Myosin Light Chain Phosphatase regulates the transcription of Wingless target genes in Drosophila

by

Elizabeth-Ann Hoesing

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Approval

Name: Elizabeth-Ann Hoesing
Degree: Master of Science
Title: Myosin Light Chain Phosphatase regulates the transcription of Wingless target genes in Drosophila

Examinining Committee:

Chair: Dr. Timothy Audas
   Assistant Professor

Dr. Esther Veheyen
   Senior Supervisor
   Professor

Dr. Nicholas Harden
   Supervisor
   Professor

Dr. Nancy Hawkins
   Supervisor
   Associate Professor

Dr. Harald Hutter
   Internal Examiner
   Professor
   Department of Biology

Date Defended/Approved: March 28, 2017
Abstract

Canonical Wnt, or Wingless (Wg) in Drosophila, is an evolutionarily well-conserved signalling pathway that is important for a wide range of processes, including cell fate determination, axis formation and stem cell renewal. Wg signalling primarily functions to regulate the cytosolic stability of the key effector β-catenin (Armadillo, Arm, in Drosophila). Arm promotes the transcription of Wg target genes but also is required for the formation of stable adherens junctions. Previously, the Verheyen lab identified the non-muscle myosin II regulator Myosin Light Chain Phosphatase (MLCP) as a putative regulator of Wg signalling. Here we find that reducing the expression MLCP components leads to the attenuation of Wg target gene expression. I present our evidence that MLCP knock down directly regulates Wg signal transduction and that this regulation is through Arm localization. Thus, our work supports mounting evidence of a regulatory relationship between the adherens junctions and the Wg signalling pathway.

Keywords: Non-Muscle Myosin II; flapwing; Wingless; Armadillo; β-catenin; MYPT
To my Mother and Father who always had more faith in my skills than I did.
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# Table of Contents

Approval .......................................................................................................................... ii  
Abstract .......................................................................................................................... iii  
Dedication ......................................................................................................................... iv  
Acknowledgements .......................................................................................................... v  
Table of Contents .............................................................................................................. vi  
List of Figures .................................................................................................................... vii  
List of Tables .................................................................................................................... vii  
List of Acronyms ............................................................................................................... viii

## Chapter 1. Introduction .............................................................................................. 1
  1.1. Signal Transduction .............................................................................................. 1  
  1.2. Drosophila as a model organism ........................................................................... 1  
  1.3. Canonical Wnt Pathway ....................................................................................... 6  
      1.3.1. Wg’s role in Drosophila wing development .................................................... 8  
      1.3.2. Identification of Wg regulators through an RNAi screen ............................... 9  
  1.4. Non-muscle Myosin II ......................................................................................... 10  
  1.5. Adherens Junctions .............................................................................................. 13  
  1.6. The interplay between Wnt and Adhesion ........................................................... 16

## Chapter 2. Materials and Methods ......................................................................... 18
  2.1. Fly Lines and Crosses ............................................................................................ 18  
  2.2. Antibody Staining ............................................................................................... 19  
  2.3. Proximity Ligation Assay .................................................................................... 20

## Chapter 3. Results .................................................................................................. 21
  3.1. MLCP interacts with Wg signal transduction ......................................................... 21  
  3.2. MLCP affects Wg signal transduction directly ....................................................... 22  
  3.3. PP1 β (flw) interaction with Axin ......................................................................... 25  
  3.4. Testing the genetic interactions of Myosin phosphatase and components of the Wg pathway ........................................................................................................ 27  
      3.4.1. Testing the putative genetic interaction between MLCP and the Wg receptors ............................................................ 28  
      3.4.2. Testing the putative genetic interaction between MLCP and Axin .......... 31  
      3.4.3. Testing the putative genetic interaction between MLCP and Arm .......... 34

## Chapter 4. Discussion .............................................................................................. 37
  4.1. MLCP genetically interacts with Arm .................................................................... 37  
  4.2. MLCP influences Arm localization ....................................................................... 38  
  4.3. MLCP impacts Arm localization through NMII ..................................................... 40  
  4.4. Future directions and lingering questions ............................................................. 41  
  4.5. Conclusion ............................................................................................................ 43

References ...................................................................................................................... 44
List of Figures

Figure 1. Drosophila Imaginal discs and corresponding adult structures. ....................... 2
Figure 2. The UAS/GAL4 system .................................................................................. 4
Figure 3. Wg Signalling Pathway .................................................................................. 7
Figure 4. Wingless expression during Drosophila imaginal wing disc development .................. 9
Figure 5. Structure of Non-muscle Myosin II ................................................................ 11
Figure 6. Structure of the Adherens Junction. ............................................................. 14
Figure 7. Knock-down of MLCP components reduces the expression of Wg target genes .......................................................... 22
Figure 8. MLCP knock-down does not impact Hedgehog signalling ................................. 24
Figure 9. MLCP does not impact Dpp target gene expression ........................................ 25
Figure 10. Testing a direct interaction between Axin and PP1β ..................................... 27
Figure 11. Knock-down of PP1β rescues Wg receptor fusion overexpression phenotype .......................................................... 31
Figure 12. MLCP does not rescue the effects of the Axin null allele ............................... 34
Figure 13. MLCP knock down partially rescues the armS10 phenotype .......................... 36

List of Tables

Table 1 Epistasis Crosses .............................................................................................. 19
# List of Acronyms

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
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<tr>
<td>A/P</td>
<td>Anterior-Posterior</td>
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<td>Adherens Junctions</td>
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<td>Ap</td>
<td>Apterous</td>
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<td>APC</td>
<td>Adenomatous polyposis coli</td>
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<td>Armadillo</td>
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<tr>
<td>ATP</td>
<td>Adenosine tri-phosphate</td>
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<tr>
<td>Ci</td>
<td>Cubitus interruptus</td>
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<tr>
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<td>Distal-less</td>
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<tr>
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<tr>
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<td>Epithelial Growth Factor Receptor</td>
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<tr>
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<tr>
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<td>Lymphoid Enhancer factor</td>
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<td>LDL receptor-related protein</td>
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<td>MARCM</td>
<td>Mosaic Analysis with a Repressible Cell Marker</td>
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<td>MLCK</td>
<td>Myosin light chain kinase</td>
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<tr>
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<tr>
<td>MLCP</td>
<td>Myosin light chain phosphatase</td>
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<td>Myosin phosphatase targeting subunit</td>
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Chapter 1.

Introduction

1.1. Signal Transduction

All multicellular life originates from a single cell that proliferates and gives rise to many daughter cells that differentiate into a multitude of distinct specialized tissues. In order for this process to be successful extensive highly coordinated communication must be conducted between cells. In metazoan development, these processes are largely coordinated by a handful of highly evolutionarily conserved signalling networks that, with appropriate temporal and spatial expression, pattern tissue. These pathways are often tightly regulated as their disruption or inappropriate expression commonly leads to disease.

In this thesis I will discuss one of these fundamental pathways: Canonical Wnt signalling. Well-characterized in the fruit fly, Drosophila melanogaster, the Canonical Wnt pathway has roles in all stages of development and adult homeostasis.

1.2. Drosophila as a model organism

For over a century, Drosophila melanogaster has been a predominant model organism in genetic research. It is favoured largely for its practicality (Jennings, 2011). Flies are easy and inexpensive to maintain with short lifespans and a catalogue of tools available for genetic manipulation. Additionally, the simplistic yet highly organized nature
of tissue and organ development in Drosophila has provided significant insight into many developmental processes.

One of the major advantages to using Drosophila in genetic research is the presence of primordial adult structures termed ‘imaginal discs’. Imaginal discs are single layer epithelial cell sacs that act as precursors to many of the adult structures including the eyes, wings and legs. During embryogenesis, small subsets of cells are allotted to each presumptive structure and little difference exists in the protein expression profiles between different tissues. These few cells then rapidly proliferate and grow during the larval stages (Neto-Silva et al., 2009).

![Figure 1](image.png)

**Figure 1.** Drosophila Imaginal discs and corresponding adult structures.
A) A schematic of imaginal disc position in a third instar larvae left, and the adult structure that will form after pupation on the right. (Modified from Aldaz and Escudero 2010). B) A third instar wing imaginal disc with Anterior-Posterior and Dorsal-Ventral boundaries indicated with orange and green respectively. This disc is stained against Cubitus interruptus and Armadillo in red and green respectively. Ci helps indicate the anterior compartment where as Arm expression is enriched in cell flanking the D/V boundary.

