THE ROLE OF PROTEIN O-MANNOSYLTRANSFERASE IN THE DEVELOPMENT OF *DROSOPHILA* TORSION PHENOTYPES

A Dissertation

by

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ABSTRACT

Congenital muscular dystrophies (CMD's) are serious diseases affecting muscle, brain, eye, and other tissues and often result in premature death of patients. These forms of muscular dystrophy are largely underlain by defects in the glycosylation of dystroglycan, and specifically by defects in the O-mannosylation pathway. The fruit fly *Drosophila melanogaster* is a good model system for studying many genetic diseases, including CMD's, as they utilize many of the same molecular processes as mammals. They have homologues of mammalian Protein O-MannosylTransferase (POMT) 1 and 2 which have been shown to O-mannosylate dystroglycan.

In this dissertation I studied the biological defects associated with POMT mutations primarily by using a live imaging approach in *Drosophila*. In *Drosophila* the most prominent defect associated with *POMT* mutations is a clockwise torsion of posterior abdominal segments relative to anterior segments. The mechanism by which this torsion arises was not previously known.

Here I characterized the gross physiological mechanism by which torsion arises. I showed that it is present at the embryonic stage, that embryos undergo chiral rolling within their shells during peristaltic contractions, and that abnormal contraction patterning in POMT mutants leads to differential rolling that gives rise to torsion of the dorsal midline.

I next demonstrated the cellular requirements for POMT in maintaining proper posture. I showed that POMT is required in the peripheral nervous system to mediate

proper feedback to the central nervous system for regulation of contraction patterning. Aberrations in the development of the peripheral nervous system can also cause torsion, even when POMT is functional. Additionally I showed that muscle tissue and possibly epidermis and central nervous system cells require POMT expression to maintain posture.

Finally, I investigated the molecular targets of O-mannosylation. I showed that O-mannosylation of dystroglycan is involved in the rise of torsion, but that it is not the only relevant target. The receptor protein tyrosine phosphatase RPTP69D is also O-mannosylated and plays a role in both neuronal and muscle tissues in regulating posture.

These results shed light on the biological mechanisms underlying muscular dystrophy, and may lead to new targets for diagnosis and therapy in human patients.

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Contributors

Faculty committee recognition

My dissertation committee consisted of my advisor and committee chair, Dr. Vladislav Panin, as well as Dr. Gary Kunkel and Dr. Lanying Zeng of the Biochemistry and Biophysics department and Dr. Paul Hardin of the Biology department.

Student/collaborator contributions

Dr. Nao Nakamura and Dr. Dmitri Lyalin preceded me in this project. They provided the initial data at the beginning of Chapter II and some of the data in Chapter III that provided the framework for my project. Dr. Zhaokai Meng of the Biomedical Engineering department collaborated with me in obtaining Brillouin spectra shown in Chapter III and Chapter IV. Ishita Chandel performed dissections and staining of larval brains, which I analyzed. Michelle Alfert assisted in dissecting and staining larvae for the G14 expression pattern in Chapter III. All data from others or involving collaboration are identified in the text. All other work was completed independently by me. Other help was provided by Dr. Andreas Holzenburg, who sat on my committee until summer of 2015, and by Dr. Agustin Guerrero-Hernandez of CINESTAV in Mexico City.

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CHAPTER I

INTRODUCTION

1.1 Overview of Protein Glycobiology

Organisms ranging from bacteria to mammals depend on a variety of post-translational modifications (PTM) of proteins in order to properly regulate their myriad biological processes. One of the most prominent forms of PTM, and until recently one of the most overlooked, is glycosylation (1). It consists of the linkage of monosaccharides or oligosaccharides (glycans) to specific amino acid side chains of secretory pathway proteins, as well as the subsequent modifications of those glycan chains. Because an individual monosaccharide can be linked to another at multiple positions, complex branching is possible, allowing for a wide variety of glycan structures to exist (2). Glycan diversity is believed to account for much of the difference between genetically similar organisms such as humans and chimpanzees, where a wider array of glycan linkages in humans may provide the molecular underpinnings for more complex social interactions (3).

Protein glycosylation takes place predominantly in the compartments of the secretory pathway: the endoplasmic reticulum (ER) and the Golgi apparatus. Glycans are assembled by enzymes called glycosyltransferases, and individual sugars can be removed by glycosidases. These processes follow the general rule “one enzyme, one linkage.” Thus a single enzyme will usually only make one specific transfer or break one glycosidic bond, and a specific linkage in a glycan chain may be accounted for by a

single enzyme. Glycosylation of proteins can be broken down into two major subsets: N-linked glycosylation, and O-linked glycosylation, which bear important similarities and differences to each other.

N-linked glycosylation involves the linkage of glycan chains to the amide group of asparagine residues. Glycans of this type always start as an invariant core glycan common to all N-linked glycosylation sites. Prior to any carbohydrate transfer to asparagine residues, the core glycan is assembled one monosaccharide at a time on a dolichol diphosphate molecule on the membrane of the ER (4). Once fully assembled, this core glycan is transferred as a single unit (*en bloc*) to target proteins by a single ER enzyme, oligosaccharyltransferase (5,6). Thus mutations in any of the enzymes involved in production of the core glycan can entirely inhibit N-linked glycosylation. Because the initial transfer is always catalyzed by the same enzyme, nearly all N-linked glycans can be found within a specific consensus sequence: Asn-X-Ser, Asn-X-Thr, and in rare cases, Asn-X-Cys, where X can be any amino acid other than proline (7). After *en bloc* transfer of the core glycan, other enzymes within the ER and the Golgi modify specific glycans, providing a substantial variety of N-glycan end products (6) (Fig. 1A).

O-linked glycosylation refers to the transfer of carbohydrates to the hydroxyl group of serine and threonine residues, and follows a different pathway. Rather than *en bloc* transfer of a preassembled glycan to a nascent protein, O-linked glycans are built directly onto the target protein, one monosaccharide at a time. This system allows for a greater diversity of initial linkages to proteins, with modifications including O-linked N-acetylgalactosamine (GalNAc), mannose, glucose, fucose, and others (8) (Fig. 1B).

Multiple enzymes are involved in these initial linkages, so unlike N-linked glycans, there is no clear consensus sequence for O-glycosylation. Thus unlike N-linked glycosylation, mutations in downstream enzymes generally do not preclude assembly of partial glycans on proteins. Rather, assembly only stops downstream of the affected enzyme.

Despite the differences in N-linked and O-linked glycosylation mechanisms, these two PTM's often function in similar ways. For example, folding and maturation within the secretory pathway are generally mediated by glycan modifications that can only occur once the protein of interest has adopted a particular conformational state, allowing it to be recognized by glycosyltransferases or glycosidases. Upon modification, the protein is then able to continue to the next step in its pathway. While this pathway has classically been associated with N-linked glycans (9), recent reports have shown similar roles for O-linked glycans (10).

Both O-linked and N-linked glycans provide structural support at cellular, tissue, and organismal levels. Proteoglycans, for example, are large proteins with several unbranched O-linked glycans in the extracellular space in animals. They bind important biological molecules such as water, and by so doing can help cells to withstand compression (11). As another example the glycocalyx, which consists of all of the glycans observed on the surfaces of eukaryotic cells, both N- and O-linked, serves as a physical barrier against proteases, antibodies, and some pathogens (12), helping to prevent auto-immune responses.

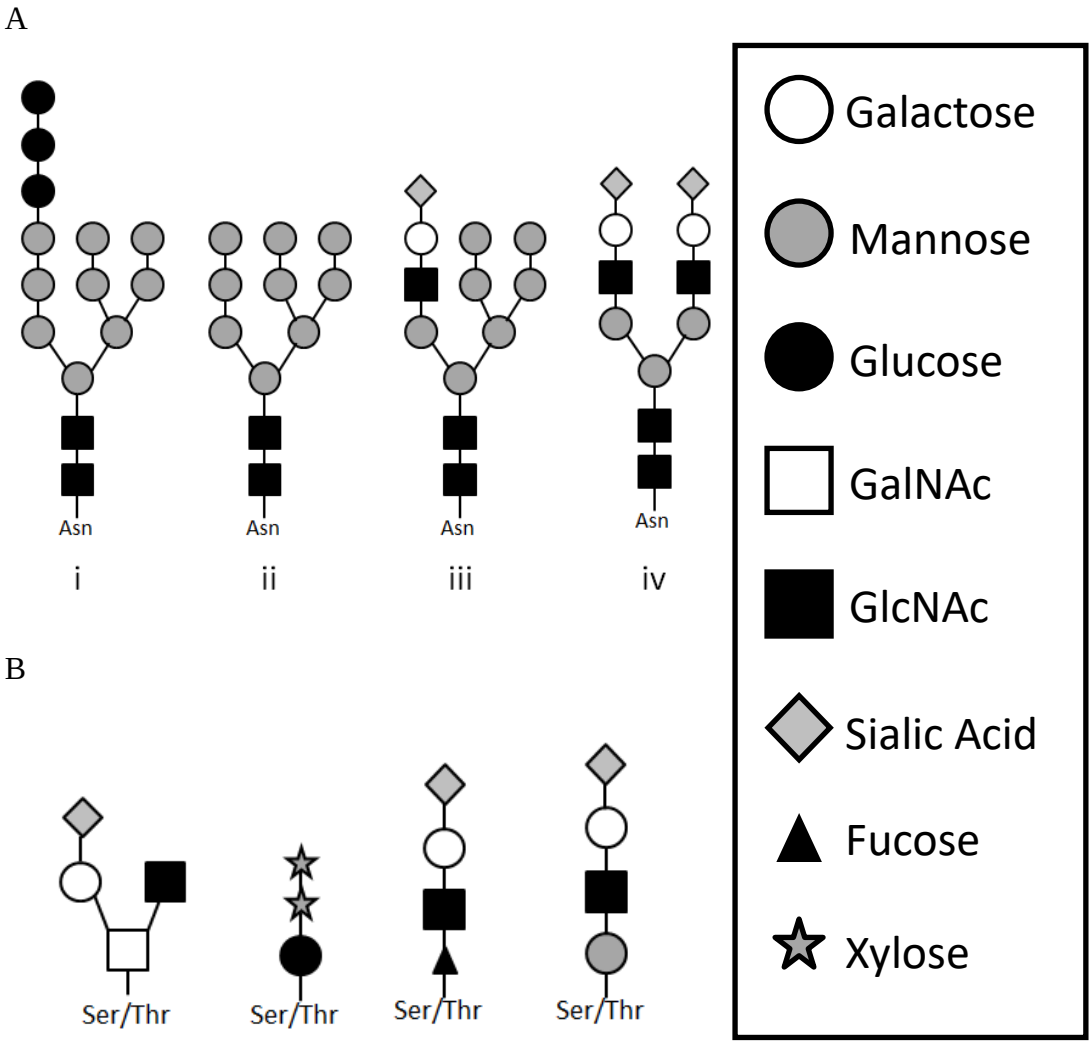


Figure 1—Glycan diversity.

A) N-linked glycans. (i) is the core glycan that initially gets transferred. (ii)-(iv) show examples of modified glycans, from which diversity is derived: “high mannose,” “hybrid,” and “complex,” respectively. B) Examples of O-linked glycans. Many more combinations exist.

Glycans can also modulate protein activity and interactions. As with many PTM's, glycosylation of proteins may serve as a molecular "on/off" switch for some protein activities, but more frequently they work as molecular "attenuators," increasing or decreasing interactions. This "tuning effect" can occur through multiple mechanisms. Some growth factors, for example, have a range of Kd values for their ligands governed directly by the size and composition of their glycans (13). Whereas that mechanism is direct, other glycans may interfere with protein interactions by physically separating proteins from their targets, sometimes even attenuating activities of proteins to which the glycans themselves are not attached, as in the case of neural cell adhesion molecules (14).

In addition to glycans themselves, an important aspect of glycobiology is the lectin family of proteins. Lectins are carbohydrate-binding proteins and protein domains. They participate in the folding and maturation of glycoproteins by recognizing distinct glycoforms, often in association with subsequent enzymatic modifications of the recognized glycan. Secretory pathway proteins in the wrong glycosylation state are not recognized, and are thus either prevented from continuing through the pathway until they are properly glycosylated, or are targeted for destruction (15). Lectins also mediate cell-cell interactions as well as interactions between cells and the extracellular matrix (ECM). Such interactions are important for maintaining membrane integrity, facilitating communication between cells, and recognition of many pathogens. Thus glycans and lectins play many important biological roles, and aberrations in an organism's glycobiology may lead to severe disease states.

1.2 Glycobiology in Human Pathology

Glycobiology has been connected to several pathological states. Many kinds of cancer cells, for example, exhibit distinct glycomes from their healthy counterparts (16-18). Currently there is no evidence that a change in glycosyltransferase or glycosidase activity underlies oncogenesis. Some evidence suggests, however, that altered glycosylation may play a role in cancer progression and metastasis (18,19). Whatever its biological role, the presence of a unique glycome in cancer cells may serve as a diagnostic marker and possibly even a target for therapeutics.

Pathogenic microorganisms frequently employ some aspect of glycobiology in infecting hosts. Viruses often use lectins to bind to host cell receptors and initiate endocytosis, the best-known example being influenza virus binding to sialic acid on cell surfaces (20). These same lectins may also bind the surface of a lysed cell when newly formed viruses exit, and thus viruses such as influenza use neuraminidase to cleave glycans and facilitate efficient release. The drug oseltamivir (Tamiflu®) is a neuraminidase inhibitor that helps shorten the duration of influenza infections by impeding viral release from compromised cells (21). HIV enters cells using glycoproteins to mediate fusion of its envelope with the cell membrane (22), and lectins such as griffithsin have been the subject of study as antivirals against enveloped viruses including HIV, coronavirus (SARS), and Hepatitis C (23-25).

Bacterial pathogens also exploit principles of glycobiology to cause disease. Like viruses, some bacteria bind to host cells via lectins (26). Such interactions may be pathogenic or beneficial to the host organism. Host organisms may fend off bacterial

infections or keep beneficial bacteria properly localized using lectins to recognize bacterial glycans (27). Additionally, many bacterial toxins, such as those associated with cholera, whooping cough, and dysentery, as well as some plant-derived toxins such as ricin, are lectins (28,29).

Several other pathological states are also underlain by aberrant glycobiology, including pathological immune responses (as seen, for example, in rejection of xenografts) (30) and spontaneous glycation of biological molecules (often seen in diabetics when blood sugar levels are elevated) (31). Here, however, I will focus on genetic disorders of glycosylation. These disorders occur when glycosyltransferases or glycosidases become mutated, leading to improper, incomplete, or absent glycosylation of target proteins.

Some of the first genetic disorders of glycosylation to be understood are known as congenital disorders of glycosylation (CDG's). The first CDG's described were in twin sisters in 1980, and included symptoms such as reduced psychomotor skills, ataxia, impaired blood clotting, cardiomyopathy, and morphological defects (32). By 1995 the underlying cause of these cases was determined to be a deficiency in phosphomannomutase (PMM2) (33). This enzyme is responsible for interconversion of mannose-6-phosphate and mannose-1-phosphate, and impaired activity ultimately leads to shortages of GDP-mannose, a necessary precursor in synthesis of the N-linked core glycan. Similar pathology has been described when the upstream enzyme phosphomannose isomerase (PMI), which catalyzes conversion of fructose-6-phosphate to mannose-6-phosphate, is inactivated. This disease can be largely relieved by mannose

supplementation in the diet because hexokinases can convert mannose to mannose-6P. Currently this is the only CDG for which a truly effective treatment is known (34). The CDG's related to enzymes involved in core glycan assembly and transfer are collectively known as the type I CDG's, and all manifest with similar symptoms. Aberrations in the N-glycanase enzyme, responsible for removing N-linked glycans from misfolded proteins and thus helping lead to their degradation, also result in similar phenotypes. This was the first characterization of deglycosylation in CDG's (35). Mutations in enzymes involved in modification of N-linked glycans underlie type II CDG's. In all there are 42 currently identified CDG's associated with N-linked glycans (36).

Disorders arising from aberrations in O-linked glycosylation have also been described. Defects in the O-Xylosylation pathway, for example, can lead to incorrect synthesis of heparan sulfate and other proteoglycan sugar chains. This in turn can cause diseases such as multiple exostoses and Ehlers-Danlos Syndrome, which are characterized by bone spurs and defects in connective tissue, respectively. The mechanism by which failed O-xylosylation can lead to abnormal bone growth is not currently known, though it arises when there are defects in heparan sulfate proteoglycan synthesis (37). Ehlers-Danlos Syndrome is somewhat better understood. It is generally associated with collagen and the proteins that interact with it. The enzyme B4GalT7 adds galactose to O-linked xylose in dermatan sulfate proteoglycans, which help link collagen fibrils (38). Loss of this linkage can lead to the syndrome. A related, unusual

type of O-glycosylation involves modification of collagen lysines. In Gly-X-Lys triplets, the lysines are often modified by lysyl hydroxylase, which puts a hydroxyl group on the delta carbon of the lysine side chain. This hydroxyl group can be modified with the disaccharide Glucose-Galactose, which helps in crosslinking of collagen fibers (39). The O-fucosylation pathway is related to such diseases as Peter's Plus Syndrome, associated with eye abnormalities as well as dwarfism and developmental delays, and to spondylocostal dysostosis, characterized by morphological defects of the abdomen. Peter's Plus Syndrome arises when transfer of glucose to O-linked fucose is disrupted, while spondylocostal dysostosis stems from inability to modify O-linked fucose with GlcNAc (40,41). The biological mechanisms by which these misglycosylations translate to disease are unknown.

1.3 Glycobiology of Muscular Dystrophy

A better-understood disorder of O-linked glycosylation is the set of muscular dystrophies known as dystroglycanopathies. Muscular dystrophy is a set of diseases associated with progressive deterioration and weakness of the muscles (42). Several forms of muscular dystrophy exist, with varying underlying causes and degrees of severity. By far the most common form of muscular dystrophy in humans is Duchenne Muscular Dystrophy (DMD), an X-linked disorder not directly associated with glycobiology that affects approximately 1 in 3500 males (43). Although DMD is not itself related to protein glycosylation, an understanding of this disease sheds light on the less common and more severe dystroglycanopathies.

DMD is caused by mutations in the gene encoding the protein dystrophin (43). The discovery of dystrophin and its link to DMD subsequently led to the discovery of several other proteins that form what is now known as the Dystrophin Glycoprotein Complex (DGC) (44). This complex consists of cytosolic components such as dystrophin and the syntrophins, extracellular components such as laminin, and transmembrane components such as dystroglycan (Dg), a glycoprotein responsible for linking dystrophin to the ECM (Fig. 2). Thus the DGC is believed to facilitate communication between the inside of the cell and its surrounding environment.

The DGC is required for muscle fiber integrity, and abnormalities of several DGC components are associated with various forms of muscular dystrophy. Some of these forms include Limb-Girdle Muscular Dystrophy (LGMD), and congenital muscular dystrophies such as Fukuyama Congenital Muscular Dystrophy (FCMD), Muscle-Eye-Brain disease (MEB), and Walker-Warburg Syndrome (WWS) (45). The Congenital Muscular Dystrophies (CMD's) manifest at or shortly after birth, and are particularly severe. WWS, the most severe form of CMD that arises from mutations in the Protein O-Mannosyltransferase (POMT) genes, is associated with defects in the brain, eyes, and muscle and typically causes death by the age of 3 (46).

Interestingly, unlike DMD, the CMD's often result not from mutations in the DGC itself, but from mutations in glycosyltransferases that act on Dg. In mammals, Dg is post-translationally modified by a series of enzymes including POMT's, Protein O-

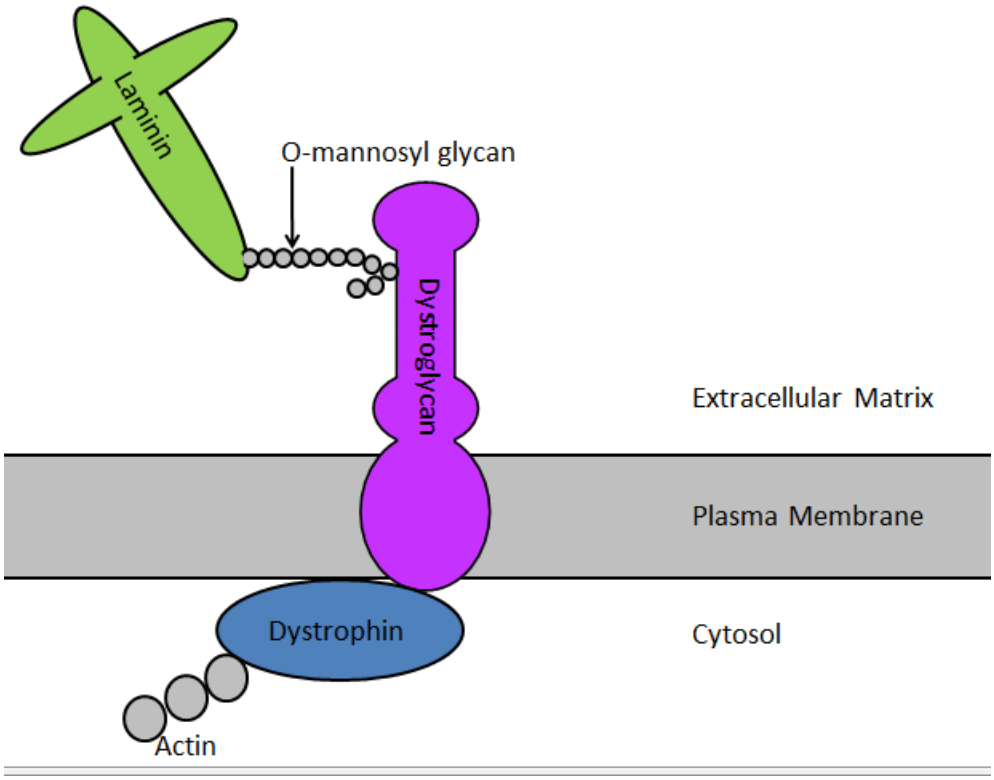


Figure 2—The Dystrophin Glycoprotein Complex (DGC).

This is a simplified depiction of the DGC. Dystrophin binds Dg and Actin on the cytosolic side, and Dg binds laminin through an O-mannosyl glycan in mammals, facilitating communication between cytosol and the ECM.

mannose Kinase (POMK), Fukutin, Fukutin Related Protein (FKRP), and like-acetylglucosaminyltransferase (LARGE) (47-50). These enzymes are all associated with at least one form of CMD, and collectively produce a glycan on Dg that is required for Dg binding to laminin. Thus laminin interacts with Dg via lectin activity. Several studies have implicated failed Dg binding to laminin as an underlying mechanism for CMD's (51-53).

In humans, the first step in the production of the laminin-binding glycan is O-mannosylation of one or more serine or threonine residues on Dg, including Thr 317 and 319 (50). O-mannosylation requires the joint activity of the Protein O-MannosylTransferases POMT1 and POMT2, glycosyltransferases colocalized in the ER. It is subsequently modified by several enzymes including POMGnT2, B3GalNT2, POMK, fukutin, FKRP, B4GAT1, and LARGE (47-50) (Fig. 3). Since the terminal [—3-xylose—a1,3-glucuronic acid-b1—] repeating unit added by LARGE is required for laminin binding (50,54), mutations in any of these enzymes can abolish Dg-laminin interactions, resulting in a CMD.

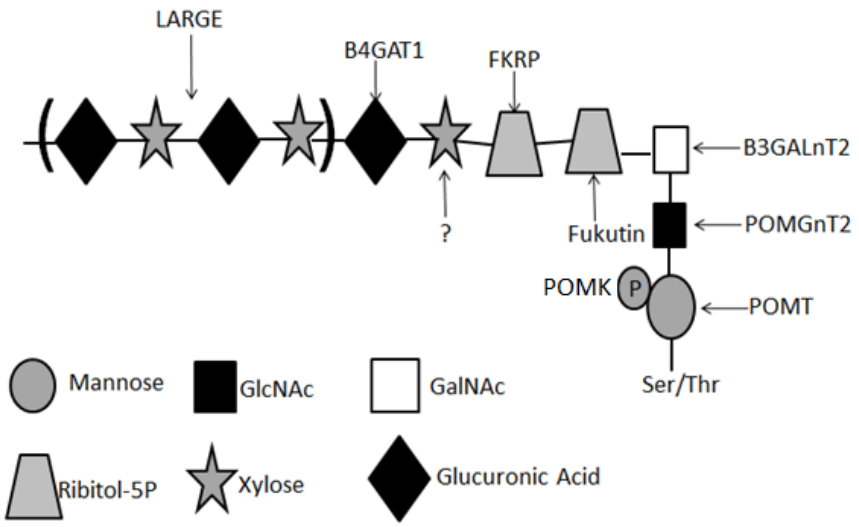


Figure 3—The laminin-binding O-mannosyl glycan and its associated enzymes.

1.4 *Drosophila melanogaster*: A Model for Dystroglycanopathies

The fruit fly *Drosophila melanogaster* has long served as a model for many genetic disorders. Many of the developmental pathways found in higher organisms are also present in *Drosophila*, though the *Drosophila* system is frequently less complex. The sialylation pathway in humans, for example, consists of some 20 different sialyltransferases (55), compared to one in *Drosophila* (56). Thus although the *Drosophila* system does not always perfectly echo the pathways of higher organisms, its relative similarity combined with its simplicity makes it an attractive system for initial studies.

Additionally, *Drosophila* have several practical advantages as a model system. Since *Drosophila* have been studied for over a century, many genetic tools have been developed that allow easy genetic manipulation. Genes can be selectively removed from or ectopically expressed in the organism as a whole, or genetic manipulation can be easily limited to a specific set of cells or tissues, or to a specific developmental period. Their ~10 day life cycle allows data to be gathered quickly. Together these advantages make *Drosophila* a powerful model for studying a variety of developmental pathways and pathological mechanisms.

Among other things, *Drosophila* provides a useful system for understanding the biological consequences of POMT mutation. Indeed, the *Drosophila* model expresses orthologues of both POMT1 and POMT2, and as in mammals both are required for O-mannosylation of Dg (57). The *Drosophila* genes *rotated abdomen (rt)* and *twisted (tw)* correspond to POMT1 and POMT2, respectively, and derive their names from the

characteristic misalignment or rotation of the abdominal segments in adult flies that results in a clockwise rotation of posterior abdominal segments relative to the anterior (Fig. 4). *Drosophila* dystroglycan interacts with dystrophin as well as extracellular matrix proteins such as perlecan and possibly laminin (58-60), though the latter is unverified. This suggests the presence of a complex similar to the mammalian DGC, though some of the enzymes required for extension of the O-mannosyl glycan that binds laminin, such as LARGE, are not known to be present in *Drosophila*.

