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The role of Chmp1 in Drosophila melanogaster

Meagan E. Valentine

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THE ROLE OF CHMP1 IN *DROSOPHILA MELANOGASTER*

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by
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LIST OF ABBREVIATIONS

Chmp1	Chromatin Modifying Protein 1, Charged Multivesicular Protein 1
ESCRT	Endosomal Sorting Complex Required for Transport
MVB	Multivesicular Body
Cy	Curly
St	Stubble
Sco	Scutoid
HEK	Human Embryonic Kidney
EGF	Epidermal Growth Factor
Aos	Argos
Spry	Sprouty
Kek-1	Kekkon-1
Ve/Rho	Veinlet/Rhomboid
Vn	Vein
Chmp1IR	Chmp1 RNAi line
hpRNA	hairpin loop RNA
PCP	planar cell polarity
Fz	Frizzled
Dsh	Disheveled
Pk	Prickle
Vang/Stbm	Van Gogh/Strabismus
Stan/Fmi	Starry night/Flamingo

ABSTRACT

THE ROLE OF CHMP1 IN *DROSOPHILA MELANOGASTER*

Meagan E. Valentine

Chmp1A has recently been linked to pancreatic cancer, a leading cause of cancer death in humans. Pancreatic tumors have lowered Chmp1A expression, and it has been described as a tumor suppressor. Chmp1A is also a member of ESCRT III (Endosomal Sorting Complex Required for Transport), a conserved protein complex involved in the degradation and recycling of activated transmembrane receptors. There is a single Chmp1 protein in *Drosophila* that is homologous to vertebrate Chmp1A; however, Chmp1 hasn't been studied in *Drosophila*. The objective of this study was to characterize Chmp1 in *Drosophila* using gene knockdown and over-expression. We used an RNAi fly line to knockdown *Chmp1* in the wing of the fly and created a transgenic fly line to look at over-expression. Our results suggest that Chmp1 may be regulating the Epidermal Growth Factor pathway and Notch-Delta signaling, as well as the Frizzled-Planar Cell Polarity pathway.

BACKGROUND

i. ESCRT

Many proteins in the cell membrane, such as ion channels and receptors, are constantly fluctuating; these proteins can be endocytosed, and then recycled or degraded. The ESCRT complexes (Endosomal Sorting Complex Required for Transport 0, I, II, III) play a role in a major pathway used for the targeted degradation of transmembrane receptor proteins. These complexes are required for control of cell signaling, down regulation of receptors, as well as other normal and pathological cell processes (1, 2, 3). In this pathway, activated receptor proteins are usually targeted for degradation by monoubiquitination. They are then endocytosed and transported to the early endosome (4). At the early endosome, proteins are sorted into multivesicular bodies (MVBs), which fuse to the lysosome and are degraded (5, 6). This makes the MVBs very important for receptor down regulation (7). The MVBs are also important for intercellular communication and antigen presentation, as they are also able to fuse with the plasma membrane, expelling their contents into the extracellular matrix (8, 9, 10). This pathway is highly conserved in eukaryotic organisms.

At least eleven proteins in mammals have been identified as components of ESCRT-III and are collectively referred to as charged multivesicular proteins (Chmps) (1, 2). All of these Chmps have similar characteristics: they are about 200 amino acids long, contain a coiled-coil region and charged residues, and they have a basic N-terminus and an acidic C-terminus (Figure 1) (1, 2).
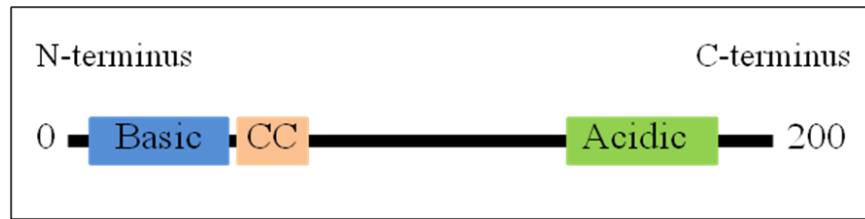
Figure 1. Chmp protein structure.

Chmps are rather small proteins about 200 amino acids in length with a basic N-terminus, an acidic C-terminus, and a coiled-coil (CC) domain.

ii. Chmp1A

Chmp1A is a highly conserved protein in both complex and simple eukaryotes. In different organisms it is known by several different names: Chmp1[1] (Chromatin Modifying Protein1), Chmp1A[2] (Charged Multivesicular Protein1), VPS46p/Did2p[3], and Sal1[4] (Supernumerary Aleurone Layers 1) (4, 11, 12, 13). In humans, there are two different isoforms of Chmp1A: a 35 kDa Chmp1A, which localizes to the nucleus, and a 32 kDa Chmp1A, which is located in the cytoplasm (11). The functional and structural differences between these two Chmp1A species are most likely due to differential post-translational modification.

It has been shown that the larger and nuclear form of Chmp1A is tightly associated with the nuclear matrix and has been suggested to play a role in stable gene silencing within the nucleus (13). In the nucleus, Chmp1A is associated with condensed chromatin and it has been reported that Chmp1A affects nuclear structure by increasing nuclear DNA concentration through chromatin condensation (13). Over-expression of

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[1] *Drosophila melanogaster* – NP_649051/CG4108
[2] *Homo sapiens* – NM_002768, this is a variant of Chmp1. There is also a Chmp1B
[3] *Saccharomyces cerevisiae* – NP_012961
[4] *Zea mays* - NP_00110521

Chmp1A affects DNA replication by halting cells in the S-phase of the cell cycle, possibly by way of its effects on chromatin structure (13). Chmp1A was also found to physically interact with the Polycomblike (Pcl) protein, and to recruit BMI1 protein, both of which are members of the Polycomb group (PcG) of transcriptional repressors responsible for gene silencing during development (13, 14, 15).

The cytoplasmic form of Chmp1A is a member of the ESCRT-III complex. Chmp1A localizes at the early and late endosomes, where it is involved in protein sorting and MVB formation (11). The Chmp1A protein has also been shown to bind to the VPS4 protein, which is shown to mediate the ATP-dependant disassociation of the ESCRT complexes and complete MVB formation (2, 11).

Loss of function of several the ESCRT components has been shown to give rise to over-proliferative phenotypes that are probably a consequence of failure of protein sorting. In *Drosophila,* genetic defects in Vps25 activity cause loss of cell polarity in epithelial tissue, followed by cell over-proliferation (16, 17). Tsg101, the mammalian homologue of Vps23 induces cell transformation and tumor formation in mice (2, 18, 19). Also, HCRP1, the human homologue of Vps37 is associated with hepatocellular carcinoma (HCC) (20, 21). In addition, mutations in the *sal1* gene*,* the maize homologue of *Chmp1A*, cause multiple layers of aleurone cells to form, a phenotype which may attributed to problematic receptor degradation (12). As a component of ESCRT, Chmp1A may be important for the control of cell growth by participating in the regulation of membrane receptor and signaling proteins.

iii. Pancreatic Cancer

Chmp1A has been linked to pancreatic cancer in humans (22). Pancreatic cancer is a leading cause of cancer death, with a five-year survival rate of only four percent. The mortality associated with pancreatic cancer is due to its aggressive malignancy, its high resistance to treatment, and that it is often not diagnosed until it is quite advanced (23). Although much research is in progress, still little is known about its molecular pathogenesis. However, specific patterns of expression have been identified and associated with pancreatic adenocarcinomas. Mutations in several genes, including *KRAS,* are characteristic of the disease (24). *KRAS* is a member of the *RAS* family oncogenes, of which activating mutations cause over-proliferation and cell survival (24). *KRAS* mutations are present in nearly 100% of adenocarcinomas (24).

iv. *Drosophila* as a Model

In this study, *Drosophila melanogaster* was used as a model for studying Chmp1 activity. *Drosophila* is a model organism that is often used for studying the function of human proteins. This species has a rather short generation time, is easy to work with, has elegant genetics, and it is inexpensive; all of these characteristics make *Drosophila* a good model. Additionally, the *Drosophila* genome has been sequenced, providing an important resource to biologists and identifying over 13,000 genes. There has also been over 100 years of work on *Drosophila*, which provides researchers with an extensive base of knowledge of this species (Flybase). There are many sophisticated genetic and molecular tools that have been developed for studying gene and protein function, many of which are unique to this organism. However, probably most importantly, there is a great

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deal of homology between human genes and *Drosophila* genes. Of about 300 known human disease genes, almost 200 have homologues in *Drosophila* (25). We also share common pathways, making many of the results in *Drosophila* transferable.

There have been no published studies of Chmp1 function in *Drosophila* and our knowledge of vertebrate Chmp1A is incomplete. It is known that there is only one copy of *Chmp1* in *Drosophila*, which will make it easier to study because we will not have to worry about the activity of homologous gene products. From previous work in the Collier lab, it is also known that Chmp1 is expressed in all embryonic tissues. Probably most importantly, we know that the protein sequence of *Drosophila* Chmp1 is 49% identical to Chmp1A in humans, allowing for the conclusions drawn about *Drosophila* Chmp1 function to be applicable to Chmp1A function in humans.

v. UAS-Gal4 System

The UAS-Gal4 system (Figure 2) is a sophisticated genetic tool that is widely used in *Drosophila* studies (26). It was first identified in yeast, but has been modified and is now used in other systems, including *Drosophila* and mammalian cell culture. This system allows for very fine control of the location and the intensity that a gene of interest will be expressed. When a transgene is under the control of a UAS (Upstream Activating Sequence) promoter, it will only be expressed in the presence of Gal4. Conveniently, there are thousands of fly lines that have been designed to express Gal4 in specific areas of the fly, at different intensities, and sometimes at specific times during development. This makes controlling gene expression quite easy, as with a single generation cross you can knock down or over-express a gene where you choose and to the extent you choose.

This efficacy of this system is temperature related, with 30°C producing strongest expression. There is higher UAS-Gal4 activity in a fly line at 30°C than the same fly line at 18°C, and therefore a stronger phenotype is exhibited (27).

Tissue-specific promoter in Gal4-expressing flies activates *Gal4* expression

Figure 2. UAS-Gal4 system in *Drosophila*
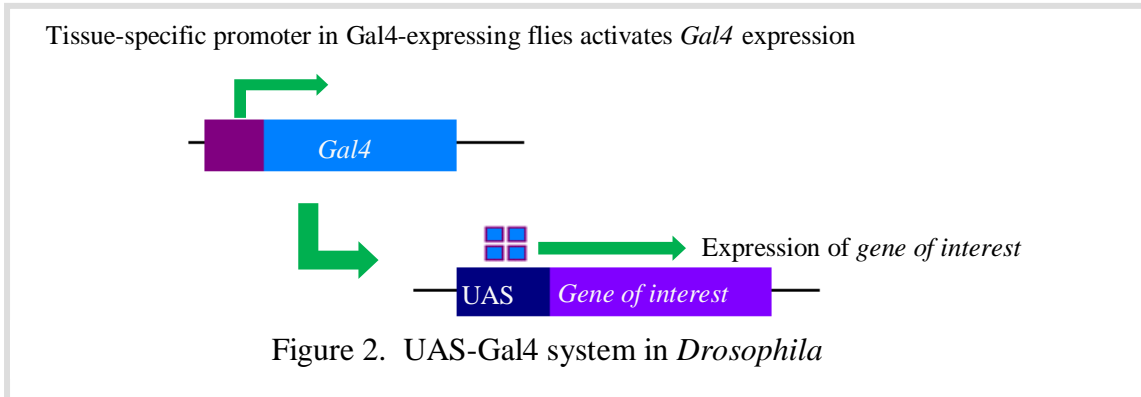
vi. *Drosophila* **Wing Anatomy**

The *Drosophila* wing (Figure 3) is like a flattened balloon. It has a dorsal and a ventral side that oppose each other, each one cell-layer thick. Cuticular structures called veins are distributed in a distinctive pattern, contributing rigidity to the wing. The pattern of these veins is nearly identical between all wild-type wings. The space between veins is called the intervein tissue. There are four longitudinal veins, L2-L5. These veins cross the length of the entire wing. There are also two transverse veins, the anterior cross vein and the posterior cross vein (acv, pcv), which are much shorter and connect the L3 and L4 veins, and the L4 and L5 veins, respectively. Veins L3, L5, and the distal part of L4 are dorsal wing veins and are located on the dorsal side of the wing (28, 29). The rest are considered ventral. Two other veins, L1 and L6 exist as well, however they do not extend into the wing blade (28).
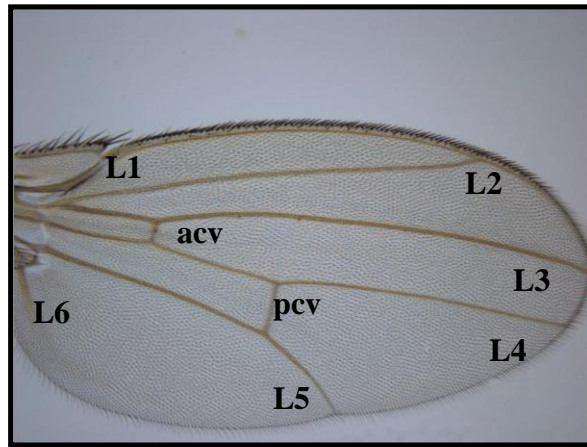
Figure 3. *Drosophila melanogaster* wild-type female wing.

10X, mounted dorsally in GMM.

A wild-type *Drosophila* wing is a quite regular structure. It is decorated with short cuticular hairs that generally point distally and are resultant of prehairs, which are composed of F-actin and microtubules (30). The cells of the developing wing are polarized within the plane of epithelium, and are packed regularly as hexagons. One wing prehair is produced at the most distal vertex of each cell (Figure 4) (30).
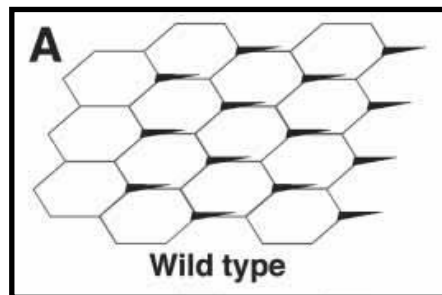
Figure 4. Wild-type *Drosophila* wing cells

Each cell is hexagonally shaped with a single wing prehair produced at the distal vertex of the cell (31).

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vii. Balancer Chromosomes

There are many advantageous genetic tools available when using *Drosophila*. One quite useful and widely used tool is the balancer chromosome. In the fly, these chromosomes have several characteristics that make doing the genetics easier. Firstly, and maybe most importantly, these chromosomes contain multiple inversions, which suppress homologous recombination. If the balancer chromosomes do recombine, the recombination products may contain duplications or may even lack a centromere. Because of these mutations, homologous recombination involving balancer chromosomes produces progeny that are not viable. The balancer chromosomes are also homozygous lethal, so flies receiving two copies of the balancer do not survive. Additionally, they have a dominant phenotype, so if a fly carries the balancer chromosome, it can be easily identified.

There are four chromosomes in *Drosophila melanogaster*. The first chromosome is the X, and is used with balancers called First Multiple (FM). Chromosome 4 is not used with balancers, as no balancer for it exists. The third chromosome balancers are called Third Multiple (TM) and may contain the dominant marker *Stubble* (*Sb*). When a fly carries this balancer, the bristles on its head and thorax are shortened. The second chromosome balancer that is often used is Curly of Oster (CyO), and its dominant marker is *Curly* (*Cy*). Flies that carry this balancer have curly wings.