As they grow, the tissues begin to organize into distinct cell fates. This patterning is aided by the formation of 4 discrete compartments divided by vertical and horizontal axes that define the disc into anterior-posterior (A/P) and dorsal-ventral (D/V) regions respectively (Brook et al., 1996; Neto-Silva et al., 2009). The boundaries at these compartments are often sharp and prevent the mixing of cells from different compartments (Martin and Wieschaus, 2010). The formation of compartments is a
common tactic used in metazoan development to help designate cell fate and tissue patterning. In imaginal discs, the compartment boundaries are often organization centers where long-range signals, termed morphogens, turn on genes in a concentration-dependent manner and aid in designating cells populations to different tissue identities (Brook et al., 1996; Neto-Silva et al., 2009; Milan and Cohen 2000). By the third instar stage of larval development, signalling between the developmental pathways is robust and expression profiles of distinct cell populations can be visualized by antibody staining of proteins or through expression of reporter genes. Thus, the consequences of any genetic manipulation can be visualized in vivo and causes for identifiable adult phenotypes can be determined.

A powerful genetic tool often used in Drosophila research is the UAS-GAL4 system (Brand and Perrimon, 1993; Duffy, 2012). Originally co-opted from Sacchaomyces cerevisiae, this system utilizes GAL4, a transcription factor, to induce expression of a target gene by binding to Upstream Activating Sequence (UAS) cis-regulatory sites. Both these elements are foreign to the Drosophila genome. As a consequence of this, when a UAS regulated gene or GAL4 transcription factor gene is present in the genome alone there are no endogenous transcription factors or transcriptional regulatory sites that the elements can interact with. Thus, GAL4 and UAS regulated genes can be carried in separate genetic lines almost indefinitely and target gene transcription will only occur when the genetic lines are crossed to produce offspring that carry both the GAL4 and UAS element. Spatial control of target gene expression is achieved through the use of specific GAL4 driver lines. In driver lines, GAL4 is inserted into the genome such that a tissue-specific promoter dictates its expression and as such these lines are commonly referred to by the promoter GAL4 expression is under (e.g. Hedgehog driver). Due to the large number of GAL4 and UAS lines, there are nearly unlimited numbers of combinations that are possible for use in genetic research.
Figure 2. The UAS/GAL4 system
A) A schematic of the UAS/GAL4 system as used in flies. The GAL4 transcription factor is expression is mediated by a tissue specific promoter. GAL4 protein binds UAS sequences to drive the transcription of an adjacent gene of interest (GFP) (Taken with permission from Duffy, 2012). B) A schematic of heat shock flip out cassette clones. FRT sequences flank a transcriptional termination sequence that prevents an Actin promoter from driving the expression of GAL4. Following the heatshock of offspring, FIp recombinase is transcribed and targets FRT sites excising the termination sequence from DNA. As a result, GAL4 is expressed and the gene of interest is transcribed.
The FLP/FRT system is another Sacchaomyces cerevisiae system used in Drosophila research that utilizes expression of Flp recombinase to recognize and mediate recombination between FRT sites in the genome (Duffy, 2012). A heat shock promoter commonly controls Flp recombinase expression so that the recombinase will only be active when the organism is placed a high temperature. One kind of clonal system that utilizes this system is Flip out cassette clones. In these lines FRT sites flank transcription termination sequences that are placed between a ubiquitous promoter and a GAL4 gene. These termination sequences block the transcription of the GAL4 gene until the organism is shifted to a higher temperature. In these conditions, Flp recombinase is expressed and targets the FRTs in cell actively undergoing mitosis, excising termination sequences in the process. This allows for the expression of GAL4 by the ubiquitous promoter thus activating any UAS transgenes also present in the system. The nature of the heat shock promoter means that only some of the cells undergoing mitosis will be successful in removing the stop cassette. Daughters of these individual cells will then continue to divide and proliferate leading to patches of mutant tissue (called clones) within a largely wild type background. Mutant tissue in this system is detected with a fluorescent marker such as UAS-GFP indicating successful induction of GAL4 expression. As clones originate from a single progenitor cell, analysis on growth and proliferation can be readily conducted.

The Mosaic Analysis with a Repressible Cell Marker (MARCM) clone system is also commonly used in Drosophila research. This system generates cells that are homozygous for a particular mutant in a heterozygous animal using FLP/FRT system. To do this, mitotic recombination is induced using a heat-shock inducible Flp recombinase and FRT sites located near the centromere of the chromosome containing the mutation of interest. When the organism is heat shocked, heterozygous chromosomes with proximal FRT sites undergo recombination. This exchange results in two homozygous daughter cells: one homozygous mutant, one homozygous wild type. In the MARCM system, mutant daughter cells are positively marked by a UAS-GFP that is not produced in heterozygous cells. This is due to the presences of the Gal80 gene in the wildtype chromosomal arm, which produces a GAL4 antagonist that prevents GAL4 activity. After successful mitotic recombination, loss of Gal80 allows GAL4 to recognize and bind to the UAS-GFP gene. Mitotic clonal systems such as MARCM are often implemented
when the lethal nature of a mutant makes study of specific genes difficult. In this technique, an additional transgene can also be expressed in the mutant clonal tissue, such as cDNA or RNAi constructs.

1.3. Canonical Wnt Pathway

In 1983 Int-1 was identified as a proto-oncogene in mouse mammary tissue (Nusse and Varmus, 1983). Later studies in Drosophila revealed Int-1 to be highly homologous to the Drosophila gene termed wingless (Wg), which previously had been described as being integral to larval development. Thus, this family of proteins derives its names from the merger of Int-1 and Wg. The Wnts are a family of cysteine-rich secreted glycoproteins that are involved in a number of biological events including cell fate determination, motility, polarity primary axis formation and organogenesis (Clevers and Nusse, 2012; Clevers, 2006; Koyima and Habas, 2008). As a consequence of their extensive involvement in development and homeostasis of organisms, their mis-regulation often results in developmental defects and diseases, including, but not limited, to cancer. One of the founding members of this family is the Wg protein. Wg is a key player in many Drosophila developmental processes. Over decades of research, characterization of Wg has informed much of what is known about Wnt functionality in general (Swarup and Verheyen, 2012). Wnt signal transduction can be loosely categorized into either canonical or non-canonical based on whether the pathway uses β-catenin as a key effector protein. The Canonical Wnt signalling pathway primarily functions to regulate cytosolic levels β-catenin (β-cat) in cells. In most cell types β-cat, named Armadillo (Arm) in Drosophila, is continuously transcribed, and it plays an integral role in adherens junctions (AJs) through its interaction with E-cadherin (see below). Non-junctional Arm, however, does not accumulate in the cytosol in the absence of a canonical Wnt ligand as it is targeted for proteasomal degradation via a protein complex referred to as the ‘Destruction Complex’. The destruction complex comprises Adenomatous polyposis coli (APC), the scaffold Axin, casein kinase (CK)-1 and glycogen synthase kinase (GSK)-3β. When Arm associates with the destruction complex, CK-1 and GSK-3β phosphorylate Arm on a series of N-terminal residues that create a phospho-degron motif that primes Arm for recognition by an E3 ligase complex.
Ubiquitination by the E3 ligase complex targets Arm for degradation by the proteasome. The destruction complex does not engage E-cadherin-associated Arm.

After binding of the Wg ligand to its receptors, the destruction complex becomes inactivated, disassembles and some components are recruited to the receptor complex. Wg engages Frizzled through Frizzled’s N terminal cysteine rich domain and a co-receptor low-density lipoprotein (LDL) receptor-related protein (LRP) encoded by *arrow* in Drosophila. This resulting complex causes the recruitment and phosphorylation of the protein Disheveled (Dsh) which then subsequently recruits Axin to the receptor complex. Without Axin serving as a scaffold, the destruction complex is unable to form. Arm is then free to accumulate in the cytosol and translocate to the nucleus where it binds to T-cell factor (TCF) / Lymphoid Enhancer factor (LEF) transcription factors. This leads to the displacement of the repressor Groucho and allows Wg target genes to be transcribed (Figure 3).

**Figure 3. Wg Signalling Pathway**

A) In the absence of the Wg ligand, Arm that is not associated with the adherens junctions is bound and phosphorylated on a series of N-terminal residues that mark the effector for proteasomal degradation. B) When Wg ligand is present, it engages the Wg receptor Fz, and its co-receptor Arr. This association triggers Dsh mediated recruitment of the destruction complex to the cytoplasmic regions of the Wg receptors leading subsequently leading to the complexes inactivation. Without the action of the destruction complex, Arm freely accumulates in the cytosol where it then translocates to the nucleus and drives the transcription of Wg pathway target genes.
1.3.1. Wg’s role in Drosophila wing development

As the name indicates, Wingless signalling is key to proper Drosophila wing development. Like most other Drosophila genes, wg was named for the adult phenotype of a hypomorphic allele, wg\textsuperscript{1}, which caused the transformation of wings into thoracic notum (Sharma and Chorpa, 1976).

The wing disc contains cells that form the wing proper, the wing hinge and part of the thoracic notum. During the 2\textsuperscript{nd} larval instar, Wg expression in the ventral region of the discs designates the presumptive wing field. This is complemented by expression of the Epithelial Growth Factor Receptor ligand, vein, which designates the dorsal region of the disc as the presumptive notum. Both wg and vein expression are required to maintain this division of wing and notum structures (Baonza et al., 2000). Thus, in the case of the wg\textsuperscript{1} mutant allele, attenuation of the Wg expression in the wing disc allows vein to pattern the entire imaginal disc as part of the thoracic notum.