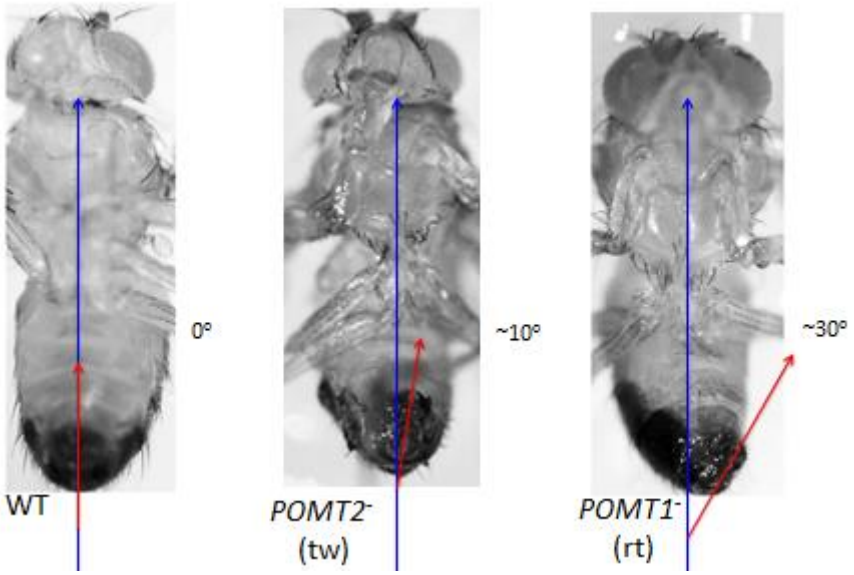


Figure 4—Abdominal rotation in *POMT* mutant *Drosophila.*

In POMT mutant flies, the abdominal segments rotate clockwise relative to the thoracic axis of symmetry and can be scored quantitatively. Blue arrows: thoracic axis, red arrows: abdominal axis.

Further establishing *Drosophila* as a model for dystroglycanopathies, mutants for Dg or dystrophin display progressive muscle degeneration and mobility problems, similar to symptoms seen in human MD patients (58,60,61). Neurological abnormalities have also been reported in connection with both human and *Drosophila* POMT mutations, including decreased synaptic transmission and changes in post-synaptic glutamate receptor composition (62), and aberrant axonal pathfinding (46,63-65). Here I will characterize and analyze the rise of rotation in *Drosophila* in both the embryonic and adult stages, and I will putatively connect the biological mechanisms underlying rotation in *Drosophila* to phenotypes in human WWS patients to shed light on the pathology of *POMT* mutations.

1.5 Dissertation Overview

In this dissertation I will focus on the mechanisms giving rise to torsion in *Drosophila*. I will examine the mechanical mechanism by which rotation may arise, I will elucidate the cellular requirement for POMT in maintaining proper posture, and I will begin to characterize a new POMT molecular target and its role in the rise of rotation.

In chapter II I will focus on the gross physiological mechanism that gives rise to rotation in POMT mutants. I will primarily focus on the embryonic stage, where my predecessor Dr. Nao Nakamura discovered a torsion of the embryonic dorsal midline that, like the adult abdominal phenotype, is always in the clockwise direction as viewed from the posterior. I will characterize the embryonic contraction patterns that occur just

before hatching, and that coincide with the rise of rotation in POMT mutant embryos. Finally, I will connect these contraction patterns to torque forces applied differentially to posterior and anterior segments of the embryo, and I will preliminarily connect aberrant contraction patterning to the rise of rotation in adult abdomens.

In chapter III, having revealed that rotation arises due to abnormal contraction patterning, I will examine the cellular requirement of POMT for maintaining correct contraction patterning and, by extension, posture. I will show that POMT is required in muscles, neurons, and possibly some epidermal cells, and I will primarily focus on the neuronal component of POMT function with some focus on muscle tissue. Specifically, I will demonstrate the importance of POMT in sensory neurons, and I will show that muscles in POMT mutants stiffen relative to their wild-type counterparts in correlation with the severity of rotation. Here I will also show that ectopic expression of POMT in a pattern involving muscles, neurons, and epidermis is sufficient to fully rescue rotation in both embryos and adults, further connecting the two developmental stages.

In chapter IV, I will examine genetic interactions between POMT and Dg in the rotation phenotype, and I will show that while mutations aggravate POMT-related phenotypes, they are not sufficient to cause rotation, suggesting that other POMT targets are involved. I will examine the role of one recently discovered mammalian POMT target, receptor protein tyrosine phosphatase. I will show that it is modified in the presence of POMT in vivo, and that like Dg, it interacts genetically with POMT to aggravate rotation phenotypes.

Although human WWS patients do not experience abdominal torsion, these studies will help shed light on the symptoms they do experience. Clinical features of WWS and MEB caused by defects in the POM pathway genes commonly include abnormal muscle contractions (66,67), and significant evolutionary conservation of the POM pathway in animals suggests an intriguing possibility that its role in sensory neurons to control contractions may be conserved in vertebrates. Further studies in *Drosophila* and other organisms should shed light on evolutionary conservation of mechanisms underlying the role of O-mannosyl glycans in regulating coordinated muscle contractions and axonal connectivity, which will potentially lead to new diagnostic and therapeutic applications for treatment of diseases associated with POM abnormalities.

CHAPTER II

THE MECHANICAL PROCESS UNDERLYING THE RISE OF TORSION PHENOTYPES IN POMT MUTANT *DROSOPHILA*

2.1 Introduction

The abdominal rotation phenotype of adult Drosophila has been known since the early 1900's (68), though only recently have *rt* and *tw* genes been positively identified as encoding POMT1 and POMT2 homologues, respectively (69,70). How rotation arises and why it is always in the same direction remains largely unknown. In this chapter I will examine the gross physiological underpinnings of the rise of rotation.

Previous experiments have demonstrated that abdominal rotation in *rt* and *tw* mutants can be fully rescued by a pulse of ubiquitous overexpression of POMT1 or POMT2, respectively, during late larval to early prepupal stages (71). Since the early prepupal stage represents the latest time at which rescue can occur, these data suggest that the critical event or events related to torsion of abdominal segments occurs during this developmental stage. However, the opaque nature of the pupal shell makes examination of the biological processes underlying rotation difficult to carry out.

Drosophila embryos undergo many of the same developmental processes as pupae, including muscle formation, neurogenesis (or in pupae, remodeling), and coordinated muscle contractions (72-74). Embryos, as opposed to pupae, are nearly transparent and therefore much more amenable to detailed analysis of developmental mechanisms. Here I analyze the function of *POMT* genes in *Drosophila* embryogenesis.

In collaboration with Dr. Nao Nakamura I describe a previously unknown embryo torsion phenotype that is reminiscent of the abdomen rotation in adults.

The torsion phenotype arises during a series of peristaltic muscle contractions in the late embryo. My analyses of muscle contraction waves revealed a directional rolling behavior of embryos inside the eggshell, which uncovered a novel chirality marker in *Drosophila* development. My results indicate that at least two contraction modes exist in *Drosophila* embryos (here designated as type 1 and type 2), and that each mode of contraction results in differential rolling of anterior and posterior segments. Type 1 contractions correlate with accumulation of counterclockwise torsion of posterior segments, while type 2 contractions have the opposite effect. Thus type 1 and type 2 contractions exist in a dynamic equilibrium to maintain straight body posture. Here I show that *POMTs* are involved in coordination of muscle contractions, and based on my results, I propose a model that explains the connection between the defect in muscle contractions and the torsion phenotype of *POMT* mutant embryos. Finally, I show preliminary data indicating that a similar mechanism may exist in pupae, leading to the abdominal rotation observed in adults. These preliminary data will be further corroborated in chapter III.

2.2 Materials and Methods

Drosophila stocks

The mutant alleles for *rt* and *tw* were previously described (69,75): $tw^{1}$is a hypomorphic allele; rt^{2}, rt^{p}, and rt^{571}, are strong hypomorphic alleles that are close to

amorphs. All experiments involving *rt* mutants used rt^{p} homozygotes unless otherwise specified. *MHC-GFP* line was a gift from Cynthia Hughes (76). Other mutant and transgenic strains were obtained from the Bloomington *Drosophila* Stock Center, Indiana University.

Fluorescent staining and microscopy

Embryos were dechorionated, fixed and dissected manually from the vitelline membrane according to published protocols (77). They were stained with Alexa-488-conjugated phalloidin (Molecular Probes) using 1:200 dilution. Digital images were obtained using Zeiss Axioplan 2 fluorescent microscope with the ApoTome module for optical sectioning. AxioVision and ImageJ software were used for 3D reconstruction and Z-projections of fluorescent samples.

Live imaging of embryonic tissue

A GFP-tagged myosin heavy chain (MHC) was expressed in wild-type, *POMT* mutant, and *senseless* mutant backgrounds. On any given day, embryos were collected within 18 hours of egg laying, and those that had air-filled trachea upon initial examination were discarded. Fifteen minutes later, stage 17 embryos with air-filled trachea and no obvious midline misalignment were collected and placed on a slide with the dorsal appendages up. Muscle contractions were recorded by a Hamamatsu ORCA-Flash4.0 CMOS digital camera for 1-2 hours using a X-Cite BDX LED with an emission max of 460 nm and an ET 525/50 emission filter on a Zeiss Examiner D1 microscope. Videos were analyzed using ImageJ software.

Rolling was assessed by measuring the average distance from the trachea to the body axis of symmetry before and after each contraction, and was measured only for contractions during which no other movement, such as head wagging, was present. Muscle midline angle was measured by selecting points halfway between left and right dorsal muscles or trachea, drawing a line connecting those points, and measuring its angle relative to the body axis of symmetry. The body axis of symmetry was estimated by drawing a line between the anterior and posterior tips of the embryo. An angle greater than 2^{o} was scored as "having torsion," as the difference between WT and mutant embryos became statistically significant at this point.

For GFP-intensity analysis, cross-sections 5 microns wide and 25 microns apart each were selected along the anterior-posterior body axis. A baseline for each cross-section was established as average intensity during the time between contraction waves. The most anterior and posterior cross-sections, as well as a cross-section halfway between anterior and posterior ends of the embryo, were used for assessment of muscle shortening. Integrated fluorescence intensity was measured for every frame of each cross-section and normalized to the baseline. Muscle shortening was estimated by increase in GFP intensity that occurs as muscle contractions bring more GFP into the focal area. Anterior intensity was divided by posterior intensity to produce a ratio.

Pupal and adult *Drosophila* imaging and analysis

Wild type and mutant pupae were mounted on slides with the left lateral side up. They were imaged using a microscope-mounted Zeiss Axiocam MRc5 color camera. Adult hearts were imaged in wild type and rt^{-} flies expressing tdTomato in the heart.

Male flies were incubated for 5 days after hatching at 30^{o} C, with food being changed after 3 days and no more than 5 flies to a vial. Prior to imaging, flies were transferred to vials without food and anesthetized with FlyNap® for 1 minute. They were mounted dorsal side up and hearts were imaged directly through the cuticle by fluorescence on a Zeiss Axiovision 2 microscope with a rhodamine reflector module. Videos were taken on an Axiocam HRm camera and analyzed in ImageJ. For analysis of contraction directionality, a region 1 pixel thick was taken from the anterior portion of the heart (just after the conical chamber) and a similar region from the posterior end. Cardiograms were created for each region by lining up each frame in the video. Anterior and posterior regions were aligned and points of greatest contraction were highlighted. If greatest contraction occurred at the posterior first, the wave was scored as "forward," and it was scored as "backward" if anterior contracted first. Heart diameter was scored based on the average size of cardiograms in diastolic and systolic states in the anterior region.

Statistical analysis

Statistical analyses were performed by one-way ANOVA with Tukey-HSD post-hoc comparisons for significance using online software (statistica.mooo.com). For bar graphs and box plots, data for each bar or box in the graph were analyzed as a separate column. In all figures, 1 asterisk represents a p-value less than 0.05, 2 asterisks represent a p-value of less than 0.01, and 3 asterisks represent a p-value less than 0.001.

2.3 Results

POMT mutant *Drosophila* embryos accumulate torsion of the muscle midline over the course of peristaltic muscle contraction waves

Previous studies of *rt* and *tw* mutants have mostly concentrated on adult and larval stages, while the effect of *POMT* mutations on embryonic development has not been well characterized (60,61,69,78). With the rationale that potential phenotypes of POMT mutants at early developmental stages could shed light more directly on pathological mechanisms associated with POM defects, we decided to focus on embryonic stages. We first sought to determine whether embryos had any rotation phenotype. After fixing embryos and staining with labeled phalloidin, my predecessor Dr. Nao Nakamura reported that both *rt* and *tw* mutants manifest a torsion of the dorsal midline such that the posterior appears to be twisted in the clockwise direction relative to the anterior, just as abdominal segments are twisted in adults (Fig. 5). This "embryonic torsion" phenotype becomes most obvious in late stage 17, the last stage of embryonic development. The penetrance of the phenotype in $rt^{2/571}$ mutant embryos was found to be 32% (N=19) at an early phase of stage 17 (stages 17a-e), and 100% (N=24) at the last phase (stage 17 f (79)). Torsion is entirely absent in stage 16. Since muscle formation is already complete by stage 16, and since penetrance of the phenotype increases as mutant

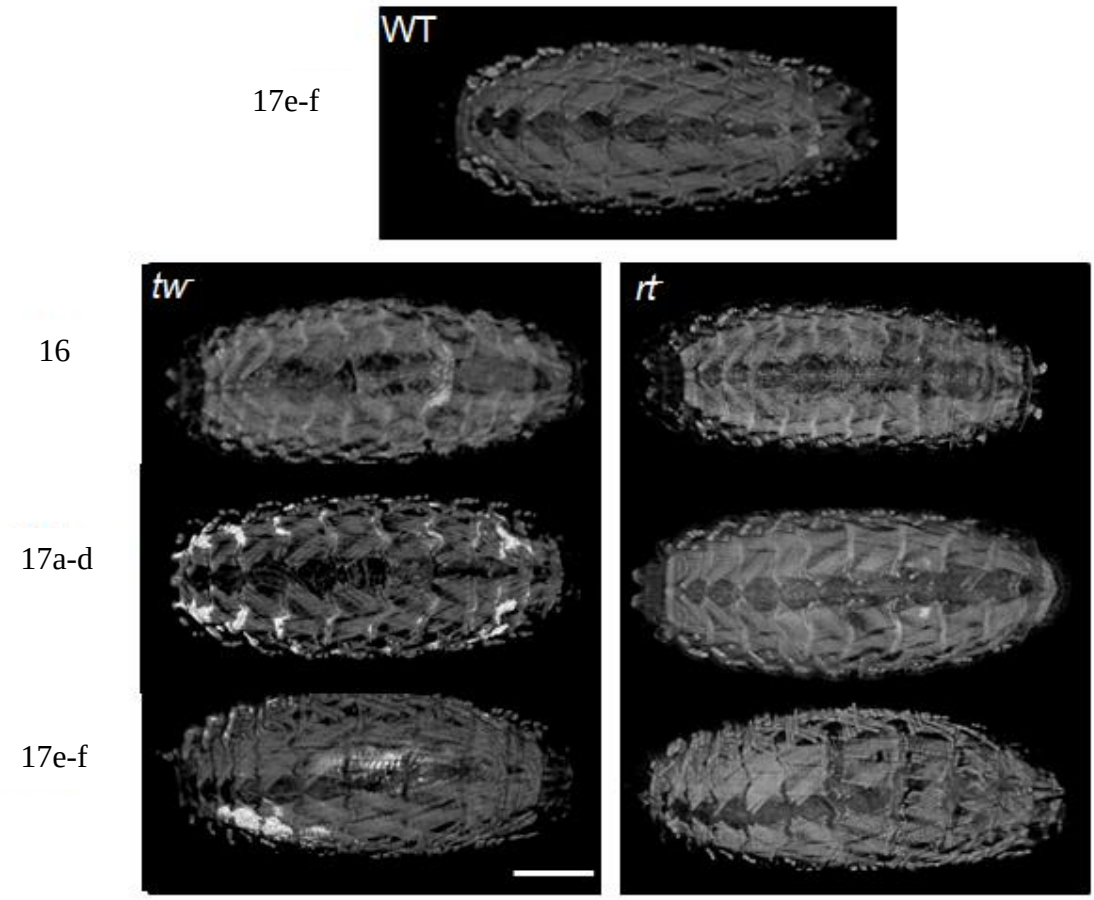


Figure 5—Torsion of embryonic dorsal midline in POMT mutants occurs in late stage 17. Data of Dr. Nao Nakamura.

Representative examples of wild type, *tw*$^{-}$, and *rt*$^{-}$ embryos at various developmental stages. In wild type embryos, the dorsal midline remains straight through the latest developmental stages (top). In *POMT* mutants, the midline forms straight at stage 16 but acquires torsion at the latest embryonic stages, 17e-f. Scale bar is 100 um.

embryos age, these data suggest that torsion arises not due to a defect in muscle development, but due to a physiological defect.

During late stage 17, embryos undergo peristaltic waves of muscle contractions that can initiate either at the posterior or anterior end of the embryo, and progress to the other end. Although episodic partial contraction waves begin at about 17 hours after egg laying, the first complete waves of forward peristalsis begin at approximately 18.25 hours after egg laying (80), minutes before tracheae fill with air. Since only 3 or 4 peristaltic contractions occur before tracheae fill, and then only sporadically, I chose tracheal filling as a convenient marker to begin recording waves. I initially analyzed contraction waves in transgenic lines expressing a GFP-labeled myosin heavy chain by live imaging, and observed propagating waves of GFP intensity progressing both from the posterior to the anterior and vice versa (Fig. 6).

To test whether these contractions are involved in the rise of midline torsion, I measured the angle between the midline and the body axis of symmetry immediately before and after each contraction. I observed that in wild type embryos, although the midline position changed as the embryo rolled within its shell, it remained nearly parallel to the eggshell axis of symmetry through all waves. In POMT mutants, on the other hand, the midline generally moved further to the left at the anterior than at the posterior. This yielded the apparent clockwise torsion of posterior segments (Fig. 7), which was really counterclockwise torsion of anterior segments.

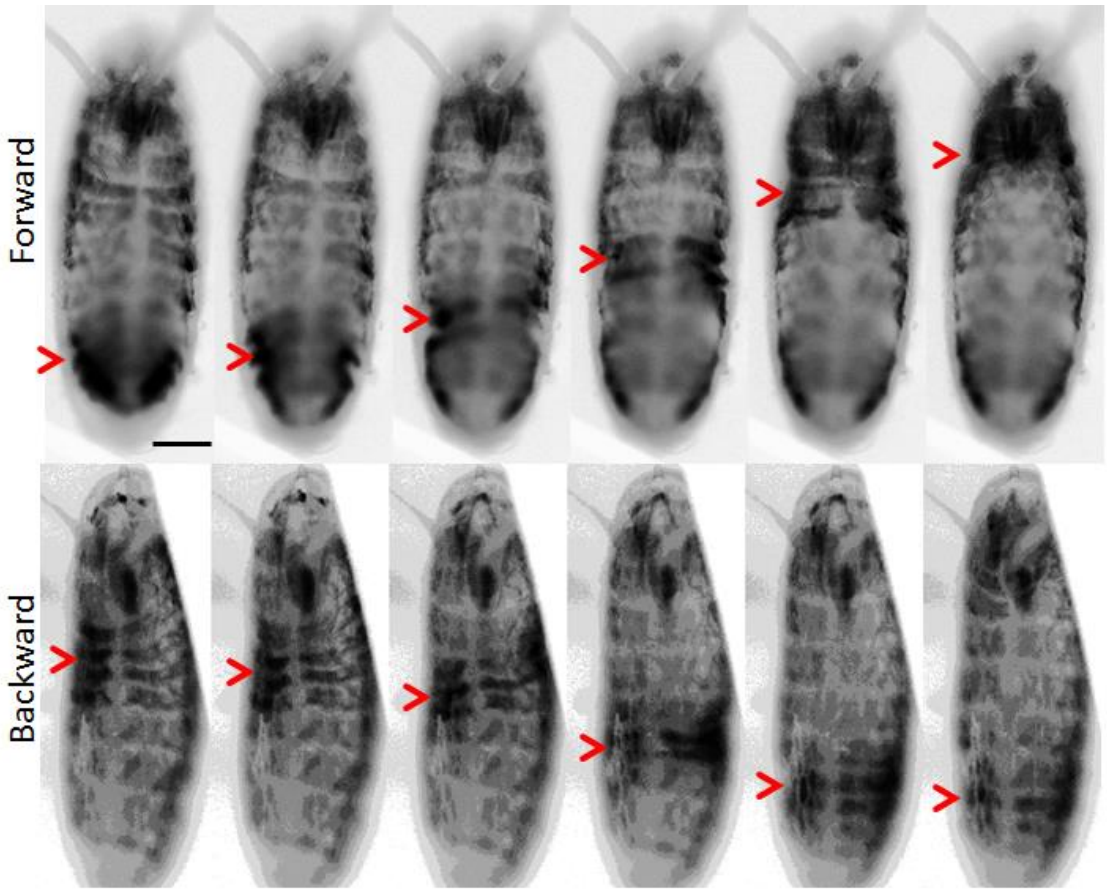


Figure 6—Forward and backward contraction waves of muscle contractions in *Drosophila* embryos.

Contractions can progress from posterior to anterior (forward) or from anterior to posterior (backward). Images are inverted monochromes. Dark pixels correspond to high GFP intensity. Red arrowheads indicate contraction wave position. Each contraction occurs over the course of ~20 seconds. Scale bar is 100 um.

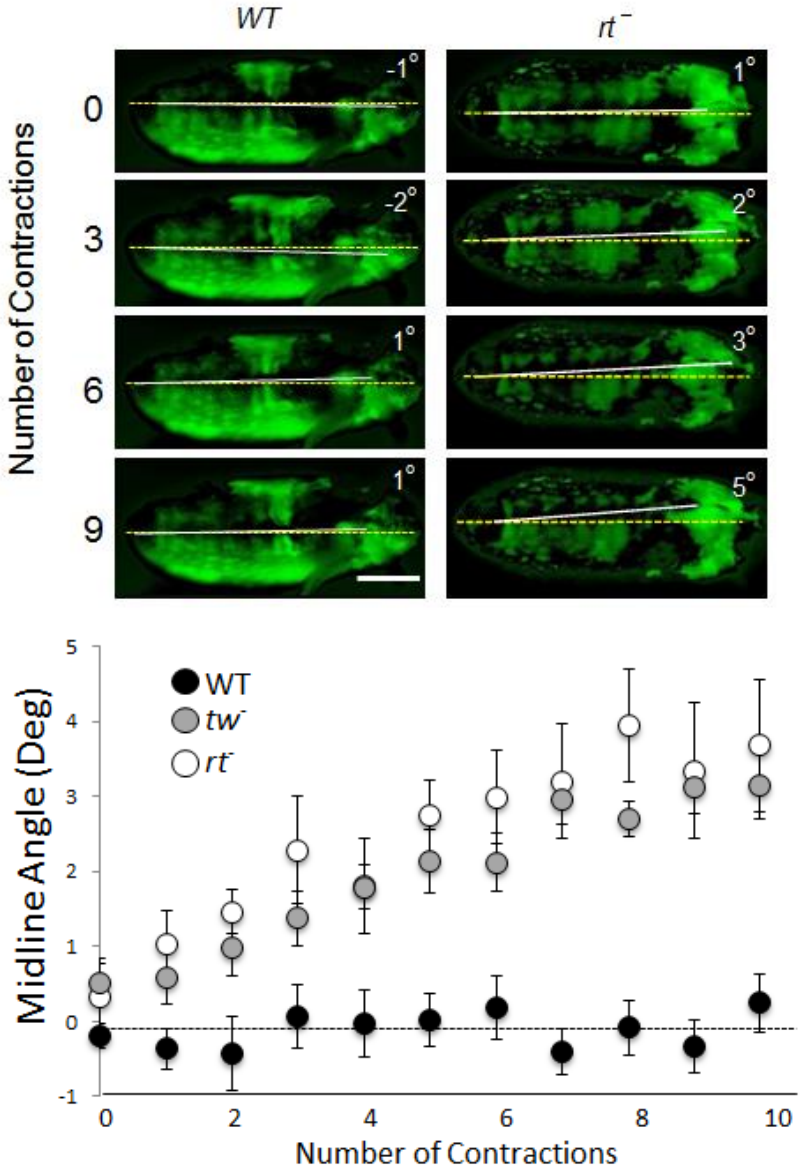


Figure 7—Torsion accumulates over the course of peristaltic contractions.

The top panel shows the angle of the dorsal midline (solid white line) relative to the eggshell axis of symmetry (dotted yellow line) after the designated number of contractions. The bottom panel is a measure of average midline angle in wild type, *tw*$^{-}$, and *rt*$^{-}$ mutants after the designated number of contractions. N = 10 embryos for WT and *tw*$^{-}$, 5 for *rt*$^{-}$. Scale bar is 100 um. Error bars are SEM. Wild type and mutants are statistically different from *POMT* mutants after 5 contractions.

The accumulated torsion remained through hatching and into the first instar larval stage (Fig. 8A), indicating that the torsion eventually becomes fixed, perhaps due to hardening of the cuticle. Interestingly, the midline torsion is relieved in 2nd instar larvae (Fig. 8B), likely due to the formation of a new cuticle that allows muscles to return to their proper posture. Consistent with previous results showing that ectopic POMT expression in early pupal stages can rescue rotation, this demonstrates that rotation in adults must rise independently from embryonic rotation, though perhaps by similar mechanisms. These results also demonstrated that the embryonic body torsion defect arises in mutants during peristaltic muscle contractions and revealed a correlation between the increase of torsion and number of contraction waves generated by mutant embryos, which suggested that contraction waves could induce torsion in mutant embryos.

Abnormal contraction patterning in POMT mutants correlates with the rise of torsion

To further test our hypothesis, I began to search for differences between contractions in wild type and mutant embryos. We hypothesized that individual waves in mutants may have defects in their progression that ultimately lead to torsion. Initially we hypothesized a left-right asymmetry of muscle contractions: if the wave on the left of the midline were more powerful at the anterior than the wave on the right, for example, rolling might result. To test this, I used GFP intensity as a marker of muscle shortening during contractions. As labeled muscles shorten, more fluorescent molecules accumulate in the same focal area, and thus increased GFP intensity can serve as a readout of muscle

shortening (81). When I plotted intensity on left and right sides of the embryo, I did find some waves with greater shortening on the left side of the embryo than the right.

