The Function of Centralspindlin in Drosophila Development

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THE FUNCTION OF CENTRALSPINDLIN IN DROSOPHILA DEVELOPMENT

By

Michael Craig Sfregola

B.A., Occidental College, 2006

A dissertation submitted to the

Faculty of the Graduate School of the

University of Colorado in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

Department of Molecular, Cellular, and Developmental Biology

2013
This thesis entitled:
The function of centralspindlin in Drosophila development
written by Michael Craig Sfregola
has been approved for the Department of Molecular, Cellular, and
Developmental Biology

Chair of Committee

Committee Member

Date ______________

The final copy of this thesis has been examined by the signatories, and we
Find that both the content and the form meet acceptable presentation standards
Of scholarly work in the above mentioned discipline.
Abstract

Centralspindlin is an essential protein complex in all metazoans well-studied for its essential role in cytokinesis. Emerging evidence suggests centralspindlin has important interphase functions as well. Through its regulation of Rho family small GTPases at the plasma membrane, the centralspindlin complex is able to effect a number of cellular process including cell adhesion and cell migration. In this thesis I explored centralspindlin’s function in development and tissue morphogenesis. In addition I investigated potential nuclear functions of the centralspindlin complex. To address these potential functions of centralspindlin I used the model organism *Drosophila melanogaster*.

I found centralspindlin is essential for migration of *Drosophila* wing disc cells and proper thorax closure in addition to its role in cytokinesis. The subcellular localization of centralspindlin is important for its function in thorax development as perturbation of centralspindlin’s cytoplasmic activity results in thorax defects. I also found centralspindlin was required for the formation of sensory organ bristles. I found no effect of centralspindlin depletion or mutation on signaling pathways involved in sensory organ differentiation indicating this is likely a secondary result of thorax closure and cell cycle defects. I used *Drosophila* salivary glands to investigate nuclear functions of centralspindlin but was unable to discern a clear function. Centralspindlin localizes to the nuclei and chromosomes of these cells but does not seem to be involved in either transcription or DNA replication.
My investigations have demonstrated centralspindlin is a multi-functional protein complex responsible for regulating various cellular events including cytokinesis, cell migration, and cell adhesion.
Acknowledgements

Thanks to my family and friends for their encouragement and support throughout this entire process.
# Table of Contents

Title page i  
Approval Page ii  
Abstract iii  
Acknowledgements v  
Table of Contents vi  
List of tables viii  
List of figures ix

## Chapter 1: Introduction
1

Epithelial morphogenesis in Drosophila development 1  
Sensory organ development in Drosophila 6  
Small GTPase Regulation 12  
Function of centralspindlin in cell division and cell morphology 15  
Scope of Thesis 19

## Chapter 2: Phenotypic analysis of dsRNA depletion of centralspindlin 22

I. Introduction 22  
II. Methods 23  
III. Results 26  
IV. Discussion 35

## Chapter 3: Structure-function analysis of centralspindlin 37

I. Introduction 37  
II. Methods 38  
III. Results 41
IV. Discussion 53

Chapter 4: Role of centralspindlin in sensory organ development and Notch signaling 59
I. Introduction 59
II. Methods 59
III. Results 71
IV. Discussion 95

Chapter 5: Localization and role of Centralspindlin in salivary gland nuclei 102
I. Introduction 102
II. Methods 103
III. Results 107
IV. Discussion 117

Chapter 6: Conclusions and Future Directions 123
Conclusions 123
Future Directions 128

References 131
List of Tables

Table 1-1. Homology of Centralspindlin and interacting proteins. 16
Table 4-2. Screen of candidates for genetic interaction with centralspindlin. 93
List of Figures

Figure 1-1. Comparison of embryonic dorsal closure and pupal thoracic closure processes. 3

Figure 1-2. Differentiation of sensory organs. 7

Figure 1-3. Notch-Delta signaling in Sensory organ precursors. 9

Figure 1-4. The Centralspindlin complex and its function during cytokinesis. 18

Figure 2-1. Centralspindlin is required for bristle formation and proper thorax closure of the adult notum. 27

Figure 2-2. Tum-dsRNA phenotype can be rescued by expression of wt transgene while mutant tum fails to rescue. 28

Figure 2-3. Disruption of centralspindlin causes thorax closure defects in the pupal notum and causes a reduction in sensory organ cells. 30

Figure 2-4. Depletion of centralspindlin by tum-dsRNA causes defects in eye and wing development. 32

Figure 3-1. Overexpression of Tum and/or Pav result in thorax closure and abdomen defects. 42

Figure 3-2. Localization of Centralspindlin proteins in mitotic and interphase thoracic cells. 44

Figure 3-3. High levels of Centralspindlin leads to plasma membrane localization. 46

Figure 3-4. Deletion of nuclear localization signals of Tum and Pav result in constitutive cytoplasmic localization during interphase. 47

Figure 3-5. Expression of Tum- and Pav- Δnls mutants causes defects in pupal development. 48

Figure 3-6. Expression of Tum and Pav transgenes in the scutellum results in bristle defects. 50

Figure 3-7. Removal of PavΔnls-GFP from the cytoplasm rescues thorax closure and bristle loss. 51

Figure 4-1. Depletion of Tum or Pav by dsRNA leads to cell fate transformation and cytokinesis defects in sensory organ lineages. 72
Figure 4-2. Pav-GFP localizes to the cleavage furrow and nuclei of sensory organ precursors.

Figure 4-3. Depletion of Tum or Pav by dsRNA leads to cell fate transformation and axon morphology defects in sensory organ lineages.

Figure 4-4. Candidate screen using luciferase reporter assay of Notch target transcription in S2 cells.

Figure 4-5. Luciferase reporter assay of potential hits to measure Notch target transcription in S2 cells.

Figure 4-6. Luciferase reporter assay of centralspindlin’s role Notch target transcription in S2 cells.

Figure 4-7. qPCR analysis of Notch target gene transcription after depletion of Tumbleweed by dsRNA with and without Notch induction.

Figure 4-8. Chromatin Immunoprecipitation from S2 cells crosslinked with formaldehyde.

Figure 4-9. Chromatin Immunoprecipitation from S2 cells crosslinked with 1% formaldehyde.

Figure 4-10. Chromatin Immunoprecipitation from 6-10 hour Pav-GFP and wildtype embryos.

Figure 4-11. Depletion of centralspindlin or expression of dominant negative constructs disrupt Notch target expression and cause cytokinesis defects in the wing disc.

Figure 4-12. Effect of Tum depletion on Notch target gene expression.

Figure 4-13. Pon-GFP localization during sensory organ cell division in wildtype and centralspindlin depleted flies.

Figure 4-14. Expression of Neuralized restores bristle number but fails to rescue thorax closure defect of tum-dsRNA phenotype.

Figure 5-1. Localization of centralspindlin in salivary gland cells.

Figure 5-2. Pav-GFP and Tum associate with polytene chromosomes.

Figure 5-3. Centralspindlin localizes to euchromatin regions but is restricted from heterochromatin.
Figure 5-4. DNA replication is unaffected by expression of Tum mutant constructs. 115

Figure 5-5. Overexpression of Pav-GFP leads to the formation of nuclear vesicles. 116

Figure 6-1. The multiple functions of centralspindin in regulation of cell division and cell morphology. 124
Chapter 1: Introduction

Epithelial morphogenesis in Drosophila development

During metazoan development the process of epithelial morphogenesis transforms tissues and cells to give rise to an organism's complex body plan (Blanchard et al., 2010; Harden et al., 1999; Martín-Blanco et al., 2000). This transformative process requires complex morphogenetic movements of epithelial sheets. Sheets of cells undergo coordinated changes in cell shape and size as they migrate (Blanchard et al., 2010; Mateus and Martinez Arias, 2011). During this process individual cell must detach from neighboring cells and form new adhesive contacts (Baum and Georgiou, 2011; Niessen et al., 2011). The regulation of these properties of epithelial sheets ultimately gives rise to the diversity of body plans among different organisms as well as the diversity of cellular structures and tissues within an organism. Epithelial morphogenesis is an essential process in all metazoans in both normal development as well as pathological processes such as wound healing (Baek et al., 2010; Parisi and Vidal, 2011). An understanding of these complex processes has been derived from studying morphogenesis in the model organism Drosophila melanogaster (Kiehart et al., 2000; Usui and Simpson, 2000).

The transformation from a syncytial embryo to an adult fly requires numerous separate but coordinated epithelial morphogenetic movements (Bloor and Kiehart, 2002; Usui and Simpson, 2000). One such event is the well-studied process of Dorsal Closure (DC) during Drosophila embryogenesis. DC occurs during stage 12 when epithelial sheets
migrate dorsally and fuse to close a gap in the epithelium (Figure 1-1A) (Kiehart et al., 2000). The dorsal migration of these epithelial sheets depends on their adhesion to the underlying sheet of cells called the amnioserosa (Fig. 1-1 A, green) (Gorfinkiel and Arias, 2007; Mateus and Martinez Arias, 2011). Amnioserosa cells undergo pulsed rhythmic contractions to pull the migratory epithelial sheets dorsally where they meet at the midline and fuse (David et al., 2010; Solon et al., 2009). The amnioserosa serves as a substrate for the migrating sheets as they alternately form and release adhesive intercellular contacts during the pulsed contractions (Gorfinkiel and Arias, 2007; Solon et al., 2009). In addition to the forces generated by the amnioserosa, leading edge cells provide forces to drive their own migration. Cells at the leading edge of the migrating sheet (Fig. 1-1A, blue) elongate and spread dorsally as they are pulled by the amnioserosa contractions (David et al., 2010). Additionally, leading edge cells contain actin and myosin bundles that form a continuous cable encircling the dorsal hole (David et al., 2010). As the actin/myosin cable contracts additional forces are provided to drive dorsal closure in a process that has been likened to the tightening of a purse string (Kiehart et al., 2000). Failure of this process can result in delayed development or embryonic lethality.

Many mutations affecting this process were isolated from genetic screens and revealed the DC process was heavily dependent on cytoskeletal regulation (Jacinto et al., 2002). Famously, the gene encoding myosin heavy chain was identified and named zipper because the failure of the epithelial sheets to fuse or “zip” together in flies mutant for the gene (Franke et al., 2005). Interestingly, in zipper null mutants, expression of the zipper protein in only the amnioserosa cells but not the epithelium is able to rescue DC (Franke et al., 2005). This result demonstrates the importance of amnioserosa contractions to pull
Figure 1-1. Comparison of embryonic dorsal closure and pupal thoracic closure processes.

A. Embryonic dorsal closure at stage 12 showing the “zippering” of the epithelium. Leading edge cells are represented by the blue line while amnioserosa is colored light green. B. Migration and fusion of thoracic discs around 6 hr after pupal formation. Leading edge cells are shown in blue while the underlying larval tissue is represented in light green.
together the epithelia as well as provide a substrate for the epithelia to adhere and migrate over. The genetic tractability of Drosophila has allowed the identification of many mutations that cause DC defects or failure. Analysis of these mutations has led to an improved understanding of this complex process and the cell signaling networks involved such as JNK, DPP, and Wg in addition to many cytoskeletal elements (Jacinto et al., 2002; Jankovics et al., 2011; Zecchini et al., 1999).

Knowledge of DC has informed research of other morphogenetic movements in Drosophila development. In Drosophila and other arthropods metamorphosis requires many complex transformative movements to convert the immature worm-like larvae into the adult fly with wings and legs (Martín-Blanco et al., 2000; Usui and Simpson, 2000). Metamorphosis starts with expansion and migration of imaginal discs (Usui and Simpson, 2000). Imaginal discs are groups of cells that are the progenitors of adult structures such as the wings, legs, or eyes. The individual imaginal discs migrate and fuse in a characteristic manner to form a continuous closed epithelium encasing the internal cells and tissues (Usui and Simpson, 2000). Migration and fusion of the wing imaginal discs gives rise to the wings and also the adult thorax in a process called thorax closure (Figure 1-1B)(Agnès et al., 1999; Martín-Blanco et al., 2000; Usui and Simpson, 2000; Zeitlinger and Bohmann, 1999). Despite differences in size, scale, and timing, TC is remarkably similar to DC (Agnès et al., 1999; Martín-Blanco et al., 2000; Zeitlinger and Bohmann, 1999). Both DC and TC use the same general signaling pathways during epithelial migration over a substrate, however, there are some differences between the two processes.

The Drosophila thorax begins to take form around 3-4 hours after pupal formation (APF) when the wing discs begin to detach from the underlying larval epidermis (Usui and
Simpson, 2000). After detaching from the epidermis the wing disc epithelium expands and migrates towards the dorsal midline. During disc migration, cells present at the leading edge (Fig. 1-1B, blue) extend filopodia over and across the larval epidermis (Fig. 1-1B, green) and contact leading edge cells of the contralateral disc. The migratory leading edge cells are called S-cells and are defined by their enlarged nuclei, spread morphology, and filopodial extensions (Usui and Simpson, 2000). At ~5 hours APF the S-cells at the anterior end of the wing disc extend filopodia and span the gap between the separated wing discs to form actin-rich bridges connecting the two wing discs (Martín-Blanco et al., 2000; Usui and Simpson, 2000). This is followed by S-cells of the posterior extending filopodia at ~5.5 hours APF (Usui and Simpson, 2000). Finally, at ~6 hours APF the filopodia originating from centrally located leading edge cells extend to form actin bridges with the contralateral disc (Martín-Blanco et al., 2000). Following the formation of the actin bridges the leading edge cells of each disc undergo changes in cell shape, elongating gradually towards the dorsal midline as the actin bridges contract to pull the two discs together (Martín-Blanco et al., 2000). This process of disc spreading and fusion, much like dorsal closure, is highly dependent on cytoskeletal regulation as well DPP and JNK signaling (Agnès et al., 1999; Zeitlinger and Bohmann, 1999). Perturbation of any of these can lead to thorax closure defects.

While DC and TC share many of the same cellular mechanisms, they differ in one key way. DC relies on contractions of the amnioserosa and the actin/myosin purse string (Kiehart et al., 2000). TC is also dependent on amnioserosa contractions, however, instead of a continuous actin/myosin cable leading edge cells of the migrating disc extend multiple
individual actin/myosin rich filopodia to bridge the gap to the disc on the opposite side (Martín-Blanco et al., 2000).

**Sensory organ development in Drosophila**

Following completion of thorax closure, cells within the recently formed epithelium are specified to differentiate into mechanosensory organs or bristles. Sensory bristles are part of the peripheral nervous system that allows the adult fly to respond to physical stimuli like touch or air currents (Gaiano and Fishell, 2002; Simpson et al., 1999). Sensory bristles come in two varieties: microchaete and the larger macrochaete. Each of these mechanoreceptors are made up of four cell types: the shaft cell, the sheath cell, the socket cell, and the neuron (Figure 1-2B) (Gaiano and Fishell, 2002; Mummery-Widmer et al., 2009). The shaft, socket and sheath cells form the physical structure of the mechanoreceptor while the neuronal cell connects it to the peripheral nervous system allowing transmission of sensory inputs (Fabre et al., 2008). Mechanosensory bristles develop from a single cell which undergoes successive rounds of asymmetric cell division (Fig. 1-2A, Manning and Doe, 1999; Wu et al., 2008). These rounds of cell division give rise to the four unique cell types of the mature sensory bristle (Fig. 1-2). The position and number of bristles vary between insect species; however within a single species the patterning of bristles is fairly uniform among individual flies (Simpson et al., 1999; Usui et al., 2008). The largely invariant bristle arrangement results from the specification of a sensory organ precursor cell at regular patterned intervals through genetically encoded mechanisms (Usui et al., 2008).
Figure 1-2. Differentiation of sensory organs.

A. Asymmetric division of sensory organ precursors. The SOP undergoes three rounds of asymmetric division to give rise to four differentiated cell types: the socket cell, shaft cell, neuron, and sheath cell (the glia cell undergoes apoptosis). The cell receiving the Notch signal is denoted by a “N”. B. Schematic of a mature wild-type sensory organ.
The precursors to macrochaete bristles and the smaller and later to develop microchaete bristles are specified by two different but related mechanisms. Macrochaete precursors are thought to be specified by the expression pattern of transcriptional activators and repressors (Calleja et al., 2002; Heitzler et al., 1996a). These transcription regulators are each expressed in unique but overlapping domains and are thought to bind the promoter region and regulate the expression of the *achaete-scute* complex of genes (Heitzler et al., 1996b; Skeath and Carroll, 1991). *Achaete-scute* genes are themselves transcription regulators that influence cell fate through their regulation of proneural gene networks. The overlapping expression domains of activators and repressors results in a reproducible pattern of clusters of cells in which *achaete-scute* is expressed and fields of cells in which *achaete-scute* is repressed (Calleja et al., 2002; Skeath and Carroll, 1991). Expression of the *achaete-scute* complex confers neural potential in these cells thus specifying the proneural cluster that will give rise to the sensory organ (Parks et al., 2008; Skeath and Carroll, 1991).

From the proneural cluster a single cell is selected as the sensory organ precursor (SOP) while the rest remain epithelial (Figure 1-3A). SOP selection depends on the stochastic expression levels of *achaete-scute* within the proneural cluster (Simpson et al., 1999; Usui and Kimura, 1993). *Achaete-scute* upregulates *Delta*, thus the cell expressing the highest levels of *achaete-scute* also expresses high levels of Delta protein (Fig. 1-3B, Heitzler et al., 1996b; Parks et al., 2008; Skeath and Carroll, 1991). The Delta expressing cell is the putative SOP and is able to activate Notch signaling in the surrounding cells (Figure 1-3A,B)(Heitzler et al., 1996b; Simpson et al., 1999). Once Notch binds its ligand Delta on a neighboring cell, the Notch intracellular domain (NICD) is cleaved and
Figure 1-3. Notch-Delta signaling in Sensory organ precursors.

A. Lateral inhibition. A single sensory organ precursor (SOP) from the proneural cluster is specified by a Notch-Delta signaling feedback loop with neighboring cells. Cells receiving the Notch signal are denoted with a “N” while the cell where Delta is active is denoted by “DI”. B. Schematic of the regulation of achaete-scute in SOP vs. epithelial cells established through Notch-Delta signaling. C. Schematic of Notch-Delta signaling between two cells. (1) Notch binds Delta on a neighboring cell. (2) Notch intracellular domain (NICD) is cleaved. (3) NICD translocates to the nucleus where it binds Su(H) at gene promoters to activate E(spl) transcription.
transported to the nucleus (Figure 1-3C). Once in the nucleus NICD forms a complex with Su(H) (Bray, 2006). Normally a transcriptional repressor, Su(H) becomes a transcriptional coactivator upon binding NICD (Fig. 1-3C, Bray, 2006; Furriols and Bray, 2000; Krejci and Bray, 2007; Schweisguth and Posakony, 1994). This protein complex is present at the promoter regions of Notch target genes where it recruits transcriptional machinery to activate their transcription (Krejci and Bray, 2007). The main targets of Notch signaling are the genes of the E(spl) locus (Fig. 1-3C) (Heitzler et al., 1996b). These mainly consist of the basic helix-loop-helix class of transcriptional repressors whose expression is able to repress various genes associated with differentiation, including achaete-scute, leading to the adoption of epithelial cell fate (Heitzler et al., 1996b; Parks et al., 2008; Simpson et al., 1999). On the other hand, in the putative SOP internalization of Delta upregulates achaete-scute transcription which in turn maintains expression of the proneural Delta gene (Fig. 1-3B) (Bray, 2006; Parks et al., 2008). In this manner, Notch-Delta signaling establishes a feedback loop that reinforces the SOP vs. epithelial cell fate decision (Fig. 1-3B) (Heitzler et al., 1996b; Simpson et al., 1999; Wu et al., 2008). This process of precursor selection is called lateral inhibition and allows the selection of a single cell to delaminate and become the sensory organ precursor (Fig. 1-3A) (Gaiano and Fishell, 2002; Wu et al., 2008).

Microchaete are evenly spaced in longitudinal rows but do not seem to form in precise genetically encoded positions (Cohen et al., 2010; Usui and Kimura, 1993). Microchaete specification, like macrochaete, depends on Notch-Delta signaling and lateral inhibition however it is unclear how the uniform spacing between individual microchaete is achieved (Usui and Kimura, 1993). A new model has been proposed where a proneural cluster is able to interact with a neighboring proneural cluster. Through lateral inhibition
Notch-Delta signaling between the cells of the two clusters creates a zone in which neural cell fates are repressed between neighboring SOP’s, thus leading to the observed uniform spacing between microchaete. However, since Notch/Delta signaling requires cell-cell contact this model must also explain the observation that microchaete spacing often spans multiple cell diameters (Cohen et al., 2010). The new model incorporates long range Notch/Delta signaling between proneural clusters by the extension of filopodia (Cohen et al., 2010). Filopodia containing the membrane-bound Notch and Delta proteins can extend multiple cell diameters and may allow lateral inhibition to proceed for SOP’s that are separated by longer distances and multiple cells (Cohen et al., 2010).