CHAPTER 1 – CHMP1 AND REGULATION OF GROWTH

Section 1. Introduction

Section 1.1: Overview of Chmp1

Cell proliferation, growth, and migration are regulated by a myriad of different proteins and pathways, the regulation of which is essential to proper cell behavior. One small divergence of this tight regulation can cause severe problems in the cell, including uncontrolled cell growth, or tumors. Tumor suppressors are a set of genes that govern a variety of normal activities in the cell, ranging from cell cycle checkpoint control to protein turnover to DNA damage (32). When tumor suppressors are absent or expressed at low levels, problems such as over-proliferation can occur. In many types of cancers, tumor suppressor genes have low expression levels or are mutated.

Recent work at the Marshall University School of Medicine has shown that Chmp1A regulates proliferation in zebrafish and in mammalian cell culture. Both the over-expression and knockdown of *Chmp1A* in zebrafish embryos causes hyperplasia formation, suggesting that Chmp1A is involved in the regulation of growth (33). When looking at HEK 293 (Human Embryonic Kidney) cultured cells, *Chmp1A* over-expression significantly inhibits growth by arresting cells in S-Phase (13), while *Chmp1A* knockdown promotes growth (33). Additionally, HEK 293 cells with reduced *Chmp1A* activity form tumors when injected into nude mice, while control HEK 293 cells do not (33).

Chmp1A has been linked to pancreatic cancer in humans, as pancreatic tumors show a considerable reduction of Chmp1A activity (33). A recent study shows that

knockdown of *Chmp1A* in a pancreatic tumor cell line (PanC-1) promoted growth, while over-expression inhibited growth, and was also associated with an increase of p53, an extremely important tumor suppressor (22). These results provide evidence that Chmp1A functions in the regulation of growth, since when it is lost growth control is abnormal. This suggests that Chmp1 may function as a tumor suppressor, at least in the human pancreas (22).

To date, there are no published Chmp1 (homologue of human Chmp1A) function studies in Drosophila. As this is the first study on Chmp1 function, we began at a classical starting point by simply looking at *Chmp1* knockdown and over-expression. *Chmp1* was knocked down and over-expressed in the *Drosophila* wing. *Chmp1* knockdown resulted in oversized wing veins that looked as if they had overgrown. The phenotype obtained in the *Chmp1* knockdown wings is very similar to phenotypes obtained in wings with over-active EGF, suggesting a possible role for Chmp1 in regulating EGF activity. When *Chmp1* was over-expressed in the *Drosophila* wing, the phenotypes obtained were similar to reduction of Delta (34), a protein involved in Notch signaling. This suggests a possible role for Chmp1 in the regulation of Notch signaling as well. As altering Chmp1 activity produces phenotypes which are suggestive of Notch and EGF regulation, it is appropriate to overview these pathways.

Section 1.2: Epidermal Growth Factor Pathway (Figure 5)

In humans, EGF signaling plays an important role in the regulation of cell growth, migration, differentiation, and proliferation (35). In *Drosophila,* proper EGF signaling is crucial in many developmental processes including oogenesis (36), eye development (37,

38), growth of imaginal discs (39), and wing vein development (40, 41). The EGF receptor is a transmembrane protein, which functions a receptor tyrosine kinase. Binding of the EGF receptor activates the Ras signaling pathway, which ultimately results in altered gene expression (42, 43). The EGF pathway is regulated by a several feedback mechanisms. Many different inhibiting and activating molecules regulate the EGF receptor pathway, which are often induced by EGF receptor activity (44).

The three known negative regulators of EGF signaling in *Drosophila* are Argos (Aos), Sprouty (Sty), and Kekkon-1 (Kek-1). Aos is a secreted molecule specific for the EGF receptor. It blocks ligand binding and can affect many surrounding cells (44, 45). Sty is an intracellular inhibitor, which inhibits Ras signaling, thereby inhibiting EGF signaling (46). Kek-1 is a transmembrane protein that interacts directly with the EGF receptor to inhibit ligand binding (47, 48).

There are several different activating ligands of EGF receptor: Spitz, Gurken, Vein, and Keren. Gurken is expressed only in the oocyte and is important for oogenesis (49). Vein (Ve) is a secreted factor that binds and activates the EGF receptor (50). Keren is a transmembrane protein that must be cleaved in order to become active (51). Lastly, Spitz is the major EGF ligand, which, like Keren, is a transmembrane protein that is inactive until cleaved (52). The transmembrane protein called Rhomboid (Rho), also known as Veinlet (Vn), is not a ligand of the EGF receptor; however it is an important activator of EGF in that both Spitz and Keren are present, but do not become active until cleaved by Rho (53, 54).

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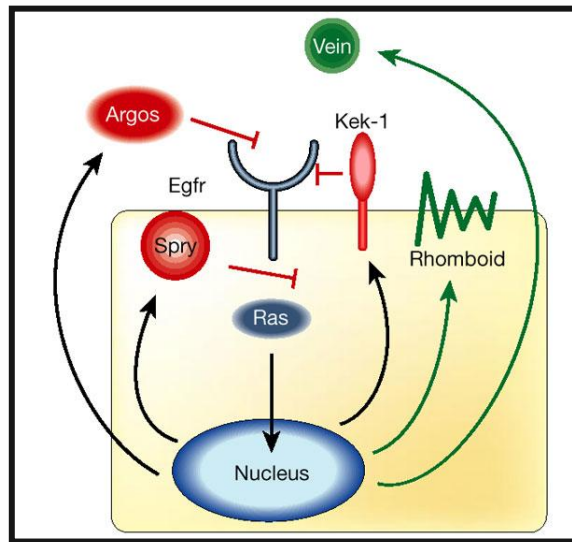
Figure 5. Regulators of the *Drosophila* EGF receptor (EGFR).

The three known inhibitors (red) are Spry, Kek-1 and Aos. The two known activators (green) are Ve and Rho (Vn) (107).

Section 1.3: Notch-Delta Signaling

Notch-Delta signaling is conserved in animals and has multiple essential activities during development such as lateral inhibition, boundary formation and cell fate decisions (55). Notch is a single pass transmembrane receptor protein, which was originally identified in *Drosophila* (56). In *Drosophila*, there is only one Notch protein, and it is expressed as a heterodimer[5] at the membrane (57, 58). It has an ectodomain called the Notch Extracellular Domain (NECD), which is involved in binding interactions, a Notch Intracellular Domain (NICD) critical for protein-protein interactions and transcriptional activation (59, 60, 61). Notch responds to two ligands, Delta and Serrate, which are also membrane-bound proteins (62, 63, 64). Ligand binding of the NECD leads to cleavage of

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[5] The Notch protein forms a homodimer at the membrane. However, for Notch to be active, one of the units is cleaved, leaving a heterodimer at the membrane (58).

the NICD, which translocates to the nucleus where it interacts with Suppressor of Hairless [Su(H)], a DNA-binding protein, and regulates the transcription of its target genes (65, 66). As both the ligands and receptors in Notch-Delta signaling are transmembrane proteins, signaling is short range.

As wings go, Notch and EGF signaling work together to promote correct wing vein formation (Figure 6). Delta is expressed in the center of wing vein territory while Notch is expressed in cells bordering the veins (34). Delta expression activates Notch, which activates Su(H) (65). Su(H) then activates expression of a gene called *Enhancer of split* [*E(spl)mβ*] (67). E(spl)mβ then goes on to repress *rho* transcription (68), confining *rho* expression to the vein, where it activates EGF signaling (40).
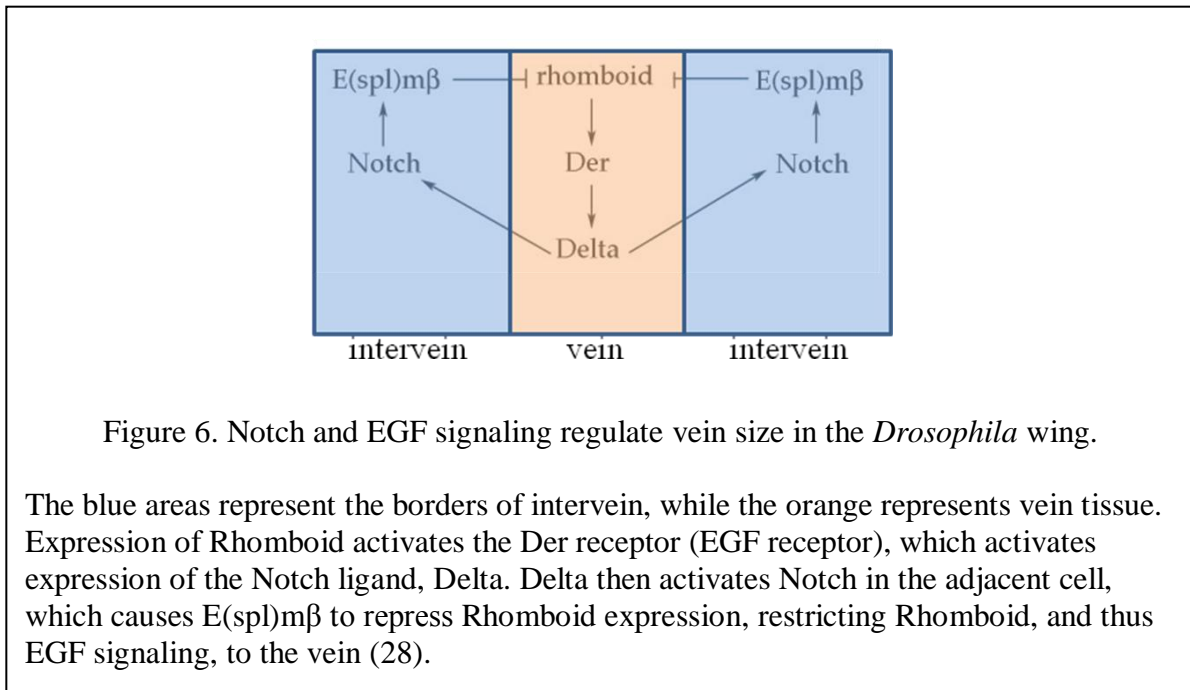
Figure 6. Notch and EGF signaling regulate vein size in the *Drosophila* wing.

The blue areas represent the borders of intervein, while the orange represents vein tissue. Expression of Rhomboid activates the Der receptor (EGF receptor), which activates expression of the Notch ligand, Delta. Delta then activates Notch in the adjacent cell, which causes E(spl)mβ to repress Rhomboid expression, restricting Rhomboid, and thus EGF signaling, to the vein (28).

Section 2. Objective and Hypothesis

Section 2.1: Objective

The objective of this study was to characterize the function of Chmp1 in *Drosophila* by observing the effects of knockdown and over-expression. *Chmp1* knockdown was achieved using RNAi, and over-expression was achieved by creating a transgenic fly line.

Section 2.2: Hypothesis

We hypothesize that Chmp1 in *Drosophila* will function in the same developmental processes as Chmp1A in vertebrates.

Section 3. Materials and Methods

Section 3.1: Gels

All gels were 0.8% agarose gels containing ethidium bromide. In a 50mL Erlenmeyer flask, 0.4g of agar was added to 50mL of 1X TBE (Tris-Borate-EDTA) buffer from a 10X stock solution. The mixture was heated in a microwave for about 35 seconds, until the agar had dissolved. One uL of ethidium bromide was added to the mixture, and it was swirled until well mixed. The gel was poured and allowed to cool. All gels were run at 120 volts for approximately 90 minutes alongside 1KB DNA ladder. They were run in 1X TBE buffer, and analyzed on a GelDoc (Biorad, Hercules, CA, USA).

Section 3.2: Transformation of *E. coli* cells

The competent cells were thawed on ice, and 50uL was added to a 1.5mL

microcentrifuge tube. 0.85uL of β-mercaptoethnol was added to the cells, and then they

were incubated on ice for 10 minutes, swirling every 2 minutes. 10uL of DNA was added

to the cells, and they were incubated on ice for 30 minutes. In the meantime, 100uL of

LB Broth was heated in a $42^{o}C$ water bath. At the end of the incubation period, the cell

tube was heat pulsed in a $42^{o}C$ water bath for 45 seconds, and then placed on ice for 2

minutes. The cells were then added to the broth tube and incubated at $37^{o}C$ for 30

minutes. The transformed cells were plated (~75uL/plate) onto LB agar plate containing

chloramphenicol (final concentration: 2uL/mL) or ampicillin (stock concentration:

50ug/mL, final concentration: 1uL/mL), depending on the vector's resistance, and grown

overnight at $37^{o}C$. The next day, individual colonies were selected[6] and cultured in

200mL of LB broth containing their specific antibiotic, and shaken overnight at 150rpm

at $37^{o}C$. The DNA was then purified using a Qiagen HiSpeed Plasmid Midi Kit

(Valencia, CA, USA), following the manufacturer's procedures. The DNA was separated

on a gel and analyzed.

Section 3.3: Plasmid preparations

All large plasmid preparations were made using 200mL of LB broth and either

2ul/mL of chloramphenicol or 1uL/mL of ampicillin, depending on the vector's

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[6] The pBluescript vector allowed for blue/white color selection. When using this vector, the plates were covered with a mixture of IPTG (72ug/mL) and X-Gal (40ug/mL) before plating. The white colonies that grew represented transformed cells, while blue colonies represented non-transformed cells.

resistance. They were shaken overnight at 37°C and purified using the Qiagen HiSpeed

Plasmid Midi Kit (Valencia, CA, USA).

All small plasmid preparations were made using 3mL of LB broth and either

ampicillin or chloramphenicol. They were shaken overnight at 37°C and purified using

the Qiagen QIAprep Spin Miniprep Kit (Valencia, CA, USA).

Section 3.4: Digests

All digests were 100uL solutions in a 1.5mL microentrifuge tube. Digests were

always performed in sets of two so they could be combined and used in a

phenolchloroform extraction. The digests consisted of 10uL of DNA (~5ug) solution,

10uL of buffer, 70uL of water and 10uL of enzymes (Table 3). The digests were

incubated at 37°C for 1 hour, then run on a gel and analyzed.

Section 3.5: Phenolchloroform extraction

In a 1.5mL microcentrifuge tube, 200uL of phenol chloroform was added to

200uL[7] of DNA solution and vortexed for 10 seconds, and then centrifuged for 4 minutes

at maximum speed. The top layer (~200uL) was removed and transferred into a new

microcentrifuge tube. Then 20uL of 3M pH5.2 sodium acetate was added and mixed by

vortexing. 400uL of isopropanol[8] was added and mixed by vortexing. The sample was

incubated for 30 minutes on ice, and then centrifuged at maximum speed for 8 minutes to

pellet the DNA. The supernatant was discarded and the pellet was washed with 70%

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[7] For digests and PCR products, combine two vials (100uL each) to make a 200uL
sample of DNA solution.
[8] When purifying cDNA, 100% ice cold ethanol was used.

ethanol and microcentrifuged again. The supernatant was again discarded, and the pellet was air dried overnight. The next day, 20uL of buffer EB from Qiagen's QIAprep Spin Miniprep Kit (Valencia, CA, USA) was added to resuspend the pelleted DNA. The DNA solution was then run on a gel and analyzed.

Section 3.6: *Chmp1* insertion into pUAST and pUASHM

Chmp1 cDNA was digested out of the pBluescript vector in 8 - 100uL digests of each construct containing 40uL (~20ug) of DNA solution, 10uL of their respective enzymes (Table 3), and 10uL of their respective buffer and 40uL of water. Then 4 – 200uL phenol chloroform extractions of each construct. The samples of each construct were loaded on gel. Eight wells were used, and each well contained 10uL of sample and 2uL of 10X loading dye, and were run alongside a 1kb DNA ladder for about 90 minutes in 1X TBE buffer

Section 3.7: Ligation

All ligations were 10uL of solution prepared in a 1.5mL microcentrifuge tube. They consisted of 1uL of T4 DNA ligase, 1uL ligase buffer, 3uL of vector (~1ug/uL), 4uL of cDNA (~1ug/uL) and 1uL of water. They were incubated at 4^oC overnight.

