Title
The Localizations and Functions of Nonmuscle Tropomyosin Isoforms in Drosophila melanogaster

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Publication Date
2013

Peer reviewed|Thesis/dissertation

The Localizations and Functions of Nonmuscle Tropomyosin Isoforms in *Drosophila melanogaster*

by

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DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Biochemistry and Molecular Biology

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

Acknowledgements

This work was made possible through the support I have had the good fortune to receive from many individuals during my years in graduate school. However, this work would have been completely impossible without the support of my mother, who has nurtured my interest in science from a young age and provided me with constant love and encouragement throughout graduate school. She is and always will be my hero and I miss her very much. She passed away just a few months before I completed my graduate work, but I know that she is watching over me and will forever be in my heart.

Dyche Mullins has been an inspiration, both for his thoughtful dedication and infectious enthusiasm for science, as well as his remarkable aesthetic and artistic sense. I have often turned to him for guidance, for both research and personal advice. He provided unwavering support and patience, both for my scientific research and personal goals. For these things, and many others I do not have the words to properly describe, I will always be grateful.

The Mullins lab has been an incredible place to work, due entirely to the talent, kindness and generosity of its members. I have grown both personally and professionally from my interactions with labmates, and I consider each of its members, past and present, a valuable friend. In chronological order, I thank Mark, Ethan, Orkun, Margot, Lawrence, Janet, Chris, Brad, Chris-ton, Elena,

Scott, Brittany, Jessica, Jenny, Peter, Natalie, and Lil. I also thank Phoebe Grigg for her cheerful and kind assistance throughout my time at UCSF. I would like to especially thank Jenny Hsiao for allowing me to mentor her. It has been an honor and a thrill.

I am so lucky to have several fabulous “unofficial mentors” including Carol Gross, who has been an incredible source of encouragement and advice, a shoulder to cry on, and a person to share my excitement with, and Henry Bourne, a source of straight-talk and funny anecdotes to help uplift me. Carol Gross has especially supported me in many of my endeavors and I will be forever grateful.

I am grateful for the thoughtful advice of my thesis committee members, Ron Vale and Orion Weiner. Ron and members of the Vale lab, particularly Steve Rogers, Nico Stuurman, Antonina Roll-Mecak, Eric Griffis, Thomas Huckaba, Sarah Goodwin, and Susana Ribeiro have been great sources for advice and friendship. I am also thankful for Kurt Thorn and the Nikon Imaging Center, where I have had access to some of the best microscopes, without which this work would not be possible. I am also grateful for all the help I have received from other labs, especially Susanne Rafelski from the Theriot & Marshall labs, she has been a friend and taught me key imaging skills.

And last, but certainly not least, I want to thank all my friends and family who have constantly supported and encouraged me along the way. My family,

especially my brother Jules and sister Rachel have been fantastic siblings. We have been through a lot together but we remain siblings and friends and I am so lucky to have them! In graduate school I was lucky to have so many friends who always listened to my terrible analogies, helped me through the hard times, and have just be exceptional friends. I am especially grateful for Erin Pearson who may have started as my college roommate but has become a lifelong friend, and also my writing but…writing parties! And of course the “fantastic four – plus” gang: Ashley Robinson, Cat Foo, Eric Chow, Veronica Zepeda, Kim Kempadoo, and Georgette Charles and my best friend, Don Casimere II.

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Abstract

Tropomyosin (TM) is an actin binding protein that has been implicated in a variety of developmental and biological processes including neuronal morphogenesis, cell transformation, and cell motility (Gunning et al., 2008; Michelot and Drubin, 2011). The precise roles of TM in these processes has remained murky, largely because of the great diversity of TM isoforms; human cells for example express up to forty TM isoforms from four genes, most of which are poorly characterized (Gunning et al., 2005). In *Drosophila* there are two TM genes (Tm1 and Tm2) to which various functions have been attributed, but splicing complexity and nomenclature changes have inhibited identifying specific TM isoforms and their cellular roles (Bautch and Storti, 1983; Erdélyi et al., 1995). Here, we have carefully identified and characterized three nonmuscle TM isoforms expressed in *Drosophila* S2 cells. We found that all three TM isoforms have both overlapping and distinct functions that are cell cycle specific, including localizations and functions at the lamellum, Golgi, and cortex during interphase, and the cortex, kinetochores, and spindle apparatus during mitosis. Using GFP-tagged TM chimeras, we have also identified regions of individual TM isoforms

that are responsible for the localization and functional differences. Together, these experiments have led to functional insights that, combined with previously known functions of tropomyosin isoforms, allow us to propose a model of how different nonmuscle TM isoforms function together in S2 cells throughout the cell cycle. Our results implicate *Drosophila* TM in cell cycle control, chromosome segregation, cellular contractility, and Golgi morphogenesis.

Contributions

This work began as a continuation of work done by Janet Iwasa. I collaborated with Juan Oses in the Burlingame lab for the mass spec analysis of endogenous TM isoforms. I received and shared several constructs, reagents, and cell lines from Janet Iwasa, Elena Ingerman, Eric Griffis, Sarah Goodwin, Brittany Belin, and Susana Ribeiro.

I performed all experiments and created all figures in this thesis dissertation under the supervision and guidance of R. Dyche Mullins.

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Chapter One

Introduction

The actin cytoskeleton

The actin cytoskeleton must be spatially and temporally regulated in order to drive fundamental cellular processes including: cell motility, morphological shape changes, and cell division. The actin cytoskeleton is impressively complex as actin filaments assemble into various networks that associate with over fifteen different subcellular structures (Chhabra and Higgs, 2007). Furthermore, the architecture and function of these structures, along with the associated actin networks, differ in form and function throughout the cell cycle. These actin networks contain diverse arrays of actin filament structures, which are built through the coordinated action of numerous actin-binding proteins, each with similar but often complementary properties (Chhabra and Higgs, 2007; Pollard and Borisy, 2003).

The role of tropomyosin

One actin binding protein in particular, the actin filament side-binding protein tropomyosin, has been shown to localize to a variety of different subcellular structures from filopodia and membrane ruffles to microvilli and stress fibers (Gunning et al., 2005; Lin et al., 1997). Each of these structures often contains a specific mixture of tropomyosin (TM) isoforms, presumably with distinct characteristics and biochemical properties. Through localization and functional studies, TM has been implicated not only in the formation and maintenance of

these subcellular structures, but also in their function during different cellular processes such as cell migration and invasion, cell division, and apoptosis (Gunning et al., 2008). Past studies have found that the proper localization of both actin and tropomyosin is essential to proper cellular and organism function. And although we know that actin and tropomyosin isoforms are differentially distributed in cells and tissues, we lack a mechanistic understanding of how this patterning is accomplished (Bryce et al., 2003; Gunning et al., 2008; Perrin and Ervasti, 2010; Schevzov et al., 2005). This study attempts to bridge this gap in knowledge by specifically studying the localizations and functions of tropomyosin isoforms expressed in *Drosophila melanogaster* nonmuscle S2 cells.

How tropomyosin functions

The tropomyosin (TM) protein forms coiled-coil dimers that assemble head to tail (end to end) along the actin filament, and in so doing, occlude or reveal binding sites for other actin binding proteins. Because TM regulates access to the actin filament, it is thought to be a master regulator of actin filament function and architecture (Michelot and Drubin, 2011). Much is known about how tropomyosin functions in skeletal muscle, where tropomyosin, in conjunction with the troponin regulatory complex, mediates muscle contractility by regulating access of myosin motors to the actin filament in response to intracellular calcium changes (Perry, 2001; Wang and Coluccio, 2010). Much less is known about how tropomyosin functions in nonmuscle cells or why there is so much diversity in TM isoforms.

Tropomyosin isoform diversity

Through the alternative splicing of four tropomyosin genes, vertebrates are known to express over forty different tropomyosin isoforms that are differentially expressed during development (Gunning et al., 2005; Lees-Miller and Helfman, 1991; Martin and Gunning, 2008). Furthermore, some individual cells in vertebrates are known express up to eight different TM isoforms from all four TM genes, most of which localize to different actin filament structures and exhibit distinct biochemical properties (Gunning et al., 2008). However, the mechanisms by which tropomyosin localizes to specific cellular structures, and then in combination with other actin binding proteins, influence actin filament diversity remains poorly understood. Why so many TM isoforms exist, how they differ in structure and function, and what roles they play in regulating the actin cytoskeleton are some of the most important outstanding questions in the field of tropomyosin (Wang and Coluccio, 2010).

The sheer number of TM isoforms expressed in vertebrates, and the different combinations expressed in different cell types and tissues, makes it difficult to dissect the contribution of any one TM isoform in vertebrate cell lines and systems. And so, much of the work characterizing the fundamental roles of “nonmuscle” tropomyosin has been done in the unicellular organism, yeast. Fission and budding yeast have been used for studies focusing on the

fundamental roles TM plays in biology because yeast express only one or two TM isoforms, and because the yeast cytoskeleton is known to assemble into only four distinct actin structures that govern diverse processes throughout the cell cycle (La Carbona et al., 2006; Skau et al., 2009).

TM isoforms in yeast

In the fission yeast S. pombe, for instance, this has allowed thorough characterization of the only tropomyosin expressed, cdc8p. Studies in S. pombe have shown that the tropomyosin cdc8p is required for contractile ring assembly and regulates endocytosis and cytokinesis kinetics (Balasubramanian et al., 1992; La Carbona et al., 2006; Skau and Kovar, 2010; Skau et al., 2009; Wu et al., 2006). On the other hand, studies in the budding yeast S. cerevisiae, which express two TM isoforms, Tpm1 and Tpm2, have shown that while Tpm1, but not Tpm2, is essential, both TM isoforms have overlapping but distinct biological functions (Drees et al., 1995). While Tpm1 promotes actin cable function with the class V myosin, myoVp, Tpm2 regulates retrograde actin flow with the conventional type II myosin, myo1p (Coulton et al., 2010; Hodges et al., 2012; Huckaba et al., 2006). But it remains unclear which, if any, of the characteristics of these TM isoforms from unicellular organisms are applicable to multicellular organisms, which possess both muscle and nonmuscle tissues. In multicellular organisms distinctions between different tissues and cell types may drive the need for diversity of TM isoforms and functions. Furthermore, in multicellular

organisms, additional control mechanisms regulate the cell cycle to specify the size of the organism as well as its constituent organs (Björklund et al., 2006).

TM isoform diversity in Drosophila melanogaster

Similar to yeast, the expression of tropomyosin (TM) isoforms has been shown to be cell cycle-regulated in mammalian cells (Percival et al., 2000). But, because so many mammalian TM isoforms are expressed in the same cell, efforts to dissect specific roles for each TM isoform in cell division, and other cell cycle stages, has been more difficult to determine. In contrast to mammals, *Drosophila melanogaster* (Drosophila) have only two TM genes and express a limited number of nonmuscle TM isoforms (Hanke et al., 1987; Karlik and Fyrberg, 1986). Indeed, before this study, Drosophila were thought to express only one TM isoform in nonmuscle cells (Gunning et al., 2005). This Drosophila nonmuscle TM isoform (“cTm”, “DcTm”, “Tm1”, “TmII”, “Tm1A”) has been implicated in a variety of important developmental and biological processes included oskar mRNA localization and head morphogenesis during development, neuronal dendritic field size and border cell migration (Erdélyi et al., 1995; Kim et al., 2011; Li and Gao, 2003; Tetzlaff et al., 1996). The limited number of TM genes, combined with the intrinsic advantages of Drosophila genetic and cellular techniques, make Drosophila the ideal organism in which to study the mechanisms by which TM isoforms are localized and influence actin cytoskeleton function. In particular, Drosophila nonmuscle S2 cells provide a system where

localization, biochemistry, mutational analysis, and depletion techniques can be combined to study the mechanisms by which different nonmuscle TM isoforms function (Iwasa and Mullins, 2007; Martin and Gunning, 2008; Rogers et al., 2003).

A major challenge in the field is to determine how subsets of actin regulatory proteins localize to specific structures and collectively influence actin filament dynamics, architecture, and function on a cellular level. In this study, we have not only identified two additional TM isoforms expressed in Drosophila nonmuscle S2 cells, but we have also gone further and characterized their function at different cellular structures, during different cell cycle stages, and under different cellular conditions.

In chapter two, I describe how we identified the three TM isoforms expressed in Drosophila nonmuscle S2 cells: Tm1A, Tm1J, and Tm2A (designation by flybase.org) using a combination of cDNA analysis, immunoblots with commercially available antibodies, partial purification of endogenous tropomyosin, and mass spec identification. Then, in chapter three, I explain how we used spinning disk confocal microscopy to determine the localization of each TM isoform tagged with fluorescent proteins and compared them to other actin binding proteins. These localization studies, in tandem with RNAi depletion methods, allowed us to characterize the roles that each TM isoform plays in cell spreading and shape maintenance, cell cycle progression, and cell division. We

found that Tm1A and Tm1J co-localize to the lamellum in spreading cells. But, in non-spreading cells, Tm1A, and not Tm1J, localizes to the cortex, while Tm1J and Tm2A both localize to the Golgi apparatus.

We also found that during mitosis, Tm1A co-localizes and functions with myosin II to maintain cortical contractility, while Tm1J displays a strikingly different pattern of localization and function. We report for the first time, the presence of a tropomyosin isoform, Tm1J, on kinetochores and centrosomes during mitosis. During prophase and metaphase, Tm1J localizes to the centrosomes and kinetochores. During late anaphase and telophase, Tm1J relocates to the central spindle and midpoint. These dramatic movements are similar to those seen for chromosomal passenger complex proteins, which have been shown to play key roles in chromosome segregation and mitosis progression. Indeed, using TM chimera constructs and mutational analysis, we found that Tm1J, in conjunction with troponin, play key roles in chromosome segregation and spindle morphology during cell division.

After carefully identifying and thoroughly characterizing the localization and function of the three TM isoforms expressed in Drosophila nonmuscle S2 cells in various cellular conditions as well as cell cycle stages, we can now propose a model of how the three TM isoforms function together, and separately, to perform overlapping but distinct functions in the cell. This study has laid the groundwork for future studies to identify additional mechanisms of tropomyosin isoform-

specific functions and, in so doing, more of the principles underlying the sorting of TM isoforms. We have also now established Drosophila S2 cells as an excellent model system in which to address the role of tropomyosin in regulating the diversity of functions of the actin cytoskeleton and how this integrates with signaling systems that regulate actin filament organization and dynamics. Misexpression of tropomyosin isoforms has been seen in a variety of human diseases from cancer to developmental defects, and this study brings us one step closer to understanding how the diversity of TM isoforms effects key cellular processes, and therefore, what roles each TM isoform could be playing in human diseases (Disanza et al., 2005).

