an enhancement of the $TKV$ knock-down phenotypes, as would be expected if DPP signaling was elevated (Figure 22C).
Table 14 Summary of genetic interaction between *CMI*, *TKV* and *SAX*

<table>
<thead>
<tr>
<th>Genotype</th>
<th>( cmi^1 / sax^4 )</th>
<th>( sax_{KG0725} / sax_{KG0725} )</th>
<th>( cmi^1 / sax^4 )</th>
<th>( sax_{KG0725} / sax_{KG0725} )</th>
<th>( tkv^1 / tkv^1 )</th>
<th>( cmi^1, tkv^1 / tkv^1a )</th>
</tr>
</thead>
<tbody>
<tr>
<td>(# of flies scored)</td>
<td>(122)</td>
<td>(108)</td>
<td>(180)</td>
<td>(8)</td>
<td>(102)</td>
<td>(164)</td>
</tr>
<tr>
<td>Phenotypes</td>
<td>Percentage showing phenotype</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inc. L2 with branches</td>
<td>10</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ectopic/ thick L2</td>
<td>-</td>
<td>50</td>
<td>13(^{14})</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Incomplete L4</td>
<td>-</td>
<td>13</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ectopic/ thick L4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>Incomplete L5</td>
<td>-</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ectopic L5/ thick</td>
<td>-</td>
<td>6</td>
<td>-</td>
<td>-</td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td>Incomplete PCV</td>
<td>1</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ectopic/ thick PCV</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>70</td>
<td>28(^{15})</td>
</tr>
<tr>
<td>Ectopic ACV</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>Incomplete/ missing ACV</td>
<td>-</td>
<td>56</td>
<td>-</td>
<td>100(^{16})</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

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\(^{13}\) All crosses with \( tkv^1 \) were carried out 18 °C

\(^{14}\) Suppression of \( sax_{KG0725} \) phenotype

\(^{15}\) Suppression of \( tkv^1 \) phenotype

\(^{16}\) Enhancement of \( sax_{KG0725} \) phenotype

\( sax^4 / sax^4 \) flies are lethal
Wing-specific knockdown or overexpression of *CMI* in combination with knockdown of *TKV*. (A) *TKV-IR/+; c765 GAL4/+*. Note the rounded wings with extensive ectopic wing

*tkv*/*cmi* flies did not show any visible wing patterning defects
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.
We next assayed for the activation of DPP downstream effector MAD (MOTHERS AGAINST DPP) by immunostaining with antibodies against phosphorylated MAD (anti-pMAD). MAD (mammalian R-Smad) is downstream of both DPP and TKV and is responsible for the activation of DPP target genes (Newfeld, Chartoff et al. 1996; Wiersdorff, Lecuit et al. 1996; Kim, Johnson et al. 1997). In the third instar wing imaginal disc, pMAD staining is observed along the A-P boundary (Figure 23A) (Newfeld, Chartoff et al. 1996). As expected, the knock-down of CMI results in a decrease in the amount of pMAD staining (Figure 23B), presumably as a result of reduced DPP signaling. In contrast, the over-expression of HA-CMI results in diffused pMAD staining, likely due to the spreading of the DPP signal (Figure 23C). Within the pupal wings pMAD is localized to the developing wing veins (Figure 23D). The knock-down of CMI results in decreased pMAD staining (Figure 23E) and the over-expression of HA-CMI results in ectopic pMAD staining towards the distal wing margin (Figure 23F). Since the phosphorylation of MAD depends on DPP signaling, our results suggest that CMI regulates MAD phosphorylation indirectly through regulation of DPP.
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 hr 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 reduced 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.
4.15 CMI regulates DPP transcription through the 3’disk regulatory region during larval development

The interactions between CMI and TKV suggested that CMI was affecting DPP signaling by regulating the expression of DPP. The DPP gene 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 (reviewed in (O’Connor, Umulis et al. 2006)). Since we observed a genetic interaction between CMI and a mutation in the DPP 3’ disk enhancer region, we tested a DPP enhancer fusion to the LACZ gene (DPPBS3.0-LACZ (Blackman, Sanicola et al. 1991) to determine whether CMI could regulate DPP through the 3’ region (Figure 19). The DPPBS3.0-LACZ transgene construct contains 10kb of disk 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, Sanicola et al. 1991; Raftery, Sanicola et al. 1991; Sanicola, Sekelsky et al. 1995) (Figure 24A). 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 over-expressed and reduced 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. Knock-down of CMI resulted in strongly reduced DPPBS3.0-LACZ expression in approximately 70% of the wing imaginal discs examined (Figure 24B). Depletion of TRR using the wing-specific C765-GAL4 driver
resulted in strongly reduced \textit{DPPBS3.0-LACZ} expression in approximately 50\% of the wings examined, consistent with cooperation between \textit{CMI} and \textit{TRR} regulating \textit{DPP} transcription (Figure 25C). Conversely, overexpression of \textit{HA-CMI} resulted in a strong increase in \textit{DPPBS3.0-LACZ} expression (Figure 24C). While the overall level of \textit{DPPBS3.0-LACZ} expression was modulated in response to increasing or decreasing \textit{CMI}, the pattern was unchanged despite ectopic expression of \textit{CMI} in the \textit{HA-CMI} background. This result suggests that \textit{CMI} is necessary though not sufficient for \textit{DPP} transcription in the larval wing disc to modulate expression within its normal domain.
Figure 24 CMI positively regulates transcription from the *DPP* 3’ disk 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’ disk enhancer reporter. (D-F) Wings from 25-46 hr old pupae were examined using the *DPP^{shv}-LACZ.RD2* 5’ shortvein enhancer reporter. (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’ disk 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 DPPshv-LACZ.RD2 expression in all the longitudinal veins (L2-L5), the PCV and the wing margin. (E) DPPshv-LACZ.RD2, CMI-IR pupal wings show incomplete veins along with reduced LACZ staining in the longitudinal veins and wing margin. (F) DPPshv-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.
Figure 25 CMI and TRR positively regulate transcription of the *DPPBS3.0 LACZ* reporter during larval development in the wing imaginal disc