Following specification the SOP is extruded from the epithelium in a process similar to epithelial-mesenchymal transition (Gaiano and Fishell, 2002; Wu et al., 2008). This transformation depends on the release of adherens junctions with the cells of the epithelial layer and reorganization of cell morphology from columnar to rounded (Bastos et al., 2012; Matthews et al., 2012). The SOP is now ready for asymmetric division. During and after delamination the SOP remains associated with the epithelium through protein complexes located on its apical surface (Yu et al., 2006). These complexes consist of apical-basal polarity proteins including Bazooka, aPKC, and Par-6 (Wu et al., 2008). This apical protein complex is able to mediate the asymmetric segregation of multiple cell fate determinants to the basal cortex. Although still unclear, it appears phosphorylation of scaffold proteins by aPKC occludes the apical binding sites for cell fate determinants (Atwood and Prehoda, 2009). These determinants are thus retained exclusively at the basal cortex throughout cell division. Two well-studied cell fate determinants are Numb and Partner of Numb. These proteins are basally localized and influence cell fate by endocytosis of the Notch
receptor, thus preventing its activation by Delta (Frise et al., 1996; Roegiers et al., 2001). This establishes a bias between the two resulting daughter cells, with one receptive to Notch signaling and the other primed to receive the Delta signal. The cell fate following asymmetric cell division depends on the activation of Notch signaling in one of the cells and Delta in the other (Wu et al., 2008; Yu et al., 2006).

Once specified the SOP undergoes multiple rounds of asymmetric division (Figure 1-2A, Mummery-Widmer et al., 2009). The daughter cell fates are determined immediately following cytokinesis through Notch-Delta signaling (Remaud et al., 2008). The SOP divides to give rise to the pIIa and pIIb cells. The pIIa cell then divides to give rise to the socket and hair cells while the pIIb cell divides to give rise to the sheath and neuron cells (Fig. 1-2A). Mutations that affect Notch activity or localization of asymmetric determinants yield various classes of cell fate transformations within the SOP lineage. Loss-of-function Notch mutants can cause two pIIb cells and result in the differentiation of multiple neuron and sheath cells (Mummery-Widmer et al., 2009). Mutations to Numb, a Notch inhibitor, result in formation of multiple pIIa cells and cause all sensory organ cells to be transformed into socket cells (Mummery-Widmer et al., 2009).

**Small GTPase Regulation**

Both DC and TC are a transformative events which require the reorganization of the cytoskeleton and plasma membrane through coordinated regulation of Rho family small GTPases, including Rho and Rac (Bloor and Kiehart, 2002; Harden et al., 1999). The Rho family of small GTPases (Rho, Rac, and Cdc42) function as molecular switches that regulate
the cytoskeleton (Bloor and Kiehart, 2002; Hall, 1998; Schmidt, 2002). Small GTPases change conformation depending on the status of their bound nucleotide (Schmidt, 2002). Small GTPases when bound to GDP are inactive but upon binding GTP they take on a conformation that allows interaction with and activation of downstream effectors (Hall, 1998). The nucleotide status of a small GTPase is determined by the presence of various GAPs and GEFs that allow further refinement of small GTPase activity (Hall, 1998; McCormack et al., 2013; Schmidt, 2002). For example, regulation can occur temporally through the expression of GAPs and GEFs and spatially through the subcellular localization of those GAPs and GEFs. This property of small GTPases allows them to serve as a molecular toggle, switching on and off downstream effector proteins depending on their nucleotide state (Schmidt, 2002). There are many Rho and Rac effectors with diverse functions. Rho kinase (ROCK) and Diaphanous (Dia) are two well characterized Rho effectors known to regulate the actomyosin cytoskeleton (Raftopoulou and Hall, 2004). Rho-GTP binds to and activates Rho kinase (ROCK) which in turn is able to phosphorylate the regulatory light chain of myosin II (spaghetti squash, Sqh) to activate its ATPase activity and enhance cytoskeleton contractility (Raftopoulou and Hall, 2004). Additionally, Rho-GTP is able to bind the formin family member Dia leading to actin filament formation (Raftopoulou and Hall, 2004). p21-activated kinase (PAK) is a well known Rac effector. PAK regulates a number of other proteins through phosphorylation including LIMK and JNK (Raftopoulou and Hall, 2004). LIMK activation enhances actin polymerization while JNK activation leads to the phosphorylation of transcription factors and regulation of gene expression (Raftopoulou and Hall, 2004).
The Rho family of proteins is important for regulating many integral cellular functions. Coordination of Rho and Rac activity provides the fine control needed for cellular functions that require regulation of multiple processes at once. For example, cell division requires the coordination of cell adhesion release, change in cell shape, and cleavage furrow ingression for cytokinesis to proceed successfully (Bastos et al., 2012; Davies and Canman, 2012; Guillot and Lecuit, 2013). In addition to cell division, the regulation of Rho family GTPases is important for many cellular processes during interphase including filopodial and lamellipodial extension, cell migration, and cell adhesion (Baum and Georgiou, 2011; McCormack et al., 2013; Yamada and Nelson, 2007). Activation of Cdc42 and Rac lead to filopodial and lamellipodial extension whereas Rho activation produces an opposite effect leading to neurite retraction and cell rounding (Hall, 1998; Raftopoulou and Hall, 2004). During cell migration Cdc42 and Rac are both found at the leading edge of migrating cells where they induce actin polymerization (Raftopoulou and Hall, 2004). Rho on the other hand is active only in the cell body where it regulates detachment and cell body contraction to facilitate forward migration (Raftopoulou and Hall, 2004). For cell adhesion it appears that active Rac and Rho are needed sequentially for initial formation and maturation of cadherin-based adhesion complexes (Davies and Canman, 2012; McCormack et al., 2013; Yamada and Nelson, 2007). Mutation or inactivation of either Rho or Rac disrupts the formation of adhesive contacts between cells as evidenced by the lack of cadherin at cell-cell junctions resulting in a loss of tissue integrity (Hall, 1998; Michael and Yap, 2013; Ratheesh et al., 2012).

Proper epithelial migration in DC and/or TC relies on the regulation of cell migration and cell adhesion by Rho and Rac. In addition to Rho and Rac, several GAP and
GEF regulatory proteins known to regulate Rho and Rac have been shown to be important in DC and/or TC. These include the centralspindlin component i Tumbleweed (MgcRacGAP) and its interacting protein Pebble (Ect2) (Azevedo et al., 2011; Jankovics et al., 2011; Mummery-Widmer et al., 2009).

**Function of centralspindlin in cell division and cell morphology**

The centralspindlin complex is largely known for its role in metazoan cell division. However, at least one of its components was first discovered for its regulation of dendritic arborization in Drosophila neurons (Gao et al., 1999). It was not until later that centralspindlin’s essential role in cytokinesis was realized (Somers and Saint, 2003; Somma et al., 2002). The centralspindlin complex is composed of two proteins: Tumbleweed and Pavarotti. Centralspindlin shows broad conservation across metazoans (Mishima et al., 2002). The human and worm homologs of centralspindlin components and related proteins are given in Table 1-1. Tumbleweed and Pavarotti bind each other to form a dimer. Research in *C. elegans* revealed centralspindlin is a heterotetrameric complex formed by two of these dimers (Mishima et al., 2002). Mutation or knockdown of either component can lead to cytokinesis failure in cell culture and in vivo (Adams et al., 1998; Goldstein et al., 2005; Somers and Saint, 2003; Somma et al., 2002). Pavarotti is a mitotic kinesin like protein that binds microtubules and is +-end motor (Adams et al., 1998). This motor activity allows transport and targeting of the centralspindlin complex to different parts of the cell along microtubule networks. During cytokinesis this allows targeting of centralspindlin to the cell cortex and eventually the cleavage furrow along equatorial microtubules (Figure 1-4A)(Adams et al., 1998). The other centralspindlin component,
<table>
<thead>
<tr>
<th>Drosophila protein</th>
<th>C. Elegans</th>
<th>Human</th>
<th>Activity/Function</th>
</tr>
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<tr>
<td>Tumbleweed (Tum)</td>
<td>CYK-4</td>
<td>MgcRacGAP</td>
<td>RacGAP</td>
</tr>
<tr>
<td>Pavarotti (Pav)</td>
<td>ZEN-4</td>
<td>MKLP1</td>
<td>plus-end microtubule motor</td>
</tr>
<tr>
<td>Pebble (Pbl)</td>
<td>LET-21</td>
<td>Ect2</td>
<td>RhoGEF</td>
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<td>RhoA</td>
<td>small GTPase</td>
</tr>
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</table>

**Table 1-1.** Homology of Centralspindlin and interacting proteins.
Tumbleweed, is a GTPase activating protein (GAP) (Sotillos and Campuzano, 2000). GAPs inhibit the activity of small GTPases by enhancing their ability to hydrolyze GTP (Schmidt, 2002). Tumbleweed has GAP activity for both Rho and Rac GTPases in vitro, however in vivo tumbleweed primarily regulates Rac activity (Figure 1-4C) (Bastos et al., 2012; Minoshima et al., 2003). Centralspindlin interacts with the protein Pebble (Ect2) another key regulator of small GTPases (Yuce, 2005). Pebble is a guanine nucleotide exchange factor (GEF) (Prokopenko et al., 1999). Pebble activates Rho by allowing the exchange of GDP for GTP (Figure 1-4C). Centralspindlin together with Pebble allows the coordinated regulation of small GTPase activity (Figure 1-4C) (Bastos et al., 2012; Bustos et al., 2008; D’Avino, 2004; Ratheesh et al., 2012; Somers and Saint, 2003).

During cell division the centralspindlin complex provides the temporal and spatial control of Rho and Rac activity (Bastos et al., 2012; Canman et al., 2008). The centralspindlin complex has intrinsic GAP activity towards Rac and interacts with the RhoGEF Pebble (Mishima et al., 2002; Yuce, 2005). Centralspindlin also has a highly dynamic localization pattern that depends on the cell cycle stage. During interphase Tumbleweed, Pavarotti, and Pebble are primarily localized to the nucleus (Somers and Saint, 2003). As cells enter mitosis and the nucleus breaks down, Tumbleweed and Pebble are transported along microtubules by Pavarotti to the cell cortex where they activate Rho and inhibit Rac (Figure 1-4B,C). Inhibition of Rac also prevents the recruitment of the inhibitory p190RhoGAP, thus further enhancing Rho activity (Bustos et al., 2008; Ratheesh et al., 2012). The association of centralspindlin and its binding partner Pebble with the cell cortex is important for activation of Rho leading to cell “rounding” in preparation for cytokinesis (Matthews et al., 2012). Centralspindlin and pebble then accumulate at the
Figure 1-4. The Centralspindlin complex and its function during cytokinesis.

A. Cells undergoing cytokinesis. Centralspindlin is transported along equatorial microtubules to the cleavage furrow where it regulates the contractile ring. B. Schematic of centralspindlin at the cleavage furrow and regulation of actin and myosin. C. Centralspindlin coordinates regulation of small GTPases during cytokinesis. Tum and Pav recruit Pebble to activate Rho at the cleavage furrow. Additionally, Tum also prevents p190RhoGAP inhibition of Rho by inhibiting Rac.
cleavage furrow where they simultaneously activate Rho and inhibit Rac. Rho in turn regulates actin/myosin contractility through its effector Rho Kinase driving the closing of the contractile ring and eventually cell abscission and the completion of cytokinesis (Figure 1-4B)(Somers and Saint, 2003). Inhibition of Rac at the cleavage furrow, on the other hand, prevents adhesion with neighboring cells allowing cleavage furrow ingestion to proceed freely (Bastos et al., 2012).

While known for their role in cytokinesis, other studies have hinted at additional interphase functions of centralspindlin and centralspindlin associated proteins. The centralspindlin protein complex and its interacting proteins have been implicated in epithelial morphogenetic process including DC (D’Avino, 2004; Harden et al., 1999; Jacinto et al., 2002). Mutation of Pebble causes defects during dorsal closure (Jankovics et al., 2011). Tumbleweed and Pavarotti have also been implicated in dorsal closure. Recently it was shown that RNAi of Pebble during TC leads to the classic cleft thorax phenotype (Jankovics et al., 2011). The mammalian homolog of pebble, Ect2, has been implicated in many processes including cell migration, neuronal morphogenesis, and metastasis of tumor cells (Kanada et al., 2008; Matthews et al., 2012; Murray et al., 2012). In neuronal systems Tumbleweed and Pavarotti can both localize to axons and dendrites and were shown to be necessary for proper axon routing (Goldstein et al., 2005). Aside from their involvement in these developmental processes, little is known about the function of Tum and Pav in interphase cells.

**Scope of Thesis**
From studies described above it is clear centralspindlin plays an important role during interphase. However, the predominantly nuclear localization of centralspindlin during interphase raises the question of what that role could be. One study found MgcRacGAP, the human homolog of tumbleweed, localizes briefly to nuclear puncta leading up to cytokinesis (Lagana et al., 2010). These puncta were found to contain markers for centromeres including CENPA, the centromere specific histone variant. Although its role is unclear, Tum seems to be necessary for CENPA incorporation and/or maintenance at the centromere (Lagana et al., 2010). Additionally, centralspindlin was also detected using mass spec analysis from immunoprecipitated chromatin regulatory complexes (Moshkin et al., 2009). These two studies hint at a potential role for centralspindlin within the nucleus but fall short of proving their function. These observations raise an interesting possibility: does centralspindlin act in the nucleus to regulate cell morphology and cell migration?

Given centralspindlin’s regulation of cell morphology during cytokinesis and the preliminary evidence showing the importance of Tum/Pav function in neuronal morphogenesis and dorsal closure, I began investigating centralspindlin’s function in epithelial morphogenesis during thorax closure. Centralspindlin is a known regulator of Rho and Rac activity and actin/myosin but if and how it regulates the cytoskeleton during thorax closure is currently unknown. During the course of my thesis research I sought to answer the following questions. Does centralspindlin have functions during interphase in addition its mitotic function? How does centralspindlin influence tissue patterning, cell migration, and differentiation during pupal development? How does centralspindlin affect lateral inhibition and asymmetric division processes following completion of thorax closure? Is centralspindlin involved in regulating the Notch pathway during notum
development as implicated in previous studies? Does centralspindlin have a nuclear function during interphse and is it important for thorax closure? The following experiments will address these questions.
Chapter 2: Phenotypic analysis of dsRNA depletion of centralspindlin

I. Introduction

Centralspindlin has an essential role in cytokinesis but may also be involved in other cellular functions. Evidence from late stages of Drosophila brain development have shown centralspindlin may be important for morphogenesis of post-mitotic neurons (Goldstein et al., 2005). Essential genes can often cause embryonic lethality when mutated, precluding genetic analysis at later stages of development. Null mutations in either of the centralspindlin components, Tumbleweed and Pavarotti, result in early embryonic lethality presumably due to the accumulation of cytokinesis defects as maternally contributed protein and transcripts are depleted (Adams et al., 1998; Zavortink, 2005). Researchers have used various strategies in order to examine centralspindlin's contribution to later stages of development.

Techniques are available to perturb gene function during later stages of development allowing researchers to bypass complications due to early lethality. Mitotic recombination has been applied in the Drosophila nervous system to conditionally create clones of cells that contain homozygous mutations of centralspindlin (Goldstein et al., 2005). However, this approach suffered from cytokinesis defects as clones were unable to proliferate (Goldstein et al., 2005). Depletion of gene products through RNAi knockdown results in a partial loss of function and has proven to be an invaluable tool in deducing a protein’s function and understanding its biological role (Mummery-Widmer et al., 2009; Somma et al., 2002). In Drosophila the GAL4/UAS system has been adapted to allow tissue
specific expression of a variety of transgenes including mutant alleles and dsRNA constructs (Calleja et al., 1996). GAL4 drivers whose expression is restricted to later stages allow one to bypass the early lethality associated with mutation of essential genes.

To evaluate the role of centralspindlin in cell migration and its contribution to adult development I used a variety of GAL4 drivers to express dsRNA constructs targeting Tumbleweed and Pavarotti, the two components of centralspindlin, in developing pupal tissues. One centralspindlin component, Tumbleweed, had previously been implicated in thorax closure in a genome wide dsRNA screen for genes involved in thorax development (Mummery-Widmer et al., 2009). Flies carrying the pnr-GAL4 driver restrict expression of UAS constructs to the hinge region of wing imaginal discs, which are the migratory regions that expand and fuse to form the adult thorax (Calleja et al., 1996; Mummery-Widmer et al., 2009). Additionally, I used GAL4 drivers specific to the eyes and wings to investigate the contribution of centralspindlin to the development of these organs.

The experiments I present here demonstrate centralspindlin is required for the normal development of various adult structures including the thorax, wings, and head. I show that defects in development increase in severity with increased levels of dsRNA constructs. Additionally, I show centralspindlin is needed for thorax closure and requires the intact GAP domain of Tumbleweed.

II. Methods

Drosophila stocks used

The following fly stocks were used: Pnr-GAL4 (Heitzler et al., 1996), UAS-pav-dsRNA-strong (VDRC, 46137 (Dietzl et al., 2007), UAS-Tum-MYC (Goldstein et al., 2005),
UAS-TumΔGAP (Sotillos and Campuzano, 2000), UAS-tum-dsRNA (UAS-RacGAP50c-dsRNA (29), and UAS-tum-dsRNA-strong (Valium, TRiP.JF01639 (22). Flies were maintained at 27 °C unless otherwise specified.

**Bristle and cytokinesis defect quantification**

*Pnr-GAL4* was used to drive the following UAS-dsRNA constructs in the dorso-central region of the adult notum: UAS-*tum*-dsRNA, UAS-*tum*-dsRNA-strong, and UAS-*pav*-dsRNA. Total remaining microchaete contained within the *pnr* expression domain were counted for individual adult flies. n > 100 for all genotypes except for control (n = 30). Average bristle counts for each genotype were normalized to the average bristle counts for *pnr-GAL4/+* (control) flies.

For rescue experiments *pnr-GAL4* was used to drive the UAS-*tum*-dsRNA (strong) in combination with the following constructs: UAS-*luciferase*-dsRNA, UAS-Tum-MYC, and UAS-TumΔGAP.

**Immunohistochemistry**

Pupal nota were dissected in PBS 24 hours after pupal formation. Pupae were moistened with a small amount of water to facilitate scraping from the side of a glass vial. Intact pupae were then placed in a deep well glass slide containing PBS. Using forceps to grip the posterior spiracles the operculum was slowly peeled off to open the pupal case. The rest of the pupal case was then carefully peeled away while holding the pupa stationary at the posterior end. Once completely free from the pupal case both the head and posterior end were carefully removed using micro dissection scissors. The remaining tissues were then blasted repeatedly with ~10 μl of PBS to remove excess fat body and inner organs leaving the intact thorax with notum and partial abdomen.
The tissues were fixed in PBST (PBS + 0.3% Triton X-100) + 5% formaldehyde for 20 minutes. Tissues were then blocked in PBST + 10% normal goat serum (Jackson ImmunoResearch) for 1 hour then incubated with primary antibody overnight. The following primary antibodies were used: anti-Elav, 7E8A10 (rat, 1:1000, DHSB), anti-Prospero, MR1A (mouse, 1:500, DHSB), and anti-Su(H) (rabbit, 1:500, Santa Cruz). For all immunostaining the appropriate Alexafluor conjugated secondary antibody was used at 1:2000 (Invitrogen).

Following staining the ventral tissues were trimmed with micro dissection scissors to leave only the dorsal portion of the thorax. These were then mounted dorsal side up on a glass slide and cover slip with fluoromount G.

**Image processing and analysis**

All imaging was performed with an inverted fluorescence microscope (TE2000-U; Nikon) equipped with an electron-multiplying charge-coupled device camera (Cascade II;Photometrics) and a Yokogawa spinning disc confocal system (CSU-Xm2; Nikon). Images were acquired using Metamorph (version 7.0; MDS Analytical Technologies). Image stacks were assembled using average intensity Z-projections in ImageJ. The resulting images were adjusted and merged using Photoshop (Adobe) or ImageJ (NIH). Scale bars were generated using ImageJ.

Adult nota, eyes, and wings were mounted in glycerol and imaged using an Olympus SZX12 microscope equipped with a SPOT Insight 2 CCD camera and SPOT imaging software. Images from multiple focal planes were combined using Helicon Focus software.
III. Results

Centralspindlin is required for normal thorax development

To test the role of centralspindlin in thorax closure, I used the pnr-GAL4 driver to express UAS-dsRNA constructs targeting the centralspindlin components tumbleweed and Pavarotti in the developing thorax. Knockdown of either component leads to a mild cleft thorax phenotype (Fig. 2-1C,D). In addition to the cleft thorax I observed a reduction in the number of sensory bristles compared to wildtype (Fig. 2-1B). Since the severity of the thorax closure defect seemed to be correlated with magnitude of the loss of bristles I attempted to measure and compare dsRNA phenotypes by counting the remaining bristles after knockdown of Tum and Pav (Fig. 2-1D). Knockdown of Tum results in ~90% reduction in bristle number while knockdown of Pav is slightly more effective, causing ~95% reduction in bristles. Thorax development defects are greatly enhanced by doubling the copy number of the UAS-dsRNA constructs. The result is in an extreme cleft thorax phenotype and/or lethality due to the failure of pupal ecdision. Tum or pav-dsRNA pupae that failed to eclose displayed extremely malformed notums indicating the observed lethality is likely a result of thorax closure defects.

I was able to rescue the thorax closure defect associated with dsRNA knockdown of Tumbleweed through the expression of a Tum-MYC transgene. Introduction of the Tum-MYC transgene in the background of tum-dsRNA is able to restore Tum protein levels and partially restore wildtype bristle numbers to around 50% of wildtype levels (Fig. 2-2). Deletion of amino acids 405-407 abolishes the GAP activity of Tumbleweed (Sotillos and Campuzano, 2000). Expression of this TumΔGAP transgene alone causes thorax closure
**Figure 2-1.** Centralspindlin is required for bristle formation and proper thorax closure of the adult notum.