From this broad pattern, Wg expression is further refined to serve as an organizing center for wing tissue patterning. Like all imaginal discs, wing discs develop A/P compartments during embryogenesis followed by the formation of the D/V compartments later in the larval stages (Brook et al., 1996; Baonza et al., 2000). This second division is partially marked by the expression of apterous which overlaps with wg expression during second instar. At the boundary of the ap region, ap triggers the expression of transmembrane proteins Serrate and Delta which help designate the dorsal region of the disc (Zecca and Struhl, 2007; Brook et al., 1996). Serrate and Delta serves as ligands for Notch signalling, which induces wg ligand expression in cells along the D/V boundary (Swarup and Verheyen, 2012). From the thin line of Wg producing cells at the D/V boundary, the morphogen diffuses across the wing pouch proper to turn on Wg target genes such as Distal-less (Dll), achaete (ac), and vestigial (vg) in a dose dependent manner in order to pattern the rest of the wing and initiate proliferation in the wing pouch (Swarup and Verheyen, 2012; Zecca and Struhl, 2007) (Figure 4).
Figure 4. **Wingless expression during Drosophila imaginal wing disc development.**

The left image depicts the expression of Wg, Ap, and EGFR in 2\textsuperscript{nd} instar larval discs. At this stage Wg (blue) and levels of high EGFR signaling (brown) mark the presumptive wing field and notum respectively. Ap expression in the 2\textsuperscript{nd} instar disc eventually induce expression of Notch receptors Ser and Dl. Notch signalling in turn, induces Wg expression in cell flanking the D/V boundary.

1.3.2. **Identification of Wg regulators through an RNAi screen**

Like all major cell signalling pathways, the canonical Wg pathway is tightly regulated during normal development to avoid developmental defects and disease. This regulation relies heavily on phosphorylation events to moderate the activity of its components (Verheyen and Gottardi, 2010). Phosphorylation events are among the most commonly occurring post-translation modifications. The effects of addition or removal of a phosphate group by kinases and phosphatases, respectively, are varied and can regulate properties from activity, protein interaction, localization and degradation of the target protein depending on where the phosphorylation event has occurred within that protein.

In order to explore this regulation, former PhD students of the Verheyen lab, S. Swarup and T. Pradhan-Sundd, conducted a screen to identify novel phospho-regulators...
of the Wnt pathway using a collection of RNAi strains specific for genes encoding kinases, phosphatases and their accessory proteins (Swarup et al., 2015). Third instar larvae were dissected and immunostaining for Armadillo, Senseless (Sens) and Distalless (Dll). These proteins served as read-outs of Wnt pathway activity (Arm levels) and transcriptional activity (Sens, Dll). Any RNAi lines that caused changes in normal protein levels or distribution of these read-outs were further studied. Putative effectors [hits] were then validated using RNAi lines encoding different hairpin structures to reduce the likelihood that results were due to off target effects.

From these putative hits, knock down of three components of non-muscle myosin II phosphatase were identified to cause disruption of Wg target gene expression and stabilized Armadillo (Arm) levels. These components were the two targeting subunits myosin phosphatase targeting subunit (MYPT) and myosin binding subunit (Mbs), and the catalytic subunit Protein Phosphatase 1 isoform β (PP1β).

1.4. Non-muscle Myosin II

The myosin superfamily is a group of protein motors that utilize ATP hydrolysis as an energy source (Vicente-Manzanares et al., 2009; Betapudi et al., 2014; Conti and Adelstein, 2008). The family can be subdivided into 25 different classes with class II containing the largest number of myosins. Class II myosins can be broadly characterized as actin related motor proteins that cause contraction by allowing actin filaments to slide by one another. This class includes skeletal, cardiac, and smooth muscle myosins, which make up the most of the contractile force in their respective tissues. Like muscle myosin II, non-muscle myosin II (NMII) works with the actin cytoskeletal network to provide contractile forces in the cell. NMII is ubiquitously expressed in all cell types and plays major roles in cell shape, migration, cell division, and adhesion. Like all class II myosins, NMII is a hexamer composed of three sets of chains: a pair of Heavy Chains (HC), a pair of Regulatory Light Chains (RLC) and a pair of Essential Light Chains (ELC).

The HC of NMII comprises the bulk of motor protein mass and can be separated into three functional regions: A globular head domain, which houses the ATP dependant
motor and actin binding site; a neck region, which binds both ELC and RLC; and a helical coiled tail region, that permits the formation of homodimers (Figure. 5) (Newall-Litwa et al., 2015). In mammals three genes, MYH9, MYH10, and MYH14, encode the HCs for the different HC isoforms A, B and C respectively, which differ in motor kinetics and durability. For example, NMIIA hydrolyzes ATP more quickly than either NMIIB or C, whereas NMIIB can stay contractile longer then the A isoform can. This means that NMII isoforms differ in distribution throughout the cell depending on physiological need in that location. In Drosophila, a single gene called zipper encodes the HC of NMII (Vicente-Manzanares et al., 2009).

The activity of the ATP motor contained within the HC is positively controlled by phosphorylation of the RLC (encoded by spaghetti squash [sqh] in Drosophila) (Vicente-Manzanares et al., 2009; Conti and Adelstein, 2009). Phosphorylation of the key Threonine and Serine residues (Thr 18 and Ser 19 in mammals and Thr 20 and Ser21 Drosophila) in the RLC cause a conformational change in NMII and significant increase
in the activity of the ATPase. Once active, NMII is able to form myosin filaments through its alpha-helical coiled-coil domain and interact more readily with actin filaments through the HC, crosslinking them and driving their contraction. Though there are over a dozen kinases that can phosphorylate the RLC, Myosin light chain kinase (MLCK) and RhoA/ROCK/Rho kinases (ROCK) appear to be the two most common positive regulators of NMII activity (Vicente-Manzanares et al., 2009).

A class of proteins called MYPTs or Myosin Phosphatase targeting proteins mediate dephosphorylation of the key residues in the RLC (Conti and Adelstein, 2009). As the name implies, MYPTs function to target the catalytic subunit from the Protein Phosphatase 1 class (PP1) to the RLC. In this thesis I will be referring to the complex of these two subunits as the Myosin light chain phosphatase (MLCP) (Vereshchagina et al., 2004). In Drosophila there are 4 genes encoding isoforms of PP1, named for their chromosomal location: PP1β9C, PP1α13C, PP1α87B, and PP1α96A. Despite the broad acting nature of the PP1 class of phosphatases, the PP1β9C catalytic subunit encoded by flap-wing (flw) has an essential and non-redundant role in the dephosphorylation of NMII. Similarly, though Drosophila has two MYPT proteins, Mbs and MYPT-75D, MYPT-75D alone functions to target PP1β9C to the NMII RLC (Vereshchagina et al., 2004)(Bennett et al., 2006). PP1β9C has a mammalian homolog (PP1δ) but MYPT75D does not, and is only similar to the mammalian MYPT3. However, it is speculated that a similar NMII RLC specific MYPT is likely to exist in mammals as well.

Along with its function in targeting an associated catalytic subunit, MYPT is also subject to phosphorylation catalyzed by ROCK, which decreases the activity of MLCP complex. A net result of negative regulation of MLCP is the subsequent enhanced NMII activity. ROCK activity acts as a strong positive regulator of NMII activity because it both activates NMII activity and negatively regulates NMII’s negative regulator (Vicente-Manzanares et al., 2009).

NMII plays several roles in the cell. In morphogenesis, NMII activity can shape the three-dimensional arrangement of tissue. For example, during Drosophila gastrulation, apical constriction of cells, driven by NMII contraction, leads to tissue invagination. Recently NMII was found to play roles in tissue organization in the formation and maintenance of compartments during development. In both the embryo
and in wing discs NMII has been shown to accumulate at the boundary of compartments (Major and Irvine, 2006; Monier et al., 2010). In wing discs, this enrichment occurs at the D/V and A/P axes where NMII and actin appear to form a thick super-cellular cable. Genetic knock down of NMII shows that loss of NMII from compartment boundary results in increased boundary roughness or in cells traversing the boundary divide (Major 2006; Landsberg et al., 2009). Though it is still unknown how NMII contributes to the separation of boundaries there is speculation that the process might be through tension as analyses conducted in live embryos have shown that the NMII cables seem to resist against the movement of newly generated cells (Monier and Irvine, 2010).