Imaginal discs dissected from third instar larvae carrying the *DPPBS3.0-LACZ* reporter gene were stained for β-galactosidase activity. (A-C) Wing imaginal discs. (D-F) Eye imaginal discs. (A) Expression of *DPPBS3.0-LACZ* at the anterior-posterior boundary in wild type *OregonR* wing discs. (B) *CMI-IR/+; c765 GAL4*. (C) *TRR-IR/+; c765 GAL4/+*. Note the strongly reduced *DPP-LACZ* expression in wing imaginal discs upon knockdown of *CMI* and *TRR* using the *c765-GAL4* driver. Also, the size of the discs in (B) and (C) are larger than in (A). (D-F) Expression of *DPPBS3.0-LACZ* in eye imaginal discs obtained from the same larvae shown in (A-C). The *c765-GAL4* driver expresses GAL4 protein exclusively in the larval wing disc and salivary glands, allowing the eye
disc to serve as a control for LACZ staining upon knockdown of CMI and TRR. All pictures \{(A-C) and (D-F)\} were taken at the same magnification.
4.16 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 LV fate as well as at a distance to specify the positions of the CVs (Posakony, Raftery et al. 1990; de Celis 1997). These events are controlled by DPP signaling during the pupal stage by the regulation of DPP transcription through the 5’ shv regulatory region (Figure 24D and Figure 19) (Christoforou, Greer et al. 2008). The knock-down and over-expression 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 DPPshv-LACZ.RD2 reporter transgene that contains an 8.9kb region of the DPP 5’ region adjacent to the coding exons, linked to a LACZ gene (Hursh, Padgett et al. 1993). The expression of LACZ in this construct mimics normal DPP transcription in the pupal wing veins (Figure 24D) (Christoforou, Greer et al. 2008). Recombinants carrying the DPPshv-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 (Figure 24E). 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 (data not shown). In contrast, ectopic
overexpression of HA-CMI resulted in an elevated level of LACZ staining throughout the wing veins and margin (Figure 24F). 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 through the 5’ transcript regulatory region at the pupal stage.
CHAPTER-5

DISCUSSION

In the present study we focus on the identification and characterization of CMI, a novel putative component of the nuclear receptor/coactivator complex (Chauhan, C., data not shown and Zraly, CB., unpublished data) and the N-terminal half of mammalian MLL2, using bioinformatics and genetic approaches. We demonstrate a critical role for CMI 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. Although one report indicates a possible role for the estrogen receptor (ER) in regulating BMP-2 transcription (Zhou, Turgeman 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 BMP/DPP transcription through the 5’ shv regulatory region.
While ALR is present as one protein in *Tribolium* (Coleoptera), our bioinformatics and phylogenetic analysis has revealed that in Drosophila, both CMI and TRR are orthologs of the N-terminal and the C-terminal sequences respectively, of the mammalian ALR and MLL3. It is likely that the *CMI* and *TRR* genes ‘split’ in Drosophila during the course of evolution, possibly to perform independent functions in response to hormone signaling. It is interesting that *CMI* and *TRR* are separate genes in the genus Drosophila (Diptera, Brachycera), but, seem to be part of only one gene (*ALR*) in mosquitoes (Diptera, Nematocera), which suggests that the split happened less than 200 million years ago, or even more recently. Drosophila undergoes metamorphosis (an event controlled by hormone signaling) while insects lack metamorphosis, suggesting that CMI and TRR have independent hormone mediated functions and hence ‘the split’.

We have established that *CMI* is an essential gene; CMI has broad and important functions in tissue patterning throughout development using analyses of null alleles, expression of silencing RNAs and ectopic overexpression in vivo. 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 patterns, 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 phenotypic analysis of the homozygous mutant *CMI* larvae demonstrates molting defects like the retention of cuticle, malformed mouth-hooks, prolonged larval stages and
inability to molt into the pupal stage; phenotypes similar to those observed in mutations in genes like \textit{ECR}, \textit{USP}, \textit{ECD} and \textit{RIG} that have been shown to be components of the hormone signaling pathway (Perrimon, Engstrom et al. 1985; Oro, McKeown et al. 1992; Hall and Thummel 1998; Schubiger, Wade et al. 1998; Li and Bender 2000; Carney, Robertson et al. 2004; Gates, Lam et al. 2004; Davis, Carney et al. 2005). These phenotypes suggest a defective hormone signaling pathway in \textit{CMI} null animals.