A-D. *pnr*-GAL4 was used to drive UAS-dsRNA constructs in the developing thorax. A. wildtype notum. B. Quantification of bristle loss phenotype. C,D. Expression of single dsRNA constructs targeting *tum* (C) or *pav* (D) results in loss of bristles phenotype. C’,D’. Doubling the copy number of the dsRNA constructs targeting tum(C’) or pav(D’) increases the severity of the loss of bristles and leads to a severe thorax closure defect.
Figure 2-2. *Tum*-dsRNA phenotype can be rescued by expression of wt transgene while mutant tum fails to rescue.

A-D. All constructs were driven by *pnr*-GAL4. A. UAS-*tum*-dsRNA. B. UAS-TumΔGAP. C. UAS-*tum*-dsRNA; UAS-Tum-MYC. D. UAS-*tum*-dsRNA; UAS-TumΔGAP. E. Quantification of bristle numbers for the above conditions.
and loss of bristle phenotypes similar to Tum knockdown. This indicates the TumΔGAP transgene likely behaves as a dominant negative. Additionally, this TumΔGAP transgene is unable to rescue thorax defects in a tum-dsRNA background indicating Tumbleweed’s GAP activity is essential for centralspindlin’s role in thorax development. Interestingly the thorax phenotype is unchanged when TumΔGAP and tum-dsRNA are expressed together. The lack of an additive or synergistic effect with the two constructs further strengthens the case that TumΔGAP behaves as a dominant negative.

**Centralspindlin depletion causes thorax closure defects**

To further characterize the effect of centralspindlin knockdown in the adult thorax I dissected pupae 24 hrs APF and examined thorax morphology as well as sensory organ lineages through immunohistochemistry. *Tum* and *pav*-dsRNA pupae were removed from their pupal casings ~24 hours after pupal formation (APF) and their thoraxes dissected. I then stained these tissues using antibodies for the cell fate markers Elav (neuronal cell maker) and Su(H) (socket cell marker). I used the DNA dye DAPI to visualize cell nuclei and observe gross morphology of the thoracic tissues. Ventral portions of the pupal thorax were removed and remaining tissue was placed on a slide with the dorsal portion facing up. Knockdown of Tumbleweed or Pavarotti (not shown) causes a failure in thoracic closure as evidenced by the inability of the two migrating wing imaginal discs to properly fuse at the dorsal midline (Fig. 2-3. B,C). Migrating wing discs are arrested mid-migration leaving a sizable gap between the leading edges of the epithelial sheets. Occasionally the two discs are able to meet and partially fuse, however, the regions surrounding the fused area are unable to complete “zippering”. The remaining sensory organ clusters are primarily found
**Figure 2-3.** Disruption of centralspindlin causes thorax closure defects in the pupal notum and causes a reduction in sensory organ cells.

A-D. Pupal nota were dissected ~24 hrs APF and stained for cell fate markers elav (neuron, green) and Su(H) (socket cell, red). dsRNA treated pupae show a discontinuous thoracic epithelium compared to the intact wildtype notum highlighted by DAPI (blue) staining. A. wildtype. B. tum-dsRNA (5xUAS). C. tum-dsRNA (10xUAS). D. TumΔGAP.
in these partially fused areas (Figs. 2-3. B-D). Cells bordering the thoracic cleft show various defects including presence of multinucleate cells, deformed cell morphology, and increased cell and nuclear size. The extended morphology and nuclear size of these cells is reminiscent of the migrating stalk cells during wing disc migration as reported in Martin-Blanco et al. 2000. This indicates that the cleft thorax phenotype is due to the arrest or delay of wing disc migration and fusion.

**Centralspindlin depletion leads to defects in eyes, wings**

Given the role of centralspindlin in the development of the thorax, I tested additional GAL4 drivers to see if centralspindlin functions in the development of other tissues and organs. I used *ey*-GAL4 and *ptc*-GAL4 to express dsRNA constructs targeting centralspindlin in the eyes and wings respectively. Expression of *tum*-dsRNA construct in the developing eye causes no observable phenotype. However, when *tum*-dsRNA is expressed in a heterozygous null background (*tum*347) there is a severe reduction in the size of the ommatidia and distortion of surrounding tissues. Occasionally one or both of the eyes are missing altogether resulting in a severely deformed adult head (Fig. 2-4B). This phenotype indicates Tumbleweed may be involved in both the development and organization of the eye as well as head involution, a process known to require complex migration of epithelial sheets. *Ptc*-GAL4 drives expression using the promoter of the patched gene, a segment polarity gene that is expressed in alternating stripes in the segmented embryo as well as the adult fly. One of these segments transverses the wing imaginal disc and allows knockdown of centralspindlin in a stripe through wing discs
Figure 2-4. Depletion of centralspindlin by tum-dsRNA causes defects in eye and wing development.

A,B. The ey-GAL4 driver was used to drive UAS constructs in the developing eyes. A. wild-type eyes. B. UAS-tum-dsRNA; tum347. C-D. ptc-GAL4 was used to drive UAS constructs in the developing wings. C. wild-type wing. D. UAS-tum-dsRNA. E. Quantification of wing phenotypes. Percent of adult wings displaying wildtype, tuft, or notched phenotype.
during larval development. Knockdown of centralspindlin in the wing disc results in disorganization and altered spacing of adult wing hairs compared to wildtype (Fig. 2-4C,D). Small tufts of hairs are visible on the wing margin as well as occasional notching (Fig. 2-4E).

IV. Discussion

Analysis of centralspindlin dsRNA phenotypes

The above results demonstrate the importance of centralspindlin in the development of the Drosophila thorax, wings, eyes, and head. Knockdown of Tumbleweed had already been shown to cause cytokinesis failure and tissue defects in wings and eyes (D'Avino, 2004; Somers and Saint, 2003). The role of centralspindlin in cytokinesis is well supported by studies in various model organisms and cell culture systems (Mishima et al., 2002; Somers and Saint, 2003; Somma et al., 2002). Consistent with this role in cell division I also detect multinucleate cells after knockdown of either centralspindlin component. However, I present evidence that these tissue defects may not necessarily be dependent on cytokinesis failure but are likely the result of a tissue migration defect associated with downregulation of centralspindlin.

I used dsRNA knockdown of centralspindlin components to examine the effect of a partial loss-of-function of centralspindlin. Homozygous null mutants are embryonic lethal precluding the examination of later developmental stages while the phenotypes due to haploinsufficiency are subtle or non-existent (Adams et al., 1998; Zavortink, 2005). Partial knockdown of centralspindlin components in the adult notum resulted in thorax closure defects. Interestingly, the process of thorax closure has been shown to proceed in the
absence of cell division (Zeitlinger and Bohmann, 1999). Since centralspindlin had also been shown to be involved in regulation of neuronal morphology and migration, I hypothesized centralspindlin may be involved in the migration of cells and tissues during thorax closure (Goldstein et al., 2005). Thorax closure depends on filopodia extensions that span the thoracic cleft and connect with the opposite migratory wing disc (Martín-Blanco et al., 2000). These initial filopodial connections facilitate the fusion of the wing discs to form the continuous epithelium of the adult thorax. Examination of pupal thoraxes from *tum*-dsRNA treated flies revealed the wing discs are arrested mid migration and fail to meet and fuse at the midline. Adding an extra copy of the UAS-dsRNA construct can enhance the severity of the thorax closure phenotype. This may also provide a hint at centralspindlin's role in additional cellular processes. Tumbleweed was initially shown to be important for neuronal morphology, adult wing morphology, and bristle polarity (Goldstein et al., 2005; Somers and Saint, 2003). These phenotypes were hypothesized to be due to cell cycle defects. However, since thorax closure proceeds normally in the absence of cell division, the ability to titrate the TC phenotype by depleting centralspindlin hints at the importance of regulating levels and/or activity of centralspindlin during thorax closure. This also suggests a function independent from its role in cell division.

I rescued the *tum*-dsRNA phenotype by addition of a TumMYC transgene to restore tum protein levels. A TumΔGAP transgene was unable to rescue, indicating Tum's GAP activity is essential for thorax closure. Previous studies have disagreed about the target of Tumbleweed's GAP activity and even the importance of GAP activity for Tum function (Goldstein et al., 2005; Sotillos and Campuzano, 2000). These disagreements likely stem from the different constructs being used and the nature of the experiments performed. An
in vitro study of Tum’s GAP activity towards small GTPases show a preference for Rac and Cdc42 with very little activity towards Rho activation (Minoshima et al., 2003). In this study, the specificity of Tum’s GAP activity changes from Rac to Rho after the GAP domain is phosphorylated by aurora kinase, however these claims have been refuted by another more thorough examination of Tum’s GAP specificity (Bastos et al., 2012). While the earlier model may be compelling, it is based solely on the fact that both Rho and Tumbleweed are required for cytokinesis. There is no in vivo evidence demonstrating Tumbleweed’s inhibitory GAP activity towards Rho whereas genetic evidence has repeatedly shown Tumbleweed inhibits Rac activity and actually enhances Rho activity in vivo (Bastos et al., 2012; D’Avino, 2004; Murray et al., 2012). Additionally, some studies have found Tumbleweed’s GAP activity to be dispensable for its function while others find GAP activity to be essential (Canman et al., 2008; Glotzer, 2009; Goldstein et al., 2005; Jones et al., 2010). The studies finding no effect by deletion of GAP activity use a single point mutation that is predicted to disrupt activity (Goldstein et al., 2005). The other studies as well as the experiments described here delete three catalytic residues in the GAP domain (Goldstein et al., 2005; Sotillos and Campuzano, 2000). This deletion presumably results in a more complete disruption of GAP activity and easily explains discrepancies between studies.

In addition to the failure of thorax closure, I observed a reduction in mechanosensory bristles in adult flies and a notable absence of sensory organ precursor cells in the developing pupa. Potential reasons for loss of sensory organs are: 1) the disruption or loss of epithelial tissue from which SOP’s are able to develop, or 2) defects in cytokinesis prevent the delamination and division of sensory organs. The experiments presented above are unable to rule out the latter option, however the areas of missing SOP’s coincide
with areas where the wing discs have failed to fuse. For this reason I believe the reduction in SOP number is mainly due to the lack of a mature, functional epithelium from which to develop.

Since centralspindlin is required for thorax closure, a process dependent on epithelial morphogenesis, I looked at other developmental processes and tissues to see if there is a general requirement for centralspindlin in tissue migration. I looked at the development of eyes and adult wings. The depletion of Tumbleweed in the eyes and head resulted in severe defects to head morphology. Flies are missing one or both eyes and there is significant disruption to the structures of the mouthparts and/or antennae. In the wing, depletion of Tumbleweed causes planar polarity defects and some cytokinesis defects. These wing phenotypes are identical to those observed for planar cell polarity network components (Adler, 2012). The head, wing, and thorax defects all indicate that centralspindlin may play a general role in development of tissues and organs in addition to its role in cytokinesis.
Chapter 3: Structure-function analysis of centralspindlin

I. Introduction

The severe defects in the head, wings, and thorax caused by dsRNA knockdown of centralspindlin components demonstrate the importance of centralspindlin in *Drosophila* development. However, the essential role of centralspindlin in cytokinesis may offer an easy but rather uninteresting explanation for the cause of these developmental deformities. Indeed other groups have observed dsRNA-mediated knockdown of centralspindlin results in cytokinesis failure in various *Drosophila* tissues as well as in cell culture (D’Avino et al., 2008; Goldstein et al., 2005; Somers and Saint, 2003; Somma et al., 2002). This cytokinesis defect could be the root cause for the tissue deformities I have observed in different fly tissues. While my analysis of dsRNA phenotypes suggests cytokinesis failure is not solely responsible for the thorax closure defect this possibility has not been completely ruled out.

Even though TC has been shown to proceed without active cell proliferation (Zeitlinger and Bohmann, 1999) I wanted to see if the cleft thorax phenotype was a secondary consequence of cytokinesis failure or due to defects in cell migration and morphology. To distinguish between secondary effects of cytokinesis failure and the putative role of centralspindlin in *Drosophila* pupal development I turned to the tissue specific expression of wildtype and mutant centralspindlin transgenes. Many of these transgenes have low to zero incidence of cytokinesis failure and allow the examination of centralspindlin’s developmental role independent from its cytokinetic functions.

The experiments I present here demonstrate the importance of subcellular localization for centralspindlin function in thorax development. I show centralspindlin can localize to
cell-cell junctions and that accumulation of cytoplasmic centralspindlin can cause thorax closure defects. I show deletion of the nuclear import signals of tumbleweed or pavarotti leads to thorax closure defects. These defects can be rescued by restoring the nuclear localization of centralspindlin. Together these results point to centralspindlin's necessary role in regulation of small GTPase signaling at the plasma membrane during epithelial morphogenesis.

II. Methods

*Drosophila* stocks used

The following fly stocks were used: *w1118* (wildtype), *pnr-GAL4* (Heitzler et al., 1996), *ptc-GAL4* (Speicher et al., 1994), UAS-Tum-MYC (Goldstein et al., 2005), UAS-TumΔGAP (Sotillos and Campuzano, 2000), UASp-PavGFP(Minestrini et al., 2002), UAST-Pav-GFP (1X*NLS, Minestrini et al., 2003), UAS-TumΔnls-MYC(Jones et al., 2010), UAST-PavΔnls-GFP (GFP-PavNLS(4-7)*, Minestrini et al., 2003), UAS-*sqh*-dsRNA(VDRC, 31542, Ni et al., 2007), and UAS-*Notch*-dsRNA (Ni et al., 2007). Flies were maintained at 27 °C unless otherwise specified.

Dissection

Pupal nota were dissected in PBS 24 hours after pupal formation. Pupae were moistened with a small amount of water to facilitate scraping from the side of a glass vial. Intact pupae were then placed in a deep well glass slide containing PBS. Using forceps to grip the posterior spiracles the operculum was slowly peeled off to open the pupal case. The rest of the pupal case was then carefully peeled away while holding the pupa stationary at the posterior end. Once completely free from the pupal case both the head
and posterior end were carefully removed using micro dissection scissors. The remaining tissues were then blasted repeatedly with ~10 ul of PBS to remove excess fat body and inner organs leaving the intact thorax with notum and partial abdomen.

**Immunohistochemistry**

The tissues were fixed in PBST (PBS + 0.3% Triton X-100) + 5% formaldehyde for 20 minutes. Tissues were then blocked in PBST + 10% normal goat serum (Jackson ImmunoResearch) for 1 hour then incubated with primary antibody overnight. The following primary antibodies were used: anti-c-Myc (chicken, 1:500, Molecular Probes), anti-GFP, ab13970 (chicken, 1:1000, Abcam), anti-Rho1, p1D9 (mouse, 1:500, DHSB) For all immunostaining the appropriate Alexafluor conjugated secondary antibody was used (Invitrogen).

Following staining the ventral tissues were trimmed with micro dissection scissors to leave only the dorsal portion of the thorax. These were then mounted dorsal side up on a glass slide and cover slip with fluoromount G.

**Time-lapse Microscopy**

Live cell imaging of pupal nota was carried out according to published protocols (Zitserman and Roegiers, 2011). *Pnr*-GAL4 was used to drive the following reporters: UASp-Pav-GFP and UAST-PavGFP(3X*NLS).

Pupae were staged by circling with a marker larvae just beginning pupariation at the white pre-pupae stage excluding those that with a cuticle that had already begun to turn brown. This time point was counted as 0hr APF. After 18hr incubation at 27 °C selected pupae were moistened with a small amount of water and scraped from the side of the vial. The pupae were attached dorsal side up to a glass microscope slide using double-
sided tape and allowed to dry for ~1 minute. Using forceps to grab the spiracles the operculum was carefully peeled off to reveal the head. The remaining pupal case was then carefully peeled until the majority of the pupa was exposed. Using a small paint brush the pupa was placed on a new glass slide. A small square of moist whatman filter paper (about 1cm x 1cm) with a hole punched through the center was placed around the pupa. Silicone lubricant was loaded into a 10ml syringe and deposited along the edges of the filter paper square creating a continuous border. A drop of 2ul of water was placed on a cover slip and then placed on the pupa with the water drop contacting the thorax. The cover slip was pressed down gently creating a seal with the silicone lubricant but making sure not to burst the pupa. The slide was then imaged on an inverted confocal microscope using oil immersion objectives making sure to not put excessive pressure on the cover slip. Single images or image stacks were taken approximately every 45 seconds throughout the time course and assembled using ImageJ.

**Image processing and analysis**

All imaging was performed with an inverted fluorescence microscope (TE2000-U; Nikon) equipped with an electron-multiplying charge-coupled device camera (Cascade II; Photometrics) and a Yokogawa spinning disc confocal system (CSU-Xm2; Nikon). Images were acquired using Metamorph (version 7.0; MDS Analytical Technologies). Image stacks were assembled using average intensity Z-projections in ImageJ. The resulting images were adjusted and merged using Photoshop (Adobe) or ImageJ (NIH). Scale bars were generated using ImageJ.
Adult nota and abdomens were mounted in glycerol and imaged using an Olympus SZX12 microscope equipped with a SPOT Insight 2 CCD camera and SPOT imaging software. Images from multiple focal planes were combined using Helicon Focus software.

III. Results

Overexpression of centralspindlin causes thorax closure defects

While depletion of centralspindlin leads to TC phenotypes, pnr-GAL4 driven overexpression of Tum and Pav in the presence of endogenous protein can also lead to similar TC defects. Overexpression of Tum-MYC in the developing notum leads to a minor thoracic cleft defect (Fig. 3-1B) while expression of Pav-GFP alone causes no significant phenotype (data not shown). However, when Tum-MYC and Pav-GFP are overexpressed simultaneously the penetrance and severity of the TC defect is significantly enhanced resulting in an extremely malformed notum (Fig. 3-1C). Interestingly I do not observe binucleate cells upon centralspindlin overexpression, indicating cytokinesis is unaffected. The lack of cell division defects indicates the thorax closure phenotype is independent of centralspindlin’s function in cytokinesis.

The severity of TC defects is dependent on titrating increasing levels of Tum or Pav protein. A very highly expressed version of the Pav-GFP construct was driven alone by pnr-GAL4. The relative expression level between the two pav-GFP constructs is readily apparent by GFP intensity in the pnr expression domain (data not shown). The result of the highly expressed Pav-GFP construct is the most severe TC defect observed (Fig. 3-1D). In addition to the thoracic cleft, these flies display a darker pigmented notum in the region of Pav-GFP expression. This brown color may be due to lesions of necrotic tissue.
Figure 3-1. Overexpression of Tum and/or Pav result in thorax closure and abdomen defects.

A-D. Tum and Pav were expressed in the developing thorax using pnr-GAL4. Thoracic clefts are denoted by arrows. A. wildtype. B. Tum-MYC expression causes mild thorax closure defect. C. Tum-MYC and Pav-GFP expressed together result in a severe thoracic cleft. D. High expression levels of Pav-GFP alone results in a severe thoracic cleft. E, F. dsRNA constructs were expressed with pnr-GAL4. E. Notch-dsRNA causes a complete loss of bristles. F. Sqh-dsRNA results in a mild thorax closure defect marked by an arrow. G. Abdomen defects caused by high expression levels of Pav-GFP. Abdomen segments fail to fuse properly (arrows) and surrounding areas have disrupted polarity of bristles.
The mild TC defect seen with Tum-MYC expression is highly similar to the TC defect observed with *Spaghetti Squash* (*Sqh*) dsRNA in which the posterior portion of the thorax has failed to fuse properly (Fig. 3-1B,F). *Sqh* is the Drosophila homolog of myosin II regulatory light chain, a protein known to be important for cytoskeletal regulation during dorsal closure and thorax closure. *Notch*-dsRNA results in a loss of thoracic bristles but has minimal to no effect on thorax closure and adult notum morphology (Fig. 3-1E).

The *pnr*-GAL4 expression domain, in addition to the thorax, extends down the length of the adult abdomen. High expression of Pav-GFP causes planar polarity defects of sensory bristles and a failure of the abdominal segments to fuse properly in addition to the TC defect (Fig. 3-1G).

**Centralspindlin undergoes dynamic localization during cell division**

Tumbleweed and Pavarotti undergo dynamic localization depending on the cell cycle. During interphase Tum and Pav are almost exclusively nuclear (Goldstein et al., 2005; Somers and Saint, 2003). During mitosis, centralspindlin localizes to the cell cortex following nuclear breakdown and are then recruited to the cleavage furrow (Somers and Saint, 2003). Using live cell imaging I examined the localization of Pav-GFP ~18h APF during the development of the thorax. As expected Pav-GFP localized to the nucleus during interphase (Fig. 3-2A). I also observed bright spots that appear to be in either the intercellular space or between cell junctions. These are likely remnants of the midbody. As mitosis progresses, Pav-GFP localizes to the cell cortex during cell rounding leading up to cytokinesis (Fig. 3-2A). Pav-GFP then concentrates at the cleavage furrow as the cells undergo the final steps of cytokinesis (Fig. 3-2A). I next examined the localization of
Figure 3-2. Localization of Centralspindlin proteins in mitotic and interphase thoracic cells.