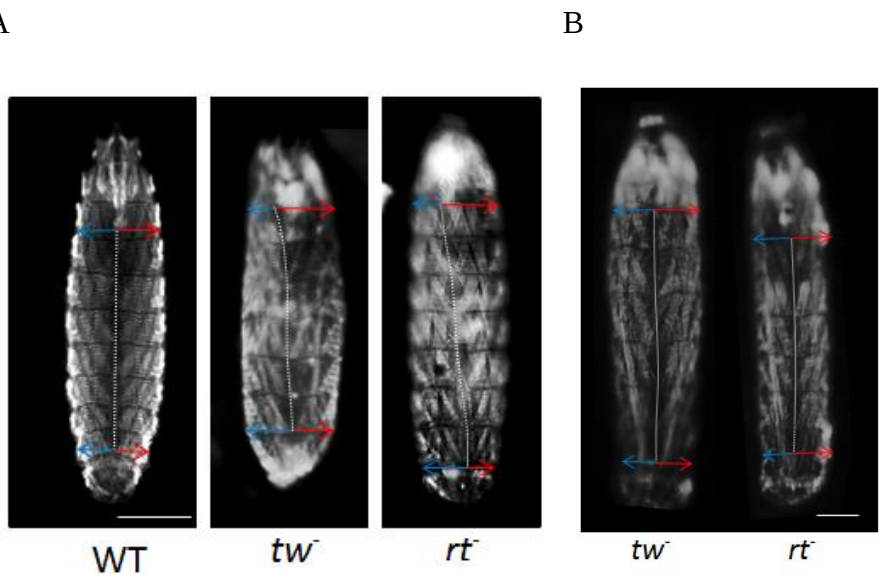
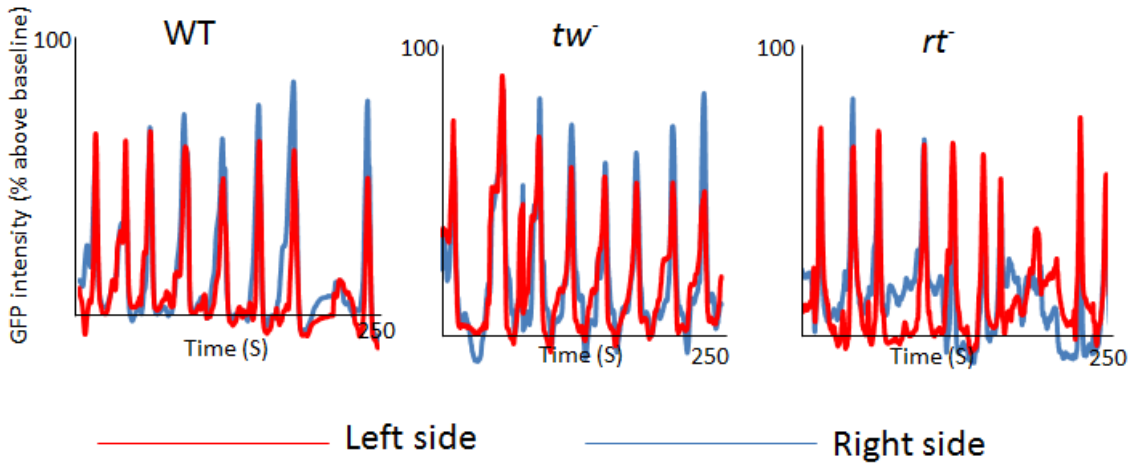


Figure 8—Persistence of torsion into larval stages.

A) Recently hatched 1st instar larvae. POMT mutants continue to have midline torsion with ~90% penetrance. Arrows indicate distance from the midline to the edge of the body on right and left sides to help illustrate midline torsion. B) At the second instar stage, torsion is gone. Scale bars are 100 um.

However, waves more frequently progressed with similar strength on both sides of the midline and were just as frequently overly strong on the right side as on the left. Additionally, wild type embryos experienced similar frequencies of left-strong and right-strong contractions as mutants, and no embryos experienced significant abnormalities in contraction synchronization on left and right sides (Fig. 9). To further verify this result, I directly measured the length of muscle segments in relaxed and contracted states on the

A



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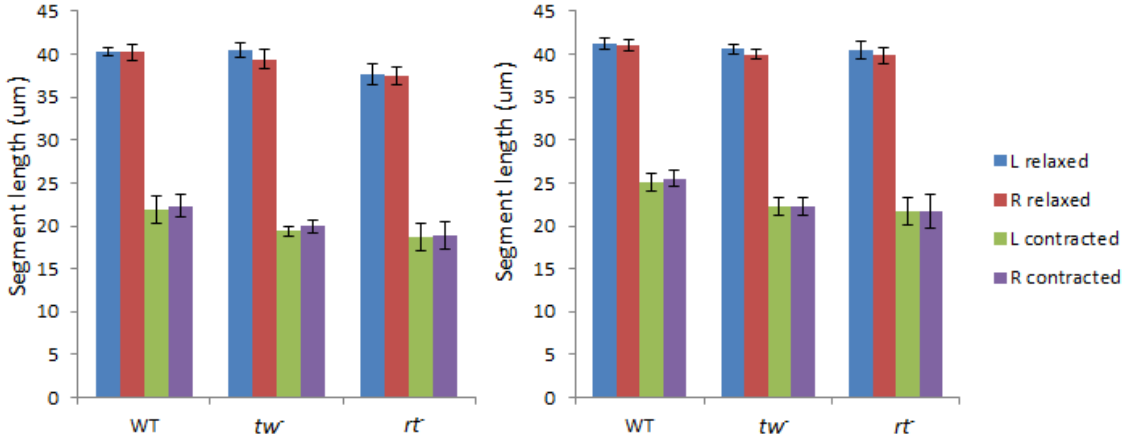


Figure 9—Left-right synchronization of contractions.

A) Representative graphs of GFP intensity over several contractions on left and right sides (red and blue, respectively) of the embryo. The two sides are synchronized. B and C) Graphs of average segment lengths in relaxed or contracted states on left and right sides for posterior segment A7 (C) and anterior segment A2 (D). No significant differences were observed within a genotype. Error bars are SEM. N = 5 embryos per genotype, and 5 waves per embryo at both posterior and anterior positions.

left and right side of the midline in both anterior and posterior segments. I found no difference in muscle shortening on left or right sides in wild type or mutant embryos (Fig. 9).

Having found no discernible differences between individual contractions in wild type and mutant embryos, I next hypothesized that a difference in contraction patterning, rather than in characteristics of individual contractions, may help explain torsion. As discussed previously, contraction waves can propagate in the forward direction (posterior to anterior) or the backward direction (anterior to posterior). I quantified percentages of backward and forward waves in wild type and mutant embryos and found that indeed mutant embryos have a slight but significant increase in percentage of backward waves (Fig. 10). Interestingly, during this analysis I also noted that while some waves simply progressed from one end of the embryo to the other (here designated "type 1 waves"), other waves would initiate in the backward direction, halt after partial propagation, and would then be met and swept back to the anterior by a forward wave (Fig. 11A-B). I designated these more complex wave forms "type 2 waves."

Type 2 waves are much more abundant in mutant embryos than in wild type, with approximately one type 2 wave per type 1 wave in mutants, as opposed to one type 2 wave per three type 1 waves in wild type (Fig. 11C). The backward component of type 2 waves appears to account for the increase in overall percentage of backward waves in mutants. To examine whether the type 2/type 1 ratio might be related to torsion, I again measured the midline angle relative to the body axis of symmetry before and after each wave as in figure 1, but this time I discriminated between wave types. I calculated the

average change in the angle that occurs after a wave and found that irrespective of genotype, type 1 waves correlate with a slight apparent counterclockwise torsion of posterior segments, while type 2 waves strongly correlate with apparent clockwise

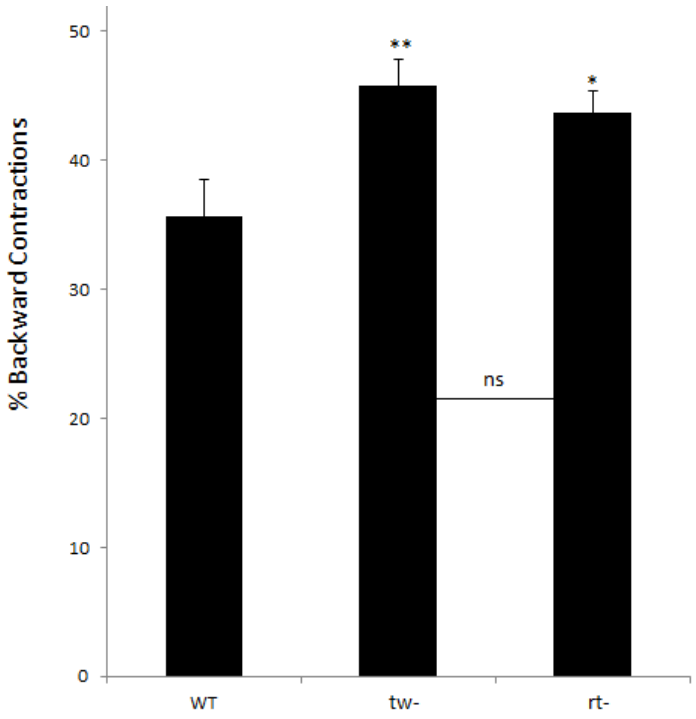
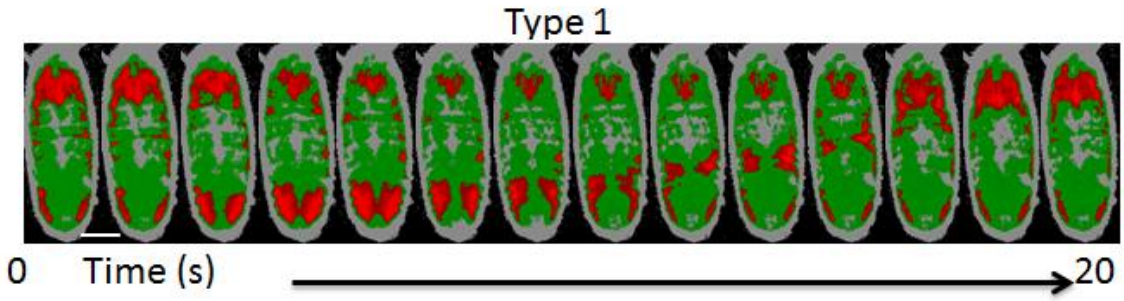


Figure 10—Difference in backward/forward contraction patterning in wild type vs. mutant embryos.

POMT mutants have increased frequency of backward contractions relative to wild type. Asterisks indicate statistical difference between the marked genotype and wild type. N = 10 embryos per genotype and at least 10 contractions per embryo.

A



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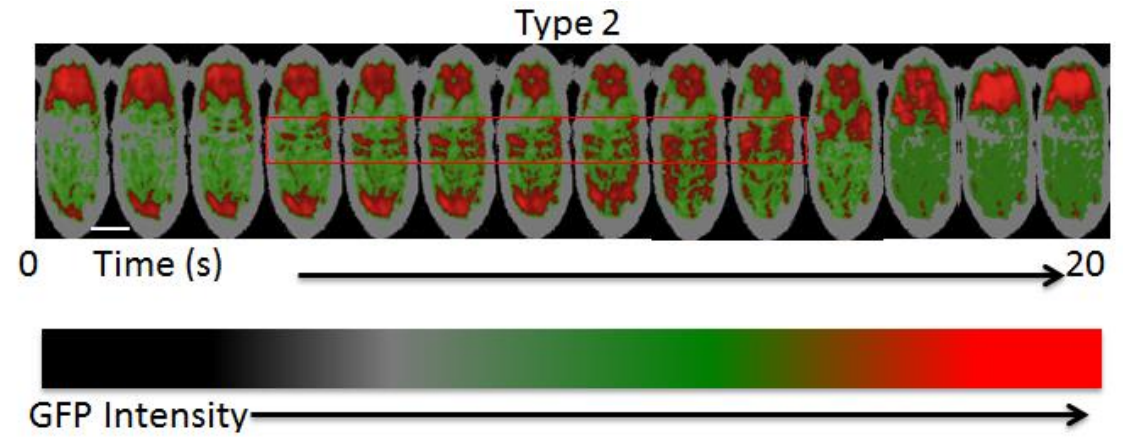


Figure 11—Embryos experience two wave modes, with "type 2" waves being overly abundant in POMT mutants.

A and B are representative examples of type 1 and type 2 waves, respectively. Type 1 contractions proceed from one end of the embryo to the other. Type 2 contractions initiate at the anterior, halt before reaching the posterior, and are swept back to the anterior by a secondary wave. Red box in B indicates static overcontraction. Scale bars are 100 um.

C

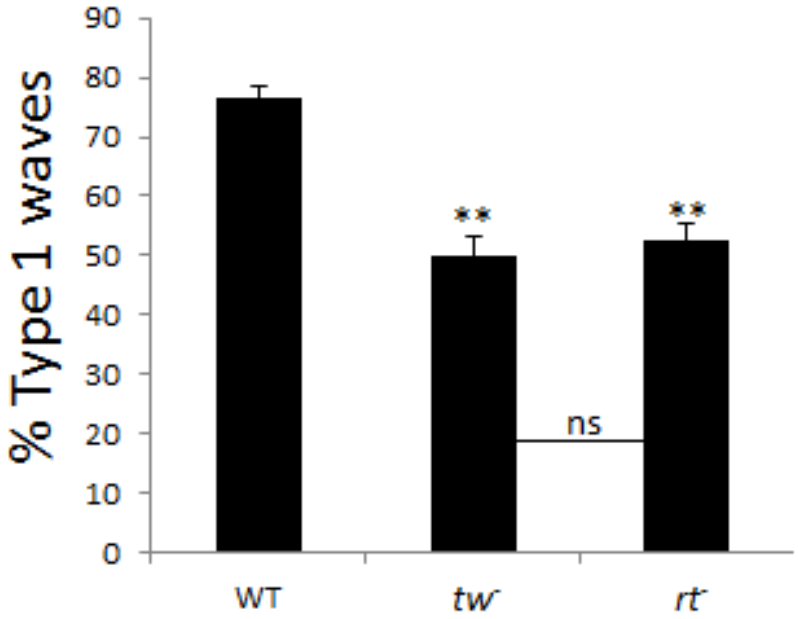
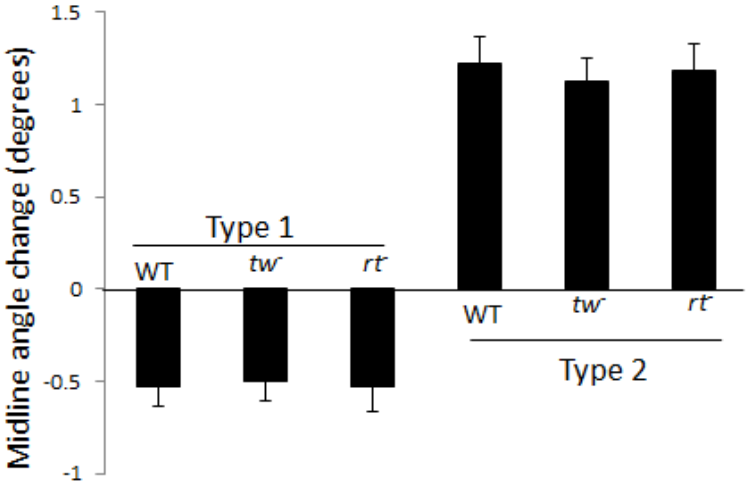


Figure 11—Continued.

C shows percentage of waves that are type 1 in each genotype. Error bars are SEM. Asterisks are statistical significance relative to wild type. N = 10 embryos for each genotype and at least 130 total contractions per genotype.

torsion (Fig. 12A). The correlation between type 1 waves and apparent counterclockwise torsion was present even when waves were subdivided into forward and backward directionality (Fig. 12B). I observed no “backward” type 2 waves- in which a halted forward wave is swept to the posterior by a backward wave- in any genotype, so no correlation could be made between “backward” type 2 waves and change in midline angle.

A



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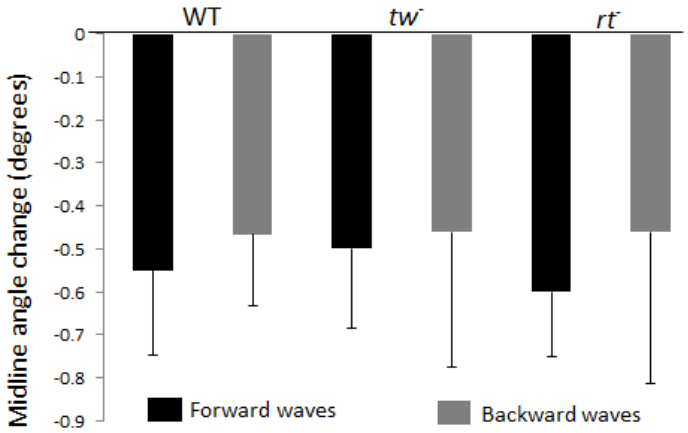


Figure 12—Correlation between contraction type and torsion.

Type 1 contraction waves yield "counterclockwise" torsion (indicated by negative numbers) while type 2 contractions yield "clockwise" torsion, regardless of genotype or contraction direction. A) depicts type 1 vs. type 2 contractions, where both contraction types are statistically identical with themselves across genotypes, while each contraction type is significantly different from the other for all genotype comparisons. B) compares forward and backward type 1 waves, which always yield counterclockwise torsion. There are no statistical differences between genotypes or directions. Error bars are SEM. In A and B, N = 10 embryos for WT and tw-, and 7 for rt-, with a total of at least 20 contractions per wave type for each genotype.

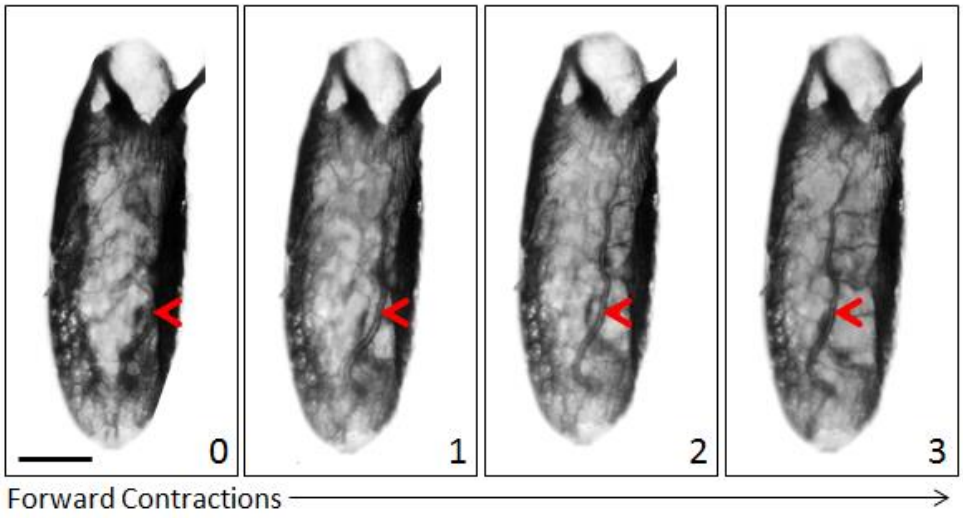
Together these results indicate that torsion arises not as a result of changes in properties of individual contractions, but as a result of changes in the patterning of those contractions. While wild type embryos preserve correct embryo posture by maintaining a ~3:1 type1/type2 ratio, an overabundance of type 2 waves in mutants correlates with accumulation of torsion.

Torsion accumulates as embryos roll differentially due to chiral interactions and differential contraction strength

Next I wanted to better understand how each wave type might induce torsion in one direction or the other. I previously saw that both wild type and mutant embryos experience a shift in midline position during contractions as the embryo rolls within its shell (see Fig. 7). Additionally, I saw that both types of contractions typically result in a change in the midline angle, suggesting that some amount of rolling occurs in any given contraction, and that it is differential- that is, the rolling is stronger at either the anterior or posterior end of the embryo than at the opposite end.

To better characterize embryonic rolling, I measured the change in embryonic positioning within the shell during type 1 contractions as measured from the middle of the anterior-posterior axis. I found that regardless of genotype, embryos rolled in correlation with the direction of contraction waves. During forward waves embryos roll to the left as viewed from the posterior, while during backward waves they roll to the right (Fig. 13 A-B). When I quantified the average distance rolled per contraction, I found that both forward and backward waves correlated with an average distance of ~6

A



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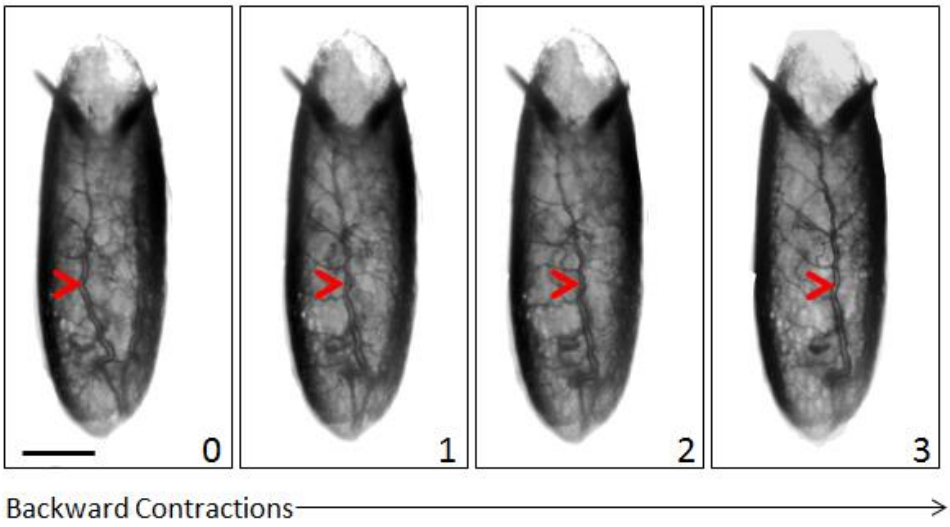

Figure 13—Embryos roll within their shells in a direction that depends on contraction directionality.

Representative images of embryos rolling to the left during forward contractions (A) and to the right during backward contractions (B). Red arrowheads indicate tracheal position. Scale bars are 100 um.

C

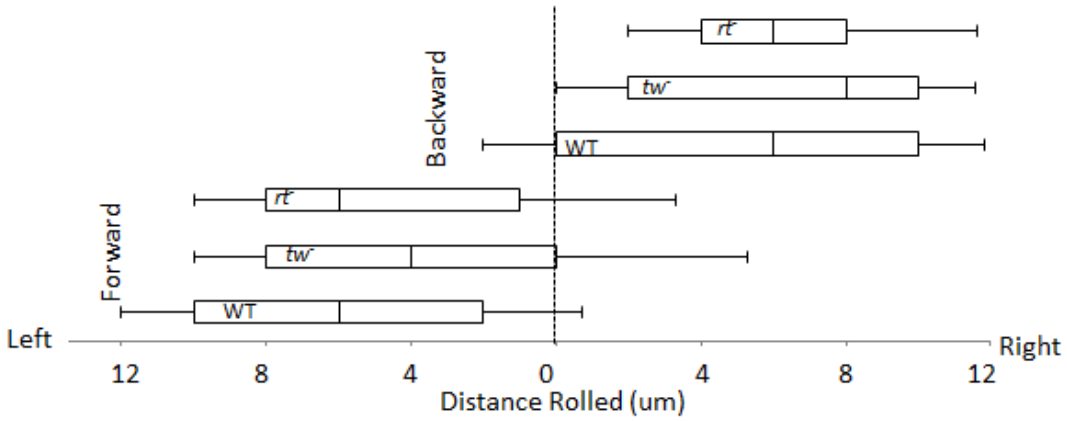

Figure 13—Continued.

C is average distance rolled per contraction in all genotypes for both forward and backward contractions. Boxes represent interquartile range, lines represent mean values, and error bars are standard deviation. N = 10 embryos for WT and *tw*-, and 7 for *rt*-, and 5 contractions of each type per embryo.

um, but in opposite directions (Fig. 13 C). It thus appears that during contractions, embryos experience a chiral interaction, perhaps between cuticle and eggshell, which induces rolling.

If indeed a chiral interaction is responsible for rolling, one would expect stronger contractions, or contractions with greater muscle shortening, to correlate with a greater distance rolled. Thus given that type 1 contractions induce leftward rolling as viewed from the point of wave initiation, and that they correlate with apparent counterclockwise torsion of posterior segments, I would expect decreasing amplitude as the wave progresses. In forward waves, such a decrease would produce greater rolling to the left at the posterior (where the wave is stronger) than at the anterior, resulting in clockwise

torsion of the anterior. Backward waves of this form would induce anterior segments to roll a greater distance to the right than posterior segments, giving the same net result on torsion as forward waves (See model in Fig. 17 at the end of the chapter). The backward component of type 2 waves could have a cancelling effect on motion of posterior segments, allowing anterior segments to roll further to the left than posterior segments, resulting in a net apparent clockwise torsion (See Fig. 17).

Given these predictions, I measured intensity of GFP at posterior, middle, and anterior segments during type 1 and type 2 waves. In general type 1 waves did in fact diminish in intensity as they progressed, regardless of genotype or wave direction (Fig. 14). Interestingly, the forward component of type 2 waves most frequently either remained relatively constant in intensity or else increased slightly as waves progressed (Fig. 14). Thus in addition to any potential cancelling effect of the backward component of these waves, it appears that type 2 waves may induce stronger leftward rolling at the anterior than at the posterior. Cumulatively these results are consistent with the hypothesis that embryos roll differentially due to changes in strength of chiral interactions as contractions progress.

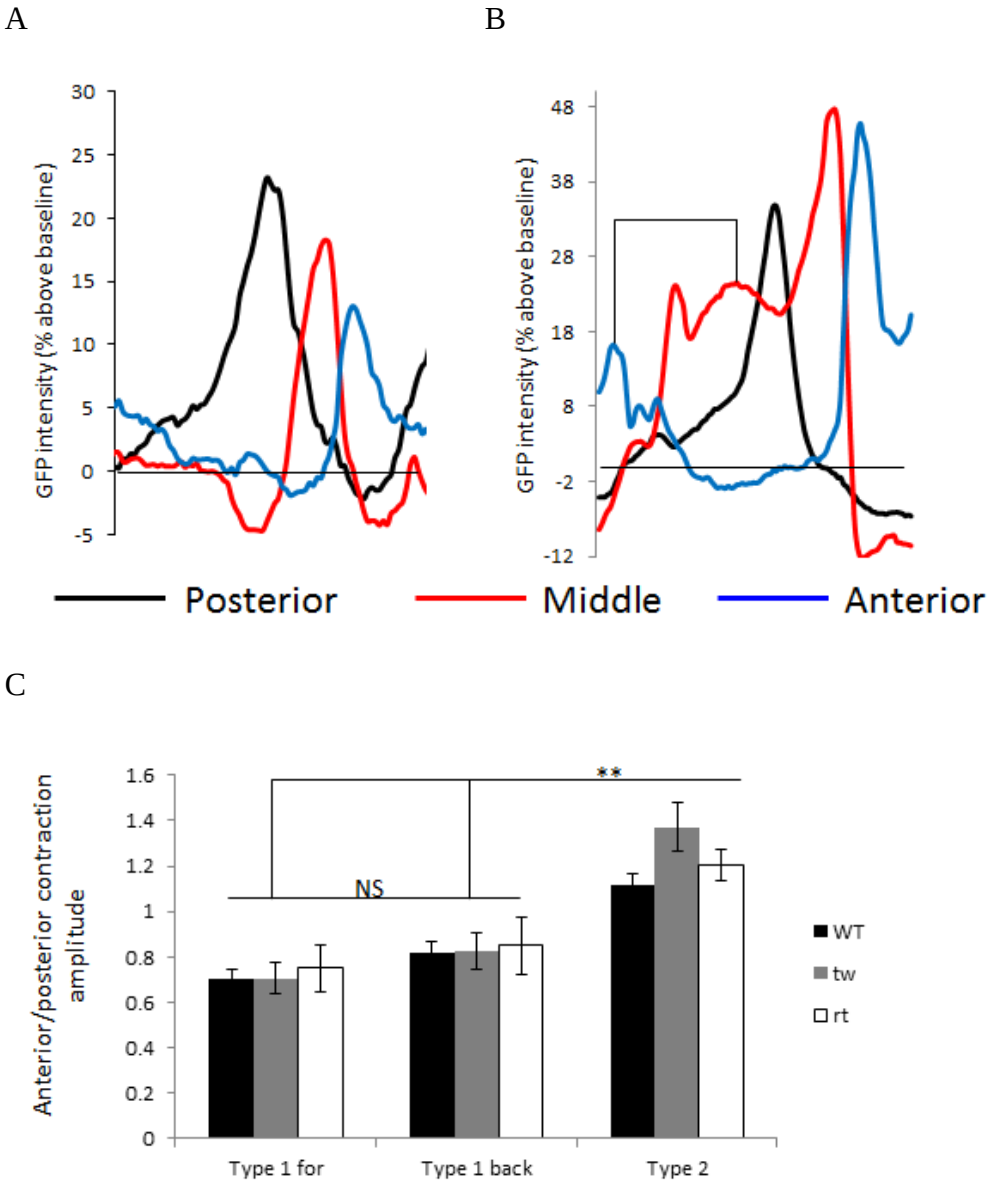

Figure 14—Contraction strength changes as waves progress.