Section 3.8: Fly food preparation

In a large pot over a burner, 1000mL of distilled water was mixed with 18 grams of agar. The mixture was heated and stirred. In the meantime, 500mL of water was added to 30 grams of Brewer's yeast, along with 120 grams of cornmeal in a 1000mL beaker.

Once the water/agar mixture began to boil, 225mL of molasses was added while stirring,

followed by the cornmeal/yeast mixture. While stirring frequently, the mixture was

brought to a boil. It was cooled with lid on for 10 minutes, and cooled with the lid off,

stirring occasionally, for 30 minutes (vials) to an hour (bottles). After the cooling period,

13.2mL of propionic acid and 42.75mL of hydrobenzoic acid were added. The mixture

was stirred and poured into either bottles or vials. The bottles/vials were stored overnight

at 18°C to completely cool and plugged the next day with cotton.

Table 1. Primers used for PCR
Restriction enzyme sites in bold. GGATCC: *Bam*H1, CATATG: *Nde*1, CTCGAG: *Xho*1,
GAATCC: *EcoR*1

Name	Sequence
pUASHM forward	GGGCCC**GGATCC**ACGTCG**CATATG**TCTACGAGTTCCATGG
pUASHM reverse	TACCAC**CTCGAG**TTATTCAGCCTGGCGGAGACG
pUAST forward	ACGTCG**GAATCC**ATGTCTACGGAGTTCCATGG
pUAST reverse	TACCAC**CTCGAG**TTATTCAGCCTGGCGGAGACG

Table 2. Primers used for sequencing

Name	Sequence
pUAST, pUASHM forward	TGCAACTACTGAAATCTGC
pUAST, UASHM reverse	CCAATTATGTCACACCACAG

Table 3. Enzymes used.
The enzymes next to the number one were used with the first construct (*Chmp1* insertion into pUASHM) and enzymes next to the number two were used with the second construct (*Chmp1* insertion into pUAST).

Vector	Enzymes
Digest of *Chmp1* PCR product	1. *Bam*H1 and *Xho*1 2. *Eco*R1 and *Xho*1
Digest of pBluescript	1. *Bam*H1 and *Xho*1 2. *Eco*R1 and *Xho*1
Digesting *Chmp1* out of pBluescript	1. *Nde*1 and *Xho*1 2. *Eco*R1 and *Xho*1
Digest of pUASHM	1. *Nde*1 and *Xho*1
Digest of pUAST	2. *Eco*R1 and *Xho*1

Table 4. Genotypes of flies used

Name	Genotype
Oregon R (wild-type)	Oregon R-C
Chmp1IR (VDRC)	w^{1118}; $P\{GD11219\}v21788/CyO$
Chmp1IR (TriP)	$y^1\ v^1$; $P\{TRiP.HM05117\}attP2$
MS1096-Gal4	$w^{1118}\ P\{w^{+mW.hs}=GawB\}Bx^{MS1096}$
argos$^{\Delta7}$	$argos^{Delta7}/TM3,\ Sb^1$
argos^{w11}	w^8; $P\{w^{+mW.hs}=lwB\}argos^{W11}/TM3,\ Sb^1$
UAS-argos on 1 &2	$y^1,w^*P\{w^{+mC}=UASargos.M\}301021;P\{w^{+mC}=$ *UAS argos.M}30-85-1*
sty$^{\Delta5}$	w^*; $sty^{Delta5}/TM3,\ Sb^1\ P\{w^{+mC}=35UZ\}2$
argos$^{r/t}$	$argos^{r/t}$
kek-1	$y^1\ w^{p67c23}$; $P\{y^{+t7.7}\ w^{+mC}=wHy\}kek1^{DG23812}$
ve vn	$rho^{ve-1},\ vn^1$

Section 4: Results

To date, there are no published studies on Chmp1 function in *Drosophila*. Classically, protein and gene studies begin with over-expression and knockdown. Because no one has studied Chmp1, no classical mutant exists. So in order to obtain *Chmp1* knockdown, a transgenic RNAi fly line was obtained from the Vienna Drosophila Resource Center (69). This fly line will allow for control of *Chmp1* knockdown, because the *Chmp1* RNAi transgene is downstream of a Gal-4 responsive UAS promoter. In order to look at *Chmp1* over-expression, transgenic fly lines had to be created. Two expression vectors were used: pUAST and pUASHM, both of which mediate random insertion of the transgene into the *Drosophila* genome. Each of these vectors allowed for *Chmp1* transgene insertion downstream of a Gal4-responsive UAS promoter, which permits fine control of *Chmp1* over-expression once in the fly. The pUASHM vector will tag the Chmp1 protein with an N-terminal HisMyc (HM) tag that will allow for visualization and localization studies in the fly.

Section 4.1: *Chmp1* knockdown

Chmp1 function has not been studied in the *Drosophila* system. A classical *Drosophila* Chmp1 mutant does not exist, so to study *Chmp1* knockdown, RNAi was used. A *Chmp1* RNAi line was obtained from Vienna Drosophila RNAi Collection (VDRC) (69). The *Chmp1* RNAi (*Chmp1IR)* fly line obtained expresses *Chmp1* hairpin loop RNA (hpRNA), which is complementary to *Chmp1* mRNA. The hpRNA is expressed under a Gal4 responsive UAS promoter, which allows for very fine control over *Chmp1* knockdown. Expression of *Chmp1* hpRNA initiates the RNAi pathway and

mediates the destruction of *Chmp1* mRNA, knocking down *Chmp1* expression (Figure 7). The *Chmp1* hpRNA is located on the second chromosome of the fly, and is balanced with CyO.
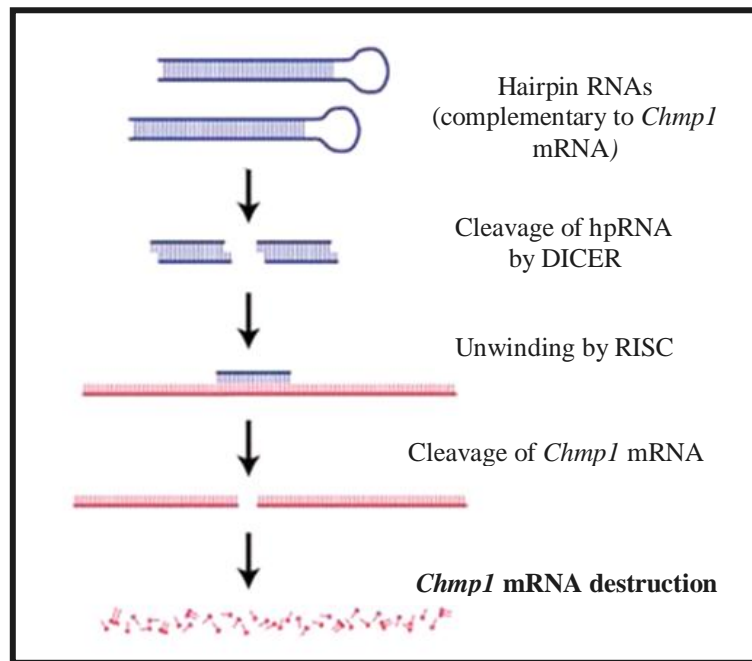
Figure 7. Mechanism of *Chmp1* RNAi in flies.

The hpRNA expressed is double stranded RNA, which is recognized by the cell. An enzyme called DICER is recruited to the site and cleaves the hpRNA into 20-22 nucleotide pieces called small interfering RNAs (siRNA). Then a complex called RNA-Induced Silencing Complex (RISC) unwinds the double stranded siRNAs. When this complex comes into contact with *Chmp1* mRNA, the siRNA binds, and RISC cleaves the mRNA. The mRNA is then destroyed and recycled in the cell.

Section 4.2 (A-E): Generation of Transgenic Flies (Figures 18 and 19)

As Chmp1 has not been studied in *Drosophila*, there was no available fly line that would allow for *Chmp1* over-expression. Thus, transgenic *UAS-Chmp1* fly lines were

created. This was accomplished by inserting the *Chmp1* coding sequence into a vector downstream of a UAS promoter. The vectors that contain the UAS promoter and allow for *Chmp1* insertion are called pUAST and pUASHM. pUASHM adds a HM tag to the Chmp1 protein.

Section 4.2 A: cDNA preparation

Four different vectors were used: GH26351 (pOT2 vector (Figure 20)) containing *Chmp1* cDNA,) has chloramphenicol resistance, pUAST (Figure 21), pUASHM (Figure 22) and pBluescript (Figure 23) all have ampicillin resistance. The GH26351 plasmid was received from *Drosophila* Genomics Resource Center (DGRC). Preparations of the plasmids were made using XL-1 Blue Competent Cells (Stratagene, La Jolla, CA, USA), which were separately transformed by each vector. To check that the vectors were correct, they were digested and analyzed on a gel.

To prepare the vectors (pBluescript, pUAST, pUASHM) for *Chmp1* insertion, they were digested with the appropriate enzymes (Table 3). The vectors were then purified and concentrated using a phenolchloroform extraction. During the phenolchloroform extraction, isopropanol was used, removing the linker DNA that was digested out of the vector to prevent re-insertion. The DNA was then run and analyzed on a gel. At this point, the vectors were ready for *Chmp1* insertion.

Now that the vectors were ready for *Chmp1* insertion, a *Chmp1* cDNA needed to be prepared from GH26351. The *Chmp1* cDNA was amplified from the vector using the PCR Extender System Kit (5 Prime Inc, Maryland, USA) and a Biometra Tgradient Thermoblock (Biometra Biomedizinische Analytik GmbH, Rudolf-Wissell, Goettingen,

Germany). Two sets of primers (Invitrogen, Carlsbad, CA, USA) were used to amplify *Chmp1* from the pOT2 vector (Table 1). The set of primers for the first construct were pUASHM forward, which added restrictions cut sites for *Bam*H1 and an *Nde*1, and pUASHM reverse, which added the restriction cut site for *Xho*1, to allow for insertion into the pBluescript and the pUASHM vectors. The set of primers for the second construct were: pUAST forward, which added a restriction cut site for *Eco*R1, and pUAST reverse, which added the restriction cut site for *Xho*1, to allow for insertion into the pBluescript and the pUAST vectors. The cycling parameter for amplifying PCR products was 30 cycles of 94°C for 30 seconds, 55° for 30 seconds and 72°C for 2 minutes. Eight individual but identical PCR vials were run. DNA from PCR was run and analyzed on a gel.

Section 4.2 B: Preparing *Chmp1* for insertion into pBluescript

As the pUAST and pUASHM vectors are quite large and less ready to be taken up during a transformation, the PCR-amplified *Chmp1* cDNA was first inserted into the much smaller pBluescript vector. The cDNA from PCR was purified and concentrated by a phenol-chloroform extraction. The concentrated DNA was then digested with enzymes respective to which vector it would be inserted. The DNA of the first PCR product[9] was digested with *Bam*H1 and *Xho*1, while the DNA of the second PCR product[10] was digested with *Eco*R1 and *Xho*1. The DNA was then purified and concentrated using a phenol-chloroform extraction. *Chmp1* was now ready for insertion into pBluescript.

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[9] Product of PCR performed with pUASHM primers
[10] Product of PCR performed with pUAST primers

Section 4.2 C: *Chmp1* in pBluescript

The precipitated pBluescript vector and each *Chmp1* preparation were combined in a ligation. XL-1 Blue Competent Cells were transformed. The pBluescript vector allowed for blue/white color selection. When using this vector, the plates were covered with a mixture of IPTG (72ug/mL) and X-Gal (40ug/mL) before plating. The white colonies that grow represent transformed cells, while blue colonies represent non-transformed cells. So the next day, individual white colonies were selected and made into small preparations and grown overnight. The plasmid was then purified using the Qiagen QIAprep Spin Miniprep Kit (Valencia, CA, USA). To check that the transformation was successful and that the plasmids were correct, the DNA was digested with enzymes that should release the insert, and run on a gel and analyzed. Large preparations of DNA were made with the samples that appeared to have the correct vector and insert size, and then sequenced, to ensure that the *Chmp1* sequence was correct, by the Genomics Core Facility of Marshall University's Joan C. Edwards School of Medicine, using the M13 forward and reverse primers.

Section 4.2 D: Preparing *Chmp1* for insertion into pUAST and pUASHM

When the correct *Chmp1* sequence was obtained, it was then removed from the pBluescript vector, and inserted into the pUAST and pUASHM vectors. Very high cDNA concentrations were required for this section of the protocol because the last step was a gel extraction, which was not extremely efficient in recovering DNA. The *Chmp1*cDNA was digested out of the pBluescript vector. A large amount of plasmid DNA was digested in order to maximize the amount of digested *Chmp1* cDNA. To concentrate the cDNA,

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the digests were precipitated in a phenol-chloroform extraction. The samples of each

construct were loaded and run on gel. The gels were then analyzed under UV light and

the inserts were cut out and collected using the QIAquick Gel Extraction Kit (Qiagen,

Valencia, CA, USA). The DNA solution obtained from the gel extraction was run on a

gel to ensure that the procedure was successful. When a high concentration of *Chmp1*

was obtained, it was then ready to be inserted into the pUAST and pUASHM vectors.

Section 4.2 E: *Chmp1* in pUAST and pUASHM

Now that *Chmp1* was ready for insertion into pUAST and pUASHM, the cDNA

could now be ligated into the vectors. Two ligations were performed, one for each

construct. XL1-Blue Ultra Competent Cells (Stratagene, La Jolla, CA, USA) were

transformed separately by each ligation. The next day, individual colonies were selected

made into small preparations, and grown overnight. The plasmid was then purified using

Qiagen's QIAprep Spin Miniprep Kit (Valencia, CA, USA), and digested to check for the

correct vector and insert size. The samples that appeared to be correct were made into

larger preparations and purified with the HiSpeed Plasmid Midi Kit (Qiagen, Valencia,

CA, USA). The plasmids were then sequenced by the Marshall University Genomics Core

Facility with custom primers (Invitrogen, Carlsbad, CA, USA). The same primers were

used for both pUAST and pUASHM sequencing (Table 2).

When the correct sequence was obtained, the samples were then prepared for

insertion into the *Drosophila* genome. A commercial generator of transgenic flies called

BestGene Inc. (Chino Hills, CA, USA) was used. They required at least 50uL of DNA

with a concentration of 1ug/uL. The concentrations of pUAST and pUASHM vectors

containing the correct *Chmp1* sequence were measured using a ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). A concentration of 1ug/uL was needed, but was not obtained. The Qiagen HiSpeed Plasmid Midi Kit produces about 1mL of purified plasmid in solution; so, to obtain a higher concentration, 400uL of each plasmid solution was concentrated by a phenolchloroform extraction and resuspended in 50uL of water. The concentrations were measured again using a ND-1000 spectrophotometer, and each had reached a concentration of 1ug/uL or higher. The plasmids were then sent to BestGene Inc. (California, USA).

BestGene Inc. provided the service of integrating the *Chmp1* transgene into the *Drosophila* genome. Both the pUAST and pUASHM vectors have P elements, which are transposons that are often used in *Drosophila* to create genetically modified flies. These P elements function to insert the *Chmp1* transgene and a *white* gene together into the genome of the fly (Figure 8). This process is random, and therefore gives rise to the possibility of insertion within a gene, or multiple insertions. The *white* gene, which gives the eye of the fly a red color, serves as a marker so that only flies with the *white*$^+$ phenotype have the *Chmp1* transgene.