A. Pav-GFP was expressed in the thorax using pnr-GAL4 and localization was followed by live-cell microscopy. Pav-GFP is nuclear during interphase. As the nucleus breaks down Pav-GFP is transported along spindle microtubules to the cell cortex and cleavage furrow. Finally Pav-GFP accumulates in the midbody at the final stage of cytokinesis. Cell outlines are indicated by dashed lines in the final frames. B-D. Tum-MYC and Pav-GFP localize to the nuclei of stationary interphase thoracic cells. Tum-MYC and Pav-GFP were expressed in the thorax using pnr-GAL4. Tissues were fixed and stained with antibodies to Tum, GFP, and MYC. B. Endogenous Tum protein. C. Pav-GFP. D. Tum-MYC.
endogenous Tum, Tum-MYC, and Pav-GFP in the 24-hour APF thorax by immunohistochemistry. In the absence of an antibody for native Pav I used low expression levels of Pav-GFP to examine Pav's localization pattern. As expected, endogenous Tum localizes predominantly to the nucleus during interphase (Fig. 3-2B). The same interphase localization pattern was observed for Pav-GFP as well as Tum-MYC (Fig. 3-2C,D). I next examined the localization of Pav-GFP at high expression levels. When overexpressed Pav-GFP is detected predominantly in the nucleus as well as in the cytoplasm at the cell cortex (Fig. 3-3A). This cytoplasmic pool of Pav-GFP co-localizes with Rho at the plasma membrane at cell-cell junctions. Co-expression of Tum-MYC and Pav-GFP (low expression) resulted in cytoplasmic localization as well. Both Tum-MYC and Pav-GFP localize to the nucleus but are also present in filopodia-like structures that span empty spaces between cells (arrows) (Fig. 3-3B).

**Mutation of Centralspindlin causes constitutive cytoplasmic localization, thorax closure defects**

Tum and Pav each contain nuclear localization signals (NLS). Deletion or mutation of these nuclear localization signals results in predominantly cytoplasmic localization of Tum and Pav throughout the cell cycle (Jones et al., 2010; Minestrini et al., 2002). In the pupal thorax, PavΔnls-GFP is excluded from the nucleus and localizes to the cell cortex as well as to microtubules (Fig 3-4B). Despite this cytoplasmic localization, many cells expressing PavΔnls-GFP are able to divide although duration of mitosis is increased (Liu and Erikson, 2007, Fig. 3-4B). Similarly, TumΔnls-MYC also localizes to the cell cortex and
Figure 3-3. High levels of Centralspindlin leads to plasma membrane localization.

A. *Pnr*-GAL4 was used to drive Pav-GFP (high expression) in the pupal thorax. Tissues were fixed and stained for GFP (green), Rho (red), and DAPI (blue). Pav-GFP localizes to nuclei and to cell-cell junctions under these conditions (arrows). B. Co-expression of Tum-MYC and Pav-GFP (low expression) leads to cytoplasmic localization and the formation of filopodia-like structures (arrows). Tissues were fixed and stained for GFP (green), Rho (red) and MYC (blue).
Figure 3-4. Deletion of nuclear localization signals of Tum and Pav result in constitutive cytoplasmic localization during interphase.

A. Examination of Pav-GFP and PavΔnls-GFP localization in live cells of the pupal notum. Pav-GFP localizes to the nuceli of interphase cells and is found at the cell cortex and cleavage furrow of mitotic cells. PavΔnls-GFP localizes exclusively to the cytoplasm and is associated with microtubule networks at the cell cortex. B. Examination of Tum-MYC and TumΔnls-MYC in fixed pupal notum. Ptc-GAL4 was used to drive expression in order to avoid lethality associated with TumΔnls-MYC expression. Tum-MYC localizes to nuclei marked by DAPI. TumΔnls-MYC localizes to the cell cortex and is excluded from most cell nuclei marked by DAPI.
Figure 3-5. Expression of Tum- and Pav- Δnls mutants causes defects in pupal development.

A-C. pnr-GAL4 was used to drive expression of Δnls constructs in the developing pupae. A. Wildtype thorax. B. PavΔnls-GFP expression causes thorax closure defects and loss of sensory bristles. C. Examples of TumΔnls-MYC adult nota. TumΔnls-MYC results in lethality and severe thorax closure defects. D. PavΔnls-GFP also results in abdominal segment fusion defects (arrows) as well as bristle polarity defects compared to wildtype. E. pnr-GAL4 driven PavΔnls-GFP in the pupal notum. Tissues were fixed and stained for GFP and DAPI. PavΔnls-GFP expression disrupts integrity of thorax causing “holes” in the epithelium (arrows). F. Magnification of boxed area in E showing filopodia-like structure spanning a “hole” in the epithelium (arrow). Neurons are marked by Elav (red).
is mostly excluded from the nucleus when compared to Tum-MYC (Jones et al., 2010, Fig. 3-4C,D).

\textit{Pnr-GAL4} driven expression of Tum or Pav NLS deletions causes thorax closure defects. Expression of PavΔnls-GFP causes thorax closure and loss of bristles similar to Tum or Pav knockdown (Fig. 3-5A). Additionally, abdomen segment fusion is disrupted along with planar polarity of bristles (Fig. 3-5B). TumΔnls-MYC is almost completely lethal due to eclosion failure. The escapers that do manage to survive to the adult sage display severe thorax closure defects (Fig 3-5C,C'). I dissected pupae~24 hours APF to examine the effect PavΔnls-GFP has on cell and tissue morphology. Although not as severe as the cleft thorax defects observed with dsRNA treatments, I observed large “holes” in an otherwise continuous epithelium (Fig. 3-5D, arrows). These holes are restricted to the \textit{pnr} expression domain and are never observed in wildtype flies. Occasionally cells exhibit filopodial protrusions that span these holes or gaps (Fig. 3-5D,E).

\textbf{Cytoplasmic centralspindlin can lead to extra macrochaete}

I also tested the effect of expressing NLS mutants in a more limited expression domain. I used the \textit{ptc-GAL4} driver to overexpress wildtype and mutant centralspindlin constructs in the scutellum of the developing thorax. \textit{Ptc-GAL4} by itself causes a low incidence of extra scutellar macrochaete in adult flies (~6%). I observed no significant defects beyond the baseline \textit{ptc-GAL4} phenotype upon over expression of either Tum or Pav alone. Thorax morphology was essentially normal in nearly all flies examined. However, expression of TumΔnls-MYC results in almost 90% of adult flies having 1-2 extra
Figure 3-6. Expression of Tum and Pav transgenes in the scutellum results in bristle defects.

A. Ptc-GAL4 was used to drive expression of Tum-MYC, TumΔnls-MYC, TumΔGAP, Pav-GFP, and PavΔnls-GFP. A. Percent of flies expressing centralspindlin constructs with greater than four (wildtype) scutellar macrochaete. TumΔnls-MYC causes 90% of flies with extra macrochaete. B. Percent of flies expressing centralspindlin constructs with less than four scutellar macrochaete. TumΔGAP and PavΔnls-GFP result in 15% of flies missing one or more macrochaete. C. Stained scutella of Tum-MYC and TumΔnls-MYC flies. TumΔnls-MYC displays three macrochaete per heminota compared to just two observed in wildtype or Tum-MYC flies.
Figure 3-7. Removal of PavΔnls-GFP from the cytoplasm rescues thorax closure and bristle loss.

A-C. pnr-GAL4 was used to drive PavΔnls-GFP alone or in combination with Tum transgenes. Tissues were fixed and stained for GFP (green), Rho (red), and DAPI (blue). A. PavΔnls-GFP expression causes bristle loss and minor thorax closure defects. PavΔnls-GFP localizes to the cell cortex and microtubule networks. B. PavΔnls-GFP is pulled into the nucleus when expressed in combination with Tum-MYC. Tum-MYC expression rescues thorax closure and partially restores bristle loss in PavΔnls-GFP expressing flies. C. PavΔnls-GFP is pulled into the nucleus when expressed in combination with TumΔGAP. TumΔGAP expression rescues thorax closure and bristle loss in PavΔnls-GFP expressing flies. D. wildtype thorax for reference.
scutellar macrochaete (Fig. 3-6A). Examination at the cellular level revealed three adjacent fully formed extra macrochaete on each half of the scutellum. Each macrochaete consisted of each of the four cell types and had wildtype morphology. On the other hand, expression of PavΔnls-GFP does not cause extra macrochaete. In fact PavΔnls-GFP results in a loss of macrochaete in ~15% of flies (Fig. 3-6B). Expression of TumΔGAP produces the same result, a loss of bristles in ~15% of flies. (Fig. 3-6B).

**Removal of centralspindlin from the cytoplasm restores normal thorax development**

Previous studies have shown the expression of one centralspindlin component can influence the subcellular localization of the other (Goldstein et al., 2005). This observation allowed me to further examine the thorax closure phenotype caused by PavΔnls-GFP expression. I expressed a Tum-MYC transgene with an intact NLS in combination with PavΔnls-GFP. PavΔnls-GFP alone causes mild thorax defects (Fig. 3-7A) while co-expression with Tum-MYC is able to rescue the phenotype resulting in a normal thorax with wildtype bristle numbers (Fig. 3-7B). I attempted the same experiment with TumΔGAP. Unexpectedly, co-expression of PavΔnls-GFP and TumΔGAP results in a wildtype thorax despite the mutation to the essential GAP domain (Fig. 3-7C). To better understand this result I dissected and stained pupal nota to examine the sub-cellular localization of centralspindlin transgenes. As expected, PavΔnls-GFP is restricted from the nucleus (Fig. 3-7E). Additionally, cells appear larger in size and with more cytoplasmic area between nuclei. Upon introduction of the Tum-MYC transgene the nuclear localization
of PavΔnls-GFP is completely restored (Fig. 3-7F). This demonstrates the ability of Tum-MYC with an intact NLS to bind and transport PavΔnls-GFP into the nucleus. In addition, Tum-MYC expression restores wildtype cell size and ratio of nucleus : cytoplasm. I also observed two large bright foci of PavΔnls-GFP within each nucleus that seem to exclude DAPI staining (Fig. 3-7F). Next I examined the effect of co-expressing PavΔnls-GFP and TumΔGAP. TumΔGAP restores nuclear localization of PavΔnls-GFP and wildtype cell size despite the lack of a functional GAP domain (Fig. 3-7G). Curiously, TumΔGAP expression does not result in two bright nuclear foci but rather several smaller bright foci throughout the nucleus of about half of the cells observed.

IV. Discussion

While RNAi depletion was able to reveal the requirement of centralspindlin for thorax closure, the cytokinesis defects and the severe disruption of tissue structure and integrity complicated a detailed investigation and analysis of the phenotype. I turned to overexpression of wildtype and mutant centralspindlin constructs in the presence of wildtype protein to further probe the role of centralspindlin in thorax development.

Overexpression of Tum-MYC results in a mild cleft thorax phenotype with incomplete penetrance while expression of Pav-GFP at low levels causes no visible phenotype. This difference in ability to induce thorax phenotypes results from Tum’s GAP activity towards Rac. While Pav is necessary for localization of centralspindlin it does not itself regulate small GTPase activity (Adams et al., 1998). In addition, Tum-MYC overexpression and Sqh-dsRNA result in highly similar thorax phenotypes suggesting a
common molecular defect between the two. Tum-MYC overexpression likely results in defects to cytoskeletal regulation in a similar manner to depletion of Sqh (myosin II). Co-expression of both Tum-MYC and Pav-GFP (low expression) results in a severe thoracic cleft. The synergy is the result of Tum-MYC’s misregulation of small GTPases combined with the ability of excess Pav-GFP to localize the centralspindlin complex along microtubules to the cell periphery and regulate membrane morphology. This result mirrors the study of centralspindlin in axon guidance and also in wing development (Goldstein et al., 2005; Jones et al., 2010). Goldstein et al. found co-expression of Tum and Pav results in severe misrouting of axons while expression of either alone causes no major defects.

In contrast to RNAi depletion, overexpression of either component did not result in binucleate cells indicating cell division proceeds normally. This lack of cytokinesis failure upon overexpression of centralspindlin components alone or in combination indicates the observed thorax closure defects are independent of defects in cell division. Additionally this indicates the thorax closure defects observed with centralspindlin depletion are also likely independent of the cytokinesis failure phenotype. These findings support the model that too much (overexpression) or too little (RNAi depletion) centralspindlin can disrupt thorax closure independent from centralspindlin’s essential role in cytokinesis and highlight an important role of centralspindlin in addition to cell division.

Centralspindlin is normally sequestered in the nucleus during interphase. During a normal cell division cycle centralspindlin moves from the nucleus to the cell periphery and then midbody. Centralspindlin’s localization at the cell periphery and recruitment of Pbl is responsible for the release of adhesion from surrounding cells and remodeling of the
plasma membrane in preparation for cell division (Matthews et al., 2012). Both Tum and Pav contain NLS’s to facilitate the sub-cellular localization of centralspindlin. Normally centralspindlin is undetectable in the cytoplasm of stationary interphase cells. Even upon overexpression, Tum and Pav transgenes with intact NLS’s are usually only found in the nucleus. Deletion of centralspindlin’s NLS’s results in constitutive cytoplasmic localization and the expression of these constructs in the developing notum results in thorax closure defects. The cytoplasmic localization of TumΔnls-MYC and PavΔnls-GFP likely causes the misregulation of small GTPases during thorax development. This is evident from the failure of wings discs to properly migrate and aberrant filopodia structures seen with PavΔnls-GFP expression. I also observe filopodia-like structures upon co-expression of Tum-MYC and Pav-GFP. Under these conditions Tum-MYC and Pav-GFP are both detected in the cytoplasm and wing discs cells have severe migration defects resulting in a severe thoracic cleft. The filopodia-like protrusions may be the result of delay or arrest of normal cell migration processes. Alternatively, these protrusions could also represent aberrant membrane structures caused by cytoplasmic localization of centralspindlin. In a previous study the authors noted PavΔnls-GFP expression in the adult fly brain causes no observable defects in axon morphology. However, the authors also noted significant localization of Pav-GFP in axons as well as the nucleus, indicating Pav may normally function in the cytoplasm of neuronal cells. In non-migrating interphase thoracic cells I do not observe Pav-GFP localization in the cytoplasm when expressed at low levels. The absence or low levels of cytoplasmic centralspindlin present in this system makes these cells especially sensitive to perturbation of centralspindlin’s nuclear localization. Interestingly, the highly expressed Pav-GFP construct with an intact NLS also resulted in a severe cleft phenotype.
This was puzzling as Pav-GFP alone has no detectable phenotype, mild or otherwise. I examined the localization of this construct and although mostly found within the nucleus, under these conditions the highly expressed Pav-GFP construct was also present at cell-cell junctions in non-migratory interphase cells. This may be the result of overwhelming the nuclear import of centralspindlin resulting in excess Pav-GFP in the cytoplasm. Similarly, co-expression of Tum-MYC and Pav-GFP (low expression) results in cytoplasmic centralspindlin and severe thorax defects. These experiments demonstrate excess cytoplasmic Pav-GFP may cause constitutive recruitment of centralspindlin and its interacting proteins to the cell cortex and misregulation of Rho and Rac. If centralspindlin normally regulates cell migration and cell adhesion its constitutive plasma membrane localization could negatively impact cell migration during thorax closure.

A previous study showed one subunit of centralspindlin is able to influence the localization of the other through their direct interaction. This study found expression of Tum-MYC is able to restore nuclear localization of Pav\textsubscript{\textit{nls}}-GFP in mushroom bodies of adult Drosophila brains. The same observation was made with expression of Tum\textsubscript{\textit{GAP}}. The authors observe no phenotype caused by Pav\textsubscript{\textit{nls}}-GFP alone. Interestingly in this system the authors find co-expression of Pav\textsubscript{\textit{nls}}-GFP and Tum-MYC results in a severe axon misrouting phenotype. However, co-expression of Pav\textsubscript{\textit{nls}}-GFP and Tum\textsubscript{\textit{GAP}} in this system results in wildtype axon morphology\cite{(Goldstein et al., 2005)}. These results are slightly different from my observations in the developing thorax. I found that Pav\textsubscript{\textit{nls}}-GFP expression alone causes mild thorax defects. I also found that both Tum-MYC and Tum\textsubscript{\textit{GAP}} restore nuclear localization of Pav\textsubscript{\textit{nls}}-GFP and can rescue the thorax defects caused by pav\textsubscript{NLS} alone. There is a simple explanation for this discrepancy. The authors
observe PavΔnls-GFP is able to “pull” a fraction of Tum-MYC and TumΔGAP proteins out of the nucleus of mushroom body neurons. They argue the cytoplasmic localization of Tum-MYC results in axonal defects because of misregulation of small GTPases. However TumΔGAP in the cytoplasm results in no phenotype because its mutated GAP domain prevents misregulation of small GTPases. These results mirror the severe defects observed when Tum-MYC and Pav-GFP are co-expressed in the thorax. Build up of centralspindlin in the cytoplasm seems to cause defects in both neurons and in the developing thorax. In the developing notum I observe no “pulling” of Tum-MYC or TumΔGAP into the cytoplasm when co-expressed with PavΔnls-GFP. Since PavΔnls-GFP, Tum-MYC, and TumΔGAP are all exclusively nuclear under these conditions no defects due to cytoplasmic build up of centralspindlin would be expected.

Interestingly, a study of centralspindlin in human cell culture cells showed MgcRacGAP (human homolog of Tum), MKLP-1 (human homolog of Pav), and Ect2 (human homolog of Pbl) are present at adherens junctions (Ratheesh et al., 2012). The localization depended on transport along microtubules by MKLP-1. These proteins all regulate Rho signaling at the zonula adherens and interact with the cell adhesion protein E-cadherin.

During the preparation of this thesis, two studies have discovered novel characteristics about centralspindlin and its interactors. Lekometsev et al. demonstrated the presence of a C1 domain in MgcRacGAP that binds polyanionic phosphoinositide lipids. This C1 domain confers plasma membrane tethering activity to the centralspindlin complex. Additionally, Murray et al. found that the Drosophila RhoGEF pebble contains a pleckstrin homology (PH) domain, a motif known to bind phosphatidylinositol phosphates. Deletion of the PH domain prevented localization of Pbl to the cell cortex and caused
migration defects in mesodermal cells. Both of these studies demonstrated the ability of both centralspindlin and Pbl to associate directly with the plasma membrane and regulate the structure of the underlying cytoskeleton. Murray et. al. demonstrated the localization of Pbl at the cell cortex during cell migration and importance of small GTPase regulation by Pbl during cell migration. Together with the experiments I have presented here, this evidence clarifies the essential role of centralspindlin in regulating the cytoskeleton at the cell cortex in all cell types examined. Centralspindlin’s interaction with the plasma membrane, cadherin, and the cytoskeleton provides a direct physical link between the cytoskeleton and the plasma membrane. In addition the multi-functional centralspindlin complex allows the coordinated regulation of small GTPases during cell migration and cell division.
Chapter 4: Role of centralspindlin in sensory organ development and Notch signaling

I. Introduction

Sensory organ precursor cells are specified and begin to differentiate into mature mechanosensory bristles following the completion of thorax closure. This differentiation process employs asymmetric stem cell division and the Notch-Delta signaling pathway to determine the individual cell fates of the sensory organs. I observed deformed sensory organ clusters after the dsRNA knockdown of Tumbleweed or Pavarotti. This observation raised the possibility that centralspindlin may have an alternative function during sensory organ differentiation in addition to cytokinesis and cell migration.

The experiments presented here examine centralspindlin’s role in sensory organ differentiation and Notch-Delta signaling. I show depletion of centralspindlin is able to cause cell fate defects independent of cytokinesis failure. However, through transcription reporter assays and chromatin immunoprecipitation I show centralspindlin is likely not involved directly in the regulation of Notch target transcription. Through genetic analysis I also show that phenotypes associated with centralspindlin depletion are neither enhanced nor suppressed by perturbation of Notch signaling indicating the absence of a genetic interaction between the centralspindlin and the Notch-Delta pathway.

II. Methods

Drosophila stocks used
The following fly stocks were used: w1118 (wildtype), pnr-GAL4 (Heitzler et al., 1996), ptc-GAL4 (Speicher et al., 1994), UASp-PavGFP (Minestrini et al., 2002), UAST-PavΔnls-GFP (GFP-PavNLS(4-7)*, Minestrini et al., 2003), UAS-pav-dsRNA (VDRC, 46137, Dietzl et al., 2007), UAS-Tum-MYC (Goldstein et al., 2005), UAS- UAS-TumΔGAP (Sotillos and Campuzano, 2000), UAS-tum-dsRNA (UAS-RacGAP50c-dsRNA, (Billuart et al., 2001), UAS-tum-dsRNA-strong (Valium, TRiP.JF01639, Ni et al., 2007), UAS-N-dsRNA-strong (Valium, TRiP.JF02959, Ni et al., 2007), UAS-Su(H)-dsRNA-strong (Valium, TRiP.HM05110, Ni et al., 2007), UAS-Pon-GFP (Lu et al., 1999), UAS-mCD8-RFP (B. Pfeiffer), UAS-luciferase-dsRNA-strong (Valium, TRiP. JF01355, Ni et al., 2007), ubi-Pav-GFP (Minestrini et al., 2003), UAS-Neuralized (Lai and Rubin, 2001), E(spl)m4-GFP (Barolo, 2004) and UAS-2xEGFP (A. Michelson). Flies were maintained at 27 °C unless otherwise specified.