A and B are examples of type 1 and type 2 waves, respectively. In B the bracket indicates the first (backward) phase of the wave. C is amplitude of the latter portion of the wave normalized to the posterior amplitude. N = 10 embryos for WT, and 8 each for *tw*$^{-}$ and *rt*$^{-}$, with at least 20 waves of each type per genotype.

Drosophila experience abnormal muscle contractions during the pupal and adult stages

Finally I wished to elucidate what, if anything, is the connection between the embryonic and adult phenotypes. Since the adult phenotype can be rescued by ectopic expression of POMT up through early prepupal phases (71), we first examined mutants at this developmental stage. Although it is difficult to directly observe contractions in pupae due to their opaque nature, we did find that mutant pupae develop a bulge of mid-posterior lateral muscles (Fig. 15). This bulge was 100% penetrant in mutants and underlain by bulges in the corresponding muscles (not shown). The bulge was entirely absent in wild type pupae. It is reminiscent of the overcontraction in the initial phase of type 2 waves (see Fig. 11 A, red box).

To further examine the effect of POMT on muscle contractions in late stages, we analyzed *Drosophila* adult hearts. The red fluorescent protein tdTomato was expressed using the heart-specific dHand-Gal4 driver in both wild type and *rt* mutant flies. This fluorophore is easily visible through the adult cuticle, allowing heart analysis in live, anesthetized flies. *Drosophila* hearts beat regularly without external stimulation, making them a good model for muscle contraction patterning. They take the form of a tube running along the anterior-posterior axis on the dorsal side, and like embryonic muscle, they undergo peristaltic contractions that can progress from anterior to posterior or vice versa.

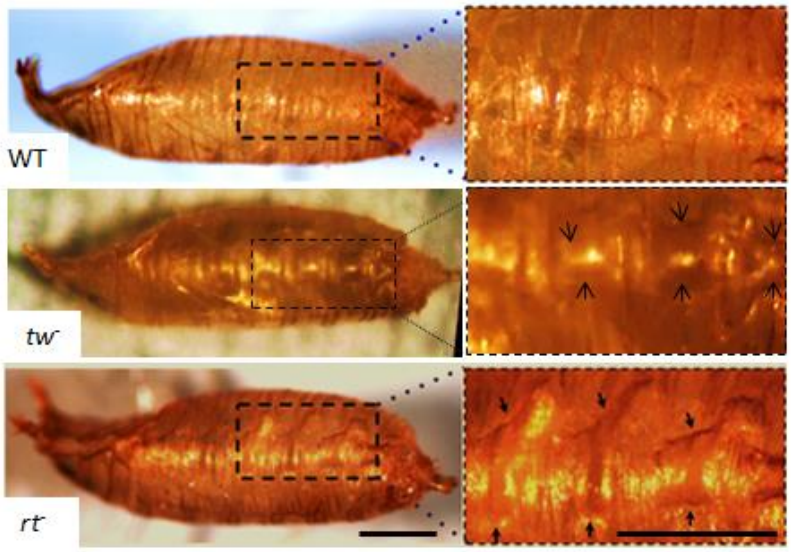

Figure 15—POMT mutant pupae experience muscle bulging. Data obtained in collaboration with Dr. Nao Nakamura.

Bulging in POMT mutant pupae, indicated by the dashed box, is 100% penetrant. Arrows indicate bulging region in close-ups. Scale bars are 500 um.

Previous studies have shown that in wild type flies, heart beats progress from posterior to anterior in ~60% of contractions, and in the opposite direction in roughly the remaining 40% (82). My analysis returned similar results, but found that in *rt* mutants, hearts beat from *anterior* to *posterior* nearly 60% of the time (Fig. 16). Additionally, when I measured heart diameters I found that *rt*$^{-}$ hearts are significantly smaller than their wild type counterparts in both the diastolic (relaxed) and systolic (contracted) states (Fig. 16). Although it is currently unclear exactly why hearts might be smaller in POMT mutants, I hypothesize that they are perpetually overcontracted, or in other words, they never fully relax. Together my data show that *Drosophila* experience abnormalities of muscle contractions at various developmental stages. It may be, then, that similar

aberrant contraction patterns contribute to abdominal rotation in adults as in embryos, though more data are needed to verify this hypothesis. Further evidence connecting adult and embryonic phenotypes will be presented in chapter III.

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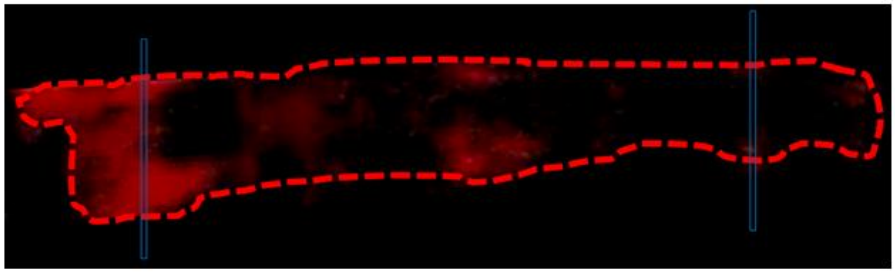

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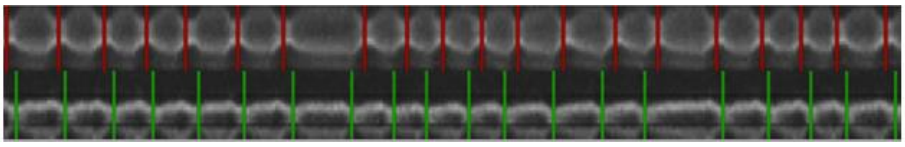

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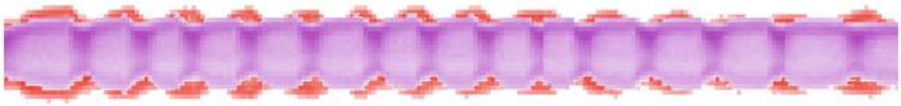
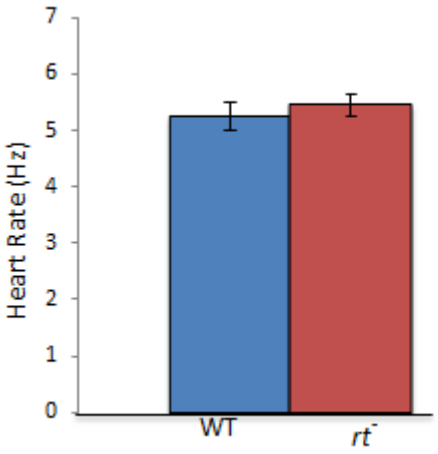


Figure 16—Heart contraction defects in POMT mutant *Drosophila.*

A is a representative fluorescent heart imaged through the cuticle of a live adult fly. Blue boxes represent cross-sections analyzed in B. B is a stack of anterior (top) and posterior (bottom) sections of the heart. Red and green lines are moments of full contraction. If red precedes green, the contraction is posterior to anterior, vice versa if green precedes red. C is an overlay of wild type (red) and *rt*$^{-}$ (purple) hearts demonstrating smaller diameter of POMT mutant hearts.

D



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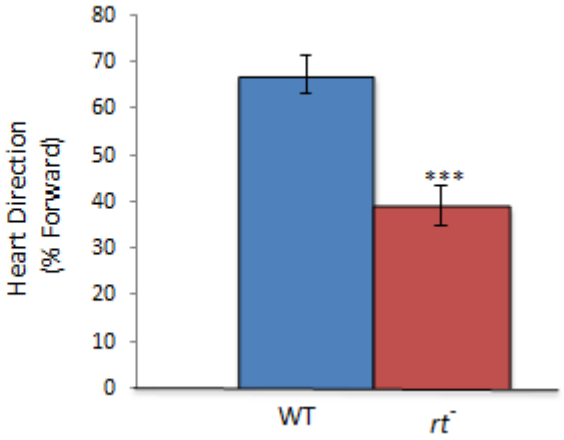

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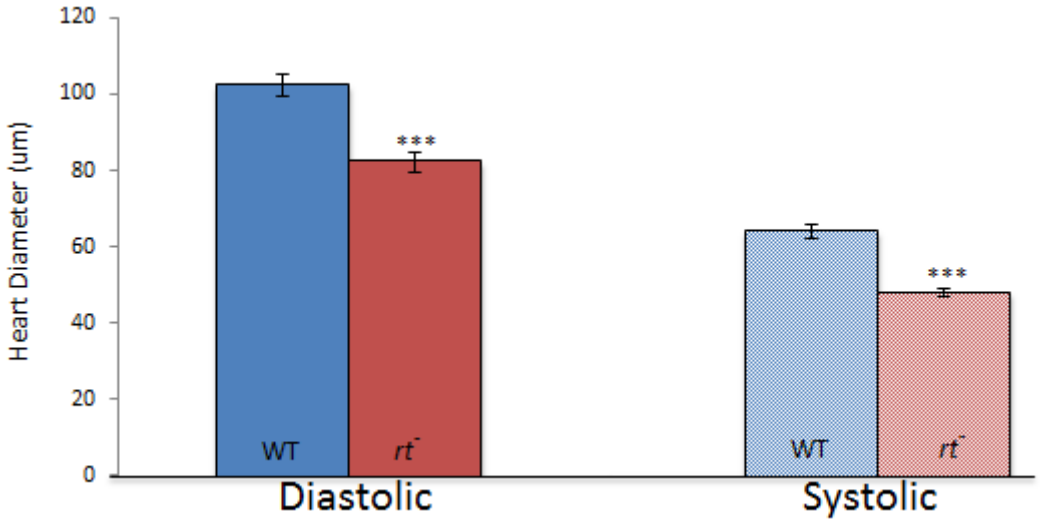


Figure 16—Continued.

D demonstrates that heart rate is not changed in mutants. E shows a significant change in directionality of heart beats and F shows a significant reduction in both diastolic and systolic diameters for mutants. N=10 hearts for each parameter and at least 20 beats each. Error bars are SEM.

2.4 Discussion

In this chapter I investigated the mechanism underlying embryonic torsion in POMT mutants using a live imaging approach. I discovered a role for POMT in regulation of contraction wave patterning, arriving at the conclusion that the embryo torsion phenotype arises due to aberrant coordination of individually normal contraction waves. The data indicate that muscle contractions dynamically control embryo body posture in all embryos, regardless of genotype, by inducing differential rolling of anterior and posterior segments relative to one another. Abnormal contraction patterning, rather than abnormal characteristics of individual contractions, gives rise to an apparent counterclockwise torsion of posterior muscle segments in POMT mutants. I refer here to "apparent counterclockwise torsion of posterior segments" several times in keeping with the original observation of rotated adult abdomens. I use this phrasing, however, because it appears that in reality it is the anterior, not the posterior, that moves. Thus here we have an example of the proverbial "tail wagging the dog." It is unclear whether anterior or posterior segments move more in adult torsion.

We found that embryonic muscles are properly aligned when they initially form, even in mutants. Torsion only arises once peristaltic contractions begin in stage 17, and rises rapidly thereafter (Figs. 5-7). This suggested that there is a difference between contractions in wild type and mutant embryos. However, my analysis suggests that individual contractions of the same type proceed similarly and have similar effects in both wild type and mutant embryos (Figs. 9, 11-14). Left-right asymmetry in contraction waves is rare even in mutants, equally present on both sides, and equally present in wild

type and mutant embryos (Fig. 9), indicating that left-right asymmetry of contractions is not responsible for the rise of torsion. Indeed, mutant embryos appear capable of generating entirely normal contraction waves on an individual basis.

The difference, then, seems to lie in wave patterning. Here I have classified two different types of waves, designated type 1 and type 2 (Fig. 11). Each individual contraction wave has an effect on embryonic posture, with type 1 waves corresponding to an apparent counterclockwise torsion of posterior segments and type 2 waves corresponding to apparent clockwise torsion, irrespective of genotype (Fig. 12). I therefore propose that type 2 waves exist in wild type flies as a mechanism to compensate for the counterclockwise torsion that would accumulate over the course of several type 1 contractions. The overabundance of type 2 waves in POMT mutants underlies the accumulation of clockwise torsion.

Why might different wave types cause torsion in different directions? While examining the effects of contraction waves, I noted that during contractions the position of the muscle midline changes in both wild type and mutant embryos showing that during peristalsis embryos roll within their shells. Further analysis showed that the direction of contractions correlates with the direction of rolling, with forward contractions causing a roll to the left and backward contractions causing a roll to the right (Fig. 13). Thus embryonic rolling is always left-handed when viewed in the direction of wave propagation. This observation unveiled an intrinsic chiral interaction that governs embryo rolling during peristaltic muscle contractions. I liken these interactions to the turning of a threaded screw. As the screw is pushed forward the thread

applies a torque and causes the screw to turn in one direction. If the screw is pulled out, it will turn in the opposite direction even though the threading has not changed, because its direction of propagation has changed. Although the exact biological mechanism behind this observation in *Drosophila* embryos remains unknown, it has been observed in *Lepidoptera* and other insects that the eggshell exhibits a left-handed chirality of protein structures on the macro scale (83), and that *C. elegans* have a similar left-handed chirality in the cuticle (84). A comparable chiral structure in *Drosophila* eggshell and/or cuticle could account for the left-handed rolling observed during contractions.

Here I propose a model that explains the relationship between rolling and torsion based on the assumption that rolling forces correlate with the strength of contractions. If each muscle segment behaves as a semi-independent screw, and contraction strength varies from one segment to another, then differential rolling could result. Type 1 waves show on average greater muscle shortening in the beginning of a wave than at the wave's end (Fig. 14), and thus my model predicts that these waves should induce stronger rolling in the beginning and weaker rolling in the end of propagation. Importantly, while opposite-direction waves generate rolling in different directions, the differential torque force is predicted to induce apparent counterclockwise torsion of posterior segments, irrespective of wave direction (Fig. 17). Indeed, this prediction is in agreement with my observations that type 1 waves propagating in any direction correlate with overall accumulation of a counterclockwise torsion of the embryonic posterior (Fig. 12). This model is also consistent with the fact that biphasic (type 2) waves generate a clockwise torsion of posterior (or counterclockwise torsion of anterior) segments. According to my

observations, type 2 waves induce differential rolling mostly at the anterior part of the embryo, during the end of the wave. This is presumably because the anterior experiences stronger contractions in the second phase of the wave, as compared to the initial phase that induces rolling in the opposite direction. Rolling is essentially halted at the posterior end of the embryo, where the backward and forward moving waves collide and cancel each other's effect, inducing prolonged static contractions. (Fig. 11, 17). As a result, only the anterior part of the embryo experiences a significant net roll, producing a cumulative apparent clockwise torsion of the embryonic posterior (Fig. 17).

Our experiments revealed that *POMT* mutations affect body posture by changing the overall pattern of contraction waves, while rolling behavior and differential torque associated with individual waves remain essentially unaffected. Therefore, *POMT* mutations do not break the symmetry of processes that control body posture. Instead, they uncover the intrinsic chirality underlying these processes by failing to compensate properly for it to ensure proper alignment of body segments. Our results shed light on a unique physiological strategy employed by an animal to counterbalance an undesired asymmetry when the overall developmental outcome needs to be symmetrical. The dynamic correction for chiral torsion described here provides, to my knowledge, the first example of a "chirality-neutralizing" mechanism that monitors and maintains the symmetry of a developing system affected by asymmetric forces. Thus I have identified a new asymmetric marker associated with *Drosophila* development, an embryo rolling behavior induced by peristaltic waves of contraction (Fig. 13).

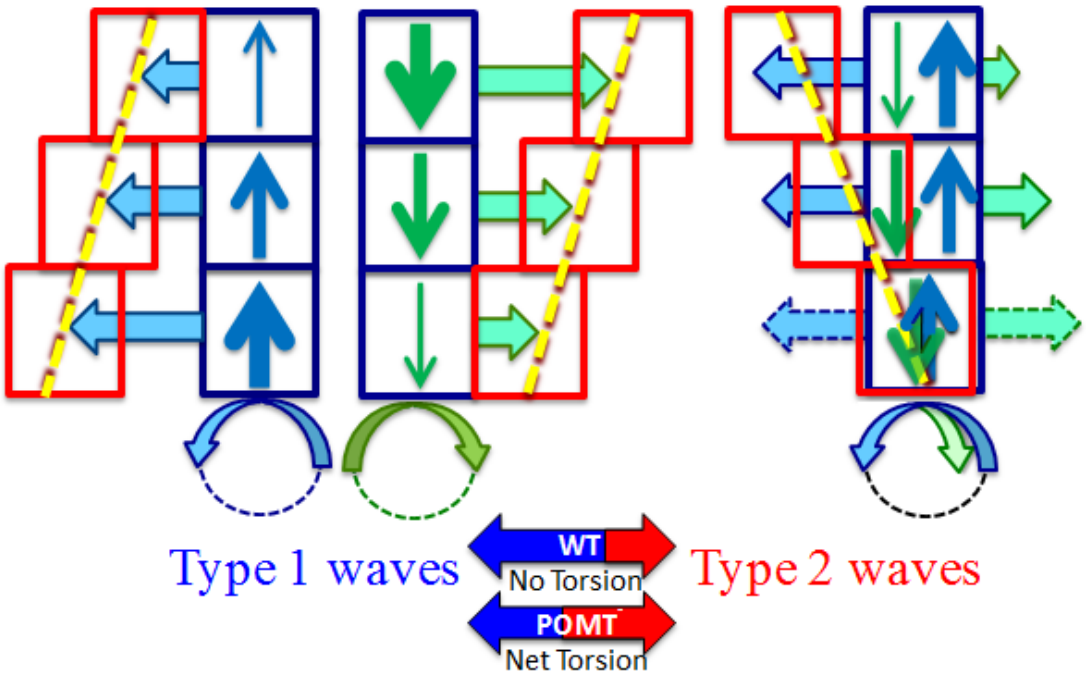


Figure 17—Model for the rise of torsion.

Type 1 contraction waves decrease in strength as they progress, yielding differential rolling. Segments at the beginning of the wave roll further than segments at the end, leading to counterclockwise torsion of posterior segments. In type 2 waves, posterior rolling is effectively canceled, leading to net leftward roll of anterior segments and apparent clockwise torsion of posterior segments.

How is this chirality related to other known markers of left-right asymmetry? While LR asymmetry affects numerous aspects of the development and physiology of more complex animals, LR asymmetry in *Drosophila* is relatively simple and its effects appear to be limited to morphogenesis of some tubular structures, such as male terminalia rotation and looping of gut and testis (85). These LR asymmetries are thought to depend on cellular interactions and common molecular regulators such as Myosin ID

(85). It is tempting to speculate that the rolling asymmetry may be "hardwired" in intrinsic molecular chirality of some structures of the eggshell, cuticle, or muscles, and thus it is possible that embryo rolling and other known LR markers have different mechanistic bases. However, further studies are required to reveal the relationship between mechanisms of asymmetric rolling and other LR markers.

Up to this point I have shown the mechanism by which torsion arises in embryos. This torsion has the same directionality as the adult phenotype, suggesting that the two may arise due to similar processes. To assess this possibility, I first monitored the progression of torsion through the developmental stages of the *Drosophila* life cycle. The torsion that arises in the embryonic stage persists into the 1st instar larval stage, though by the 2nd instar stage it has essentially disappeared (Fig. 8). I hypothesize that although midline angle is initially dynamic during embryonic peristalsis, it becomes static as the cuticle thickens and hardens prior to hatching (86). The hardened cuticle holds the midline in its torqued posture throughout the first instar stage. The 1st instar cuticle is lost upon molting during the transition to the second instar stage, and the new cuticle does not harden until after ecdysis (87), potentially allowing muscles to resume normal posture. Thus it seems that while embryonic and adult torsion may arise via similar mechanisms, they do so during two independent events. In other words, an embryo could develop torsion while going on to have a straight abdomen as an adult, or an embryo could hatch with a straight midline and still have a twisted abdomen as an adult, if POMT is ectopically expressed during the relevant developmental stage. Consistent with this hypothesis is the previous observation that rotation in adult

abdomens can be rescued by a ubiquitous pulse of POMT expression limited to late larval or early prepupal stages (71).

To assess whether there is any similarity between the rise of torsion in embryonic and adult stages, I examined wild type and POMT mutant *Drosophila* at the latest developmental stage during which ectopic POMT expression can rescue adult torsion- the early prepupal stage. Intriguingly, coordinated waves of abdominal muscle contractions that are thought to facilitate the prepupal-pupal transition occur during metamorphosis (88-90), suggesting the possibility that abnormal muscle contractions could be present at this critical stage. I found that both tw^{-} and rt^{-} pupae exhibit a bulge of lateral muscles near the posterior, while this bulge is not present in wild type pupae, suggesting that there may indeed be abnormal contraction coordination in pupae as well as embryos.

Further corroborating the notion of abnormal contraction patterning across multiple developmental stages, I observed that adult *Drosophila* hearts spend an inordinate amount of time beating from anterior to posterior in mutants. This phenomenon is not directly related to torsion, since abdominal torsion was already present when these observations were made. Nevertheless, these results together strongly indicate that contraction patterning is compromised in late stages of the *Drosophila* life cycle, and this may be responsible for abdominal torsion in adults. Further studies are needed to test this hypothesis.

O-mannosyl glycans play important developmental roles in organisms ranging from *Drosophila* to humans. Clinical feature of WWS and MEB syndromes caused by

defects in POM pathway genes commonly include abnormal muscle contractions (66). Interestingly, downregulation of POMK, a kinase that phosphorylates O-linked mannose and is critical in the formation of laminin-binding extended O-mannosyl glycans on Dystroglycan, causes abnormality in coordinated muscle contractions in zebrafish embryos (67). Thus further studies in *Drosophila* and other organisms may shed light on evolutionary conservation of mechanisms underlying the role of O-mannosyl glycans in regulating coordinated muscle contractions, which will potentially lead to new diagnostic and therapeutic applications for diseases associated with POM abnormalities.

CHAPTER III

CELL-SPECIFIC REQUIREMENTS OF POMT EXPRESSION FOR PROPER DEVELOPMENT OF POSTURE

3.1 Introduction

In the previous chapter I discussed the mechanical means by which rotation arises in POMT mutant *Drosophila* embryos: an abnormal pattern of peristaltic contractions leading to differential rolling of anterior segments relative to posterior. I also tentatively connected that mechanism to the adult phenotype, showing that at the critical time for maintaining proper abdominal posture, POMT mutant pupae and adults experience abnormal contractions. In this chapter I will investigate the cellular POMT requirement for maintaining normal contraction patterning and posture.

Peristaltic contractions are controlled by a circuit in which the central nervous system (CNS) sends signals to the muscles, while the peripheral nervous system (PNS) returns information about the result to the brain, allowing the brain to determine what to do next. In the absence of PNS feedback, contractions soon halt entirely at both embryonic and larval stages (91). When PNS feedback is present but impaired, embryos experience abnormal contraction patterning and locomotion (76,91,92). Additionally, some studies have shown that Dg is O-mannosylated in the PNS (93,94), though the role of POMT functionality in the PNS as it relates to muscular dystrophy or other physiological abnormalities has not been well studied. Thus I hypothesize that POMT in the PNS facilitates feedback to the CNS and is required for proper contraction

patterning. I will therefore examine the role of POMT in the PNS and other tissues by ectopically expressing POMT in mutant backgrounds in various expression patterns. My results demonstrate that POMT is required in several tissues, including PNS, muscles, and possibly epidermis and CNS to maintain proper posture. Di Antonio previously showed that mutations in *dPOMT1* (*rt*) lead to defects in synaptic transmission at the neuromuscular junction, suggesting an additional requirement for POMT in the CNS (63). However, we were not able to duplicate these results in *dPOMT2* (*tw*) mutants, indicating that while POMT may be important in the CNS for other biological functions, it may not be directly involved in torsion. Thus while I do not rule out the necessity of a CNS component of POMT expression, here I will focus primarily on other cellular requirements.

I will first focus on the role of POMT in the PNS. I report that axons of class IV dendritic arborization (da) neurons experience morphological defects in *POMT* mutant backgrounds, and that these defects can be rescued by POMT overexpression limited to those neurons. These results are consistent with the role of class IV and other da neurons in proprioception, mechanosensation, and ultimately larval locomotion (95,96). I also demonstrate that compromising the PNS by inhibiting its development leads to abnormal embryonic contraction patterns and corresponding posture defects, as well as an aggravation of abdominal rotation in adult *POMT* mutants.

Interestingly, while I will show that ectopic POMT expression in muscle is not sufficient to rescue posture on its own, it is required for complete rescue. Occasional deranged and missing muscles were reported in *POMT* mutants, but these defects are not

fully penetrant, present at low frequency and not bilaterally biased (60,61,69), and thus it was previously concluded that the misalignment of body segments is unlikely to be caused by these muscle defects (69). Some forms of muscular dystrophy are associated with a stiffening of muscles (97-99), so I will examine muscle stiffness in POMT mutants in collaboration with Zhaokai Meng and Dr. Vladislav Yakovlev of the Biomedical Engineering department using a technique known as Brillouin spectroscopy. Here I report a correlation between muscle stiffness and rotation severity. In chapter IV I will demonstrate that stiffness does not cause rotation, however, suggesting that muscle stiffening may aggravate torsion, but is not the only underlying cause. The results in this chapter are shown for both embryonic and adult torsion, further suggesting a connection between the underlying mechanisms of the two stages.