Further, the data from our genetic epistasis test reveals an allele specific genetic interaction between \textit{CMI} and \textit{ECR} in flies transheterozygous for mutant alleles. Our hypothesis is supported by the data from the GST-fusion and co-immunoprecipitation based assays where TRR and CMI show physical interaction in response to 20E as well as binding to ECR and USP in a hormone dependent manner \{Chauhan, C., data not shown; Zraly, CB., unpublished results; and (Sedkov, Cho et al. 2003)\}; RT-PCR analysis and reporter assays that demonstrate that \textit{CMI} positively regulates \textit{E74}, an ecdysone inducible gene in vivo and reporter genes fused to an upstream \textit{ECRE} in vitro, respectively \{Chauhan, C., data not shown; Zraly, CB., unpublished results\}.

The appearance of \textit{CMI} gain/loss of function phenotypes is largely dependent on other presumed components of the Drosophila ALR nuclear receptor coactivator complex (Chauhan, C., data not shown and Zraly, CB., unpublished results). Mutations in \textit{CMI} and \textit{TRR} genetically interact to give rise to wing patterning defects. TRR is a histone H3K4 methyltransferase and is important for positive regulation of \textit{HH} transcription both in the eye imaginal disc and in cultured Drosophila S2 cells, where \textit{HH} transcription is
dependent on ecdysone (Sedkov, Cho et al. 2003). However, we have not observed any ecdysone dependent regulation of HH in S2 cells (Zraly, CB., unpublished results).

5.1 CMI is required in wing vein patterning through the DPP signaling pathway

Wing vein patterning is governed by multiple signaling pathways that include HH, EGFR, BMP/DPP, WNT/WG and N (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 (KN) (Farkas and Knopp 1997) in the L3 provein and the L3/L4 intervein, respectively (Gomez-Skarmeta and Modolell 1996; Mullor, Calleja et al. 1997; Vervoort, Crozatier et al. 1999; Mohler, Seecoomar et al. 2000; Crozatier, Glise et al. 2002). 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 SAL, KNL, and IRO gene complexes (de Celis, Barrio et al. 1996; Gomez-Skarmeta and Modolell 1996; Biehs, Sturtevant et al. 1998; Entchev, Schwabedissen et al. 2000; 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 (Spencer, Hoffmann et al. 1982; Segal and Gelbart 1985).

Based on our observations, the wing phenotypes observed upon mis-regulation of CMI could arise as a consequence of CMI having normal functions in regulating the HH signaling pathway upstream of DPP, through direct 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 epistasis 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 in 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 regulated by ecdysone in cultured Drosophila S2 (late embryonic) cells as well as the eye imaginal disc (Sedkov, Cho et al. 2003), it is not a significant target in Drosophila Kc167 cells (mid-embryonic) that differentiate in response to ecdysone, nor is it a significant target for in vivo regulation during metamorphosis (Gauhar, Sun et al. 2009).

### 5.2 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, Hoffmann 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 disk specific (disk/d) based on mutant phenotypes (Spencer, Hoffmann et al. 1982; Segal and Gelbart 1985) (Figure 19). The shv region is located 5’ of the coding exons and it controls expression of \( DPP \) during pupal development. The 3’ disk region is located 3’ of the coding exons and controls larval expression of \( DPP \). We have provided evidence for a positive genetic interaction
between \textit{CMI}^I and various loss of function mutations both in the 3’ as well as the 5’ regulatory regions of \textit{DPP} and the DPP type I receptors, \textit{TKV} and \textit{SAX}.

In the larval wing imaginal disc, \textit{DPP} transcription is regulated through the 3’ regulatory region in a HH-dependent manner \cite{Blair2007}. Mutations in the 3’ regulatory disk region result in defects in the imaginal discs and the adult derivatives of the imaginal discs \cite{Spencer1982, Bryant1988, Masucci1990, Blackman1991}. In the wing imaginal disc, DPP activates \textit{SAL} in early L2 development. Low levels of \textit{SAL} in turn activate \textit{KNI} expression that is required for L2 formation. Higher levels of \textit{SAL}, on the other hand, repress \textit{KNI} leading to loss of L2 \cite{deCelis2000}. We observed a loss of proximal L2 upon \textit{CMI} over-expression that may be explained by an increase in \textit{SAL} expression as a result of increased DPP signaling leading to repression of \textit{KNI} in wing imaginal disc and hence the loss of proximal L2. Our \textit{LACZ} reporter assays support this view, showing a positive temporal regulation of \textit{DPP} transcription by \textit{CMI} through the 3’ regulatory region at the larval stage.

During the early pupal stage, \textit{DPP} is expressed independent of HH within all the presumptive veins leading to increased BMP signaling \cite{Segal1985, Yu1996, deCelis1997, Ralston2005}. The mechanism of this highly specific regulation is not well understood, though it has been reported that mutations in the \textit{DPP} shv region manifest as incomplete veins that fail to reach the wing margin \cite{Segal1985, St Johnston1990, deCelis1997, Sotillos2006}. We found a similar shortened vein phenotype
associated with \textit{CMI} knock-down that is consistent with reduced \textit{DPP} expression within presumptive vein cells near the distal wing margin. Our results from genetic epistasis tests using a \textit{CMI} \textsuperscript{1} null allele are consistent with this view as we observed an enhancement of the \textit{DPP} shv phenotype.