The *Chmp1* constructs that were created, along with a helper plasmid, were injected near/into the germ cells of *white*$^-$ (white eye) embryos. The helper plasmid encodes a transposase, which is required to insert the transgene into the genome. Some of the germ cells take up the plasmids, and the *Chmp1* transgene and the *white* gene get inserted into the genome of those cells. When the injected *white*$^-$ embryo develops into an adult fly, it can be crossed to another *white*$^-$ fly. The successful BestGene lines had the *Chmp1* transgene, which was evident by its *white*$^+$ phenotype.
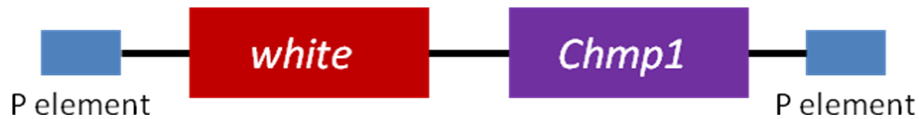
Figure 8. P-elements and transgenes.

Both the pUAST and the pUASHM use P elements to insert the *Chmp1* transgene into the genome of the fly. The P elements also insert the *white* gene, which functions as a indicator that a fly has the *Chmp1* transgene.

Twenty separate and successful fly lines (ten for each vector preparation) were returned from BestGene Inc. In order for these fly lines to be very useful, it will be necessary to determine the chromosome of *Chmp1* insertion. The lines will also have to be balanced to ensure that the stock remains stable.

Section 4.3 (A & B): Over-expression and knockdown of *Chmp1* in the wing

When beginning a study on protein function, the usual place to begin is to observe the results of loss of function, or losing protein activity, as well as gain of function, or over-expression of that protein. The results and phenotypes of these first two studies can give insight to the protein's function. *Drosophila* is a very well studied model organism. So, if mis-expression of a protein interrupts a signaling pathway, the phenotypes observed may give a clear indication as to which pathways that protein is involved.

Section 4.4 A: Knockdown of *Chmp1* in the wing

Now the tools were available to both over-express and knock down *Chmp1* in the fly. Achieving *Chmp1* knockdown was very simple; using the RNAi fly line, it only required a single generation cross. In the RNAi line, the *Chmp1IR* transgene is under the

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control of a UAS promoter, meaning *Chmp1* hpRNA is only expressed in the presence of the Gal4 protein. A single generation cross between *Chmp1IR* virgin females and males that express Gal4 in the wing will result in offspring with *Chmp1* knockdown in the wing (Figure 9).

Fly expressing
Gal4 in wing
♂

X

Chmp1IR fly
♀

Fly with *Chmp1* knockdown
in wing
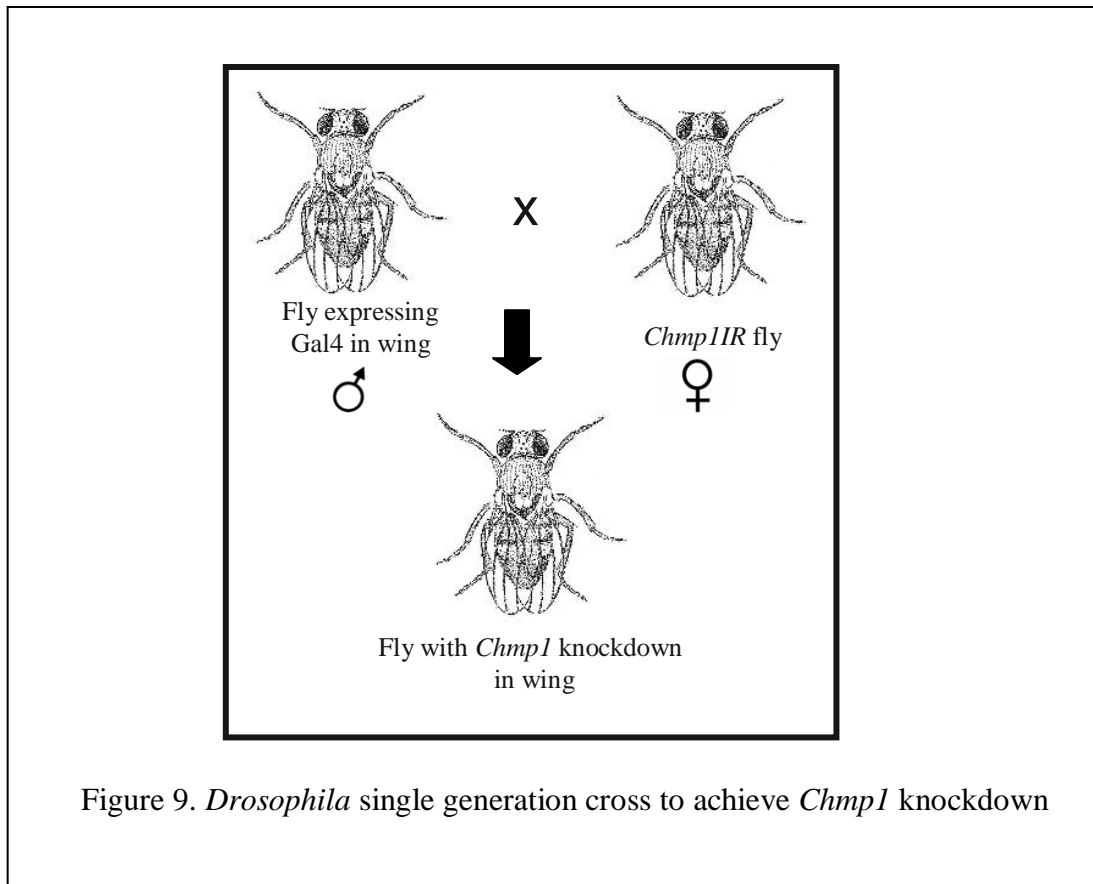
Figure 9. *Drosophila* single generation cross to achieve *Chmp1* knockdown

The Gal4 line that was used is called *MS1096-Gal4*. *MS1096-Gal4* only drives Gal4 expression in certain parts of the fly, most strongly on the dorsal side of the developing wing. Three separate crosses were set up, each with ten virgin *Chmp1IR/Cy* females to ten *MS1096-Gal4/Y*; *Cy/Sco* males. One cross was kept at 25°C, one at 28°C, and one at 30°C. The parent generation was moved to a new vial with fresh fly food every two to three days. When the first generation of these crosses was fully developed, the

right wing of *MS1096-Gal4/X; Chmp1RNAi/Sco* female flies was dissected off, and mounted dorsally onto a microscope slide in GMM (70). It should be mentioned that for all of the crosses completed in these studies, the first generation was quite large (>100 flies). For every cross that was performed, at least 50 flies were analyzed and at least 10 wings were mounted. The results presented for each cross were consistent and representative of the relative first generations.

When *Chmp1* was knocked down in the wing of the flies, the result was overgrowth of dorsal wing veins L3 and L5 (Figure 10B). Overgrowth of wing veins is a phenotype that is often associated with over-active EGF signaling. The results from this initial *Chmp1* knockdown suggest that Chmp1 may be involved in regulation of growth in *Drosophila,* as it seems to be so in zebrafish and mammalian cell culture. More specifically, it seems that Chmp1 may be involved in regulating EGF signaling. As knockdown of *Chmp1* results in phenotypes similar to over-active EGF, Chmp1 may negatively regulate the EGF pathway.

In order to investigate this possibility further, *Chmp1* was knocked down in the wing, while at the same time reducing activity of the positive and negative regulators of the EGF pathway. Some only slightly more complicated crosses were performed to check for Chmp1 involvement in EGF signaling. *MS1096-Gal4/X; Cy/Sco* virgin females were again crossed to *Chmp1IR* males. From that cross, first generation males that were *MS1096-Gal4/Y; Chmp1IR/Sco* were collected and used for six separate crosses. They were crossed to *vevn, kek-1, aos^{Δ7}, aos^{w11}* and *sty^{Δ5}* virgin females[11]. All of these

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[11] In *Drosophila*, fly lines are often named for their mutation. So an Argos fly is deficient in Argos protein, or an Argos mutant. All of the EGF mutants used in this study were heterozygous mutants, with the exception of *vevn*.

mutations are heterozygous, and therefore only reduce the activity of the protein/gene for which they are specific. Three vials of each cross were prepared with one kept at 25°C, one at 28°C, and one at 30°C. When the flies from these crosses were fully developed, the right wing of *MS1096-Gal4; Chmp1IR*; heterozygous EGF mutant female flies was dissected off and mounted dorsally on a microscope slide in GMM.

EGF signaling is very important for the formation of wing veins. When EGF is over-active, the result is over-sized wing veins. On the contrary, when EGF is reduced, wing veins are reduced in size or even missing. Wings lacking both *ve* and *vn* (*rho*) (activators of EGF) have no veins (71), because when the activators of EGF are missing, the only regulators of EGF present are repressors, and thus EGF signaling is significantly repressed. However, *Drosophila* wings heterozygous for *vein* and *rho (ve)* alleles are wild type, suggesting that reduced activity of *vein* and *rho* is sufficient to achieve proper EGF signaling. *Chmp1* knockdown in the wing results in overgrowth wing veins, suggesting that EGF becomes over-active in the absence of *Chmp1* (Figure 10).

So what happens when *Chmp1* is knocked down at the same time as reducing Ve and Vn? When *Chmp1* was knocked down in the wing in heterozygous for alleles of the activators of the EGF pathway, *rho (ve)* and *vein*, the result was a normal sized wing vein (Figure 10C). This is very interesting, as it seems that the *Chmp1IR* phenotype requires EGF activators because without them, the *Chmp1IR* phenotype is extinguished and the wing appears to be wild-type. This suggests that *Chmp1IR* phenotype is dependent upon the EGF pathway and that Chmp1 may be regulating EGF signaling.

Chmp1 was also knocked down in the wing in combination with reduced activity (heterozygous mutants) of each of the negative regulators of EGF (*kek-1*, *sty^{A5}*, and *aos^{A7}*)

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separately (Figure 10 D-F). The result was wing veins that were much larger than those acquired by sole *Chmp1* knockdown. Knocking down *Chmp1* and reducing just one of the EGF negative regulators greatly enhances the *Chmp1IR* phenotype. However, wings that were heterozygous for *kek-1*, *sty$^{\Delta 7}$*, or *aos$^{\Delta 7}$* separately appeared to be wild type. This result also suggests that the *Chmp1IR* phenotype is dependent on the EGF pathway and offers more evidence that Chmp1 is specifically important for proper EGF signaling, and thus wing vein development.
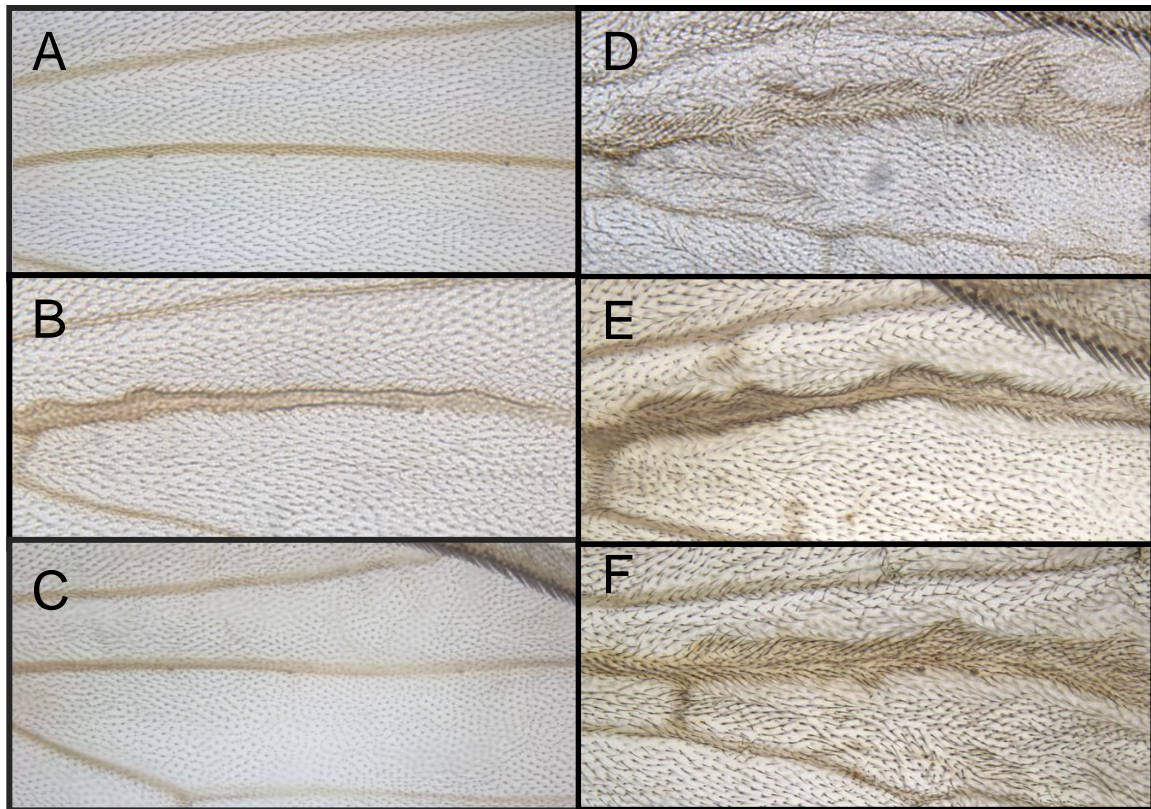
Figure 10. *Chmp1* knockdown in the wing.

All wings were developed at 28°C. **A.** Oregon R : a wild-type wing with normally sized wing veins. **B.** *MS1096-Gal4; UAS-Chmp1IR*: *Chmp1* knockdown in the wing results in wider wing veins. **C.** *MS1096-Gal4; UAS-Chmp1IR; rho^{ve-1}, vn^1/+*: *Chmp1* knockdown, along with reduced activity of EGF positive regulators *rho* and *vein* results in wild-type sized wing veins. **D.** *MS1096-Gal4; UAS-Chmp1IR; argos^{Δ7}/+*: Knockdown of *Chmp1* along with reduced activity *aos*, a negative regulator of EGF, results in veins much wider than those observed with sole *Chmp1* knockdown. **E.** *MS1096-Gal4; UAS-Chmp1IR; sty^{Δ5}/+*: Knockdown of *Chmp1* along with reduced activity of *sty*, a negative regulator of EGF, results in much wider veins. **F.** *MS1096-Gal4; UAS-Chmp1IR; kek1/+*: Knockdown of *Chmp1* along with reduced activity of *kek-1*, a negative regulator of EGF, also results in much wider veins.

RNAi fly lines express hpRNA, specific to a gene of interest, under the control of a Gal4 responsive UAS promoter. The creation of an RNAi line is not a foolproof process, and there are several problems of which to be aware. For example, when the

transgene coding hpRNA is inserted into the genome of the fly, the insertion is completely random. This could be problematic, as insertion within or near a gene could affect or even disrupt that gene's expression. Additionally, when the hpRNA is expressed, it is supposed to cause destruction of the mRNA for which it is specific. However, there is the possibility that the hpRNA does not target the mRNA well, or that it could target a different mRNA in addition to *Chmp1* mRNA. Therefore, it is important to assess whether the phenotypes we observe are actually due to Chmp1 knockdown, rather than a result of the transgene's position within the genome of the fly, or a malfunction of the hpRNA. There is another *Chmp1* RNAi fly line available from Harvard Medical School's Transgenic RNAi Project (TRiP) (72). This *Chmp1* RNAi line is different from the VDRC line in that the hpRNA is on a different chromosome, and it targets a different portion of the *Chmp1* mRNA (Flybase). Therefore, observing similar phenotypes from both the TRiP *Chmp1IR* line and the VDRC *Chmp1IR* fly line would be good evidence that the phenotypes previously obtained were in fact due to *Chmp1* knockdown.