Chapter Two

Identification of tropomyosin isoforms expressed in Drosophila nonmuscle S2 cells

Drosophila nonmuscle S2 cells express three tropomyosin isoforms

Most nonmuscle cells, including budding yeast (S. cerevisiae), express at least two nonmuscle tropomyosin isoforms with many organisms expressing up to eight different nonmuscle tropomyosin (TM) isoforms in the same cell type (Bryce et al., 2003; Gunning et al., 2005). In contrast, it is currently accepted that *Drosophila melanogaster* express only one nonmuscle TM isoform, termed here "Tm1A", a "short" isoform predicted to be 252 amino acids in length (flybase.org). However, several papers in the last ten years suggest that additional nonmuscle TM isoforms may be expressed in the developing Drosophila embryo and may play key roles in various development processes, but efforts to identify these isoforms were inconclusive (Erdélyi et al., 1995; Li and Gao, 2003; Tetzlaff et al., 1996).

Therefore, we first sought to determine if only one TM isoform is expressed in nonmuscle S2 cells. Using the commercially available TM antibody E-17, which was previously used by our lab (Iwasa and Mullins, 2007), we identified one reactive band in S2 cell lysates (Figure 2A, left). That band was approximately 32 kDa in size, the predicted molecular weight of Tm1A. When we probed the same lysates with a different commercially available TM antibody, ab11190 from Abcam, we still found only one reactive band, but it was approximately 38 kDa in size, the predicted molecular weight of a long TM isoform (Figure 2A, right). To confirm this surprising result, we probed the lysates with a third commercially

available TM antibody, TM311. TM311 is a monoclonal tropomyosin antibody widely used in the tropomyosin field as a gold standard for identifying long tropomyosin isoforms in many different species (Schevzov et al., 2011). Again, the TM311 antibody recognizes only one band approximately 38 kDa in size, confirming the presence of at least one long TM isoform, in addition to Tm1A, in nonmuscle S2 cells (Figure 2A, middle).

To identify which long and short TM isoforms are expressed in Drosophila S2 cells, we purified and reverse transcribed mRNA from S2 cells into cDNA and amplified the cDNA using different combinations of primers to individual exons of the Tm1 or Tm2 genes. Using these methods, we found the entire coding sequence of Tm1A as a single unit and that of Tm1J as two large overlapping units, including portions of the 5' and 3' UTRs (Figure 2B, red and blue circles). Surprisingly, we also found the entire coding sequence of Tm2A from the Tm2 gene, previously thought to produce only muscle TM isoforms (Figure 2B, orange circle). We confirmed the presence of Tm1J through mass spec analysis after partially purifying endogenous tropomyosin from S2 cells (data not shown). We found 5 unique peptides present in Tm1J that are not present in Tm1A or Tm2A as well as several peptides throughout the coding sequence shared with other isoforms, like Tm1A. We used cDNA sequence analysis to eliminate several of the "non-canonical" TM isoforms that are predicted to be much larger than the two bands identified in immunoblots and have previously been shown to be expressed only in non-flight muscles. While immunoblots using the E-17 antibody

show that Tm1L is not likely expressed in S2 cells. While the absence of evidence for these other TM isoforms does not rule out their presence, together, our results from immunoblots, cDNA analysis, partial purification, and mass spec show that Drosophila S2 cells express at least three nonmuscle tropomyosin isoforms: Tm1A, Tm1J, and Tm2A.

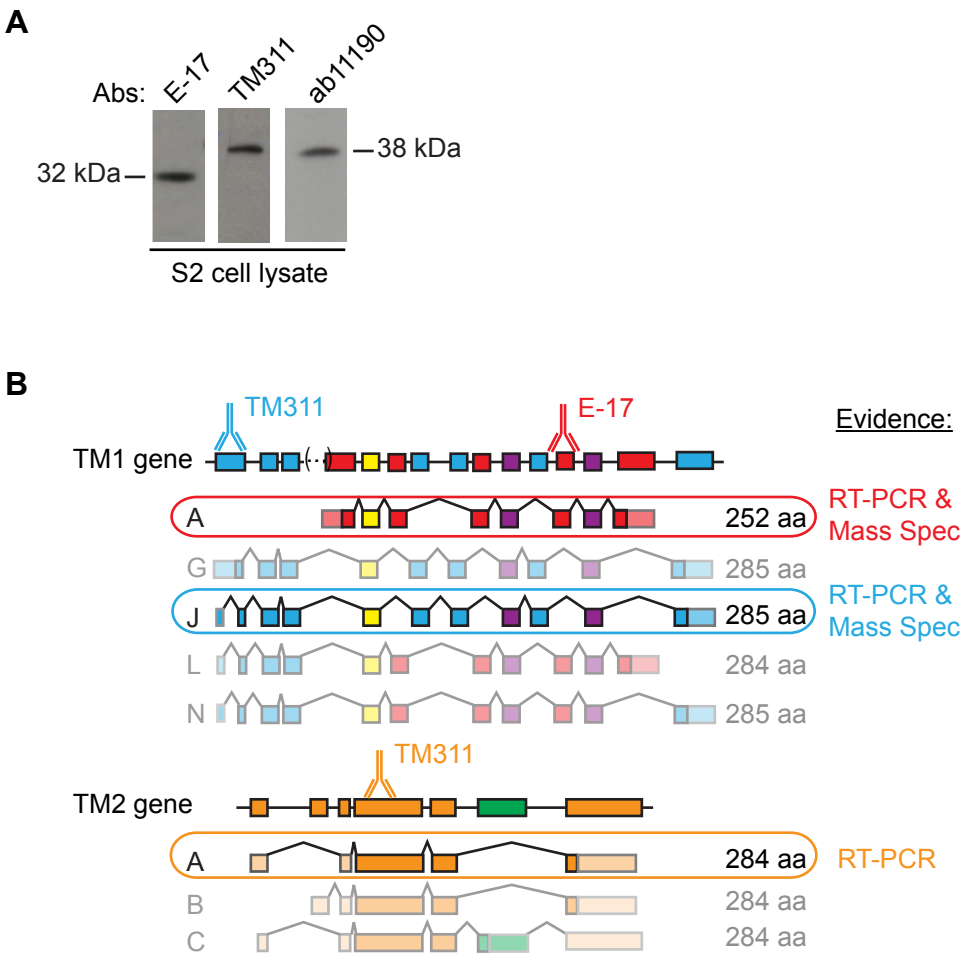


Figure 2. Drosophila S2 cells express three non-muscle tropomyosin isoforms: Tm1A, Tm1J, and Tm2A

(A) Immunoblots of S2 cell lysates probed with three commericially available antibodies with different isoform specificities (E-17, TM311, and ab11190) demonstrate that S2 cells express both “short” and “long” isoforms of tropomyosin. Conditions: see Materials and Methods. The TM311 and ab11190 antibodies recognize a protein with an apparent molecular weight of 38 kDa, characteristic of “long” TM isoforms, while the E-17 antibody recognizes a shorter polypeptide with an apparent molecular weight of 32 kDa, characteristic of “short” TM isoforms. (B) Exons and

introns of the TM1 and TM2 genes and the “canonical” isoforms predicted by FlyBase. For the complete list of possible splice variants, see flybase.org. A combination of techniques (reverse transcriptase PCR; mass spectrometry; and immunoblotting) indicate that three TM isoforms are expressed in Drosophila S2 cells: two isoforms from the TM1 gene (TM1A, TM1J) and one from the TM2 gene (TM2A).

Chapter Three

Determination of the localizations and functions of the three tropomyosin isoforms expressed in Drosophila nonmuscle S2 cells

During interphase, Tm1A and Tm1J co-localize to the lamellum in S2 cells spreading on ConA.

In vertebrates and yeast, nonmuscle tropomyosin (TM) isoforms have been shown to localize to a variety of cellular structures and participate in distinct biological processes {Gunning:2008hv}. To determine what roles TM isoforms play in Drosophila nonmuscle S2 cells, we expressed Tm1A, Tm1J, and Tm2A as GFP-tagged constructs (Figure 3-1) under the pMT promoter, a copper inducible promoter, and monitored their localization in live cells using spinning disk confocal microscopy. S2 cells are often used for their ability to spread out on Concanavalin A (ConA) coated surfaces and form large thin flat projections around the periphery of the cell, termed lamella. The lamella of S2 cells has been shown to recapitulate many of the properties of the leading edge of migrating cells {Rogers:2003jf}. Within the lamella, three main actin networks reside: the lamellipod, a densely packed and highly branched actin network, the lamellum, a less packed network with longer unbranched actin filaments, and the convergence zone where actin is depolymerized and recycled {Gupton:2002ur, Vallotton:2004kz, Gupton:2005gb}.

Previous work from our lab showed that Tm1A localizes to and plays a role in the formation and maintenance of the lamellum {Iwasa:2007ep}. One of the well-known biochemical activities of nonmuscle TM isoforms is the ability to inhibit actin branching by the Arp2/3 complex, a resident protein of the lamellipod

{Blanchoin:2001vh}. To determine if the other two Drosophila TM isoforms expressed in S2 cells also localize to the lamellum, or show an entirely different pattern of localization, we co-expressed actin-mRFP, a marker of the lamellipod, with GFP-tagged Tm1A, Tm1J, or Tm2A. S2 cells were imaged using a spinning disk confocal microscope as live cells spread on ConA coated glass surfaces. We found that both eGFP-Tm1A (Figure 3-2, left column) and eGFP-Tm1J (Figure 3-2, middle column) localize to the less branched lamellum network and were excluded from the highly branched lamellipod network, visualized by actin-mRFP (Figure 3-2). Surprisingly, eGFP-Tm2A showed no significant localization in the lamella (Figure 3-2, right column).

In non-spreading cells, Tm1A localizes to the cortex while Tm1J and Tm2A localize to the Golgi apparatus during interphase.

Plating S2 cells on ConA forces the cells to spread out and adhere to the glass surface, but Drosophila S2 cells normally grow as semi-adherent spherical cells in tissue culture plates. When S2 cells are plated on poly-D-lysine coated glass, however, S2 cells do not spread out but rather maintain their normal spherical shape. To determine what roles the TM isoforms play in non-spreading cells, we plated S2 cells, expressing GFP-tagged Tm1A, Tm1J, or Tm2A, onto poly-D-lysine coated glass and imaged live cells using spinning disk confocal microscopy. In order to determine the cell cycle stage, we co-expressed mCherry-alpha-tubulin with each GFP-tagged TM isoform. In contrast to S2 cells

spreading on ConA, we found that in non-spreading cells, Tm1A and Tm1J do not seem to co-localize but rather Tm1A localizes to the cortex, while Tm1J localizes to ring-shaped structures scattered throughout the cytoplasm (Figure 3-3A, left and middle). And while eGFP-Tm2A showed no significant localization in the lamellar region of spreading cells, in non-spreading cells, Tm2A, like Tm1J, localizes to ring-shaped structures in the cytoplasm (Figure 3-3A, right). To verify that Tm1A and Tm1J do indeed localize to different networks in non-spreading cells, we co-expressed eGFP-Tm1J with mCherry-Tm1A in the same cell. We found that Tm1A, but not Tm1J, does indeed localize to the cortex while Tm1J, but not Tm1A, does form ring-shaped structures in the cytoplasm (Figure 3-3B).

To our knowledge, ring-shaped structures in the cytoplasm of S2 cells, such as those seen with eGFP-Tm1J and eGFP-Tm2A, have not been described before. But, we believed that these structures were not simply aggregated nonfunctional protein because the structures disappeared before mitosis and reappeared after mitosis. This suggested to us that Tm1J and Tm2A likely associate with an intracellular structure that is disassembled during mitosis. To determine which intracellular structure eGFP-Tm1J associates with, we first used different lipid moiety dyes such as ER-tracker, Mito-tracker, and NBD-ceramide to stain the endoplasmic reticulum, mitochondria, and Golgi apparatus, respectively. We found that while eGFP-Tm1J partially overlaps with ER-tracker, the most striking resemblance is between eGFP-Tm1J rings and NBD-ceramide, a marker of the Golgi (data not shown).

Unlike in vertebrates, in Drosophila, the Golgi apparatus does not form into ribbons of linked ministacks but rather remain as single or paired ministacks that are associated with the trans-ER network {Feinstein:2008hq, Kondylis:2007iw}. These Golgi ministacks resemble blobs by light microscopy {Kondylis:2007iw}. To confirm that the ring-shaped structures seen with eGFP-Tm1J and eGFP-Tm2A were associated with the Golgi apparatus, we performed immunofluorescence in S2 cells using an antibody against a resident Golgi protein, GM130. We found that both eGFP-Tm1J (Figure 3-4, top row) and eGFP-Tm2A (Figure 3-4, middle row) ring-shaped structures are indeed Golgi-associated networks, which seem to wrap around and encompass the Golgi blobs or ministacks.

During mitosis, Tm1A localizes to the equatorial cortex during early anaphase and retracting blebs at the cell poles during late anaphase.

In both yeast and vertebrates, nonmuscle TM isoforms have been shown to participate in cell division. Based on these studies, the primary role of TM isoforms is thought to be participation in the contractile ring and formation of the cleavage furrow at the site of division {Skau:2009gl, Wong:2000ju}. To determine what, if any, roles the three Drosophila nonmuscle TM isoforms play in cell division, we co-expressed eGFP-tagged TM isoforms with mCherry-tagged alpha-tubulin and imaged dividing cells using spinning disk confocal microscopy.

We again imaged S2 cells plated on poly-D-lysine (PDL) because S2 cells maintain their native spherical shape and can complete cell division, unlike on ConA where their adherence to the surface prevents complete cytokinesis {Rogers:2008jz, Uehara:2010gp}.

We found that eGFP-Tm1A localizes to the entire cell cortex during prophase. But during early anaphase, eGFP-Tm1A specifically localizes to the equatorial cortex and is absent from the cell poles (Figure 3-5A). Then, as the cell elongates and intracellular pressure causes blebbing at the cell poles {Sedzinski:2011ky}, we found that eGFP-Tm1A localizes specifically to blebs as they begin retracting, suggesting a role for Tm1A in cortical contractility (Figure 3-5A). Similar to eGFP-Tm1A, we found that the actin filament marker, lifeact-eGFP, localizes to the cortex throughout cell division and especially concentrates at the equatorial cortex during anaphase (Figure 3-S1A).

Before and after mitosis, Tm1A and Tm1J both resume their interphase localization patterns at the cortex and Golgi, respectively. But, while we saw Tm2A at the Golgi before and after mitosis, we saw no significant localization pattern of eGFP-Tm2A during mitosis (Figure 3-5B), indicating that Tm2A does not likely play a role in cell division.

During mitosis, Tm1J localizes to centrosomes, kinetochores, the central spindle, and midpoint.