1.5. Adherens Junctions

Cell-cell adhesion is essential for the health and maintenance of epithelial tissue. Along with linking individual cells together to form barriers between environments, adhesion also allows cells to react in a coordinated matter to stimuli (ie. wound healing) and to resist external and internal mechanical stresses (Nanes and Kowalczyk, 2012). In general, three types of cell-cell junctions exist in epithelial cell: tight junctions (or septate junctions in Drosophila), adherens junctions, and desmosomes (Dejana, 2004). Though all three of these junctions are important to epithelial health, this thesis will be focused on the Adherens Junctions (AJs) specifically, which are classically defined as circumferential belts of adhesive contacts that are closely associated with the actin cytoskeleton. These cellular adhesions are key to embryogenesis and to epithelial integrity and their disruption drives formation and progression of diseases like metastasis, inflammation and tumour cell invasion.

AJs are not static structures and are formed, degraded, remodelled and reformed during normal physiological events in response to the needs of cells and tissues. Adhesion itself, and the strength of adhesion, in these junctions are controlled by the levels of classical cadherins (in this case Epithelial cadherin [E-cad]) that associate with the plasma membrane (PM) (van Roy and Berx, 2008; Harris and Tepass, 2010).
Figure 6. Structure of the Adherens Junction.
A simplified diagram of an epithelial adherens junction. E-cadherin is a Ca^{2+} dependent cell adhesion molecule that dimerizes with E-cadherins on the neighbouring cell through well conserved extracellular cadherin domains. The E-cadherin cytoplasmic tail associates with p120 catenin and β-catenin. β-catenin subsequently interacts with the actin cytoskeleton associated α-catenin. β-, α-catenin function to connect the actin cytoskeleton and E-cadherin; an association that is vital to the stability of the Adherens Junction complex.

Classical cadherins are characterized as single-pass transmembrane cell adhesion molecules that typically form homophilic interactions in a Ca^{2+} dependent manner with a cadherin on a neighbouring cell. Adhesion with other cadherin molecules and Ca^{2+} binding occurs through the 5 extracellular cadherin repeats (EC1-EC5) within the N-terminal domain. Binding of Ca^{2+} allows E-cad to adopt a rigid conformation to maintain adhesion with its neighbour on the opposing cell (van Roy and Berx, 2008; Nanes and Kowalczyk, 2012). The availability of E-cad associated with the AJ can be regulated by transcription to a certain amount, but it does not account for rapid changes in AJs often seen during physiological processes as the half-life of classical cadherin molecules are often long. Instead, cadherin turnover is controlled by a combination of endocytosis, degradation, cadherin recycling and transcription. Rapid changes in AJ are attributed to endocytosis and there are several factors that control the rate of cadherin endocytosis. In this thesis, I will be referring to factors that decrease E-cad endocytosis as ‘stabilizing’.

One well-characterized key factor in E-cad stabilization at the PM is its association with the actomyosin network and stable Filamentous actin (F-actin) (Harris
and Tepass, 2010; West and Harris, 2016). E-cadherin associates with the actin cytoskeleton in an indirect manner (Figure 6). Contained within the highly conserved C-terminal of classical cadherins is the catenin-binding domain (CBD) that interacts with β-cat. As well as directing proper trafficking of newly synthesized E-cad, β-cat also connects the cadherin with the actin-associated α-catenin (α-cat) (van Roy and Berx, 2008; Miyashita and Ozawa, 2007; Curtis et al., 2008). There is some contention about whether α-cat physically bridges the cadherin-catenin complex and the cytoskeleton directly (Yamada et al., 2005). However, it is clear that α-cat holds important significance to the stability of the E-cad (Harris and Tepass, 2010). α-cat also acts as a tension sensor and recruits the F-actin binding protein vinculin in response to NMII driven tension. Vinculin recruitment allows for the subsequent association of more F-actin with the AJ complex further stabilizing the complex under tension (le Duc et al., 2010).

Also contained within the conserved C-terminal region of E-cad is the juxtamembrane binding site for p120 catenin. p120 catenin functions as a powerful master regulator of E-cad stability by negatively regulating E-cad endocytosis and degradation. Additionally, p120 catenin has been shown to positively influence the transport of E-cad to the AJś. Despite its importance in vertebrates, however, p120 appears to be dispensable in Drosophila melanogaster development and null mutant animals are both viable and fertile (Harris and Tepass, 2010; Harris, 2012).

Another possible factor in E-cad stabilization is cadherin clustering. Cadherin clustering occurs when cadherins on the same cellular membrane associate laterally forming dense homophilic clusters on the PM. This lateral association of E-cad occurs after the E-cad molecules are already engaged in trans dimerization (i.e. interacting with E-cad on a neighbouring cell membrane) and creates stable adhesive microdomains, (Hong et al., 2013; Cavey et al., 2008). Both F-actin and NMII have been shown to play a role in E-cad clustering, however the mechanism of neither has yet been completely resolved (Shewan et al., Ivanov et al., 2007; Hong et al., 2013).
1.6. The interplay between Wnt and Adhesion

β-cat/Arm protein is mainly made up of 12 (13 in Drosophila) copies of a ~42 amino acid repeat called Armadillo repeats. These repeats form a rigid central domain that serves as the binding site for both TCF and E-cad and is flanked by distinct N- and C- terminal regions (Valenta et al., 2012). Thus, Wnt signalling and cell-cell adhesion have an inherent relationship due to the shared effector protein, β-cat/Arm.

It has been shown that under certain circumstances AJs and Wnt signal transduction can directly impact each other through β-cat. For example, transcriptional overexpression of E-cad has been shown to decrease Wg target gene expression via sequestration of available β-cat by excess E-cad (Orsulic et al., 1999; Heasman et al., 1994). Likewise, under conditions where destruction complex formation or activity is impaired, dissolution of the AJ and E-cad/β-cat binding has been shown to result in increased expression of Wnt target genes, presumably due to more free β-cat that can enter the nucleus to activate target gene expression (Eger et al., 2000). How the function of β-cat/Arm is determined appears to be largely regulated by phosphorylation of β-cat/Arm or its binding partners. Though TCF and E-cad bind the same domain in β-cat/Arm, the conformation β-cat/Arm adopts with each is different and thus phosphorylation sites on the proteins can impact TCF/E-cad binding in different ways (Xu and Kimelman, 2007). For example, phosphorylation of three E-cad cytoplasmic serine residues by GSK3β and CK2 increase the association of the cadherin and β-cat/Arm by nearly 100 fold by increasing the catenin-cadherin interaction. However, phosphorylation of β-cat/Arm Tyrosine residue 654 by Src or EGFR appears to weaken the interaction between E-cad and β-cat/Arm exclusively, as tissue with phosphomemetic 654Yβ-cat constructs have increased signalling (van Veelen et al., 2011). Phosphorylation of residue 654Y disrupts a key interaction between E-cad and β-cat Arm repeat 11-12 where as TFC does not interact with this Arm repeat (Xu and Kimelman, 2007).

In this thesis I describe experiments conducted to determine genetically where PP1β acts to affect Wnt target gene expression. This thesis is part of a larger project
exploring the mechanism of Arm interaction with PP1 β and how this relationship affects both adherens junctions and Wg signalling.
Chapter 2. Materials and Methods

2.1. Fly Lines and Crosses

Flies were grown and crossed at 25°C unless otherwise noted. Genetic strains used in the experiments include: *omb-lacZ*, *UAS-salm-lacZ*, *UAS-MYPT*, *UAS-flw-HA*, *en-Gal4*, *Dll-lacZ (on II #BL10981)*, *UAS-GFP (on II, #BL5430)*, *UAS-GFP (on III, #BL5431)*, *armS10 (on II #BL4782)* (obtained from the Bloomington Drosophila Stock Center); *flw-RNAi (on III #VDRC 29622)*, *flw-RNAi (on II #VDRC 104677)* (obtained from the Vienna Drosophila Resource Center); *yw; tub>FLAG-axin/CyO; axinS044230, FRT82/TM6B, fz-myc-arr/CyO* (a gift from Marcel Wehrli, Oregon Health & Science University, USA); *MARCM82B* (a gift from Bruce Edgar), *Hh-Gal4* (a gift from Konrad Balser).