**Cloning of Tumbleweed-HA and transfection**

Tumbleweed was cloned into the the pIZ/v5-his vector using restriction sites Spe1 and Mlu1. An HA tag was inserted using restriction sites KpnI and Spe1 to produce and N-terminally tagged Tum-HA construct. This construct was used to transfect S-2 cells using Qiagen effectene reagent according to manufacturers recomendations. 2μg plasmid DNA was mixed with 150μl of EC buffer. 16μl of enhancer solution was added and after mixing the solution was incubated at room temperature for 5 minutes. 20μl of Effectene solution was added and the solution was then vortexed for ~10 seconds and incubated at room temperature for one hour. 1ml of Schneider’s cell culture media + 10% FBS was added. The transfection mix was then added to 5ml of 1x10^6 cells with mixing. Transfected cells were then incubated at 25°C for 48-72 hours.
**Dissections**

Pupal nota were dissected in PBS 24 hours after pupal formation. Pupae were moistened with a small amount of water to facilitate scraping from the side of a glass vial. Intact pupae were then placed in a deep well glass slide containing PBS. Using forceps to grip the posterior spiracles the operculum was slowly peeled off to open the pupal case. The rest of the pupal case was then carefully peeled away while holding the pupa stationary at the posterior end. Once completely free from the pupal case both the head and posterior end were carefully removed using micro dissection scissors. The remaining tissues were then blasted repeatedly with ~10 ul of PBS to remove excess fat body and inner organs leaving the intact thorax with notum and partial abdomen. The nota were fixed in PBST (PBS + 0.3% Triton X-100) + 5% formaldehyde for 20 minutes. Tissues were then blocked in PBST + 10% normal goat serum (Jackson ImmunoResearch) for 1 hour then incubated with primary antibody overnight.

Wing discs were dissected from wandering third instar larvae. The larvae were submerged in PBS in a glass depression slide. The larval head was grasped firmly just above the trachea. Using a second pair of forceps the larval body was grasped at the approximate midpoint and separated away from the anterior portion of the larva in a gentle by firm fashion. The remaining larval head was fixed in PBST (PBS + 0.3% Triton X-100) + 5% formaldehyde for 20 minutes. Tissues were then blocked in PBST + 10% normal goat serum (Jackson ImmunoResearch) for 1 hour then incubated with primary antibody overnight.

**Wing disc experiments**
PTC-GAL4 was used to drive UAS-2xEGFP in combination with the following UAS-dsRNA constructs in larval wing discs: UAS-tum-dsRNA-strong, UAS-pav-dsRNA-strong, and UAS-N-dsRNA-strong, and UAS-luciferase-dsRNA (control). Discs were dissected from third instar larvae and stained for Cut expression. Approximately 25% of the cells in the tum-dsRNA-strong expression domain were binucleate, while the remaining had wild-type cell morphology.

**Immunohistochemistry**

The following primary antibodies were used: anti-Elav, 7E8A10 (rat, 1:1000, DHSB), anti-Prospero, MR1A (mouse, 1:500, DHSB), anti-Su(H) (rabbit, 1:500, Santa Cruz), anti-HRP (rabbit, 1:500, Jackson ImmunoResearch), anti-Spectrin, 3A9 (mouse, 1:500, DHSB), anti-Cut, 2B10 (mouse, 1:500, DHSB), anti-c-Myc (chicken, 1:500, Molecular Probes), anti-Tubulin, E7 (mouse, 1:500, DHSB). For all immunostaining the appropriate Alexafluor conjugated secondary antibody was used (Invitrogen).

**Luciferase Reporter Assay**

The transcription reporter assay was carried out as in the genome-wide RNAi screen for modifiers of Notch target transcription. S2 cells were harvested and resuspended to a density of 1 x 10^6 cells/ml in 75% serum free Schneider’s media and 25% conditioned Schneider’s media. Cells were treated with 8 μg dsRNA (Ambion Megascript kit) per million cells for 1 hr at 25 °C. An additional 50% volume of Schneider’s media with 20% FBS was added and cells were incubated for 4 days at 25 °C.

The dsRNA treated S2 cells were split into three separate aliquots and transfected (4μl Effectene, Qiagen) with respective combinations of luciferase reporter constructs and Nicd expression (400 ng total DNA each). The final cell concentration after transfection for
each replicate was 1 x 10⁶ cells/ml. The following plasmid combinations were used: a. The promoter of the Notch transcription target HLHm3 driving luciferase expression (HLHm3-luciferase) b. The HLHm3-luciferase reporter cotransfected with a construct that constitutively expresses activated Notch (pIZ-Nicd) c. A control luciferase reporter that is driven by the viral Ople2 promoter (pIZ-luciferase). Signal from this constitutively active construct was used to normalize the first two transfections for variation in cell viability and general transcriptional effects. Cells were assayed using Steady Glo Luciferase Assay System (Promega) 18 h after transfection. Data displayed are from a single biological replicate, the results of which are representative of multiple independent experiments.

**Primer sets for dsRNA generation**

The following primer pairs were used in the initial PCR for the construction of dsRNA:

- **tum-5’**: taatacgactcactatagggGAGACGGCCAGGATACAAAA
- **tum-3’**: taatacgactcactatagggTTTCAGTCCAGTGCTCAATCG
- **pav-5’**: taatacgactcactatagggGGCAGATGCTGATGAACCTT
- **pav-3’**: taatacgactcactatagggAAACTGGGCAGATCTTCGG
- **Su(H)-5’**: taatacgactcactatagggTATCCAAGCCGTCAAAAAAG
- **Su(H)-3’**: taatacgactcactatagggCcGGTGATATGATGCTCC
- **rad(control)-5’**: taatacgactcactatagggTTCTCTGCGGTAAGAGGT
- **rad(control)-3’**: taatacgactcactatagggATTGCTGGGTGAAGGACTG
Transcription Assay

N-S2 cells are a Drosophila cell culture line that stably express Notch protein under the control of a metallothionein promoter (Krejci and Bray, 2007). The cell line was grown and passaged with 10uM methotrexate to select for cells retaining the Notch expression construct. Prior to Notch induction, cells were treated with rad dsRNA (negative control) or tum dsRNA constructs as described above for luciferase assay experiments. After 2-3 days of dsRNA treatment N-S2 cells were treated with 600uM CuSO4 overnight to induce Notch protein expression. Cells were spun down at 2000xg for 4 min and washed in PBS + 5mM CaCl2. After washing cells were treated with PBS + 2mM EDTA (Induced) or with PBS
+ 5mM CaCl2 (control, non-induced) to trigger cleavage of the Notch receptor and activate Notch signaling. Samples were taken 30-120 min after EDTA induction of Notch. Cells were spun down and frozen in liquid nitrogen and stored at -80°C. To analyze Notch target expression frozen cell pellets were thawed and RNA extracted using the Qiagen RNeasy Kit utilizing a narrow gauge syringe to disrupt the cell pellet during lysis. 200ng of purified RNA was loaded into each RT reaction containing M-MLV Reverse Transcriptase (Ambion) and run according to manufacturer's instructions. Expression levels were to rp49 transcript levels.

**qPCR**

qPCR was performed using SYBR Green Master Mix (Fermentas). Reactions were run on a 7500 Real-Time PCR system (Applied Biosystems).

Primers list:

- **mβ**
  - left: GGATCAAGCTATGGTCACCTCC
  - right: CTCTCAGCTATGGTCACCTCC

- **m6**
  - left: GGGCAGCTATGGTCACCTCC
  - right: GCTGCTGCTTACTGGTCACCTCC

- **m3**
  - left: ATGGTCATGGGAGATGTCCAAGA
  - right: CGTCTGGAGGCCCCGTGGTAA

**Chromatin Immunoprecipitation**

Chromatin IP's were performed in a variety of different manners. Chromatin extracts were prepared from S2 cells (a Drosophila cell-culture line) and from developmentally staged embryos. Additionally, a number of different antibody/antigen strategies were used.
Initially a rabbit polyclonal antibody directed at full-length tumbleweed was used. I then tagged tumbleweed with and HA-tag and used a high affinity mouse monoclonal antibody directed at HA. Additionally I used a variety of polyclonal antibodies to GFP to attempt to IP the GFP-tagged pavarotti.

**S2 cells:** S2 cells were grown to a density of 2-4 x 10^6 cells/ml and harvested by spinning at 1000 x g for 5 minutes. The cells were then washed in PBS and spun again. Cells were resuspended in PBS + 1-3% Formaldehyde and incubated at room temperature for 10 minutes with gentle rocking. After crosslinking the formaldehyde was quenched by addition of glycine to a concentration of 125 mM and rocked gently for 5 minutes. Crosslinked cells were spun down at 1000 x g and resuspended in hypotonic lysis buffer (~2-4 ml) containing freshly added PMSF, protease inhibitor cocktail, NP-40, and BME and incubated for 3 minutes. The resuspended cells were passed through a ~26 gauge or greater syringe needle 10-15x to break open cells and release nuclei. Nuclei were pelleted at 500 x g at 4C for 10 minutes and resuspended in cold nuclear lysis buffer (~0.5-1ml). The nuclear lysate was then sonicated for 15 sec followed by 15 seconds of rest. This was repeated for a total of 16 cycles and yielded fragmented chromatin of about 250-500bp in size. The nuclear lysate was cleared by spinning at full speed at 4C for 10 minutes. The protein concentration of the resulting extract was measured using a Bradford assay and normalized to 1mg/ml of protein by dilution with RIPA buffer containing PMSF, protease inhibitor cocktail, and BME. Normalized extract was split into 1ml aliquots and frozen in liquid nitrogen and stored at -80C.

**Embryos:** Flies expressing PavGFP driven from the ubiquitin promoter were scaled up to stock large population cages containing large grape juice agar plates spread with a thin
layer of yeast paste. After laying, grape juice plates were moistened with distilled water and large amounts (~1-5 grams) of staged embryos (6-10 hours after egg laying) were collected with a medium sized paint brushed (~0.5 in wide) and placed on a nitex membrane rinsed with distilled to water to remove excess yeast paste. The collected embryos were dechorionated in 50% bleach for 3 minutes and then rinsed thoroughly with distilled water and finally PBT (PBS + 0.1% Triton X-100). The washed dechorionated embryos were transferred to a 50 ml Falcon tube containing 30 ml of heptane and 10 ml of crosslinking solution (1mM EDTA, 0.5mM EGTA, 100mM NaCl, 1.8% formaldehyde, 50mM HEPES, pH 8.0) and incubated at room temperature for 15 minutes with rocking. The crosslinked embryos were spun at 500 x g for 5 minutes and the supernatant was replaced with 30 ml of stop solution (PBS + 125mM glycine, 0.1% Triton X-100) to quench the crosslinking reaction and rocked at room temperature for 5 minutes. The embryos were again pelleted at 500 x g for 5 minutes and washed with 50 ml PBT. At this point crosslinked embryos were stored in 1.5ml tubes and frozen in liqued nitrogen and stored at -80 C. Frozen crosslinked embryos were suspended in ice cold PBT containing PMSF and protease inhibitor cocktail and homogenized on ice in a pre-chilled Dounce homogenizer with a loose-fitting pestle. The resulting lysate was spun at 500 x g for 1 min to pellet large debris and the supernatant was spun again at 1000 x g. The pelleted cells were resuspended in cold cell lysis buffer and homogenized in a cold Dounce homogenizer with a tight-fitting pestle. Nuclei were pelleted from the resulting lysate by spinning at 2,000 x g for 5 min at 4C. The nuclear pellets were resuspended in cold nuclear lysis buffer containing PMSF and proteasae inhibitors and incubated at room temperature for 20 minutes. Nuclear lysates were sonicated using the same conditions and protocol as used
for S2 cell chromatin preparation. Extracts were normalized to 1mg/ml protein based on a Bradford Assay, aliquoted, frozen in liquid nitrogen, and stored at -80C.

*Sequential Dual-crosslinking:* Ethylene glycolbis(succinimydylsuccinate) (EGS) was used as a crosslinking reagent because the longer linker sequence allows crosslinking of proteins over a greater distance. The ChIP procedure was carried out the same as above except EGS was dissolved in DMSO at 200mM and added to the cells resuspended in PBS to a final concentration of 2mM. This crosslinking reaction was allowed to proceed for 30 minutes. The cells were then crosslinked with formaldehyde as described above.

*Immunoprecipitation:* All chromatin extracts were immunoprecipitated using the following protocol. Chromatin extracts were thawed and 12µl gammabind beads/IP were pooled and washed with .5ml RIPA two times. Beads were spun at 1000xg for 2 min and resuspended in 50µl RIPA/IP. 50µl of bead suspension was added to each aliquot of chromatin extract and incubated at 4°C for 1 hour on rotator to preclear the extract of proteins binding to the beads non-specifically. The extracts were then spun down at 1000xg for 2 min and put in new siliconized tubes. A 10.5µl sample of extract was set aside in separate tube for 1% input sample. Antibodies were then added to each tube and incubated overnight at 4°C on a rotator. The antibody amount varied depending on concentration and background and was determined empirically. In a separate tube 25µl of gammabind beads/IP were pooled and rinsed with RIPA. The beads were resuspended in .5ml RIPA+1mg/ml BSA and .3mg/ml salmon sperm DNA and incubated 1-2 hours. The blocked beads were then washed with 1ml of RIPA buffer twice and resuspended in 50µl RIPA/IP and added to each chromatin sample and subsequently incubated for 3 hours at 4°C with mixing. Each IP was then pelleted and washed two times with 1ml RIPA for 10 min
each. Each IP was then washed four times with RIPA500 for 10 min each. Each IP was then washed one time with LiCl buffer and two more times with TE buffer for 10 min each. The following buffer was used to elute immuoprecipitated protein/DNA complexes from beads: 1% SDS, 100 mM NaHCO3, in distilled water. 100ul of elution buffer was added to each immunoprecipitation and incubated at room temperature for 15 min. The beads were pelleted and the supernatant was moved to fresh tube. The elution was repeated for a second time with 100ul of elution buffer and combined with the first elution. 8ul of 5M NaCl was added to each IP and input sample and incubated at 65C overnight to reverse crosslinks. 1ul of RNase A (10mg/ml) was added and incubated for 30 min at 37C. 4ul of 0.5M EDTA, 8ul of 1M Tris-HCl, and 1ul of Proteinase K (10mg/ml) was added to each tube and incubated for an additional 2hours. The immunoprecipitated DNA was then purified by phenol/chloroform extraction using phase lock gel tubes. Finally, the DNA was ethanol precipitated and analyzed by qRT-PCR.

Chip primers:

mβ:

promoter

aaatcggtgcgaacagt

cctctctctctcgtatgtgtgtgtgt

left primer

right primer

orf

GGATCAAGCTATGGTCACTCC

CTCTCAAGCTGTCGCATTC

left

right

m6:

promoter

Cccttggctgagaccta

Gagacggtgagcagtttgt

left

right
orfs		GGGCAGCTTCTACTGGCAGCTGCTGCTCCTCCGTTT		left				right

**Time-lapse Microscopy**

Live cell imaging of pupal nota was carried out according to published protocols (Zitserman and Roegiers, 2011). *Pnr*-GAL4 was used to drive the following reporters: UAS-Pav:GFP, UAS-mCD8:RFP and UAS-Pon:GFP.

Pupae were staged by circling with a marker larvae just beginning pupariation at the white pre-pupae stage excluding those that with a cuticle that had already begun to turn brown. This time point was counted as 0hr APF. After 18hr incubation at 27 °C selected pupae were moistened with a small amount of water and scraped from the side of the vial. The pupae were attached dorsal side up to a glass microscope slide using double-sided tape and allowed to dry for ~1 minute. Using forceps to grab the spiracles the operculum was carefully peeled off to reveal the head. The remaining pupal case was then carefully peeled until the majority of the pupa was exposed. Using a small paint brush the pupa was placed on a new glass slide. A small square of moist whatman filter paper (about 1cm x 1cm) with a hole punched through the center was placed around the pupa. Silicone lubricant was loaded into a 10ml syringe and deposited along the edges of the filter paper square creating a continuous border. A drop of 2ul of water was placed on a cover slip. The cover slip was inverted and then placed on the pupa with the water drop contacting the thorax. The cover slip was pressed down gently creating a seal with the silicone lubricant but making sure not to burst the pupa. The slide was then imaged on an inverted confocal
microscope using oil immersion objectives making sure to not put excessive pressure on the cover slip with the microscope objective. Single images or image stacks were taken approximately every 45 seconds throughout the time course and assembled using ImageJ.

III. Results

**Centralspindlin RNAi causes cell fate transformation and cytokinesis failure**

The loss of thoracic bristles phenotype due to depletion of centralspindlin was reminiscent of Notch signaling defects and prompted me to investigate potential cell fate transformation defects.

Close examination of cell types and cell morphology within dsRNA treated sensory organ clusters revealed a range of cell fate defects. Often Tum or Pav depletion causes cytokinesis failure leading to multinucleate sensory organ cells (Fig 4-1 B). Multinucleate cells were identified through spectrin staining, a protein found on the intracellular side of the plasma membrane. Tum depleted cells show multiple nuclei contained within spectrin rings compared to a single nucleus per spectrin ring found in wildtype sensory organs (Fig. 4-1 A,B). Surprisingly some sensory organ cells are able to complete cytokinesis, resulting in multiple neuronal cells each outlined by a spectrin ring (Fig. 4-1 C). The cell division defect was expected considering centralspindlin’s essential role in cytokinesis and the observed dynamic localization from the nucleus to cleavage furrow in dividing sensory organ precursors (Fig 4-2 A,B).
Figure 4-1. Depletion of Tum or Pav by dsRNA leads to cell fate transformation and cytokinesis defects in sensory organ lineages.

A-C. Sensory organ cells are stained with Elav (green) a neuron marker, Spectrin (red) a membrane associated protein, and DAPI (blue). A. wildtype sensory organ. B. A tum-dsRNA treated sensory organ displaying multinucleate cells and containing two Elav+ cells. C. A tum-dsRNA treated sensory organ displaying four mononucleate cells three of which are Elav+. 
Figure 4-2. Pav-GFP localizes to the cleavage furrow and nuclei of sensory organ precursors.

A. Live-cell imaging of Pav-GFP (green) shows localization to the cleavage furrow in dividing sensory organ cells. Plasma membrane is marked by mcd8-RFP (red). B. Fixed cells stained with anti-GFP antibody (green) show the nuclear localization of Pav-GFP in immature sensory organ cells. The neuronal cell is marked by Elav (red). The socket cell is marked by the arrow. Notably Pav-GFP is absent from the sheath cell (arrowhead).
I next examined cell fates defects within the sensory organ lineage and compared them to phenotypes associated with Notch signaling defects, a known regulator of sensory organ development. By staining for the neuron cell fate marker Elav or the socket cell marker Su(H) I discovered dsRNA depletion of Tum or Pav results in defects in the differentiation of sensory organs similar to those found with Notch depletion (Fig. 4-3 A). Both neurons and socket cells appear to be either absent or greatly reduced in the pnr-GAL4 expression domain. Additionally, whereas each wildtype sensory organ contains one of each cell type, dsRNA treated sensory organs display missing or duplicated cell types (Fig. 4-3A). Most tum- or pav-dsRNA sensory organs contain multiple neurons ranging from one to four and are often missing socket and bristle cells. Sheath cell numbers also vary from zero to three. These cell fate transformations are reminiscent of those seen with notch- dsRNA, and prompted me to further examine the relationship between centralspindlin and Notch signaling.

**Centralspindlin RNAi may disrupt Notch target gene transcription in luciferase assay**

Previously, a genome wide RNAi screen was performed using a cell culture based luciferase assay (Mourikis et al., 2010). This screen provided preliminary evidence that centralspindlin components may influence the activation of Notch target transcription. Additionally, mass spectroscopy analysis revealed centralspindlin may interact with chromatin regulators known to affect Notch signaling(Moshkin et al., 2009). To confirm the genome wide screen data I synthesized 500bp dsRNA constructs targeting centralspindlin components and its potential interacting proteins (Fig. 4-4 B,D). I also synthesized 500bp dsRNA constructs targeting proteins involved in chromatin regulation
Figure 4-3. Depletion of Tum or Pav by dsRNA leads to cell fate transformation and axon morphology defects in sensory organ lineages.

A. Sensory organs stained for cell fate markers Elav (green) and Su(H) (red). Centralspindlin or Notch depleted sensory organs contain multiple neuronal cells. 
B. Sensory organs stained for cell fate markers Elav (green) and Prospero (red). Centralspindlin or Notch depleted sensory organs contain multiple neuronal cells and multiple sheath cells. 
C. Sensory organs stained for cell fate markers Elav (green) and HRP (red, neuron cell membrane marker). Centralspindlin or Notch depleted sensory organs contain multiple neuronal cells each projecting axonal extensions.
**Figure 4-4.** Candidate screen using luciferase reporter assay of Notch target transcription in S2 cells.