The localization of Tm1A to the equatorial cortex and retracting polar blebs during cell division is similar to that of myosin II, a known tropomyosin interaction partner {Vale:2009bu, Dean:2006hv}. We found that eGFP-Tm1J, on the other hand, shows an entirely different and novel localization pattern throughout cell division (Figure 3-5C). We found that during prophase, a subset of Tm1J localizes to centrosomes and is excluded from the nucleus (Figure 3-5C, prophase). At the onset of mitosis, upon nuclear envelope breakdown (NEBD), the subset of Tm1J that is associated with the Golgi disperses and Tm1J appears at kinetochores (Figure 3-5C, metaphase). Tm1J remains at both centrosomes and kinetochores throughout metaphase while the chromosomes align at the metaphase plate. Then in early anaphase, as the chromosomes begin to separate, Tm1J abruptly dissociates from the kinetochores and instead localizes to the central spindle, the area midway between the separating chromosomes where spindle microtubules overlap (Figure 3-5C, early anaphase and late anaphase). As the cleavage furrow ingresses, the microtubules in the central spindle begin to bundle and Tm1J concentrates at the midpoint of the central spindle (Figure 3-5C, telophase), which will become the midbody after cytokinesis is complete. But, unlike other midbody proteins that remain associated with the midbody long after cell division is complete {Maiato:2003iq}, Tm1J does not stay associated with the midbody. Instead, eGFP-Tm1J relocates from the midpoint back into the cytoplasmic pool and reassembles into ring-

shaped structures surrounding the Golgi soon after the nuclear envelope begins to reform (Figure 3-7B).

The actin nucleator Diaphanous and long actin filaments are also associated with the central spindle during mitosis.

To our knowledge, this is the first time a TM isoform has been shown to localize to the kinetochores or centrosomes during mitosis, two locations not generally thought to contain actin filament networks {Sandquist:2011cx}. But, several members of the formin family, a different type of actin binding protein, have been shown to localize to various parts of the spindle during mitosis. In particular, mDia2 is known to localize to the central spindle and midbody {Watanabe:2010cp}, while mDia3 has been shown to localize to kinetochores during mitosis, similar to what we have found for Tm1J {Wiggan:2011ji}. Indeed, even mDia1, the third member of the mDia family, has also been shown to participate in the dynamics of the Golgi network, another structure we have shown Tm1J localizes to {Zilberman:2011bi}. These similarities between the mDia family proteins and the localization we see for Tm1J led us to hypothesize that the Drosophila homolog of mDia1/2/3, Diaphanous, may function with Tm1J in an isoform-specific manner.

To determine if Diaphanous (Dia) does function with Tm1J in an isoform-specific manner, we imaged Drosophila S2 cells expressing Dia-eGFP and compared its

localization to that of eGFP-Tm1J. We found that, similar to Tm1J and Tm2A, Dia-eGFP localizes to ring-shaped structures surrounding the Golgi during interphase, before and after mitosis (Figure 3-4, bottom row). Similarly, Dia-eGFP also localizes to the central spindle and midpoint, similar to eGFP-Tm1J (Figure 3-5C & D). But, in contrast to Tm1J, Dia did not localize to centrosomes during prophase or kinetochores during metaphase (Figure 3-5C & D). These results suggest that while Diaphanous may function with Tm1J and Tm2A at Golgi during interphase, during mitosis, Dia may function with Tm1J at the central spindle and midpoint during anaphase and telophase, but not at centrosomes or kinetochores during metaphase.

Initially, the presence of two actin binding proteins associated with the central spindle, in the absence of actin filaments, seemed strange. And while initial characterization of lifeact-eGFP localization showed primarily cortical localization during mitosis, we found that when the fluorescence signal of lifeact-eGFP is high enough, we can visualize long intracellular filaments of lifeact-eGFP that seem to be associated with the central spindle, specifically during anaphase (Figure 3-S1B). The fluorescence signal from lifeact-eGFP was always highest in the cortex but, in a subset of S2 cells, long filaments containing lifeact-eGFP could be seen throughout the cytoplasm during metaphase (Figure 3-S1B, top row). Then, as the cell elongates during anaphase, these long intracellular actin filaments seem to align with the central spindle (Figure 3-S1B, bottom row).

Diaphanous is required for Tm1J localization to the central spindle but not to kinetochores.

We showed earlier that Dia also localizes to Golgi-associated cytoplasmic ring-shaped structures similar to Tm1J and Tm2A (Figure 3-4, bottom row). Because Diaphanous (Dia) and Tm1J both localize to the central spindle, midpoint, and Golgi, we reasoned that Dia may be important for Tm1J localization to one or all of these structures. To determine if Dia plays a role in the localization of Tm1J at Golgi after mitosis or the central spindle and midpoint during mitosis, we depleted Diaphanous from S2 cells using RNAi and imaged dividing cells expressing eGFP-Tm1J and mcherry-Tm1A (Figure 3-S2). We found that, in the absence of Diaphanous (Dia), Tm1J still localizes to Golgi before and after mitosis (Figure 3-S2B, A) and to kinetochores during metaphase (Figure 3-S2A, yellow arrow). But Tm1J does not localize to the central spindle and midpoint during mitosis in the absence of Dia (Figure 3-S2A, asterisk). Instead, Tm1J relocalizes directly from kinetochores to Golgi during anaphase (Figure 3-S2A, late anaphase). These results indicate that Tm1J requires Diaphanous to localize to the central spindle and midpoint but localizes to kinetochores and Golgi independent of Dia.

Tm1A functions with myosin II during mitosis and interphase.

Tropomyosins are well known for their ability to enhance or modulate myosin activity in cells {Hodges:2012dt, Clayton:2010bf, Perry:2001vw}. Nonmuscle

myosin II plays several key roles during cell division, including maintaining cortical contractility to balance intracellular pressure and maintain cell shape {Sellers:2000ub, Sedzinski:2011ky}. Due to the cortical localization of Drosophila Tm1A, we hypothesized that Tm1A and myosin II would co-localize and work together during interphase and mitosis to maintain cortical contractility. To determine if Tm1A and myosin II do function together, we co-expressed mCherry-Tm1A with Sqh-GFP (the nonmuscle myosin II regulatory light chain in Drosophila, tagged with GFP) and imaged S2 cells during mitosis and interphase. We found that during mitosis, mCherry-Tm1A and Sqh-GFP co-localize to the equatorial cortex and are absent from the cell poles during early anaphase (Figure 3-6A, anaphase). During late anaphase, as the cell elongates and the intracellular pressure builds causing blebbing at the cell poles, mCherry-Tm1A and Sqh-GFP co-localize specifically to retracting blebs at the cell poles (Figure 3-6A). Likewise, we found that mcherry-Tm1A and Sqh-eGFP co-localized to the cortex and retracting membrane blebs in spherical S2 cells during interphase (Figure 3-6B, C). Together, these results indicate that Tm1A and myosin II function together to maintain cortical contractility during mitosis and interphase in Drosophila S2 cells.

Tm1A localizes to the equatorial cortex prior to Diaphanous.

Formins have also been shown to be crucial components of the contractile ring and cleavage furrow in various cell types including yeast and S2 cells

{Kovar:2003kq, Castrillon:1994te, Goode:2007eq}. In S. pombe, Pollard and colleagues have shown that Bni1p (a homologue of the Drosophila formin, Diaphanous) localizes to the site of cell division and first polymerizes the actin network that is subsequently bound by the tropomyosin Cdc8p, and clustered together to form a contractile ring through the activity of tropomyosin and myosin II {Laporte:2010jb, Skau:2009gla}. Tm1A's co-localization with myosin II suggested to us that, in Drosophila,Tm1A might be the TM isoform that, like Cdc8p in S. pombe, binds to and stabilizes the actin network nucleated by formins. To determine if Tm1A does indeed coordinate with the Drosophila homologue of Bni1p, Diaphanous, during cell division, we co-expressed eGFP tagged Diaphanous (Dia-eGFP) with mcherry-Tm1A and followed their localization during cell division (Figure 3-S3). We expected that Tm1A and Dia would co-localize to the site of furrow ingression as they both likely play roles in constriction during cytokinesis. Surprisingly, we found that while their localization patterns were similar, mcherry-Tm1A and Dia-eGFP displayed different spatial and temporal dynamics (Figure 3-S3). While both Tm1A and Dia localize to the equatorial cortex during early anaphase, Tm1A seems to arrive at the cortex prior to Diaphanous (Figure 3-S3A, metaphase). In addition, while the region of the cortex occupied by mcherry-Tm1A expands over time as anaphase progresses, Dia-eGFP localization seems to contract and accumulate in a more focused region near the site of division, which overlaps with but is not restricted to the cortex, as mcherry-Tm1A does during cell division (Figure 3-S3A, bottom row schematic). Similarly, during interphase, mcherry-Tm1A, but not Dia-eGFP

localized to retracting membrane blebs around the periphery of the cell (Figure 3-S3B).

Diaphanous is not required for the recruitment of Tm1A to the cleavage furrow, but is needed to stably maintain Tm1A at the midzone.

To further verify that Tm1A localization to the equatorial cortex was not dependent on Diaphanous, we depleted Diaphanous protein using RNAi in S2 cells stably expressing mcherry-Tm1A and eGFP-Tm1J and imaged S2 cells during division using spinning disk confocal microscopy (Figure 3-S2). Similar to previous studies, we saw that depletion of Diaphanous (Dia) causes an obvious multinucleate phenotype as the cells fail to complete cytokinesis {Rogers:2003jf, Somma:2002fh}.

When we followed Tm1A localization during cell division, we found that even after depletion of Diaphanous protein from S2 cells by RNAi, mcherry-Tm1A is still recruited initially to the equatorial cortex, but this localization is not stable (Figure 3-S2C). In the absence of Dia, we found that Tm1A does not remain at the equator of the cell but rather delocalizes from the equatorial cortex and undergoes highly dynamic appearances and disappearances as patches around the cell cortex, often associated with areas of clear cortical contraction (Figure 3-S2, blue arrows). These findings are very similar to those seen by Dean et al. 2005 where they found that while f-actin and Diaphanous are not necessary for

initial recruitment of myosin II to the equatorial cortex, both proteins are required to stably maintain myosin II at the furrow. Thus, these results further support our model that Tm1A functions with myosin II to maintain cortical contractility during cell division.

Tm1J and Tm2A localize to the Golgi in a cell cycle-dependent fashion.

While the presence of Tm1A at the cortex was expected, the localization of Tm1J and Tm2A to the Golgi apparatus was quite surprising. To determine what role Tm1J and Tm2A play at the Golgi, we first imaged S2 cells expressing GFP-tagged Tm1J or Tm2A before and after mitosis. We found that the localization of both Tm1J and Tm2A to the Golgi apparatus is cell cycle-dependent (Figure 3-7). During late G2 phase, Tm1J and Tm2A remain associated with Golgi ministacks, but immediately after nuclear envelope breakdown (NEBD) and the onset of mitosis, Golgi-associated Tm1J and Tm2A networks rapidly disperse throughout the cytoplasm (Figure 3-7A). As noted previously, during metaphase, Tm1J localization shifts to the spindle apparatus, while Tm2A shows no significant localization during mitosis (Figure 3-5B, C). At the end of mitosis, after the nuclear envelope reforms, we found that both Tm1J and Tm2A rapidly reassemble around the Golgi ministacks (Figure 3-7B). When we imaged S2 cells expressing eGFP-tagged fringe, a resident Golgi protein, we saw very similar spatiotemporal dynamics, where fringe containing Golgi blobs disperse after NEBD and reform immediately after the nuclear envelope reforms during

telophase (Figure 3-7B). Interestingly, while Tm1J and Tm2A rapidly disperse after NEB, with full dispersal seen just a few minutes after NEB, fringe blobs seem to disperse much more slowly and can still be seen over 15 minutes after NEB (Figure 3-7A).

Tm1J and Tm2A, the Golgi-associated TM isoforms, influence cell cycle progression through G0/G1 and G2/M.

The cell cycle dependent localization pattern of Tm1J and Tm2A at the Golgi, in addition to the role Golgi are known to play in cell cycle regulation {Rabouille:2007vi, Kondylis:2007iw}, suggested to us that Tm1J and/or Tm2A may play roles in cell cycle progression through their role(s) at the Golgi. To determine what, if any, roles Tm1J and Tm2A play in the cell cycle, we depleted Tm1J and Tm2A, singly and together, from S2 cells using RNAi. We then used flow cytometry to analyze the cell cycle status of S2 cells fixed and stained with propidium iodide, a DNA stain. We found that depletion of Tm1J or Tm2A resulted in an increase in the percentage of cells in the G1/G0 stage with a relative decrease in the percentage of cells in G2/M stage of the cell cycle (referred to henceforth as “G1/G2 phenotype”) (Figure 3-8). When we depleted both Tm1J and Tm2A, we saw a stronger “G1/G2 phenotype” than either protein depleted alone. In contrast, depletion of Tm1A, the cortical TM isoform, did not cause a significant change in the ratio of cells in G1 in comparison to G2 (Figure 3-8). The Golgi apparatus is the only cellular structure we have found that

contains both Tm1J and Tm2A but not Tm1A. Therefore, we believe that the "G1/G2 phenotype" seen with Tm1J and Tm2A depletions is related to their function at the Golgi. This G1/G2 phenotype is similar to that seen after depletion of the resident Golgi kinase, myt1, which has been shown to play roles in Golgi maintenance, disassembly, reassembly, and cell cycle progression {Nakajima:2008jp, Jin:2008gt, Cornwell:2002wy, Villeneuve:2013cc}.

The N-terminus and C-terminus of Tm1J influence localization to the Golgi during interphase.

The differential localization of Tm1A, Tm1J, and Tm2A indicated to us that each TM isoform likely possesses certain sequence elements or biochemical activities that influence its localization and function in an isoform-specific manner. To determine which regions were important for localization to the Golgi during interphase, we created tropomyosin chimeras containing regions of different TM isoforms. When we designed the chimera constructs, we chose to use naturally occurring exon boundaries in order to maintain the coiled-coil structure of each TM isoform and so that each chimera would maintain the canonical long or short isoform length. Both of these parameters are known to be crucial for tropomyosin binding to actin {Brown:2005fz, Murakami:2008en, Brown:2001bg}. When we imaged S2 cells during interphase, we found that regions in both the N and C terminus influenced the localization of tropomyosin isoforms to ring-shaped structures at the Golgi (Figure 3-9). While wildtype Tm1A did not localize to the

Golgi, the chimera 1A-CtermJ, where we replaced the C terminus of Tm1A with that of Tm1J, did localize to the Golgi during interphase (Figure 3-9). Likewise, the chimeras Tm1J-Tm1A, where we replaced the N terminus of Tm1A with that of Tm1J, and Tm1Ains42aa, where we inserted forty-two amino acids from the N –terminus of Tm1J into the N terminus of Tm1A, both localize to Golgi during interphase (Figure 3-9). These results indicate that sequences in both the N and C termini of Tm1J influence the localization of tropomyosin to the Golgi apparatus in Drosophila S2 cells.

The C-terminus of Tm1A influences localization to the cortex during interphase.

In contrast, when we looked at cortical localization during interphase, we found that the C-terminus, but not the N-terminus, of Tm1A was required for localization to the cortex during interphase (Figure 3-9). Tm1A, Tm1JCtermA, and Tm1J-Tm1A, all containing the C-terminus of Tm1A but different N-termini, all localized to the cortex during interphase, but Tm1ACtermJ, which did not contain the C-terminus of Tm1A, did not localize to the cortex (Figure 3-9). These results indicate that the C terminus of Tm1A is necessary for localization of tropomyosin to the cortex in Drosophila S2 cells. On the other hand, Tm2A-CtermA, where we replaced the C terminus of Tm2A with that of Tm1A did not localize to the cortex, indicating that the C-terminus of Tm1A is not sufficient to localize tropomyosin to the cortex (Figure 3-9).