The *UAS-flw-RNAi, Dll-lacZ/CyO* recombinant chromosome was made by me by recombining *Dll-lacZ (on II #BL10981)* and *flw-RNAi (on II #VDRC 104677)* onto the same chromosome. Additionally, all lines listed in the table below under the male genotype and actin flip-out cassette lines were lines generated by me.
### Table 1 Epistasis Crosses

<table>
<thead>
<tr>
<th>Male genotype</th>
<th>Female genotype</th>
<th>Cross handling</th>
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<tbody>
<tr>
<td><code>flw − RNAl, fz − myc − arr&lt;sub&gt;cyo&lt;/sub&gt;; TM6B</code></td>
<td><code>hsflp; Dll − lacZ, Act &gt;&gt; Gal4, RFP&lt;sub&gt;cyo&lt;/sub&gt;; TM6B</code></td>
<td>Vials are heat-shocked 72hrs A.E.L for 12 mins.</td>
</tr>
<tr>
<td><code>GFP fz − myc − arr&lt;sub&gt;cyo&lt;/sub&gt;; TM6B</code></td>
<td><code>hsflp; Dll − lacZ, Act &gt;&gt; Gal4, RFP&lt;sub&gt;cyo&lt;/sub&gt;; TM6B</code></td>
<td>Vials are heat-shocked 48hrs A.E.L for 45 mins</td>
</tr>
<tr>
<td><code>flw − RNAl, Dll − lacZ, FRT82axinS04423&lt;sub&gt;cyo&lt;/sub&gt;; TM6B</code></td>
<td><code>hsflpUAS − GFPtubGal4; +&lt;sub&gt;4&lt;/sub&gt;; FRT82BtubGal80&lt;sub&gt;TM6B&lt;/sub&gt;</code></td>
<td>Vials are heat-shocked 48hrs A.E.L for 12 mins</td>
</tr>
<tr>
<td><code>armS10 flw − RNAl&lt;sub&gt;cyo&lt;/sub&gt;; TM6B</code></td>
<td><code>hsflp; Dll − lacZ, Act &gt;&gt; Gal4, RFP&lt;sub&gt;cyo&lt;/sub&gt;; TM6B</code></td>
<td>Vials are heat-shocked 48hrs A.E.L for 12 mins</td>
</tr>
<tr>
<td><code>armS10 GFP&lt;sub&gt;cyo&lt;/sub&gt;; TM6B</code></td>
<td><code>hsflp; Dll − lacZ, Act &gt;&gt; Gal4, RFP&lt;sub&gt;cyo&lt;/sub&gt;; TM6B</code></td>
<td>Vials are heat-shocked 48hrs A.E.L for 12 mins</td>
</tr>
<tr>
<td><code>flw − RNAl, GFP&lt;sub&gt;cyo&lt;/sub&gt;; TM6B</code></td>
<td><code>hsflp; Dll − lacZ, Act &gt;&gt; Gal4, RFP&lt;sub&gt;cyo&lt;/sub&gt;; TM6B</code></td>
<td>Vials are heat-shocked 48hrs A.E.L for 12 mins</td>
</tr>
<tr>
<td><code>GFP flw − RNAl&lt;sub&gt;cyo&lt;/sub&gt;; TM6B</code></td>
<td><code>hsflp; Dll − lacZ, Act &gt;&gt; Gal4, RFP&lt;sub&gt;cyo&lt;/sub&gt;; TM6B</code></td>
<td>Vials are heat-shocked 48hrs A.E.L for 12 mins</td>
</tr>
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</table>

Note: Above are the genotypes used in the pseudo epistasis experiments and conditions in which the crosses where handled.. To induce heatshock in these crosses, vials were placed in 38°C water bath for the above stated time. All crosses were then grown at 29°C until they reached the late L3 larval stage.

#### 2.2. Antibody Staining

Imaginal wing discs from wandering third instar larvae were dissected with 1x phosphate-buffered saline (PBS). Wing discs were fixed in 4% paraformaldehyde at room temperate for 20min then washed with PBS three times. Discs are then blocked for 45 mins in 5% normal donkey serum followed by incubation overnight at 4°C with the primary antibodies. PBS 0.1% Triton x-100 (PBT) is then used to wash the discs three times followed by an 1.5h incubation at room temperature with secondary antibodies. Discs are then washed three more times in PBT then mounted in 70% glycerol solution.
The following antibodies were used in this study: mouse anti-β-galactosidase (1:2000, Promega), mouse anti-Arm (1:50, DSHB), rabbit anti-FLAG (1:200, Sigma), mouse anti-GFP (Cell Signaling, 1:500), mouse anti-HA (Sigma 1:500), mouse anti-Ptc (DSHB 1:50) and rat anti-Ci (DSHB, 1:50). All secondary antibodies were used at a dilution of 1:200 (Jackson ImmunoResearch).

2.3. Proximity Ligation Assay

The Proximity Ligation Assay (PLA®) system [Duolink® by Sigma-Aldrich] is used to detect in vivo interactions between proteins. This method utilizes unconjugated primary antibodies from two different species specific for the two proteins whose interaction is being tested. The primary antibodies are then detected by oligonucleotide labeled secondary antibodies. If the primary antibodies against the two proteins of interest bind in close proximity to one another, the addition of primers allows for rolling circle replication creating multiple iterations of a specific sequence which is then detected by a fluorescent DNA probe. A cluster of probes positively indicates close proximity of the two proteins.

In this thesis, PLA was performed using the Duolink® in situ PLA kit. Late third instar Drosophila wing discs were dissected in PBS and then fixed in 4% paraformaldehyde at room temperature for 20min. Following three PBS washes, discs were blocked for 1hr in 1% BSA dissolved in PBS. Primary antibody incubation was done overnight at 4°C. The discs were then incubated for 1 hour at 37 ° C with PLA probes diluted in 1%BSA. Followed by Ligation at 37 ° C for 30 minutes and amplification at 37 ° C for 100 minutes. After a final wash, the discs were mounted in 70% glycerol and imaged the next day.
Chapter 3. Results

3.1. MLCP interacts with Wg signal transduction

The data shown in Figure 7 were provided by Eric Hall.

After MYPT-75D and flw were identified as putative regulators of the Wg pathway, the Verheyen lab sought to further characterize the effects of knock-down of MLCP components. MYPT-RNAi and flw-RNAi (not shown) were expressed in the posterior region of L3 wing discs using the Hh-GAL4 driver. Expression of either RNAi resulted in mild reduction in both cytosolic Arm levels and Dll-lacZ expression (Figure 7F-H’’). Note that cytosolic Arm refers to non-junctional free Arm that is stable in the cytosol and likely participating in transcriptional activity after entering the nucleus. This reduction did not appear to be the result of cell death as a cleaved caspase 3 stain, a commonly used marker for apoptosis, showed no activation (Figure 7B’’ & F’’).

It is possible that MLCP may negatively impact Wg signalling in a non-autonomous manner, such as by regulating Notch pathway transduction, and prevent the transcription of the Wg ligand gene itself. To test this, levels of Wg protein expression and transcription were analyzed in L3 wing disc expressing MYPT-RNAi and flw-RNAi in heat shock flip out clones and in the posterior of the disc using an anti-Wg antibody staining and wg-lacZ reporter line respectively. We found that MYPT-RNAi and flw-RNAi did not affect Wg ligand distribution or transcription (Figure 7A-D’’). Thus, it is likely that MLCP regulates Wg signal transduction within the pathway itself.
Figure 7. Knock-down of MLCP components reduces the expression of Wg target genes.

A) Transcription of the Wg ligand in a wild type larvae as shown by staining for wg-lacZ expression. B-B'') Expression MYPT75D-RNAi in the posterior of L3 wing discs does not impact Wg ligand transcription (wg-lacZ), nor does it trigger apoptosis indicated by normal cleaved caspase 3 levels. C-D'') Wg ligand expression remains normal in heat shock inducible clones expressing MYPT75D-RNAi (D-D'') when compared to the wild type distribution (C). E-F'') Expression of MYPT75D-RNAi in the posterior of the wing disc shows a mild reduction in the transcription of Dll-lacZ compared to Dll-lacZ transcription in wild type wing imaginal discs. G-H') Additionally, cytosolic Arm levels decrease in MYPT75D-RNAi expressing tissue. Note the altered cytosolic Arm distribution in these discs (H') relative to the cytosolic Arm distribution in a wild type larvae (G).

3.2. MLCP affects Wg signal transduction directly

As mentioned in section 1.4, NMII is a core component of the actin cytoskeleton and has multiple key roles in the cell. It is possible that the effects seen from
overexpression and knockdown of myosin phosphatase components were the result of altering the basic function of a cell, such as transcription. To test this, I utilized UAS-flw-RNAi and UAS-MYPT-75D transgene lines to increase or decrease NMII activity respectively in heat shock induced flip-out clones within the imaginal wing disc. In this tissue, I then examined target gene transcription or protein expression of the Dpp and Hedgehog pathways. These pathways are both important in wing disc patterning and are not known to be regulated by MLCP. The target genes that were selected to monitor Dpp and Hedgehog (Hh) are commonly used readouts of signal transduction of these pathways. (Figure 8) Manipulating the levels of MLCP components appeared to have little effect on Hh pathway targets Cubitus interruptus (Ci) and Patched (ptc). Antibody staining against Ci and Ptc protein showed expression of these targets appeared to be unaltered by UAS-flw-RNAi and UAS-MYPT-75D expression, as stain signal intensity was similar between clones and unmarked control tissue (Figure 8B-C’). This is despite apparent perturbation in the expression of pattern of both Ci and Ptc that is likely the result of bulk tissue deformation rather than altered Hh pathway transduction (Figure 8C’-C”). Similar results were also found for expression of Dpp targets (Figure 9). Optomoter-blind (omb) and spalt-major (salm) lacZ reporter lines show no change in transcription when compared to wild type omb-lacZ and salm-lacZ expression outside the mutant clones (Figure 8 C-F”). These data are further support for a specific interaction with Wg pathway transduction rather than a general disruption in cellular physiology.
Figure 8. MLCP knock-down does not impact Hedgehog signalling
A-A") These panels represent the normal protein expression and distribution of the Hedgehog targets Ptc and Ci respectively. B-B") flw-RNAi expression in GFP marked flip out clones does not appear to impact the protein expression of Ci and Ptc when compared to wild type unmarked tissue in about 17 discs examined. C-C") The overexpression of MYPT-75D in flip out clones does not appear to consistently lead to a decrease (indicated by white arrows) in the protein expression of Hh target genes ci and ptc despite causing significant tissue deformation in approximately 20 discs examined.
Figure 9. MLCP does not impact Dpp target gene expression
A-B) Wild type expression patterns of the salm and omb lacZ reporter trap lines. C-D’) The transcription Dpp target salm does not appear to be impacted by either knock down of flw-RNAi or overexpression of MYPT-75D (approximately 20 discs each). MYPT75D overexpression does cause significant tissue distortion. E-F’) The transcription of Dpp target omb also appears to be minimally affected by flw-RNAi or UAS-MYPT75D in ~ 17 discs examined.

