THE ROLE OF DISCO IN DLL-DEPENDENDENT PD AXIS SPECIFICATION

THE ROLE OF DISCO
IN DLL-DEPENDENT PROXIMAL DISTAL AXIS SPECIFICATION
OF DROSOPHILA APPENDAGES

By

EMMANUEL POPO-OLA, B.Sc.

A Thesis

Submitted to the School of Graduate Studies

In Partial Fulfillment of the Requirements

For the Degree

Master of Science

McMaster University

MASTER OF SCIENCE (2008) McMaster University

(Biology) Hamilton, Ontario

TITLE: THE ROLE OF DISCO IN DLL-DEPENDENT PROXIMAL DISTAL AXIS SPECIFICATION OF DROSOPHILA APPENDAGES

AUTHOR: Emmanuel Popo-Ola, B.Sc. (McMaster University)

SUPERVISOR: Dr. Ana Campos

NUMBER OF PAGES: ix, 105

Abstract

Distal-less (Dll) is a master regulator gene responsible for proximal-distal axis formation as well as distal appendage identity. Previous research showed that the expression of *Dll* is maintained through a feedback loop with Disco, a C_2H_2 zinc finger transcription factor. In this project I investigate recent suggestions that *disco* may play additional roles as a cofactor or downstream target of *Dll* during appendage development. I confirm previous research that the presence of *Dll* is sufficient to turn on *disco*. I found that the presence of ectopic *Dll* in the wing discs activates *Dll* subordinate genes in cells where they are not normally expressed. I again performed experiments confirming previous reports that ectopic expression of *Dll* in the wing tissue is sufficient to cause the appearance of ectopic legs. I then showed that when *Dll* is expressed ectopically in the absence of *disco*, there ectopic appendages similar to those formed in the presence of *disco*. Put together, my results suggest that *disco* is does not function as a cofactor or downstream target required for the development and differentiation of *Drosophila* ventral appendages.

Acknowledgements

Firstly, I would like to thank Dr. Ana Campos for giving me the opportunity to work in her lab and for the knowledge she has imparted upon me throughout these short two years. I would also like to thank Xiao Li for all her help, her humour, and delicious Chinese food without which the lab would not be the same. I would like to thank my labmates Maria, Nadia, Veronica, Anu, Stacy, Allison, my homeboy Aidan and also the entire Jacobs lab. Finally I thank my parents and my brothers for their love and support in everything.

TABLE OF CONTENTS

List of Figures

List of Tables

CHAPTER 1

INTRODUCTION

Drosophila disconnected gene

Disconnected (*disco*) is a *Drosophila melanogaster* gene that was first studied for its function in the nervous system. It was first identified as mutants in which axons of the photoreceptors are unable to make a connection with the optic lobes of the brain (Steller et al., 1987). During development, *disconnected* is first expressed at the posterior pole of a stage 5 embryo (Lee et al., 1991). Later, *disco* expression can be found in several different areas of the embryo including neural and non-neural cells (Lee et al., 1991; Robertson et al., 2002). Particularly, *disco* is expressed in many parts of the CNS and PNS. Immunohistochemistry experiments showed that *disco* gene product is localized to the nucleus (Lee et al., 1991).

Drosophila visual system

Drosophila larvae are photophobic. During the first two instars and the early part of the third instar, they retreat away from light. The larvae are usually found buried deep within their food source in the early stages of development. During the late third instar known as the wandering stage, they leave the dark environment of their food source to find a suitable location to enter into the pupal stage. *Drosophila* larvae detect light by means of two light sensitive structures located bilaterally at the anterior portion known as Bolwig's organs (Steller et al., 1987). Each structure is made up of a cluster of photoreceptor cells. A nerve, known as Bolwig's nerve, is formed by the axons of the photoreceptor cells and extends to the brain where it locates and connects to the larval

optic lobe (Steller et al., 1987). However, it is unknown how the Bolwig's nerve is able to recognize and synapse with its targets in the brain.

The adult compound eye is made up of approximately 800 ommatidia. Every ommatidia contains eight photoreceptor neurons known as R-cells which are numbered R1 to R8. Each photoreceptor neuron projects in a predictable manner to the optic lobes. There are two types of R-cells, the outer R-cells corresponding to R1- R6, and the inner R-cells made up of R7 and R8 cells. The compound eye has a repetitive architecture, such that there are several hundreds of repeated projections (reviewed by Mast et al., 2006).

disco in the Larval Nervous System

The *disco* gene codes for a transcription factor containing a zinc finger DNA binding domain (Lee et al., 1991). The zinc finger is a small stretch of amino acids that forms a unique secondary structure stabilized by a zinc ion. The secondary structure contains two β–sheets at the N-terminal end and one α–helix at the C-terminal end, with conserved linker regions of about eight amino acids between each finger. The fingers are used by a variety of regulatory proteins to bind to DNA and control target genes. Affinity of a zinc finger containing protein is dictated by the specific sequence of the α–helix of its fingers (reviewed by Iuchi, 2001). The *disco* protein possesses two fingers in its zinc finger domain. The first finger contains all the necessary amino acids required for a zinc finger domain. However, the second finger appears to be missing some

residues and there are only six amino acids in the linker region between the fingers (Heilig et al., 1991).

Homozygous mutations of the *disconnected* gene cause defects in the larval visual system so that Bolwig's nerve is unable to connect to its synaptic partners in the *Drosophila* brain (Steller et al., 1987). In these mutants, the nerve is able to extend in the proper direction just as they do in wild-type organisms. However, once they reach the region of the brain where they must connect to the optic lobes, the nerves keep growing and do not recognize or connect to their targets (Steller et al., 1987). This means that the *disco* gene is not required for the development of the nerves but is only required by them for target recognition (Steller et al., 1987). Campos et al (1995) conducted studies on a group of cells known as optic lobe pioneer (OLP) cells that are contacted by the larval optic nerve during projection towards the brain. They reported that in *disco* mutants, these cells are unable to differentiate and do not provide the necessary guidance cues required for Bolwig nerve connectivity.

Disco is required for proper development of other areas of the nervous system as well. Shepherd and Glossop (1998) looked at the central projections of thoracic and abdominal sensory neurons and found that in most *disco* mutants they are severely defective. In addition, abnormal circadian rhythmicity can be observed in *disco* mutant flies (Dushay et al., 1989). Hardin et al. (1992) reported that *disco* mutants still have an active circadian pacemaker. Their results suggest that *disco* mutation affects the output pathway between the pacemaker and its effectors.

Apart from the nervous system, *disco* is required for pattern formation during development of structural parts of the *Drosophila* larva. Previous experiments showed that during embryogenesis, *disco*, along with head Hox genes such as *Deformed* (*Dfd*), and *Sex Combs Reduced* (*Scr*), plays a role in specifying identity of larval head segments (Mahaffey et al., 2000). *Disco* expression is detected in the primordia of the gnathal lobes prior to their development. The absence of *disco* and a related gene known as *disco-r* causes abnormal development of all three gnathal lobes including the mandibular, maxillary and labial lobes (Mahaffey et al., 2000).

Appendage Development in *Drosophila melanogaster*

Adult limbs and other head and thoracic structures of *Drosophila* are derived from clusters of cells that are present in the larvae, called imaginal discs. These cells do not form a structural part of the larvae, but are set aside to develop into adult structures during metamorphosis. Each of the different imaginal discs has a different size and shape and they are named for the adult appendage that they will develop into (reviewed by Morata, 2001). In adult flies, wings are located at the dorsal side of the body and legs on the ventral side. However, in the early embryo, they arise from a common set of cells in the embryonic ectoderm called the embryonic limb primordium (Cohen et al., 1993). The limb primordium first appears at stage 11 of embryonic development spanning the parasegment boundary in response intercellular activities of Wg and Dpp proteins. Later, the wing disc primordium separates from the leg disc primordium by migrating dorsally to the dorsolateral position while cells of the leg primordium stay at the ventrolateral

position (Cohen et al., 1993). Each limb disc primordium invaginates to form its respective imaginal disc (reviewed by Brook et al., 1996).

From the earliest stages, the leg and wing disc primordia are subdivided into anterior and posterior cell populations. This is accomplished through the action of the homeobox gene *engrailed (en)* which functions as a selector gene to distinguish anterior and posterior cells from one another. *engrailed* is expressed in the posterior compartment, and causes the cells there to be immiscible with cells in the anterior compartment. Secondly, it triggers the expression of *hedgehog (hh)* in the posterior cells. Hedgehog (Hh) activity is blocked in the posterior compartment by *en*. However, Hh diffuses from the posterior compartment, across the anterior-posterior (A/P) compartment boundary, into the anterior compartment. Here Hh activates the expression of the genes *wingless (wg)* and/or *decapentaplegic (dpp)* (reviewed by Morata, 2001).

Wingless (wg) and *decapentaplegic (dpp)* are morphogens. Morphogens are molecules that are expressed and originate from a localized source. The molecules spread away from the source and this establishes a concentration gradient that is highest at the source. Cells respond to this gradient based on their distance from the source of the morphogen (reviewed by Affolter and Basler, 2007). In these target cells, the morphogen triggers a signal that is transduced from the cell surface to the nucleus which leads to the activation of genes. The specific genes turned on by each cell are dependent on its distance from the source and the concentration of the morphogen received by the cell (reviewed by Morata, 2001).

The primary target of Hh in the wing disc is *decapentaplegic (dpp)*. *dpp* activation and expression takes place in a small group of cells that are present along the anterior-posterior compartment boundary of the wing imaginal disc. *dpp* is a member of the TGF–β super family. It sends signals to cells of the wing disc instructing them of their fate and controls the growth of the disc along the anterior-posterior axis. In the absence of *dpp* signalling, the size of the wing is severely reduced, producing only a little stump. On the other hand overexpression of *dpp* causes an overgrowth of the wing. This shows that proper expression of *dpp* is required for the wing to attain a normal size (reviewed by Affolter and Basler, 2007).

In the second instar larva, another axis specification event occurs in the wing disc that distinguishes the dorsal and ventral (DV) regions from one another (reviewed by Morata, 2001). The gene responsible for the dorsal-ventral axis specification is *apterous (ap)*. Apterous is a LIM-homeodomain protein that is expressed in the dorsal compartment of the wing disc. It acts as a selector gene whereby ON specifies a dorsal fate and OFF specifies a ventral fate (Diaz-Benjumea and Cohen, 1993; Blair et al., 1994). Hence *ap* is turned on in the presumptive dorsal cells. *ap* expression leads to the activation of *vestigial (vg)* and other genes in the cells of the dorsal-ventral (DV) compartment boundary. These molecules are important for proper growth of the wing along the DV axis (Diaz-Benjumea and Cohen, 1993; Blair et al., 1994).

Distal-less in early Limb Development

In the leg disc primordium, Hh triggers the expression of *wingless* (*wg)* along the anterior-posterior compartment boundary (Diaz-Benjumea et al, 1994). *Wg* is a member of the Wnt protein family, and it directs the growth of the leg along the dorsal-ventral axis (Neumann and Cohen, 1997). Secondly, *wg* expression triggers the expression of *Distal-less (Dll)* (Cohen et al., 1989). *Distal-less (Dll)* is the gene responsible for proximal-distal (PD) axis pattern formation during appendage development. *Dll* expression in the leg disc primordium represents the first sign of leg identity (Cohen et al., 1989). In the second instar and later, *Dll* is expressed in the center of the leg imaginal disc. These are the cells that correspond to the distal-most portion of the adult leg, made up of the tarsus, tibia, femur and the trochanter. Therefore, as the limbs develop, Dll activity directs the growth and differentiation of distal leg parts (Diaz-Benjumea et al, 1994).

At stage 14, proximal-distal axis formation begins, with the separation of distal parts from proximal parts. This is accomplished by expression of *homothorax* (*hth*), and the nuclear translocation of *extradenticle* (*exd*) within cells at the periphery of the imaginal discs (which correspond to proximal structures). At the same time, the cells of the central region (corresponding to distal structures) continue to express *Dll*. Translocation of *exd* into the nucleus requires the activity of Hth. Also, Hth is stabilized by Exd function (Abu-Shaar and Mann, 1998). The proximal parts of the leg include the coxa and part of the body wall. In these parts, *exd* and *hth* specify proximal fate by

preventing response to *wg* and *dpp*. This prevents activation of *Dll* in the proximal domains of the leg (Abu-Shaar and Mann, 1998).

Dll mutant phenotypes

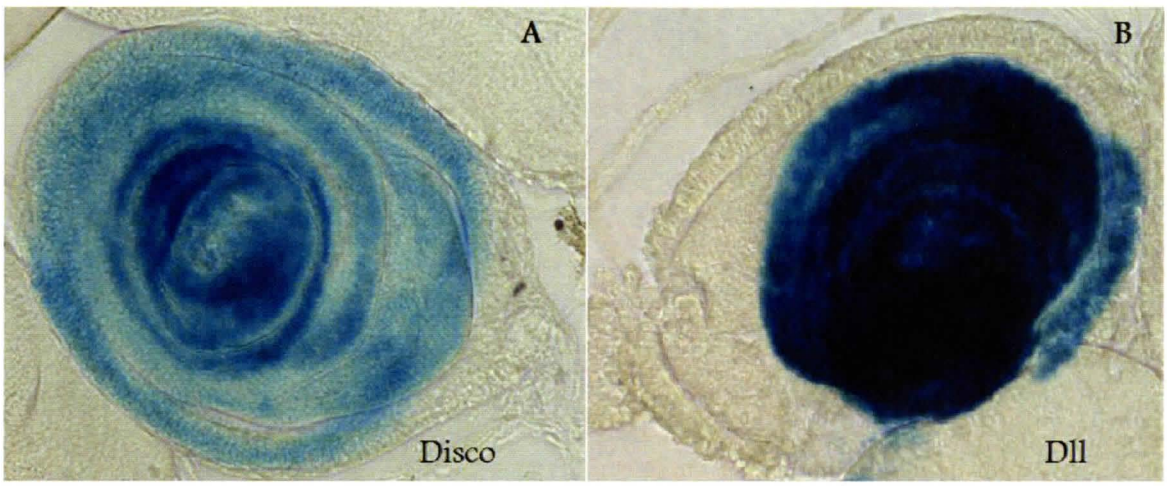
Homozygous null mutation of *Dll* gene causes embryonic lethality. However, less severe mutations that reduce the activity of *Dll* but not completely eliminate it allow embryos to develop to adulthood. These adults display developmental malformations in their limbs (Cohen, et al. 1989). Strong mutant alleles that have a severe reduction of *Dll* function show a greater loss of structures occurring at different regions of the leg, especially the distal parts. The leg is truncated and the tarsi and tibia are missing (Cohen, et al. 1989; reviewed by Panganiban and Rubenstein, 2002). Alleles of intermediate *Dll* function cause a loss of tarsal segments of the leg. Weak alleles only cause a fusion of leg segments. The entire leg is still present but each region is reduced in size and fused together. Furthermore, lack of *Dll* function leads to the loss of the larval rudimentary limbs known as Keilin's organs (Cohen and Jurgens, 1989; reviewed by Panganiban and Rubenstein, 2002). The second role of *Dll* in the developing *Drosophila* is in specifying antennae identity as well as being responsible for proximal-distal axis patterning of the antenna (reviewed by Panganiban, 2000). Mutations in the *Dll* gene result in antennae phenotypes where distal portions of the antennae are truncated, while stronger alleles cause antennae to leg transformations (Cohen and Jurgens, 1989).

Analysis of the *Distal-less* gene shows that it codes for a homeodomain containing protein, which suggests that the activity of this gene involves regulation of downstream

target genes (Cohen, et al. 1989). The homeodomain is 60 amino acids in length and is coded for by a 180 bp long sequence of DNA called the homeobox. The homeobox gene is extremely well conserved throughout evolution and can be found in a large variety of organisms (reviewed by Hueber and Lohmann, 2008). Homeodomain containing proteins were first discovered through mutations that cause a transformation of one structure into a homologous structure from another part of the body. This phenomenon is known as a homeotic transformation. Researchers discovered that the Hox genes are important for patterning along the anterior-posterior axis. They are responsible for assigning different morphologies to the different segments of the body. Their role is primarily to function as master regulators, and bind via the homeodomain to specific sequences on DNA known as Hox response elements (HREs) (reviewed by Hueber and Lohmann, 2008).