3.2 Materials and Methods

UAS-GAL4 mediated rescue experiments and embryonic torsion scoring

In experiments investigating spatial requirements of POMT activity, the expression of a *UAS-tw* or a *UAS-rt* transgenic construct (71) was induced with each of the drivers described. Flies were scored for torsion at embryonic and adult stages, and for contraction patterning at the embryonic stage as described in chapter II. All driver-mediated rescue experiments included controls confirming that neither *GAL4* drivers alone nor *UAS-tw* or *UAS-rt* alone influence the mutant phenotype. All flies were reared at 25^{o}C in light and humidity-controlled environments as described previously (100).

Fluorescent staining and microscopy

Sens^{-} embryos were fixed, stained with Alexa-488 phalloidin, and imaged as described in chapter II. First and third instar larvae expressing GFP in the G14 expression pattern with the muscle component removed by MHC-Gal80 (101,102) were dissected, fixed, and stained with rabbit α-GFP in a 1:800 dilution. Subsequently they were stained with goat α-rabbit in a 1:250 dilution, and imaged.

For imaging of ventral ganglia, First and third instar larvae expressing tdTomato or tdGFP in the Ppk pattern (103), either in a wild-type, *tw*$^{-}$, or *rt*$^{-}$ mutant background, were collected. Brains were dissected, fixed in PBS with 4% paraformaldehyde, and stained with rabbit anti-dsRed or anti-GFP antibody (Clontech) in a 1:1000 dilution. Alexa-546 or Alexa-488-conjugated goat anti-rabbit was used as a secondary antibody in a 1:125 dilution. Stained brains were mounted on slides and imaged. To minimize potential errors, control and experimental samples were stained using the same master-mix of antibodies and imaged with the same settings for camera, illumination, and microscope. Commissural branch thickness was measured for every branch in a given brain using ImageJ and averaged, and the collective average from a given genotype reported. Longitudinal tracts were scored as "missing" if the region where a tract was expected to be was less than 10% brighter than the average background of the image.

Digital images were obtained using a Zeiss Axioplan 2 fluorescent microscope with the ApoTome module for optical sectioning. AxioVision and ImageJ software was used for 3D reconstruction and Z-projections of fluorescent samples.

Brillouin spectroscopy

Third instar larvae were dissected dorsally and immediately mounted on slides in ice cold PBS. Embryos were mounted whole. Mounted samples were analyzed by a background-free Brillouin spectrometer developed by our collaborators in biomedical engineering (104-106). The Brillouin peak was detected by a CCD camera with a 60 s exposure time. Since the Stokes peaks were overlapped with an absorption band of the iodine cell used in the optical setup, they could not be measured. We therefore assessed the shift of anti-Stokes peaks only.

3.3 Results

POMT function is required in muscles, neurons, and possibly epidermis for proper body posture

Since coordination of muscle contractions is partially controlled by PNS cells (91,92), we decided to investigate the role of PNS neurons in the embryonic muscle misalignment phenotype. To test the requirement for POMT function in different tissues, we applied a rescue strategy using UAS-GAL4-driven expression of a transgenic *tw* or *rt* construct in *tw* and *rt* mutants, respectively. Because simultaneous expression of MHC-GFP in these embryos proved difficult, we instead scored torsion by the angle of tracheae relative to the eggshell axis of symmetry (Fig. 18 A). Similar to measurements of muscle midline, tracheae in wild type embryos all ran essentially parallel to the eggshell axis, while nearly all tracheae in tw embryos experienced apparent clockwise torsion at the posterior (Fig. 18 B). Adult torsion was scored quantitatively by measuring

the angle of the most posterior segment (A8) relative to the thorax axis of symmetry (See Fig. 4).

In embryos we found that a general neuronal driver (ELAV-Gal4) and a driver specific for class IV da sensory neurons (Ppk-Gal4) were able to significantly rescue the torsion phenotype, while three different muscle-specific drivers that we tested (DMEF2-GAL4, MHC-GAL4, and HOW-GAL4) could not mediate rescue (Fig. 18 B) (76,107-109). These results indicated that POMT function is required in neurons, and specifically in class IV da sensory neurons, which supported our hypothesis that POMTs function in the PNS to ensure a proper feedback from these cells to maintain normal body posture.

Interestingly G14-gal4, a driver normally associated with muscles (110), was able to fully rescue torsion (Fig. 18 B). G14 was also able to mediate full rescue of abdominal torsion in both tw^{-} and rt^{-} adults (Fig. 18 C-D), indicating that a similar POMT expression pattern may be required for proper posture in both embryos and adults. However, neither neuronal drivers nor muscle drivers alone (with the exception of G14) were sufficient for even partial rescue in adults, indicating some difference in the mechanisms underlying each stage, a possibility which I will consider further in the discussion section.

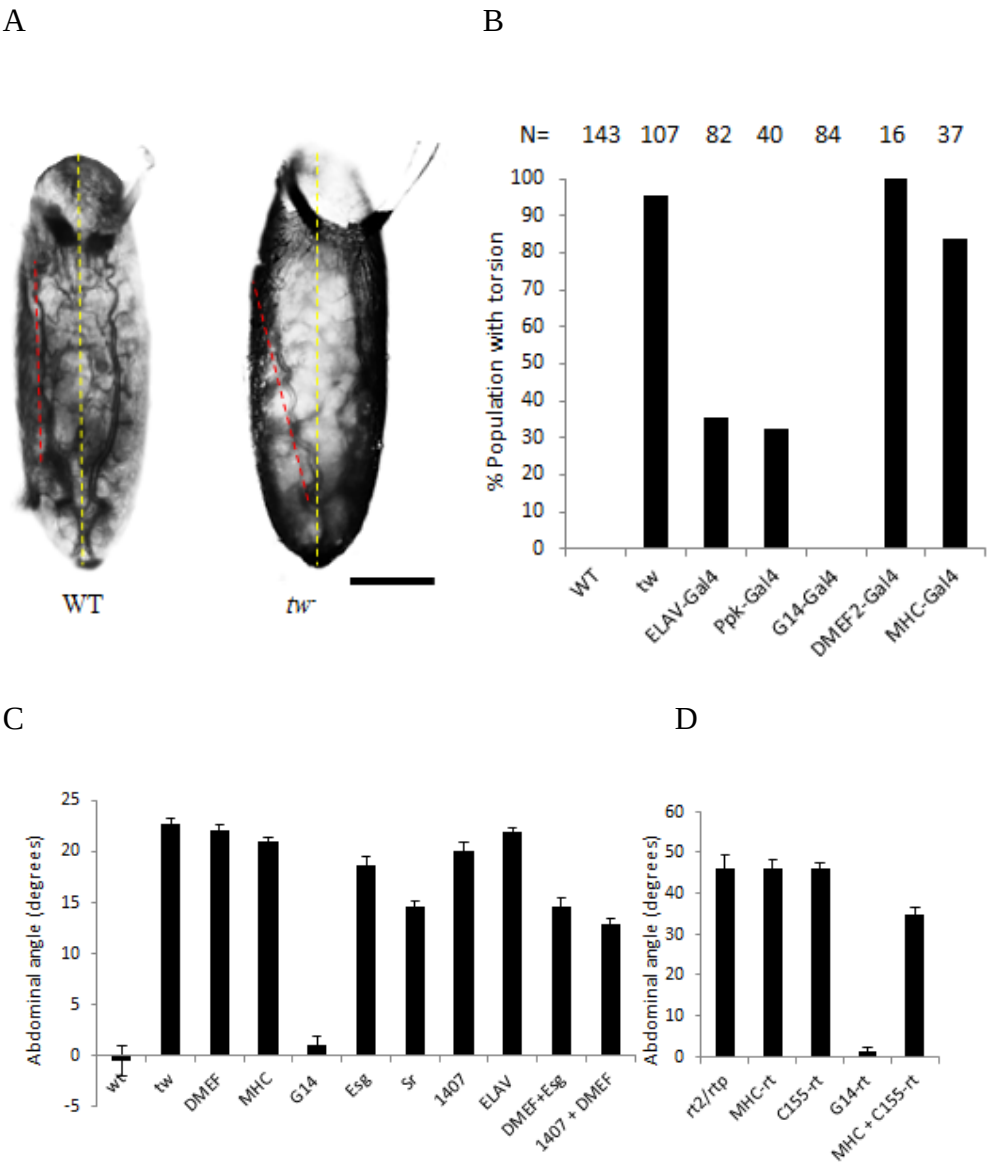


Figure 18—Rescue of torsion. Data obtained in collaboration with Dr. Dmitri Lyalin and Dr. Nao Nakamura.

A) depicts a method of scoring embryonic torsion using tracheae. Yellow line is eggshell axis of symmetry, red line follows right trachea. B) shows percentage of embryos with torsion when UAS-tw is expressed in a *tw*$^{-}$ background by various drivers. All embryos were *tw* mutants expressing UAS-tw, but only drivers are listed. The bar labeled "tw" had no driver. C) and D) show rescue in adults for both *tw*$^{-}$ and *rt*$^{-}$. As in B), only drivers are listed. N is at least 20 flies per genotype. Error bars are SEM. Drivers could not rescue in the absence of UAS-POMT, nor could UAS-POMT in the absence of a driver. Scale bar is 100 um.

Because G14 is known to be expressed in muscles, and because neuronal drivers could mediate only partial rescue in embryos and no rescue in adults, we hypothesized that POMT expression in muscles may be necessary in combination with other tissues for full rescue. We further hypothesized that G14 may have a neuronal component of expression that was previously unknown, and that helps account for the full rescue observed.

To test whether G14 is indeed expressed in neurons in addition to muscle, we induced expression of GFP using G14-gal4, and blocked muscle expression using MHC-gal80. Both first and third instar larvae were dissected, fixed, stained with anti-GFP, and imaged to approximate expression conditions at the critical developmental stages for torsion (embryonic and late third instar (71)). In both cases we found that G14 is expressed in PNS cells including chordotonal organs and, importantly, multidendritic neurons, though the neurons we observed appeared to be class I rather than IV. I could not detect overlap between GFP expressed in the G14 pattern and tdTomato expressed in the Ppk pattern, further suggesting that the two drivers work in different da neurons (not shown). We also found that G14 was expressed in histoblast nests at the third instar stage (Fig. 19). There was also a CNS component of expression (not shown). Taken together, the data so far indicate roles for POMT in muscle, PNS neurons including class IV da neurons, and possibly epidermal cells and CNS neurons. Here I will focus primarily on the former two.

We attempted to reconstruct G14-mediated rescue by using combinations of drivers to induce simultaneous POMT expression in muscles and neurons in adults. We

found that while muscle or neuronal drivers alone were incapable of mediating rescue, a combination of the two was able to mediate partial rescue (see Fig. 18 C-D). Further screening revealed that drivers typically associated with epidermal expression were able

A B

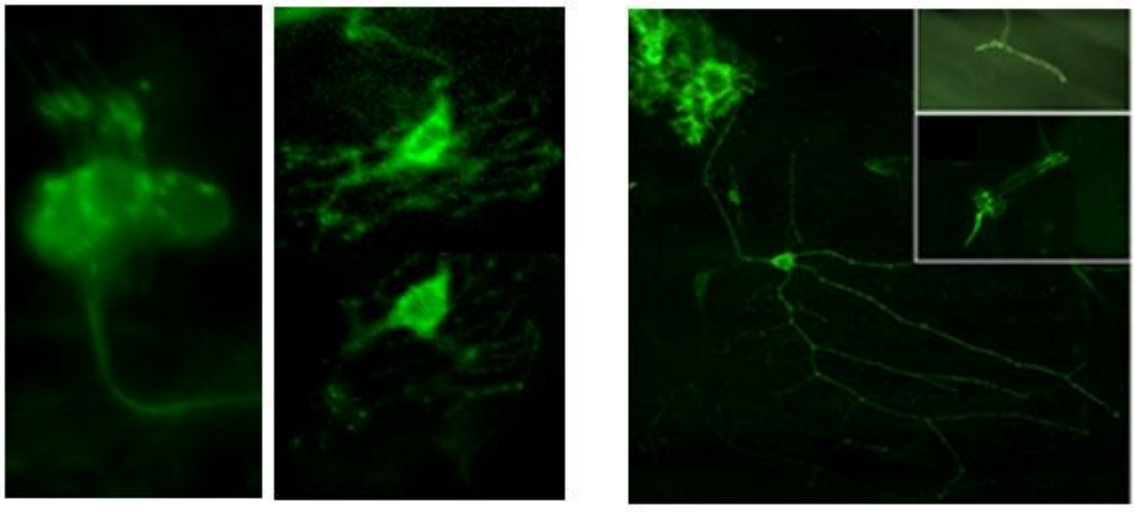


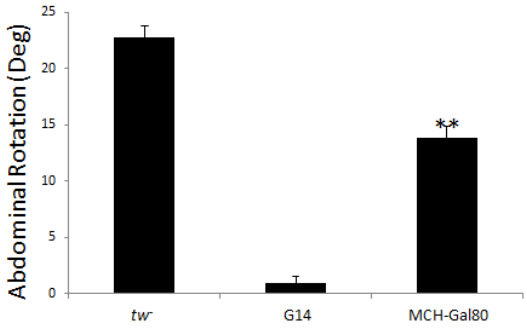
Figure 19—G14 non-muscle expression pattern. Data obtained in collaboration with Dr. Dmitri Lyalin and Michelle Alfert.

A) depicts chordotonal organ (left) and multidendritic neurons (right) in first instar larvae, while be depicts histoblasts and MD neurons (main box, histoblast is in top left corner) and chordotonal organs as well as a motor neuron (insets, bottom and top, respectively) in third instar larvae

to contribute to rescue as well (see Fig. 18 C). However, these epidermal drivers included neuronal components (111)(personal communication from D. Lyalin), so it is not possible to rule out the additional contribution to neuronal POMT expression as the reason for increased rescue. We also found that removal of the muscle or neuronal

component of G14 expression using MHC-gal80 or ELAV-gal80, respectively, significantly inhibited rescue (Fig. 20), demonstrating the importance of POMT in both muscles and neurons for posture maintenance.

A



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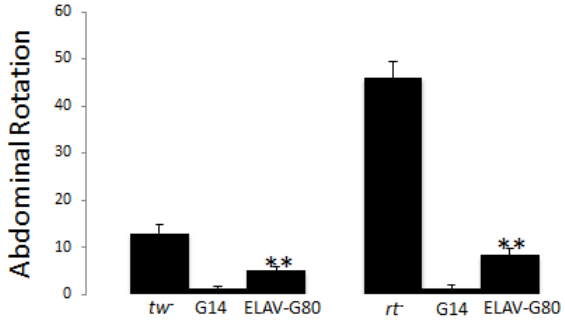


Figure 20—Muscular and neuronal components of G14-expression are necessary for rescue. Data obtained in collaboration with Dr. Dmitri Lyalin.

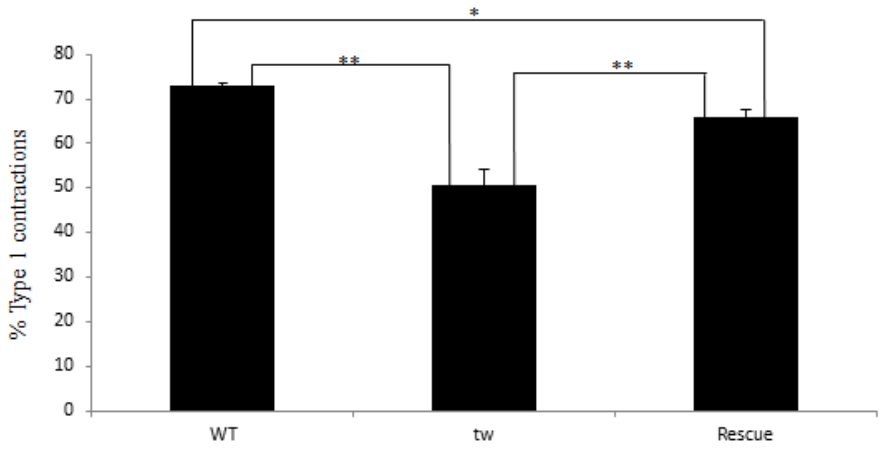
A) Suppression of G14-mediated rescue by removal of the muscle component. B) Removal of neuronal component. "G14" indicates that G14-mediated UAS-POMT expression is in place, and "ELAV-Gal80" or "MHC-Gal80" indicates addition of that construct to everything that was previously in place. Error bars are SEM. Asterisks represent statistical significance of rescue suppression relative to G14-rescue. N is at least 20 flies per genotype.

To further confirm the role of POMT in class IV da neurons, I scored the contraction pattern of *tw*$^{-}$ embryos with ectopic expression of UAS-tw in the Ppk expression pattern. The percentage of type 1 contractions in these embryos was between that of wild type and *tw* (Fig. 21 A), demonstrating rescue of contraction pattern as well as posture (see Fig. 18 B), and further confirming the relation between the two phenomena. I also attempted to induce embryonic torsion in a wild type or *tw* heterozygous background by expressing an RNAi against *tw* in the Ppk expression pattern. This approach failed to alter the contraction pattern from that of wild type or induce any torsion in embryos or adults (not shown). However, expression of the same RNAi in class IV da neurons of *tw* mutants was able to significantly aggravate the rotation phenotype (Fig. 21 B). Since our *tw* allele is a hypomorph, this result suggests that the RNAi was able to knock down residual tw activity in da neurons and thus increase phenotypic severity.

Defects in PNS neurons lead to abnormal contraction patterns and posture

My data so far have demonstrated a requirement for POMT in several tissues to maintain proper posture. Herein I will examine potential biological roles for different cell types. First, I examined the role of PNS neurons in embryonic and adult torsion. If the PNS is important in embryonic posture, then inhibiting PNS development should result in embryonic torsion. Thus to confirm the intrinsic importance of PNS neurons in maintaining proper body posture, I examined torsion phenotypes in senseless mutants (*sens*$^{-}$). *Sens*$^{-}$ embryos lose most of the peripheral sensory neurons by embryonic stage 16 through cell-specific apoptosis (112), and thus the feedback from the PNS to the CNS

A



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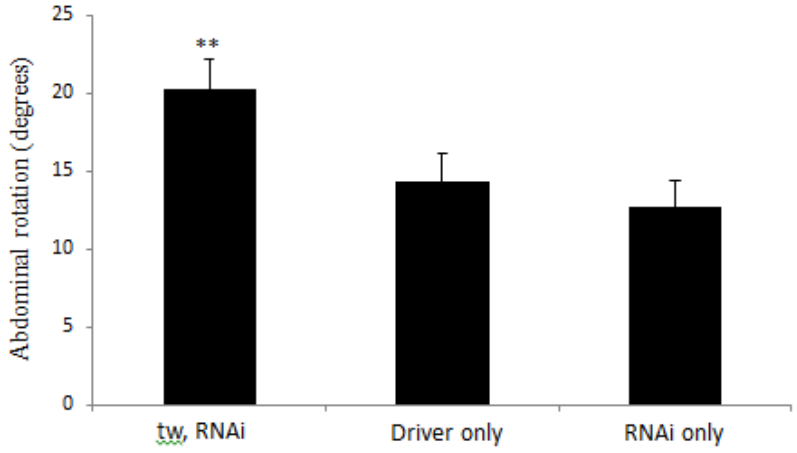


Figure 21—Class IV da neurons in torsion.

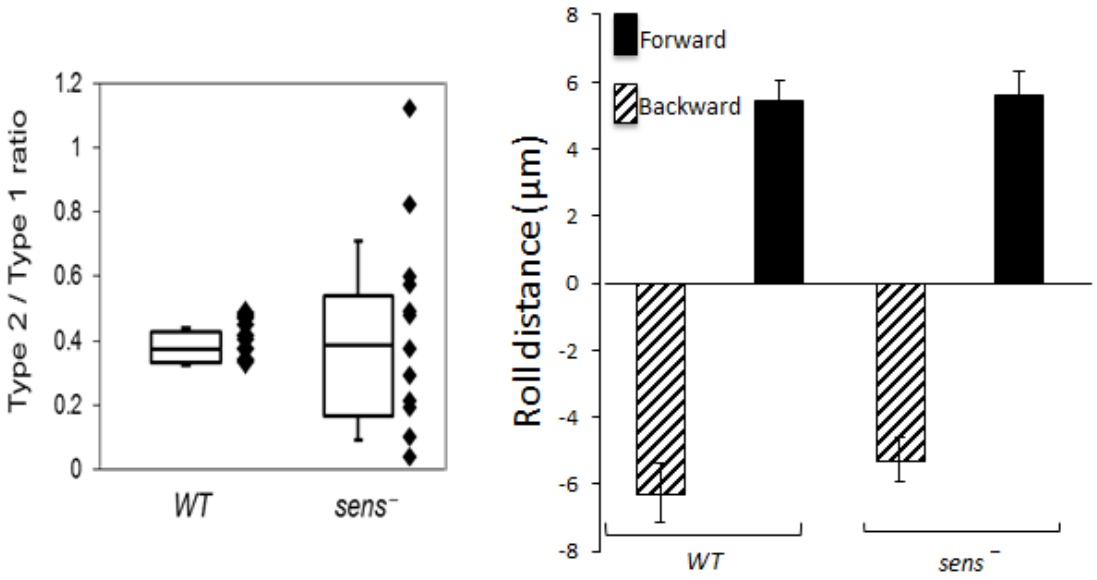
A) Contraction patterning in wild type embryos, *tw*$^{-}$ embryos, and *tw*$^{-}$ embryos expressing UAS-tw in class IV da neurons ("rescue"). N = 10 embryos for wt and *tw*$^{-}$, 8 for rescue, and at least 120 total contractions per genotype. B) Effect of RNAi against *tw* in class IV da neurons in a *tw*$^{-}$ background compared to *tw*$^{-}$ with Ppk-gal4 or RNAi only ("Driver only" and "RNAi only" respectively). Error bars are SEM. tw, RNAi is significantly different than either control. N is at least 20 flies per genotype

is impaired in these mutants. *Sens*$^{-}$ embryos are still able to generate contraction waves, presumably due to the fact that some dendritic arborization (da) sensory neurons are still present in these embryos, though the frequency of waves is significantly affected (91,92). I analyzed *sens*$^{-}$ mutants and found that they generate apparently normal type 1 and type 2 contraction waves, but the pattern of waves is highly variable in these embryos. Whereas wild type embryos stay within a type 2/type 1 ratio range of ~0.35-0.55, in sens mutants the ratio ranges from 0 to ~1.1 (Fig. 22 A). Despite these differences in the pattern of wave generation, our analysis confirmed that overall characteristics of individual type 1 and type 2 waves in *sens* mutants are essentially the same as in wild type embryos, including the effects of these waves on rolling and body posture (Fig. 22 B-C). Interestingly, *sens*$^{-}$ mutants did manifest backward type 2 contractions, which correlated with clockwise torsion (Fig. 22 C).

Considering the variability of contraction patterns in *sens* mutants, our model in chapter II predicts that some embryos should accumulate clockwise torsion (embryos with substantially fewer than 70% of waves being type 1, or a type 2/type 1 wave ratio significantly above ~0.33, as in the case of POMT mutants), some should acquire a counterclockwise torsion (type 2/type 1 wave ratio significantly below ~0.33, or much greater than 70% type 1 waves), and yet other embryos should have no torsion (type 2/type 1 ratio is roughly equal to ~0.35, as in wild type). Indeed I found that all three types of torsion existed within the *sens*$^{-}$ population, with ~40% of embryos twisted clockwise, ~40% twisted counterclockwise, and ~20% showing no torsion (Fig. 23 A-B). These data indicated that PNS cells participate in generating the proper pattern of

A

B



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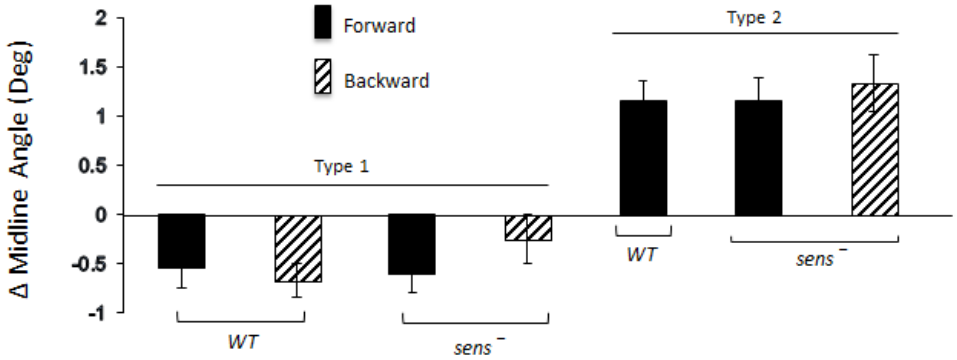
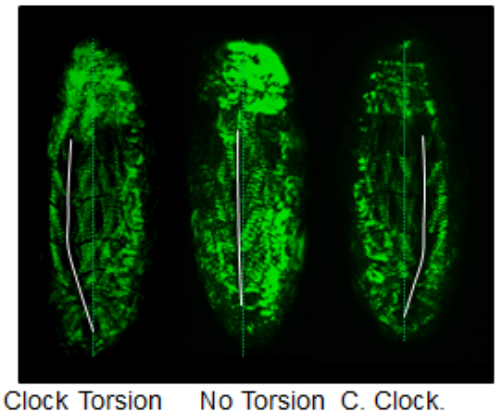


Figure 22—Contraction pattering in *senseless* mutants.

A) Ratio of type 2 to type 1 contractions has the same average in WT and *sens*$^{-}$ embryos, but *sens*$^{-}$ is more variable (black diamonds represent individual embryos). B) Rolling compared to contraction direction C) Changes in midline angle, which are the same for individual contractions in both *sens*$^{-}$ and wild type. There were no significant differences within given contraction types. N = 12 flies per genotype and at least 20 contractions per wave type.

A



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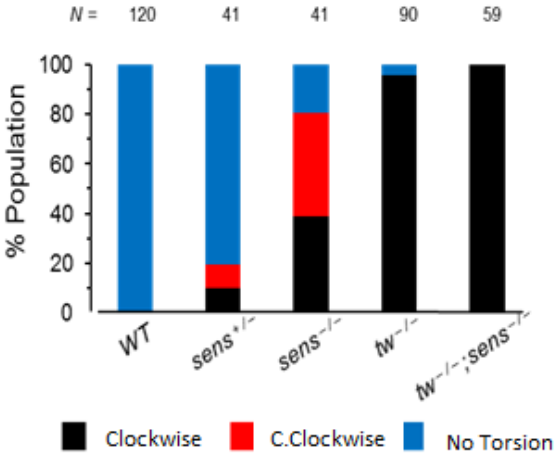

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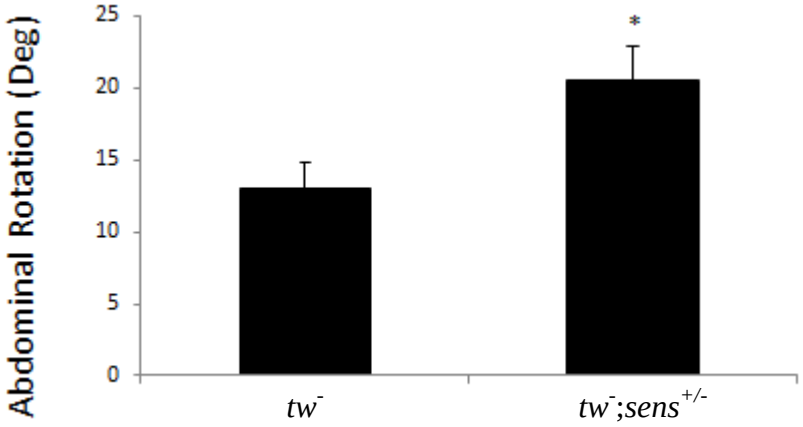


Figure 23—Torsion in *senseless* mutants.