3.3. PP1 β (flw) interaction with Axin

Though MLCP is thought to primarily negatively regulate the activity of NMII there are previously published data that NMII regulation is not MLCP’s sole function (Yang et al., 2012). In 2012, a publication by Yang et al., found that an exogenously expressed flw-HA transgene in Drosophila S2 cells was able to associate with two members of the ERM family of proteins (ezrin-radizin-moesin) Merlin (Mer) and Moesin (Moe). Mer has been shown to regulate cell proliferation and there is evidence that its function is needed for the formation of AJs. Moe has been shown to negatively regulate Rho activity to maintain epithelial integrity. This publication also found correlations between the knockdown of flw and an increase in phosphorylation of both Mer and Moe as well as altered Mer and Moe localization.
Previous research done by Luo et al. (2009) also has suggested that several isoforms of PP1 may act as positive regulators of the Wg pathway. Though none of the PP1 isoforms were sufficient to induce Wg target gene transcription alone, co-expression of many of the PP1 isoforms together with Wg and Fz resulted in greater Wg target gene expression than was seen with Wg and Fz expression alone. Luo et al. (2009) found that the increased activity of the Wg pathway was likely due to negative regulation of the destruction complex scaffold Axin by MLCP. To test if Axin and PP1β are in close proximity with one another, I conducted a proximity ligation assay (PLA) between a flag-tagged Axin (yw;tub>FLAG-axin) transgene and HA tagged flw transgene (UAS-flw-HA) (Figure 10). It is important to note that the yw;tub>FLAG-axin construct does not use the UAS/GAL4 system to drive ubiquitous expression of FLAG-axin, rather FLAG-axin is directly controlled by a tubulin promoter. Expression of this construct alone does not disrupt Drosophila development extensively and this genotype is able to produce fertile and viable offspring that are wild type in appearance. The positive control for this experiment was the interaction between FLAG-Axin and endogenous Arm (a confirmed direct interaction) and the negative control was between GFP and FLAG-Axin (Figure 10B&A, respectively)(Price, 2006). The positive control shows puncta covering the whole of the disc surface. This is best visualized in the cross section (Figure 10B). In the experimental cross the engrailed-Gal4 driver confines UAS-flw-HA expression to the posterior region of the wing disc, while FLAG-Axin is expressed throughout the disc. A comparison of posterior and anterior domains of this tissue shows no difference in puncta formation and resembles the negative control (Figure 10C). Thus, based on this PLA experiment, there is no close (<45nm) interaction of Axin and the β isoform of PP1 encoded by flw.
Figure 10. Testing if Axin and PP1β are in close association with one another. A-A') The negative control for the PLA is between Flag-Axin and GFP. There are only minor background speckles formed randomly in this genotype indicating that these proteins do not interact. B) The positive control for the PLA tests the interaction between endogenous Arm and a Flag-tagged Axin. Speckles on the surface of the disc are a positive indication that both proteins are in close proximity with one another. C) Like the negative control, PLA conducted between Axin and PP1β shows dispersed speckles in the tissue indicating these proteins are not in close proximity with one another (<45nm).

3.4. Testing the genetic interactions of Myosin phosphatase and components of the Wg pathway

The importance of NMII in a multiple processes within the cell means there are multitudes of different ways that its activity could affect Wg pathway components. Additionally, it is possible that MLCP itself could regulate Wg pathway components directly through dephosphorylation. Thus, identifying where in the Wg pathway MLCP genetically interacts was key to elucidating the mechanism of how MLCP impacts Wg signal transduction.
In order to identify where MLCP and the Wg pathway genetically interact, I designed a series of pseudo-epistasis experiments utilizing the *flw-RNAi* transgene. I term these experiments ‘pseudo epistasis’ because I elected to largely use transgenes to measure the interaction between MLCP and the Wg pathway, rather than traditional loss of function or null alleles. In the case of MLCP, the decision to use a MLCP transgene rather than an NMII constitutively active allele was largely because the transgenes was less severe, thus allowing me to examine mutant tissue. Additionally, *flw-RNAi* lines were commercially available on both the second and third chromosomes simplifying the genetic crosses that were needed for each experiment. It should be also noted that I did not perform this analysis or any subsequent analysis with MLCP overexpression transgenes as MLCP overexpression often induced cell death and also lead to decreased expression of Wg target genes. The overexpression of genes can often create artificial environments that are not always representative of normal biological processes. We therefore decided to pursue MLCP knockdown, as it was more likely to yield data relevant to development. In these experiments the activity of Wg pathway components was genetically altered at different “levels” of signal transduction to result in pathway hyperactivity, as assayed by elevated *Dll-lacZ* expression. The *flw-RNAi* transgene was then expressed in these different genetic backgrounds. If introduction of the *flw-RNAi* altered *Dll-lacZ* overexpression then it is likely that the *flw-RNAi* expression was exerting its effects “below” the level of the manipulated Wg pathway component. However, if the presence of *flw-RNAi* caused no change *Dll-lacZ* expression, then the result was interpreted to mean that *flw-RNAi* impacted signal transduction “above” the manipulated Wg pathway component. Three different Wnt pathway components were tested in the epistasis analysis: The receptors Fz and Arr, the destruction complex scaffold Axin, and the major effector protein Arm.

3.4.1. Testing the putative genetic interaction between MLCP and the Wg receptors

Evidence in Figure 7 indicated that MLCP was likely acting within the Wg-signal receiving cell to affect Wg targets (reviewed in section 3.1). Thus, Fz and Arr were tested as the first possible sites of genetic interaction between the Wg pathway and MLCP. For this analysis, a transgene was used in which the sequences encoding the
two receptors were fused together, with a myc tag (fz-myc-arr). Fusion of these two receptors forces interaction between them, mimicking Wg ligand engagement, and initiating signal transduction (Tolwinski et al., 2003).

Figure 11B-B’ shows increased Dll-lacZ expression when fz-myc-arr and GFP are co-expressed within RFP marked clones. GFP expression is used to rule out possible GAL4 titration effects that may occur from the expression multiple transgenes in the experimental crosses. Insignificant increases of Dll-lacZ expression in clones occurring outside of the D/V boundary region is result the result of the weak driver used to generate transgene expression in clones. Co-expression of flw-RNAi with fz-myc-arr (Figure 11D’) leads to an appreciable suppression of this overexpression phenotype. Comparison between mutant clones and wildtype tissue showed similar levels of Dll-lacZ expression. This indicates that flw-RNAi affects the Wg pathway below the “level” of Wg receptor and co-receptor engagement, since it is needed for the full effects of pathway activation that are triggered by Fz-myc-Arr.
Figure 11. Knock-down of PP1 β rescues Wg receptor fusion overexpression phenotype
A) Expression of Dll-lacZ in wild type larval wing discs. B-B’) Co-expression of a fz-myc-arr fusion construct with GFP (not shown) successfully induces increased expression of Dll-lacZ in RFP marked clones in 26 discs examined. C-C’) flw-RNAi co-expression with GFP (not shown) in RFP marked clones causes cell autonomous decreased Dll-lacZ expression in 16 discs examined. D-D”) flw-RNAi co-expression with the fz-myc-arr construct causes a complete rescue of the Dll-lacZ overexpression phenotype seen in fz-myc-arr;GFP expression in 29 discs examined.