Homeodomain containing proteins in *Drosophila* have been grouped into two homeotic complexes known as the *Antennapedia* complex (ANT-C) and the Bithorax complex (BX-C). The ANT-C contains five genes while the BX-C contains three genes (reviewed by Morata, 2001). Many other homeodomain containing proteins exist that are not grouped into these two categories and *Dll* falls into this criteria (reviewed by Morata, 2001). There are many genes that are downstream of *Dll* that could be candidates for regulation by it. Some of these genes include *aristaless (al), bric a brac (bab), dachshund (dac), spineless (ss), spalt (sal)* and *disconnected (disco).*

Figure 1: Lac Z staining showing *disco* expression (A) and *Dll* expression (B) in 3rd instar *Drosophila* leg discs. *disco* and *Dll* expression overlap in appendage discs. *Dll* expression is confined to the cells of the inner parts of the disc which correspond to distal parts of the appendage. *disco* expression is present in the entire disc.


A
Disco
B
Dll

Interaction between *Dll* and *disco*

disco expression is found in *Dll* domains in the leg and antennae discs (Figure 1). The expression of *disco* is turned on soon after the expression of *Dll* begins and depends on prior activity of *Dll* (Cohen et al, 1991). *disco* and *Dll* expression in the leg discs overlap in the regions that develop into the trochanter (tr), femur (fe), tibia (ti), tarsi (ts) and claw (cl). In the antenna discs, their expression overlap in the regions corresponding to antennal segments II, III, and arista (Cohen et al, 1991). In *Dll* mutants, *disco* expression is markedly reduced, and in *disco* mutant background, there is reduction in *Dll* expression. In addition, ectopic expression of *Dll* is sufficient to turn on *disco* expression. However, ectopic expression of *disco* is not sufficient to turn on *Dll* activity (Dey, 2006). This shows that *Dll* is upstream of *disco* but requires *disco* activity to maintain its expression. These observations suggest that the two genes regulate each other through a positive feedback loop (Dey, 2006).

Previous experiments demonstrated that adult escapers of *disco* mutants have antennae and leg phenotypes similar to those of *Dll* mutants. Furthermore, knocking down *disco* gene function using UAS-*disco*^{RNAi} or UAS-*disco*^{DN} results in phenotypes similar to those of *Dll* mutant flies (Dey, 2006). These phenotypes include loss of arista and partial antennae to leg transformations, as well as abnormalities in leg development characterized by reduction of the femur, tibia and deletions of distal tarsi and claws (Dey, 2006). In order to establish whether *disco* has a role in *Dll*-dependent development of leg and antenna, experiments were performed where *disco* gene expression was upregulated

in *Dll* domain of flies carrying hypomorphic recessive alleles of *Dll*. This resulted in partial reduction of lethality and almost normal antenna and leg in all escapers (Dey, 2006). Secondly, down-regulation of *disco* gene product in *Dll* domain of *Dll* mutant flies resulted in increased lethality, decreasing the number of escapers to almost zero (Dey, 2006).

These findings led to the suggestion that Disco function is required concomitantly with Dll for proximal-distal axis formation in *Drosophila* leg development. However, the results were insufficient to establish whether *disco* function is only in regulation of *Dll* or whether it functions in the axis specification pathway along with *Dll* to regulate downstream targets or both. This question was raised because the *Dll* mutants used were hypomorphs and not complete loss of function mutants. Up-regulation of *disco,* driven by *Dll* promoter in these experiments causes an increase in expression of these *Dll* hypomorphs. Since these hypomorphs can still function in the *Dll* pathway, this may account for the reduced lethality and near normal phenotypes that were observed in those experiments. Down-regulation of *disco* also causes a reduction in the expression level of the *Dll* hypomorphic alleles, which may result in the increased lethality that was observed. Therefore, it still remained to be determined whether *disco* function during appendage development is simply, to maintain *Dll* expression through a positive feedback loop. This would make *Dll* alone responsible for PD axis specification and appendage development. On the other hand, *disco* may function downstream of *Dll*, or as a cofactor

along with *Dll* in the same pathway to regulate genes responsible for PD axis formation and leg development.

The aim of this project is to determine which of these two possible roles *disco* plays during *Dll*-dependent appendage development in *Drosophila melanogaster*. A method with which to determine the precise function of *disco* and *Dll* is through experiments involving ectopic formation of appendage tissues. Previous experiments by Gorfinkiel et al (1997) showed that ectopic expression of *Dll* results in the formation of ectopic leg structures. Dong et al (2000) also reported ectopic antennae formation when *Dll* expression is driven ectopically by *dpp*-Gal4. Furthermore, it had been previously established that ectopic expression of *Dll* is sufficient to turn on *disco*. Therefore it is very likely that *disco* is ectopically activated in the tissues that were transformed by the ectopic expression of *Dll*. Here we devise experiments that involve ectopic expression of *Dll* while preventing the accompanying activation of *disco* expression. If we observe ectopic appendage formation in these flies, then we can conclude that *Dll* alone directs axis specification and appendage development while the sole function of *disco* is to maintain *Dll* expression during this process. However, if the absence of *disco* gene product results in the suppression of ectopic appendage phenotypes, then *disco* is required along with *Dll*, in the genetic pathway of axis specification and appendage development. With these experiments, we hope to distinguish the roles of *disco* and *Dll* from one another and determine the role that *disco* plays in proximal-distal axis specification and appendage development.

CHAPTER 2

MATERIALS AND METHODS

Drosophila strains

The fly strains used in this project include, *disco*^{1} (Fishbach and Heisenberg, 1984), C50.1S1 (Cohen et al., 1997), *Dll*-Gal4 (Calleja et al., 1996), UAS-*Dll*/CyO, UAS-*Dll*/UAS-*Dll* (Gorfinkiel et al., 1997), UAS-*disco* (Dey, 2006), UAS-*disco*^{DN} (Dey, 2006), UAS-*disco*^{RNAi} (Dey, 2006), *omb*-Gal4/FM6, *ptc*-Gal4, *ap*-Gal4/CyO (Gorfinkiel et al., 1997).

Crosses

See appendix for genetic schemes of all crosses performed for this project.

Calculation of %Lethality

Theoretical yield = Mean of 100% viable F1 flies

% Viability = (Actual yield / Theoretical yield) *100

% Lethality = 100% - % Viability

Statistical equation

$t_s = (\arcsin \sqrt{p1} - \arcsin\sqrt{p2}) \div [\sqrt{820.8\ (1/n1 + 1/n2)}]$

Electron Microscopy

All scanning electron images were taken with ESEM2020 Electro Scan electron microscope with a large feild detector operating at 20Kv. Vacuum was set at 1 Tour and condenser at 50%.

Confocal Microscopy

All images were taken with a Bio-Rad MRC 600 krypton/argon laser confocal microscope.

X-Gal Staining (adapted from Ghysen and O'Kane, 1989, Development 105:35-52)

Wandering third instar larvae were collected from the walls of vials. The larvae were placed in a Petri dish containing a small amount of 1X PBS. A small test tube containing PBS was placed on ice. The larvae were dissected and the brains, with all the imaginal discs still attached were removed and placed in the cold PBS. Once the dissection is complete, the PBS was removed and the brains were fixed in 4% paraformaldehyde solution for 1 hour. After fixation, the brains were washed with 1X PBS three times for 10 minutes each and with 0.2% PBT once, for 10 minutes. The brains were incubated in 0.2% x-gal solution at 37°C overnight. To make the x-gal solution, 1ml of staining solution was heated to 65°C. The staining solution is made up of 10mM $NaPO_4$, 150mM NaCl, 1mM $MgCl_2$, 3.1mM $K_4(Fe(CN)_6)_1$, 3.1 mM $K_{39}Fe(CN)_6$. 15ul of 8% x-gal in DMSO solution was added to the staining solution and allowed to cool to room temperature. After overnight incubation, the brains were washed in 1X PBS three times for 10 minutes at room temperature and one time with 0.2% PBT. The brains and imaginal discs were mounted in 70% glycerol in 1X PBS.

dac Immunohistochemistry of larval imaginal discs

Wandering third instar larvae were collected and dissected as described above. The brain and imaginal discs are removed and fixed in 4% paraformaldehyde for 1 hour at room temperature. After fixing, the brains were washed three times, 10 minutes each with 1X PBS and once with 0.3% PBT for 10 minutes. The tissues were blocked in 10% Goat serum in PBT (0.3%) for 1 hour at room temperature. After blocking, the blocking solution was removed and the tissues placed in primary anti-dac (mAbdac2-3) antibody solution. The antibody solution is 1:100 Ab:blocking solution. The samples are placed at 4°C overnight. After overnight incubation, the brains were washed with 0.3% PBT and tissues were blocked again for 1 hour at room temperature. After blocking the blocking solution is removed and a 1:200 secondary antibody:blocking solution was added to the brains. The secondary antibody was Alexa 488 anti-mouse antibody. The samples were placed at 4°C overnight. After overnight incubation the samples were washed with 1X PBS and mounted in 70% glycerol.

α–β–gal immunohistochemistry

Third instar larvae were collected and dissected as described above. The brains and discs were fixed, washed, and the tissues were blocked as described above. Primary mouse anti–β–gal solution of 1:200 antibody:blocking was added to the brains and discs and placed at 4°C overnight. After incubation, the tissue were washed, and blocked as previously described. After blocking, secondary anti-mouse HRP in glycerol was added

to the tissues and incubated at 4°C overnight. The next day, a 2:1 PBT:DAB solution was prepared and allowed to sit for 1 minute. All secondary antibody solution was removed from tissues and DAB solution was added to tissues. After 1 minute, a 3% H_2O_2 solution was added to the tissues and mixed. The reaction takes place and the tissues are stained. After the tissues are satisfactorily stained, PBT solution was added to stop the reaction. The brains and discs were transferred to a new test tube and washed with PBT two times and once with PBS for 5 minutes each. The brains and discs were mounted in 70% glycerol.

CHAPTER 3

RESULTS

Ectopic Expression of *Dll* Results in Formation of Ectopic leg tissue

One of the functions of Disco in the developing *Drosophila* is to maintain the expression of *Dll* through a positive feedback loop. The aim of this project is to determine whether in addition to this role, Disco participates as a cofactor and/or downstream target in *Dll*-dependent appendage development. To answer this question, an approach was chosen that takes advantage of previously reported data which demonstrated that ectopic *Dll* expression results in the formation of ectopic appendages (Gorfinkiel et al., 1997; Dong et al., 2000). However, the details of these experiments such as temperature settings, percentage of escapers from crosses, etc, were not provided in the publications. Also, two UAS-*Dll* lines were used by Gorfinkiel et al (1997) in their experiment and only one resulted in the phenotypes described in the publication. However, it was not mentioned which line it was that resulted in the reported phenotypes and which line resulted in one hundred percent lethality.

Therefore, the first step in this project was to recreate the experiments by Gorfinkiel et al (1997) and Dong et al., (2000). These experiments were repeated in order to find out which fly lines, at which experimental conditions gives rise to the highest amount of escapers bearing ectopic structures. Hence all the Gal4 lines used in the previous experiments were obtained to be crossed to the two UAS-*Dll* fly lines described by Gorfinkiel et al (1997). The Gal4 lines are *patched (ptc)*-Gal4, *apterous (ap)*-Gal4, *optomotor-blind (omb)*-Gal4, and *Dpp*-Gal4. In the F1 offspring of each cross, *Dll* would be expressed ectopically based on the promoter under which the *Dll* gene is placed.

These experiments were carried out at different temperatures in order to determine the temperature settings with which the best results could be obtained. Performing the experiment at different temperatures modulates the Gal4 activity and increases or decreases the level of ectopic *Dll* expression at a higher or lower temperature respectively. The best results will be a high number of escaped flies with a high percentage of the escapers having ectopic appendage structures as reported by Gorfinkiel et al (1997) and Dong et al (2000).

The temperature settings are the following, (i) keeping the crosses at 25°C or 18°C for the duration of development, (ii) placing the crosses at 25°C for the first five days of development and then transferring to 18°C until eclosion, (iii) placing the crosses at 25°C for the first 3 days and then transferring to 18°C, (iv) placing the crosses at 29°C for the first 4 days and then transferring to 18°C, and finally, (v) placing the crosses at 29°C for the first 3 days and then transferring to 18°C.

Ectopic expression of Dll at 25°C

UAS-*Dll* flies were crossed to each of the Gal4 lines listed above (refer to appendix for scheme). F1 adults from each cross were separated by genotype and each genotype counted. Table 1 shows the results of this experiment. There were nearly no escapers when the experiment was performed with this temperature setting. Virtually all the flies with the Gal4>UAS-*Dll* genotype died during development. There was one escaper each for *ap*-Gal4 and *ptc*-Gal4 and none for *omb*-Gal4 and *dpp*-Gal4. Some

larvae developed to reach the pupal stage but none of the pupae eclosed as there were many dead pupae on the walls of the test tubes in which the crosses were carried out.

Ectopic expression of Dll at 18°C

When the crosses were kept at 18°C for the entire duration of development, UAS-*Dll* crossed to *dpp*-Gal4 yielded the highest number of escapers. There were 18 escapers with the genotype *dpp*-Gal4>UAS-*Dll* that developed to adult stage out of 1569 F1 flies (Table 2). This represents 96.5% lethality. There were only 8 *ap>Dll* escapers, 1 *ptc>Dll* escaper and 5 *omb>Dll* escapers. Again, many flies developed to pupal stage but died before eclosing from their pupal cases. Therefore we conclude that this temperature setting is most favourable for experiments with flies in which *dpp*-Gal4 is driving the expression of *Dll* ectopically.

Ectopic expression of Dll at 25°C (5 days) then shifted to 18°C

When crosses were placed at 25°C for 5 days and then transferred to 18°C for the remainder of development, crosses with UAS-*Dll* and *ap*-Gal4 yielded the most escapers (Table 3). Most of the flies died during development but there were 22 *ap>Dll* flies that survived to adults out of 2001 flies. This represents 96.7% lethality. There were no escapers from any of the other crosses. This temperature setting therefore favours experiments with flies in which the expression of *Dll* is driven by *ap*-Gal4.

Ectopic expression of Dll at other temperature settings

Tables 4 shows the results of crosses placed at 25°C for 3 days and transferred to 18°C for the remainder of development. Table 5 shows results for crosses at 29°C for 4 days then shifted to 18°C. Finally Table 6 shows results for crosses at 29°C for 3 days and shifted to 18°C for the rest of development. At these settings the numbers of escapers were too low. Most of the flies carrying Gal4>UAS-*Dll* genotype did not survive to adult stage. There were many dead pupae on the test tube walls indicating some survived until pupal stage but died before eclosion.

From these results we were able to determine that for *ap*-Gal, the optimal temperature setting is 25°C for the first 5 days of development and then transferred to 18°C till adult stage while for *dpp*-Gal4, it is keeping the crosses at 18°C for the duration of development. The other Gal4 lines did not yield a good enough number of escapers and so were eliminated from further experimentation.

All escapers from each experiment were collected and stored in test tubes which were placed in a freezer (-4°C). The flies were then photographed with a scanning electron microscope to observe and document any ectopic structures. Flies with the genotype *ap*-Gal4/UAS-*Dll* appear normal overall except at the wings. Most flies show a severe malformation of the wing. There is only a stump where the wing should be and there are very coarse bristles on these structures resembling bracted bristles of the leg. A small percentage of the flies possess what looks like ectopic leg structures at the hinge region of the wing. The haltere is also affected by ectopic expression of *Dll*. The haltere

on all flies possess coarse bristles, suggesting what looks like a partial transformation towards leg. Figure 2A shows a fly from F1 generation of *ap*-Gal4 and UAS-*Dll* cross. The fly has wings that have been transformed and there is an ectopic leg formation due to ectopic *Dll* expressions under the *ap* promoter.

When *Dll* is ectopically driven by *dpp* promoter, the results are escaper flies that are severely deformed. Figure 2C shows a fly with the genotype *dpp*-Gal4>UAS-*Dll.* The wings look un-inflated and possess very coarse bristles as well as arista-like bristles that suggest ectopic leg and/or antenna formation. Furthermore, the legs are severely truncated. All segments are shortened and fused together and the coxa, trocanter, femur and tibia are either missing or misshapen. This explains why many flies survive to pupal stage but do not eclose as viable adults. Many flies were observed unable to crawl out of open pupal cases due to their misshapen legs and die soon afterwards.

Ectopic expression of Dll with a second UAS-Dll line

Another UAS-*Dll* stock was obtained (henceforth designated as UAS-*Dll**) and the experiments above were repeated with this new line. The optimal temperature settings established for *ap*-Gal4 and *dpp*-Gal4 were used for crosses with this new line. Since *ptc*-Gal4 and *omb*-Gal4 had been removed from further experimentation, they were not included in these new sets of crosses. With the new UAS-*Dll** flies, each experiment yielded more escapers than with the previous UAS-*Dll* stock. When *ap*-Gal4 was crossed to UAS-*Dll**, there were 280 escaper flies with the genotype *ap*-Gal4/UAS-*Dll** out of 1421 flies representing a 75.4% lethality (Table 7). The phenotypes of these escapers

were different from those described for UAS-*Dll*. Escapers in this group displayed a range of phenotypes that go from mild to severe. The mild phenotype is characterized by wings that are almost wildtype except for some "crumpled" areas around the hinge region (Figure 3A). The halteres look normal in these flies. Amongst flies with the intermediate phenotype, the wings are roughly the same size as wild type, but they are severely malformed and some look un-inflated. There is also a mild deformation in the thorax area (Figure 3B). Flies with severe phenotypes have no wings at all and the thorax, including the scutellum, is extremely deformed (Figure 3C).