A panel of candidate genes were re-screened using dsRNA treatment and an assay for activity of a luciferase reporter driven by the m3 promoter, a known Notch target. A,B. Basal activity of the m3-luciferase reporter normalized to cell number for (A) known transcriptional regulators and for (B) centralspindlin and its interacting proteins. C,D. Notch-induced activity of the same m3-luciferase reporter for (C) known transcriptional regulators and for (D) centralspindlin and its interacting proteins. Su(H), a known Notch regulator served as a positive control. All samples were normalized to rad-dsRNA treated cells, which served as a negative control. Error bars represent the standard deviation of technical replicates performed for each dsRNA treated sample.
of Notch (Fig. 4-4 A,C). Using this targeted candidate approach I looked at the role of these components in basal transcription of Notch target genes as well as Notch induced transcription. The assay was performed by first incubating S2 cells at 30°C in the presence of dsRNA for 3-4 days. Subsequently cells were transfected with the luciferase reporter constructs and allowed to incubate overnight. Luciferase activity was normalized to cells treated with radish dsRNA, a negative control (Mourikis et al., 2010). Cell numbers were normalized to the luciferase signal produced from a constitutively active viral promoter (Mourikis et al., 2010). Su(H), a known component of the Notch pathway, serves dual functions as an activator and repressor of Notch signaling and served as a positive control in the luciferase assay (Fig. 4-4 A,C). Of the candidates in the chromatin regulator group only hira and lid had a reproducible effect on basal Notch transcription, resulting in ~40% decrease compared to control (Fig. 4-4 A). Notch induced transcription was affected only by hira and asf1 dsRNA treatment, preventing full activation of transcription upon Notch induction (Fig. 4-4 C). Of the centralspindlin group tum, pbl, and rho treated samples all showed a decrease in basal Notch target transcription (Fig. 4-4 B). Upon Notch induction only pav resulted in a decrease in transcription (Fig. 4-4 D). Repetition of the luciferase assay using identical methods and dsRNA constructs showed inherent variability with the assay. While some trends remained consistent, most results were highly variable. For example, while asf1 or hira dsRNA treatment showed no effect on basal transcription in initial experiments, subsequent replicates showed robust increases (Fig. 4-5 A). For centralspindlin, dsRNA treatment consistently results in decreased basal transcription (Fig. 4-5 B). However, in subsequent experiments Notch induced transcription was mostly unaffected in contrast to the decrease seen previously with pav-dsRNA (Fig. 4-4 D, and Fig.
Figure 4-5. Luciferase reporter assay of potential hits to measure Notch target transcription in S2 cells.

Potential hits from the candidate screen were selected for repetition to confirm their effect on Notch-reporter activity. A. Basal activity of the m3-luciferase reporter normalized to cell number. B. Notch-induced activity of the same m3-luciferase reporter. Su(H), a known Notch regulator served as a positive control. All samples were normalized to rad-dsRNA treated cells, which served as a negative control. Su(H), asf1, and lid show similar trends to results of the previous targeted candidate screen. However tum, pav, and hira deviate from the trends observed previously showing the variability and irreproducibility of the assay. Error bars represent the standard deviation of technical replicates performed for each ds-RNA treated sample.
Figure 4-6. Luciferase reporter assay of centralspindlin’s role Notch target transcription in S2 cells.

Centralspindlin components were selected for repetition to confirm their effect on Notch-reporter activity. A. Basal activity of the m3-luciferase reporter normalized to cell number. B. Notch-induced activity of the same m3-luciferase reporter. Su(H) and H, known Notch regulators served as a positive controls. All samples were normalized to rad-dsRNA treated cells, which served as a negative control. Centralspindlin components again show some similarity but also some deviation from previous experimental results. Error bars represent the standard deviation of technical replicates performed for each ds-RNA treated sample.
4-5 B). In a third and final round of the luciferase assay using identical methods I focused only on *tum* and *pav* and compared the results to two known Notch regulators, *Su(H)* and *Hairless* (Fig. 4-6). Again, *tum* and *pav*-dsRNA resulted in decreased basal Notch transcription (Fig. 4-6 A). *Tum* and *pav*-dsRNA also resulted in a modest decrease in Notch induced transcription by ~35% (Fig. 4-6 B). While the result of *Su(H)*-dsRNA followed expected trends, *hairless* -dsRNA showed little to no effect. This was puzzling as *hairless* is a known transcriptional repressor of Notch target transcription. I would expect Hairless knockdown to result in increased Notch target transcription regardless of Notch activation state. The high variability of the assay can be the result of a number of problems. The effectiveness of dsRNA knockdown is inconsistent between experiments. Additionally, the transfection step requires the introduction of two individual plasmids into the same cell, the luciferase reporter plasmid and the NICD expressing plasmid. Transfection of S2 cells with one plasmid is notoriously inefficient let alone transfection of two constructs simultaneously. The inherent variation of dsRNA treatment and reporter plasmid transfections led me to consider other potentially more robust assays.

**Transcription assay reveals centralspindlin is needed for Notch induced transcription**

To circumvent the problems with the luciferase assay I attempted to use qPCR to quantify endogenous mRNA transcripts of Notch target genes thus eliminating the variation inherent with transfecting multiple reporter plasmids. Additionally, I used a cell line containing an inducible Notch expression construct under the control of a metallothionein promoter (N-S2 cells, Krejci and Bray, 2007). Once Notch expression is induced, the pathway can be activated by incubating cells in EDTA. EDTA chelates Ca^{++} needed to
Figure 4-7. qPCR analysis of Notch target gene transcription after depletion of Tumbleweed by dsRNA with and without Notch induction.

Assays were performed in N-S2 cells with EDTA induction of Notch (see methods). Rad-dsRNA treatment served as a negative control. Transcript levels are normalized relative the rp49 levels. A. mβ transcript levels. Depletion of Tum results in a ~50% decrease in mβ transcript. B. m6 transcript levels. Depletion of Tum results in a ~60% decrease in m6 transcript.
stabilize the Notch transmembrane receptor. Elimination of Ca++ enhances the cleavage of the Notch receptor and subsequent translocation to the nucleus (Rand et al., 2000). N-S2 cells were treated with dsRNA targeting either rad or tum and transcripts of Notch targets m6 and mβ of the E(spl) locus were measured with qPCR. The transcript levels of m6 and mβ were unaffected by tum-dsRNA (Fig. 4-7 A,B). However, the levels of m6 and mβ were significantly decreased following addition of EDTA indicating Tum is needed for Notch activated transcription (Fig. 4-7 A,B).

The results of the luciferase assays and qPCR of endogenous transcripts indicate centralspindlin may be needed for Notch induced transcription. However these assays do not offer any insight into the potential mechanism by which centralspindlin influences Notch target transcription. The predominant nuclear localization of centralspindlin during interphase and its interaction with chromatin regulators prompted me to see if centralspindlin participates directly in transcription. I employed chromatin immunoprecipitation (ChIP) to detect the presence of centralspindlin at Notch target genes.

**ChIP assays reveal no direct association with Notch target genes**

Using standard procedures I attempted to ChIP native Tum at the promoters and open reading frames of m3 and mβ, two Notch-inducible genes of the E(spl) locus. I used Su(H) as a positive control as it is a known DNA binding protein with known binding sites in the promoters of both m3 and mβ. I was able to detect Su(H) at both promoters at up to 2% of input (Fig. 4-8 A). Tumbleweed on the other hand showed very little enrichment when compared to the GFP negative control antibody (Fig. 4-8 A). Following this result I attempted to modify the ChIP protocol to enhance the Tum signal.
Figure 4-8. Chromatin Immunoprecipitation from S2 cells crosslinked with formaldehyde.

ChIP signals at m3 and mβ promoter and gene regions are expressed as % input. Chromatin extracts were immunoprecipitated with a Su(H) antibody to serve as a positive control and a GFP antibody to serve as a negative control. A. ChIP performed with 1% formaldehyde. ChIP signals for Tumbleweed show no enrichment compared to the positive control. B. ChIP performed with 6% formaldehyde. ChIP signals for Tumbleweed show no enrichment compared to the positive control.
First I increased the concentration of formaldehyde crosslinking reagent from 1% to 6%. Under increased concentrations of crosslinker I was able to slightly enhance the signal of Su(H) but I was still unable to detect Tum at m3 and mβ (Fig. 4-8 B). The inability to detect Tum could be due to the poor quality of the polyclonal antibody. I then cloned an HA-tagged Tum (Tum-HA) and transfected this construct into S2 cells. I then performed the ChIP experiment using standard protocols employing a high affinity monoclonal antibody to the HA tag. With this antibody I was unable to detect any enrichment of Tum-HA at Notch target genes beyond the levels of the negative control (Fig. 4-9). I hypothesized the inability to ChIP Tum could be due to its physical distance from the DNA. Tum may bind chromatin indirectly through intermediary proteins. To test this possibility I used a dual crosslinking method consisting of sequential use of EGS and formaldehyde. EGS is a bifunctional crosslinking reagent with a long linker sequence (~16 Å) allowing crosslinking over a much greater physical distance than formaldehyde. Using this protocol I was unable to detect either native or Tum-HA at levels above background.

I then attempted to ChIP centralspindlin from embryos instead of cells. I hypothesized centralspindlin may only be present at Notch target genes during Notch induction. To test this I staged embryos from 6-10 hours. At this developmental stage the nervous system, in which Notch signaling highly active, is beginning to develop. I used embryos that expressed Pavarotti-GFP under the control of the ubiquitin promoter to prepare chromatin extracts. I then used standard ChIP procedures and employed a polyclonal GFP antibody to attempt to detect Pav at Notch targets. Again I was unable to detect any signal above the level of background (Fig. 4-10 A). I repeated the protocol again this time omitting the high-salt wash steps during the purification of immunoprecipitated
Figure 4-9. Chromatin Immunoprecipitation from S2 cells crosslinked with 1% formaldehyde.

S2 cells were transfected with a plasmid encoding HA-Tumbleweed. ChIP signals at m3 and mβ promoter and gene regions are expressed as % input. Chromatin extracts were immunoprecipitated with a GFP antibody to serve as a negative control. ChIP signals for HA show no enrichment compared to the negative control.
Figure 4-10. Chromatin Immunoprecipitation from 6-10 hour Pav-GFP and wildtype embryos.

ChIP signals at m3 and mβ promoter and gene regions are expressed as % input. Chromatin extract derived from wildtype fly embryos and was ChIPed using the GFP antibody to serve as a negative control. A. ChIP from Pav-GFP embryos using standard protocols. The signal for the Pav-GFP chromatin extract (red) shows no significant enrichment compared to the negative control (blue). B. ChIP from Pav-GFP embryos omitting the stringent wash steps (high salt). Pav-GFP signal (red) shows no enrichment over the negative control (blue).
chromatin. Under these conditions I was able to detect the highest occupancy of Notch targets by Pav-GFP of about 1% input (Fig. 4-10 B, red bars). Sadly these ChIP conditions also led to high levels of background as the negative control gave enrichment of up to 2% input (Fig. 4-10 B, blue bars).

**Centralspindlin RNAi prevents Notch target expression in vivo and causes cytokinesis failure**

The negative ChIP results indicate centralspindlin does not directly associate with Notch target genes. The results offer no insight into how centralspindlin influences Notch target transcription. To clarify the role on centralspinlin in Notch target transcription I turned to an in vivo reporter to analyze the effect of Tum depletion on endogenous Notch transcriptional activity. *Ptc-GAL4* drives expression in a stripe along the length of the wing disc (Fig. 4-11, dashed lines). The *ptc* expression domain bisects a stripe of cut expression (Fig. 4-11, red staining). The *cut* gene is a direct target of the Notch pathway and is used as an indirect readout of Notch activity. *Cut* defines the wing margin at the border between the dorsal half and the ventral half of the wing disc. I used *ptc-GAL4* to drive expression of dsRNA targeting centralspindlin components as well as mutant constructs of the centralspindlin components (Fig. 4-11, dashed lines). dsRNA knockdown of Tum and Pav leads to a slight disruption to the stripe of Cut protein expression (Fig. 4-11 B, C). This disruption is minimal when compared to knockdown of the Notch receptor (Fig. 4-11 F). Additionally, knockdown of Tum and Pav both result in ~25% of binucleate cells in the *ptc* expression domain (Fig. 4-11 G,H). Overexpression of Tum or Pav in the wing disc had no effect on cut expression. Additionally, expression of TumΔGAP, PavΔnls-GFP, or TumΔnls-
Figure 4-11. Depletion of centralspindlin or expression of dominant negative constructs disrupt Notch target expression and cause cytokinesis defects in the wing disc.

A-H. Ptc-GAL4 was used to express UAS-constructs in a narrow stripe in the wing disc. A-F. Cut expression (red) marks the dorsal ventral margin of the wing. Dashed lines marked ptc-GAL4 expression regions. B,C. Cut expression is disrupted by dsRNA depletion of Tum or Pav. D,E. Cut expression is unaffected by expression of TumΔGAP or PavΔnls-GFP. F. Severe disruption of Cut expression caused by Notch depletion. G,H. Cytokinesis defects in ptc-GAL4 expression regions (red) caused by centralspindlin depletion. Multinucleate cells are identified by tubulin (green) marked by asterisks. tum-dsRNA causes a 25% cytokinesis failure rate while pav-dsRNA results in 20% cytokinesis failure.
**Figure 4-12.** Effect of Tum depletion on Notch target gene expression.

The E(spl)m4-stinger (eGFP) fly line was used to examine in vivo expression of E(spl)m4 in the adult notum following depletion of Tum by dsRNA. E(spl)m4 is expressed in broad vertical stripes along the length of the notum (shown in red). eGFP intensity is indistinguishable from areas outside of pnr-GAL4 expression domain (marked by dashed lines). Sensory organs cells display cell fate defects. Multiple neuronal cells marked by Elav staining show some sensory organs suffer cell fate defects.
MYC has no noticeable effect on Cut protein levels (Fig. 4-11 D,E). If anything, TumΔGAP results in small expansion in the stripe of cut expression (Fig. 4-11 D), however this may be an artifact due to tissue fixation and processing.

**In vivo Notch reporter shows no effect on target transcription**

I then used the m4-stinger reporter to look at the effect of tum-dsRNA on m4 levels in vivo (Fig. 4-12). The m4-stinger reporter fly line carries a P-element insertion of a construct containing a nuclear localizing GFP under the control of the endogenous m4 promoter sequence, another known Notch target. m4 is expressed in broad vertical stripes along the length of the notum (Fig. 4-12, A). Analysis of GFP intensity in the pupal thorax shows that the expression of m4 is not affected by pnr-GAL4 driven tum-dsRNA. m4 driven expression of GFP appears normal and is indistinguishable from regions outside of the pnr-GAL4 expression domain (marked by dashed lines).

**Centralspindlin RNAi does not affect asymmetric Partner of Numb localization**

Sensory organ cell fate can be determined by Notch signaling. However there are a number of events leading up to activation of Notch in sensory organ cells. The sensory organ precursors must undergo asymmetric division prior to specification of cell fate. I hypothesized that these upstream events may be disrupted by centralspindlin depletion thus leading to cell fate transformations. To test this possibility I examined the asymmetric segregation of Partner of Numb (Pon), an known asymmetric determinant essential for inhibition of Notch in one of the daughter cells. Using a GFP-tagged Pon I was able to follow Pon localization in live cells during sensory organ precursor divisions in both wildtype and centralspindlin depleted flies (Fig. 4-13). Pon localizes properly to the anterior cell after
Figure 4-13. Pon-GFP localization during sensory organ cell division in wildtype and centralspindlin depleted flies.

Pon-GFP localization was followed in live cells during asymmetric division of sensory organ cells. A. In wildtype cells Pon-GFP is asymmetrically localized to the anterior cell as the cells begin mitosis. B. In tum-dsRNA cells Pon-GFP is segregated to the anterior cell normally. C. After Pav depletion Pon-GFP localizes to the anterior cell initially. However, as cytokinesis fails Pon localization spreads along the cell cortex in the arrested cell.
tum-dsRNA treatment throughout cell division. (Fig. 4-13 B). Depletion of Pav on the other hand causes mislocalization of Pon-GFP. Initially Pon-GFP localization appears normal. As cell division progresses however, pav-dsRNA treated cells arrest in late stages of cytokinesis. In these cases Pon-GFP becomes uniformly distributed around the cell cortex (Fig. 4-13 C). In cells that divide successfully, Pon-GFP localizes properly throughout the cell cycle.

**Centralspindlin does not interact genetically with the Notch signaling pathway**

As molecular biology techniques failed to offer insight into centralspindlin’s role in Notch signaling I turned to genetics to clarify the interaction of centralspindlin with the Notch pathway. Using a candidate approach I crossed various alleles and UAS-constructs of Notch pathway components to a fly expressing tum-dsRNA driven by pnr-GAL4. Pnr-GAL4 driven tum-dsRNA results in a thoracic closure defect as well as a loss of thoracic bristles. I then examined adult flies to check for suppression or enhancement of the tum-dsRNA phenotype. Of the ~20 candidates tested only one construct, UAS-Neuralized, showed any significant change to the tum-dsRNA phenotype (Table 4-1). Expression of neuralized in addition to tum-dsRNA leads to an increase in the total number of bristles remaining (Fig. 4-14 A).

I chose to examine this interaction in detail by dissecting and staining pupae for cell fate markers. Neuralized is a known Notch regulator. Expression of Neuralized alone leads to an increase in the number of bristles (Fig. 4-14 A). I found that Neuralized expression combined with Tum depletion does lead to an increase in the total number of sensory organs as compared with tum-dsRNA alone (Fig. 4-14 A). However, Neuralized
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**Table 4-2.** Screen of candidates for genetic interaction with centralspindlin.
Figure 4-14. Expression of Neuralized restores bristle number but fails to rescue thorax closure defect of tum-dsRNA phenotype.

A. Adult nota showing bristle phenotypes of wildtype, UAS-Neuralized, tum-dsRNA, and UAS-Neuralized; tum-dsRNA in combination. Neuralized expression results in a doubling of bristle number while tum-dsRNA results in a loss of bristles. When expressed in combination there is an intermediate number of bristles. B. Pupal nota of tum-dsRNA and tum-dsRNA; Neuralized flies stained for Elav (green). tum-dsRNA results in thorax closure defects highlighted by the arrows. tum-dsRNA; Neuralized display an increase in sensory organ number but still show cell fate and thorax closure defects (arrows). C. Sensory organs of tum-dsRNA; Neuralized treated flies show cell fate defects. Elav (green) shows multi-neuronal clusters as well as excess socket cells (Su(H), red).
expression neither enhances nor suppresses the thorax closure phenotype (Fig. 4-14 B). Additionally, sensory organs display identical cell fate and cytokinesis failure phenotypes seen with tum-dsRNA alone (Fig. 4-14 C). Together these results indicate the tum-dsRNA phenotype is epistatic to that of neuralized overexpression as Neuralized expression is able to increase bristle number by specifying excess sensory organ precursors but does not effect thorax closure or cell fate phenotypes of Tum depletion (Fig. 4-14 B,C). With the exception of neuralized expression, perturbation of the Notch pathway fails to either rescue or enhance the tum-dsRNA phenotype (Table 4-1). This indicates the cell fate transformation defect may not be a direct result of a defect in Notch signaling but rather a combination of defects, including cytokinesis failure and epithelial cell organization defects.

IV. Discussion

The cell fate transformation caused by dsRNA depletion of centralspindlin was reminiscent of classic phenotypes of Notch pathway mutants. I used a variety of techniques and approaches to further investigate the relationship between centralspindlin and Notch signaling during the differentiation of sensory organs.

Examination of the multi-neuronal clusters resulting from tum- or pav-dsRNA revealed a fraction of cells displayed cytokinesis defects while some show normal cell division. Additionally, examination of Pav-GFP localization in real time in dividing SOP’s revealed centralspindlin behaves much like it does in other cell types such wing disc cells(Somers and Saint, 2003). Centralspindlin cycles from the nucleus of interphase cells to the cleavage furrow of dividing cells. Unlike neuronal cells, however, centralspindlin is not present in the cytoplasm in amounts detectable by immunofluorescence or live-cell
microscopy (Goldstein et al., 2005). One interesting observation is the lack of Pav-GFP signal in each nucleus of the mature sensory organ cells. The absence of Pav-GFP from the sheath cell can be explained in a couple of ways. Pavarotti may be selectively degraded in the sheath cell following cytokinesis. Centralspindlin is a component of the midbody (Hutterer et al., 2009; Lekomtsev et al., 2012; Zhu et al., 2005). The processing of the midbody is known to vary in different cell types (Schiel et al., 2013). In some cells the midbody is expelled into the extracellular space and eventually degraded (Schiel et al., 2013). In other cells the midbody is selectively inherited by one cell following an asymmetric division (Schiel et al., 2013). Another possibility is that the pnr-GAL4 driver is selectively turned off only in the sheath cell. This is highly possible however I would still expect residual Pav-GFP to be present after cell division even without renewed transcription of the transgene. Further investigation is needed to characterize the processing and inheritance of the midbody and Pav-GFP during SOP divisions before we can understand this potential function of centralspindlin.

Comparisons of cell fate transformations between centralspindlin and Notch knockdowns showed highly similar phenotypes. Both treatments result in multi-neuronal clusters and occasionally extra sheath cells. However, nearly identical phenotypes were observed in thoracic sensory organ cells when septin or diaphanous are mutated (Founounou et al., 2013). Both proteins are involved in regulation of the cytoskeleton and cell adhesion during cytokinesis indicating cell fate transformation may by secondary consequences of cell division defects (Founounou et al., 2013; Guillot and Lecuit, 2013).
I turned to Notch reporter assays to determine if the cell fate transformation phenotype could be due to perturbation of Notch signaling. My attempts to validate results from the genome-wide RNAi screen were highly variable and irreproducible (Mourikis et al., 2010). The published assay and the one used here required the treatment of cells with dsRNA for 3-4 days followed by the simultaneous transfection of multiple plasmids encoding reporter contracts. Transfection with Qiagen effectene reagent yields poor transfection efficiencies of ~10% even with optimization of the protocol (Mosher and Crews, 1999). Both dsRNA treatment and reporter transfection introduce unavoidable problems and potential for variation. dsRNA depletion of some genes, including centralspindlin, can have pleiotropic effects resulting in cell lethality or slow growth. Although I attempted to normalize cell counts across all samples, the varying health of cells following dsRNA treatment could easily affect transfection efficiency between samples. I also could not determine if dsRNA uptake by cells is uniform across the entire population. Additionally the simultaneous transfection of multiple plasmids introduces further complications. Assuming a 10% transfection efficiency for one plasmid means the probability of a single cell receiving one copy each of two individual plasmids is 1% in a worst case scenario. The design of this assay limits me to measuring transcription, at worst, in just 1 out of 100 cells that may or may not have received dsRNA.