Overexpression of \textit{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 \textit{DPP} through the 5’ shv enhancer region in our reporter based assays.

Similar to ALR/MLL2 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 TGF\(\beta\)/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 \textit{DPP} transcription in cultured Drosophila Kc167 or S2 cells through hormone dependent pathways \{(Zraly, CB., unpublished observations and (Gauhar, Sun et al. 2009)}\} and the \textit{CMI} wing phenotypes are not substantially modified by reduced \textit{ECR} function. 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 \textit{DPP}. In this regard, it is important to note that despite widespread expression of \textit{HA-CMI}, the ectopic veins and \textit{DPP-LACZ} expression are restricted to certain distal regions of the wing, suggesting
that CMI is necessary but not sufficient for \textit{DPP} transcription. Since CMI and its associated cofactors are presumably recruited to specific target sites through binding of specific DNA binding transcription factors, it is likely that the phenotype associated with overexpression of CMI is the result of misregulation of target genes by that unknown factor. A strong candidate for this DNA binding transcription factor is USP, the Drosophila ortholog of the vertebrate RXR receptor, as CMI directly interacts with USP and targeted depletion of \textit{USP} in the wing generally suppresses the \textit{HA-CMI} ectopic vein phenotype (Chauhan, C. and Zraly, CB., unpublished results). If \textit{DPP} is directly regulated by USP it would likely be ECR independent, as there is no significant binding of ECR/USP heterodimers in the \textit{DPP} genomic region (Gauhar, Sun et al. 2009). Thus, regulation of \textit{DPP} by USP most likely involves a different USP partner from ECR and possibly is ligand independent.

5.3 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, Jackson et al. 1992; Khalsa, Yoon et al. 1998). DPP and GBB signal through two Type I TGFβ receptors, SAX and TKV and a type II receptor, PUT {reviewed in (Affolter and Basler 2007)}. Misexpression of DPP, GBB or an activated form of TKV leads to ectopic venation (Terracol and Lengyel 1994; de Celis 1997; Bangi and Wharton 2006; Sotillos and de Celis 2006). Similarly, reducing \textit{TKV} function leads to vein thickening, due to spreading of the DPP signal (de Celis 1997; Marenda, Zraly et al. 2004). Our hypothesis that CMI positively regulates DPP signaling
is further supported by the enhancement of TKV knock-down phenotypes upon CMI over-expression and the reciprocal suppression of these phenotypes upon CMI knock-down.

An alternate scenario is that CMI contributes to the regulation of downstream components of the DPP signaling pathway. We have also found that selective GAL4-dependent RNAi depletion of genes encoding presumed Drosophila ASCOM/ALR-1 complex components, such as WDS, and UTX, enhanced the CMI-IR shortened vein phenotype and suppressed the ectopic veins associated with HA-CMI overexpression while the knock-down of ASH2 suppressed the CMI-IR shortened vein phenotype (Figure 27 and Tables A1-4). ASH2 is known to positively regulate intervein specific genes NET and BS, and negatively regulate the L2 specifying gene KNI (Angulo, Corominas et al. 2004). Knock-down of mammalian ALR in Hela cells results in a decrease in MADH6 expression, a downstream effector of BMP signaling (Issaeva, Zonis 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 {reviewed in (Affolter and Basler 2007)}. In contrast, increasing CMI levels results in reduced tissue growth while knockdown of CMI produces larger animals, presumably through increased growth. When DPP is overexpressed simultaneously with the CMI-IR using GAL4, we observed only a few rare escapers (flies that survive to reach adulthood) that displayed strong ectopic DPP phenotypes (data not shown). Overexpression of both DPP and CMI together resulted in flies that survived with pattern defects similar to both DPP and CMI
over-expression phenotypes (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 ASCOM/ALR complex function to control downstream DPP targets, phenotypes associated with GAL4-directed over expression of \textit{DPP} were not suppressed by removing \textit{CMI} function (Figure 27 and Tables A1-4).

We can envisage two models of how CMI affects wing patterning through the DPP signaling pathway based on our findings that CMI regulates \textit{DPP} transcription (Figure 26). The first model suggests a regulation of both \textit{DPP} and its downstream effectors by CMI. In this model, the up-regulation of \textit{CMI} should result in an enhanced up-regulation of DPP effectors as they are activated both by increased DPP signal as well as increased levels of CMI. The second model suggests the regulation of \textit{DPP} alone by CMI. In this case, the over-expression of \textit{CMI} leads to an increase in \textit{DPP} transcription that in turn results in increased down-stream effectors. In both models, the transcriptional control of \textit{DPP} by CMI plays an important role. However, the roles of \textit{CMI}, \textit{DPP} and the down-stream effectors need to be uncoupled to fully reject Model A. Using somatic clonal analysis, we can selectively mis-regulate \textit{CMI} in a small population of cells and test the expression of the downstream targets to test Model A.
Figure 26 Models of CMI function in regulating the DPP signaling pathway

(A) CMI regulates transcription of both DPP and its downstream effector genes. CMI may directly control the expression of both DPP as well as its downstream effectors in parallel with DPP itself (left panel). In this scenario, an increase in CMI would be expected to result in an increase in DPP transcription and a much higher increase in the transcription of the downstream effectors as they are positively regulated by both DPP and CMI (right panel).  