Together, these results indicate that sequence elements in the C-terminus of Tm1A are necessary but not sufficient to determine cortical localization, while sequence elements in both the N and C-termini of Tm1J influence the localization of TM isoforms to the Golgi in Drosophila S2 cells. Indeed, while the localization to the Golgi or cortex seemed to be mutually exclusive in Tm1J and Tm1A, respectively, the chimera Tm1J-Tm1A, which contained the N-terminus of Tm1J with the C-terminus of Tm1A, localizes to both the Golgi and cortex in the same cell (Figure 3-9). These results show that localization to the Golgi and cortex are not mutually exclusive and that separate elements in the N-terminus of Tm1J and C-terminus of Tm1A control localization to the Golgi and cortex, respectively.

The C-terminus of Tm1A is necessary for localization to the equatorial cortex, while the C-terminus of Tm1J is necessary and sufficient for localization to kinetochores during mitosis.

We next determined which regions of Tm1A and Tm1J determine the localization of TM isoforms during mitosis. Using the same chimeras mentioned above, we co-expressed mCherry-alpha-tubulin in S2 cells and imaged cells during mitosis. We found that Tm1J-CtermA but not Tm1A-CtermJ localizes to the equatorial cortex, while Tm1A-CermJ, but not Tm1J-CtermA, localizes to the kinetochores (Figure 3-10). These results show that the C-terminus of Tm1A is necessary for localization to the equatorial cortex while the C-terminus of Tm1J is necessary for

localization to the kinetochores during mitosis. Furthermore, when we replaced the C-terminus of Tm2A, an isoform that shows no significant localization during mitosis, with that of Tm1J, we found that Tm2A-CtermJ localizes to kinetochores during mitosis (Figure 3-10). In contrast, replacement of the C-terminus of Tm2A with that of Tm1A, Tm2A-CtermA, did not induce localization to the equatorial cortex (Figure 3-10). Together, these results show that the C-terminus of Tm1A is necessary for localization to the equatorial cortex while the C-terminus of Tm1J is both necessary and sufficient for localization of tropomyosin to kinetochores during mitosis.

The C-terminus of Tm1J, but not Tm1A or T2A, contains a consensus-binding site for troponin T.

We reasoned that the regions of each TM isoform that conferred their localization may contain specific binding sites for partner proteins and would therefore give more insight into how each TM isoform functions at its respective subcellular locations. And so we compared the protein sequences in the C termini of Tm1A, Tm1J, and Tm2A to determine which sequence elements may influence localization or function. Because the localization of Tm1J to kinetochores was most surprisingly and since we found that the C-terminus of Tm1J is sufficient for localization to kinetochores (Figure 3-10), we chose to focus on sequence elements in the C-terminus of Tm1J that may influence localization to kinetochores during mitosis. After aligning the C-terminus of Tm1A, Tm1J, and

Tm2A, we then looked for sequences that are present in the sequence of Tm1J, but not Tm1A and Tm2A, as candidates for kinetochore functional relevance. We found that Tm1J, but not Tm1A or Tm2A, contained a consensus binding site for troponin T (Figure 3-11A). The troponin (Tn) regulatory complex is composed of three subunits: calcium-binding troponin C (TnC), inhibitory troponin I (TnI) and the tropomyosin binding troponin T (TnT) {Murakami:2008en}. TM isoforms that are regulated by the Tn regulatory complex contain both primary and secondary TnT binding sites in the N and C termini, as well as in internal exons. We found that the C terminus of Tm1J contains a consensus “strong TnT binding” sequence while the C terminus of Tm1A and Tm2A contains sequences indicative of “weaker TnT binding” (Figure 3-11A).

Mutation of the troponin binding site in Tm1J causes defects in chromosome segregation and spindle morphology during mitosis.

The primary Tn binding site in the C-terminus of tropomyosin contains a consensus binding motif that, when mutated, displays decreased binding of, and therefore regulation by, the Tn regulatory complex {Murakami:2008en, HitchcockDeGregori:1987th, Hammell:1996vd}. We reasoned that the presence of a strong Tn binding site in the C-terminus of Tm1J might mean that the Tn regulatory complex plays a role in the function of Tm1J at kinetochores. To determine if troponin does play a role in Tm1J’s localization to or function at the spindle during mitosis, we mutated two amino acids in the consensus troponin T

binding site of Tm1J, Y267 to N and I270 to L (“YNIL”). The equivalent “YNIL” mutation in skeletal muscle tropomyosin decreases the binding affinity of skeletal muscle troponin T {Murakami:2008en}. N267 and L270 are also the amino acids found in the equivalent positions of Tm1A, one of the TM isoforms we have shown does not localize to kinetochores in S2 cells (Figure 3-5A, 3-10).

We found that the Tm1J-YNIL mutant seems to localize to centrosomes and kinetochores as well as wildtype Tm1J (Figure 3-11B). But, surprisingly, we also found that expression of the Tm1J-YNIL mutant, even in the presence of endogenous wildtype Tm1J, resulted in obvious defects in chromosome segregation and spindle morphology (Figure 3-11B). Live imaging analysis revealed that cells expressing the Tm1J-YNIL mutant show a range of kinetochore attachment defects as well as spindle pole focusing problems, which often leads to a delay in anaphase progression. In addition, those that did enter anaphase, often show chromosome segregation defects such as the formation of binucleate cells after cell division failure or micronuclei surrounding unaligned and therefore missegregated chromosomes (Figure 3-11).

To quantify the severity of these defects, we imaged dividing cells expressing GFP-tagged Tm1J-wildtype or Tm1J-YNIL mutant proteins and grouped them into “normal” or “abnormal” mitoses. Abnormal mitoses were defined as any mitotic cell that took greater than 45 minutes to progress through metaphase and into anaphase or that showed clear chromosome segregation defects such as

the creation of micronuclei or binucleate cells. We found that, on average, 51% of cells expressing the Tm1J-YNIL mutant exhibit abnormal mitoses, compared to just 25% of cells expressing wildtype Tm1J (Figure 3-11C). In almost all cells that took greater than 45 minutes to progress into anaphase, clear kinetochore attachment or spindle pole focusing defects were seen earlier in mitosis (Figure 3-11B). Together, these results indicate that Tm1J, in conjunction with the troponin regulatory complex, play a critical role in chromosome segregation through its localization to the kinetochores and centrosomes during mitosis.

Model of TM isoform localization and function during the cell cycle

After determining which TM isoforms are expressed in Drosophila S2 cells and then carefully analyzing their localizations and functions in S2 cells during various stages of the cell cycle, we can now construct a model of how TM isoforms function at various locations in the cell throughout the cell cycle (Figure 3-12). We found that both Tm1J and Tm2A localize to the Golgi in late G2 and early G1 and together function to influence G1/G2 cell cycle progression (Figure 3-12G-H, A). An increase in the relative percentage of G1 to G2 cells could indicate that S2 cells are progressing more slowly through G1 or faster through G2. After the nuclear envelope breaks down (NEBD) at the onset of mitosis, a subset of Tm1J also localizes to the centrosomes, while Tm1A remains at the cortex (Figure 3-12A). During pro-metaphase, soon after NEBD, Tm1J and Tm2A disassemble from ring-shaped structures surrounding the Golgi while Golgi

ministacks fragment and disperse throughout the ctyoplasm. Tm2A and Golgi remain scattered around the dividing cell during mitosis (Figure 3-12B). During metaphase, Tm1J remains at centrosomes at the spindle poles and also localizes to kinetochores while chromosomes align at the metaphase plate (Figure 3-12C). During early anaphase, Tm1A and myosin II co-localize at the equatorial cortex and are absent from the cell poles (Figure 3-12D). Tm1J remains at kinetochores and spindle poles as chromosomes begin to separate (Figure 3-12D). But during late anaphase, as the cell elongates and intracellular pressure builds, Tm1A and myosin II co-localize specifically to retracting blebs at the cell poles to maintain cortical contractility and cell shape (Figure 3-12E). On the other hand, the localization of Tm1J shifts from the centrosomes and kinetochores to the central spindle during late anaphase (Figure 3-12E). Then during telophase, Tm1A remains at the cortex as the cell completes cytokinesis. But as the central spindle microtubules bundle together, Tm1J initially localizes to the midpoint (Figure 3-12F), but as the nuclear envelope reforms, Tm1J, Tm2A, and Dia reassemble into ring-shaped structures surrounding the Golgi as the Golgi ministacks also reform (Figure 3-12G). During interphase, the associated-associated TM isoforms, Tm1J and Tm2A, function to influence G1/G2 cell cycle progression (Figure 3-12H, A).

During both interphase and mitosis, we found that Tm1A, along with myosin II, localizes to the cortex and specifically to sites of cortical contraction. Future

experiments should be able to determine what roles Tm1A and myosin II play in each other's localization and function at the cortex and sites of contraction.

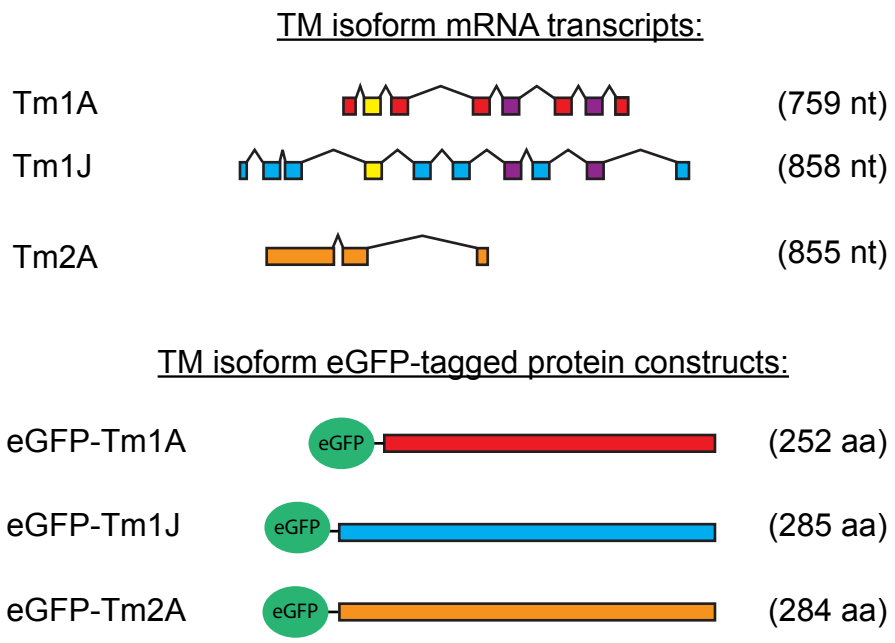


Figure 3-1. Cartoon depiction of TM isoform constructs expressed in Drosophila nonmuscle S2 cells.

Top panel illustrates mRNA splice variants determined to be present in Drosophila S2 cells. Left column: name used to designate each splice variant based on the names found in flybase.org at the time of the experiments (2008). Right column: size of mRNA splice variant is in parentheses. Bottom panel illustrates contructs used for TM isoform localization studies. TM isoform protein products are tagged with eGFP on the N-terminus. Left column: name used to designate each eGFP-tagged TM isoform protein construct based on the names found in flybase.org at the time the constructs were made (2010). Right column: size of TM isoform protein without eGFP is in parentheses.

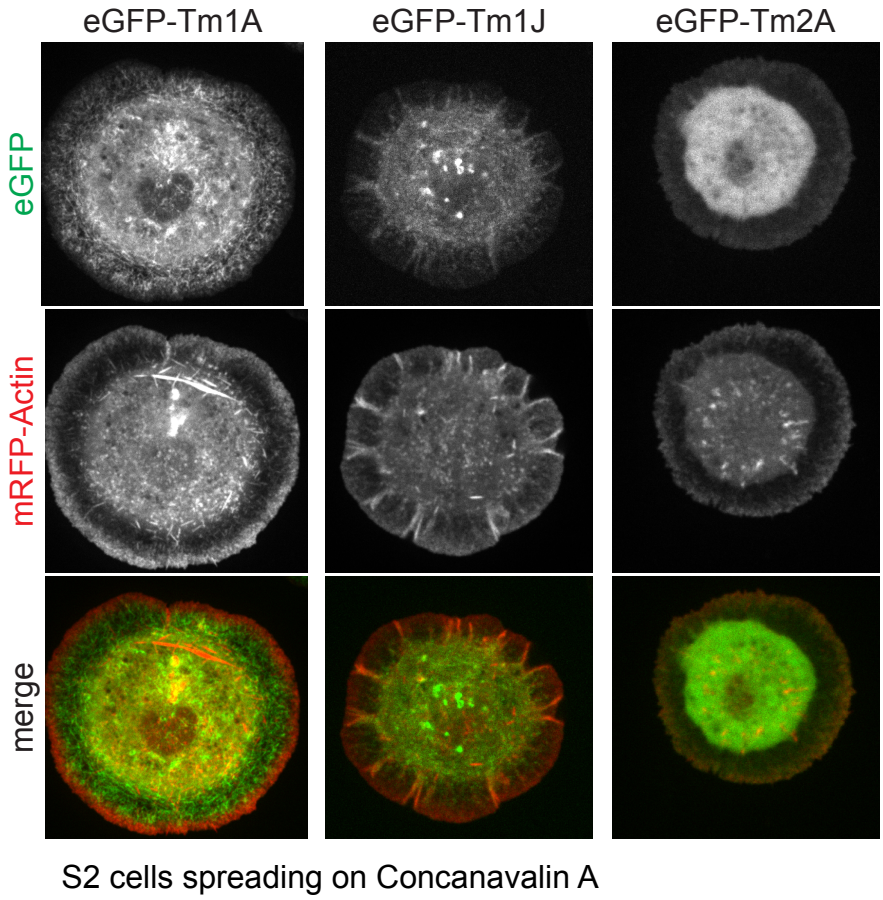


Figure 3-2. During interphase, Tm1A and Tm1J co-localize to the lamellum in S2 cells spreading on ConA.

During interphase, in S2 cells spreading on Concanavalin A (ConA), both eGFP-Tm1A (left column) and eGFP-Tm1J (middle column) localize to the lamellum actin network, but are excluded from the highly branched lamellipod network, visualized with mRFP-Actin, at the leading edge of the cell. In contrast, eGFP-Tm2A (right column) shows no significant localization in the lamellar region of S2 cells spreading on ConA. Top row: eGFP. Middle row: mRFP-Actin. Bottom row: merged images (eGFP, green; mRFP-Actin, red).