A) Representative images of torsion (or lack thereof) in both directions in *sens*$^{-}$ mutant embryos. B) Quantitative data regarding torsion in various genotypes. C. clock = "counterclockwise." C) Abdominal rotation in adult *tw*$^{-}$ flies heterozygous for *sens*$^{-}$ releative to tw mutants alone. Error bars are SEM. N = at least 20 flies per genotype.

alternating type 1 – type 2 contractions, and corroborates the hypothesis that these contractions influence body posture. In further experiments, I analyzed *tw–sens* double mutants and revealed that *tw* is epistatic to *senseless*, as all double mutant embryos were found to have torsion in the clockwise direction (Fig. 23 B). Although mutants homozygous for *sens*$^{-}$ do not survive past the embryonic stage, I also assessed the effect of a single defective copy of *sens* on *tw* mutants and found a genetic interaction in the adult stage as well (Fig. 23 C).

Since da cells represent the only type of peripheral sensory neurons present in *sens* mutants (92,112), this result suggests that downregulation of POMT activity in da neurons is sufficient to generate an abnormal feedback from these cells to the CNS and affect muscle contractions in *tw-sens* double mutants. However, these cells do not suffice to maintain proper contraction patterning in *sens* mutants, presumably because normal contractions also require other PNS cells that are missing in *sens* mutants (76,92). Taken together, these results suggest that PNS neurons, while normally supporting the proper pattern of contraction waves, generate an aberrant feedback to the CNS in POMT mutants. This abnormal feedback affects the pattern of muscle contractions, thus underlying the pathogenic mechanism of the torsion phenotype.

POMT mutations cause morphological defects in PNS neurons, leading to aberrant contraction signaling

Since POMT expression in the PNS is required for proper contraction patterning and posture, I next focused on the morphology of class IV da neurons that develop characteristic laminar axonal projections in the ventral ganglion during embryogenesis

(113). To do this, I expressed the fluorescent protein tdTomato (114) or tdGFP (115) in the Ppk expression pattern in wild type, *tw*$^{-}$, and *rt*$^{-}$ embryos. Brains were dissected from recently hatched first instar larvae and from late stage third instar larvae, and axonal connections from class IV da neurons to ventral ganglia were imaged. I found that in both *tw* and *rt* mutants, commissural branches display significant thickening relative to their wild type counterparts in both developmental stages, and that *rt* mutants also have several missing longitudinal tracts (Fig. 24). Branch thickness could be rescued by ectopic POMT expression in the Ppk pattern, expression of which was also able to partially rescue torsion and contraction patterning in embryos (see Figs. 18, 21). These data suggest that aberrant PNS morphology may, in this case, be linked to abnormal signaling, which may in turn give rise to the abnormal contraction pattern observed in mutants.

POMT mutations cause muscle stiffness, which may aggravate but not cause torsion

My previous evidence indicated that POMT expression in muscle cells also plays a role in *Drosophila* torsion. To explore this further, I focused first on third instar larval muscle. Previous work has shown that larval muscles in POMT mutants experience morphological defects including missing, split, thin, abnormally attached, and damaged muscles (60,61,69). We examined muscles in mutant larvae and found that while

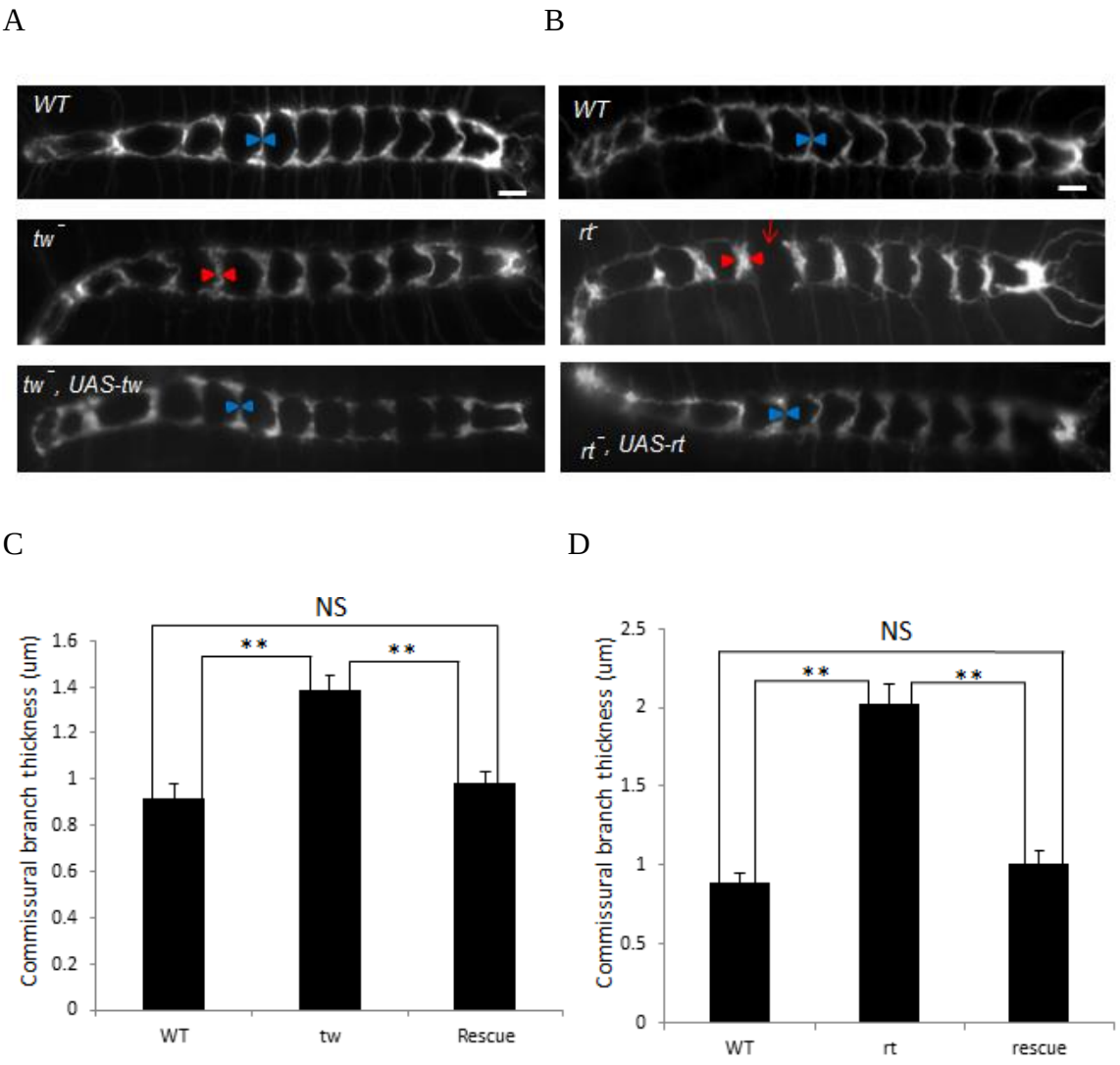


Figure 24—POMT effect on class IV da neuron axonal morphology. Data obtained in collaboration with Ishita Chandel.

A and B show labeled axons of class IV da neurons in ventral ganglia. UAS-tw and UAS-rt were driven by Ppk-gal4, specific to those neurons, for rescue. Red arrowheads indicate examples of thickened commissural branches. Blue arrowheads show normal thickness for corresponding branches in wild type. Red arrow indicates missing longitudinal tract. C and D show quantitative data regarding branch thickness. N = at least 10 embryos per genotype. Error bars are SEM.

E

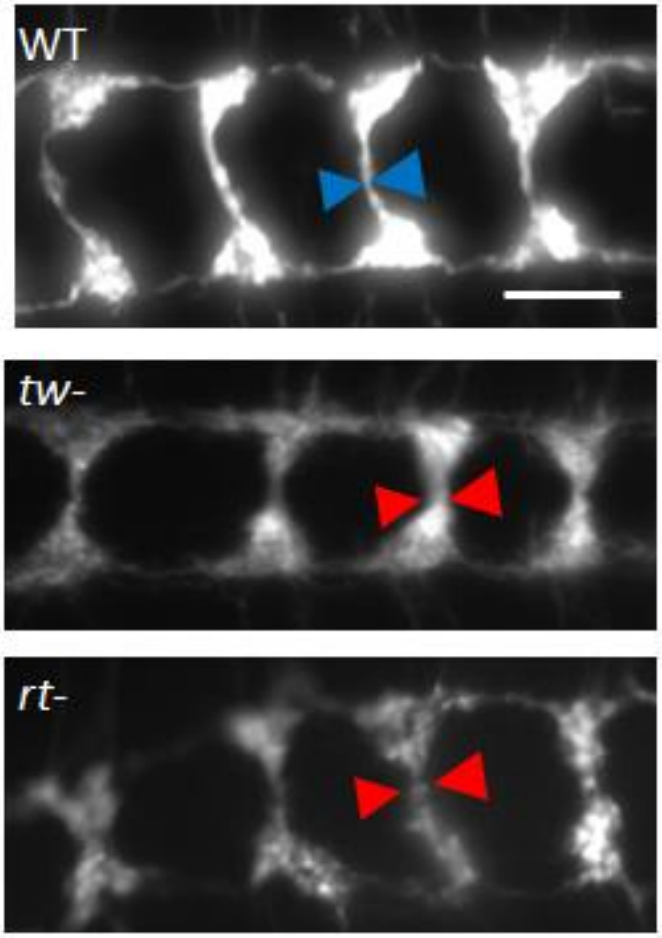


Figure 24—Continued.

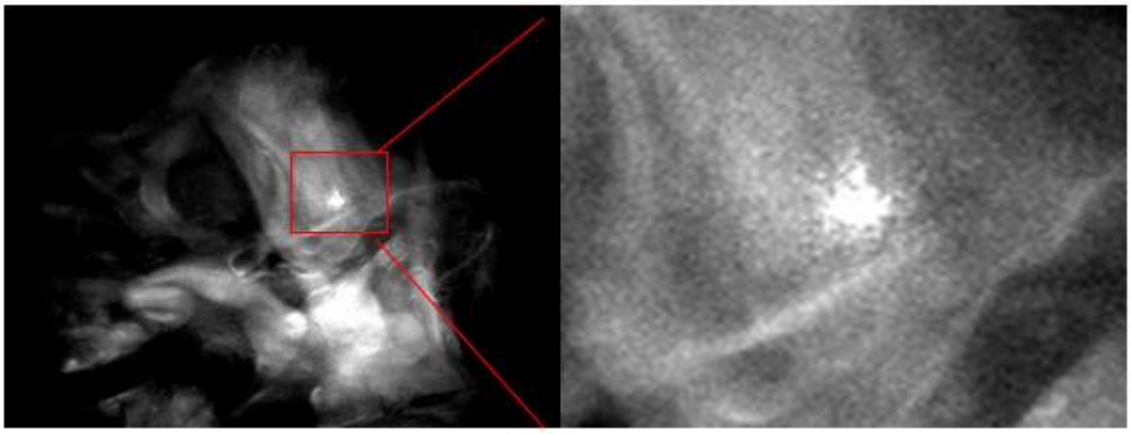
Third instar larval brains show similar defects of axonal morphology in *POMT* mutants as first instar larvae.

there are indeed such defects in POMT mutants, in our experiments we could detect similar levels of defects in wild type larvae (not shown). The lack of obvious gross morphological defects suggested that something more subtle may be at play in muscles. Various forms of muscular dystrophy are associated with muscle stiffness (97-99), so I hypothesized that a similar stiffening may occur in *Drosophil*a POMT mutants. Such stiffening may decrease the organism's ability to properly sense posture, and could thus aggravate torsion phenotypes.

To assess muscle stiffness in *Drosophila* we used Brillouin spectroscopy. Brillouin spectroscopy is a powerful tool for microscopic, non-invasive material characterization. It provides unique information on viscoelasticity properties and is widely used in remote sensing, material science and, more recently, biomedical applications (106,116,117). Brillouin scattering originates from the inelastic interaction between the incident light and the "acoustic phonons" in the material of interest (118). The incident light experiences a shift in frequency proportional to the speed of sound in the medium. Thus the medium's elastic modulus, which is also directly related to the speed of sound in the medium, can be determined by measuring the Brillouin shift. A greater shift corresponds to decreased elasticity (or increased stiffness).

I collected wild type, *tw*$^{-}$, and *rt*$^{-}$ third instar larvae expressing MHC-GFP and dissected their muscles. In collaboration with the biomedical engineering department, I measured the elasticity of the muscles in each genotype, first using MHC-GFP to ensure that incident light was focused on muscles (Fig. 25 A). We found that the Brillouin shift was greater in tw muscle than in wild type, and greater still in rt muscle (Fig. 25 B-C).

A



B C

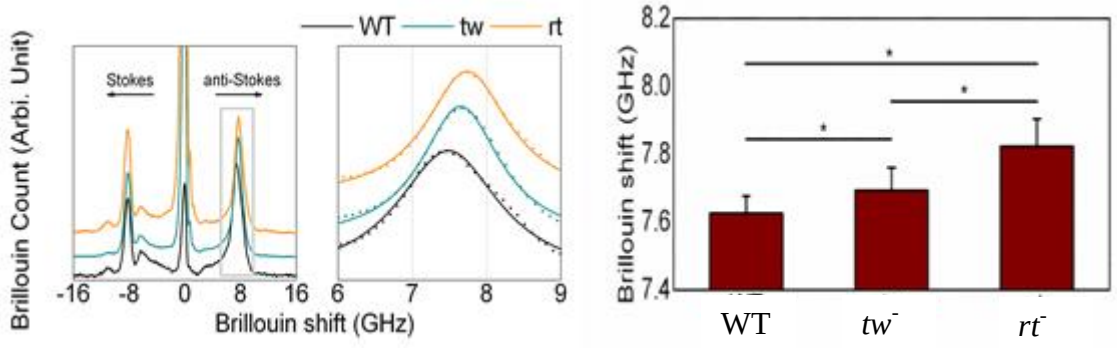
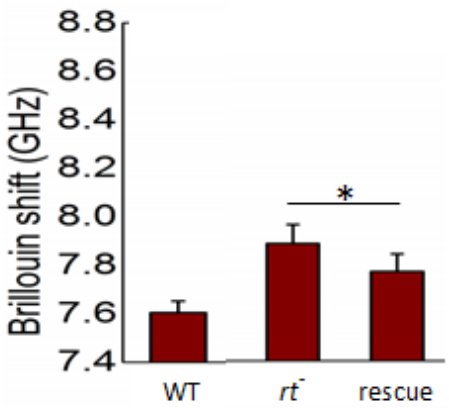


Figure 25—POMT effect on larval muscle stiffness. Data obtained in collaboration with Dr. Zhaokai Meng.

A) Representative example of fluorescent larval muscle exposed to Brillouin laser. B) Typical Brillouin shifts for WT, *tw*$^{-}$, and *rt*$^{-}$ muscle. C) Average Brillouin shift for each genotype. *tw* mutants have stiffer muscle than wild type, and *rt* mutants have stiffer muscles than *tw* mutants. N = 10 larvae per genotype, error bars are SEM.

D



E

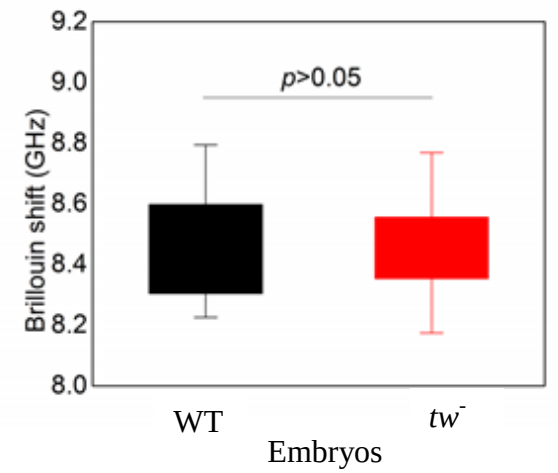


Figure 25—Continued.

D shows rescue of muscle stiffness in *rt*$^{-}$ mutants by UAS-rt driven by the muscle driver How-Gal4. E shows that *tw*$^{-}$ embryos are no stiffer than WT. We have not been able to gather data on *rt*$^{-}$ embryos. N = 5 larvae per genotype in D, 10 embryos per genotype in E.

Similar results were obtained for non-fluorescent larvae (not shown). Interestingly, stiffness correlates with the severity of torsional angles in adult abdomens, since *rt* mutants are significantly more severely twisted than *tw* mutants (75), suggesting a potential connection between stiffness and torsion. Stiffness could be partially rescued by ectopic POMT expression in muscles using a How-Gal4 driver (Fig. 25 D). *Tw*$^{-}$ embryonic muscle, on the other hand, was no stiffer than its wild type counterpart (Fig. 25 E). This may help explain why there is no notable difference in torsion between *tw*$^{-}$ and *rt*$^{-}$ embryos, and also why neuronal drivers by themselves are able to partially rescue torsion in embryos but not adults.

3.4 Discussion

In chapter II I proposed a model in which embryonic contraction wave patterning (and possibly patterning at the pupal stage) dynamically governs posture based on some chiral interaction between cuticle and egg or pupal shell. The torsion phenotype arises due to alteration in the pattern of contraction waves, while the effects of individual waves on posture are not significantly altered in *POMT* mutants. These data suggest that the torsion phenotype is caused primarily by a neurological abnormality, though in this chapter I also show that body posture is governed at least in part by POMT expression in other tissues, including muscles and possibly CNS neurons and epidermal cells.

First, I demonstrated that the proper pattern of embryonic contraction waves requires a PNS-mediated feedback that relays information on muscle contractions and body posture to the CNS. I demonstrated this in several ways. First, I showed that ectopic expression of POMT in class IV da neurons is sufficient to partially rescue embryonic torsion. I simultaneously demonstrated that the contraction pattern in rescued mutants is closer to wild type than that of *tw* mutants in the absence of Ppk-Gal4-mediated rescue (Fig. 18, 21). Thus it appears that the pattern of waves depends on POMT activity in the nervous system, including class IV sensory da neurons. I also showed that while POMT expression by neuronal drivers is insufficient to rescue adult torsion, it does mediate partial rescue in the presence of muscle drivers (Fig. 18). Indeed, the G14 driver, which expresses in both muscles and neurons, as well as histoblasts, can mediate full rescue of torsion at any stage (Fig. 18-19). Thus while the underlying physiology of adult torsion may have a stronger requirement for POMT expression in

muscles, it appears that there is a similar biological mechanism governing the rise of torsion at both embryonic and adult stages.

To determine whether the effect of POMT in class IV da neurons was cell autonomous, I expressed RNAi against *tw* in both wild type and tw^{-} heterozygote backgrounds. This experiment had no effect on contraction patterning in embryos or on torsion in embryos or adults. These data alone are consistent with a few potential hypotheses. It is possible that RNAi failed to induce phenotypes even if a true knockout of POMT in class IV da neurons would have sufficed. For example, maternal contribution of POMT, persistence of maternally contributed O-mannosylated targets or of targets that were O-mannosylated before RNAi expression was sufficiently induced, or presence of POMT in other cell types that could compensate for class IV da neurons may all explain this result. The latter option posits that the effect of POMT is cell non-autonomous. In that case expression of POMT in Ppk neurons could rescue the *tw* phenotype by compensating for loss of function in other cells. Consistent with this hypothesis is the fact that the G14 neuronal expression pattern appears to include class I da neurons and chordotonal organs (see fig. 19) but not class IV neurons. Conversely, class I da neurons and/or chordotonal organs may not normally be involved in body posture but compensate for the lack of POMT in class IV neurons when POMT is expressed in the G14 pattern. Additionally, all three classes of neurons or some combination thereof may normally express POMT, and expression in any of them could be sufficient to mediate at least partial rescue in embryos. However, since Ppk-mediated expression of RNAi in *tw* mutants is sufficient to aggravate rotation, I submit that POMT

expression in class IV da neurons is directly involved in contraction patterning and posture. This does not rule out the involvement of POMT in other PNS neurons such as those seen in the G14 expression pattern.

Next, I posited that in *POMT* mutants, sensory cells generate an aberrant signal to the CNS, which results in the observed abnormal pattern of contraction waves and leads to the embryo torsion phenotype. The analyses of *sens* mutants and interactions between *sens* and *tw* highlighted the role of PNS cells in the etiology of the body torsion phenotype. My experiments demonstrated that a defect in specification of PNS neurons alone could cause muscle contraction and body posture phenotypes similar (though not identical) to those in *POMT* mutants (Fig. 22-23). In this case, torsion could accumulate in either direction, consistent with contraction patterns that ran the spectrum from type 1-heavy to type 2-heavy. This suggests that in these mutants the PNS sends limited or delayed feedback to the CNS, causing the CNS to essentially "guess" which type of torsion is being experienced and how to compensate appropriately. In POMT mutants, on the other hand, the contraction pattern is always type-2 wave-heavy and torsion is always clockwise as viewed from the posterior. This suggests that rather than sending limited feedback to the CNS, the *POMT*⁻ PNS sends incorrect feedback, sensing or conveying counterclockwise torsion when no torsion or even clockwise torsion is present. Epistatic interaction between *tw* and *sens* also suggested that da sensory neurons, which remain partially intact in *sens* mutants, are sufficient to propagate the incorrect feedback generated by the PNS lacking *POMT* activity. Although senseless mutants die prior to adulthood, preventing analysis of senseless-mediated torsion in

adults, I was able to observe an increase in torsion in *tw* mutants heterozygous for *sens*$^{-}$ (Fig. 23), further indicating a mechanistic connection between embryonic and adult phenotypes.

Finally, with regard to PNS neurons, I examined the morphology of axonal connections between class IV da neurons and the CNS and found that *tw* and *rt* are required for normal axon patterning of class IV da neurons (Fig. 24). Significantly thickened commissural branches were observed in all *POMT* mutants at both early and late larval stages, again corroborating the hypothesis that torsion rises in early and late stages via similar mechanisms. This phenotype, as well as the phenotype of missing longitudinal tracts in the more severely affected *rt* mutants, could be rescued by Ppk-mediated POMT expression. This further suggested that POMT expression, while likely functionally important in other cell types as well, is both expressed and functionally important in class IV da neurons. These data led me to propose a model in which aberrant connectivity between sensory and CNS neurons may underlie the mechanism of PNS-generated abnormal feedback in *POMT* mutants (Fig. 26). This role of da neurons in the POMT-mediated effect on muscle contractions and body posture is consistent with the proposed role of these polymodal neurons in proprioception and mechanosensation, and their involvement in regulation of larval locomotion (95,96,119,120).

As important as the PNS is in body posture, it is clear that other tissues are also involved in POMT-related torsion, as even a pan-neuronal driver is not sufficient to mediate full rescue at any developmental stage, while removal of the muscle component from G14 suppresses rescue (Fig. 18, 20). The possibility of CNS and epidermal

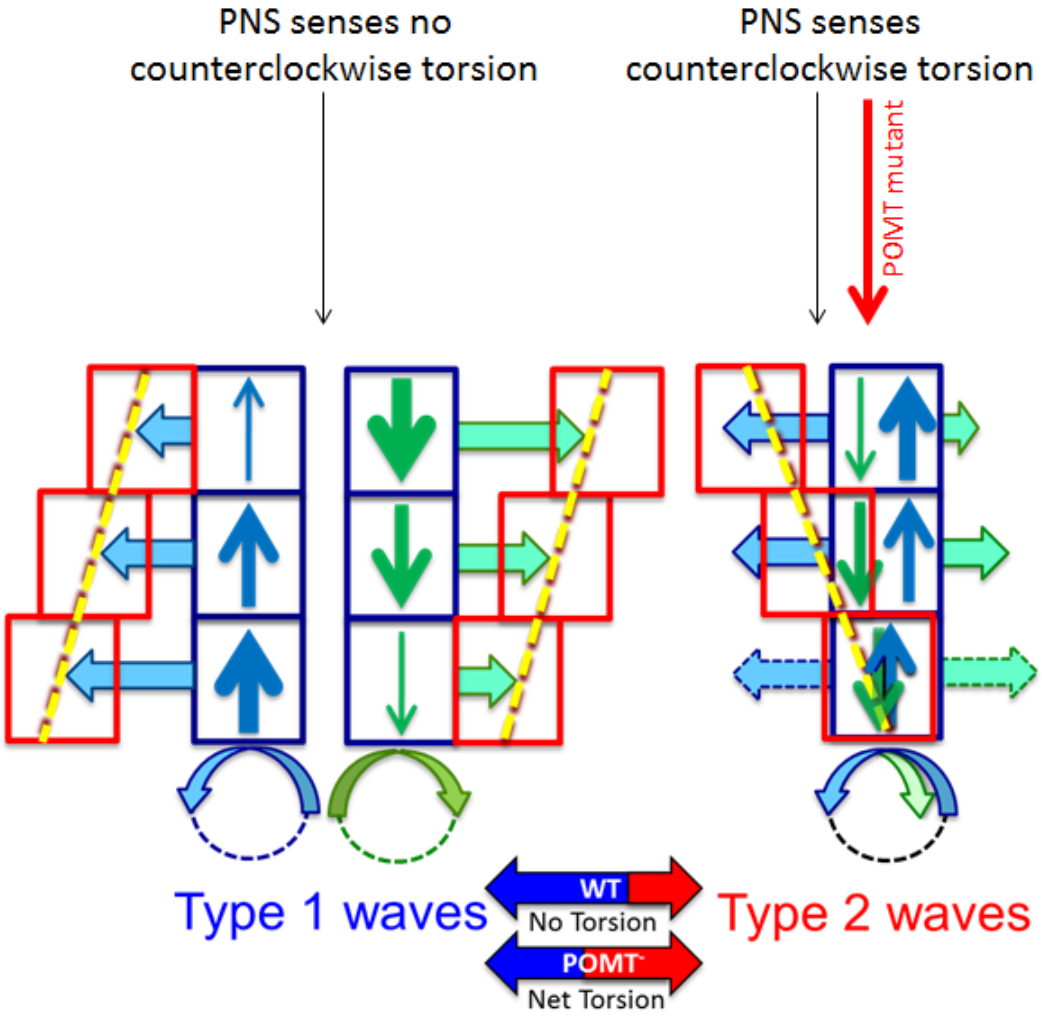


Figure 26—Updated model of torsion.