These complications led me to pursue a similar assay using a stable-transfected Notch inducible cell line (Krejci and Bray, 2007). This eliminated the problems associated with transfection efficiency and allowed uniform conditions in a population of cells. Using this assay I was able to show tum-dsRNA has little to no effect on Notch target transcription in the absence of active Notch signaling. Upon induction of Notch signaling however, tum-
dsRNA results in about 60% decrease in transcript levels compared to controls. This may indicate Tum is required for the full activation of Notch signaling. Another possibility is that Tum depletion causes a change in the cell cycle profile of the assayed cell population. Notch signaling is known to vary with cell cycle stage (Remaud et al., 2008). Tum depleted cells could have lower Notch transcription simply because of cell cycle arrest. These data point to a potential role in regulation of Notch target transcription but fail to offer insight into how centralspindlin might be involved in Notch signaling.

Given the interaction of centralspindlin with chromatin regulators, I used ChIP to determine if centralspindlin is bound to chromatin at Notch target genes. I exhausted all realistic options for ChIP techniques and modifications including various crosslinking conditions and multiple antibodies. Ultimately I was unable to detect Tumbleweed or Pavarotti on or near Notch target genes. These negative results do not conclusively rule out centralspindlin regulation of Notch at the DNA or chromatin level. However the lack of a detectable ChIP signal under any of the conditions tested is a glaring weakness in this model.

By examining the differences among phenotypes caused by centralspindlin depletion or dominant negative expression I sought to glean information about the relationship between the thorax closure phenotype and disruption of Cut expression. Depletion of centralspindlin causes severe thorax defects and relatively minor disruption of Cut expression, while expression of TumΔGAP, TumΔnls-MYC, and PavΔnls-GFP all cause thorax defects but fail to disrupt expression of Cut in the developing wing disc. The absence of a phenotype in the wing disc with these potent dominant negative centralspindlin mutants indicates the disruption of Cut expression, although relatively
minor, is specific only to dsRNA knockdown of centralspindlin. This leads me to conclude the disruption of Cut expression is probably a secondary effect of centralspindlin depletion, probably due to cytokinesis defects. Analysis of m4 transcription in pupal nota supports this conclusion as expression of the m4 reporter, a Notch target gene, is unaffected by tum-dsRNA.

The conflicting results from cell culture based assays and in vivo reporters in the thorax and wing disc are difficult to explain. Perhaps the fluorescent reporter used in vivo is not sensitive enough to detect small changes in Notch target transcription. Alternatively, cells grown in isolation without cell-cell contacts necessary for native Notch-Delta signaling may not provide an accurate or relevant model for Notch signaling. Either way the highly contrived nature of the cell culture system led me to focus on the more biologically relevant system of pupal development. While I did not observe changes in Notch target transcription in the pupa, I do consistently see cell fate transformations in the sensory organ lineages.

In asymmetric cell division Pavarotti is localized primarily to the apical cell cortex indicating Pavarotti may have role in establishing cell polarity either through regulating localization of asymmetric determinants or the regulation of cell size asymmetry during cell division (Cabernard et al., 2010). Given centralspindlin's role in cytokinesis and spindle formation and orientation I asked if the observed cell fate defects could be due to defects in these events (Cabernard et al., 2010). Surprisingly, examination of Pon-GFP localization during SOP division revealed mostly normal cell division. Pon-GFP, an asymmetric determinant, is properly localized to the anterior cell and restricted from the posterior cell regardless of tum-dsRNA. Occasionally tum-dsRNA causes delay or complete
failure of cell division eventually allowing the anterior crescent of Pon-GFP begins to diffuse along the rest of the cortex. It is unknown if this type of incident results in cell fate transformations. While localization of asymmetric determinants and spindle orientation appear normal the kinetics of cell division may be altered slightly by centralspindlin depletion. Following cell division there is a 30 minute window when sensory organ cells are especially receptive to Notch signaling (Remaud et al., 2008). Delaying cell division by *tum*-dsRNA could effect Pon retention in the anterior cell and/or disrupt the kinetics of Notch-Delta signaling during SOP specification leading to cell fate transformation. Alternatively, defects in cell morphology could affect ligand presentation to neighboring cells. Notch and Delta ligand interactions are predicted to be present in filopodial extensions from sensory organ cells (Cohen et al., 2010). The disruption of filopodia structures due to cell morphology defects could prevent Notch-Delta signaling and could explain cell fate transformations.

Molecular techniques failed to clarify the role of centralspindlin in Notch signaling. I performed a targeted screen for genetic interactions with the *tum*-dsRNA cell fate phenotype. I demonstrated that perturbation of the Notch pathway, either inhibition or activation, has no effect on the loss of bristles or cell fate phenotypes observed with *tum*-dsRNA. I was able to find one construct that modified the *tum*-dsRNA phenotype. Overexpression of Neuralized with *pnr*-GAL4 was able to partially restore lost bristles. However, Neuralized overexpression by itself leads to about twice the number of bristles compared to wildtype. When combined with *tum*-dsRNA, Neuralized expression resulted in not only more bristles but also more incidences of cell fate transformation while leaving the thorax closure defect unaffected. The lack of a genetic interaction with Notch pathway
components indicates centralspindlin probably functions primarily in an alternative pathway during thorax development. This pathway presumably involves proteins involved in cell migration that are essential for thorax closure. Centralspindlin was recently shown to coordinate the regulation of the small GTPases Rho and Rac (Bustos et al., 2008; Ratheesh et al., 2012). Thus, it will be interesting to see if the tum-dsRNA phenotype can be suppressed or enhanced through mutations in Rac and/or Rho.

The results presented here demonstrate that Notch regulation is not a major role of centralspindlin in Drosophila thorax development. Centralspindlin depletion, despite having a modest inhibitory effect on Notch activated transcription in cell culture experiments, seems to mainly cause cell migration and cell division defects. The preponderance of evidence suggests centralspindlin plays an essential role in thorax development, mainly through the regulation of wing disc migration. Although centralspindlin depletion does lead to cell fate transformations that are reminiscent of Notch mutants, these appear to be secondary consequences of thorax closure and cell division defects and are unaffected by activation or inhibition of Notch activity.
Chapter 5: Localization and role of Centralspindlin in salivary gland nuclei

I. Introduction

In a screen for proteins involved in centromere assembly, MgcRacGAP, the human homolog of tumbleweed, was found to localize to centromeric regions in live HEla cells prior to cell division. MgcRacGAP and Ect2, the human homolog of pebble) were found to be needed for centromeric histone loading and maintenance. centralspindlin was also found associated with histone chaperone proteins in a mass spec characterization of chromatin remodeling complexes. These findings hint at a nuclear function and prompted me to investigate centralspindlin in a system to clarify its role in the nucleus.

Drosophila salivary glands are specialized larval secretory organs serving two main functions: secretion of both digestive enzymes and of glue proteins. The secretion of digestive enzymes allows external digestion of complex food substrates prior to consumption. During pupariation larvae secrete glue proteins allowing attachment of the pupal case to a substrate during the process of metamorphosis. Salivary glands consist of duct cells and secretory cells. Duct cells form the tubes connecting the glands to the larval mouth while secretory cells are responsible for protein production and secretion. Salivary glands consist of cells that are highly polyploid, with each cell containing up to ~1000 genomic copies. The DNA within these cells is organized in large bundles called polytene chromosomes. Polytene chromosomes are easily visible under a light microscope. The large size of cells and the ability to easily visualize and stain polytene chromosomes have
made salivary glands an excellent tool to study nuclear biology. In particular polytene chromosomes are an excellent system to study transcriptional regulation.

Polyplloid cells undergo DNA replication without cell division, a process called endoreplication. The absence of cell division allowed me to examine the functions of centralspindlin independent from its role in cytokinesis and circumvent the pleiotropy observed with mutation or knockdown of centralspindlin. Salivary glands complete their morphogenesis and take on their mature structures quite early in larval development. I took advantage of this to isolate and examine centralspindlin’s interphase function independent from a role in cell and/or tissue migration. Salivary gland cells have a very simple morphology lacking specialized membrane structures such as filopodia or axons. Thus, Drosophila salivary glands are an ideal system to study the nuclear role of centralspindlin and independently from its role in cytokinesis and cell migration.

II. Methods

Polytene Chromosome Preparation

Salivary glands were dissected from third instar larvae raised at 18C. The larva was submerged in PBS in a deep well glass slide. Using forceps the larva was grasped firmly by the mouth hooks and at the approximate midpoint of the larva. In one motion the anterior portion of the larva was removed with the salivary glands attached. Only large, healthy salivary glands were chosen to proceed due to ease of chromosome spreading. The fat body was delicately peeled away from the glands with forceps. The glands were then placed in PBS + 4% formaldehyde and 1% Triton X-100 and incubated for ~2 min. The glands were then transferred to a solution of 50% acetic acid and 4% formaldehyde in
water for ~ 2-4 minutes. The glands were then placed in a ~20ul drop of 50% acetic acid and 4% formaldehyde in water on a siliconized cover slip. The glands were pressed between the cover slip and a poly-lysine coated glass slide and placed cover slip side-up. Using a pencil eraser the glands were squashed with gentle tapping. Then using increased pressure and force the glands were squashed further. The cover slip was rotated slightly with the pencil eraser to enhance spreading. The spreading of the chromosomes was monitored with a phase contrast microscope. If spreading was poor attempts were made to further squash and enhance chromosome spreads. Then holding the cover slip in place on one edge using a gloved finger, I used a round pencil to squeeze excess liquid out from under the cover slip on the side opposite my finger. This step helps with spreading and with elimination of cellular debris that results in high background. Only well-spread squashes were selected for subsequent staining. The slide was then frozen in liquid nitrogen and the cover slip was immediately “flicked off” with the edge of a razor. The slide was quickly placed in cold PBS. After 30 min in PBS the slide was transferred to 95% ethanol and stored at 4C for up to 1 week. Prior to immunostaining the slides were rehydrated in PBST for 30 min. The slides were moved to a fresh jar of PBST and incubated again for 30 min. The slides were then dried by placing an edge on a paper towel. 50 ul of blocking buffer (PBST + 5% NGS, 0.2% BSA) was applied to the slide and a small square of parafilm applied on top. The slide was then placed in a humid chamber and allowed to block for 1-2 hours. Primary antibodies were diluted 1:300 in blocking buffer. The blocked slide was dried with a paper towel and 30ul of primary antibody solution was applied with a glass cover slip placed on top and incubated overnight at 4C. For antibody incubation a cover slip with a sliver of scotch tape applied to one side was used as a spacer and to allow
easy cover slip removal. The cover slip was removed and the slide gently washed in PBST with very gentle shaking 3 times for 10 min each. Secondary antibodies and DAPI were diluted 1:750 in blocking buffer and applied identically to the primary antibody and incubated for 4 hours. The slide was again washed three times for 10 min in PBST. After drying with a paper towel fluoromount-G was applied along with a fresh cover slip. After an overnight incubation in the dark at room temperature the cover slips were sealed to the slides with nail polish.

To visualize intact salivary gland cells the extensive squash steps were skipped. A cover slip was gently placed on the fixed glands prior to freezing in liquid nitrogen. Subsequent staining steps were performed the same as described above.

**Acid-Free Polytene Chromosome Preparation**

In order to visualize GFP-tagged proteins in salivary gland squashes a modified protocol was used to preserve GFP fluorescence through the fixation process (Johansen et al., 2009). The glands were dissected and fixed in acid-free fixation buffer (0.15M PIPES, 3mM MgSO4, 1.5mM EGTA, 1.5% NP-40, pH 6.9) in 2-3 min. The glands were then transferred to PBST (PBS + 0.3% Triton X-100) for ~2 min and then to 50% glycerol where they incubate for 5 min. The glands are then placed on a siliconized cover slip in a ~20ul drop of glycerol. The glands were then squashed and stained according to the above protocol. Slides were stained fresh to avoid ethanol washes and storage.

**Drosophila stocks used**

The following fly stocks were used: UAS-Tum-MYC (Goldstein et al., 2005), UAS-TumΔGAP (Sotillos and Campuzano, 2000), UASp-PavGFP (Minestrini et al., 2002), UAST-PavΔnls-GFP(3X*NLS, Minestrini et al., 2003), UAS-TumΔnls-MYC (Jones et al., 2010),
UAST-PavΔnls-GFP(1X*NLS, Minestrini et al., 2003), sgs3-GAL4 (Cherbas, 2003), H3.3a-FLAG (Sakai et al., 2009).

**DNA replication and chromatin assembly assays**

DNA replication and chromatin assembly were monitored by Brdu incorporation. UAS-TumΔGAP and UAS-TumΔnls-MYC were driven by sgs3-GAL4. Dissected salivary glands were incubated for 1 hour in 200ug/ul in Ringers solution. The glands were then fixed for 30 minutes in Ringers solution plus 4% paraformaldehyde. Glands were then treated for 30min with 2N HCl. Chromosomes were stained with anti-Brdu antibody and polyclonal Tum antibody.

Ringers Solution:  
180mM KCl  
50mM NaCl  
3mM CaCl2  
10mM Tris ph 7.2

**Live cell microscopy**

Salivary glands were dissected and incubated in PBS supplemented with DAPI for 10 minutes. Glands were then placed in a drop of PBS + DAPI between a slide and cover slip and imaged by confocal microscopy.

**Immunohistochemistry**

Whole mount salivary glands were stained according to typical protocols for Drosophila tissues. Glands were fixed in PBST (PBS + 0.3% Triton X-100) + 5% formaldehyde for 20 minutes. Tissues were then blocked in PBST + 10% normal goat serum (Jackson ImmunoResearch) for 1 hour then incubated with primary antibody overnight. The following primary antibodies were used: anti-c-Myc (chicken, 1:500,

**Pav-GFP overexpression experiments**

Constructs were expressed using the *sgs3*-GAL4 driver. All experiments contain a single copy of *sgs3*-GAL4 unless otherwise specified. Glands were dissected and stained for GFP and LaminC using whole-mount protocols described above.

**III. Results**

**Centralspindlin localizes to nuclei in *Drosophila* salivary glands**

I examined the localization of centralspindlin components in isolated salivary gland cells. Using native GFP fluorescence I examined the localization of Pav-GFP and PavΔnls-GFP. Pav-GFP localizes primarily to the nucleus while PavΔnls-GFP localizes exclusively to the cytoplasm where it associates with the cell cortex and with microtubule networks (Fig 5-1 A,B). Using a polyclonal antibody to stain fixed salivary gland cells, I was able to detect endogenous Tumbleweed in the nuclei of fixed cells (Fig. 5-1 C). Tum is also present in the cytoplasm and appears to be associated with microtubules and the cell periphery. In flies expressing Pav-GFP I dissected intact salivary glands and co-stained for GFP and endogenous Tum. Using a whole-mount approach, I was able to detect both Tum and Pav primarily in the nuclei of these cells (Fig. 5-1 D). However, some cytoplasmic signal is
Figure 5-1. Localization of centralspindlin in salivary gland cells.

A. Pav-GFP is primarily nuclear in isolated salivary gland cells. B. PavΔnls-GFP is restricted to the cytoplasm and associates with microtubule networks at the cell cortex. C. Tum localizes primarily to the nucleus with some cytoplasmic staining. D. Pav and Tum localize to the nucleus and to cell-cell junctions in intact salivary glands.
apparent along with a significant population located at the cell periphery at cell-cell junctions (Fig. 5-1 D).

**Centralspindlin associates with euchromatin of polytene chromosomes**

Given the primarily nuclear signal of Tum and Pav I sought to examine their sub-nuclear localization and determine if they are associated with the polytene chromosomes. Traditional fixation and mounting protocols for preparing polytene salivary gland squash preps call for a formaldehyde/acetic acid fix followed by physical disruption of the cells by squashing the tissue between a glass slide and cover slip. These methods are extremely harsh and can denature antigens or result in high background staining among other problems. Initial attempts at localizing Pav-GFP proved difficult as GFP signal was disrupted during the fixation and staining process (Fig. 5-2 A). I used a modified protocol that removes acetic acid from the fixation buffer (Johansen et al., 2009). This protocol preserved the GFP signal throughout the staining process and revealed an association with the chromatin of polytene chromosomes (Fig. 5-2 B). The signal from these preparations was dim and the spreading of the polytene chromosomes was poor, preventing a detailed co-localization analysis (Fig. 5-2 B). Finally, through trial and error, I found a commercially available polyclonal antibody for GFP that was able to recognize the GFP antigen following the harsh fixation methods of polytene squash preparations (Abcam, ab13970). Using traditional protocols I found sgs3-GAL4 driven Pav-GFP associates broadly with polytene chromosomes (Fig. 5-2 C). Using a polyclonal antibody directed to full-length Tum I was able to detect Tum along polytene chromosomes however not nearly as widespread as Pav-GFP (Fig. 5-2 C, red). The intensity of Tum staining varies from band to band with some
Figure 5-2. Pav-GFP and Tum associate with polytene chromosomes.

A. Native GFP signal in Pav-GFP (green) expressing salivary glands. Traditional acid fixation degrades most of the GFP signal. B. Acid-free fixation methods preserve some native GFP signal in Pav-GFP expressing salivary glands. C. Detection of Pav-GFP by polyclonal GFP antibody shows broad association of Pav-GFP with polytene chromosomes. Pav-GFP localizes to euchromatin regions and shows some co-localization with Tum (red). D. Expression of Tum-MYC (green) in salivary glands leads to broad Tum association with polytene chromosomes and increase in staining by Tum antibody (red).
displaying bright signal while others are quite dim. The co-localization of Tum and Pav-GFP is highlighted in Fig. 5-3 A. However, only some Tum bands show co-localization with Pav-GFP under these conditions. This is surprising considering the proteins are usually found together in a complex and may indicate expressing UAS-Pav-GFP beyond endogenous protein levels disrupts the stoichiometric balance between Tum and Pav. Interestingly both proteins are excluded from heterochromatic regions marked by DAPI stained bands whether they are overexpressed or not. To examine the ability of the overexpressed centralspindlin components to associate with chromatin, either specifically or non-specifically, I expressed a Tum-MYC transgene and used a monoclonal MYC antibody to stain polytene chromosomes. Tum-MYC localizes broadly to euchromatin as detected by MYC antibody (Fig. 5-2 D, green). Expression of the TumMYC protein also enhances the staining of euchromatin by the polyclonal Tum antibody (Fig. 5-2 D, red vs 5-2 C, red). This results in spreading of Tum signal across all euchromatic bands and a general increase in the brightness or intensity of Tum staining at individual bands.

Centralspindlins co-localizes with active chromatin marks on polytene chromosomes

Tum and Pav association with polytene chromosomes was examined further through staining for histone methylation marks. H3K4 trimethylation marks open, actively transcribed gene regions. Pav-GFP, expressed beyond endogenous levels, showed broad association with H3K4 trimethyl staining (Fig. 5-3 B). Pav-GFP is also excluded from regions positive for H3K27 trimethylation (Fig. 5-3 C). H3K27 trimethyl is a marker for heterochromatic regions of polytene chromosomes. Tum and Pav also show co-localization with a subset of bands marked by a FLAG-tagged H3.3a construct (Fig. 5-3 D). H3.3a is a
Figure 5-3. Centralspindlin localizes to euchromatin regions but is restricted from heterochromatin.

A-C. Localization of centralspindlin and histone methylation marks. Arrows mark areas of clear co-localization. Inset shows a chromosome regions split for each of the two signals to highlight co-localization or regions that clearly do not co-localize. A. Co-localization of Tum and Pav-GFP at euchromatin regions. B. Co-localization of Pav-GFP with H3K4me3. H3K4me3 marks sites of active transcription. C. Pav-GFP does not co-localize with H3K27me3. H3K27me3 marks heterochromatin regions. D. Tum co-localizes with some H3.3a-FLAG bands. Arrows mark bands displaying a high degree co co-localization.
histone variant that is involved in transcriptional regulation typically found at actively transcribed genes. Together these results demonstrate Tum and Pav both co-localize on euchromatic regions of polytene chromosomes but are excluded from all heterochromatic regions.

I next attempted to deplete centralspindlin with dsRNA to look at the effect on histone methylation and transcription in salivary glands. I used the Sgs3-GAL4 driver, whose expression is specific to mature salivary glands, to drive dsRNA constructs targeting either tum or pav. Squashing and staining these depleted salivary glands revealed no change in histone methylation and no gross changes in chromosome or tissue morphology. However, I also did not see a difference in Tum staining between control samples and dsRNA treated samples. This indicates dsRNA treatment was unable to deplete Tum protein levels. This may be due to a number of factors. The Sgs3-GAL4 driver is only expressed at later stages of salivary gland development at relatively low levels. The Sgs3 driver may not be strong enough for efficient knockdown of centralspindlin. Alternatively, the pool of centralspindlin proteins may not undergo appreciable turn over in mature salivary glands because of their stable association with chromatin and the lack of cell division. From these results I conclude centralspindlin is resistant to dsRNA mediated knockdown probably due to its low turnover rate in salivary glands.