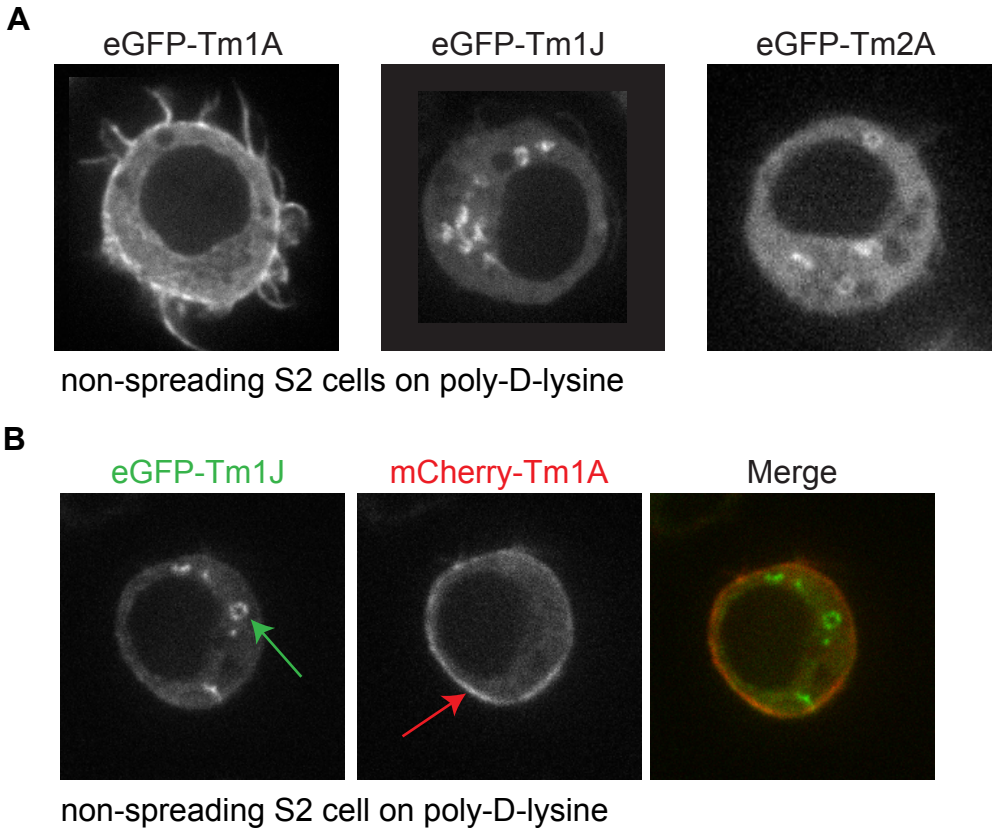


Figure 3-3. In non-spreading S2 cells, Tm1A localizes to the cortex, while Tm1J and Tm2A both localize to the Golgi during interphase. In non-spreading S2 cells, plated on poly-D-lysine ,(A) eGFP-Tm1A (left) localizes to the cell cortex while eGFP-Tm1J (middle) and eGFP-Tm2A (right) localize to ring-shaped structures in the cytoplasm. (B) eGFP-Tm1J (green; left) and mCherry-Tm1A (red; middle) clearly localize to distinct structures when both expressed in the same cell (merge, right). (green arrow, ring-shaped structure. red arrow, cortex.)

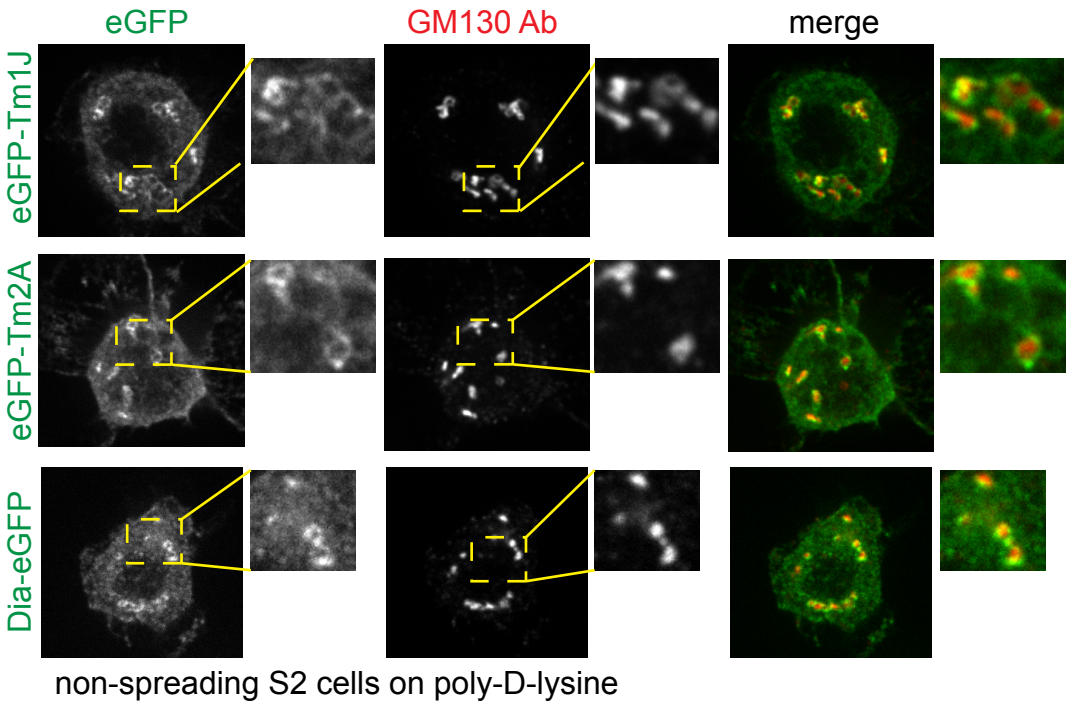


Figure 3-4. The ring-shaped patterns formed by non-cortical tropomyosin isoforms, Tm1J and Tm2A, as well as Diaphanous, are associated with the Golgi apparatus.

Non-spreading S2 cells plated on poly-D-lysine, expressing eGFP-tagged Tm1J, Tm2A or Diaphanous, were fixed and stained with an antibody against the cis-Golgi marker, GM130 (”GM130 Ab”). Top row: eGFP-Tm1J rings (left column, green in merge) encircle the cis-Golgi marker, GM130 (middle column, red in merge). Middle row: eGFP-Tm2A rings (left column, green in merge) also encircle the cis-Golgi marker, GM130 (middle column, red in merge). Bottom row: Diaphanous-eGFP rings (left column, green in merge) also encircle the cis-Golgi marker, GM130 (middle column, red in merge). Areas outlined in yellow dashed box in left-hand images are blown up in the right-hand images.

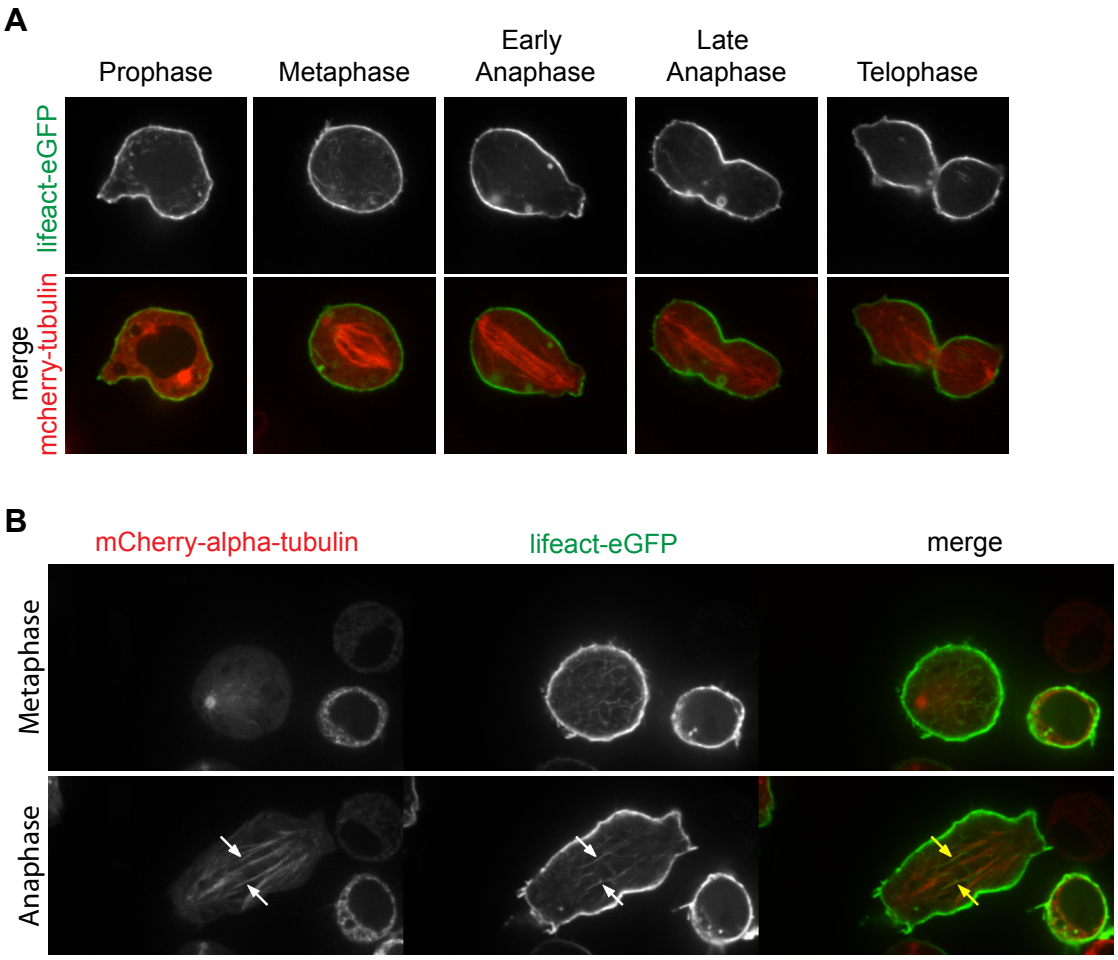


Figure 3-S1. The actin-binding reporter, lifeact-eGFP, localizes to the cortex and cleavage furrow during mitosis and aligns with the central spindle during anaphase.

Dividing S2 cells plated on poly-D-lysine surfaces (A) Top: lifeact-eGFP at various stages of mitosis, as indicated above the images. Bottom: merged images of lifeact-eGFP (green) and mCherry-alpha-tubulin (red). (B) When lifeact-eGFP fluorescence is high, long f-actin filaments can be seen all throughout the cytoplasm during metaphase (top row) but specifically associated with mictrotubules of the central spindle (arrows) during late anaphase (bottom row).

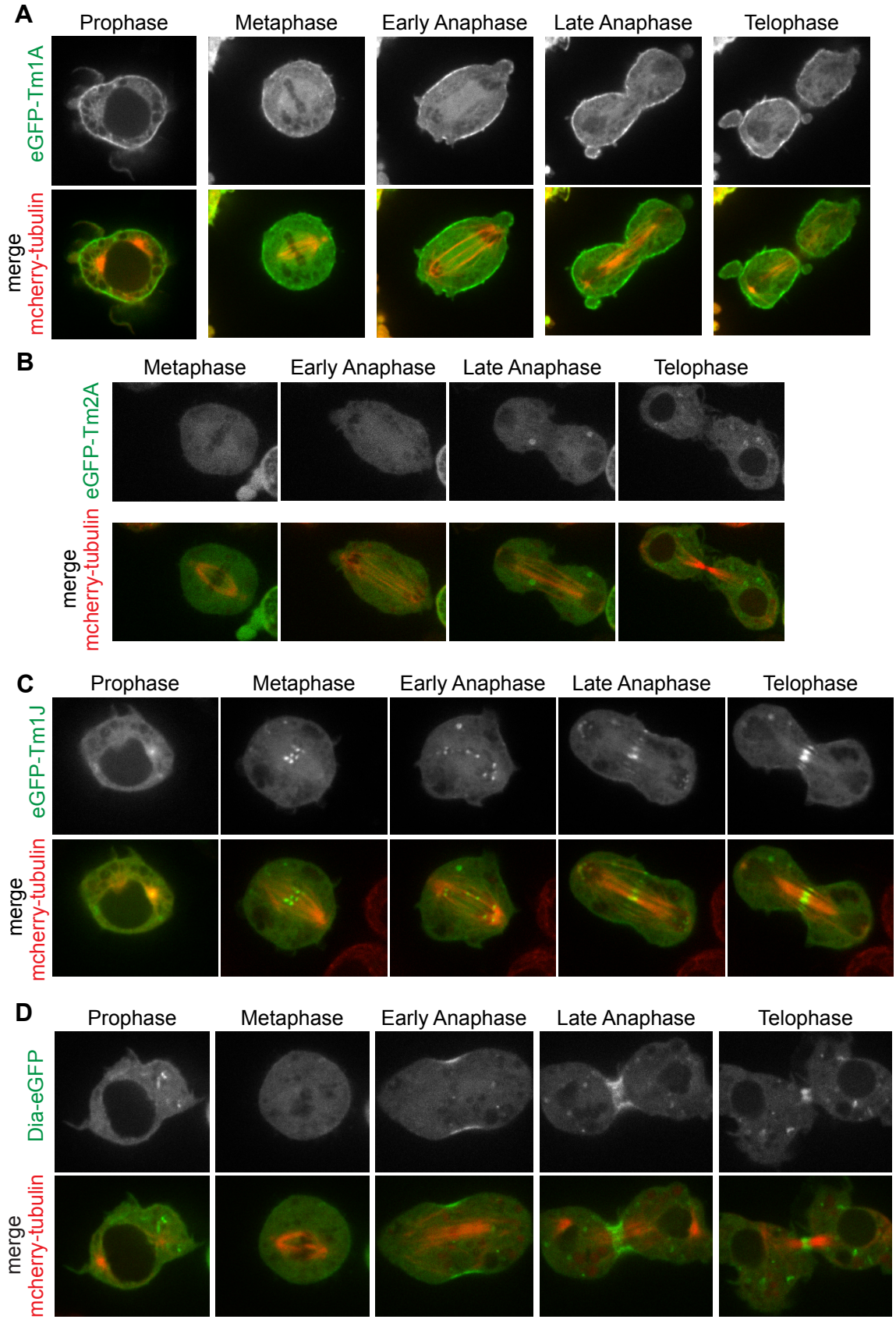

A
Prophase
Metaphase
Early Anaphase
Late Anaphase
Telophase
eGFP-Tm1A
merge
mcherry-tubulin
B
Metaphase
Early Anaphase
Late Anaphase
Telophase
eGFP-Tm2A
merge
mcherry-tubulin
C
Prophase
Metaphase
Early Anaphase
Late Anaphase
Telophase
eGFP-Tm1J
merge
mcherry-tubulin
D
Prophase
Metaphase
Early Anaphase
Late Anaphase
Telophase
Dia-eGFP
merge
mcherry-tubulin

Figure 3-5. During mitosis, Tm1A localizes to the equatorial cortex and retracting polar blebs, while Tm1J localizes to the centrosomes, kinetochores, central spindle, and midpoint during different stages of mitosis in non-spreading Drosophila S2 cells.