Added to the model is the idea that POMT mutations alter PNS-CNS connections, causing aberrant feedback to the CNS and a subsequent abnormal patterning of contraction waves.

involvement in torsion are interesting and not without merit. Indeed WWS patients are known to have CNS defects, and similar phenotypes have been observed in fruit flies. However, my data suggest that the individual contraction waves generated by *POMT* mutants do not have significantly different characteristics from their wild type counterparts, and the CNS defects seen in *rt* mutants did not appear to be present in *tw* mutants. Thus it seems that in POMT mutants the CNS is capable of sending normal signals to the muscles, and that the known CNS defects in POMT mutants are not required for torsion to occur. It is possible, however, that the CNS defects in *rt* mutants partially explain the more severe torsion observed in adult *Drosophila* relative to *tw* mutants. Additionally, POMT in the CNS may aid PNS axons in forming proper connections with CNS cells.

POMT expression may be necessary in the epidermis for proper posture, since drivers classically associated with epidermal cells could, in some cases, mediate partial rescue on their own. Additionally, epidermal drivers in combination with muscle drivers were able to increase rescue. Consistent with the idea of epidermal involvement, some recent studies have reported that dendrites sense stretching of the epidermis, are connected to epidermal tissue, and that laminin is required for correct patterning of dendrites in da neurons (114,121). If laminin must interact with O-mannosylated Dg on the surface of epidermal cells for proper dendritic connections to occur, for example, it may be that POMT is required in both da neurons and epidermis for correct stretch sensation. As intriguing as this possibility is, so far we could observe no morphological defects in the dendrites of class IV da neurons (not shown), and all epidermal drivers

used in this study had other components of expression as well, including neuronal components. Thus while there is a strong possibility of epidermal involvement, further studies will be needed to confirm or falsify this hypothesis.

Whatever the influence of CNS and epidermis on torsion, it is clear that POMT expression in muscle is involved. Previous studies have indicated a number of muscle defects in *POMT* mutant *Drosophila* (60,61,69). However, these results have not been definitively linked to torsion. Furthermore, our observations suggested that under certain conditions, these phenotypes are no more prevalent in mutants than in wild type larvae (not shown), yet mutant larvae invariably develop rotated abdomens as adults. This led us to propose that the defects observed in other studies, while most likely real, are probably a result of muscle torsion rather than a cause. This is supported by the fact that the torsion in first instar larvae is only observed when larvae are killed and their muscles forced to relax (see Fig. 8). This suggests that while alive, larvae may exert muscle strength to force the twisted cuticle into a straight posture. Under particular conditions of food composition, genetic background, and/or population density, muscle damage may result.

Taken together, our data show that muscles develop normally in *POMT* mutants from a morphological standpoint, and experience torque over time. Since we have shown that proprioception is required to maintain correct contraction patterning and posture, we hypothesized that POMT expression in muscles might be required to facilitate proper stretch sensing by PNS neurons. It is possible, for example, that some dendrites interact with muscle cells either directly or indirectly through the ECM. In either case, a laminin-

Dg interaction or some other interaction involving O-mannosylated protein may be required. Whether this particular mechanism is involved in torsion will require further study. It is known, however, that dystrophic muscle is frequently stiffer than wild type (97-99). I thus reasoned that stiffening of muscle in POMT mutants could be involved in torsion. Stiff muscle may interact differently with PNS neurons than normal muscle, and may contribute to the perception of counterclockwise torsion when there is none. Brillouin spectroscopy revealed that indeed, *POMT*$^{-}$ muscle in third instar larvae is stiffer than that of wild type. Furthermore, *rt*$^{-}$ muscle is stiffer than *tw*$^{-}$, providing a correlation between muscle stiffness and ultimate torsional severity in adults.

Interestingly, increased muscle stiffness was not observed in *tw*$^{-}$ embryos relative to wild type. This again points to a potential difference between the mechanisms of torsion in embryonic and adult stages. Worthy of note here is the fact that in embryos the difference, if any, between *tw*$^{-}$ and *rt*$^{-}$ torsion is negligible (see fig. 7). This may be accounted for, at least in part, by a lack of any difference in muscle stiffness between genotypes at this stage, though embryonic muscle stiffness in *rt* mutants remains to be tested. This also demonstrates that relative muscle stiffness is not required for torsion to occur, and as I will show in the next chapter, increasing muscle stiffness does not, on its own, cause torsion.

In *Drosophila*, POMT is required in multiple tissues to maintain correct posture at both the embryonic and adult developmental stages. Here I have indicated a need for POMT in muscles, as well as the possibility of CNS neurons and epidermis. More importantly, I showed a requirement for POMT in PNS neurons. In *Drosophila*, a defect

of POMT in the PNS is associated with the rise of torsion due to chiral interactions, presumably between cuticle and egg or pupal shell, during peristaltic contraction waves. Human WWS patients do not experience such torsion, likely because no such chiral interactions are present during developmental processes. Nevertheless, WWS patients do experience abnormal muscle contractions (66). Until now, dystroglycanopathies have been associated with muscles, eyes, and CNS neurons, but PNS neurons, while known to have O-mannosylated Dg [93,94], have been largely overlooked. While other tissues are doubtlessly important, this study highlights the importance of PNS POMT expression in *Drosophila* development, and this biological functionality may translate to mammalian systems, including humans. A further study of POMT and other components of the O-mannosylation pathway in higher organisms may therefore bring to light important new pathological mechanisms in human patients, and could ultimately lead to new targets and improved treatments for dystroglycanopathies.

CHAPTER IV

RECEPTOR PROTEIN TYROSINE PHOSPHATASE AS A TARGET OF O-MANNOSYLATION

4.1 Introduction

So far I have characterized the gross mechanical means by which torsion arises and elucidated the cellular POMT requirement that governs it. In this chapter I will investigate targets of O-mannosylation with potential functional relevance to torsion. Dystroglycan is by far the best characterized POMT target, having been identified as an O-mannosylated protein in 1997 (93). Much is understood about the possible extensions of O-mannosyl glycan chains on Dg, as well as about the specific glycan that is both necessary and sufficient for laminin binding (122). Previous work has shown that Dg is O-mannosylated in *Drosophila* as well as in mammals, and that both *tw* and *rt* are required for O-mannosylation to occur *in vivo* (123).

Recent work has also shown, however, that Dg is by no means the only target of O-mannosylation in mammals. Since the turn of the millennium, several other targets have been identified, including receptor protein tyrosine phosphatase ζ (RPTPζ), four lectican proteins, and 37 different cadherins (122). Nevertheless, Dg remains the only known target of O-mannosylation in *Drosophila*.

In this chapter I first investigate the interaction of Dg and POMT using a genetic approach. I will show that while Dg is involved in the rotation phenotype, it must not be the only relevant target. For example, RPTPζ is hypoglycosylated in mouse models of

the CMD muscle-eye-brain disease (MEB) that lack POMGnT1, a glycosyltransferase that modifies O-mannosylglycans (124) (see Fig. 3). Although *Drosophila* do not have any known homologues for RPTPζ, they do have 6 RPTP's, all of which are involved in axonal guidance and at least one of which (RPTP69D) is specifically known to have an effect on the axonal guidance of class IV da neurons (113). I therefore hypothesized that one or more of these RPTP's may be both O-mannosylated and relevant to torsion, so I examined the interaction between the RPTP's and POMT. I found that RPTP69D and possibly LAR are likely O-mannosylated and appear to be involved in the rise of torsion. Surprisingly, I show preliminary evidence indicating a role for RPTP69D O-mannosylation in muscles that may be more important to torsion than RPTP69D in neurons.

4.2 Materials and Methods

RPTP stocks

All RPTP deficiency stocks, Lar RNAi, and Dg086 and 248 were obtained from Bloomington. RPTP mutants are all deficiencies of RPTP4E, 10D, 52F, 69D, 99A, and Lar, and have been previously described (125-128). Dg 086 is a point mutation that inactivates the gene, and Dg 248 is a deficiency that is homozygous inviable but is viable with Dg086 (129,130). Dominant negative RPTP69D and UAS-RPTP69D were gifts from Paul Hardin's lab (131,132).

Western blot

Western blots against RPTP69D were performed on lysed larvae overexpressing RPTP69D in muscles in wild type and *rt*$^{-}$ hetero- and homozygous backgrounds. After crossing parents, vials were incubated for 5 days at 25^{o} C and 30 third instar larvae were collected from vial walls for each genotype. Larvae were lysed by crushing in 600 uL of buffer containing 50 mM Tris-HCl, 200 mM NaCl, 0.5% Triton-X100, PMSF, and a cocktail of protease inhibitors. The lysate was centrifuged at 18,000 g for 15 minutes at 4^{o} C, and the supernatant was mixed with SDS-PAGE loading buffer with b-mercaptoethanol. Samples were boiled for 3 minutes, briefly spun down, and 15 uL of each were loaded onto a 10% SDS polyacrylamide gel. The gel was run for 1 hour at 190 V and then transferred to nitrocellulose at 200 mA for 90 minutes. RPTP69D was detected using a monoclonal mouse-derived antibody against the N-terminal portion of the processed protein (Antibody 3F11 obtained from DSHB) (133) in a 1:12 dilution. The blot was counterstained with a goat anti-mouse antibody conjugated to horseradish peroxidase and developed using a kit from Thermo Scientific. It was imaged on a BioRad GelDoc imager. As a loading control, beta-tubulin was stained with mouse-derived monoclonal anti-tubulin (E7), also obtained from DSHB.

Other techniques

All other experiments and analyses in this chapter were carried out as described in chapters II and III.

4.3 Results

Dystroglycan is not the only functionally relevant target of O-mannosylation

The best characterized target of O-mannosylation is the transmembrane protein dystroglycan (Dg). Dg interacts with dystrophin on the cytosolic side of the cell membrane and with laminin and other proteins in the extracellular matrix. O-mannosylation of Dg and subsequent glycan extension is required for binding to laminin in mammals (50), which is why the muscular dystrophies associated with defects in the O-mannosylation pathway are collectively known as dystroglycanopathies. We therefore hypothesized that genetic defects in Dg would interact with the abdominal rotation phenotype.

To test this hypothesis, we first examined the angle of abdominal rotation in adult *tw* and *rt* flies and compared them to the angle observed in tw^{-}, $dg^{086/248}$ or rt^{-}, $dg^{086/248}$ double mutants, respectively. We found that *Dg* mutations did indeed interact with POMT mutations, causing a significantly higher average rotational angle in both tw^{-} and rt^{-} backgrounds. Interestingly, however, *Dg* mutations alone were not sufficient to induce rotation (Fig. 27 A).

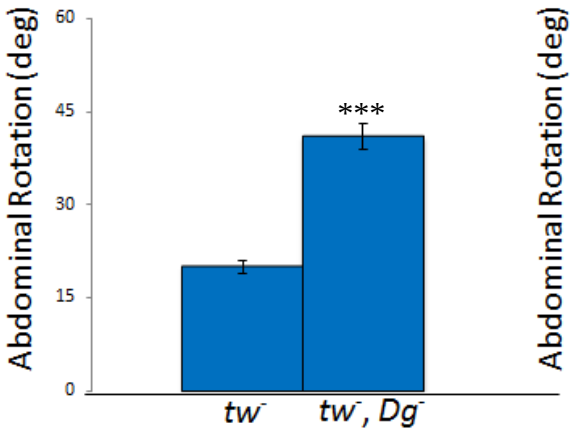
We further tested interactions between POMT and Dg using Brillouin spectroscopy in third instar larvae. We found that *Dg* mutations alone cause muscles to stiffen even more than in tw mutants, and that *tw-Dg* double mutants were even stiffer than *Dg* mutants (Fig. 27 B). Together our data so far indicate that Dg is not required to maintain correct posture, though it is required for normal muscle elasticity. Thus muscle stiffness does not cause rotation, though our results are consistent with the hypothesis

that stiffness may aggravate rotation when it is present. Furthermore, since complete absence of Dg can not induce rotation, and removal of Dg in POMT mutant backgrounds does not alleviate rotation, it appears that one or more other POMT targets are also involved in rotation phenotypes.

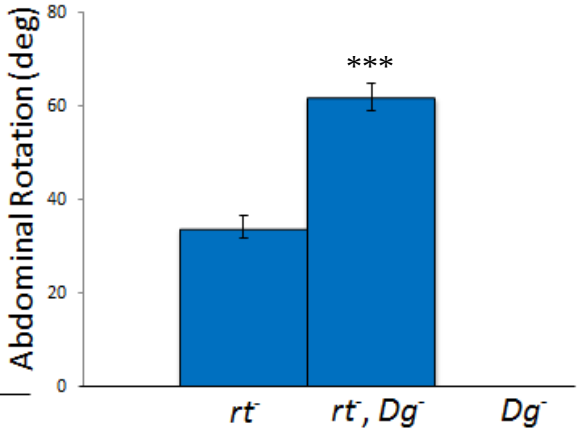
Receptor protein tyrosine phosphatases are functionally relevant targets of O-mannosylation

We previously noted that *POMT* mutations result in abnormal axonal connections from the PNS to the CNS. A similar phenotype has been observed in larvae with mutations in the receptor protein tyrosine phosphatase RPTP69D (113). Additionally, in mammals RPTPζ has been shown to be a target of O-mannosylation with potential relevance to CMD's (122,124), and we therefore hypothesized that *Drosophila* RPTP's might be involved in rotation phenotypes. To test this hypothesis, I measured the abdominal angle in double mutants for either tw^{-} and a null mutation of one of the 6 *Drosophila* RPTP's, or rt^{-} and an *RPTP* mutant. Because *Drosophila* homozygous for most of these *RPTP* alleles do not survive to adulthood, I measured the interaction in flies heterozygous for *RPTP* mutations. I found that several of the *Drosophila RPTP's* genetically interact with *POMT* (Fig. 28), the most prominent two being *RPTP69D* (hereafter simply called *69D*) and Leukocyte Antigen-Related RPTP (*LAR*), which are part of the same subfamily and the only two *Drosophila* RPTP's in that subfamily (134). RPTP 52F and 99A may have had slight genetic interactions with POMT, but these were not statistically significant. RPTP 10D had no detectable interaction, and RPTP 4E was not viable in combination with rt^{-}. Thus although 4E may

A



B



C

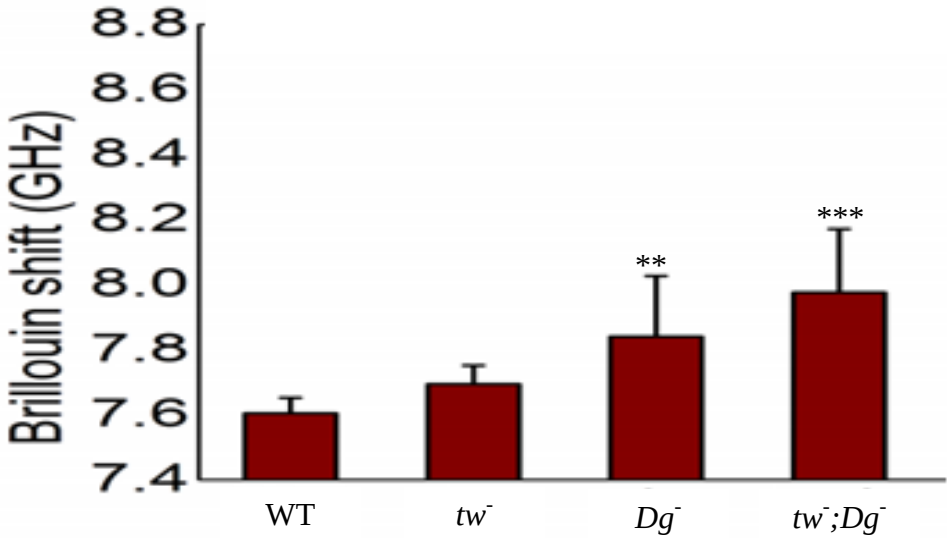
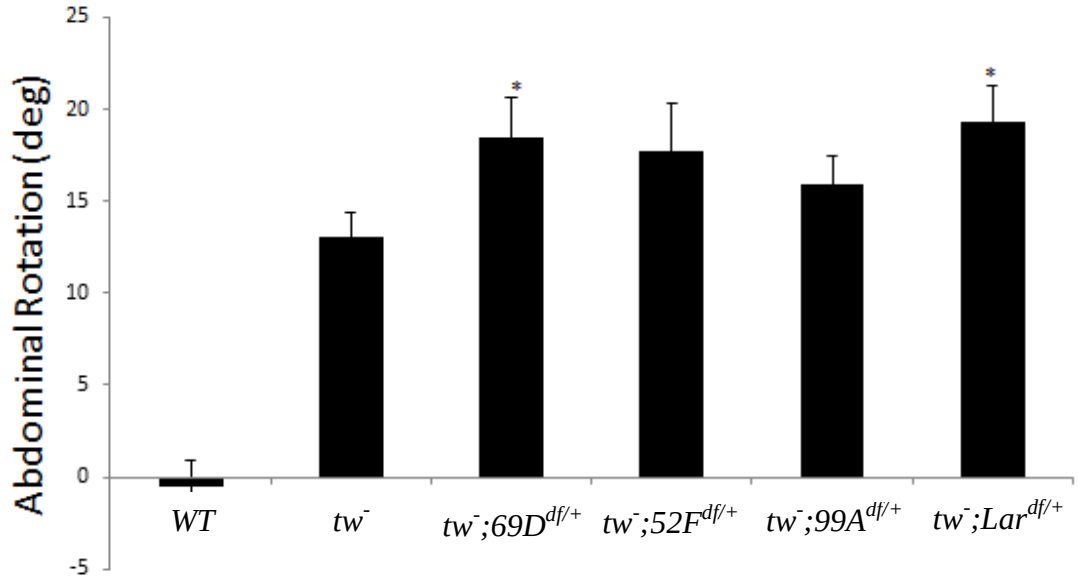


Figure 27—Genetic interactions between Dg and POMT. Data obtained in collaboration with Dr. Dmitri Lyalin and Dr. Zhaokai Meng.

A and B show that *Dg* mutations can aggravate, but not cause rotation. C shows that *Dg* mutations both cause and aggravate muscle stiffness. N = at least 20 flies per genotype in A and B, 10 larvae per genotype in C. Error bars are SEM. Asterisks in C are relative to tw. Dg and tw-Dg are also statistically different from each other, with $p < 0.05$.

A



B

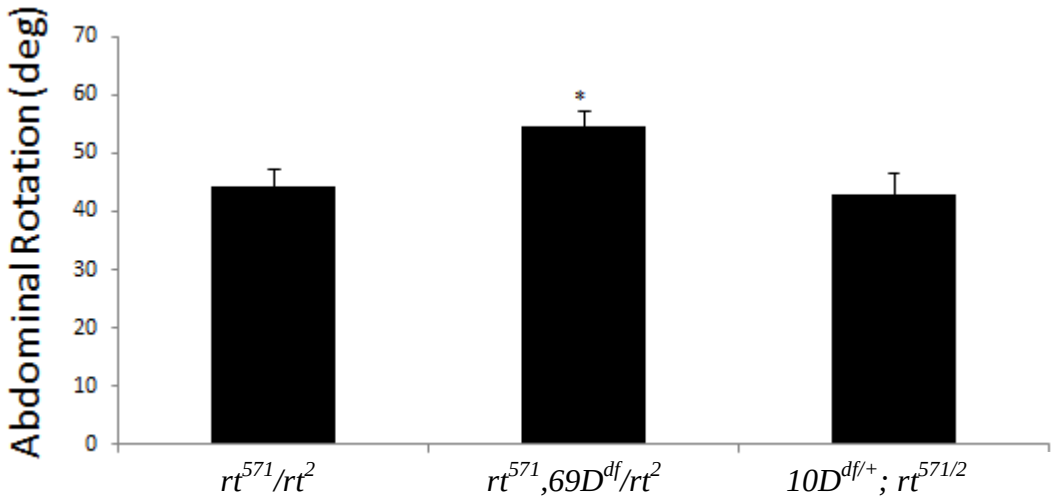


Figure 28—Genetic interactions between several RPTP's and POMT.

RPTP 69D and Lar strongly interact with POMT to aggravate (but not cause) rotation. Other RPTP's have weaker interactions or no interactions. N = at least 20 flies for each genotype, error bars are SEM. Asterisks represent significance relative to *tw*$^{-}$ or *rt*$^{-}$.

actually have the strongest interaction, it is not feasible to work with. Here I will focus primarily on *69D*, with some discussion of *LAR*.

Next I analyzed the interaction of *69D* with *rt* in embryos. When I examined embryonic double mutants homozygous for both rt^{-} and $69D^{-}$, I found that although tracheae still filled with air, peristaltic contractions were completely absent in all six embryos measured over the course of 1 hour of recording (not shown). It remains to be seen whether *69D* heterozygotes can affect contraction patterning in a $POMT^{-}$ background, or whether *69D* mutants by themselves have any contraction phenotype. I also analyzed the interaction of 69D with the POMT phenotype of axonal connections in class IV da neurons. Interestingly, I could detect no difference in commissural branch thickness in double mutants (heterozygous for $69D^{-}$) relative to *tw* mutants alone (not shown). Thus it seems that any interaction between 69D and POMT in axonal connections is weak.

69D O-mannosylation is required in muscle tissue for regulation of torsion

I was surprised to see no interaction between POMT and PTP in axonal guidance, since axonal guidance is the best-characterized role for the RPTP's. LAR has also, however, been shown to have a role in other tissues including muscle (135-137), so I hypothesized that LAR and, by extension, 69D might need to be O-mannosylated in muscle tissue. I therefore examined the tissue-specific requirement for 69D by expressing a dominant negative form of 69D ($69D^{DN}$) in various cell types in a tw^{-} background. I found that G14-mediated expression was able to induce an increase in abdominal rotation relative to tw^{-} alone. The neuron-specific driver 1407-Gal4 (pan-

neuronal) was also able to induce an increase comparable to that in G14 (Fig. 29 A), while ELAV-Gal4-induced $69D^{DN}$ expression resulted in flies failing to survive to adulthood. It therefore appears that despite my inability to detect interactions in axonal guidance, 69D may interact with POMT in neurons to help maintain posture. However, the greatest observed change in rotation occurred when I expressed $69D^{DN}$ specifically in tw^{-} muscles using an MHC-Gal4 driver (Fig. 29 A). This indicated a putative role for O-mannosylation of 69D in muscle in posture maintenance.

I also attempted to rescue torsion in *rt* mutants by overexpression of functional 69D in various tissues. I found that expression in both G14 and MHC patterns could slightly but significantly rescue the phenotype, though I could observe no such rescue when 69D was expressed only in neurons (Fig. 29 B). To test for a cell autonomous role for RPTP's in muscles, I expressed an RNAi against LAR in a tw^{-} background and again found a significant increase in abdominal torsion (Fig. 29C).

I also preliminarily tested the interaction between 69D and POMT in the heart phenotype described in chapter II by expressing $69D^{DN}$ with the dHand-Gal4 driver. I found a slight increase in the number of backward contractions relative to *rt* mutants alone, though additional data will have to be gathered to verify the significance of this difference (Fig. 30 A). I also found that the diastolic diameter was substantially different in *rt* mutants expressing $69D^{DN}$ than in *rt* mutants alone. Interestingly, systolic diameter in rt^{-}-$69D^{DN}$ flies was no different from systolic diameter in rt^{-} background alone (Fig. 30 B).

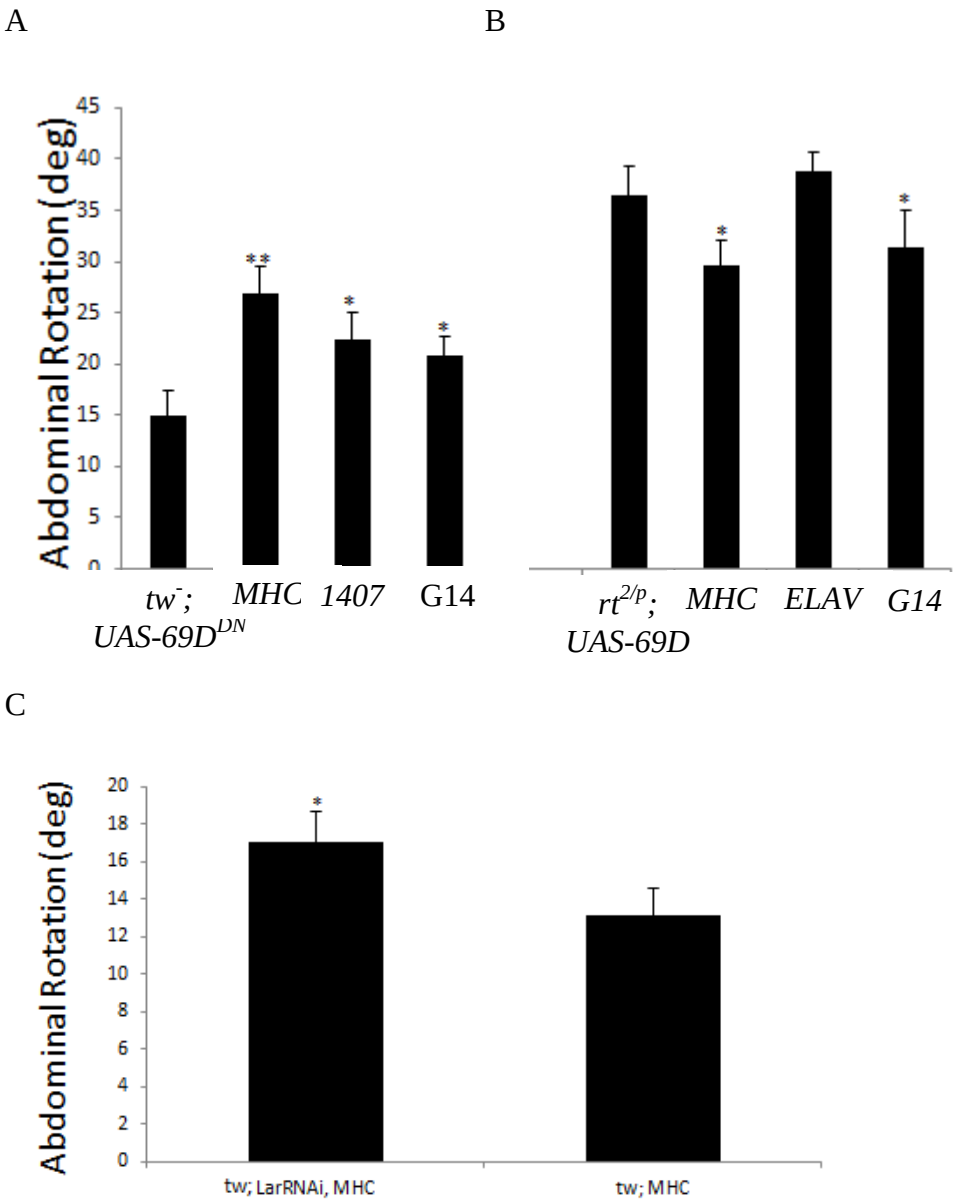


Figure 29—Tissue-specific requirements for RPTP's.