Similar experiments were carried out with *dpp*-Gal4 and UAS-*Dll** fly lines. Again, the phenotypes here were drastically different from those of the aforementioned experiments using UAS-*Dll* fly lines. There were 289 escapers out of 1023 flies representing 60.6% lethality (Table 8). The wings of these escapers appear "crumpled" along the entire length of the wing (Figure 4A) and with some flies, there are outgrowths on the surface of the wings (Figure 4C). There are also bristles on the surface of the wings that resemble bristles of the arista (Figure 4B). In addition, one fly was found that had no wing at all but a leg-like structure in its place (Figure 4D).

Ectopic expression of *Dll* activates *disco* expression in wing discs

Previous experiments demonstrated that ectopic expression of *Dll* is sufficient to activate *disco* expression ectopically (Dey, 06). However, in those experiments, *Dll* was ectopically expressed in the eye-antenna disc and activation of *disco* promoter was

observed in the developing retina. Here we wanted to determine whether ectopic expression of *Dll* in the wing tissues would cause a similar ectopic activation of *disco*.

X-Gal Staining

Flies were created carrying *disco* C50.1S1 and UAS-*Dll*. *Disco* C50.1S1 is a lacZ enhancer trap inserted near the *disco* promoter such that the expression of lacZ is activated according to a *disco* pattern of expression. This provides a means of visualizing the activation of the *disco* gene promoter within the tissues of the flies. Flies carrying the C50/C50;UAS-*dll**/UAS-*Dll** virgin females were crossed to *ap*-Gal4 and *dpp*-Gal4 males to get the F1 C50/x(y);*ap*-Gal4/UAS-*Dll** and C50/x(y);UAS-*Dll*;dpp*-Gal4 respectively (see appendix for scheme). LacZ expression will be used to locate and identify the ectopic activation of *disco* due to ectopic *Dll* expression. The F1 offspring of these crosses were allowed to develop to the wandering 3rd instar larval stage. These larvae were collected and dissected to remove the brain and immaginal discs. Figure 5A shows X-Gal staining of discs from control larvae (parental stock). There is no staining detected as expected for wildtype flies showing that there is no *disco* expression in the wing discs of wildtype flies.

When C50/C50;UAS-*Dll**/UAS-*Dll** flies were crossed to *ap*-Gal4, to obtain an F1 generation of C50/x(y);*ap*-Gal4/UAS-*Dll** genotype, wing disc from 3rd instar wandering larvae of F1 show some faint x-gal staining in various parts of the wing (Figure 5B). The same occurs when C50/C50;UAS-*Dll**/UAS-*Dll** were crossed to *dpp*-Gal4. Figure 5C shows wing discs from 3rd instar larvae of F1 flies with faint x-gal

staining. This experiment shows that ectopic expression of *Dll* in the wing is sufficient to activate *disco* expression. In addition to ectopic *disco* activation observed, it is evident that the wings discs are misshapen and do not resemble wildtype wing discs.

Anti β–gal immunohistochemistry

Another method used to visualize ectopic *disco* expression in these flies was β–galactosidase immunohistochemistry. This is a more sensitive method and could potentially detect lac-Z expression more efficiently than X-gal staining. For this experiment, the same crosses as above were performed and the larvae were collected at the third instar wandering stage. Figure 6A is an example of the anti β–gal staining for flies with the genotype C50/x(y);*ap*-Gal4/UAS-*Dll**. There is ectopic *disco* expression detected in a large area of the wing disc. Figure 6B shows an example of the staining for flies with the genotype C50/x(y);UAS-*Dll*;dpp*-Gal4. There is *disco* expression detected in these wings as well. In both instances, anti β-gal immunohistochemistry detected more lac-Z expression in the wing discs than with X-Gal staining. Both methods show that *disco* expression is activated in the wing discs when *Dll* is ectopically expressed in these tissues.

Testing of UAS-*disco*^{DN} and UAS-*disco*^{RNAi} fly lines

To downregulate the expression of *disco* while ectopically expressing *Dll*, flies were created carrying both UAS-*Dll* and UAS-*disco*^{DN} or UAS-*disco*^{RNAi}. The UAS-*disco*^{DN} and UAS-*disco*^{RNAi} fly lines were created several years earlier, and may have

accumulated mutations and lost their effectiveness in silencing the *disco* gene. Therefore, before making constructs with these fly lines, both the UAS-*disco*^{DN} and UAS-*disco*^{RNAi} lines were tested to determine if they were still capable of down regulating the expression of *disco*. To test the fly lines, virgin females were collected from each line and crossed to *Dll*-Gal4 males. Expression of *disco* gene will be down regulated in the *Dll* domain of the F1 flies from these crosses. This method was used to test the effectiveness of these constructs when these fly lines were first created (Dey, 2006). It was observed that down regulation of *disco* in the *Dll* domain resulted in partial lethality as well as antennae to leg transformations in the F1 flies.

Table 9 shows the results of the cross between *Dll*-Gal4 and UAS-*disco*^{DN} and UAS-*disco*^{RNAi} lines. There was 76.9% lethality in the F1 of the cross between *Dll*-Gal4 and UAS-*disco*^{DN} and 80.5% lethality for the cross between *Dll*-Gal4 and UAS-*disco*^{RNAi}. The escapers from each cross displayed phenotypes similar to those described by Dey (2006) where the antennae are malformed and resemble a small ectopic leg. These results showed that both the UAS-*disco*^{DN} and UAS-*disco*^{RNAi} fly lines were effective in silencing the expression of *disco* and thus can be used in experiments involving downregulation of *disco*.

Knocking down *disco* gene product while ectopically expressing *Dll* does not prevent formation of ectopic leg tissue.

The experiments described above confirmed previous reports that ectopic expression of *Dll* causes the formation of ectopic appendages (Gorfinkiel et al., 1997).

We were also able to find the optimum conditions at which ectopic *Dll* expression results in ectopic appendage formation. In addition, we show that there is an ectopic expression of *disco* during the formation of these ectopic tissues. To determine whether *Dll* alone is sufficient to induce ectopic formation of appendages, flies were created carrying both UAS-*Dll*, and UAS-*disco*^{DN} or UAS-*disco*^{RNAi}. UAS-*disco*^{DN} , designated "MutD5" is a dominant-negative allele made up of a non-functional version of the zinc finger region of the *disco* gene. This construct competes for the DNA biding site of wildtype *disco* and blocks proper function of wildtype proteins (Dey 2006). UAS-*disco*^{RNAi} was described as a construct with a sequence that targets and knocks down wildtype *disco* mRNA (Dey, 06). In the crosses carried out with these flies, ectopic expression of *disco* will be accompanied by down regulation of *disco* gene product. Hence, up regulation of *disco* due to the positive feedback loop with *Dll* will be reduced or eliminated. Table 10 shows the F1 offspring from crossing *ap*-Gal4 flies with flies carrying UAS-*Dll* and UAS-*disco*^{DN}. The results show that in the F1 generation, there are more escapers of flies carrying *ap*-Gal4, UAS-*Dll* and UAS-*disco*^{DN} than those carrying only *ap*-GAL4 and UAS-*Dll*. A statistical comparison of the percentage lethality of the two sets of flies showed that there is no significant difference between them. Also, both sets of flies display the same phenotypes, which were the same as those described above for ectopic expression of *Dll* driven by *ap*-Gal4. The wings are reduced to a stump, possessing coarse bristles, and the haltere as well, displays a partial transformation towards a leg structure (Figure 7 A-D). Table 11 shows the same cross, this time with flies carrying the UAS-*Dll**. Here the number of escapers are nearly identical between those carrying *ap*-

Gal4, UAS-*Dll** and UAS-*disco*^{DN} and those carrying just *ap*-Gal4 and UAS-*Dll**. Once again, both sets of flies show the same phenotypes (Figure 7 E,F).

Table 12 shows a similar experiment performed with *dpp*-GAL4. Flies carrying *dpp*-Gal4 were crossed to flies carrying UAS-*Dll* and UAS-*disco*^{DN}. Once again, flies carrying *dpp*-Gal4, UAS-*Dll* and UAS-*disco*^{DN} survived better and produced more escapers than those carrying *dpp*-GAL4 and UAS-*Dll*. However, statistical test showed that there is no significant difference between their percentage lethality levels. And again, both sets of flies display the same phenotypes. The legs are truncated and tarsal segments are fused together. The wings are un-inflated, possessing leg-like and antenna-like bristles (Figure 8). When flies carrying UAS-*Dll** are used in the same experiment, the results show that flies carrying the UAS-*disco* dominant negative once again have more escapers than those without the dominant negative (Table 13). Here, there is a significant statistical difference between the percentage lethality of both sets of flies, however, as before, both sets of flies display the same phenotypes. The wings have a "crumpled" look and there are arista-like bristles on the surface of the wings that suggest ectopic antenna.

These experiments were repeated with flies in which ectopic *disco* gene products are knocked down using UAS-*disco*^{RNAi}. Here *ap*-Gal4 and *dpp*-Gal4 flies were crossed to flies carrying the constructs UAS-*Dll* and UAS-*disco*^{RNAi}. When *disco* gene product is knocked down using UAS-*disco*^{RNAi}, results were similar to those of experiments where UAS-*disco*^{DN} was used. Table 14 shows the F1 generation of *ap*-Gal4 crossed to flies

carrying UAS-*Dll* and UAS-*disco*^{RNAi}. Even though there were more escapers when the offspring carried *ap*-Gal4, UAS-*Dll* and UAS-*disco*^{RNAi} compared to those without the UAS-*disco*^{RNAi} construct, this was not a statistically significant differnce. Both sets of flies displayed the same sets of phenotypes as described above (Figure 9 A,B). The same occurs when *dpp*-Gal4 is used in this cross. Flies carrying the UAS-*disco*^{RNAi} construct survived better than flies without the construct but not at a statistically significant level (Table 15). Again, both sets of flies have the same phenotypes (Figure 9 C,D).

Ectopic expression of *Dll* affects *dachshund* expression pattern

The experiments above showed that when *Dll* is expressed ectopically, there is a partial transformation of wing tissues and there are ectopic leg/antennae structures originating from the parts of the wing. Here, I attempt to determine, on a molecular level, what is occurring during ectopic expression of *Dll* and transformation of wing into ectopic leg tissue. One of the genes downstream of *Dll* that is activated during leg development is d*achshund (dac)*. *dac* is a nuclear protein, and is expressed in most of the leg, from the tibia to the tarsi and functions in the morphogenesis of these parts (Mardon et al., 1994). Hence, I used *dac* antibody to detect *dac* expression in the wing. *ap*-Gal4 and *dpp*-Gal4 flies were crossed to UAS-*Dll**/UAS-*Dll** flies to obtain the F1 carrying *ap*-Gal4/UAS-*Dll** and those carrying *dpp*-Gal4/UAS-*Dll**. These F1 larvae were collected and dissected to remove the leg and wing discs. Immunohistochemistry was performed on the discs to detect *dac* expression. *dac* expression was present in the leg discs of all flies. *dac* expression was also detected in specific areas of the wing discs of

wildtype and internal control flies. The first observation made of the experimental flies was that the shapes of the wing discs were dramatically different from those of wildtype and control flies. Secondly, in experimental wing discs, the expression pattern of *dac* is altered compared to those of control wing discs (Figure 10). Therefore, ectopic expression of *Dll* caused modifications in the expression pattern of genes controlled by *Dll* and affected the development of the wing discs.

Table 1: Ectopic Expression of *Dll*, driven by different Gal4 lines @ 25°C

Male flies from each Gal4 line were crossed to UAS-*Dll* virgin females. These crosses were placed at 25°C for the entire duration of development. There was 100% lethality of escapers from all the crosses. This temperature setting does not favour the development of any of the F1 of generation flies from any of the crosses.

Fly Lines	Total number of flies	Number of Escapers	% Lethality
apterous-Gal4	693	1	99.7
patched -Gal4	955	1	99.8
omptomotor-blind-Gal4	367	0	100
decapentaplegic-Gal4	558	0	100

Sample Calculation (*ap*-Gal4)

Theoretical yeild = 692/2 = 346

% Viability = 1/346 * 100 = 0.289%

% Lethality = 100% – 0.298% = 99.7%

Table 2: Ectopic Expression of *Dll*, driven by different Gal4 lines @ 18°C

Males from the Gal4 lines were crossed to UAS-*Dll* virgin females. The crosses were placed at 18°C for entire duration of development. At this temperature setting, crosses performed with *dpp*-Gal4 yielded the best results. There are 18 escapers out of a total of 1569. This is a 96.5% lethality which is the lowest out of all temperature settings experimented with. This is therefore the optimal temperature to carry out crosses with the *dpp*-Gal4 line.

Fly Lines	Total number of flies	Number of Escapers	% Lethality
apterous-Gal4	2971	8	99.4
patched-Gal4	2637	1	99.9
omptomotor-blind-Gal4	2681	5	99.4
decapentaplegic-Gal4	1569	18	96.5

Sample calculation (*ap*-Gal4)

Theoretical yield = 2971/2 = 1485.5

% Viability = 8/1485.5 * 100 = 0.5385%

% Lethality = 100% – 0.5385% = 99.4%

Table 3: Ectopic Expression of *Dll*, driven by different Gal4 lines @ 25°C (5 days) and shifted to 18°C AEL

Male flies from each Gal4 line were crossed to UAS-*Dll* virgin females. The crosses were placed in the 25°C incubator. After five days the crosses were moved into an 18°C incubator for the remainder of development. At this temperature setting, *ap*-Gal yielded 22 escapers out of 2001 flies. This is 96.7% lethality, the best results obtained with *ap*-Gal4. Hence this is the optimal temperature setting to carry out crosses with *ap*-Gal4 line.

Fly Lines	**Total number of flies**	**Number of Escapers**	**% Lethality**
apterous-Gal4	2001	22	96.7
patched-Gal4	1788	0	100
omptomotor-blind-Gal4	1950	0	100
decapentaplegic-Gal4	1553	0	100

Table 4: Ectopic Expression of *Dll*, driven by different Gal4 lines @ 25°C (3 days) and shifted to 18°C AEL

Males from each Gal4 line were crossed to UAS-*Dll* virgin females and placed at 25°C. Three days after egg laying the crosses were transferred to 18°C. At this temperature setting *ap*-Gal4 and *dpp*-Gal4 yielded a fairly high number of escapers. They resulted in 98.1% and 97.6% lethality respectively. These are higher lethality levels than at the temperature settings established to be best for the two Gal4 lines.

Fly Lines	**Total number of Flies**	**Number of Escapers**	**% Lethality**
apterous-Gal4	1588	10	98.1
patched-Gal4	1246	0	100
omptomotor-blind-Gal4	1438	0	100
decapentaplegic-Gal4	1267	10	97.6

Table 5: Ectopic Expression of *Dll*, driven by different Gal4 lines @ 29°C (4 days) then shifted to 18°C AEL

Here the crosses were placed at 29°C for 3 days and then transferred to 18°C after egg laying. At this temperature setting, there is 100% lethality for all except *ap*-Gal4, which has 99.1% lethality. This temperature setting does not favour the development of any of the F1 from these crosses.

Fly Lines	**Total number of Flies**	**Number of Escapers**	**% Lethality**
apterous-Gal4	1325	4	99.1
patched-Gal4	1110	0	100
omptomotor-blind-Gal4	1307	0	100
decapentaplegic-Gal4	460	0	100

Table 6: Ectopic Expression of *Dll*, driven by several Gal4 lines @ 29°C (3 days) then shifted to 18°C AEL

Here the crosses were placed at 29°C for three days and then shifted to 18°C for the remainder of development. At this temperature, there are not enough escapers from any of the crosses. There is 100% lethality for all except *ap*-Gal4 which has 98.4%. This temperature setting does not favour the development of F1 flies from these crosses.

Fly Lines	**Total number of Flies**	**Number of Flies**	**% Lethality**
apterous-Gal4	1128	6	98.4
patched-Gal4	547	0	100
omptomotor-blind-Gal4	1156	0	100
decapentaplegic-Gal4	735	0	100

Figure 2: Ectopic expression of *Dll* driven by *apterous*-Gal4 and *dpp*-Gal4.