(A) The cortical tropomyosin isoform, Tm1A, localizes to the cortex and cleavage furrow during mitosis. Prophase: eGFP-Tm1A is concentrated at the cell cortex. Metaphase: eGFP-Tm1A is no longer concentrated at the cortex and is found throughout the cytoplasm. Early Anaphase: eGFP-Tm1A is highly enriched in the equatorial cortex and absent from polar regions. Late Anaphase: as the cleavage furrow ingresses and the cell elongates, eGFP-Tm1A localizes specifically to retracting blebs at the cell poles. Top: eGFP-Tm1A. Bottom: merged images of eGFP-Tm1A (green) and mCherry-alpha-tubulin (red). (B) Tm2A has a diffuse, nonspecific cytoplasmic localization pattern during mitosis. Top: eGFP-Tm2A during various stages of mitosis. Bottom: merged images of eGFP-Tm2A (green) and mCherry-alpha-tubulin (red). (C) In contrast, Tm1J localizes to the spindle during mitosis. Prophase: eGFP-Tm1J is at the centrosomes. Metaphase: eGFP-Tm1J localizes to kinetochores and centrosomes. Early Anaphase: eGFP-Tm1J remains associated with centrosomes and separating kinetochores. Late Anaphase: eGFP-Tm1J localization shifts to the central spindle. Telophase: eGFP-Tm1J becomes tightly focused on the microtubule bundle at the midpoint of the central spindle. Top: eGFP-Tm1J during various stages of mitosis. Bottom: merged images of eGFP-Tm1J (green) and mCherry-alpha-tubulin (red). (D) The actin nucleator, Diaphanous (Dia), localizes to the equatorial cortex during early anaphase, similar to Tm1A, but localizes to the central spindle and midpoint during late

anaphase and telophase, similar to Tm1J localization. Prophase: Dia-eGFP is seen at Golgi-associated ring-shaped structures in the cytoplasm. Metaphase: Dia-eGFP disperses from ring-shaped structures and remains diffuse. Early Anaphase: Dia-eGFP localizes to the equatorial cortex. Late Anaphase: Dia-eGFP localizes to the central spindle. Telophase: Dia-eGFP localization becomes tightly focused on the microtubule bundle at the midpoint of the central spindle. Top: Dia-eGFP during various stages of mitosis. Bottom: merged images of Diaphanous-eGFP (green) and mCherry-alpha-tubulin (red).

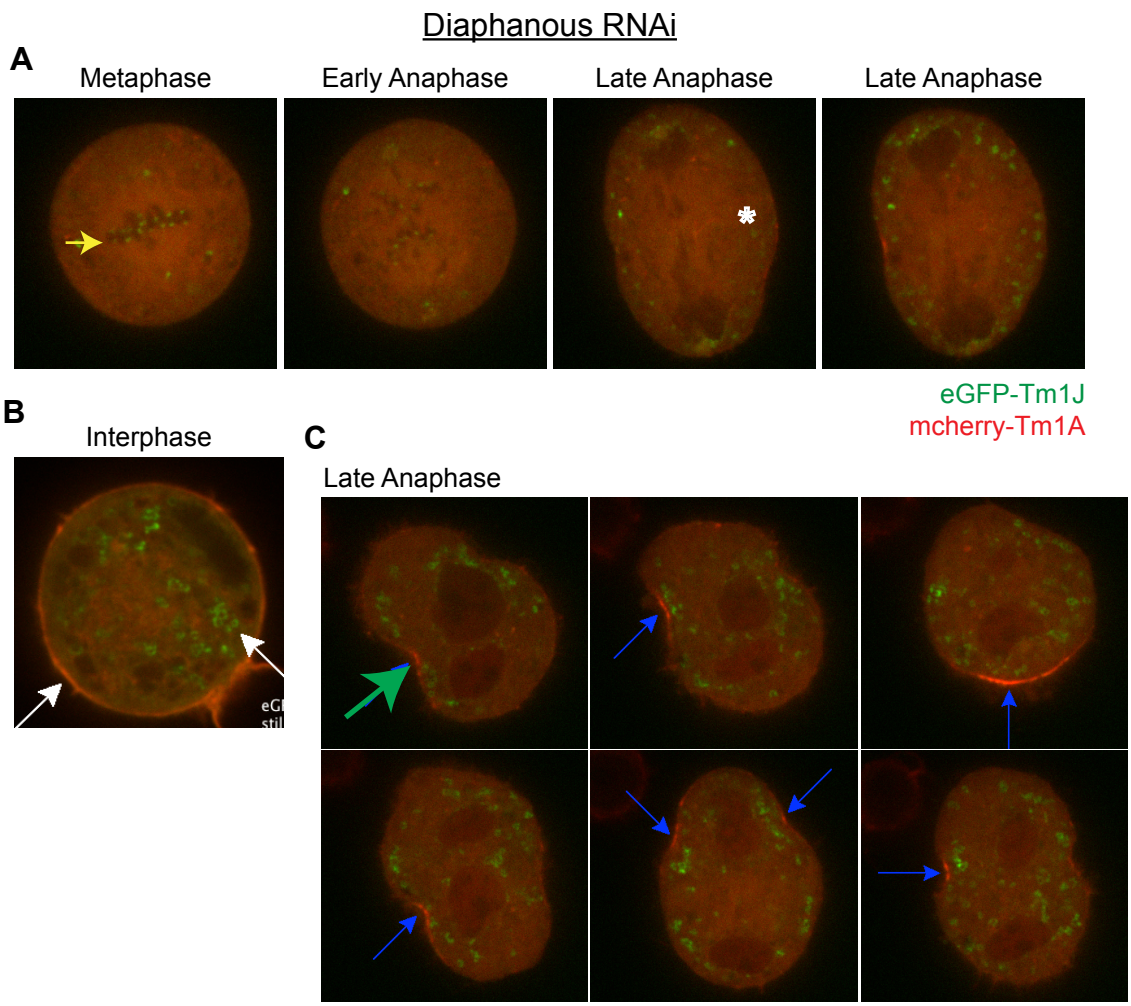


Figure 3-S2. Diaphanous is required for Tm1J localization to the central spindle and midpoint, but is not required for Tm1J localization to kinetochores during mitosis. Diaphanous is not required for initial localization of Tm1A to the equatorial cortex but is required to maintain Tm1A at the equator of the dividing cell.

Merged images of mCherry-Tm1A (red) and eGFP-Tm1J (green) in non-spreading S2 cells after depletion of Diaphanous with RNAi. (A) Dividing cell showing eGFP-Tm1J still at kinetochores (yellow arrow) in metaphase but absent from the central spindle in late anaphase (white asterisk). (B) S2 cell in interphase showing eGFP-Tm1J at golgi (right arrow) and

mCherry-Tm1A at cortex (left arrow). (C) mCherry-Tm1A initially localizes to the equatorial cortex during anaphase (green arrow) but does not stay at the equator in the absence of Diaphanous but rather relocalizes to patches all along the cortex at sites of contraction (blue arrows).

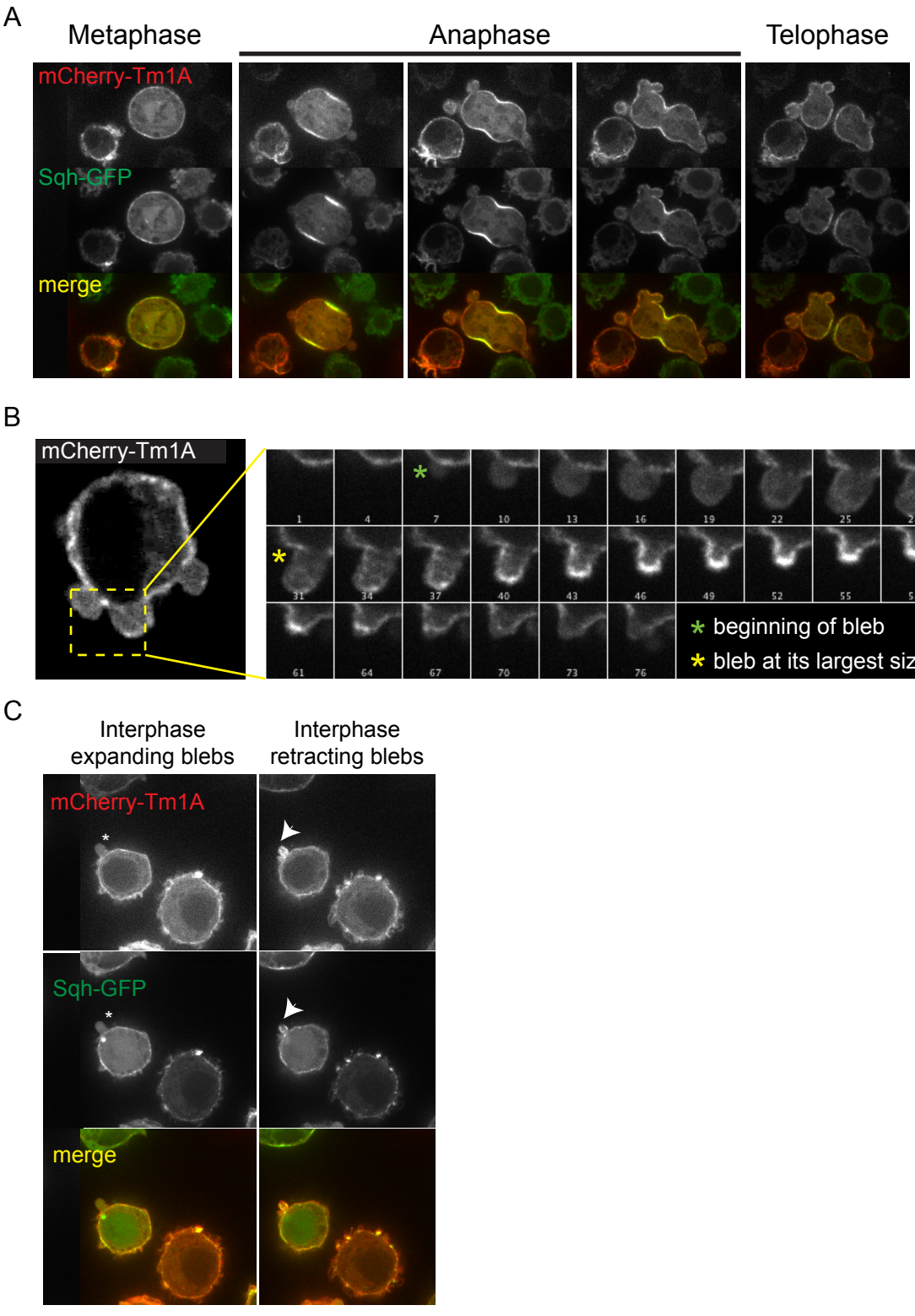

A
Metaphase
Anaphase
Telophase
mCherry-Tm1A
Sqh-GFP
merge
B
mCherry-Tm1A
1
4
7
10
13
16
19
22
25
28
31
34
37
40
43
46
49
52
55
58
61
64
67
70
73
76
* beginning of bleb
* bleb at its largest size
C
Interphase
expanding blebs
Interphase
retracting blebs
mCherry-Tm1A
Sqh-GFP
merge

Figure 3-6. Tm1A co-localizes with myosin II to sites of contraction at the cell cortex during both mitosis and interphase.

(A) During mitosis, Tm1A and myosin II (visualized with GFP-tagged myosin regulatory light chain, Sqh; “Sqh-GFP”) co-localize to the equatorial cortex during early anaphase and to retracting polar blebs during late anaphase and telophase, both sites of contraction. Top row: mCherry-Tm1A. Middle row: Sqh-GFP. Bottom: merged images of mCherry-Tm1A (red) and Sqh-GFP (green) during various stages of mitosis as indicated above. (B) Images from timelapse movies of blebbing S2 cells show that during interphase, Tm1A localizes specifically to retracting, but not expanding, blebs at the cell periphery. Image on left is representative image from timelapse. Yellow boxed region in the right image is blown up as a montage of consecutive images on the left. Green asterisk notes frame of movie when expanding bleb is first seen. Yellow asterisk notes frame of movie when bleb is largest, immediately before bleb retraction begins. (C) During interphase, Tm1A and myosin II co-localize to retracting blebs in non-spreading cells. Top row: mCherry-Tm1A. Middle row: Sqh-GFP. Bottom: merged images of mCherry-Tm1A (red) and Sqh-GFP (green). Columns shows example frame from a timelapse movie of blebbing cells, left: expanding blebs (white asterisk), right: retracting blebs (white arrowhead).