(B) CMI regulates transcription of DPP. This model suggests that CMI directly regulates the transcription of DPP (left panel). As a result, an increase in CMI produces a modest increase in the DPP
downstream effectors through increased *DPP* transcription and hence increased signaling (right panel).
CHAPTER-6

CONCLUSIONS

This study identifies a novel co-activator, CMI in *Drosophila*. CMI is an essential protein with critical role in every stage in development. CMI functions as a global co-regulator essential for tissue patterning as both the knock-down and over-expression of *CMI* results in a variety of phenotypes in different tissues such as the eye, the leg, the abdomen and the wing. The evidence we provide above suggests a role for CMI, an ALR complex component in the regulation of BMP/TGFβ mediated DPP signaling in wing patterning in Drosophila. In summary, we have demonstrated that *CMI* has a role throughout the wing when DPP patterns the wing primordium. We conclude that this role is likely through HH-independent, direct regulation of *DPP*, although the transcription factors that CMI interacts with need to be identified. We have shown a direct genetic interaction between *CMI* and *DPP* as well as components of the DPP signaling pathway. We have also established a positive regulation of *DPP* transcription by *CMI* which is temporally regulated through two independent regulatory regions in the larval and pupal development of the wing. Clearly, our data shows that *DPP* is an important target of *CMI* in both larval and pupal wings. Whether this regulation is evolutionarily conserved remains to be determined. But, the fact that the knock-down of ALR has been shown to
lead to a decrease in the transcription of BMP downstream effector, *MADH6* (Issaeva, Zonis et al. 2007), supports the hypothesis that this regulation might be evolutionarily conserved. A detailed analysis of these orthologs in developmental context will be crucial to determine whether the robustness of vertebrate BMP signaling in patterning also depends on its regulation through ALR.
APPENDIX A
MATERIALS AND METHODS
Generation of recombinants and stocks

Putting \( CMI^I \) in \( WHITE^w \) background

To put \( CMI^I \) in \( WHITE^w \) background, virgin \( w^+; CMI^I/SM6b \) females were crossed to \( w^w; CMI^I/SM6a \) flies. From the progeny, \( w^+; CMI^I/SM6b \) males were individually crossed to virgin \( w^w; Sco/SM6b \) flies. The \( SM6b \) (rough eye) containing progeny from this cross was crossed together to generate a \( w^w; CMI^I/SM6b \) stock.

Generation of recombinants between \( CMI^I \) and \( e^{22c} Gal4 \) driver in a \( w^- \) background

To generate a recombinant between \( CMI^I \) and \( e^{22c} Gal4 \) driver in a \( w^- \) background, \( w^-; CMI^I/SM6a \) virgin females were crossed to \( w^-; e^{22c} Gal4/SM5 \) (orange eyes). From the progeny, the virgin females that were \( w^-; CMI^I/e^{22c} Gal4 \) were crossed back to \( w^-; CMI^I/SM6a \) males. Individual orange eyed males that were either \( w^-; CMI^I, e^{22c} Gal4/SM6a \) or \( e^{22c} Gal4/SM6a \) from this cross were then collected and crossed to \( w^-; CMI^I/SM6a \) to test for non-complementation. In case no straight wing flies were obtained from this cross, the \( w^-; CMI^I, e^{22c} Gal4/SM6a \) flies were crossed together to obtain a stock.

A similar scheme was used to generate recombinant between \( CMI^I \) and other second chromosome \( Gal4 \) drivers.

Generation of \( UAS DICER/ UAS DICER; Sco/CyO; CMI-IR/ CMI-IR \) stock

To generate \( UAS DICER/ UAS DICER; Sco/CyO; CMI-IR/ CMI-IR \) stock, \( UAS DICER/ UAS DICER; +/+; CMI-IR/ CMI-IR \) virgin females were crossed to \( w^-; Sco/ CyO; TM3/ TM6b \) males. The progeny from this cross \( UAS DICER/ X; Sco/+; CMI-IR/ TM3 \) and \( UAS DICER/ Y; CyO/+; CMI-IR/ TM6b \) were crossed to generate \( UAS DICER/ UAS DICER; Sco/CyO; CMI-IR/ CMI-IR \) stock.

Generation of recombinants between \( CMI^I \) and chromosome 2 \( HA-CMI \) transgene

Virgin \( CMI-HA- chr2/ HA-CMI chr2 \) females were crossed to \( w^-; CMI^I/SM6a \) males. The virgin female flies from this cross with the genotype \( w^-; CMI^I/ CMI-HA- chr2 \) were crossed to \( w^-; Sco/ CyO \) males. The males from this progeny (either \( HA-CMI chr2/ CyO \) or \( CMI^I, HA-CMI chr2/ CyO \)) were individually crossed to virgin \( w^-; CMI^I/SM6a \) females. The flies from this progeny were then crossed together to generate a \( HA-CMIi chr2/ CyO \) stock if no straight wing flies were obtained from this cross.