Panel A & B: This figure shows an escaper fly with the genotype *ap>Dll*. There is a transformation of wing tissues to ectopic leg tissues. On the right (B) is a close up of ectopic leg tissue growing out of the hinge region of the wing.

Panel C & D: A scanning electron microscope image of a fly with the genotype *dpp>Dll*. These flies have malformed legs and wings. The wings have coarse leg-like bristles as well as ectopic antenna formation. (D) A close up image of the transformed wing displaying ectopic antennae on the wing surface (arrowheads).

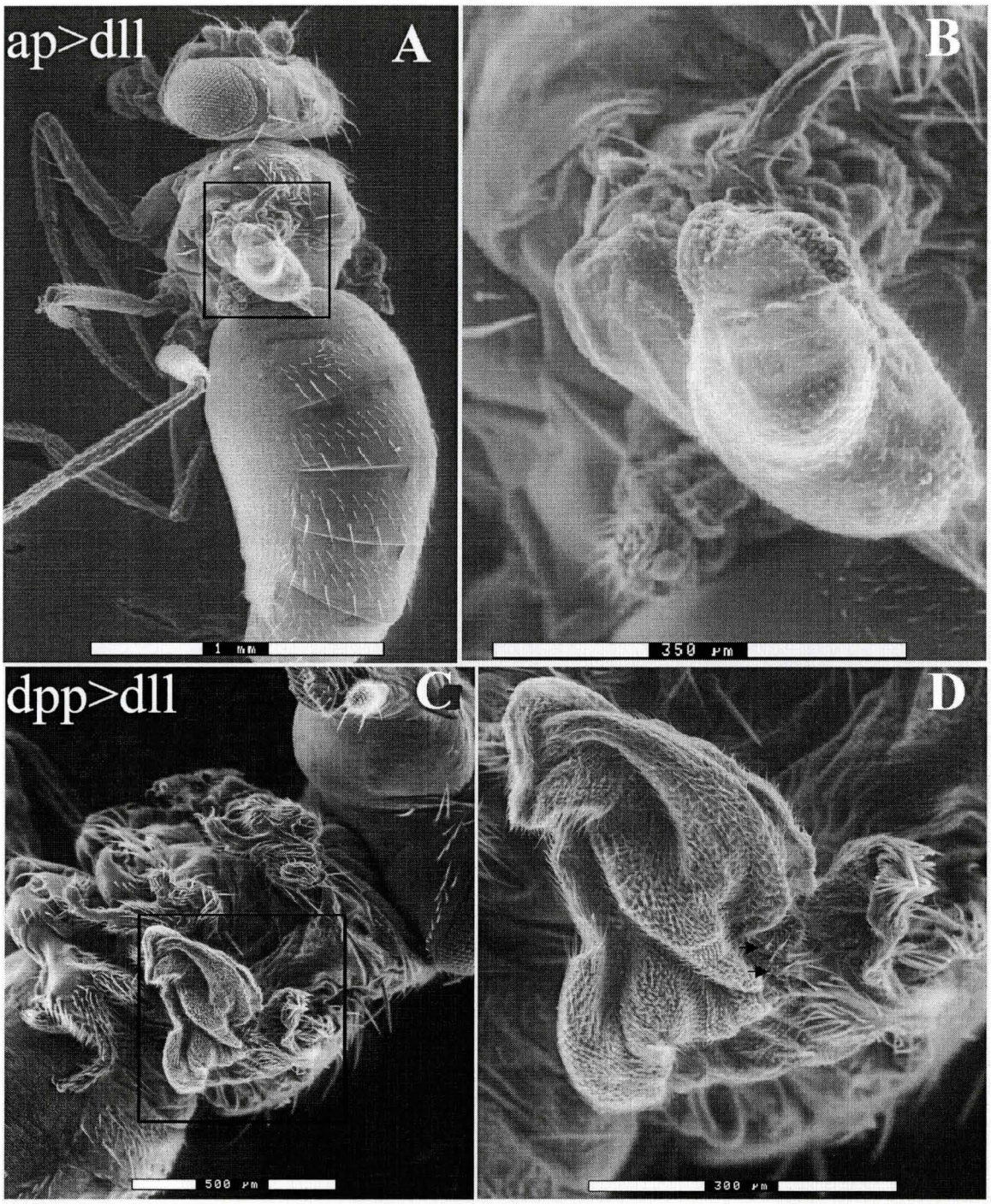

ap>dll
A
1 mm
B
350 µm
dpp>dll
C
500 µm
D
300 µm

Table 7: Ectopic expression of *Dll* driven by *ap*-Gal4 using the second UAS-*Dll line**

Here *Dll* was ectopically expressed using the new UAS-*Dll** line. The new line was crossed to *ap*-Gal4 and the experiment performed with the temperature settings 25°C for 4 days and then transferred to 18°C after egg laying. With this new UAS-*Dll** line, the lethality was reduced to 75.4% and a much more escapers were obtained.

Genotype	**Number of Flies**	
	Females	**Males**
ap-Gal4/UAS-*Dll* (escapers)	142	138
UAS-*Dll*/CyO	1141	

Table 8: Ectopic expression of *Dll* driven by *dpp*-Gal4 using the new UAS-*Dll line**

Here *Dll* was ectopically expressed by crossing UAS-*Dll** to *dpp*-Gal4. The crosses were placed at 18°C for the entire duration of development. The new UAS-*Dll** crossed to *dpp*-Gal4 results in 60.6% lethality, a 35.9% higher viability than earlier crosses made with UAS-*Dll* which had 96.5% lethality.

Genotype	**Number of Flies**	
	Females	**Males**
UAS-*Dll*/+ ; *dpp*-Gal4/+ (escapers)	191	98
UAS-*Dll*/+ ; +/TM3.Sb	407	327

Figure 3: Various phenotypes caused by ectopic expression of *Dll* driven by *ap*-Gal4 at 25°C (5 days) then shifted to 18°C AEL

UAS-*Dll** driven by *ap*-Gal4 results in a range of phenotypes. (A) Mild phenotype where the wing has a few "crumpled" areas but largely looks wildtype. (B) Intermediate phenotype. The wings here grow to wildtype size but are severely deformed. The thorax area is also mildly deformed. (C) Severe phenotype. There are no wings at all and the thorax is extremely deformed.

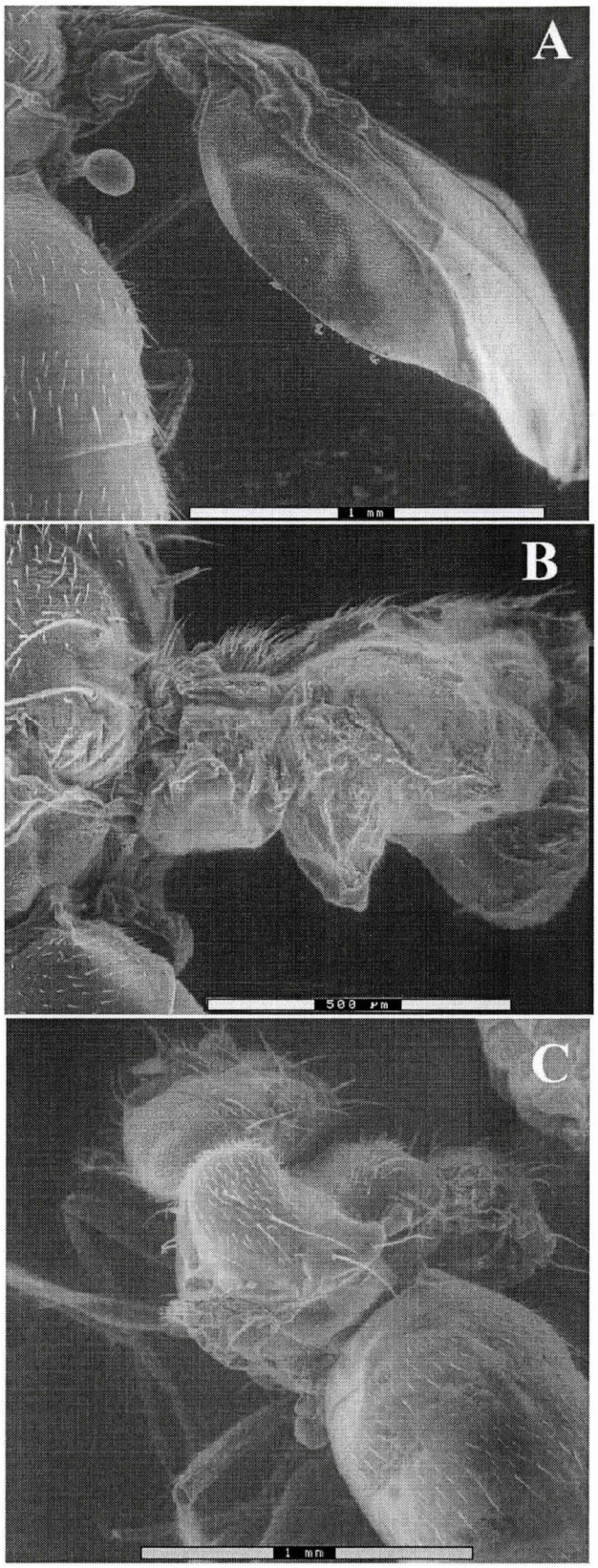

A
1 mm
B
500 µm
C
1 mm

Figure 4: Phenotypes caused by ectopic expression of *Dll* driven by *dpp*-Gal4 at 18°C AEL

UAS-*Dll** driven by *dpp*-Gal4 results in phenotypes milder than those of UAS-*Dll*. Most of the flies have wings that appear crumpled throughout the length of the wings. There are long bristles on the surface of the wings that suggest ectopic arista formation with (A). (B) A close up of ectopic arista on the wing surface (arrows). (C) A few flies have large outgrowths on the surface of their wings. (D) Ectopic leg-like structure growing in place of a wing.

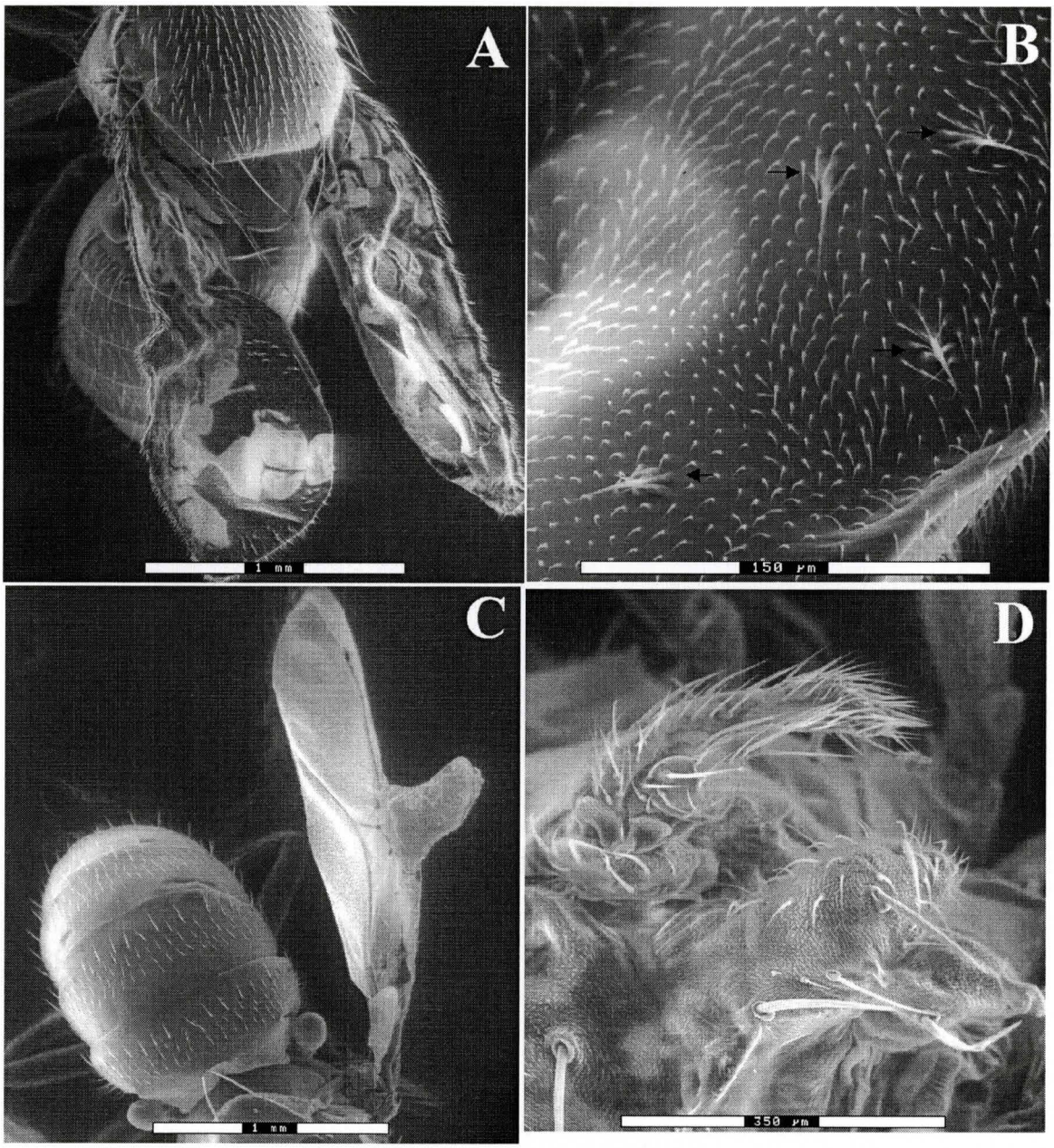

A
1 mm
B
150 µm
C
1 mm
D
350 µm

Figure 5: LacZ expression indicating ectopic disco expression in wing discs.

Panel A: This figure shows a wing disc from a wildtype fly. There is no lacZ expression and therefore no *disco* gene expression present in wing discs.

Panel B & C: These figures show wing discs from flies with the genotypes C50/x(y);*ap*-Gal4/UAS-*Dll* (B) and C50/x(y);UAS-*Dll;dpp*-Gal4 (C). Discs from both sets of flies show X-gal staining, meaning that there was ectopic *disco* gene expression in these tissues. The staining detected was very faint, suggesting that the level of *disco* expression was very low.

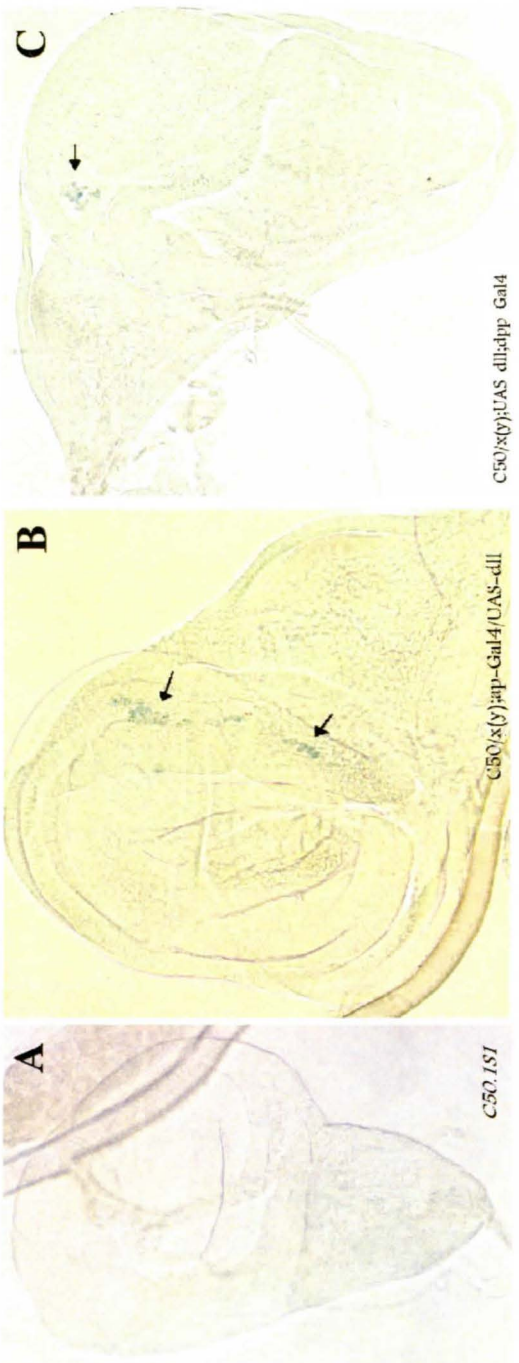

C
C50/x(y);UAS dll;dpp Gal4
B
C50/x(y);ap-Gal4/UAS-dll
A
C50.1SI

Figure 6: Ectopic expression of *Dll* results in ectopic activation of *disco*

In this experiment, a more sensitive method was used to detect the presence of β-galactosidase in the wing discs of C 50/x(y);*ap*-Gal4/UAS-*Dll* (C) and C50/x(y);UAS-*Dll;dpp*-Gal4 (D) flies. Anti β-Galactosidase antibody was used to detect the presence β-galactosidase in the wing discs and was visualized by staining with DAB. (A) Wings from C50/x(y);*ap*-Gal4/UAS-*Dll* the presence of β-gal in large areas of the wings. (B) Wings from C50/x(y);UAS-*Dll;dpp*-Gal4 show β-gal staining in a few locations (arrows).