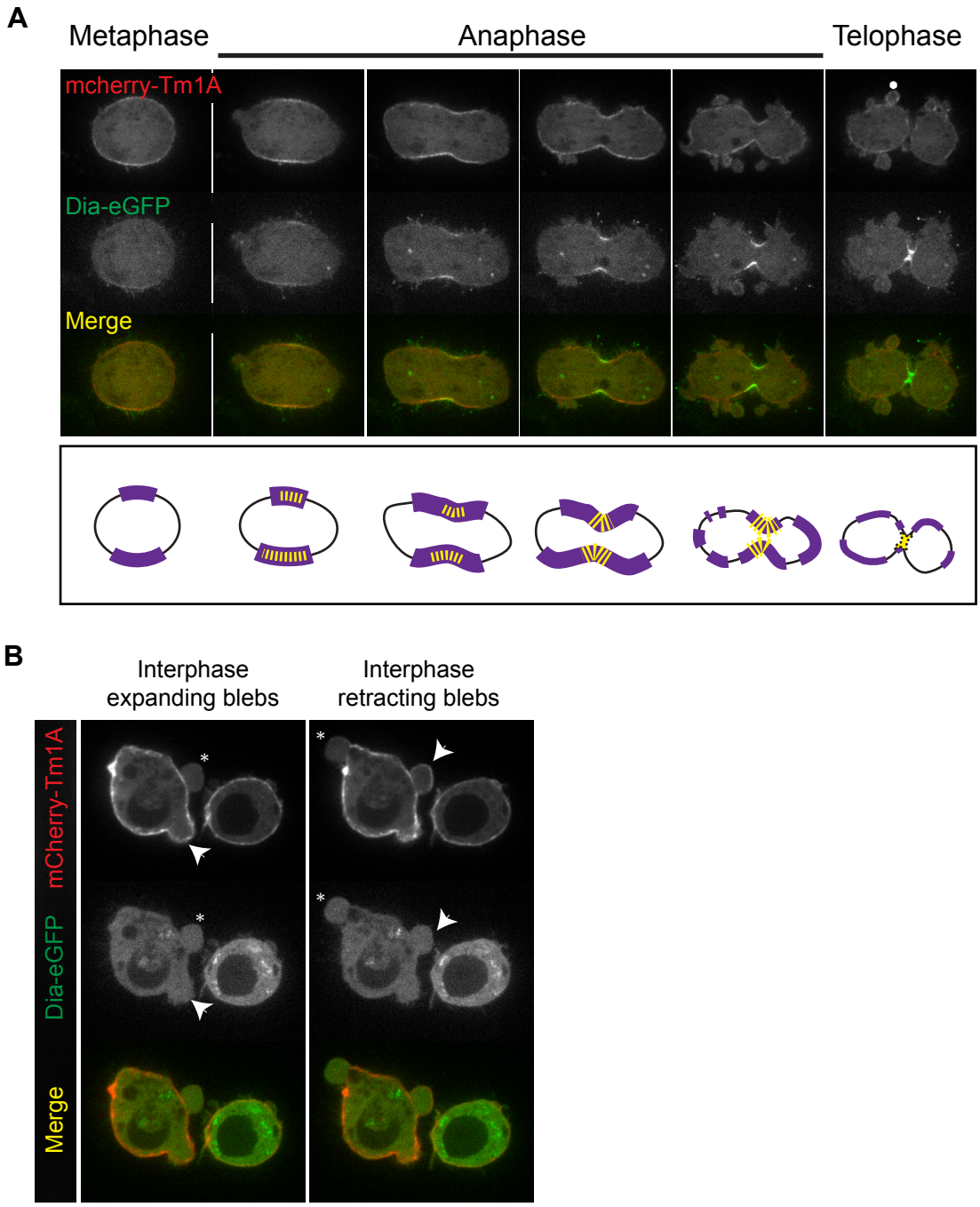
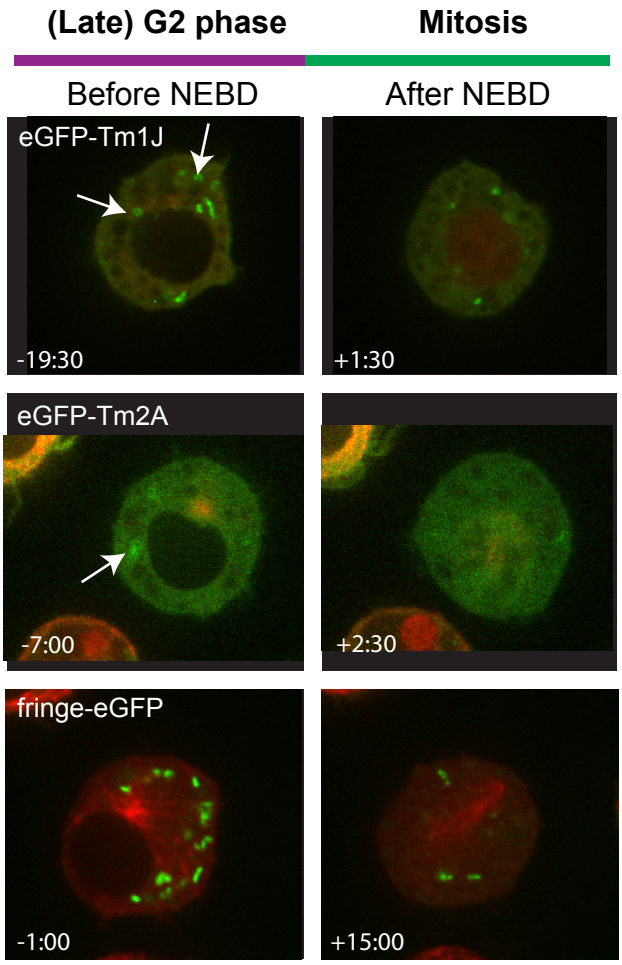


Figure 3-S3. Tm1A and Diaphanous show distinct patterns of localization when co-expressed in the same cells.

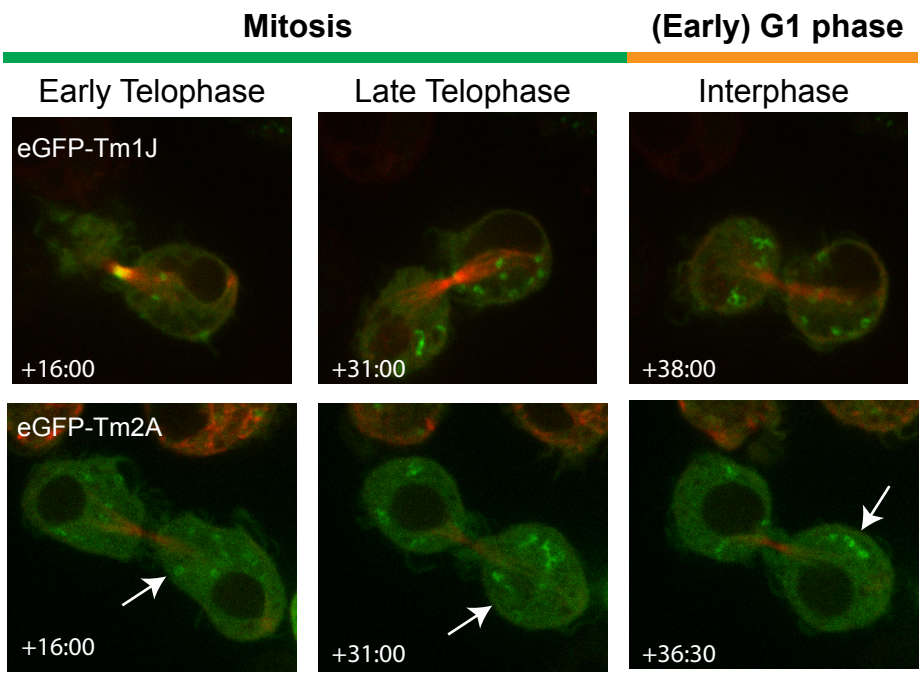
(A) During mitosis, distinct localizations of Tm1A and Diaphanous (Dia) are seen in the same cell. During metaphase, mcherry-Tm1A localizes

to the cortex prior to Dia-eGFP (left column, metaphase). As the cell progresses through mitosis, Tm1A occupies a larger expanding region of the cortex around the periphery of the cell, while in contrast, Diaphanous occupies a smaller region in the middle of the cell that seems to concentrate at the site of cleavage furrow ingression (middle columns). Top row: mcherry-Tm1A during various stages of mitosis (as noted above). Second row: Dia-eGFP. Third row: merged images of mCherry-Tm1A (red) and Dia-eGFP (green). Fourth row: schematic diagram illustrating differential localizations of Tm1A (purple) and Dia (yellow) during various stages of mitosis. (B) During interphase, mCherry-Tm1A but not Dia-eGFP localizes to the cortex and to retracting blebs (white arrowhead), not expanding blebs (white asterisk) around the periphery of Drosophila S2 cells.

A


(Late) G2 phase
Mitosis
Before NEBD
After NEBD
eGFP-Tm1J
-19:30
+1:30
eGFP-Tm2A
-7:00
+2:30
fringe-eGFP
-1:00
+15:00

B


Mitosis
(Early) G1 phase
Early Telophase
Late Telophase
Interphase
eGFP-Tm1J
+16:00
+31:00
+38:00
eGFP-Tm2A
+16:00
+31:00
+36:30

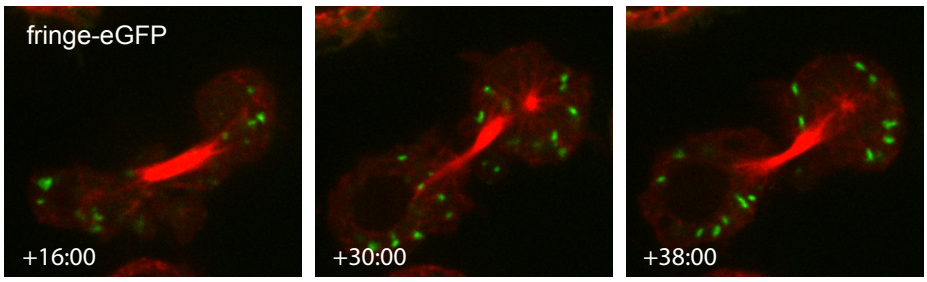


Figure 3-7. Tm1J and Tm2A localize to the golgi in a cell cycle dependent fashion.

(A) eGFP-Tm1J (top row) and eGFP-Tm2A (middle row) localize to the Golgi in late G2 phase prior to nuclear envelope breakdown (NEBD) at the onset of mitosis, but disperse after NEBD, similar to the Golgi marker fringe-eGFP (bottom row). [time before and after NEBD is noted in the bottom left corner of image] (B) At the end of mitosis, eGFP-Tm1J (top row) and eGFP-Tm2A (middle row) reform into ring-shaped structures at the Golgi during late telophase, after the nuclear envelope reforms, similar to the Golgi marker fringe-eGFP (bottom row). [time after anaphase onset in bottom left corner of image] Merged images of eGFP-tagged protein (green) and mCherry-tubulin (red) at various stages of the cell cycle immediately before and after mitosis, as indicated above the images.

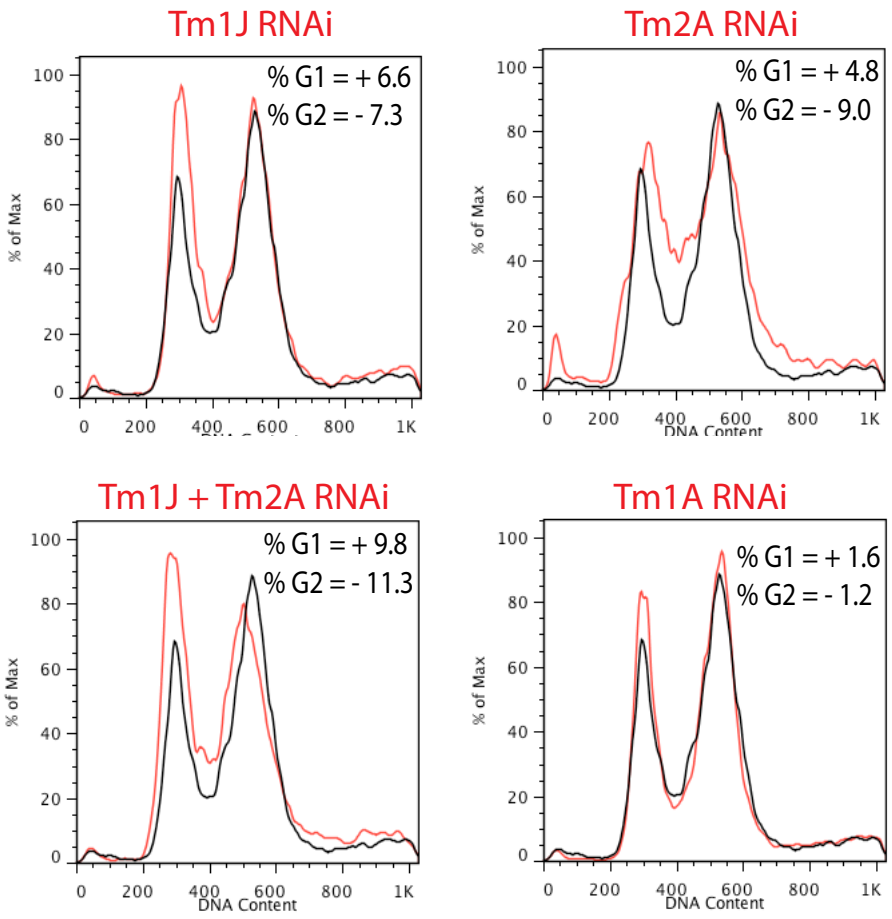


Figure 3-8. The Golgi-associated TM isoforms, Tm1J and Tm2A, influence cell cycle progression.

Cell cycle analysis using flow cytometry reveals a Golgi-related cell cycle phenotype (a relative increase in the percentage of cells in G1 in comparison to G2) in S2 cells depleted of the Golgi-associated TM isoforms, Tm1J and/or Tm2A, but not the cortical TM isoform, Tm1A. Graphs depict histograms of DNA content assessed in fixed S2 cells using propidium iodide (a DNA marker) after depletion of the indicated protein. Control histogram is shown in black while histogram of indicated experimental condition is shown in red. Relative increase or decrease in the percentage of G1 or G2 cells in comparison to controls are indicated in the upper right of graph.

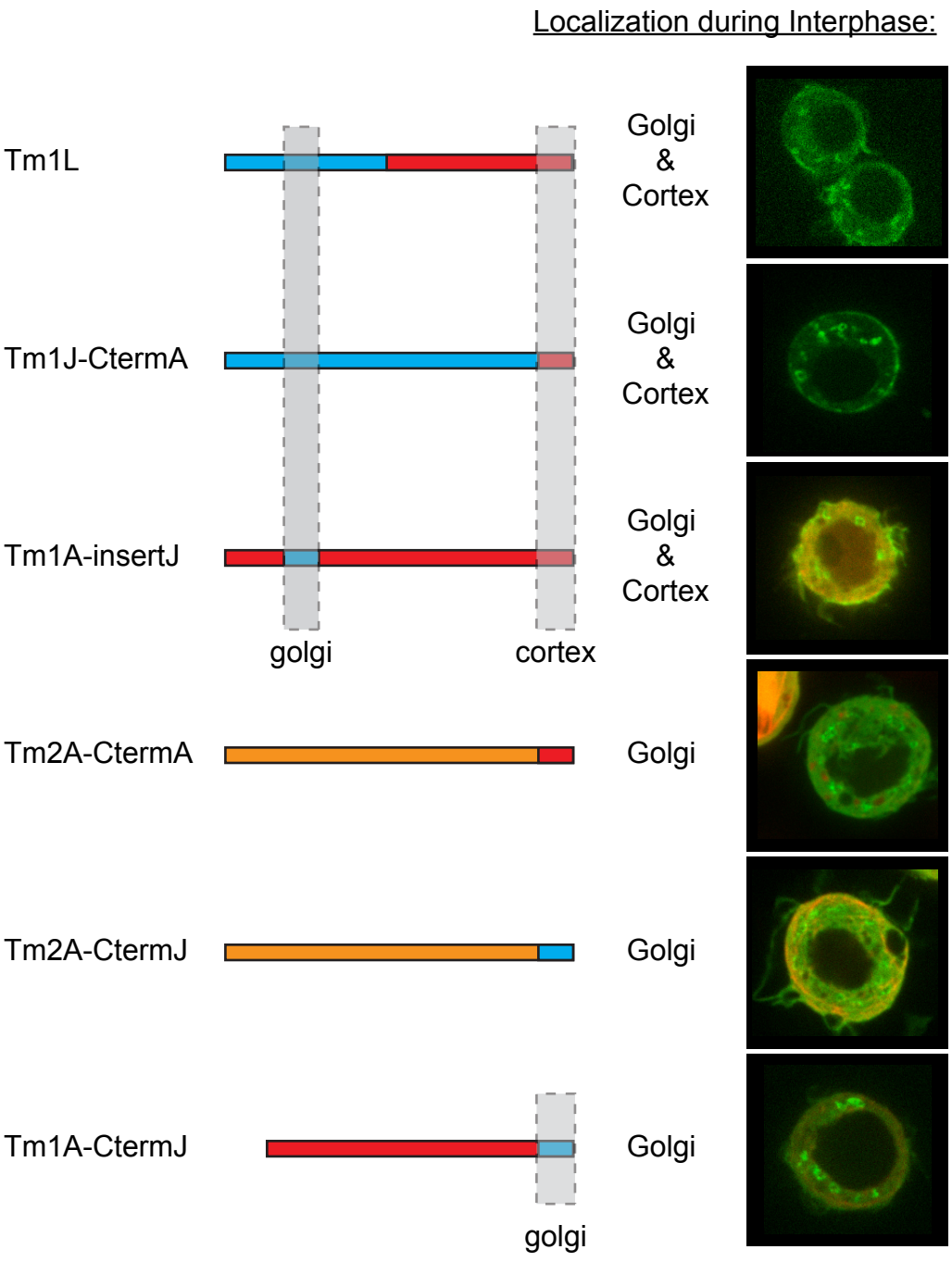


Figure 3-9. During interphase, protein sequences in both the N and C-termini of Tm1J determine the localization of TM isoforms to the Golgi apparatus, while protein sequences in the C-terminus of Tm1A determine cortical localization.