In order to test this, TRiP *Chmp1IR* female virgin flies were crossed to *MS1096-Gal4* males. The adult flies were moved to a vial of fresh food every 2-3 days and the developing first generation was incubated at 25°C, 28°C and 30°C. The right wing of the first generation adult female flies were dissected off and mounted dorsally on a microscope slide in GMM. *Chmp1* was knocked down using the TRiP flies resulted in wing vein overgrowth, a phenotype very similar to that obtained from *Chmp1* knockdown using the VDRC fly line (Figure 11). This offers evidence that the *Chmp1* knockdown

and interaction phenotypes obtained previously were in fact due to *Chmp1* knockdown, rather than off-target effects.

Figure 11. TRiP *Chmp1* knockdown in the wing at 28°C
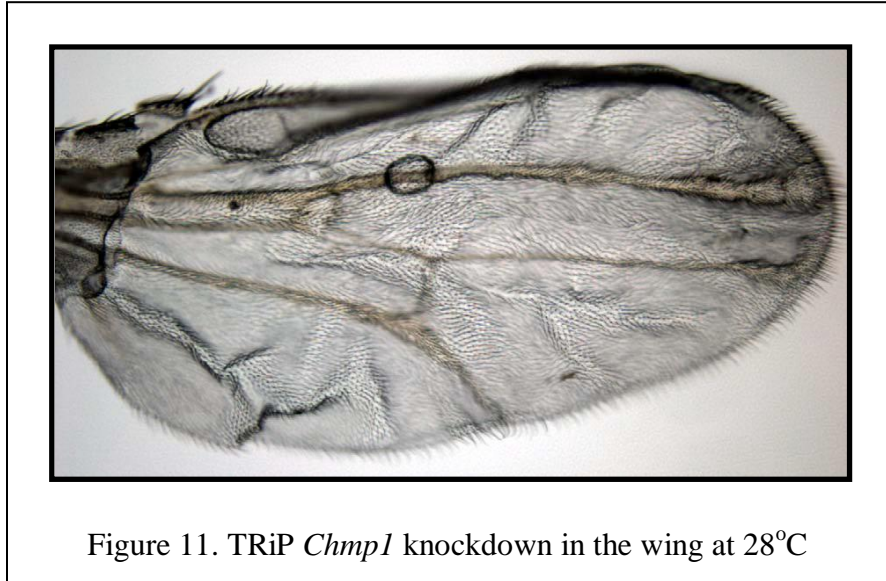
Section 4.3 B: Over-expression of *Chmp1* in the wing

When beginning a study on protein/gene function, the classical place to begin is knockdown and over-expression. We were able to look at knockdown rather easily using an RNAi line. As we have only recently acquired the resources available to study over-expression, *Chmp1* over-expression has only been very briefly investigated.

The transgenic fly lines that were created are designed to work using the UAS-Gal4 system. *Chmp1*, located downstream of a Gal-4 responsive UAS promoter, was inserted into the genome of the fly. Therefore, a simple cross of a *UAS-Chmp1* fly line to a Gal4 driver fly line should be sufficient to achieve *Chmp1* over-expression. As mentioned before, twenty different fly lines were created. Ten of the fly lines express a Chmp1 protein tagged with HM, and the other ten are untagged. It is important to know

the phenotypes of most, if not all lines for several different reasons. Firstly, the *Chmp1* cDNA was inserted randomly into the genome. This means it could have been inserted within or near genes whose mis-expression could lead to false *Chmp1* phenotypes. Also, as one of the *Chmp1* lines is tagged, it is possible that a tag could affect Chmp1 protein activity, thereby resulting in a false phenotype. If *Chmp1* is over-expressed in many of the fly lines and similar phenotypes are obtained from all of them, the phenotype observed is most likely a result of *Chmp1* over-expression. It is likely that the phenotypes will be slightly different, depending on the location of the *Chmp1* transgene insertion. If *Chmp1* was inserted into a highly expressed part of the genome, stronger phenotypes should be obtained. On the other hand, if *Chmp1* was inserted into a weakly expressed portion of the genome, expression will be hindered and weak phenotypes will result.

UAS-Chmp1 male flies were crossed to *MS1096-Gal4* virgin females. The parent generation was transferred to a new vial of fly food every 2-3 days. The developing first generation flies were incubated at 25°C, 28°C and 30°C. When the flies were fully developed, the right wings of males and females were dissected off and mounted dorsally onto a microscope slide in GMM.

To date, nineteen of the twenty lines have been investigated, and all of the phenotypes obtained have been very similar. The last line did not survive and therefore could not be investigated. *Chmp1* over-expression in the wing of the fly results in: 1.) occasional loss of the anterior cross vein (acv) and/or posterior cross vein (pcv); and, 2.) phenotypes indicative of problematic Notch-Delta signaling, specifically, reduction of Delta (M-89). The phenotypes obtained from both tagged (Figure 14) and untagged

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(Figure 13) *UAS-Chmp1* lines are similar, suggesting that the phenotype is real, and that the activity of the epitope-tagged Chmp1 protein is not altered by the HM tag.

The vein phenotypes observed are quite similar to phenotypes observed when the fly wing has decreased activity for Notch signaling ligand, Delta (Figure 12). Since altering *Chmp1* activity produces a phenotype related to faulty Notch signaling, Chmp1 may be involved in regulating the Notch pathway. These results are very recent, and further investigation into the involvement of Chmp1 with the Notch pathway is needed.
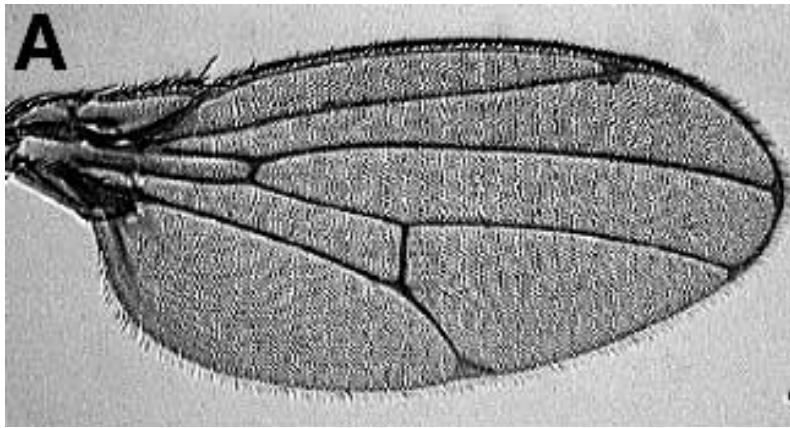
Figure 12. Loss of Delta in *Drosophila* wing

Adult wing phenotype of Dl^{PlacZ}/Dl^{RF} developed at 18°C causes reduction in Delta activity. *Chmp1* over-expression phenotypes resemble reduction of Delta phenotypes (34).
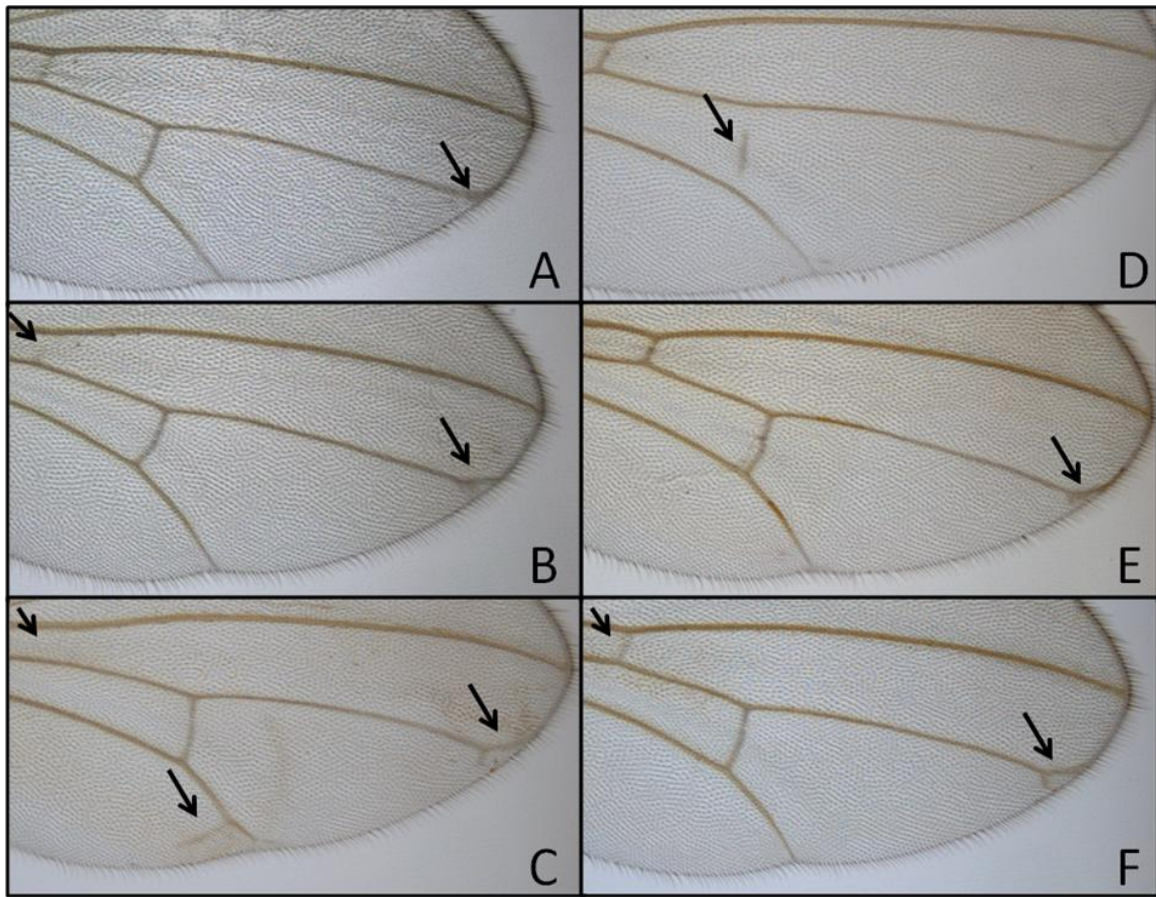
Figure 13. Over-expression of *Chmp1* in the wing.

A. *MS1096-Gal4; UAS-Chmp1 5491-2-2M* male wing developed at 25°C. **B.** *MS1096-Gal4; UAS-Chmp1 5491-2-2M* male wing developed at 28°C. **C.** *MS1096-Gal4; UAS-Chmp1 5491-2-2M* male wing developed at 30°C. **D.** *MS1096-Gal4; UAS-Chmp1 5491-2-4M* male wing developed at 25°C. **E.** *MS1096-Gal4; UAS-Chmp1 5491-2-4M* male wing developed at 28°C. **F.** *MS1096-Gal4; UAS-Chmp1 5491-2-4M* male wing developed at 30°C.

Figure 14. Over-expression of tagged *Chmp1* in the wing.

A. *MS1096-Gal4; UAS-Chmp1 5491-1-6M* male wing developed at 25°C. **B.** *MS1096-Gal4; UAS-Chmp1 5491-1-6M* male wing developed at 28°C. **C.** *MS1096-Gal4; UAS-Chmp1 5491-1-6M* male wing developed at 30°C. **D.** *MS1096-Gal4; UAS-Chmp1 5491-1-3M* male wing developed at 25°C. **E.** *MS1096-Gal4; UAS-Chmp1 5491-1-3M* male wing developed at 28°C. **F.** *MS1096-Gal4; UAS-Chmp1 5491-1-3M* male wing developed at 30°C.
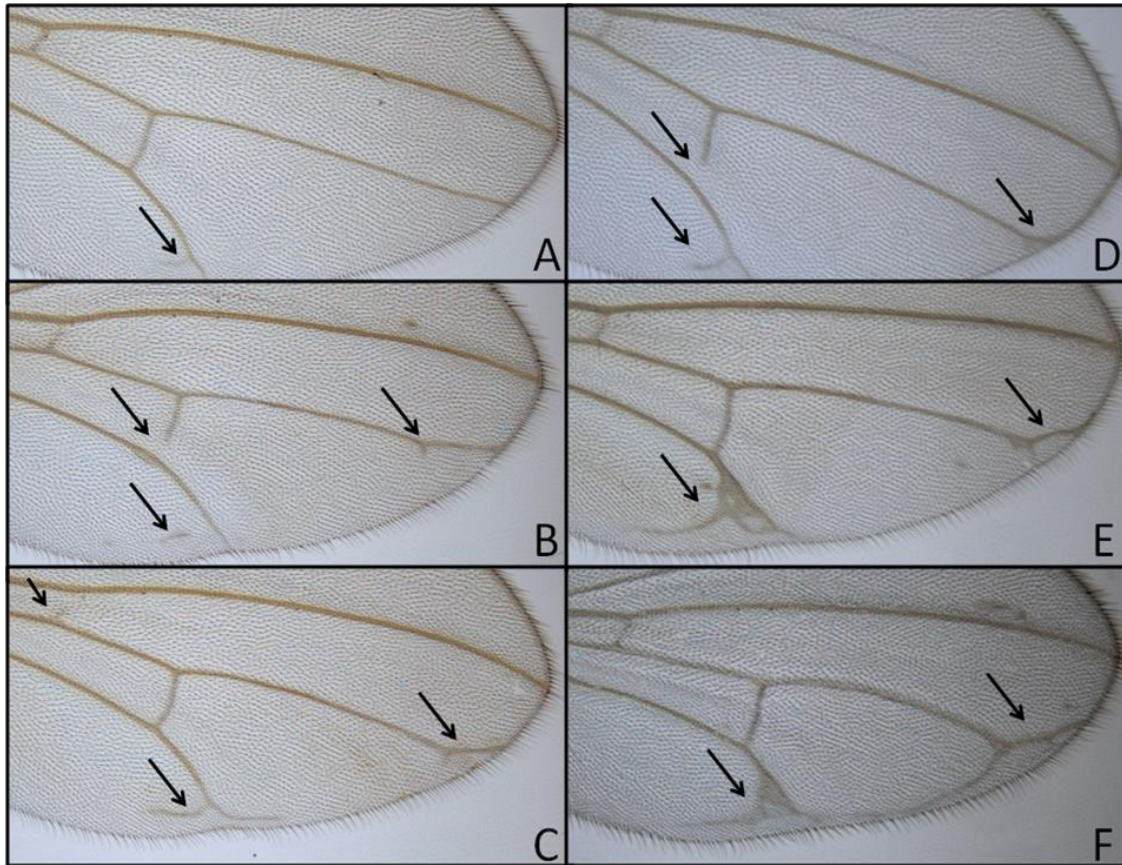
Section 5: Discussion

To date, studies with Chmp1 and its homologues show that mis-expression of *Chmp1* causes overgrowth (22, 33). *Chmp1* knockdown in the *Drosophila* wing results in oversized wing veins. This result suggests that Chmp1 is involved in regulation of growth in *Drosophila.* This is consistent with previous research and suggests that *Drosophila* is a good model for studying Chmp1 function. The establishment of wing veins in the *Drosophila* wing is dependent upon EGF and Notch-Delta signaling. Since mis-expression of *Chmp1* results in vein phenotypes, Chmp1 may be regulating these pathways.