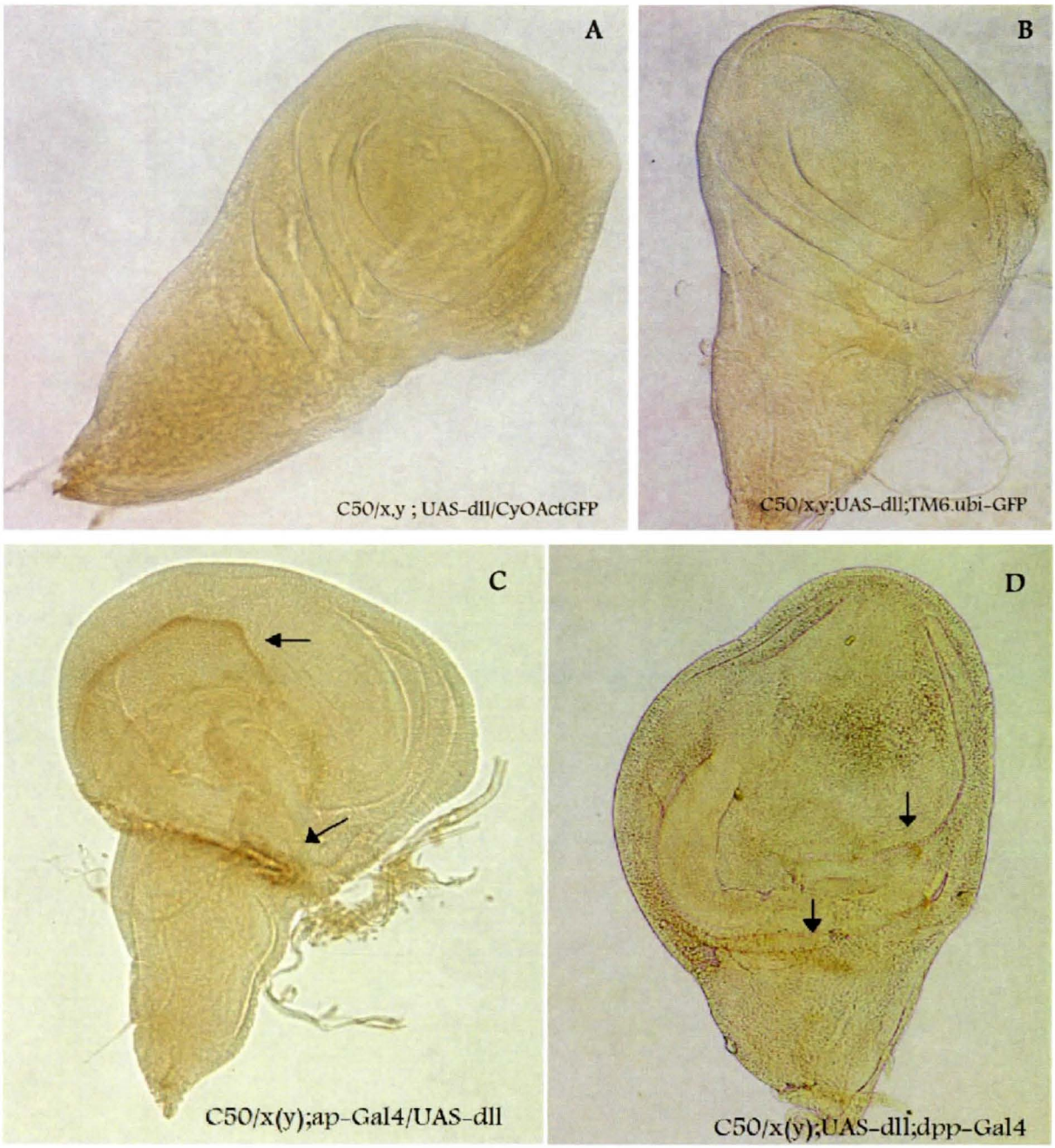

A
C50/x,y ; UAS-dll/CyOActGFP
B
C50/x,y;UAS-dll;TM6.ubi-GFP
C
C50/x(y);ap-Gal4/UAS-dll
D
C50/x(y);UAS-dll;dpp-Gal4

Table 9: Testing UAS-*disco*^{DN} and UAS-*disco*^{RNAi} fly lines

Virgin females were collected from UAS-*disco*^{DN} and UAS-*disco*^{RNAi} fly lines and crossed to *Dll*-Gal4 males. Downregulation of *disco* in the *Dll* domain leads to partial lethality as well as partial antennae to leg transformation (Dey 2006). These results were replicated successfully, therefore both fly lines are still effective in silencing *disco* expression.

Fly Lines	**% Lethality**	**% Of escapers with phenotype**
UAS-*disco* ^{DN} (MutD5)	76.9 (n=708)	100
UAS-*disco* ^{RNAi}	80.5 (n=685)	100
UAS-*disco* ^{DN} (Full length w/ ZF mutation)	97.7 (n=626)	100

Table 10: Ectopic expression of *Dll* driven *ap*-Gal4 while knocking down *disco*

The cross a*p-Gal4/CyO* X x/y;UAS-*Dll*/CyO;MutD5/TM3.Sb was performed using the temperature settings 25°C (5days) to 18°C. Flies carrying *ap*-Gal4, UAS-*Dll* and UAS-$disco^{DN}$, have a reduced lethality, at 96.1% while those carrying only *ap*-Gal4 and UAS-*Dll* have 97.7% lethality. However, both set of flies display the same phenotypes.

F1 Genotype	Number of flies	
	Females	Males
x/x,y;*ap*-Gal4/UAS-*Dll*;MutD5/+	12	10
x/x,y;*ap*-Gal4/UAS-*Dll*;+/TM3	9	4
x/x,y;*ap*-Gal4/CyO; MutD5/+	552	563
x/x,y;UAS-*Dll*/CyO;MutD5/+		
x/x,y; *ap*-Gal4/CyO;+/TM3	256	245
x/x,y; UAS-*Dll*/CyO;+/TM3	231	244

Sample Calculation

Theoretical yield = (552 + 563)/2 = 557.2

Actual yield = 12 + 10 = 22

% Viability = 22/557.2 * 100 = 3.95%

% Lethality = 100% - 3.95% = 96.1%

Stats Sample Calculation

t_s = (arcsin $\sqrt{}$ p1 - arcsin$\sqrt{}$p2) ÷ [$\sqrt{}$820.8 (1/n1 + 1/n2)]

= (78.6 – 81.3)/10.02 = -0.269

P = 0.378 therefore % Lethality 1 is not significantly lower than % Lethality 2

Table 11: Ectopic expression of *Dll* (UAS-*Dll) driven by *ap*-Gal4 while knocking down *disco* gene**

The cross *ap*-Gal4/CyO X x/y;UAS-*Dll**/CyO;MutD5/TM3.Sb was performed at the temperature setting 25°C (5days) to 18°C. Flies carrying *ap*-Gal, UAS-*Dll**, and UAS-$disco^{DN}$, have a 73.1% lethality and those without the dominant negative have a slightly higher lethality at 74.0%. However, both sets of flies have identical phenotypes.

F1 Genotype	**Number of flies**	
	Females	**Males**
x/x,y;*ap*-Gal4/UAS-*Dll**;MutD5/+	99	92
x/x,y;*ap*-Gal4/UAS-*Dll**;+/TM3	100	85
x/x,y;*ap*-Gal4/CyO; MutD5/+	741	680
x/x,y;UAS-*Dll**/CyO;MutD5/+		
x/x,y; *ap*-Gal4/CyO;+/TM3	415	391
x/x,y; UAS-*Dll**/CyO;+/TM3	427	328

Figure 7: Ectopic expression of *Dll* driven by *ap*-Gal4 while knocking down *disco* gene

Electron microscope images of flies in which ectopic expression of *Dll* is driven by *ap*-Gal4 while *disco* gene product is being knocked down.

Panel A & B: A fly with the genotype x/x,y;*ap*-Gal4/UAS-*Dll**;MutD5/+. This fly is has ectopic leg present at the hinge region of the wing even though ectopic *disco* expression is being down regulated by the presence of UAS-$disco^{DN}$. (B) A close up image of the ectopic leg structure. The presence of the UAS-$disco^{DN}$ did not suppress the phenotypes associated with ectopic expression of *Dll* driven by *ap*-Gal4.

Panel C & D: Internal control flies with the phenotype *x*/x,y;*ap*-Gal4/UAS-*Dll*;+/TM3. These flies do not have the UAS-$disco^{DN}$ in their genotype. This image shows a fly with ectopic leg at the hinge region of the wing. (C) A close up view of the hinge region with ectopic leg structure.

Panel E & F: Flies in which the UAS-*Dll* as been replaced with the new UAS-*Dll**. Panel E shows a fly with the genotype *x*/x,y;*ap*-Gal4/UAS-*Dll**;MutD5/+ and panel F shows a fly with the genotype *x*/x,y;*ap*-Gal4/UAS-*Dll**;+/TM3. There is no difference between the phenotypes of the two flies despite the presence of UAS-$disco^{DN}$ in the genotype of flies in panel E.

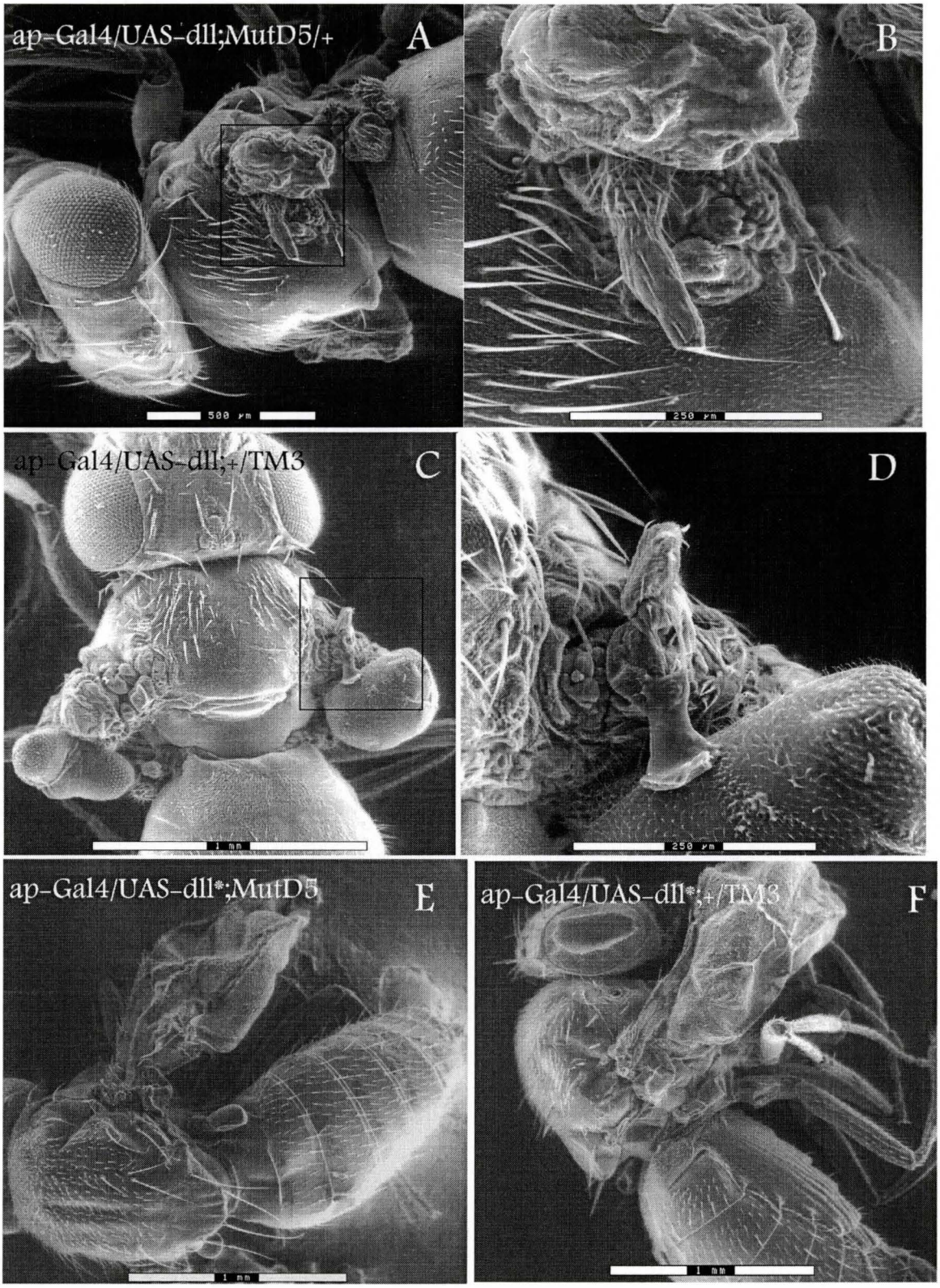

ap-Gal4/UAS-dll;MutD5/+
A
500 µm
B
250 µm
ap-Gal4/UAS-dll;+/TM3
C
1 mm
D
250 µm
ap-Gal4/UAS-dll*;MutD5
E
1 mm
ap-Gal4/UAS-dll*;+/TM3
F
1 mm

Table 12: Ectopic expression of *Dll* driven by *dpp*-Gal4 while knocking down *disco*

The cross x/x;+/+;*dpp*-Gal4/TM3.Sb X x/y;UAS-*Dll*/CyO;MutD5/TM3.Sb was performed and placed at 18°C for the entire duration of development. Flies carrying *dpp*-Gal4, UAS-*Dll* and UAS-$disco^{DN}$ have more escapers with 72.7% lethality and than those carrying only *dpp*-Gal4 and UAS-*Dll* have 81.2% lethality. However both sets of flies display the same phenotypes.

F1 Genotype	Number of flies	
	Females	Males
x/x,y;UAS-*Dll*/+;MutD5/*dpp*-Gal4	61	90
x/x,y;UAS-*Dll*/+;*dpp*-Gal4/TM3.Sb	46	58
x/x,y;UAS-*Dll*/+;MutD5/TM3.Sb	297	282
x/x,y;+/CyO;MutD5/*dpp*-Gal4	271	258
x/x,y;+/CyO;MutD5/TM3.Sb	234	159
x/x,y;+/CyO; *dpp*-Gal4/TM3.Sb	224	261

Sample Calculation

Theoretical yield = (297 + 282 + 271 +258)/2 = 554

Actual yield = 61 + 90 = 151

% Viability = 151/554 * 100 = 27.2%

% Lethality = 100% - 27.2% = 72.7%

Stats Sample Calculation

t_s = (arcsin $\sqrt{}$ p1 - arcsin$\sqrt{}$p2) ÷ [$\sqrt{}$820.8 (1/n1 + 1/n2)]

= (58.5 – 64.3)/3.65 = -1.59

P = 0.0537 therefore % Lethality 1 is not significantly lower than % Lethality 2

Table 13: Ectopic expression of *Dll* (UAS-*Dll) driven by *dpp*-Gal4 while knocking down *disco***

The cross x/x;+/+;*dpp*-Gal4/TM3.Sb X x/y;UAS-*Dll**/CyO;MutD5/TM3.Sb was placed at 18°C for the entire duration of development. Again with the new UAS-Dll* line, the level of lethality was drastically reduced. Flies carrying *dpp*-Gal4, UAS-*Dll**, and UAS-$disco^{DN}$, have 53.4% lethality while and those without the dominant negative have 63.9% lethality. The phenotypes of both sets of flies are the same.

F1 Genotype	**Number of flies**	
	Females	**Males**
x/x,y;UAS-*Dll**/+;MutD5/*dpp*-Gal4	101	98
x/x,y;UAS-*Dll**/+;*dpp*-Gal4/TM3.Sb	71	83
x/x,y;UAS-*Dll**/+;MutD5/TM3.Sb	231	184
x/x,y;+/CyO;MutD5/*dpp*-Gal4	230	209
x/x,y;+/CyO;MutD5/TM3.Sb	189	135
x/x,y;+/CyO; *dpp*-Gal4/TM3.Sb	200	189

Figure 8: Ectopic expression of *Dll* driven by *dpp*-Gal4 while knocking down *disco*

Electron microscope images of flies in which *Dll* is being ectopically expressed while *disco* is being down regulated.