3.4.2. Testing the putative genetic interaction between MLCP and Axin

Axin was utilized to test if MLCP interacted with the Wg pathway at the level of the destruction complex. As mentioned above, the destruction complex regulates Arm levels within the cytosol. Key to the function of the destruction complex is the scaffold protein Axin which acts to bring APC, β-cat and the kinases CK-1 and GSK3β together. In the absence of Axin, the other components of the destruction complex are unable to assemble. Though the PLA shows that PP1β and Axin do not directly interact, MLCP may still regulate other destruction complex components or Axin through an indirect mechanism. At the “level” of the destruction complex, an axin null allele (axin<sup>S044230</sup>) was made homozygous in GFP-positive MARCM clones. Figure 12C-C’ shows that clonal tissue mutant for axin has significant increases in Dll-lacZ reporter expression, as well as ectopic expression in areas outside of the endogenous domain of expression in 10/10 discs imaged. There appears to be little difference in Dll-lacZ expression between the presence and absence of the flw-RNAi transgene expression within axin null clones (Figure 12C-B’), suggesting that loss of flw cannot block the hyperactivation of the Wg pathway seen in axin mutant cells. However, antibody staining against cytosolic Arm shows that in axin mutant clones expressing flw-RNAi, Arm appears to tightly localize to the periphery of cells compared to Arm in axin mutant tissue not expressing flw-RNAi (Figure D’ vs E’ respectively). Recall that in Figure 7, expression of MYPT-75D-RNAi in flip out clones appeared to cause a decrease in the level of cytosolic Arm. Together this data suggests that knock down of MLCP influences Wg by changing the distribution of Arm. It is likely that there are no observable differences in Dll-lacZ expression between clone with and without flw-RNAi expression due to the strong positive effect the axin null
allele has on cytosolic Arm levels for which the flw-RNAi transgene was unable to compensate.
Figure 12. MLCP does not rescue the effects of the Axin null allele
A) Expression of Dll-lacZ in wild type larval wing discs. B-C') GFP marked clones mutant for axin induce strong etopic Dll-lacZ expression 42 discs examined. Expression of flw-RNAi with this mutant tissue does not appear to rescue this strong induction in 30 discs examined. E-E') Antibody staining against cytosolic Arm levels distributed through out the cell in clones mutant for axin. D-D') In flw-RNAi expressing axin mutant tissue, Arm is localized more closely with the periphery of the cell. Arrows in panel D-E' mark the periphery of the clone borders.

3.4.3. Testing the putative genetic interaction between MLCP and Arm

Mounting evidence suggested that MLCP likely influenced Arm directly. To test this I used a truncated Arm construct that lacks the phosphorylation sites targeted by the destruction complex (UAS-armS10). This makes the armS10 protein degradation resistance, and thus able to participate in the transcription of Wg target genes in the absence of the Wg ligand. Expression of UAS-armS10 in RFP-marked heat shock induced clones shows significant increases in Dll-lacZ (Figure 13C-C'). Co-expression of UAS-armS10 and UAS-flw-RNAi appeared to yield mix results as clonal tissue showed both increases and decreases in Dll-lacZ expression within the same wing disc (Figure13 B-B'). Closer inspection of clone location hinted that the variable influence of flw-RNAi expression in this genetic background correlate with where the clone was induced in the wing disc. Analysis of the total clone population and their impact on wild type Dll-lacZ expression, however, showed that overall clones co-expressing armS10 and flw-RNAi showed a decrease in wt Dll-lacZ expression (75% of clones impacted Dll-lacZ), whereas the armS10 and GFP expressing clones were more likely to show an increase in Dll-lacZ expression (75% of clones impacted Dll-lacZ).

Interestingly, the small number of clones that co-expressed armS10 and flw-RNAi and failed to ameliorate increases in Dll-lacZ expression stereotypically occurred in a specific region in the disc: next to the A/P and D/V boundaries in the posterior compartment of the disc (Figure 13B’ arrow). Clones occurring outside this region consistently showed either normal Dll-lacZ expression or decreases in expression. Expression of the positive control genotype arms10;GFP in RFP marked clones generated in any region resulted in increase/no change consistently.
Additionally, location specific failure of \textit{flw-RNAi} to rescue \textit{Dll}-\textit{lacZ} expression appeared to be specific to \textit{armS10} genotype and did not occur in \textit{fz-myc-arr;GFP} co-expression crosses (Figure 11). The \textit{armS10} transgene is a truncated Arm construct that removes the first 54 amino acids in the N-terminus in a region that lies adjacent to the \textit{α}-catenin binding site (Pai et al., 1996). Though this construct has previously been shown to interact with \textit{α}-catenin, the location-dependent effect of \textit{flw-RNAi} co-expression suggests that the Arm- \textit{α}-cat interaction may be attenuated by this truncation leading to an association that is sensitive to cellular tension as \textit{α}-cat is known to change conformation upon mechanical stretching (Pai et al., 1996; le Duc et al., 2010). Regardless of this confounding data, these experiments provide evidence that the MLCP impacts Wg pathway transduction through Arm localization.
Figure 13. MLCP knock down partially rescues the *armS10* phenotype

A) Expression of Dll-lacZ in wild type larval wing discs. B-B') *armS10;GFP* expression lead to ectopic expression of Dll-lacZ in 75% of RFP marked clones (n=11 clones). C-C') *armS10* and *flw-RNAi* co-expressing RFP marked clones cause a decrease in Dll-lacZ expression in 75% of clones that intercept the normal wing disc Dll expression pattern (total n=32 clones marked by white arrow). The clone indicated by a red arrow in C' is representative of the 8 clones found in the *arms10;flw-RNAi* genotype that caused ectopic expression of Dll-lacZ.
Chapter 4. Discussion

The Wg pathway is one of several key biological processes that are highly evolutionarily conserved throughout metazoan development and homeostasis. The conserved nature of these processes enables the use of Drosophila to explore questions that are otherwise difficult to research in an in vivo context. In this thesis, we have utilized Wg signalling in the Drosophila imaginal wing disc as a stereotypical model of canonical Wnt signalling to explore how the putative regulator MLCP impacts the Wg target gene expression. We show here, through genetic analysis, that MLCP appears to impact Wg pathway signal transduction in a specific manner and that MLCP likely imparts this regulation through the key Wg effector protein Arm by altering its localization.

4.1. MLCP genetically interacts with Arm

In section 3.3 I described data published by Luo et al. (2007) that suggested that isoforms of PP1 might positively regulate Arm stabilization by inhibiting the destruction complex scaffold Axin. Their data showed that exogenously expressed isoforms of PP1 in HEK293 cells could interact with dephosphorylated Axin and that PP1 knockdown resulted in decreased electrophoretic migration of Axin. Luo and colleagues suggested that this was likely the result of an increase in phosphorylated Axin species due the knockdown of PP1 isoforms. The authors of the article also noted that PP1 expression appeared to decrease the association between the key destruction complex kinase GSK3β and Axin. Luo and colleagues suggested that this association was a positive indication of Axin’s activity and therefore concluded that PP1 isoforms negatively regulate Axin through a dephosphorylation event that decreases the association of Axin and GSK3β. It is important to note that much of the Luo et al. analysis is focused on PP1 expression in general and individual isoforms of PP1 are only tested in the preliminary analysis.
As I mentioned in section 1.4, though PP1 isoforms are involved in a number of cellular activities, PP1β9C serves a non-redundant role in the dephosphorylation of NMII RLC. It is likely, however, that this RLC is not the only substrate of PP1β9C. Thus we tested the possibility that the effects of PP1β9C maybe a consequence of increased activity of the destruction complex through the increased association of Axin and GSK3β. Our data, however, casts doubt on this putative interaction between Wg pathway members and this isoform of PP1 in the wing disc. In Figure 10, results from the PLA performed between PP1β-HA and flag-Axin were negative for an interaction between these two proteins. Additionally, in Figure 13B-B′ I show that flw-RNAi expression in most cases was able to rescue the effects of armS10 expression on elevated Dll-lacZ transcription levels. If PP1β impacted Wg signal transduction through negative regulation of Axin, a rescue of Arms10 phenotype should not be possible as ArmS10 can not be targeted and degraded by the destruction complex. Therefore, our data suggests that the regulation of Axin by MLCP is unlikely.

4.2. MLCP influences Arm localization

The ability of MLCP knockdown to rescue the ArmS10 phenotype also suggests that MLCP may indirectly regulate Arm localization as Arm is still present in these cells, but possibly unable to associate with TCF/LEF transcriptional machinery. The axin null allele data supports this as the likely mechanism of MLCP/Arm genetic interaction. Though flw-RNAi expression was unable to rescue ectopic expression of Dll-lacZ induced in axin mutant tissue, antibody staining for Arm in this genotype shows a clear change in Arm localization compared to non-flw-RNAi expressing axin mutant tissue. Cross sections (Figure 12D-E′) of axin null cells with flw-RNAi expression show Arm appears to localize to the apical region of cells. axin null cells with wild type flw expression, however show a broad, homogenous distribution of Arm in the cytosol. The change in Arm localization upon flw knockdown was also seen in Yang et al. (2012). Their study showed that flw-RNAi expression within the ptc expression domain of third instar wing discs appears to cause apical accumulation of Arm within the cells.