As these pathways are quite dependent upon each other in the formation of wing veins, it is possible that Chmp1 is involved in the regulation of only one of the pathways. *Chmp1* knockdown phenotypes suggest that Chmp1 is regulating EGF signaling, while over-expression phenotypes suggest that it is regulating Notch-Delta signaling. It is not clear yet exactly how Chmp1 is acting on these two pathways. One possibility is a simple matter of ESCRT function. It is probable that *Chmp1* over-expression and knockdown would have an effect on ESCRT, as it is a functioning member of the protein complex. Significantly, both the EGF and Notch pathways are reported in the literature to be regulated by ESCRT machinery. Studies with EGF signaling and ESCRT have shown that deletion of ESCRT-III component Vps24 (also known as Chmp3) results in persistent EGF signaling (73). This information is consistent with our results that a defect in ESCRT-III component Chmp1 results in over-active EGF signaling as well. In the Notch-Delta pathway, Notch is continuously being internalized and either recycled or degraded. This seems dependent upon ESCRT, as mutations in ESCRT significantly

affect Notch activity (74, 75). Studies have shown that tumor suppressor and ESCRT-II component Vps25, regulates Notch activity. When Vps25 is deleted, Notch is improperly degraded, which leads to over-proliferation (17). The activity of Vps25 may be similar to Chmp1, as its absence leads to loss of growth control, possibly through the regulation of Notch or EGF. Additionally, it seems that in order for Delta to be active it is monoubiquitinated by ubiquitin ligases, which have been shown to physically interact with Delta and promote ubiquitination and internalization (76, 77, 78). Although the ESCRT machinery usually mediates degradation and recycling of monoubiquitinated transmembrane receptors, it seems that in this case, it works on a transmembrane ligand (75). This could be consistent with our over-expression results, if heightened Chmp1 activity lead to increased ESCRT III activity and therefore increased Delta degradation, the result may be a phenotype similar to reduced Delta activity. It has already been discussed in the literature that ESCRT machinery plays a very important role in the recycling and degradation of activated receptor proteins. The implications of this regulation are very important. Without proper ESCRT, cell signaling can be thoroughly disrupted, and may lead to considerable problems in the cell such as over-proliferation.

Chmp1 has been linked to pancreatic cancer in humans. Pancreatic tumors have lowered Chmp1expression compared to normal pancreatic cells (22). Another characteristic of many pancreatic tumors, which may be a result of Ras mutations, is an over-active Epidermal Growth Factor (EGF) pathway (24). Both the EGF receptor and its ligands have increased expression and activity in pancreatic tumors (79). Our results suggest that Chmp1 negatively regulates EGF signaling, which would be consistent with these previous findings, as lowered Chmp1 expression would enhance EGF signaling.

Although cancers, pancreatic cancer included, usually have a whole hoard of problems, there may be a very important link between EGF signaling and Chmp1 expression.

Section 6: Future Studies

We are pretty sure the phenotypes obtained are due to *Chmp1* knockdown, as two different RNAi lines exhibit similar results. It would be nice to quantify the level of *Chmp1* knockdown, which may be easy to do with a western blot and a good antibody.

The *UAS-Chmp1* lines need to be balanced. In doing this, we will find out into which chromosome the *Chmp1* transgene was inserted. Then, to help characterize the function of Chmp1, we can obtain more *Chmp1* over-expression phenotypes in the *Drosophila* wing. We will also want to over-express *Chmp1* while knocking down or over-expressing EGF pathway components and possibly Notch pathway components. Additionally, at some point we need to check that *Chmp1* is in fact being over-expressed, and possibly quantify the level of over-expression. This may be easy to do with a western blot and a good antibody, or mRNA assays.

The EGF pathway is quite active in the *Drosophila* eye. Over-expression and knockdown of *Chmp1* in the eye will be performed, to investigate whether Chmp1 functions in the same pathways in the wing as the eye. This will require uncomplicated crosses. We have recently obtained a protocol for eye fixation and sectioning that will allow for visualization of ommatidial cells. SEM images of the full eye may be useful as well.

As the pUASHM vector has tagged Chmp1 with HM, we should now be able visualize the localization of Chmp1 protein within the cells. Wing disc staining and

imaging using confocal microscopy could be useful and informative. The salivary glands of the *Drosophila* third instar larvae have polytene chromosomes, which can be easily stained and visualized under a light microscope. As previous studies have shown Chmp1 to localize with condensed chromatin, we can use these Chmp1 HM-tagged lines investigate whether the same is true in *Drosophila.*

Bridge to Chapter 2

We have shown through *Chmp1* knockdown and over-expression that Chmp1 may regulate the Notch-Delta and EGF signaling pathways. The Notch-Delta and EGF pathways are fairly dependent on each other, and actively work together to promote proper wing vein formation in the *Drosophila* wing. However, both *Chmp1* knockdown and over-expression results in another phenotype that suggests that Chmp1 regulates a different and seemingly separate pathway as well, which is known as the Frizzled Planar Cell Polarity pathway. The Frizzled Planar Cell Polarity pathway is responsible for establishing proper planar cell polarity (PCP) in the *Drosophila* cuticle. We explored the effect of Chmp1 on PCP, and found that Chmp1 may regulate this pathway through a PCP protein called Strabismus.

Chapter 2 – Chmp1 and Planar Cell Polarity

Section 1: Introduction

Cell polarity is caused by an asymmetrical distribution of molecules in a cell. The establishment of cell polarity in organisms is extremely important for cell diversity and tissue specialization. During development almost all cells become polarized in some way, and in many cases, the polarity of the cells must be correctly coordinated with the polarity of the tissue (80, 81). One quite common example of this is planar cell polarity (PCP), in which epithelial cells become polarized in a plane of epithelium not only on the apical-basal axis, but also within the plane of cells (82). This cell polarity is important for the proper function of many tissues, from the sensory hair cells in the vertebrate inner ear to hair and feather arrangement in animals (82). PCP has been studied extensively in the *Drosophila* cuticle and many polarity proteins have been identified that are required for the process (83). One of the key components for the establishment of cell polarity is a conserved pathway called the Frizzled Planar Cell Polarity pathway (Figure 15) (84).

Some of the significant members of this pathway include Frizzled (Fz), Disheveled (Dsh), Prickle (Pk), Van Gogh (Vang, also known as Strabismus [Stbm]), Diego (Dgo), and Starry night (Stan, also known as Flamingo [Fmi]). Fz is a seven-pass transmembrane receptor which localizes at the distal end of the developing wing cell (85, 86), Dsh is a cytoplasmic protein and colocalizes with Fz (87, 88), Vang/Stbm is a four pass transmembrane protein and is found at the proximal end of the developing wing cell (89, 90), Stan/Fmi is a seven-pass membrane protein with cadherin domains and localizes both proximally and distally (88, 91, 92), Pk is a cytoplasmic protein which accumulates

at the proximal edge (94, 95), and Dgo is a cytoplasmic protein which accumulates at the

distal and proximal edges (95). The asymmetric distribution of these proteins is important

for intracellular and extracellular signaling, and proper PCP establishment. Failure to

appropriately localize of all six of these proteins results in a disruption of PCP.
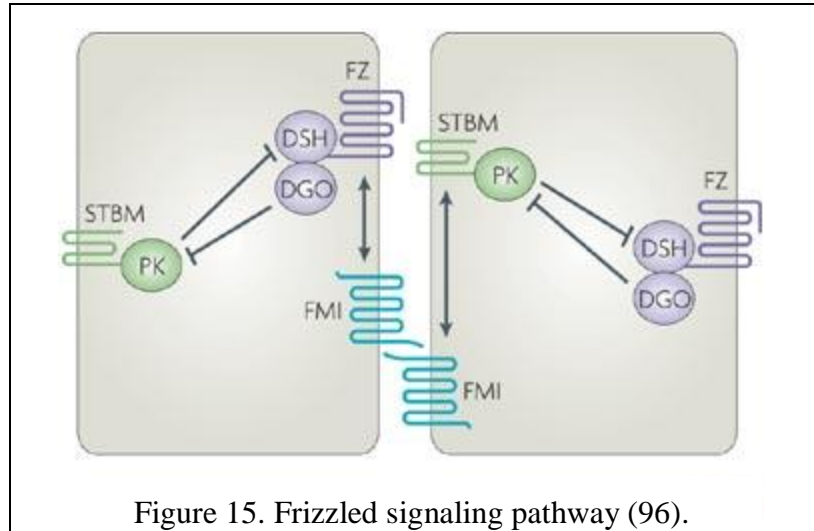

Figure 15. Frizzled signaling pathway (96).

In *Drosophila melanogaster,* PCP is required for proper organization of cuticular

structures in the adult organism, but has been best characterized in the wing, sensory

bristles and eye. In the wing, it is required for correct orientation and number of wing

hairs produced by the wing cells and mutations in any one of the PCP proteins disrupt the

wing hair polarity. Depending on the mutated protein, a wing hair may be produced in a

different area of the cell, pointing a different direction, or multiple hairs may be produced

per cell (83).

Although the Fz PCP pathway was initially characterized in epithelial structures

in *Drosophila*, it seems to be a conserved pathway in vertebrates and is required for many

diverse processes. Vertebrate PCP was first discovered to be required for convergent

extension movements in during neurulation in Xenopus and zebrafish embryos (97, 98, 99). There is now evidence that it is also involved in the process of neural tube closure (99, 100), cardiovascular development (101) and establishing the precisely aligned orientations of sensory hair cells in vertebrate ears (100, 102).

Strabismus is a transmembrane protein that has been shown to physically interact with Fz-PCP pathway proteins Pk, Dgo, and Dsh (90, 103, 104). Recent studies in zebrafish show that the PCP protein, Stbm, can physically interact with Chmp1A in a yeast two-hybrid screen as well as in a co-immunoprecipitation assay (33). A study done by Dr. Maiyon Park at Marshall University School of Medicine showed that loss of Stbm activity in zebrafish embryos causes faulty convergent extension[12]. The resulting embryo has a short and wide body, opposed to the normal long and narrow body. The same study found that loss of Chmp1A activity during zebrafish embryogenesis results in a convergent extension phenotype very similar to loss of Stbm, suggesting a physical Chmp1A-Stbm interaction that may regulate cell movement.

Section 2. Objective and Hypothesis

Section 2.1: Objective

There were two objectives of this study. The first objective was to determine if Chmp1 regulates planar cell polarity (PCP) in *Drosophila* by observing the effect of knockdown and over-expression. The second objective was to determine if Chmp1 interacts with PCP protein, Strabismus, in *Drosophila*.

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[12] Convergent extension is a process during embryogenesis where cells come together (converge) and lengthen (extend) the body. It only involves the movement of cells, not change in cell shape or cell division.

Section 2.2: Hypothesis

We hypothesize that Chmp1 in *Drosophila* regulates PCP through an interaction with PCP protein, Strabismus, like it does in zebrafish.

Section 3: Materials and Methods

Table 5. Genotypes of fly lines used

Name	Genotype
Oregon R (wild-type)	Oregon R-C
Chmp1IR/Cy	w^{1118}; *P{GD11219}v21788/CyO*
MS1096-Gal4	w^{1118} *P{$w^{+mW.hs}$=GawB}Bx^{MS1096}*
Vang^{TBS42}	*b pr cn TBS42/CyO*
en-gal4	*P {en2.4-GAL4}e16E*

Section 4: Results

As previous studies in zebrafish have shown a possible Chmp1-Stbm interaction, we wanted to see if the same was true for *Drosophila*. As we already have fly lines heterozygously mutant for Stbm, and the *Chmp1IR* line, this was rather simple.

In order to test for a Chmp1-Stbm interaction, about 10 *Chmp1IR/Cy* male flies were crossed to 10 *MS1096-Gal4; Cy/Sco* female virgins. The parent generation was moved to a new vial containing fresh food every 2-3 days. From that cross, 10 first generation virgin females that were *MS1096-Gal4; Chmp1IR/ Sco* were then crossed to 10 *Vang^{TBS42}/Cy* males. The parent flies for this cross were moved to a new vial containing fresh food every 2-3 days. The flies obtained from this cross were cultured during their development at 25°, 28° and 30°. The flies that were used were those

cultured at 28°C, as they produced the best phenotypes. Culturing at 25°C produces a rather weak phenotype, while culturing at 30°C, which is the optimal temperature for the UAS-Gal4 system, produces very strong *Chmp1* phenotypes, resulting in wings in which vein and intervein tissues are indistinguishable and rendering PCP phenotypic studies rather useless. The wings on the right side of the flies were dissected off and mounted dorsally on a microscope slide in GMM.

Flies heterozygous for *stbm* (*Vang*^{TBS42}) have a weak dominant phenotype in the proximal part of the wing, but mostly exhibit a wild-type wing phenotype (Figure 16A). There was no vein overgrowth, and hardly any PCP phenotype was observed. This indicates that low Stbm activity is sufficient for proper PCP. Ubiquitous *Chmp1* knockdown in the wing results in wing vein overgrowth (Figure 16B), but no PCP phenotypes. This result alone would suggest that PCP is unaffected by *Chmp1*. However, knocking down *Chmp1* in wings heterozygous for *stbm* results in overgrown wing veins as well as a PCP phenotype, which includes multiple hairs produced per cell and a change in hair polarity (Figure 16C). This result does suggest that Chmp1 and Stbm are somehow interacting, as PCP is only disrupted when both of the proteins are less active, and PCP is executed properly when Chmp1 and Stbm levels are normal. The PCP phenotype observed here is similar to phenotypes observed in wings homozygously mutant for *stbm*, as well as other PCP mutant wings, including Fz (105). Interestingly, the most noticeable PCP phenotype in these wings was seen in the wing hairs surrounding the wing veins. Usually, wing hairs point toward high Stbm activity (Figure 16C), suggesting that the wing veins have lower Stbm than the intervein tissue, but also that reduced activity of Stbm is associated with reduced activity of Chmp1.

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To test that these results were consequential of and specific to a Chmp1-Stbm interaction, *Chmp1* was also knocked down with other members of the PCP pathway. When *Chmp1* is knocked down along with reduced activity of Fz (*MS1096-Gal4*; *UAS-Chmp1IR; fz^{P21}/+*) or Pk (*MS1096-Gal4*; *UAS-Chmp1IR/ pk^{pk-sple14}*), no PCP phenotypes are observed. This suggests that the PCP phenotype observed is indeed due to a Chmp1-Stbm interaction, rather than a Chmp1 interaction with other PCP proteins.