Panel A & B: Flies with the genotype x/x,y;UAS-*Dll*/+;MutD5/*dpp*-Gal4 (A) and x/x,y;UAS-*Dll*/+;*dpp*-Gal4/TM3.Sb (B). Both sets of flies display similar phenotypes. The wings are un-inflated and there are arista-like bristles on the wing surfaces. The presence of the UAS-$disco^{DN}$ did not suppress the phenotypes associated with ectopic *Dll* expression driven by *dpp*-Gal4.

Panel B & C: Flies with the genotype x/x,y;UAS-*Dll**/+;MutD5/*dpp*-Gal4 (C) and x/x,y;UAS-*Dll**/+;*dpp*-Gal4/TM3.Sb (C). These flies carry the new UAS-*Dll**. The phenotypes between both sets of flies are similar. Both sets of flies have wings that are "crumpled" throughout the wing. The presence of the UAS-$disco^{DN}$ did not suppress the phenotypes.

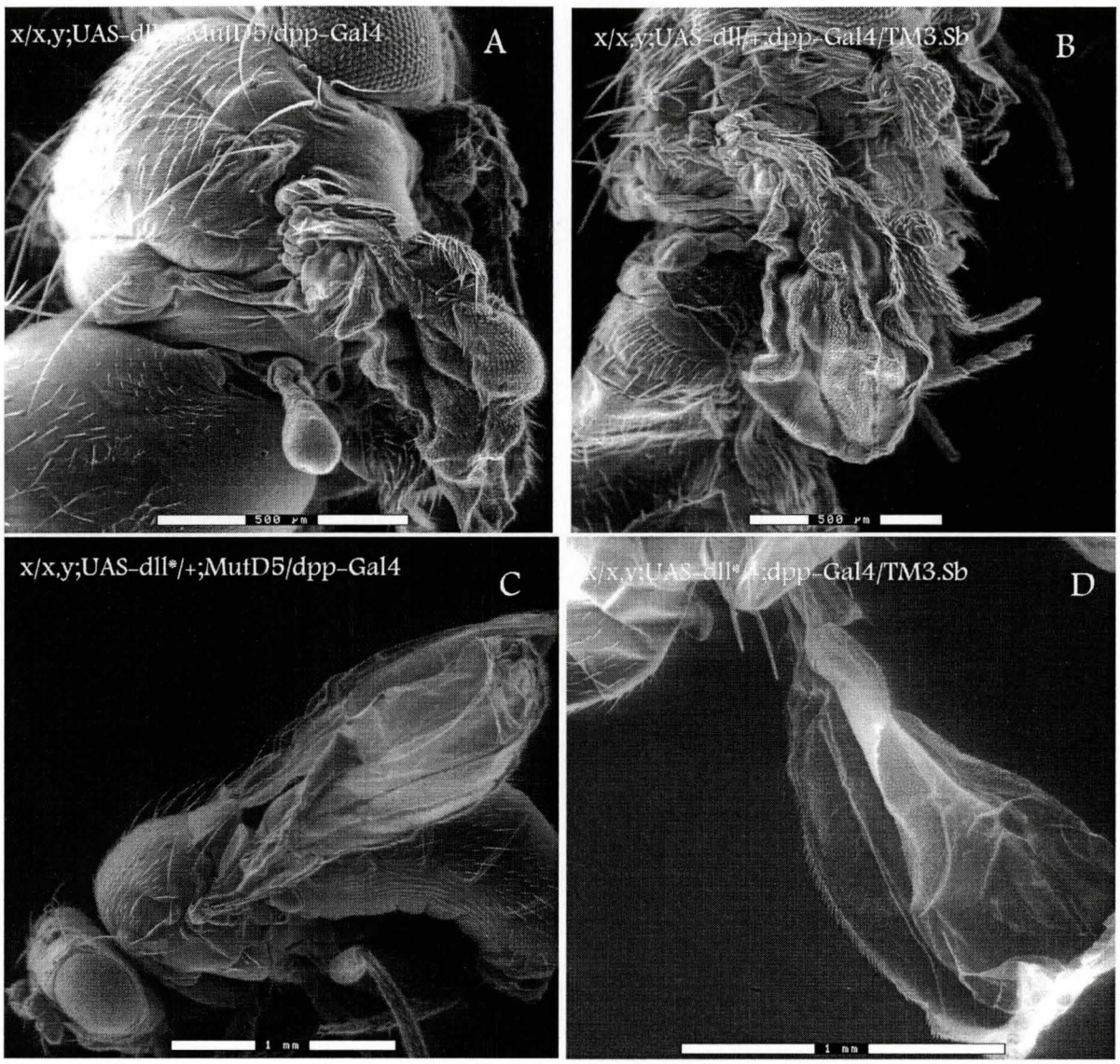

A
500 µm
B
500 µm
x/x,y;UAS-dll*/+;MutD5/dpp-Gal4
C
1 mm
D
1 mm

Table 14: Ectopic expression of *Dll* driven by *ap*-Gal4 while down regulating *disco* expression with UAS-*disco*^{RNAi}

The cross *ap*-Gal4/CyO X x/y;UAS-*Dll*/CyO;*disco*^{RNAi}/TM3.Sb was performed with the temperature setting 25°C (5days) to 18°C. Flies carrying *ap*-Gal4, UAS-*Dll* and UAS-*disco*^{RNAi} have more escapers with 90.8% while those carrying only *ap*-Gal4 and UAS-*Dll* have a slightly higher lethality level at 94.0%. Again the presence of the UAS-*disco*^{RNAi} did not suppress the phenotypes of *ap>Dll* flies.

F1 Genotype	Number of flies	
	Females	**Males**
x/x,y;*ap*-Gal4/UAS-*Dll*; *disco*^{RNAi}/+	42	23
x/x,y;*ap*-Gal4/UAS-*Dll*;+/TM3	26	16
x/x,y;*ap*-Gal4/CyO; *disco*^{RNAi}/+	749	662
x/x,y;UAS-*Dll*/CyO; *disco*^{RNAi}/+		
x/x,y; *ap*-Gal4/CyO;+/TM3	343	321
x/x,y; UAS-*Dll*/CyO;+/TM3	360	284

Sample Calculation

Theoretical yield = (749 + 662)/2 = 705.5

Actual yield = 42 +23 = 65

% Viability = 65/705.5 * 100 = 9.2%

% Lethality = 100% - 9.2% = 90.8%

Stats Sample Calculation

t_s = (arcsin $\sqrt{}$ p1 - arcsin$\sqrt{}$p2) ÷ [$\sqrt{}$820.8 (1/n1 + 1/n2)]

= (-3.48)/5.67 = -0.613

P = 0.2709 therefore % Lethality 1 is not significantly lower than % Lethality 2

Table 15: Ectopic expression of *Dll* driven by *dpp*-Gal4 while down regulating *disco* expression with UAS-*disco*^{RNAi}

The cross x/x;+/+;*dpp*-Gal4/TM3.Sb X x/y;UAS-*Dll*/CyO;*disco*^{RNAi}/TM3.Sb placed at 18°C for the entire duration of development. Flies carrying *dpp*-Gal4, UAS-*Dll* and UAS-*disco*^{RNAi} have more escapers with 71.8% while those carrying only *dpp*-Gal4 and UAS-*Dll* have 72.8%. Here also the presence of the *disco*^{RNAi} did not suppress the phenotypes of ectopic *Dll* expression.

F1 Genotype	**Number of flies**	
	Females	**Males**
x/x,y;UAS-*Dll*/+; *disco*^{RNAi}/*dpp*-Gal4	55	65
x/x,y;UAS-*Dll*/+;*dpp*-Gal4/TM3.Sb	60	56
x/x,y;UAS-*Dll*/+; *disco*^{RNAi}/TM3.Sb	231	231
x/x,y;+/CyO; *disco*^{RNAi}/*dpp*-Gal4	208	182
x/x,y;+/CyO;*disco*^{RNAi}/TM3.Sb	183	137
x/x,y;+/CyO; *dpp*-Gal4/TM3.Sb	226	201

Figure 9: Ectopic expression of *Dll* while down regulating *disco* expression with UAS-*disco*^{RNAi}

Electron microscope images of flies in which ectopic expression of *Dll* is accompanied by down regulation of *disco* by *disco*^{RNAi}.

Panel A & B: Panel A shows flies in which *ap*-Gal4 is driving UAS-*Dll* and panel B shows flies in which UAS-*Dll* and UAS-*disco*^{RNAi} are being driven by *ap*-Gal4. Both sets of flies show similar phenotypes.

Panel C & D: Panel C shows flies in which *dpp*-Gal4 is driving UAS-*Dll* and panel B shows flies in which *dpp*-Gal4 is driving UAS-*Dll* and UAS-*disco*^{RNAi}. Once again both sets of flies show similar phenotypes.

The presence of UAS-*disco*^{RNAi} did not suppress the phenotypes of driving UAS-*Dll* with *ap*-Gal4 or *dpp*-Gal4.

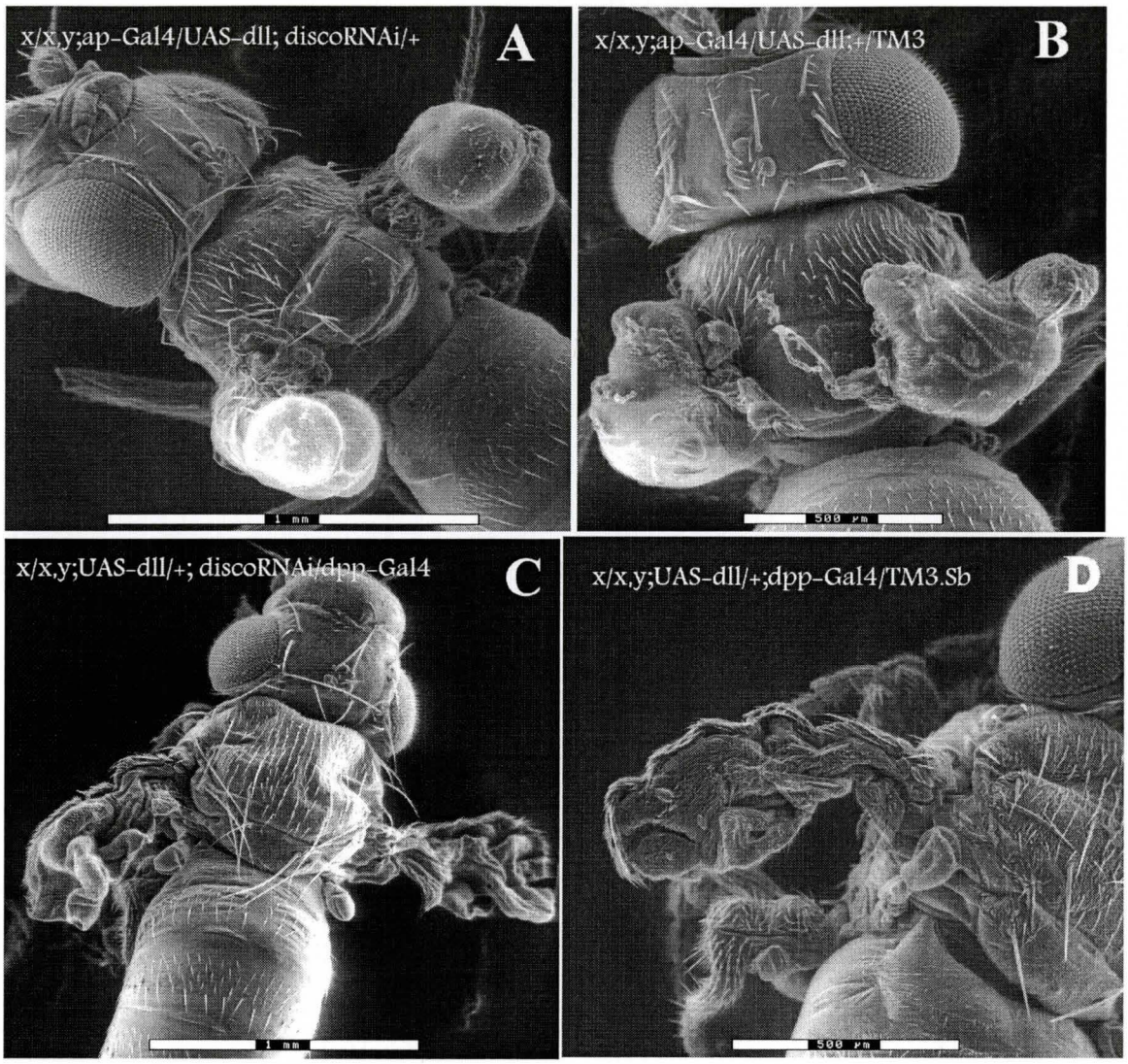
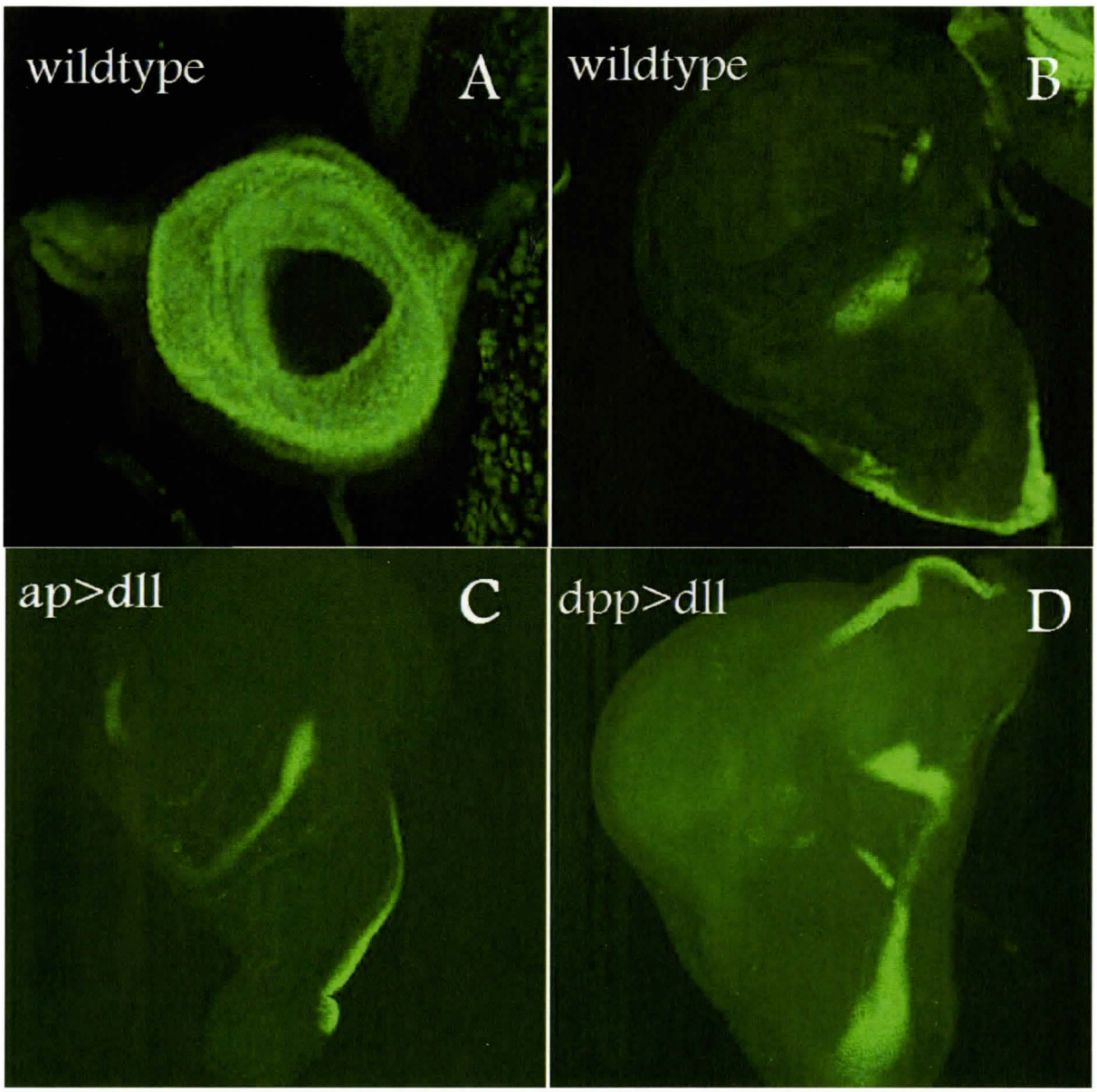

x/x,y;ap-Gal4/UAS-dll; discoRNAi/+
A
1 mm
x/x,y;ap-Gal4/UAS-dll;+/TM3
B
500 µm
x/x,y;UAS-dll/+; discoRNAi/dpp-Gal4
C
1 mm
x/x,y;UAS-dll/+;dpp-Gal4/TM3.Sb
D
500 µm

Figure 10: Confocal images of Drosophila imaginal discs showing *dac* expression

Imaginal discs were dissected and immunohistochemistry was performed to determine the activity of leg molecular marker *dac*. Images are a single taken at 20x magnification.