The apparent change in Arm localization to the apical region of discs, and MLCP’s association with NMII suggests that MLCP knockdown may be causing
increased Arm recruitment to the AJ. As mentioned in section 1.6 of the introduction, Arm has dual roles, in adhesion at AJs and as a co-transcription factor in Wg signalling. These two functions are largely regarded as using separate pools of Arm that are distinguished from one another spatially and likely by post-translational modification (Valenta and Basler, 2012). There, however, is evidence that these spatially distinct pools can compete. For example, experiments with E-cad in cell culture have shown that ectopic expression of E-cad can cause a suppression of Wg target gene transcription, presumably by sequestering Arm from the nucleus (Heasman et al., 1994; Orsulic et al., 1999). A possible mechanism by which MLCP knockdown could impact Wg target gene expression is through MLCP’s well-characterized role in negative regulation of NMII activity (Figure 14). As previously mentioned, NMII functions in cadherin clustering; a process that increases the stability of E-cad at the AJ (Shewan et al., 2005; Ivanov et al., 2007). Studies in Drosophila embryos and mammalian cell lines suggest the E-cad at AJ exists in two states: a mobile fraction and immobile fraction (Engl et al., 2013; Cavey et al., 2008). E-cad proteins that are immobilized by clustering and F-actin interaction are less likely to participate in recycling, leading to expansion and/or stability of the AJ in the process (Hong et al., 2012). As knockdown of MLCP increases NMII function, the increased NMII, likely through F-actin, could cause trans dimerized E-cad to laterally interact, increasing the immobile pool of E-cad at the AJ leading to larger AJs (Wu et al., 2014). A larger immobile pool of E-cad at the AJ would subsequently lead to increased recruitment of Arm, and less Arm that is able to take part in transcription (Figure 14).
Figure 14. Increased NMII activity indirectly impacts Arm localization. The above model is based on data generated by the Verheyen lab as well from Engl et al., 2014. We suggest that increased NMII activity stabilizes E-cad at the site of the AJs. This leads to AJ expansion and subsequently sequesters Arm from the cytosol. Though the Wg pathway is active, the engagement of Arm with E-cad prevents it from participating in Wg target gene transcription.

4.3. MLCP impacts Arm localization through NMII

In section 3.3 of the results, I mention the work of Xu and colleagues that identified PP1β as a possible regulator of the ERM proteins Mer and Moe. Like other ERM proteins Moe is a membrane-associated protein that has been shown to regulate the dynamics of cortical actin. When Moe is unphosphorylated, the protein is active and functions to regulate apical-basal polarity in the cell and encourage the maintenance of membrane apical-basal polarity and encourage cortical F-actin assembly. Mer, however, appears to function as a suppressor of proliferation and is active when it is hypophosphorylated. Mer also appears to have a role in stabilizing AJs. In the publication by Xu et al., the authors find that knocking down flw, altered the localization
of Moe and Mer. Xu et al. mention that localization of these proteins is an indication of their activity and that Mer association with the PM indicates that the protein is likely phosphorylated and inactive when flw was knocked down. The authors also noted that flw mutant cells have increased levels of both Arm and E-cad associated with their apical surface and that tissue deformation in these cells worsened during pupal development. This tissue deformation may have been partially due to cell death as cleaved caspase 3 stains in L-3 larval imaginal disc showed an increased number of cells undergoing apoptosis. The authors also showed that inactive Mer and overexpression of Moe were able to partially phenocopy flw knockdown and induce similar tissue folding. The connection between Moe activity and cortical F-actin stability suggests PP1β9C may be able to impact AJ stability through more than one protein. One would expect, however, that this AJ expansion would also be negatively impacted given the role of Mer in AJ stability. Additionally, the lack of effect on Moe and Mer when flw is overexpressed indicates a more complex relationship between PP1β9C and these proteins. Data provided by my colleague Eric Hall have shown that expression of a constitutively active form of NMII closely phenocopies the impact of MLCP knockdown on larval disc tissue morphology, cytosolic Arm levels and Dll-lacZ transcription (unpublished). Thus, as a result of our data, there is more evidence that MLCP knockdown likely impacts Wg signal transduction through NMII activity. This, however, does not exclude Moesin as a contributing factor.

4.4. Future directions and lingering questions

Members of the Verheyen lab, and in particular my colleague Eric Hall, are currently performing several experiments to provide further evidence in support of our hypothesis. Based on the above data and the data provided by Eric Hall and other lab members, we hypothesize that MLCP knockdown decreases Wg target gene transcription through changes in Arm localization. Increased NMII activity leads to a larger pool of stabilized E-cad at the AJs. This increased amount of E-cad sequesters Arm from participating in Wg target gene transcription. Some of these ongoing experiments include trying to replicate the effect of MLCP knockdown on Wnt signalling components in mammalian cells. One key experiment that has yet to be completed
utilizes an E-cad-α-cat fusion construct that does not bind nor require Arm for adhesion. With this construct we plan to replace endogenous E-cad expression in clonal cells while simultaneously inducing the knockdown of MLCP. If our hypothesis is correct, knockdown of MLCP should not reduce the expression of Wg target genes as the exogenous E-cad-α-cat doesn’t associate with Arm, which would allow Arm to continue to accumulate in the cytosol and nucleus of these mutant cells. If our hypothesis proves true, however, I believe the next question that must be asked is how this new information fits into what is already known about Drosophila wing development and the relationship between Wg signalling and E-cadherin stability therein. Cellular morphology in third instar wing disc is anisotropic. Cells near the D/V boundary in a third instar wing disc appear uniform and apically constricted (LeGoff et al., 2013). These cells contribute to an NMII supercellular cable that functions in the separation of the Dorsal and Ventral compartments. As one moves away from this point, the apical diameter of cells becomes wider and less uniform. Cells flanking this region also have the highest Wg signalling yet also appear to have high levels of NMII activity due to the apical constriction present in these cells and the supercellular cable (Widmann and Dahmann, 2009; Major et al., 2006). The D/V boundary region, however, is not enriched in E-cad or other adherens junctional proteins. This fact appears to be contradictory to our hypothesis that might predict that increased NMII activity is antithetical to proper Wg target gene expression by way of sequestering the effector Arm to more stable AJ. To complicate matters further, Wg signalling has been shown to positively regulate the E-cad transcription in the wing disc and both are needed for cells to maintain the proper wing morphogenesis (Jaiswal 2006; Widmann and Dahmann 2009).

What other factors prevent Wg target gene transcription from being disrupted when E-cad expression is increased? The answer to this question may lay in endocytosis and exocytosis rates of E-cad in MLCP mutant cells. As mentioned previously, the stability of E-cad at the AJ complex is largely controlled by its ability to resist endocytosis (West and Harris, 2016). Increased endocytosis of E-cad is a major factor in AJ remodelling and, if endocytosis is increased enough, can result in the dissolution of adhesion all together. Thus, I purpose that experiments be conducted to look at the co-localization of E-cad and the recycling markers Rab 5 and Rab 11. Rab 5 is a known indicator of E-cad endocytosis whereas Rab 11 is known to be associated
with E-cad recycling back to the membrane (West and Harris, 2016). Levels of E-cad localization with Rab 11 and Rab 5 would help determine the endocytosis rate of the cadherin and comparisons between E-cad-Rab11/Rab5 in MLCP mutant and non-mutant tissue would indicate if E-cad endocytosis is altered. Increased NMII activity suggests that this might be the case and that E-cad is more stable. This result also suggests that Wg signalling may require endocytosis of E-cad to liberate enough Arm to make Wg target gene transcription possible. Though not entirely related to our hypothesis, I also propose analysing E-cad phosphorylation state in cells engaged in Wg signalling versus in cells not engaged in Wg signalling. Phosphorylation of E-cad sites can both improve and perturb E-cad stabilization. Analysis of the phosphorylation species present in these different cell populations may elucidate how Wg gene transduction is able to resist increased E-cad transcription.

4.5. Conclusion

Dynamics of development and tissue patterning relies on a series of highly conserved signalling pathway to dictate gene expression and tissue morphology in the organism. The Wingless pathway is one of these key pathways and its dysregulation often leads to disease. In this thesis we identify MLCP as a putative regulator of the Wg signalling pathway. We provide evidence that MLCP genetically interacts with the key Wg effector protein Armadillo. Additionally, we provide evidence that MLCP knockdown likely impacts Arm localization to the Adherens Junctions.

Taking this and other evidence into consideration, I propose a regulatory relationship between Wg signalling and the AJs wherein increased E-cadherin stability can negatively impact cytosolic levels of Arm that are needed to drive Wg target gene transcription. Additional experiments are needed to confirm this model as a means of MLCP impacting Wg target gene expression. I believe, however, that determining the molecular mechanism of MLCP and Wg genetic interaction will contribute to understanding the complex relationship that Wg signalling and cellular adhesion have.
References