Generation of recombinants between \( Df(chig)230 \) and \( e^{22c} GAL4 \) driver in a \( w^- \) background

To generate a recombinant between \( Df(chig)^{230} \) and \( e^{22c} GAL4 \) driver in a \( w^- \) background, \( w^-; Df(chig)^{230}/ CyO \) virgin females were crossed to \( w^-; e^{22c} GAL4/SM5 \) (orange eyes). From the progeny, the virgin females that were \( w^-; Df(chig)^{230}/ e^{22c} GAL4 \)
were crossed to \( w^+; Sco/ CyO \) males. Individual orange eyed males that were either \( w^+; Df(chig)^{230} \), \( e^{22c} GAL4/ SM6a \) or \( e^{22c} GAL4/SM6a \) from this cross were then collected and crossed to \( w^+; CMI^I/ SM6a \) to test for non-complementation. In case no straight wing flies were obtained from this cross, the \( w^+; Df(chig)^{230} \), \( e^{22c} GAL4/ SM6a \) flies were crossed together to obtain a stock.

**Generation of recombinants between \( CMI^I \) and \( ECR^{4483T} \)**

Virgin \( w^+ \), \( CMI^I/ SM6a \) females were crossed to \( ECR^{4483T}/ SM6b \) males. The non-balanced virgin females, \( w^+; CMI^I/ ECR^{4483T} \) were then crossed to \( w^+; Sco/ CyO \) males. The curly males from this cross were crossed back to virgin \( ECR^{4483T}/ SM6b \) females to look for non-complementation for the presence of \( ECR^{4483T} \). The rough eye males from this cross were crossed to \( w^+; CMI^I/ SM6a \) virgin females to check for non-complementation for the presence of \( cCMI^I \). In case, no unbalanced flies were obtained, the non-rough eye flies were crossed together to generate a \( w^+; CMI^I, ECR^{4483T}/ SM6a \) stock.

**Generation of recombinants between \( Df(chig)^{230} \) and \( ACTIN5c GAL4 \) driver in a \( w^- \) background**

To generate a recombinant between \( Df(chig)^{230} \) and \( ACTIN5c GAL4 \) driver in a \( w^- \) background, \( w^-; Df(chig)^{230}/ CyO \) virgin females were crossed to \( w^-; ACTIN5c GAL4/ CyO \) (orange eyes). From the progeny, the virgin females that were \( w^+; Df(chig)^{230}/ ACTIN5c GAL4 \) were crossed to \( w^+; Sco/ CyO \) males. Individual orange eyed males that were either \( w^-; Df(chig)^{230}, ACTIN5c GAL4/ CyO \) or \( ACTIN5c GAL4/ CyO \) from this cross were then collected and crossed to \( w^-; cmi^I/ SM6a \) to test for non-complementation. In case no straight wing flies were obtained from this cross, the \( w^-; Df(chig)^{230}, ACTIN5c GAL4/ SM6a \) flies were crossed together to obtain a stock.

**Generation of recombinants between chromosome 3 \( CMI-IR \) and \( ECRE LACZ \)**

To generate a recombinant between \( ECRE LACZ \) and \( CMI-IR- 63 \) in a \( w^- \) background, \( ECRE LACZ/ ECRE LACZ \) virgins were crossed to \( CMI-IR- 63/ CMI-HA- 63 \) males. Virgin females from this cross were then crossed to \( w^-; TM3/TM6b \) males. The balanced dark eyed males from this cross were individually crossed back to \( w^-; TM3/TM6b \) virgins to obtain a stock. DNA was isolated from a couple of males from this cross to test for the presence of both LACZ and RNAi construct, by way of PCR using specific primers.

**To clean up \( w^-; CMI^I/ CyO \) stock of \( \Delta2,3 \) transposase**

To clean up the \( w^-; CMI^I/ CyO \) stock of \( \Delta2,3 \) transposase, \( w^-; Sco/ CyO\), virgin females were crossed to \( w^-; FRT 1928/ cmi^I; \Delta2,3/+ \) males. The virgin non-scutoid, curly flies from the progeny, \( w^-; CMI^I/ CyO \) were then crossed to \( w^-; e^{22c} GAL4/ SM5 \) (orange eyes) males. The \( w^- \) progeny was then crossed together to obtain \( w^-; CMI^I/ SM5 \) stock.
Figure 27 Conserved components of the ALR-related fly complex are required for the development and patterning of the adult epidermis

The knock-down of CMI results in incomplete longitudinal veins (A) which is enhanced by introducing UAS-DICER in the background (B), while the simultaneous knock-down
of TRR and CMI results in an enhancement of incomplete vein phenotype observed in (A) as well as upon TRR knock-down alone (G). The knock-down of ASH2 suppresses CMI-IR phenotype (D); the knock-down of WDS has no effect (E); while the knock-down of UTX enhances CMI-IR phenotype (F). The over-expression of HA-CMI results in ectopic distal veins (H) which are suppressed by the knock-down of TRR (I), ASH2 (J), WDS (K), and UTX (L). The arrows point to the various defects in each wing.
Table A1 Loss of function of \textit{ASH2} and \textit{WDS} leads to a suppression of \textit{CMI} over-expression phenotypes