A) Interaction of a dominant negative 69D with *tw*$^{-}$ in various tissues. B) Rescue of rotation by overexpression of 69D in specific tissues. X-axis in A and B describes the driver used to express 69D^{DN} and functional 69D, respectively. All drivers are in addition to either *tw*$^{-}$ and 69DDN or *rt*$^{-}$ and UAS-69D. C shows effect of LAR knockdown in muscles. Asterisks are significance relative to controls, error bars are SEM. N = at least 20 flies for all genotypes.

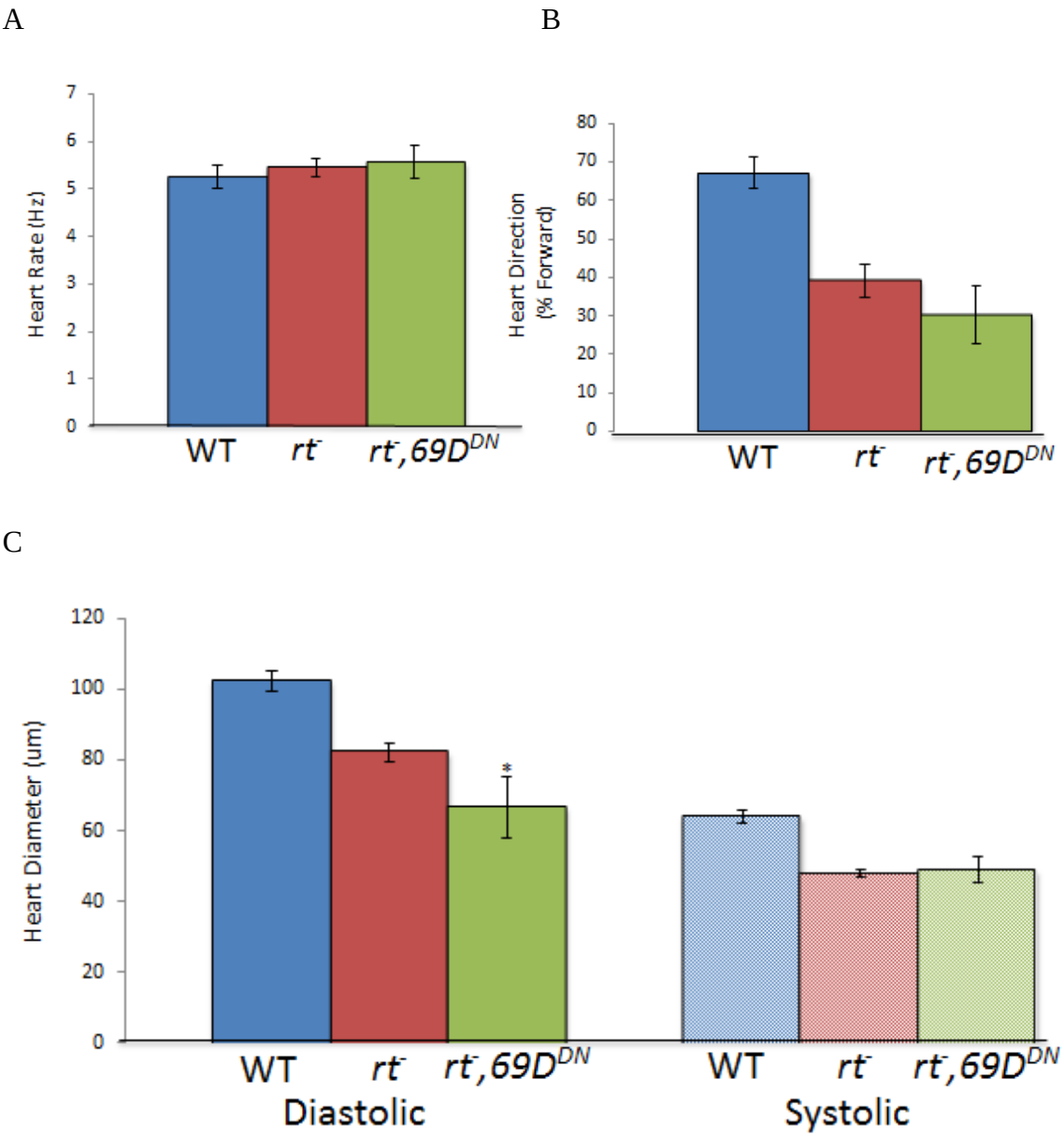


Figure 30—69D^{DN} effect on heart.

In A I show that heart rates are unaffected by mutations and transgenes. B shows that there may be a decrease in forward contractions in *rt* mutants when 69D^{DN} is present in the heart, though there are not yet enough data to assess significance. Diastolic diameter is smaller in double mutants than in *rt* mutants alone, but systolic diameter is not. WT and *rt*$^{-}$ data are identical to that in Fig. 16. N = 4 hearts for double mutants. Error bars are SEM. Asterisk indicates a difference in double mutant relative to single mutant.

So far my data indicate that POMT interacts with 69D and LAR in muscles as well as potentially in neurons. To test more fully whether 69D is O-mannosylated in muscles, I overexpressed 69D in *Drosophila* larvae with wild type and *rt*$^{-}$ backgrounds as well as a background heterozygous for *rt*$^{-}$ using MHC-Gal4 driver. I collected and lysed third instar larvae and performed a Western blot to detect 69D in the lysates of each background. In wild type larvae I found that 69D existed in two species: a high molecular weight species and a low molecular weight species. The low molecular weight species increases in relative abundance as *rt* is compromised, with more present in *rt*$^{-}$ heterozygotes and even more in *rt*$^{-}$ homozygotes. The high molecular weight species shows the opposite effect (Fig. 31). These data show a molecular interaction between 69D and POMT in *Drosophila* muscles, although further studies will be needed to verify that the interaction is an O-mannosylation event. However, given that POMT is an O-mannosyltransferase, RPTPζ is known to be O-mannosylated in mammals, and POMT interacts with 69D in such a way as to increase its molecular weight, such an outcome seems likely. Taken together, my data show that Dg is not the only POMT target involved in posture maintenance, that 69D and LAR are likely O-mannosylated in muscles, and that this O-mannosylation is relevant to torsion as well as heart phenotypes.

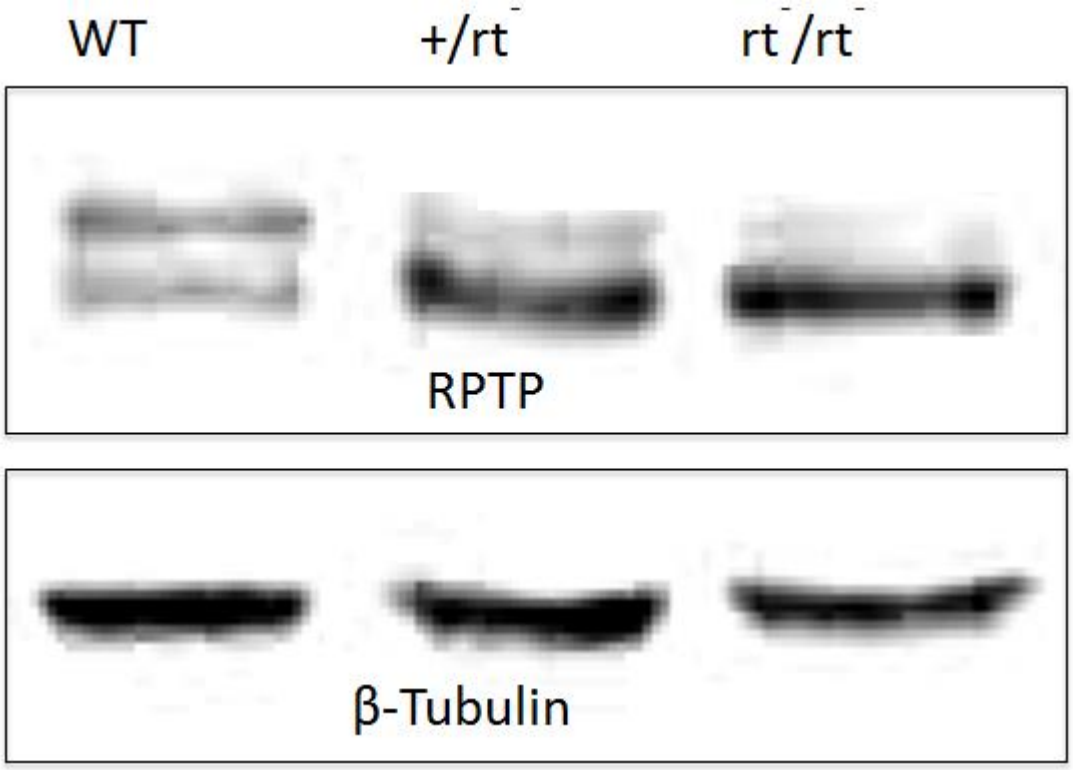


Figure 31—69D is altered, directly or indirectly, by POMT.

69D was overexpressed in muscles in various genetic backgrounds and detected by Western blot. Two molecular weight species exist when POMT is functional, only one when POMT is not, suggesting POMT modifies 69D. Tubulin is a loading control.

4.4 Discussion

In this chapter I have shown that Dg interacts genetically with POMT in such a way as to affect torsion. Dg deficiency alone caused muscle stiffness but not torsion. Double mutants for *Dg* null mutations and tw^{-} or rt^{-}, however, experience more severe rotation and muscle stiffness than *POMT* mutants alone (Fig. 27). These data are consistent with the hypothesis that stiffness can aggravate rotation but not cause it. Since *Dg* mutations are not sufficient to cause rotation, I would expect Dg removal in *POMT* mutant backgrounds to relieve rotation if Dg were the only relevant O-mannosylation target. Because this is not what I observe, I hypothesized that there must be other relevant POMT targets that contribute to rotation when O-mannosylation fails.

Dg is currently the only known POMT target in *Drosophila*, but studies in mammalian systems suggested several other potential targets. I focused on the receptor protein tyrosine phosphatases because RPTPζ is hypoglycosylated in mouse models of MEB, they are involved in axonal guidance, and 69D is specifically involved with class IV da neurons (113,124,133). In mammals RPTPζ is currently the only known RPTP to be O-mannosylated, and *Drosophila* do not have a known homologue of RPTPζ (134). Nevertheless I hypothesized that some similarity in sequence may be enough for O-mannosylation to occur. My data demonstrate that 69D does interact with POMT, as does the closely related LAR (Fig. 28-31). My western blot data also provide evidence consistent with the hypothesis that 69D is indeed O-mannosylated (Fig. 31), though further evidence will be needed to confirm this. These data introduce the possibility that

other mammalian RPTP's, such as the 69D homologues PTP-σ, PTP-δ, and hLAR (134) may be both O-mannosylated and functionally relevant to muscular dystrophy.

Classically, the RPTP's are involved in axonal guidance, and mutations in 69D can alter the morphology of class IV da neurons (113). Since I demonstrated that these neurons are involved in the rise of torsion, and since the phenotype seen in these neurons in 69D mutants is very similar to that of POMT mutants, I hypothesized that a genetic interaction between the two may exist to guide axons in class IV da neurons. Although I did find that downregulation of 69D in neurons had an effect on rotation, I could not find any interaction between 69D and POMT with respect to ventral ganglia morphology. It seems, then, that there may be an effect of 69D O-mannosylation in neurons including class IV da neurons, but any morphological effects were beyond my ability to measure. This may in part be due to the fact that I used larvae heterozygous for the 69D mutation. Perhaps a single copy of functional 69D is sufficient to avoid any obvious morphological neuronal defects. Homozygous double mutants might show a detectable interaction. I observed that these double mutants do not undergo embryonic peristaltic contractions and therefore do not hatch. However, they do develop into stage 17 embryos, so examining their ventral ganglia may still be possible.

Nevertheless, if the only relevant aspect of 69D O-mannosylation were in axonal guidance, I would be surprised to find such a weak morphological interaction and still find a significant change in torsion. I previously showed that POMT in muscle tissue is also important in maintaining embryonic posture, and LAR has been shown to have a role in muscles (135-137). Since LAR and 69D belong to the same subfamily, I

hypothesized that 69D may also be important in muscles in addition to neurons. I therefore assessed the role of 69D in muscle tissue and found a strong genetic interaction between POMT and $69D^{DN}$ expressed specifically in muscle (Fig. 30). Knockdown of LAR in muscle by RNAi also showed an interaction, further supporting a cell autonomous role for RPTP's in muscle tissue with respect to O-mannosylation and torsion.

My data also indicate that 69D and its mammalian homologues, PTP-σ, PTP-δ, and hLAR may be regulated by O-mannosylation differently than RPTPζ, which has its phosphatase activity attenuated by O-mannosylation (138). If O-mannosylation of 69D attenuated its phosphatase activity, then I would expect lower 69D expression levels to similarly attenuate dephosphorylation of tyrosine residues and thus recapitulate the effect of O-mannosylation. Such an effect should partially rescue the torsion phenotype, or at the very least not aggravate it. However, as described above, I observed the opposite (Fig. 28, 29), suggesting that O-mannosylation of 69D may upregulate phosphatase activity in *Drosophila*. Further evidence will be required to verify this. However, I also showed that increased levels of 69D in the muscles of flies can mediate a partial rescue. If O-mannosylation acts as an upregulator of activity that is already present, rather than an "on/off switch," increased 69D expression levels might mimic the effect of O-mannosylation. Curiously, upregulation of 69D in muscles alone was able to mediate partial rescue (Fig. 29 B), even though POMT overexpression in muscle was not. The simplest explanation is that POMT activity can not increase 69D activity beyond a maximum level governed by the endogenous 69D levels, and thus may not be

able to compensate for a lack of O-mannosylated POMT targets in other tissues. An increase in the actual levels of 69D, on the other hand, might increase phosphatase activity sufficiently to have an impact.

My work here has preliminarily established a new target of O-mannosylation in *Drosophila*, RPTP69D. This target is similar to a known mammalian counterpart, RPTPζ, but belongs to a different subfamily (134). Thus my work may have revealed a new target or targets of O-mannosylation for mammalian systems as well, such as PTP-σ, PTP-δ, or hLAR. I also provided supporting evidence for a role for RPTP's outside the nervous system, and specifically connected this role to O-mannosylation. These findings may be of import to the pathology of CMD's such as Walker-Warburg Syndrome by providing new putative therapeutic targets at the molecular level, particularly since upregulation of 69D helps relieve the torsion phenotype in *Drosophila*. Much research remains to be done, but mimicking this upregulation or otherwise targeting RPTP activity in WWS patients could potentially offer an effective treatment for some of the most severe forms of muscular dystrophy.

CHAPTER V

SUMMARY AND FUTURE DIRECTIONS

5.1 Summary

Protein glycosylation is one of the most prominent forms of post-translational modification. It has a wide range of biological functions and is related to a variety of pathological states including cancer biology, infectious disease, autoimmune responses, and genetic disorders related to misglycosylation. Enzymes known as glycosyltransferases attach and extend glycan chains onto asparagine (N-linked) and serine/threonine (O-linked) amino acid residues, and glycosidases remove sugars from glycan chains. Defects in any of these enzymes can cause congenital disorders of glycosylation. The enzyme complex Protein O-MannosylTransferase (POMT) links mannose to serine and threonine residues on various target proteins, and these O-mannosyl glycans are subsequently extended by various enzymes including POMGnT2, POMK, Fukutin, FKRP, and LARGE (47-50). Failure of these enzymes to properly glycosylate their targets is linked to the most severe forms of congenital muscular dystrophy, collectively known as dystroglycanopathies.

In this dissertation I used fruit flies as a model system to investigate the biological mechanisms underlying physiological and developmental defects associated with POMT mutations. The most prominent and best characterized phenotype in *Drosophila* POMT mutants is a torsion of posterior segments in the clockwise direction relative to anterior segments. This phenotype was characterized near the beginning of the

20th century, but has only relatively recently been associated with POMT defects. The mechanism by which torsion occurs has remained elusive.

Here I have focused on the role of POMT mutations in the rise of rotation in *Drosophila* embryos and adult abdomens. I have characterized the gross biological mechanism that causes rotation, demonstrating that embryos of all genotypes experience a chiral interaction during peristaltic contractions. This interaction is manifested as rolling of the developing larva within the eggshell in a direction that correlates with the direction of the contraction wave. I hypothesize that this interaction may be between the embryonic cuticle and the vitelline membrane. Simple contraction waves that propagate from one end of the embryo to the other (designated here as "type 1 waves") tend to decrease in intensity as they progress, and thus induce a smaller roll distance at the end of the wave than at the beginning. Because the rolling is left-handed in nature, this results in a stronger leftward roll at the beginning of the wave than at the end when viewed from the point of initiation, and leads to an apparent counterclockwise torsion of posterior segments relative to the anterior (See Fig. 17, 26).

If type 1 waves were the only wave form, wild type embryos would accumulate counterclockwise torsion, and presumably, so would adult abdomens. Thus some mechanism must exist to counteract the accumulation of counterclockwise torsion. This mechanism is found in the form of "type 2 waves," which have a more complex nature than type 1. Type 2 waves initiate in an anterior-posterior direction, halt part way through, and are swept back to the anterior by a second wave. I observed a strong correlation between type 2 waves and apparent torsion of posterior segments in the

clockwise direction. This presumably occurs both because the initial phase of type 2 waves cancels rolling in posterior segments and because the 2nd phase of the wave tends to increase in strength as it progresses.

POMT mutants experience an overabundance of type 2 waves, resulting in a net accumulation of clockwise torsion. This phenomenon suggested a defect in posture sensing by PNS neurons. When POMT is restored to affected class IV da sensory neurons, which play a role in proprioception, embryos experience a partial rescue of both contraction patterning and torsion. Although POMT expression limited to neurons is insufficient to rescue abdominal rotation in adults, removal of the neuronal component of G14-Gal4-mediated expression, which can fully rescue rotation at all stages, inhibits rescue. Additionally, expression of RNAi against *tw* in class IV da neurons results in an aggravation of torsion in mutants, suggesting that POMT expression in these neurons is important in maintaining posture at both embryonic and adult stages. Confirming a role for POMT in class IV da neurons, POMT mutations cause defects in the axonal connections between these neurons and CNS cells in the ventral ganglion, with thickening of commissural branches and, in some cases, missing longitudinal tracts. These defects can be rescued by ectopic POMT expression in class IV da neurons alone.

In fact, PNS function is required generally for correct contraction patterning and body posture in embryos. Removal of nearly all PNS neurons by a mutation in the *senseless* gene causes abnormal contraction patterns that can be overabundant in type 1 or type 2 waves, and correspondingly causes both clockwise and counterclockwise torsion. POMT mutations, however, always cause torsion in a single direction with

corresponding overabundance of type 2 contractions, indicating that rather than failing to send signals to the CNS, POMT mutations cause erroneous signaling. These incorrect signals can also be propagated through the PNS neurons that are present in *senseless* mutants, since double mutants always manifest clockwise torsion.

PNS neurons are not, however, the only cells involved in the rise of torsion. Rescue experiments demonstrated that POMT is also required in muscles and possibly other tissues including epidermis and CNS. Although the exact means by which failed POMT activity in these tissues may contribute to torsion is unknown, it is clear that muscles are stiff in POMT mutant late third instar larvae relative to wild type. While this stiffness is not sufficient to cause torsion, the evidence suggests that it is able to aggravate any torsion that has been induced.

At the molecular level, both the muscle and neuronal components of torsion may be underlain by hypoglycosylation of dystroglycan (Dg). Dg is an integral component of the dystrophin-associated glycoprotein complex (DGC), known to be involved in a number of muscular dystrophies, and is abundant in muscle and in CNS neurons. It has also been shown to be present and O-mannosylated in PNS neurons. In *Drosophila*, Dg genetically interacts with POMT, increasing rotational severity in double mutants. However, Dg mutations alone can not cause torsion, suggesting that other POMT targets may play a role. This fact may help explain, at the molecular level, why dystroglycanopathies are among the most severe forms of muscular dystrophy: more than just the DGC is compromised.

Receptor protein tyrosine phosphatases (RPTP's) have recently been characterized as POMT targets in mammals and are known to be hypoglycosylated in Muscle-Eye-Brain disease mouse models (124). At least some RPTP's also appear to be O-mannosylated in *Drosophila*, and play a role in torsion phenotypes. Although RPTP's are best known for their role in axonal guidance (which may well be a factor in torsion and symptoms associated with dystroglycanopathies in humans), here I show that RPTP O-mannosylation likely also plays a role in muscles.

In short, I have characterized the rise of torsion at the organismal level, elucidated the cellular requirement for POMT that governs this mechanism, and identified a new functionally relevant target of O-mannosylation. Together these findings indicate previously unknown roles for O-mannosylation at both the cellular and molecular levels. These new roles may find application in both diagnostic and therapeutic techniques to help treat a disease that currently has no good long-term solutions.

5.2 Future Directions

In this work I have shown that embryos experience a chiral interaction during peristaltic contractions. I proposed that this interaction is likely between the cuticle and vitelline membrane. However, it is currently unknown what the true basis for this interaction is. Electron microscopy might be informative as it could show whether there is any left-handed chirality of structures on the macro scale either in the cuticle, vitelline membrane, or both. Other structures may be examined as well.

My characterization of the mechanical basis for torsion was primarily in embryos. Preliminary evidence suggests that similar abnormalities of muscle contractions may exist in the pupal stage, which is the critical stage for rescue of adult torsion. I have also shown that similar but not identical sets of drivers can mediate rescue in embryos and adults, connecting the two phenotypes. Moving forward, it would be useful to examine contractions in wild type and mutant pupae directly. Previously this has been difficult as pupae are relatively opaque. However, fluorescently labeled muscle can be imaged through the pupa, and now that we know what to look for an analysis of pupal contractions may prove fruitful. It may shed light on both the similarities and differences between the rise of torsion in embryos and adults.

I am frequently asked whether there are any known mutations that cause counterclockwise torsion. In this work I showed that *sens*$^{-}$ can cause counterclockwise torsion, but not with 100% penetrance, and it can not be observed in adults because *sens* mutants do not survive to adulthood. There is, however, at least one known mutation that causes counterclockwise rotation in adults, in the gene now known as abdomen rotatum (AR) (139). This phenotype was first described in 1931 (140), and little work has been done on it since. Neither the molecular function of this gene nor the mechanism by which it causes rotation are known. I would love to see whether *ar*$^{-}$ embryos, and perhaps pupae, undergo abnormal contraction patterning in these mutants with an overabundance of type 1 contractions and whether defects in PNS morphology might be observed.

Regarding PNS morphology, further work should be done to determine how the observed PNS defects of POMT mutants translate to aberrant signaling and ultimately rotation. Similar defects of PNS morphology in other mutants do not necessarily cause rotation and may not be associated with abnormal feedback. Thus the morphological defects described here are likely a symptom rather than an underlying cause of signaling defects. Control theory in electronic circuits says that offsets in baseline feedback can result in offsets in the desired outcome (Personal communication from Alan Baker). For example, if a thermometer is miscalibrated such that it is off by 20 degrees, and is used to set the temperature of a refrigerator, the end result will be a refrigerator that is too hot or too cold due to a feedback offset. A similar offset may exist in POMT mutant neuronal circuits, and could help explain why torsion is always in the same direction. A better understanding of the relevant neuronal connections and measurements of their action potentials could be informative, though technological limitations may make such studies currently impractical.

In addition to the peripheral nervous system, muscle, epidermis, and CNS have potential roles in posture. Muscle stiffness may play a role in aggravating torsion, but can not be the cause on its own. I suspect that muscles make aberrant connections to the PNS, either directly or indirectly through the ECM, that may contribute to generation of incorrect feedback to the CNS. Similarly, I hypothesize the dendritic connections to the epidermis may be compromised, though so far we have not observed any alterations in dendritic morphology in *POMT* mutants. I also propose the possibility that although the CNS is evidently able to stimulate normal muscle contractions, axonal connections from

the PNS to the CNS may be compromised by failed O-mannosylation in the CNS. Studies examining connections between da neurons and epidermis, muscle, and CNS would therefore be enlightening.

Further work should also be done at the molecular level. Dystroglycan is evidently involved in the rise of torsion associated with POMT mutations. However, the molecular underpinnings for this role are unclear. In *Drosophila*, it is not yet even clear whether Dg binds laminin, though *Drosophila* Dg failed to bind human laminin (personal communication from N. Nakamura). Future studies should include an assessment of Dg-laminin interactions in *Drosophila*, and if such interactions exist, the necessary glycan chains should be characterized. In mammals, Dg-linked O-mannosyl glycans must be phosphorylated by POMK and subsequently extended by Fukutin, FKRP, and LARGE for laminin binding to occur (50,141). *Drosophila* do not have any known LARGE homologues, and it is unknown whether O-linked mannose is modified at all in *Drosophila*, though they do have an FKRP homologue (142). A more in-depth characterization of any O-mannosyl glycan extension that may exist in *Drosophila*, including whether O-linked mannose is phosphorylated and what role FKRP may play could help further establish both the utility and the limitations of *Drosophila* as a model system.

In this work I showed that RPTP69D and LAR are likely targets of O-mannosylation with relevance to the torsion phenotype. Functional data suggest that it plays a role in neurons and, surprisingly, muscles. However, we have so far been unable to detect an interaction between POMT and RPTP69D in terms of neuronal morphology.

Further studies involving homozygous double mutants and neuronal rescue by ectopic expression of UAS-RPTP69D may help shed light on any interactions that exist. Downregulation studies in muscles have so far only been performed for LAR, and downregulation of RPTP69D would be helpful in further establishing this model. In mammalian systems, loss of LAR has been shown to impair muscle function and results in mitochondrial defects. Further studies might therefore include examination of mitochondria in *Drosophila* muscle in *LAR* and *RPTP69D* mutants and in flies with LAR and 69D downregulated in muscles specifically.

Finally, future work could examine roles for POMT's outside of O-mannosyl transfer. POMT1 in several species has been shown have domains with a sequence similar to that of ryanodine receptors (143). Furthermore, inhibition of calcium flux in dystrophic mice has been shown to alleviate some dystrophy symptoms, while an increase in calcium flux in healthy mice can induce some symptoms seen in dystrophy (144-146). We therefore hypothesize that POMT1 may help regulate calcium flux in the ER of one or more cell types. Preliminary evidence suggests that POMT1 but not POMT2 does indeed alter calcium flux in cellulo (Personal communication from Agustin Guerrero-Hernandez). In the future we will examine the effect of POMT on calcium regulation in fly hearts both by inhibiting flux with thapsigargin and by monitoring calcium directly using the Ca^{2+}-sensitive fluorophore GCaMP (82). These studies may shed light on a new role for POMT1 and also highlight a difference in the roles of POMT1 and POMT2.

Ultimately, these data will present evidence regarding the rise of a previously mysterious phenotype in *Drosophila*, make potential links between *Drosophila* mechanisms and human pathology, and shed light on the biochemical and cellular roles of POMT and subsequent glycosyltransferases in maintaining proper development and physiology. This information could be of great value in treating patients with the most severe forms of muscular dystrophy.

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