Panel A & B: Imaginal discs from wildtype flies serving as controls. (A) Leg disc showing *dac* expression. (B) Wing imaginal disc showing *dac* expression.

Panel C & D: Wing imaginal discs from flies in which *ap*-Gal4 is driving UAS-*Dll* (B) and *dpp*-Gal4 is driving UAS-*Dll* (C). There are changes in the expression pattern of these flies compared to wildtype wing discs. In addition, the shapes of the wing discs are different from wildtype.


wildtype
A
wildtype
B
ap>dll
C
dpp>dll
D

CHAPTER 4

DISCUSSION

The gene *Distal-less (Dll)* is responsible for the proximal-distal axis specification of *Drosophila* appendages, and to specify antennal identity (Cohen et al., 1989; Dong et al., 2000). *Dll* expression is initiated very early in the limb primordium and marks the first sign of leg identity (Cohen et al, 1989). Previous experiments showed that during development, maintenance of *Dll* expression requires the activity of Disco, a C_2H_2 zinc finger transcription factor (Dey, 2006; Lee et al., 1991). In the absence of *disco*, *Dll* expression is reduced, while the absence of *Dll* leads to a reduction in the expression of *disco* (Dey, 2006). *disco* expression can be found in many different tissues during development which suggests that *disco* is involved in several developmental events (Lee et al., 1991; Robertson et al., 2002). Dey (2006) demonstrated that *disco* plays a role in the *Dll*-dependent proximal-distal axis specification pathway. However, his experiments did not distinguish whether this role is simply to maintain the expression of *Dll* or that *disco* plays a role as a cofactor and/or as a downstream target of *Dll*. In this project, we perform experiments to determine which of these roles *disco* plays. Our results suggest that apart from maintaining *Dll* expression through a positive feedback loop, *disco* is not likely to be required downstream from Dll either as a cofactor or direct regulator of Dll-dependent genes.

Dll turns on _disco_ expression ectopically

Previous experiments show that *Dll* expression is sufficient to activate the *disco* promoter and turn on *disco* expression (Dey, 2006). In previous experiments, *Dll* was

ectopically expressed in a *disco* enhancer trap line by driving UAS-*Dll* with GMR-Gal4. LacZ expression was detected in the developing retina behind the morphogenic furrow which confirmed that *disco* expression was activated by the presence of *Dll* gene products (Dey, 2006). Here, we performed a similar experiment where *Dll* was ectopically expressed in the wing tissue of the same *disco* enhancer trap line, C50.1S1. Xgal staining was performed on the wing imaginal disc of flies carrying *ap*-Gal4>UAS-*Dll* and *dpp*-Gal>UAS-*Dll* in a C50.1S1 disco enhancer trap background. In both sets of flies, faint lacZ expression was detected, showing that there was *disco* expression ectopically in the wings tissues due to the presence of *Dll* (Figure 5B and C). The faintness of the LacZ expression detected may be due to the fact that imaginal discs from wandering 3rd instar larvae were used for this experiment. At this late stage, dorsal-ventral axis patterning must be complete. The expression of the gene responsible for specifying this axis, *apterous* (*ap*), may be reduced to levels much lower than it would have been at earlier stages of development. Low *ap* expression means that Gal4 expression would be low as well. The reduced Gal4 activity would result in a decrease in ectopic *Dll* activation. Similarly, at this late stage, the expression of *dpp*, a morphogen required for the growth of the wing may have been reduced since the imaginal disc is no longer growing. Gal4 activity would be lower than it would have been at earlier stages of development and ectopic *Dll* expression would be very low. Low levels of *Dll* gene products would result in a weak activation of *disco* promoter by *Dll* resulting in the low *disco* expression that was observed.

Since the Xgal staining was so faint, an alternate method of detecting *disco* expression was attempted. Immunohistochemistry was performed, using anti-β-Gal antibodies to detect lacZ expression in the wing tissues of *ap>Dll* and *dpp>Dll* flies. This method was more successful for the detection lacZ expression in the wing discs. In *ap>Dll* flies, lacZ expression is present extensively throughout the wing discs (Figure 6C). This result showed that the presence of *Dll* gene products is sufficient to activate the *disco* promoter and presumably turn on *disco* expression ectopically in the wing discs. *Disco* expression in *dpp>Dll* wing imaginal discs was less pronounced than that of the *ap>Dll* discs. There were only a few scattered patches of the β-gal staining detected in *dpp>Dll* wing discs (Figure 6D). In the future this experiment should be performed using imaginal wing discs from earlier stages of development when the wing discs are still growing. *dpp* expression may be much higher at these stages such that the resultant high Gal4 activity will drive *Dll* expression high enough to induce a higher level of ectopic *disco* expression.

Dll turns on downstream targets ectopically in wing tissues

As part of its role in PD axis specification, *Dll* regulates subordinate genes that are responsible for the differentiation and growth of various parts of developing appendages. Examples of genes that are regulated by *Dll* are *bric a brac* (*bab*) (Gorfinkiel et al., 1997), *spineless* (*ss*) (Duncan et al., 1998), *aristaless* (*al*) (Campbell and Tomlinson, 1998), and *dachshund* (*dac*) (Dong et al., 2001). Each of these genes, along with other *Dll* target genes, have specific roles in the growth and differentiation of the developing

appendages. The absence of any one of these genes leads to phenotypes that can be described as a subset of *Dll* mutant phenotypes (reviewed by Panganiban and Rubenstein, 2002). Given that in the absence of *Dll*, the expression of all of these target genes is lost, mutations in *Dll* result in phenotypes that are a combination of the phenotypes obtained by mutations of downstream target genes.

We decided to examine the expression patterns of *dac* while *Dll* is being ectopically expressed in the wing tissues. Normally, *dac* is expressed in, and is required for proper development of the leg and antennae, and the eyes. Weak *dac* mutants have a rough and reduced eye while null mutants have eyes that are either severely reduced or completely missing (Mardon et al., 1994). *dac* mutants have legs that are reduced in overall length even though they have proper proximal distal morphology. The intermediate segments are fused together while the coxa, trochanter, the fourth and fifth tarsal segments and claws are normal (Mardon et al., 1994). *dac* is also required for proper antenna formation. Mutants have a fusion between segments of the antennae (Dong et al., 2001).

To investigate the presence or absence of *dac* expression, anti-*dac* antibody was used to detected *dac* protein in the wing imaginal discs (Figure 10). We hypothesised that there would be no expression of *dac* in wildtype wing discs and that *dac* expression would be activated in the wing discs by ectopic *Dll* expression. However, immunohistochemistry showed that *dac* is expressed in both the wing discs of wildtype and experimental flies. When the two sets of wing discs were compared, *dac* expression

patterns in the experimental flies (*ap>Dll* and *dpp>Dll*) were different from those of wildtype flies (Figure 10). *dac* was detected in areas of the mutant wing discs where there were no *dac* expressions in wildtype discs. This shows that the presence of *Dll* may have activated *dac* expression in areas of the wing where it is not normally expressed. Furthermore, the shapes of the wing discs from experimental flies were severely deformed, indicating a transformation of the wing. These results suggest that the ectopic presence of *Dll* causes transformation of wing tissues by activating its downstream genes that disrupt normal growth of the wing discs.

Disco is not required as a cofactor of *Dll* for the differentiation and growth of *Drosophila* appendages

Dey (2006) suggested that *disco* functions as a cofactor of *Dll* and that its function is required along with *Dll* to turn on subordinate genes. Dll is a homeodomain containing protein (Cohen et al., 1989; Diaz-Benjumea et al., 1994). Past experiments showed that all homeodomain proteins have very poor DNA binding specificities in vitro despite having very specific actions in vivo (reviewed by Hueber and Lohmann, 2008). In vitro, these proteins bind to similar nucleotide sequences that possess an -ATTA- core. However, in vivo, Hox proteins have the ability to target a variety of genes. A single Hox gene can regulate the expression of number of genes based on tissue or developmental stage (reviewed by Hueber and Lohmann, 2008). It is now known that other factors contribute to the specificity of Hox proteins. It is thought that Hox proteins form heterodimers with cofactors that would allow the proteins to recognize their targets with

higher specificity. Confidence for this theory grew due to the discovery of the mechanism of action of yeast transcription factors a1 and α2. Individually each transcription factor binds very poorly to DNA. However, when they form a complex with each other, they bind to DNA with much more specificity (reviewed by Hueber and Lohmann, 2008).

Zinc finger proteins may work in concert with homeodomain containing proteins to confer higher specificity for DNA binding sites. In addition to binding to DNA, zinc finger proteins have the ability to bind to other proteins or to RNA as well. Depending on the amino acid sequence and the number fingers, they can bind to multiple targets simultaneously (reviewed by Iuchi, 2001). Hence it is possible that zinc finger proteins bind to homeodomain containing proteins and work together to increase specificity for DNA binding sites and to regulate genes. An example illustrated by Roder et al., 1992 shows that *teashirt* (*tsh*), a zinc finger protein is required by trunk homeotic genes such as *sex combs reduced* (*scr*), *antennapedia* (*antp*) and *ultrabithorax* (*ubx*) for proper trunk identity. For example, in the absence of *tsh*, *scr* is unable to induce proper formation of anterior prothoracic structures (Roder et al., 1992)

Dll may work in a similar manner, in concert with a zinc finger protein to increase specificity and allow it to regulate downstream targets. If *Dll* requires the action of *disco* gene products to regulate downstream targets, then, even when expressed normally, *Dll* would be unable to turn on target genes such as *bab*, *ss*, *dac*, etc in the absence of *disco* expression. Furthermore, the absence of *disco*, would mimic a *Dll* null mutant since *Dll*

on its own would be unable to direct the development and growth of Drosophila appendages.

Results of the experiments in this project suggest that *disco* does not play such a role. We ectopically express *Dll* while knocking down *disco* gene function that would be produced due to the activation of the *disco* promoter by Dll proteins. Since *Dll* requires Disco activity to maintain its own expression, *Dll* would normally be down regulated in the absence of *disco* gene expression (Dey 2006). In our experiments, ectopic *Dll* expression was driven by a heterologous promoter. The yeast transcription factor Gal4 is expressed under the control of the promoter of *ap* or *dpp*. The Gal4 activity induces the upstream activation unit (UAS) and drives the expression of the gene next to it, in this case, *Dll*. Thus, in the absence of *disco*, *Dll* expression persists, due to the action of the Gal4 transcription factor.

We observed that ectopic expression of *Dll* while knocking down *disco* gene product still leads to the formation of ectopic leg structures, similar to those formed when *Dll* is ectopically expressed without knocking down *disco* (Figure 7). This suggests that *Dll* does not require Disco function as a cofactor to pattern and direct the growth of *Drosophila* appendages. It is able to activate the necessary downstream target genes required for this process.

Studies by Mahaffey et al. (2000) lead to the discovery of two ORF within the 14AB region that coded for the exons of a gene closely related to *disco*. They termed this gene *disco-related* (*disco-r*). More importantly they found that there is redundancy in the

functions of these two genes during gnathal lobe development. Downregulation of either *disco* or *disco-r* resulted in very minor or no detrimental effects on development. However, knocking down both genes resulted in severe developmental abnormalities in the mouthparts of unhatched larvae. It is possible that *disco* and *disco-r* have such redundancy during *Dll*-dependent appendage development as well. The absence of *disco* gene products may not be sufficient to determine that *Dll* does not require a cofactor to direct appendage development. The presence of *disco-r* products may act as a replacement, and function as a cofactor for *Dll*. Future experiments should attempt to knock down both *disco* and *disco-r* while ectopically expressing *Dll*. This will help to determine whether *Dll* alone is sufficient for regulation of target genes and proximal distal axis specification.

Disco does not act as a downstream target required to differentiate and direct growth of a subsection of Drosophila appendages

Dey (2006) also suggested that *disco* may be required downstream of *Dll* as a target gene that function in the same manner as other subordinate genes that are regulated by *Dll*. For example, *bab* is a *Dll* target gene that codes for a protein expressed in ventral imaginal discs but not in dorsal imaginal discs. It is required for proper development of the tarsi (Godt et al., 1993). *Bab* mutation causes a change in the bristle patterns on the tarsal segments of the legs and ectopic sex combs on TS2, TS3, and TS4 of male flies. Also, joints between segments TS4 and TS5 of the tarsi are missing so that there is a fusion of these tarsal segments (Godt et al., 1993). The gene *spineless* (*ss*) plays a role in

the formation of the distal regions of the antennae and leg. Mutations in *ss* lead to the deletion of distal leg and reduction in the size of most leg bristles. Also in the absence of this gene, distal parts of the antennae are transformed to leg (Duncan et al., 1998). *aristaless* (*al*) is expressed in the cells at the distal tip of adult leg and is responsible for the differentiation and growth of these cells. In *Dll* mutants, *al* expression is lost in leg imaginal discs. Mutation or loss of *al* results in loss of the tip of the adult legs leading to a deletion of adult claws (Campbell and Tomlinson, 1998).

If *disco* plays a role similar to other *Dll* target genes, it would perform a specific function in the appendage development process and would be responsible for patterning a specific section of the appendage. Its absence would lead to phenotypes that are a subset of *Dll* phenotypes i.e. appendages missing specific parts of their structure. As mentioned earlier, in the absence of *disco* gene products, the formation of ectopic leg *still* occurs when *Dll* is ectopically expressed in wings tissues (Figure 7). The ectopic leg structures resemble distal part of the leg, which is the part that *Dll* is required for. It is difficult to tell if all distal parts of a leg are present in the ectopic leg structures. Therefore we cannot conclude that the absence of *disco* results in deletion of leg parts. However, previous characterization of *disco* mutant phenotype did not indicate that it represents a subset of *Dll* mutant phenotypes. Rather disco mutant phenotype resembles the phenotype of hypomorphic *Dll* mutants suggesting that *disco* is not responsible for the development of a specific part of the leg.

Put together, results obtained in this project suggest that *disco* does not function as a cofactor or as a downstream target of *Dll*. The role of *disco* is primarily to maintain *Dll* expression through a positive feedback loop while *Dll* activates the expression of downstream targets and directs the growth of the appendages. In the absence of *disco*, *Dll* expression is reduced and *Dll* is unable to direct the growth and patterning of *Drosophila* appendages. This is what leads to the observed *Dll*-like phenotypes in *disco* mutant flies.

Future experiments

Can *Dll* activate downstream targets in the absence of *disco*?

There should be further experimentation to investigate the ability of *Dll* to activate its downstream targets ectopically in the wing discs. Due to time constraints, we were only able to investigate one downstream *Dll* target, namely *dac*. It turned out that *dac* was not the best candidate for this experiment because observation of wildtype wing discs showed that *dac* is normally expressed in the wing discs of wildtype flies. When compared to each other, the only difference between wildtype flies and *ap>Dll* or *dpp>Dll* flies was the expression pattern of *dac*. The best *Dll* target gene to use for this experiment would be ones that are only expressed in *Dll* domains of the legs and antennae but not in the wing discs. Tests can then be conducted to determine if *Dll* can turn these genes on ectopically when *Dll* is expressed in wing tissues.

This experiment should be carried a step further to test the ability of *Dll* to activate target genes in the absence of *disco*. *Dll* should be ectopically expressed in the wing discs while knocking down *disco* gene products. Immunohistochemistry should then be used to determine if *Dll*, in the absence of *disco* could activate the expression of downstream target genes. This can be accomplished by constructing flies carrying UAS-*Dll*, UAS-*disco*^{DN} or UAS-*disco*^{RNA}, and *ap*-Gal4 or *dpp*-Gal4. Wing imaginal discs from these flies can be dissected out and tested for the presence of the appropriate *Dll* targets. This experiment will be informative in determining whether *Dll* requires *disco* as a cofactor to regulate subordinate genes.

Can *Dll* rescue *disco* mutant phenotypes?

Previous experiments show that *disco* mutants display *Dll*-like phenotypes. There is approximately 97% lethality and escapers have leg and antennae phenotypes similar to those of *Dll* mutants (Dey, 2006). Future experiments should attempt to determine if these phenotypes were due to downregulation of *Dll* that results because of the absence of *disco*, or a requirement of *disco* in the genetic pathway that directs PD axis specification. To do this, experiments can be performed where expression of *Dll* is driven in the *Dll* domains of *disco* mutant flies. This experiment can be performed by constructing flies carrying *Dll*-Gal4 and UAS-*Dll* in a *disco* mutant background. In these flies, *Dll* expression will be driven by the Gal4 transcription factor, thus downregulation of *Dll* due to the absence of *disco* can be avoided. The phenotypes of these flies should then be

compared to those of *disco* mutants to see if maintaining *Dll* expression rescues the *disco* mutant phenotypes.