Expression of dominant-negative Tumbleweed does not effect DNA replication

The broad co-localization with euchromatic polytene regions can be a result of at least two different roles for centralspindlin in chromatin regulation. Centralspindlin could be involved in transcription given its localization at actively transcribed genes marked by H3K4 trimethylation. Alternatively, since polytene chromosomes actively undergo several
round of DNA endoreplication, the broad association of centralspindlin with polytene chromosomes could indicate a role in DNA replication and the chromatin assembly process. To examine this possibility I expressed mutant versions of Tum in the salivary glands. These mutant constructs, TumΔGAP and TumΔnls-MYC both result in strong phenotypes when expressed in other Drosophila tissues including the thorax. I then dissected salivary glands and incubated them in the presence of Bromodeoxyuridine (Brdu), a thymidine analog. The tissues were fixed and Brdu incorporation was examined in whole mount salivary glands. In wildtype salivary glands cells undergo asynchronous rounds of DNA replication resulting in 50-75% of nuclei incorporating Brdu in the 2 hour labeling period (Fig. 5-4 A). Expression of TumΔGAP or TumΔnls-MYC showed no significant effect on the incorporation of Brdu compared to wildtype controls (Fig. 5-4 B,C). Additionally, in wildtype squashed polytene chromosomes there was no co-localization between Tum positive bands and bands labeled with Brdu (Fig. 5-4 D). This indicates Tum is not involved in DNA replication or chromatin assembly.

**Overexpression of centralspindlin causes the formation of nuclear vesicles**

Interestingly overexpression of Pav-GFP resulted in the formation of small nuclear puncta. The size and number of these puncta depends on Pav-GFP expression levels as well as the levels of Tum expression. Expression of a single copy of UAS-Pav-GFP results in a range of 5-15 bright nuclear puncta resembling membrane-bound vesicles (Fig. 5-5 A). Doubling the copy number of the UAS-Pav-GFP construct results does not seem to alter vesicle number but appears to increase the total size and volume of each individual vesicle (Fig. 5-5 B). Expression of a high expression UAST-Pav-GFP construct causes an increase
Figure 5-4. DNA replication is unaffected by expression of Tum mutant constructs.

A-C. Sgs3-GAL4 was used to drive UAS-TumΔGAP or UAS-TumΔnls-MYC in salivary glands. Brdu incorporation was measured to assay DNA replication and chromatin assembly. A. w1118 salivary glands show ~75% of nuclei incorporate Brdu in the 1 hour labeling period. B,C. Expression of (B) UAS-TumΔnls-MYC or (C) UAS-TumΔGAP do not affect DNA replication as ~75% of nuclei incorporate Brdu. D. Endogenous Tum does not co-localize with areas of Brdu incorporation in polytene chromosomes.
Figure 5-5. Overexpression of Pav-GFP leads to the formation of nuclear vesicles.

A. Sgs3-GAL4 driven expression of Pav-GFP leads to formation of ~10 vesicles per cell. B. Doubling the copy number of Pav-GFP increases the average size of vesicle but does not effect vesicle numbers. C. The high expression Pav-GFP construct leads to an increase in vesicle number. Vesicle numbers range from 15-35. D. Co-expression of Pav-GFP and Tum-MYC causes a huge increase in vesicle numbers ranging from 30-150. E. Co-expression of Pav-GFP and UAS-TumΔGAP disrupts formation of Pav-GFP labeled vesicles. The GFP signal localize throughout the nucleus. F. Pav-GFP labeled vesicles do not co-localize with the nucleolar marker fibrillarin.
in total vesicle number varying from 15-35 vesicle per nucleus (Fig. 5-5 C). Co-expression of Pav-GFP and Tum-MYC results in a significant increase in the total number of vesicles per nucleus ranging from 30-150 (Fig. 5-5 D). These vesicles also appear much smaller than those formed by Pav-GFP expression alone. Co-expression of Pav-GFP with the mutant TumΔGAP results in the disruption of these vesicles with diffuse Pav-GFP signal throughout the nucleus (Fig. 5-5 E). These puncta/vesicles resulting from Pav-GFP expression appear to be stable and stationary, showing little to no movement over the course of a few hours. The induced vesicles show no co-localization with fibrillarin, a nucleolar marker, indicating no role in ribosomal assembly and processing (Fig. 5-5 F).

IV. Discussion

I used Drosophila salivary glands to study centralspindlin function in the absence of cell division. Salivary glands are post-mitotic allowing the isolation of centralspindlin’s role in cytokinesis from other potential functions thus limiting pleiotropic phenotypes seen in other systems such as the thorax (reported here) and the brain (Goldstein et al., 2005)

The localization of of centralspindlin components in salivary glands mirrors that seen in other Drosophila tissues such as the thorax, reported here, neuronal cells(Goldstein et al., 2005), and wing discs (Somers and Saint, 2003). Tum and Pav are primarily nuclear with some protein detected at cell-cell junctions. Deletion of Pav’s nuclear localization domains prevents the nuclear accumulation of Pav-GFP as expected resulting in exclusively
cytoplasmic localization and association with microtubule networks at the cell cortex. The presence of Tum and Pav at cell-cell junctions likely reflects the newly discovered role of centralspindlin in maintenance of cell-adhesion through its regulation of Rho and Rac signaling (Ratheesh et al., 2012).

Interestingly both Tum and Pav were associated with polytene chromosomes. Tum and Pav together showed broad co-localization with euchromatic regions but were restricted entirely from heterochromatic regions. These euchromatic regions were characterized by their lack of DAPI staining as well as the presence of the H3K4 trimethyl mark associated with open chromatin conformations and active transcriptions. Endogenous Tum was present in varying amounts with some intensely stained bands versus a number faintly stained bands. Overexpression of Tum resulted in spreading of the Tum signal and uniform association with nearly all euchromatic regions. Overexpression of Tum could result in the non-specific accumulation of protein at these open or exposed regions of chromatin. Alternatively this result may suggest a regulatory function for centralspindlin levels. Interestingly, centralspindlin was found associated with chromatin regulatory complexes by mass spec characterization of proteins that interact with histone chaperones (Moshkin et al., 2009). These include Asf1, Hira, and CAF-1. Asf1 is the canonical H3/H4 histone chaperone while Hira is the chaperone for the histone variant H3.3. CAF-1 is known to be important for chromatin assembly. Interestingly Tum was found to co-localize with a subset of H3.3a positive bands. The interaction with Hira and co-localization with H3.3 may indicate a functional role in regulating the incorporation of histone variants. However, preliminary experiments indicate expression of the potent
dominant negative TumΔGAP does not affect global H3.3a patterns. This does not rule out potential functions at specific gene loci however.

A 2010 study showed localization of MgcRacGAP at centromeric regions during the assembly of new centromeres (Lagana et al., 2010). This localization occurred briefly during S-phase leading up to cell division (Lagana et al., 2010). This finding prompted me to examine centralspindlin’s function in DNA replication and chromatin assembly using Brdu incorporation assays. Expression of the mutant constructs TumΔGAP and TumΔnls-MYC had no effect on the levels or rates of Brdu incorporation. Additionally, endogenous Tum protein did not co-localize with newly replicated DNA regions marked by Brdu incorporation. These results indicate centralspindlin does not function in DNA replication or chromatin assembly, but may play another unknown role at the centromere.

Expression of Pav-GFP resulted in the formation of vesicles in the nuclei of salivary gland cells. These vesicles were observed in other cell types including the abdominal histoblasts and in germ cells of Drosophila oocytes (Minestrini et al., 2002). The size and number of these vesicles depended on the level of Pav-GFP expression. Higher levels lead to a greater number of vesicles and vesicles of larger size. Co-expression of Pav-GFP and Tum-MYC leads to a large number of relatively small vesicles. This reduction in vesicle size suggests a role for Tum in either limiting vesicle growth or increasing vesicle turnover rate. Interestingly co-expression of Pav-GFP with TumΔGAP results in the disappearance of all vesicles. Pav-GFP signal appears diffuse throughout the nucleus indicating Tum’s GAP activity is needed for vesicle formation. These vesicles are stable and immobile. The cargo if any of these vesicles is unknown. They do not co-localize with fibrillarin, a nucleolar marker, or with other known subnuclear domains. The morphology of these vesicles is
remarkably similar to lamin coated vesicles observed in previous studies (Schulze et al., 2009; Speese et al., 2012). In these studies the authors observe nuclear vesicles containing RNP granules that are encased in membrane and a LaminC coating. The granules are exocytosed through the nuclear membrane in novel form of nuclear export. I do not observe robust LaminC staining on Pav-GFP induced vesicles nor do I observe markers of RNP cargo within the vesicles. This does not rule out the possibility that Pav-GFP induced vesicles are aberrantly formed intermediates in this newly discovered pathway. Another alternative explanation is that high levels of Pav-GFP overexpression and disruption of centralspindlin stoichiometry results in the formation of nuclear aggregates. These aggregates can be then reduced by restoring centralspindlin stoichiometry with Tum-MYC overexpression. Along this line of reasoning, TumΔGAP expression could prevent aggregate formation by binding Pav-GFP irreversibly and blocking its interaction with additional Pav-GFP molecules. These possibilities remain to be tested.

Centralspindlin has robust nuclear localization and chromatin association but no apparent nuclear functions from the experiments described above. What then is the nuclear role of centralspindlin? The localization of centralspindlin during the cell cycle is nearly identical to that of the chromosomal passenger complex (CPC). Just like the CPC, centralspindlin localizes to centromeres during S-phase leading up to mitosis. Then as the nucleus breaks down centralspindlin and CPC associate with spindle microtubules and both complexes are transported to the cleavage furrow where they are needed for contractile ring regulation. As cell enter telophase centralspindlin, like the CPC, then becomes a part of the midbody as the cells undergo mitotic exit. Given their identical localization throughout the cell cycle and their complementary role in cytokinesis, it is
highly likely that centralspindlin and CPC have similar functions in the nucleus leading up to mitosis. Interestingly depletion of CPC components by RNAi causes bristle and thorax defects (Mummery-Widmer et al., 2009). Centralspindlin, much like CPC, probably plays a role in regulating the structure of mitotic chromosomes and in regulating kinetochrome-microtubule attachments. A few studies have demonstrated the importance of small GTPase regulation during microtubule-kinetochore attachment (Narumiya et al., 2004). In one study Ect2 depletion lead to chromosome misalignment in Xenopus extracts (Tatsumoto et al., 2003). Another showed aneuploidy and chromosome segregation defects when Ect2 was mutated (Tatsumoto et al., 1999). Additionally, MgcRacGAP, the human homolog of Tum was found as a part of the pre-kinetochore complex in Hela cells (Obuse et al., 2004). Tum’s GAP activity and Pav’s +end microtubule motor activity make centralspindlin an attractive candidate for study in this process.

However, the nuclear function of centralspindlin in salivary gland cells is unclear. Salivary gland cells do not undergo mitosis and do not form kinetochrome-microtubule attachments. What then is the function of centralspindlin’s euchromatic localization? CPC is normally recruited to heterochromatic regions by interaction with HP1 (heterochromatin protein 1) (Carmena et al., 2012). Recently HP1 was found to associate broadly with many euchromatic sites on Drosophila polytene chromosomes including heat shock and developmentally induced puffs (Piacentini et al., 2009). This localization is in addition to its heterochromatic localization. HP1’s euchromatic association is able to effect transcription of these target genes (Piacentini et al., 2009). Centralspindlin could play a similar role in salivary gland cells. When not needed at the centromere centralspindlin could have additional unknown roles in transcription regulation. Alternatively, detection
of Tum and Pav on polytene chromosomes could be an artifact of overexpressed protein constructs or even antibody non-specificity in a notoriously noisy fixation, mounting, and staining procedure.
Chapter 6: Conclusions and Future Directions

Conclusions

This research project was concerned with the multiple functions of the centralspindlin protein complex and the roles they serve in Drosophila development. Centralspindlin has proven to be a multi-functional complex with roles extending well beyond its initially discovered role in cell division. Recent studies along with the research presented here have clarified our understanding of centralspindlin and revealed a much more general role for this complex that was previously thought to be active only during cell division. My research in conjunction with other studies has shown centralspindlin is a general regulator of cell and tissue structure—including cell size, cell morphology, and cell adhesion. Centralspindlin regulates these characteristics during cell division but importantly centralspindlin also regulates these characteristics during any dynamic changes to cell morphology (Fig. 6-1). This general function makes this protein complex essential for a broad range of biological phenomena ranging from epithelial morphogenesis and cell migration to the formation of highly specialized cell structures like axons or cilia (Fig. 6-1).

During Drosophila metamorphosis imaginal discs undergo complex morphological transformations to transition the organism from the larva to the adult fly. My investigation of centralspindlin's function during metamorphosis revealed a complex role for this protein complex. It is needed for development of the eye as well as in both the migration of imaginal wing discs to form the thorax and the subsequent differentiation and division of thoracic sensory organs. During
Figure. 6-1. The multiple functions of centralspindin in regulation of cell division and cell morphology.

A. Centralspindlin connects equatorial microtubules to the plasma membrane at the cleavage furrow as well as drive constriction of the contractile ring (green). Centralspindlin also releases adhesion from neighboring cells at the cleavage furrow through Rac inhibition to allow ingression. B. Centralspindlin (blue) is transported along microtubules during axon/filopodial extension. C. Centralspindlin regulates cell-adhesion and actin-myosin constriction during changes in tissue morphology.
Thorax development is the subcellular localization of the centralspindlin complex that is important for imaginal disc migration. Primarily nuclear in stationary interphase cells, centralspindlin exits the nucleus and associates with the cell cortex as cells begin to migrate. Depletion or over-expression of centralspindlin both cause similar thorax closure defects. Thorax defects appear to be independent of cytokinesis failure as centralspindlin overexpression does not disrupt cell division but still results in a thoracic cleft. Similar findings have also been observed in neuronal cells (Goldstein et al., 2005). Depletion or overexpression of centralspindlin results in either axon overextension or misrouting, respectively (Goldstein et al., 2005). This indicates an important regulatory role for centralspindlin where either too little or too much of the protein complex can cause deleterious effects. Centralspindlin regulates Rho and Rac at the cell cortex and allows the cycling of adhesive contacts to facilitate the crawling of the wing disc epithelium towards the dorsal midline over the underlying substrate (Martín-Blanco et al., 2000; Ratheesh et al., 2012; Warner and Longmore, 2009). Then as the disc approaches the midline leading edge cells extend filopodial structures to bridge the gap to the opposing disc (Martín-Blanco et al., 2000). These filopodia are actin rich and exert mechanical force to pull the two discs together (Martín-Blanco et al., 2000). The thoracic cleft caused by either Tum or Pav RNAi could be due to defects in cell-adhesion and the crawling of the disc, the disruption of filopodial extensions, or a combination of both.

Deletion of Pav’s NLS results in holes or tears in the thoracic epithelium and the formation of aberrant filopodia in areas where the discs fail to fuse. Additionally, co-expression of wildtype Tum and Pav above endogenous levels results in severe thoracic
clefts and stabilization of similar filopodial structures. The accumulation of cytoplasmic centralspindlin seems to stabilize or arrest these structures, suggesting a function in regulating dynamic filopodial extensions. Interestingly cytoplasmic accumulation of centralspindlin in neuronal cells also causes defects in axon morphology identical to Rac loss-of-function (Goldstein et al., 2005). This is consistent with Tum’s inhibitory GAP activity towards Rac. There is an alternative cell-adhesion based explanation for the observed holes in the thoracic epithelium and filopodia structures. They may be the result of cells disengaging and releasing adhesive contacts from one another.

This role in thorax development clearly demonstrates an interphase role for centralspindlin. Centralspindlin was once thought to be exclusively nuclear during interphase, however during the course of this thesis research it was discovered to have important interphase roles in the regulation of cell adhesion (Ratheesh et al., 2012). Previously it was unclear how a nuclear protein could influence cytoplasmic events. However, the experiments presented here and elsewhere clearly indicate centralspindlin exerts its regulatory activity in the cytoplasm (Murray et al., 2012; Ratheesh et al., 2012). Centralspindlin’s activation of Rho at the plasma membrane was found to be necessary for junctional integrity by maintaining cadherin based adhesion complexes (Ratheesh et al., 2012). My findings show the importance of the nuclear to cytoplasm transport of centralspindlin in interphase cells during thorax development, a process that relies heavily on cadherin based cell-adhesion (Martín-Blanco et al., 2000). Disruption of this nuclear-cytoplasmic balance causes the failure of thorax closure similar to that seen from RNAi depletion of Rac or Rho kinase, the downstream effector of Rho (Mummery-Widmer et al., 2009). Together these studies demonstrate the importance of centralspindlin in the
temporal and spatial control of small GTPases during epithelial morphogenesis in addition to cell division. Recent studies have demonstrated another new function of centralspindlin and its interacting proteins as a membrane anchor by binding directly to lipids in the plasma membrane (Lekomtsev et al., 2012; Murray et al., 2012). Centralspindlin and Ect2 bridge the plasma membrane with spindle microtubules to provide a direct physical link between the cytoskeleton and the cell membrane. This linkage allows the cytoskeleton to exert mechanical force on the membrane while also influencing actin-myosin contraction and cell-adhesion through small GTPase. These multiple functions are essential for the coordination of cell division. It is now clear that cytokinesis is just one specialized role for centralspindlin. Centralspindlin is at the center of a network of proteins present in many cell and tissue types that controls diverse aspects of cell and developmental biology.

The exact role of centralspindlin in sensory organ cell division and differentiation is still unclear. As expected depleting centralspindlin disrupts cell division in sensory organ cells. However, centralspindlin’s role in sensory organ differentiation is uncertain. While some sensory organs depleted for centralspindlin display cell fate transformation phenotypes I showed that these cell fate defects, although reminiscent of Notch pathway mutants, are not due to the disruption of Notch signaling. During the preparation of this thesis, the mutation of the cytokinesis proteins Diaphanous and Septin were shown to cause identical cell fate transformations to the ones I observed by RNAi depletion of centralspindlin (Founounou et al., 2013). This indicates the observed cell fate defect may be a secondary consequence of cell division problems, potentially due to altering the kinetics of cell division and thus the kinetics of cell fate specification. In support of this idea I observe a breakdown in asymmetric localization of Pon-GFP in delayed or arrested
mitotic sensory organ cells. This demonstrates how a cytokinesis defect could indirectly lead to disruption of downstream events or even defects in parallel pathways.

The formation of nuclear vesicles upon expression of Pav-GFP may indicate another novel function of centralspindlin. A few recent studies have shown the existence of lamin coated vesicles within the nucleus. They are present in wildtype cells and contain RNP granules while others are induced by mutation of nuclear lamin proteins (Schulze et al., 2009; Speese et al., 2012). Centralspindlin may be involved in the formation of the RNP containing vesicles, however I am unable to detect RNP components in Pav-GFP induced vesicles in preliminary experiments. Alternatively the plasma membrane binding activity of centralspindlin and its ability to modify membrane structure may induce aberrant nuclear vesicles by binding to lipids in the nuclear membrane.

**Future Directions**

The discovery of centralspindlin’s role as a key regulator of cell morphology during interphase was unexpected but will be important for understanding cytoskeletal regulation during cell migration and cell division. Centralspindlin’s ability to coordinate small GTPase regulation and thus simultaneously regulate cell adhesion and the cytoskeleton make it an extremely important and versatile regulator of cell behavior. Study of the centralspindlin complex in live cells during tissue migration will be invaluable in understanding how it controls these dynamic processes. Additionally, the use of fluorescent sensors of Rho and Rac activity in live cells will clarify the ability of centralspindlin to temporally and spatially regulate small GTPases (Goffinet et al., 2008; Pertz, 2010). It is currently unknown how Rac and Rho activity are coordinated during dynamic processes. In fact the interpretation
of many studies has been confused by the multiple functions of centralspindlin. Centralspindlin activates Rho through pebble recruitment but inhibits Rac through the GAP activity of Tum (Bustos et al., 2008; Canman et al., 2008; Ratheesh et al., 2012). For years it was hypothesized that Tum is a RhoGAP (Janstch-Plunger et al., 2000; Minoshima et al., 2003). This was thoroughly debunked when it was discovered that Tum only has GAP activity towards Rac (Bastos et al., 2012). With our current knowledge we can know revisit these studies and better understand the contribution of not only centralspindlin but also Rho and Rac coordination.

The function of centralspindlin in the nucleus is still mysterious but a few hints have emerged recently. The centralspindlin complex, much like the chromosomal passenger complex, has a highly dynamic localization (Carmena et al., 2012a). Both complexes localize to the nucleus during interphase and then to centromeres during S-phase (Carmena et al., 2012a; Lagana et al., 2010). As the cell cycle progresses both centralspindlin and the CPC translocate to the cleavage furrow (Carmena et al., 2012a; Minestrini et al., 2003; Somers and Saint, 2003). Additionally, both protein complexes play a role in the incorporation and maintenance of CENP-A at the centromere (Carmena et al., 2012a; Lagana et al., 2010). CENP-A is a histone variant involved in kinetochore assembly. Some evidence has pointed to the role of small GTPases in regulating the attachment of kinetochores to the + -ends of microtubules (Narumiya et al., 2004) k. Pav-GFP is a + -end microtubule motor so it will be interesting to see if centralspindlin is associated with any kinetochore components in addition to its association with the centromere. Also, live-cell monitoring of small GTPase activity during mitosis may reveal a role for centralspindlin regulation of Rho family proteins at the kinetochore. The identical localization of
centralspindlin and the CPC throughout the cell cycle may indicate involvement in the same molecular pathways. Given the highly similar phenotypes produced by interruption of these complexes in flies and in cell culture it would not be surprising if they are involved in the many of the same processes (Carmena et al., 2012b; Mummery-Widmer et al., 2009). Future studies investigating how centralspindlin and the CPC together can sequentially coordinate centromere assembly, kinetochore attachment, and cleavage furrow ingression will enlighten our understanding of how cell division is regulated from start to finish.
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