<table>
<thead>
<tr>
<th>Genotype</th>
<th># of progeny</th>
<th>L2\textsuperscript{p}</th>
<th>L2\textsuperscript{d}</th>
<th>L3</th>
<th>L4</th>
<th>L5</th>
<th>ACV</th>
<th>PCV</th>
</tr>
</thead>
<tbody>
<tr>
<td>c765 \textit{Gal4}, \textit{HA cmi/+}</td>
<td>24</td>
<td>48</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>15</td>
<td>19</td>
</tr>
<tr>
<td>Dicer/ \textit{X}; ash2-IR/+; c765 \textit{Gal4/+}</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ash2-IR/+; c765 \textit{Gal4/+}</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Dicer/ \textit{X}; ash2-IR/+; c765 \textit{Gal4}, \textit{HA cmi/+}</td>
<td>28</td>
<td>20</td>
<td>93</td>
<td>43</td>
<td>70</td>
<td>100</td>
<td>2</td>
<td>37</td>
</tr>
<tr>
<td>ash2-IR/+; c765 \textit{Gal4}, \textit{HA cmi/+}</td>
<td>34</td>
<td>18</td>
<td>1</td>
<td>3</td>
<td>40</td>
<td>100</td>
<td>-</td>
<td>6</td>
</tr>
<tr>
<td>c765 \textit{Gal4}, \textit{HA cmi/ash}\textsuperscript{2}</td>
<td>67\textsuperscript{18}</td>
<td>-</td>
<td>39</td>
<td>45</td>
<td>24</td>
<td>84</td>
<td>-</td>
<td>13</td>
</tr>
<tr>
<td>Dicer/ \textit{X}; wds-IR/+; c765 \textit{Gal4/+}</td>
<td>51</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>wds-IR/+; c765 \textit{Gal4/+}</td>
<td>51</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Dicer/ \textit{X}; wds-IR/+; c765 \textit{Gal4}, \textit{HA cmi/+}</td>
<td>42</td>
<td>12</td>
<td>18</td>
<td>6</td>
<td>54</td>
<td>100</td>
<td>1</td>
<td>19</td>
</tr>
<tr>
<td>wds-IR/+; c765 \textit{Gal4}, \textit{HA cmi/+}</td>
<td>49</td>
<td>14</td>
<td>14</td>
<td>10</td>
<td>54</td>
<td>91</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td>\textit{P}{wds}/\textit{X}; c765 \textit{Gal4}, \textit{HA cmi/+}</td>
<td>27\textsuperscript{19}</td>
<td>57</td>
<td>85</td>
<td>82</td>
<td>91</td>
<td>93</td>
<td>-</td>
<td>9</td>
</tr>
<tr>
<td>wds\textsuperscript{7}/\textit{X}; c765 \textit{Gal4}, \textit{HA cmi/+}</td>
<td>46</td>
<td>8</td>
<td>36</td>
<td>24</td>
<td>59</td>
<td>100</td>
<td>1</td>
<td>15</td>
</tr>
<tr>
<td>wds\textsuperscript{7}/\textit{Y}; c765 \textit{Gal4}, \textit{HA cmi/+}</td>
<td>7</td>
<td>21</td>
<td>57</td>
<td>64</td>
<td>86</td>
<td>100</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

\textsuperscript{17} All crosses were set at 25 °C
\textsuperscript{18} Phenotypes were much less severe in males
\textsuperscript{19} Over-expression of \textit{cmi} suppressed \textit{Bar} phenotype (100%) of the \textit{FM7} sibling flies
Table A 2 Loss of function of *ASH2* leads to a suppression while the loss of function of *WDS* leads to an enhancement of *CMI* knock-down phenotypes

<table>
<thead>
<tr>
<th>Genotype</th>
<th># of progeny</th>
<th>L2</th>
<th>L5</th>
<th>ACV</th>
<th>PCV</th>
</tr>
</thead>
<tbody>
<tr>
<td>c765 Gal4, cmi-IR/+</td>
<td>81</td>
<td>43</td>
<td>-</td>
<td>1</td>
<td>13</td>
</tr>
<tr>
<td>Dicer/X; ash2-IR/+; c765 Gal4/+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ash2-IR/+; c765 Gal4/+</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Dicer/X; ash2-IR/+; c765 Gal4, cmi-IR/+</td>
<td>9</td>
<td>17</td>
<td>78</td>
<td>33</td>
<td>-</td>
</tr>
<tr>
<td>ash2-IR/+; c765 Gal4, cmi-IR/+</td>
<td>42</td>
<td>24</td>
<td>8</td>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td>c765 Gal4, cmi-IR/ ash2</td>
<td>56</td>
<td>39</td>
<td>-</td>
<td>54</td>
<td>5</td>
</tr>
<tr>
<td>Dicer/X; wds-IR/+; c765 Gal4/+</td>
<td>51</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>wds-IR/+; c765 Gal4/+</td>
<td>51</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Dicer/X; wds-IR/+; c765 Gal4, cmi-IR/+</td>
<td>14</td>
<td>54</td>
<td>65</td>
<td>64</td>
<td>14</td>
</tr>
<tr>
<td>wds-IR/+; c765 Gal4, cmi-IR/+</td>
<td>44</td>
<td>65</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>P{wds}/X; c765 Gal4, cmi-IR/+</td>
<td>70</td>
<td>15</td>
<td>46</td>
<td>3</td>
<td>14</td>
</tr>
<tr>
<td>wds/Y; c765 Gal4, cmi-IR/+</td>
<td>58</td>
<td>92</td>
<td>9</td>
<td>-</td>
<td>9</td>
</tr>
<tr>
<td>wds/Y; c765 Gal4, cmi-IR/+</td>
<td>67</td>
<td>58</td>
<td>5</td>
<td>8</td>
<td>8</td>
</tr>
</tbody>
</table>