We also looked at *Chmp1* knockdown alone in the wing, both ubiquitously and in the posterior half of the wing. To look at ubiquitous *Chmp1* knockdown, *Chmp1IR/Cy* females were crossed to *MS1096-Gal4*; *Cy/Sco* males. In order to look at *Chmp1* knockdown in the posterior half of the wing, *Chmp1IR/Cy* virgin females were crossed to *en-Gal4* males. The parent generation of each cross was moved to a new vial with fresh fly food every two to three days, and the first generation was cultured at 25°C, 28°C, and 30°C. The wings used for this study were those developed at 28°C. The wings were dissected off of the flies and mounted dorsally on a glass slide in GMM. As we saw previously, when *Chmp1* was knocked down ubiquitously in the dorsal wing, the result was vein overgrowth and no PCP phenotypes were observed (Figure 16B, D). However, when *Chmp1* is knocked down in only the posterior half of the wing, we do see PCP phenotypes, such as doubled hairs and a change in hair polarity, at the boundary of *Chmp1* knockdown and normal levels *Chmp1* expression (Figure 16E). This suggests that in order for Chmp1 alone to have an effect on PCP, there must be a gradient of Chmp1 activity.
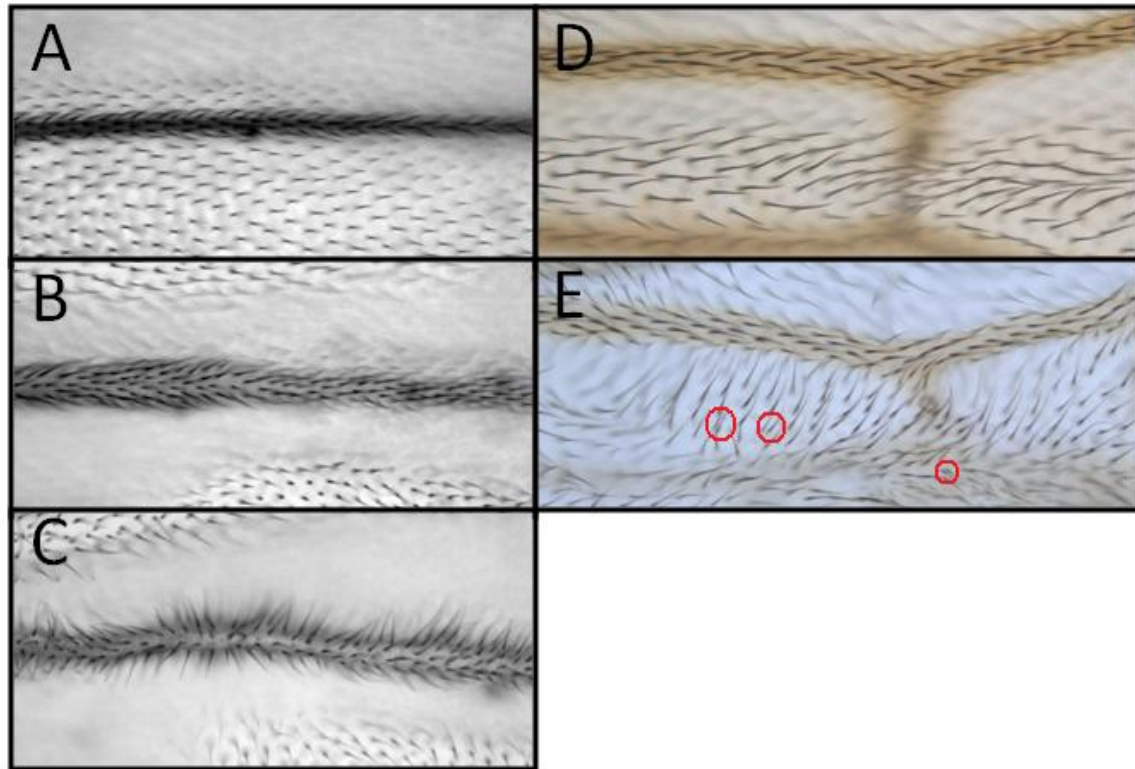
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Figure 16. Chmp1 and Stbm.

A. A wing heterozygous for Stbm (*stbm^VangTBS42^*) has an apparent wild-type wing **B.** Chmp1 knockdown (*MS1096-Gal4; UAS-ChmpIR*) results in overgrown wing veins **C.** A wing heterozygous for Stbm, as well as having *Chmp1* knockdown (*MS1096-Gal4; UAS-Chmp1IR/stbm^VangTBS42^*) results in overgrown wing veins, but also a PCP phenotype as hair polarity is abnormal **D.** Wild-type wing (*Oregon R*) **E.** Knockdown of *Chmp1* in the posterior half of the wing (*en-Gal4; UAS-Chmp1IR*) results in overgrown wing veins in the posterior half of the wing, but also PCP phenotypes such as doubled hairs (circled in red) and abnormal hair polarity at the boundary of *Chmp1* knockdown.

Unexpectedly, PCP effects were also observed in *Chmp1* over-expression wings. The over-expression lines that were created, when crossed to *MS1096-Gal4*, should over-express *Chmp1* ubiquitously in the wing. Nine out of the ten untagged over-expression lines resulted in phenotypes indicative of faulty PCP (Figure 17). The PCP effects are not as severe as those observed in regional *Chmp1* knockdown.
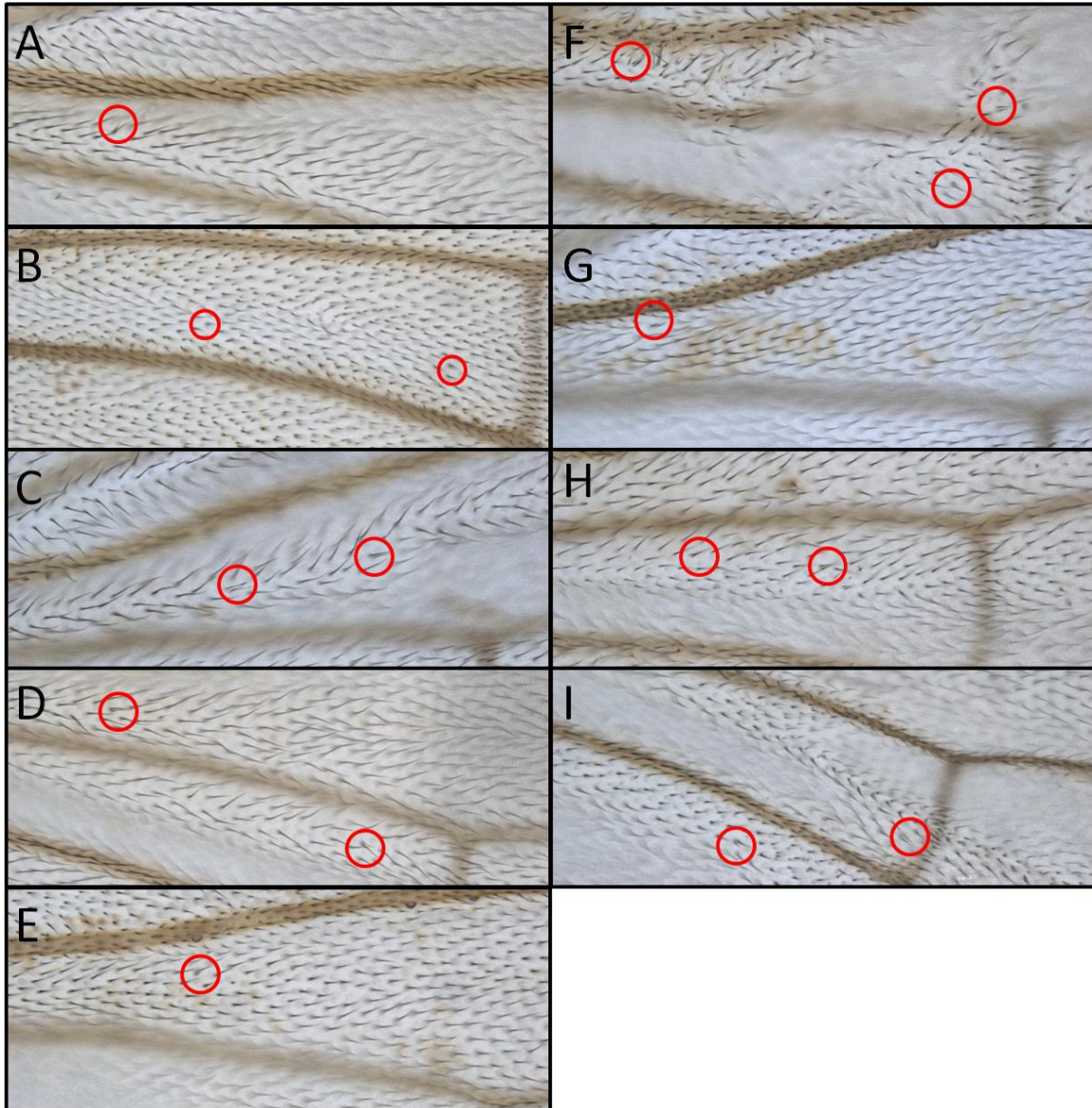
Figure 17. Untagged *Chmp1* over-expression in the *Drosophila* wing resulted in PCP effects.

Doubled hairs are indicated in the red circles. All wings were from male adults developed at 28°C. All images were taken between the L3 and L5 veins, near the PCV and ACV (usually missing in these wings). **A.)** *MS1096-Gal4; UAS-Chmp1 5491-2-1M* **B.)** *MS1096-Gal4; UAS-Chmp1 5491-2-2M* **C.)** *MS1096-Gal4; UAS-Chmp1 5491-2-3M* **D.)** *MS1096-Gal4; UAS-Chmp1 5491-2-4M* **E.)** *MS1096-Gal4; UAS-Chmp1 5491-2-5M* **F.)** *MS1096-Gal4; UAS-Chmp1 5491-2-6M* **G.)** *MS1096-Gal4; UAS-Chmp1 5491-2-7M* **H.)** *MS1096-Gal4; UAS-Chmp1 5491-2-8M* **I.)** *MS1096-Gal4; UAS-Chmp1 5491-2-9M*

Section 5: Discussion

The results of this study suggest that there is indeed an interaction between Chmp1 and Stbm in *Drosophila,* and that this interaction is important for the establishment of proper PCP. When Chmp1 and Stbm interact in the correct fashion, proper PCP is established. However, when the interaction between Chmp1 and Stbm is disrupted, PCP is disrupted as well. It is unknown how these proteins are interacting, though from previous studies, it seems that the interaction is not permanent, but is short lived (33).

Studies in zebrafish suggest that Chmp1 regulates PCP through an interaction with Stbm (33). Phenotypes obtained from *Chmp1* knockdown in zebrafish resembled *stbm* mutants, and the same seems true for *Drosophila*. It is very nice that we see the same result in *Drosophila* as was observed in zebrafish. This suggests that Chmp1 function is conserved between zebrafish and *Drosophila*, and it is possible that is conserved between other organisms as well.

Stbm is a transmembrane protein. It has recently been described as a transmembrane receptor for Fz extracellular domain (106). It has not been shown that Stbm is regulated by ESCRT machinery, nor have any results been published regarding regulation of Stbm by ubiquitination. Nevertheless, its receptor capabilities make it a potential ESCRT target. Other than being a member of ESCRT, Chmp1 has also been implicated in gene silencing. Chmp1 is normally associated with condensed chromatin and recruitment of gene silencing proteins, and it is possible that this action of Chmp1 is important in the regulation of Stbm, or genes that may regulate Stbm.

An interesting result was the way Chmp1 seems to affect hair polarity. When Chmp1 was knocked down in a background of Stbm, the strongest effect on hair polarity was seen at the wing veins. Specifically, hairs pointed away from the wing vein. Typically in the wing, hairs point toward high Stbm activity. This would suggest that there is lower Stbm in the wing vein. However, the driver (*MS1096-Gal4*) that we used should drive *Chmp1-IR* ubiquitously in the wing. If Chmp1 does regulate PCP through Stbm, it is strange that ubiquitous *Chmp1* knockdown would result in localized reduction of Stbm. One possible explanation for this is that wing vein tissue and intervein tissue have different requirements for Chmp1, and therefore each tissue is differentially affected by *Chmp1* knockdown.

Chmp1 has been linked to pancreatic cancer. Previous studies, as well as our studies, suggest that Chmp1 is involved in the regulation of growth. In *Drosophila*, *Chmp1* knockdown and over-expression result in what seem to be two differential phenotypes: 1) misregulation of growth and 2) planar cell polarity effects. These phenotypes may not be as far separated as we originally thought. There are some recent reports linking PCP to cancer. One study found that in loss of VANGL2, human homologue of Strabismus, promotes migration and invasion in human cancer cells (108). Additionally, aberrant activation of the PCP signaling pathway in human cancer cells can lead to more malignant phenotypes (109). If PCP is regulated by Chmp1, it is possible that Chmp1 misregulation could lead to cancer-related phenotypes such as over-proliferation or migration, as well as PCP phenotypes, resultant of faulty Fz-PCP signaling.

Section 6: Future Studies

Although in it was found that Chmp1 and Stbm interact in zebrafish, it might be a good idea to do an assay to make sure that the same is true in *Drosophila*. Our results do suggest the Chmp1-Stbm interaction, but we still want to be positive. This could be done using a yeast two-hybrid assay. Another method, and maybe a more informative one, would be a co-immunoprecipitation.

Now that we have the *Chmp1* over-expression lines available we can look at *Chmp1* over-expression along with reduced activity of Stbm. This simple cross could provide more information as to how Chmp1 may be involved with PCP. Additionally, one of our over-expression lines has a tagged Chmp1 protein. With some good antibodies, we could look at Chmp1 localization, along with Stbm localization, for further support of a Chmp1-Stbm interaction.

We looked at *Chmp1* knockdown along with reduced activity of Fz and Pk, but it would probably be a good idea to look at *Chmp1* knockdown along with reduced activity of Fmi, since it is a transmembrane protein.

It may be interesting to look at Chmp1 and Stbm in the eye of the fly. This would be very simple, as we already have some fly lines that drive Gal4 in the eye. The eye is a very regular structure, and the establishment of cell polarity by PCP is extremely important. This and the regular structure of the eye make the eye good place to study PCP in the fly, as small problems become very apparent.

It would be very nice find out whether Chmp1 regulates Stbm by the ESCRT machinery. We could try to find out whether Stbm is ubiquitinated at the membrane. If it is, that small bit of evidence would be very suggestive of ESCRT regulation.

Final Discussion

Our results suggest that Chmp1 regulates three different pathways in the *Drosophila* wing: the Epidermal Growth Factor Pathway, Notch-Delta signaling, and the Frizzled Planar Cell Polarity pathway. Our studies of *Chmp1* knockdown and EGF regulators in the wing indicate that *Chmp1* negatively regulates the EGF pathway during wing vein development in *Drosophila*. When we over-express *Chmp1* in the wing, we see what appears to be faulty Notch-Delta signaling, which is apparent by the "delta" wing vein phenotype. Both the EGF pathway and Notch-Delta signaling are very important for wing vein development and actively interact to promote proper wing vein size. At this point, it is unclear whether Chmp1 regulates one or both of these signaling pathways. It is also unclear at what level Chmp1 regulation comes into play. Chmp1 has two major functions in the cell: it mediates the degradation of activated receptor proteins through ESCRT, but it also is involved in gene silencing in the nucleus. It is possible that regulation is at the level of transcription. However, it is most likely that Chmp1 regulates these pathways through its ESCRT function. The EGF receptor, the Notch receptor, and the Delta ligand are all probably regulated by ESCRT machinery. Thus, if mis-regulation of Chmp1 affects ESCRT function, these signaling pathways would be affected.