Ectopic expression of *disco* while knocking down *Dll*

A recently published paper by Patel and colleagues (2007) reported that ectopic expression of *disco* leads to ectopic leg formation. It will be interesting to find out if this was due to *disco* proteins driving the expression of *Dll* ectopically. Although Dey (2006) demonstrated that ectopic *disco* expression is not sufficient to turn on *Dll*, the leg and wing discs arise from a common set of cells called the embryonic limb primordial (Cohen et al., 1993). *Dll* expression appears very early in the embryonic limb primordial prior to the separation of dorsal imaginal cells (progenitors of wing discs) from those of the ventral imaginal cells (progenitors of leg discs) (Cohen et al., 1993). The ectopic expression of *disco* may cause the expression of *Dll* to persist in the wing discs throughout development. The presence of *Dll* therefore may be responsible for the ectopic legs reported by Patel et al., (2007). To determine what happens when *disco* alone is present ectopically in the wing disc, a similar experiment like the ones carried out in this project can be utilised. For this experiment *disco* should be ectopically expressed while knocking down *Dll* gene products. To accomplish this, flies will be constructed carrying *ap*-Gal4 or *dpp*-Gal4, UAS-*disco*, and UAS-*Dll* RNAi or UAS-*Dll* dominant-negative. From these flies, it is possible to observe whether *disco* in the absence of *Dll* can cause the same ectopic structures as reported by Patel et al., (2007). This experiment

will allow us to determine if *disco* indirectly caused the formation of ectopic legs by driving the expression of *Dll*.

References

Basler K, A. M. (2007). The Decapentaplegic morphogen gradient: from pattern formation to growth regulation. *Nature Reviews* , (8):663-673.

Blair S S, B. D. (1994). The role of apterous in the control of dorsoventral compartmentalization and PS integrin gene expression in the developing wing of Drosophila. *Development* , (120):1805-1815.

Bolinger R A, B.-F. G. (2005). Distal-less functions in subdividing the Drosophila thoracic limb primordia. *Developmental Dynamics* , (232):801-816.

Brook W J, D.-B. F. (1996). Organizing Spatial Pattern in Limb Development. *Annu. Rev. Cell Dev. Biol.* , (12):161-180.

Campbell G, a. T. (1998). The roles of the homeobox genes aristaless and Distal-less in patterning the legs and wings of Drosophila. *Development* , (125):4483-4493.

Campos A R, L. K. (1995). Establishment of neuronal connectivity during development of the Drosophila larval visual system. *J Neurobiol* , (3):313-329.

Cohen B, S. A. (1993). Allocation of the thoracic imaginal primordia in the Drosophila embryo. *Development* , (117):597-608.

Cohen B, W. E. (1991). Early development of leg and wing primordia in Drosophila embryo. *Mechanisms of Development* , (33):229-240.

Cohen S M, a. J. (1989). Proximal-distal pattern formation in Drosophila: cell autonomous requirement for Distal-less gene activity in limb development. *The EMBO Journal* , (8):2045-2055.

Cohen S M, B. G. (1989). Distal-less encodes a homeodomain protein required for limb development in Drosophila. *Nature* , (338):432-434.

Cohen, S. (1990). Specification of limb development in the Drosophila embryo by positional cues from segmentation genes. *Nature* , (343):173-177.

Couso JP, a. B. (1998). Proximo-distal development in the legs of Drosophila. *Int. J. Dev. Biol.* , (42):345-352.

Dey B.K. 2006. The Role of The *Drosophila* Zinc Finger Transcription Factor *disconnected (disco)* In the Development of Ventral Appendages: a Molecular Genetic Study. pHD Thesis. McMaster University.

Diaz-Benjumea F, C. B. (1994). Cell interaction between compartments establishes the proximal-distal axis of Drosophila legs. *Nature* , (372):175-179.

Dominguez M, C. F. (2005). Organ Specification-Growth Control Connection: New In-sights From Drosophila Eye-Antennal Disc. *Developmental Dynamics* , 232:673-684.

Dong P D, C. J. (2000). Coexpression of the homeobox genes Distal-less and homothorax determines Drosophila antennal identity. *Development* , (127):209-216.

Dong PD, D. J. (2002). Distal-less and homothorax regulate multiple targets to pattern the Drosophila antenna. *Development* , (129):1967-1974.

Duncan DM, B. E. (1998). Control of distal antennal identity and tarsal development in Drosophila by spineless-aristapedia, a homolog of the mammalian dioxin receptor. *Genes and Development* , (12):1290-1303.

Galindo M I, B. S. (2002). Leg Patterning Driven by Proximal-Distal Interactions and EGFR Signalling. *Science* , (297):256-259.

Glossop N R J, S. D. (1998). Disconnected Mutants show Disruption to the Central Projections of Proprioceptive Neurons in Drosophila melanogaster. *J Neurobiol* , (36):337-347.

Godt D, C. J. (1993). Pattern formation in the limbs of Drosophila: bric a brac is expressed in both a gradient and a wave-like pattern and is required for specification and proper segmentation of the tarsus . *Development* , (119):799-812.

Gorfinkiel N, M. G. (1997). The homeobox gene Distal-less induces ventral appendage development in Drosophila. *Genes & Development* , (11):2259-2271.

Hardin P E, H. J. (1992). Behavioral and molecular analyses suggest that circadian output is disrupted by disconnected mutants in D. melanogaster. *The EMBO Journal* , (11):1-6.

Heilig J S, F. M. (1991). Isolation and characterization of the disconnected gene of Drosophila melanogaster. *The EMBO Journal* , (10):809-815.

Iuchi, S. (2001). Three classes of C2H2zinc finger proteins. *CMLS* , (58):625-635.

Kubota K, G. S. (2003). The role of Wg signalling in the patterning of embryonic leg primordium in Drosophila. *Evelopmental Biology* , (257):117-126.

Lee K J, F. M. (1991). Expression of disconnected gene during development of Drosophila melanogaster. *The EMBO Journal* , (10):817-826.

Mahaffey J W, G. C. (2001). The Drosophila genes disconnected and disco-related are redundant with respect to Larval Head Development and Accumulation of mRNAs from Deformed Target Genes. *Genetics* , (157):225-236.

Mardon G, S. N. (1994). dachshund encodes a nuclear protein required for normal eye and leg development in Drosophila. *Development* , (120):3473-3486.

Mast J D, P. S. (2006). The mechanisms and molecules that connect photoreceptor axons to their tagets in Drosophila. *Cell & Developmental Biology* , (17):42-49.

Morata, G. (2001). How Drosophila appendages develop. *Nature Reviews* , (2):89-97.

Neumann C J, C. S. (1997). Long-range action of Wingless organizes the dorsal-ventral axis of the Drosophila wing. *Development* , (124):871-880.

Panganiban G. (2000). Distal-less function During Drosophila Appendage and Sense Organ Development. *Developmental Dynamics* , (218):554-562.

Panganiban G, R. J. (2002). Developmental functions of the Distal-less/Dlx homeobox genes. *Development* , (129):4371-4386.

Patel M, F. L. (2007). The appendage role of insect disco genes and possible implications on evolution of the maggot larval form. *Developmental Biology* , (309)56-69.

Robertson L K, D. B. (2002). Expression of the Drosophila Gene disconnected Using the UAS/GAL4 System. *Genesis* , (34):103-106.

Steller H, F. K. (1987). disconnected:A Locus Required for Nueronal Pathway Formation in the Visual System of Drosophila. *Cell* , (50):1139-1153.

Wu J, C. S. (1999). Proximodistal axis formation in the Drosophila leg:subdivision into proximal and distal domains by Homothorax and Distal-less. *Development* , (126):109-117.

APPENDIX

GENETIC SCHEMES

Double Balancer Stock

1) yw/y ; 34D/CyO(y^+) ; +/TM3.Sb ♂ X yw/x ; ; D/TM3.Sb ♀

↓

yw/x ; +/CyO(y+) ; D/TM3.Sb

1a) yw/y ; +/CyO(y+) ; D/TM3.Sb ♂ X yw/x ; S/CyO(y+) ; +/+ ♀

↓

yw/y ; S/+ ; +/TM3.Sb

2) yw/x ; +/CyO(y+) ; D/TM3.Sb ♀ X yw/y ; S/+ ; +/TM3.Sb ♂

↓

yw ; S/CyO(y+) ; D/TM3.Sb

New *dpp*-Gal4 Line

1) yw/x ; +/+ ; D/TM3.Sb♀ X w ; wsp-1/CyO ; *dpp*-Gal4/TM6B ♂

↓

yw ; +/CyO ; *dpp*-Gal4/TM3.Sb

2) yw ; +/CyO ; *dpp*-Gal4/TM3.Sb♀ X yw/y ; S/+ ; +/TM3.Sb ♂

↓

yw ; +/+ ; *dpp*-Gal4/TM3.Sb

UAS-*dll* and UAS-*disco*^{DN} Construct

A) yw/x ; UAS-*dll*/CyO ; +/+ ♀ X yw/y ; S/CyO(y^+) ; D/TM3.Sb ♂

↓

yw/x ; UAS-*dll*/CyO(y^+) ; +/TM3.Sb

A') yw/x ; +/+ ; UAS-*disco*^{DN} ♀ X yw ; S/CyO(y^+) ; D/TM3.Sb ♂

↓

yw/y ; S/+ ; UAS-*disco*^{DN}/D

B) yw/y ; S/+ ; UAS-*disco*^{DN}/D ♂ X yw/x ; UAS-*dll*/ CyO(y^+) ;+/TM3.Sb ♀

↓

yw ; UAS-*dll*/S ; UAS-*disco*^{DN}/TM3.Sb

C) yw ; UAS-*dll*/S ; UAS-*disco*^{DN}/TM3.Sb ♂ X yw ; S/CyO(y^+) ; D/TM3.Sb ♀

↓

yw ; UAS-*dll*/CyO(y^+) ; UAS-*disco*^{DN}/TM3.Sb

UAS-*dll* and UAS-*disco*^{RNAi} Construct

A) yw/x ; UAS-*dll*/CyO ; +/+ ♀ X yw/y ; S/CyO(y^{+}) ; D/TM3.Sb ♂

↓

yw/x ; UAS-*dll*/CyO(y^{+}) ; +/TM3.Sb

A') yw/x ; +/+ ; UAS-*disco*^{RNA}*i* ♀ X yw ; S/CyO(y^{+}) ; D/TM3.Sb ♂

↓

yw/y ; S/+ ; UAS-*disco*^{RNAi}/D

B) yw/y ; S/+ ; UAS-*disco*^{RNAi}/D ♂ X yw ; UAS-*dll* CyO(y^{+}) ; +/TM3.Sb ♀

↓

yw/y ; UAS-*dll*/S ; UAS-*disco*^{RNAi}/TM3.Sb

C) yw ; UAS-*dll*/S ; *disco*^{RNAi}/TM3.Sb ♂ X yw ; S/CyO(y^{+}) ; D/TM3.Sb ♀

↓

yw ; UAS-*dll*/CyO(y^{+}) ; UAS-*disco*^{RNAi}/TM3.Sb

Ectopic expression of *dll*

x/y ; *ap*-Gal4/CyO ; +/+ ♂ X x/x ; UAS-*dll*/CyO ; +/+♀

↓

x/x,y ; *ap*-Gal4/UAS-*dll* ; +/+ (escapers)
x/x,y ; *ap*-Gal4/CyO ; +/+
x/x,y ; UAS-*dll*/CyO ; +/+

omb-Gal4/y ; +//+ ; +/+ ♂ X x/x ; UAS-*dll*/CyO ; +/+♀

↓

omb-Gal4/x ; UAS-*dll*/+ ; +/+ (escapers)
omb-Gal4/x ; +/cyO ; +/+
x/y ; UAS-*dll*/+ ; +/+
+/y ; +/CyO ; +/+

x/y ; +/+ ; *dpp*-Gal4/TM3.Sb ♂ X x/x ; UAS-*dll*/CyO ; +/+♀

↓

x/x,y ; UAS-*dll*/+ ; *dpp*-Gal4/+ (escapers)
x/x,y ; +/CyO ; *dpp*-Gal4/+
x/x,y ; UAS-*dll*/+ ; +/TM3.Sb
x/x,y ; +/CyO ; +/TM3.Sb

UAS-*dll and UAS-*disco*^{DN} Construct**

yw/y ; UAS-*dll*/CyO ; UAS-*disco*^{DN}/TM3.Sb♂ X x/x ; UAS-*dll**/UAS-*dll** ; +/+♀

↓

yw ; UAS-*dll**/CyO ; UAS-*disco*^{DN}/+

yw ; UAS-*dll**/CyO ; UAS-*disco*^{DN}/+ X yw ; S/CyO ; UAS-*disco*^{DN}/TM3.Sb

↓

yw ; UAS-*dll**/CyO ; UAS-*disco*^{DN}/TM3.Sb

Ectopic expression of *dll**

x/y ; *ap*-Gal4/CyO ; +/+ ♂ X x/x ; UAS-*dll*/UAS-*dll* ; +/+ ♀

↓

x/x,y ; *ap*-Gal4/UAS-*dll* ; +/+ (escapers)
x/x,y ; UAS-*dll*/CyO ; +/+

omb-Gal4/y ; +//+ ; +/+ ♂ X x/x ; UAS-*dll*/UAS-*dll* ; +/+ ♀

↓

omb-Gal4/x ; UAS-*dll*/+ ; +/+ (escapers)
x/y ; UAS-*dll*/+ ; +/+

x/y ; +/+ ; *dpp*-Gal4/TM3.Sb♂ X x/x ; UAS-*dll*/UAS-*dll* ; +/+ ♀

↓

x/x,y ; UAS-*dll*/+ ; *dpp*-Gal4/+ (escapers)
x/x,y ; UAS-*dll*/+ ; +/TM3.Sb

C50;UAS-*dll** construct

1a) C50/C50 ; +/+ ; +/+ ♀ X yw/y ; S/CyO ; +/+ ♂

↓

C50/y ; S/+

1b) FM7/FM7 ; +/+ ; +/+ ♀ X yw/y ; S/CyO ; +/+ ♂

↓

FM7/yw ; +/CyO

2) C50/y ; S/+ ♂ X FM7/yw ; +/CyO ♀

↓

C50/FM7 ; S/CyO

3) C50/FM7 ; S/CyO ♀ X yw/y ; UAS-*dll*/UAS-*dll*

↓

C50/y ; UAS-dll/CyO

4) C50/y ; UAS-*dll*/CyO ♂ X C50/FM7 ; S/CyO ♀

↓

C50/C50,y ; UAS-*dll*/CyO

GFP Constructs

ap-Gal4

x/x ; *ap*-Gal4/CyO ♀ X x/y ; wee/CyOActGFP ♂

↓

x/x,y ; *ap*-Gal4/CyOActGFP

dpp-Gal4

x/x ; +/+ ; *dpp*-Gal4/TM3.Sb ♀ X x/y ; +/+ ; D/TM6.ubi-GFP ♂

↓

x/x,y ; *dpp*-Gal4/TM6.ubi-GFP

LacZ expression experiment

<u>*ap*-Gal4</u>

C50/C50 ; UAS-*dll*/UAS-*dll* ♀ X x/y ; *ap*-Gal4/CyOActGFP ♂

↓

C50/x,y ; *ap*-Gal4/UAS-*dll*
C50/x,y ; UAS-*dll*/CyOActGFP

<u>*dpp*-Gal4</u>

C50/C50 ; UAS-*dll*/UAS-*dll* ♀ X x/y ; +/+ ; *dpp*-Gal4/TM6.ubi-GFP ♂

↓

C50/x,y ; UAS-*dll*/+ ; *dpp*-Gal4/+
C50/x,y ; UAS-*dll*/+ ; +/TM6.ubi-GFP

dac expression experiment

x/y ; *ap*-Gal4/CyOActGFP ♂ X x/x ; UAS-*dll*/UAS-*dll* ; +/+ ♀

↓

x/x,y ; *ap*-Gal4/UAS-*dll*
x/x,y ; UAS-*dll*/CyOActGFP

x/y ; ; *dpp*-Gal4/TM6.ubi-GFP ♂ X x/x ; UAS-*dll*/UAS-*dll* ; +/+ ♀

↓

x/x,y ; UAS-*dll*/+ ; *dpp*-Gal4/+
x/x,y ; UAS-dll/+ ; +/TM6.ubi-GFP