20 All crosses were set at 25 °C
21 ACV defects included ectopic ACV/ duplication (28%) and ACV absent (5%)
22 ACV defects included ACV duplication (57%) and Incomplete ACV (7%)
23 ACV defects included ACV duplication alone
24 ACV defects included incomplete ACV (3%) and ectopic/ ACV (5%)
Table A 3 The gain of *CMI* function phenotypes are affected by the conserved components of fly ALR-related complex

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>L2</th>
<th>L3</th>
<th>L4</th>
<th>L5</th>
<th>ACV</th>
<th>PCV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotype</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(no. of flies scored)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Percentage showing phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>c765, HA cmi/+</em></td>
<td>100</td>
</tr>
<tr>
<td><em>ash2-IR/Y; c765/+</em></td>
<td>n.a.</td>
</tr>
<tr>
<td><em>ash2-IR/Y; c765, HA cmi/+</em> (34)</td>
<td>1(^{27}), 18(^{28})</td>
</tr>
<tr>
<td><em>wds-IR/+; c765 Gal4/+</em> (100)</td>
<td>-</td>
</tr>
<tr>
<td><em>wds-IR/+; c765, HA cmi/+</em> (49)</td>
<td>29</td>
</tr>
<tr>
<td><em>trr-IR/Y; c765 Gal4/+</em> (62)</td>
<td>1</td>
</tr>
<tr>
<td><em>trr-IR/Y; c765, HA cmi/+</em> (9)</td>
<td>10(^{3}, 8(^{4})</td>
</tr>
<tr>
<td><em>utx-IR/X; c765 Gal4/+</em> (4)</td>
<td>-</td>
</tr>
<tr>
<td><em>utx-IR/X; c765, HA cmi/+</em> (3)</td>
<td>13(^{3})</td>
</tr>
</tbody>
</table>

\(^{25}\) All crosses were set at 25 °C

\(^{26}\) *ash2-IR/Y; c765/+* flies are pupal lethal

\(^{27}\) Ectopic L2 distally

\(^{28}\) Incomplete L2 proximally
Table A 4 The loss of CMI function phenotypes are affected by the conserved components of fly ALR-related complex

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>L2\textsuperscript{29}</th>
<th>L3</th>
<th>L4</th>
<th>L5\textsuperscript{30}</th>
<th>ACV\textsuperscript{31}</th>
<th>PCV</th>
</tr>
</thead>
<tbody>
<tr>
<td>c\textsubscript{765} Gal4, cmi IR/+</td>
<td>10</td>
<td>-</td>
<td>-</td>
<td>2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ash2-IR/Y; c\textsubscript{765} Gal4/+\textsuperscript{33}</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>ash2-IR/Y; c\textsubscript{765} Gal4, cmi IR/+ (42)</td>
<td>24</td>
<td>1</td>
<td>-</td>
<td>8</td>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td>wds-IR/+; c\textsubscript{765} Gal4/+ (51)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>wds-IR/+; c\textsubscript{765} Gal4, cmi IR/+ (44)</td>
<td>65</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>trr-IR/Y; c\textsubscript{765} Gal4/+ (62)</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>10</td>
<td>-</td>
<td>6</td>
</tr>
<tr>
<td>trr-IR/Y; c\textsubscript{765} Gal4, cmi IR/+ (50)</td>
<td>97</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>21</td>
<td>3</td>
</tr>
<tr>
<td>utx-IR/X; c\textsubscript{765} Gal4/+ (9)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>33</td>
</tr>
<tr>
<td>utx-IR/X; c\textsubscript{765} Gal4, cmi IR/+ (64)</td>
<td>97</td>
<td>-</td>
<td>-</td>
<td>42</td>
<td>-</td>
<td>23</td>
</tr>
</tbody>
</table>

\textsuperscript{29} Incomplete L2 distally
\textsuperscript{30} Incomplete/ectopic L5 distally
\textsuperscript{31} ACV defects included ACV duplication and incomplete ACV
\textsuperscript{32} All crosses were set at 25 °C
\textsuperscript{33} ash2-IR/Y; c\textsubscript{765} Gal4/+ animals are pupal lethal.
BIBLIOGRAPHY


The author, Chhavi Chauhan, was born on March 8th, 1979 in New Delhi, India to Usha and Roop Chand Chauhan. Upon completion of her Bachelor of Science in Chemistry at Delhi University, she was awarded a University scholarship to pursue her Masters degree in Bio-technology. She worked as a Research Assistant at International Center for Genetic Engineering and Bio-technology, New Delhi; followed by a teaching career at Modern School, New Delhi.

In August of 2003, Chhavi entered the program in Molecular Biology at Loyola University Chicago, as a pre-doctoral candidate. In June 2004, she joined the laboratory of Dr. Andrew Dingwall. Her research focused on studying the role of a novel nuclear receptor co-activator in Drosophila development and tissue patterning.

After completing her Ph.D., Chhavi will join Dr. Steven X. Hou’s laboratory at the National Cancer Institute (NCI), National Institute of Health (NIH) at Frederick, Maryland for a Post Doctoral position to study intestinal stem cell regulation using Drosophila and mouse as model systems.
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The thesis is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

------------------                                        ----------------------------------------
Date                                                        Director’s Signature