Our studies also suggest that Chmp1 regulates Fz-PCP signaling by an interaction with PCP protein, Strabismus. Strabismus is a transmembrane receptor protein, and therefore it is possible that it is regulated by ESCRT machinery as well. Our studies so far make it seem as though Chmp1 regulation of EGF/Notch-Delta signaling and PCP is separate, since *Chmp1* knockdown and over-expression seemed to result in two different phenotypes. However, it would be interesting if there was more of a connection than

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between these pathways than is traditionally thought. In the *Drosophila* wing, wing veins are formed by the EGF pathway. When EGF activators are absent, no wing vein forms, which suggests that EGF signaling is absolutely necessary for wing vein formation. Interestingly, hair polarity in the wing, which is directed by the Fz-PCP pathway, is often affected near the wing veins. A possibility for this wing vein effect on hair polarity is that EGF signaling affects PCP signaling. When we knocked down *Chmp1* while reducing Stbm activity, the strongest phenotype was observed at the wing vein, where the hair polarity was severely altered. This was an unexpected and rather confusing result. It is possible that the change in hair polarity is a result of an interaction of Fz-PCP and EGF signaling. There have been some reports of the need for cooperative EGF and Fz-PCP signaling to establish cell fate and planar cell polarity (110). It is possible that these pathways are interconnected and that each can influence the activity of the other. Ultimately, there is still much that is unclear, and further investigation is needed to determine exactly how Chmp1 may regulate these pathways.
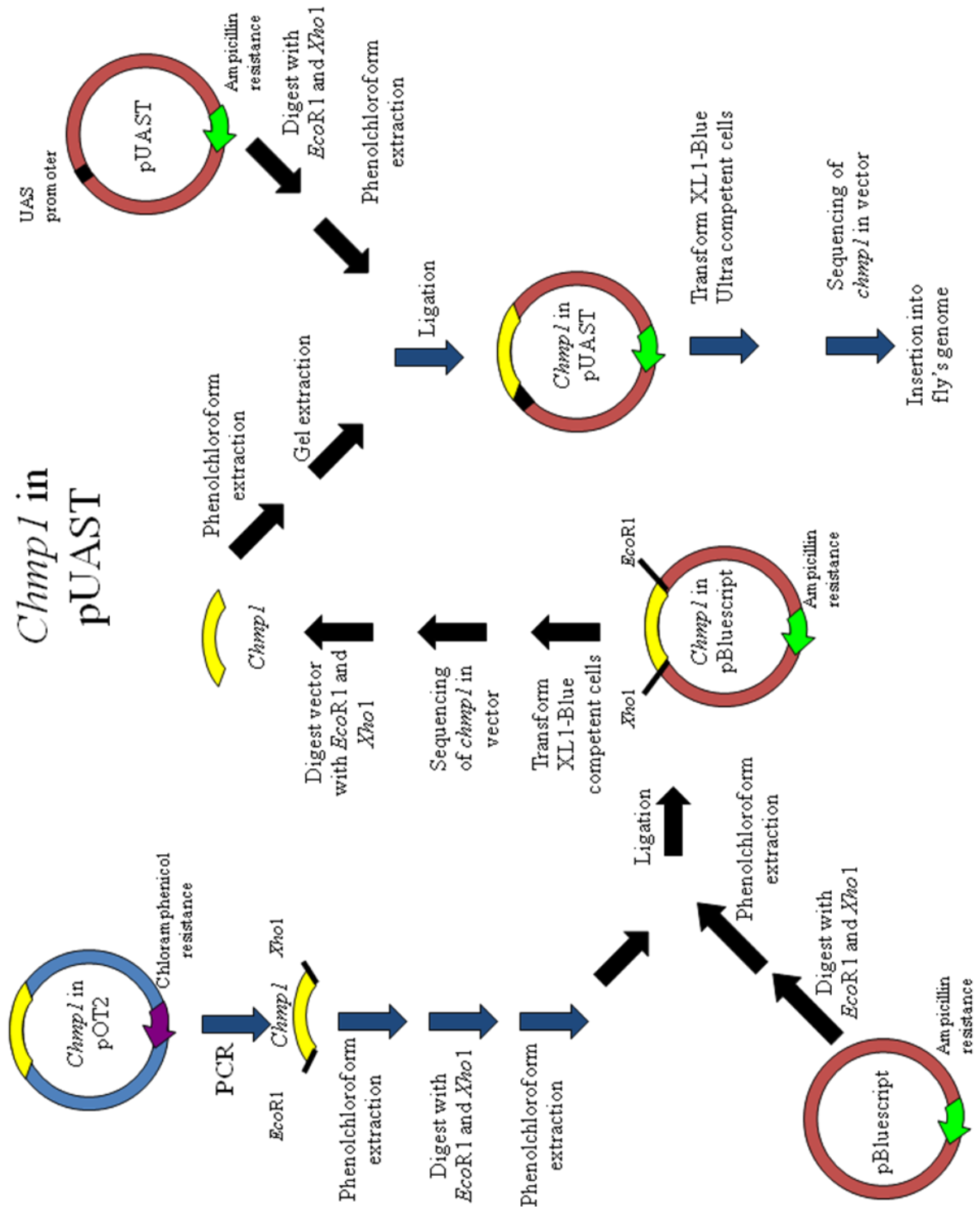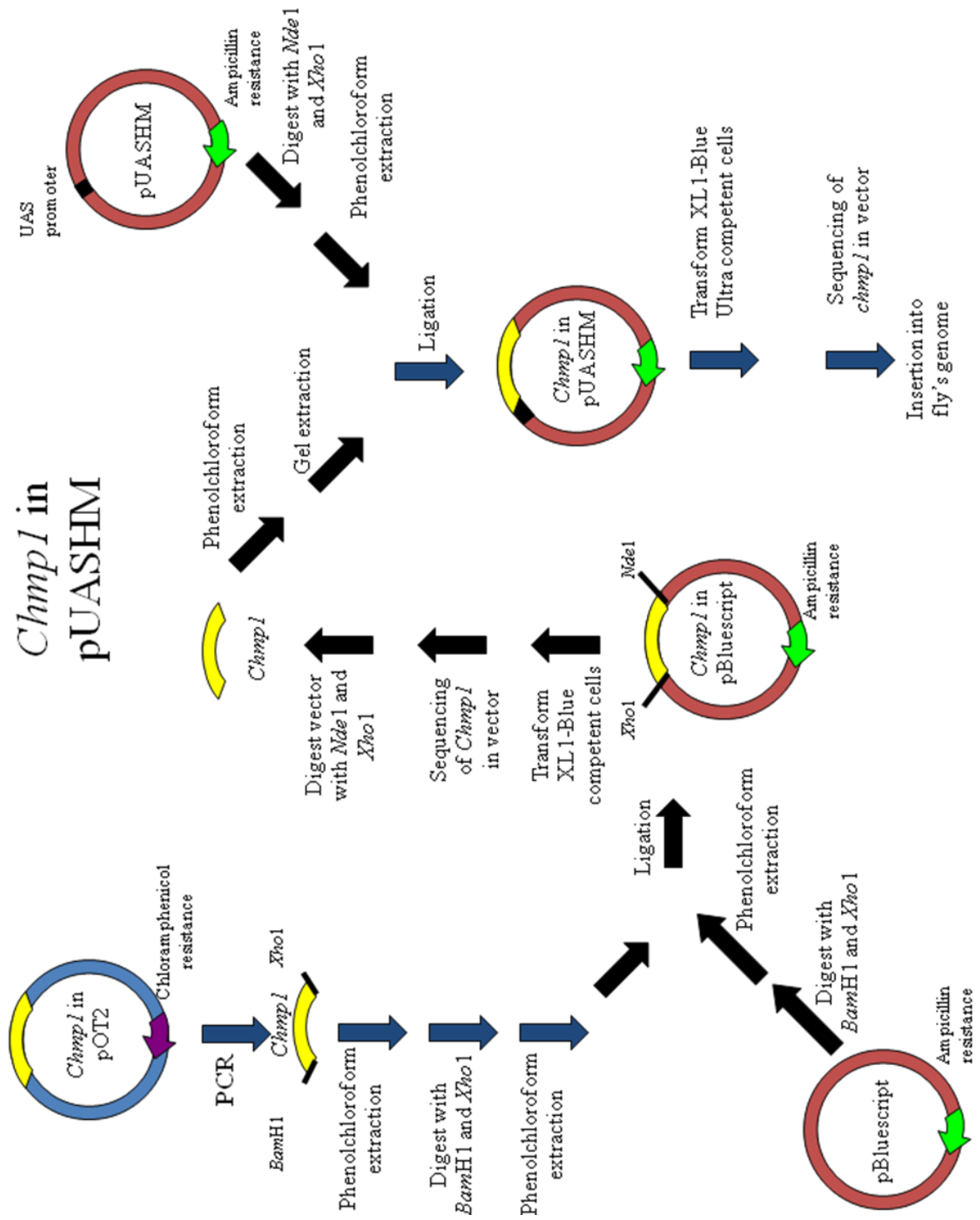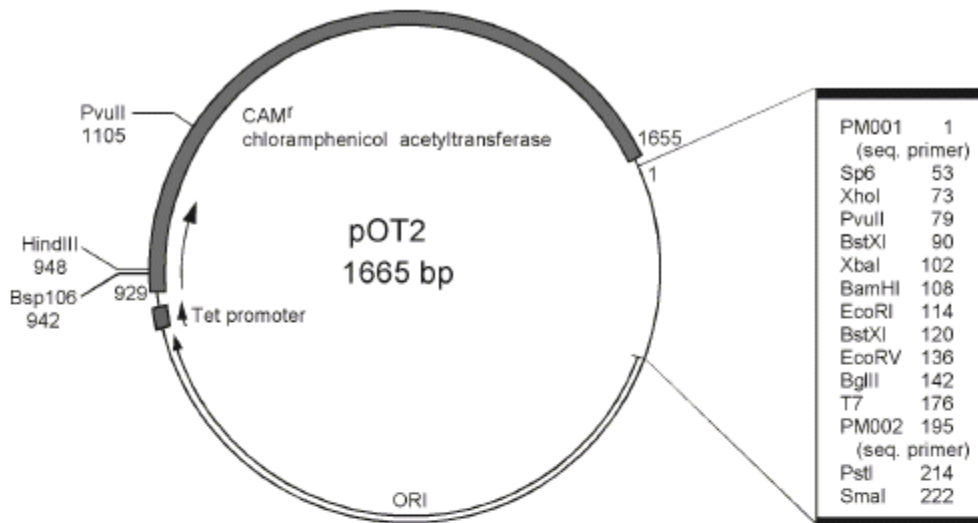
Figure 18. Inserting *Chmp1* into pUAST

Figure 19. Inserting *Chmp1* into pUASHM

Figure 20. pOT2 vector

Source: Addgene

Figure 21. pUAST vector
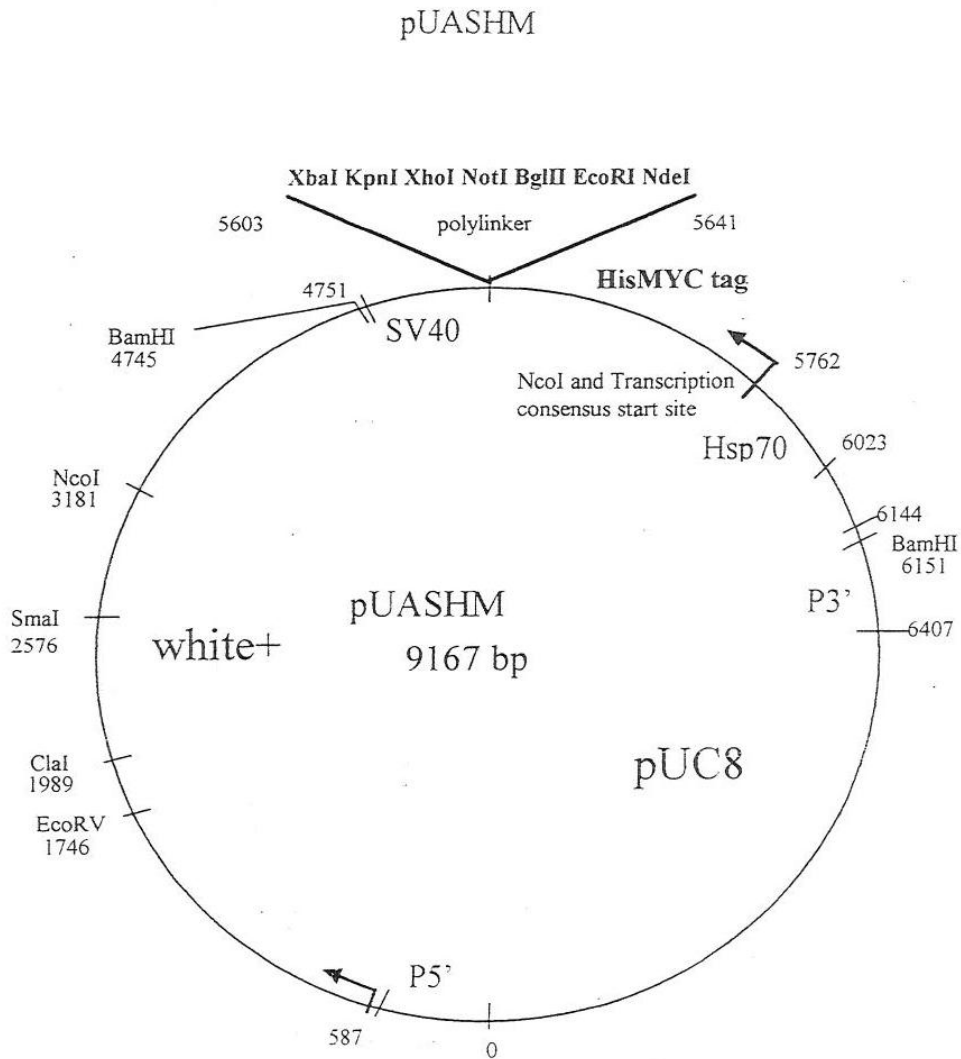
pUASHM

pUASHM was produced by modification of pUAST to allow expression of N-terminal HM-tagged proteins *in vivo*. A consensus start sequence was inserted, followed by the HM tag

In addition, an NdeI site was added to the polylinker. Subcloning of proteins should use the NdeI or EcoRI sites in the polylinker to minimise the amount of polylinker translated as protein.

Figure 22. pUASHM vector

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pBluescript II SK (+/–) Phagemids

f1 (-) ori

f1 (+) ori

ampicillin

lacZ'

Kpn I

MCS

Sac I

P lac

pBluescript II SK (+/-)
3.0 kb

pUC ori
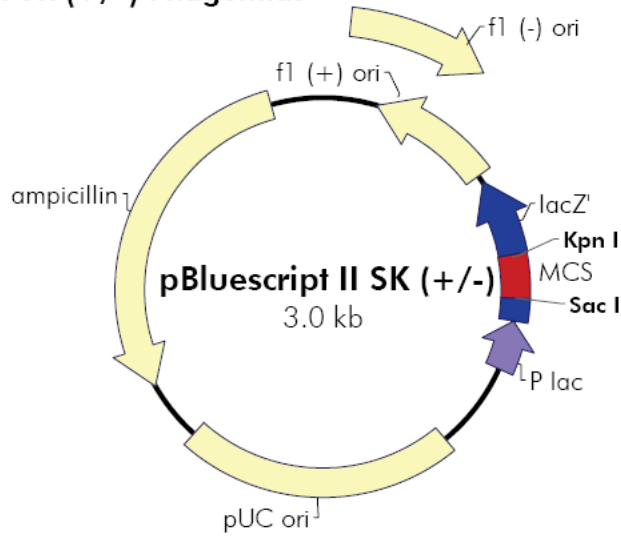
pBluescript II SK (+/–) Multiple Cloning Site Region
(sequence shown 598–826)

BssH II   **T7 Promoter**   Kpn I   Apa I / EcoO109 I / Dra II   Xho I   Hinc II / Acc I / Sal I

TTGTAAAACGACGGCCAGTGAGCGCGCGTAATACGACTCACTATAGGGCGAATTGGGTACCGGGCCCCCCCTCGAGGTCGAC...

M13 –20 primer binding site   T7 primer binding site   KS primer binding site...

Bsp106 I / Cla I   Hind III   EcoR V   EcoR I   Pst I   Sma I   BamH I   Spe I   Xba I   Not I / Eag I   BstX I   Sac II   Sac I

...GGTATCGATAAGCTTGATATCGAATTCCTGCAGCCCGGGGGATCCACTAGTTCTAGAGCGGCCGCCACCGCGGTGGAGCTC...

...KS primer binding site   SK primer binding site

T3 Promoter   BssH II   β-gal α-fragment

...CAGCTTTTGTTCCCTTTAGTGAGGGTTAATTGCGCGCTTGGCGTAATCATGGTCATAGCTGTTTCC

T3 primer binding site   M13 Reverse primer binding site

Feature	Nucleotide Position
f1 (+) origin of ss-DNA replication [pBluescript SK (+) only]	135–441
f1 (–) origin of ss-DNA replication [pBluescript SK (–) only]	21–327
β-galactosidase α-fragment coding sequence (lacZ')	460–816
multiple cloning site	653–760
T7 promoter transcription initiation site	643
T3 promoter transcription initiation site	774
lac promoter	817–938
pUC origin of replication	1158–1825
ampicillin resistance (bla) ORF	1976–2833

FIGURE 1 The pBluescript II SK (+/–) phagemid vectors. The complete sequence and list of restriction sites are available at www.stratagene.com. Genbank® #X52328 [SK(+)] and #X52330 [SK(–)].

Source: Stratagene

Figure 23. pBluescript vector

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