Left: names and diagrams of TM isoform chimera constructs tested for

localization to the cortex or cytoplasmic ring-shaped structures associated with the Golgi during interphase. Middle: Table of results indicating whether each TM isoform chimera localizes to the Golgi and/or cortex during interphase in non-spreading S2 cells. Right: Example merged images of S2 cells expressing mcherry-tubulin (red) and the indicated TM isoform chimera tagged with eGFP (green) during interphase.

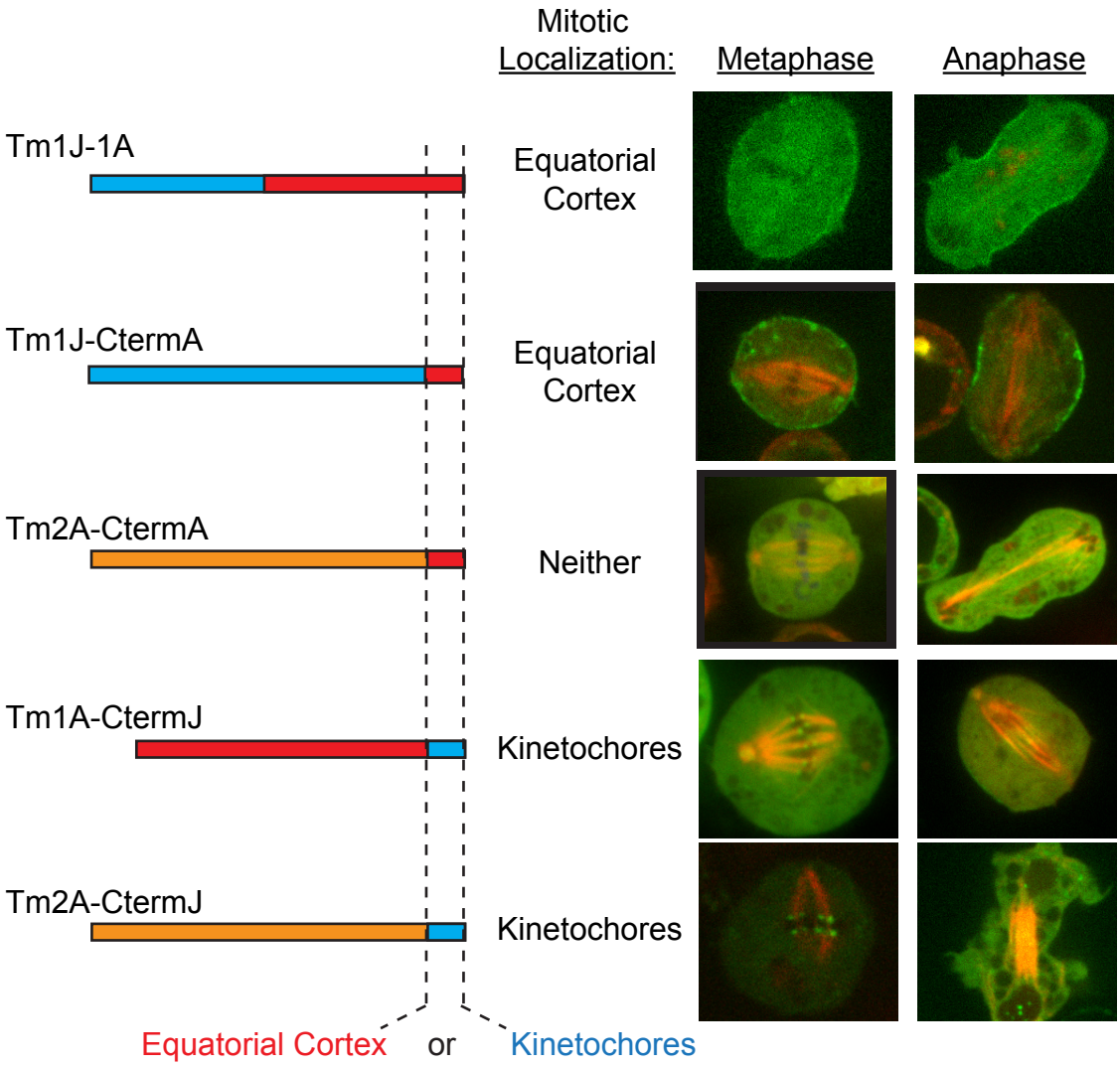


Figure 3-10. Protein sequences in the C-terminus of Tm1J and Tm1A determine the localization of Drosophila TM isoforms during mitosis. Left: names and diagrams of TM isoform chimera constructs tested. Non-spreading S2 cells were imaged during mitosis. Middle column: chart of results indicating whether each TM isoform chimera localizes to the kinetochores during metaphase, equatorial cortex during anaphase, or neither during mitosis. Right: example merged images of cells expressing mcherry-alpha-tubulin (red) and the indicated TM isoform chimera tagged with eGFP (green) during metaphase (left column) or anaphase (right column).

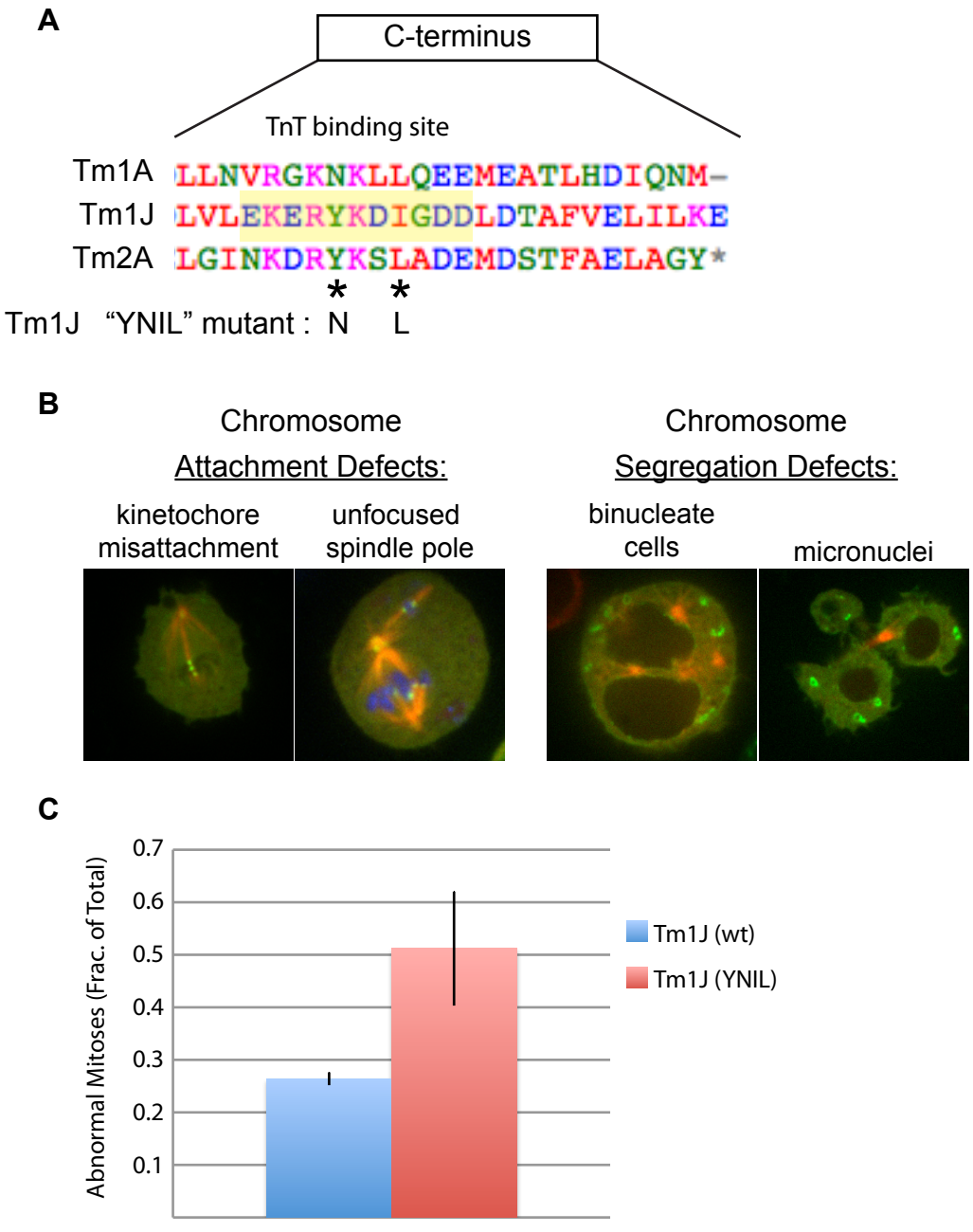
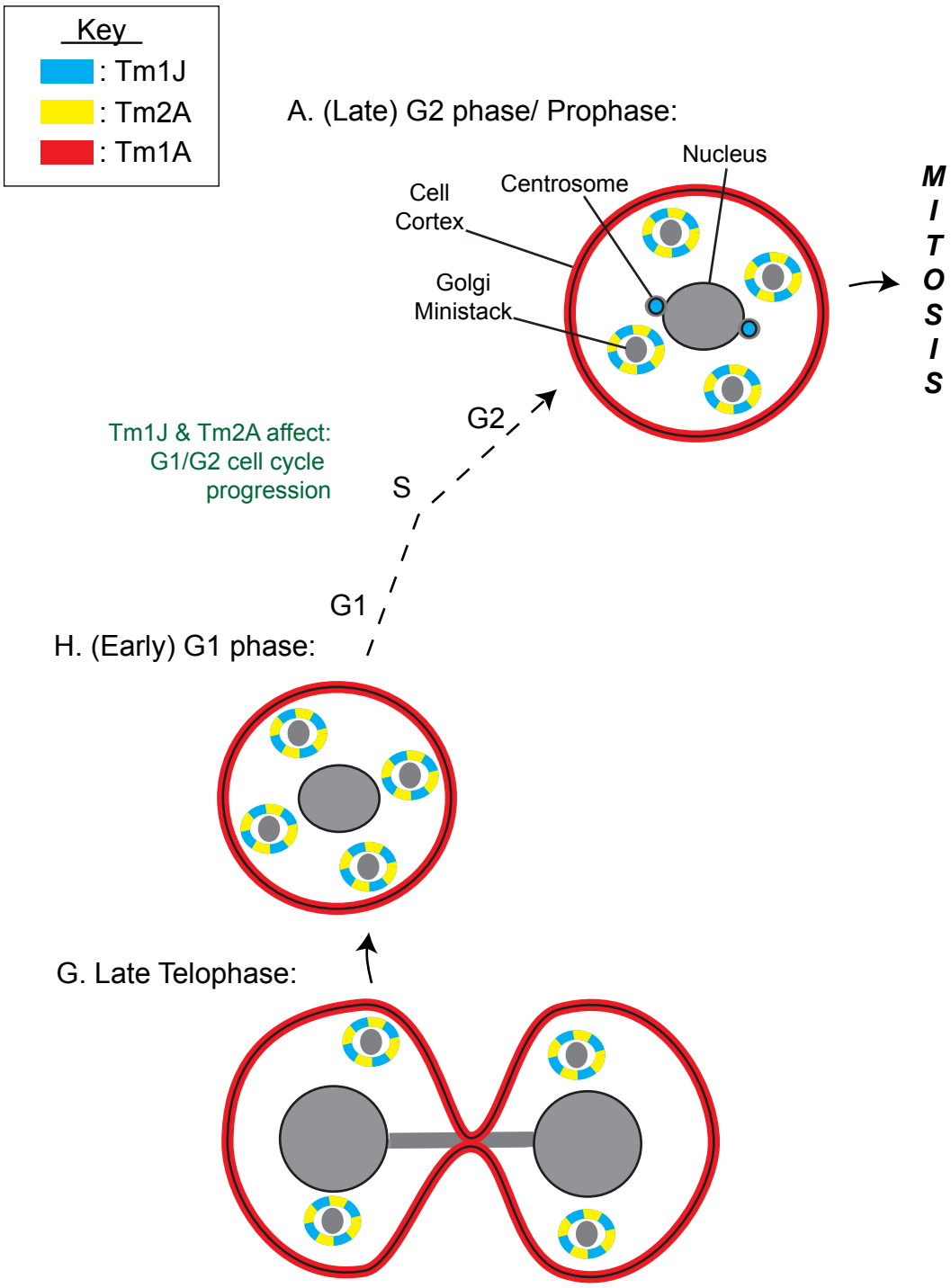


Figure 3-11. Tm1J functions with troponin at the kinetochores and spindle poles to influence chromosome segregation and anaphase progression during mitosis.

Low level induction of the Tm1J-YNIL mutant leads to chromosome attachment and segregation defects which often led to anaphase progression delay or cell division failure in dividing S2 cells. (A) Alignment of the

C-terminal regions of all three TM isoforms expressed in Drosophila S2 cells shows that Tm1J, but not Tm1A or Tm2A, contains a consensus binding site for Troponin T (TnT) (highlighted by yellow box). Asterisks illustrate the “YNIL” mutant, where two of the amino acids of this binding site are mutated, from Y267 to N and I270 to L, which has been shown to decrease the binding affinity of TnT to tropomyosin. (B) Examples of mitotic S2 cells expressing the eGFP-Tm1J-YNIL mutant which exhibit chromosome attachment defects (such as kinetochore attachment abnormalities or defects in microtubule attachment to the spindle poles, i.e. “unfocused spindle poles”) or chromosome segregation defects (such as binucleate cells or formation of micronuclei around misaligned chromosomes) Merged images shown. Red: mCherry-alpha-tubulin Green: eGFP-Tm1J (wildtype or mutant). Blue: Hoescht DNA dye. (C) Quantification of the fraction of dividing S2 cells imaged, expressing eGFP-tagged wildtype or YNIL-mutant TM isoforms, that exhibitted abnormal mitoses, defined as mitotic cells that took longer than 45 minutes to proceed into anaphase or that failed cell division and clearly formed binucleate cells or micronuclei.

Model of TM isoform localizations and functions during the cell cycle



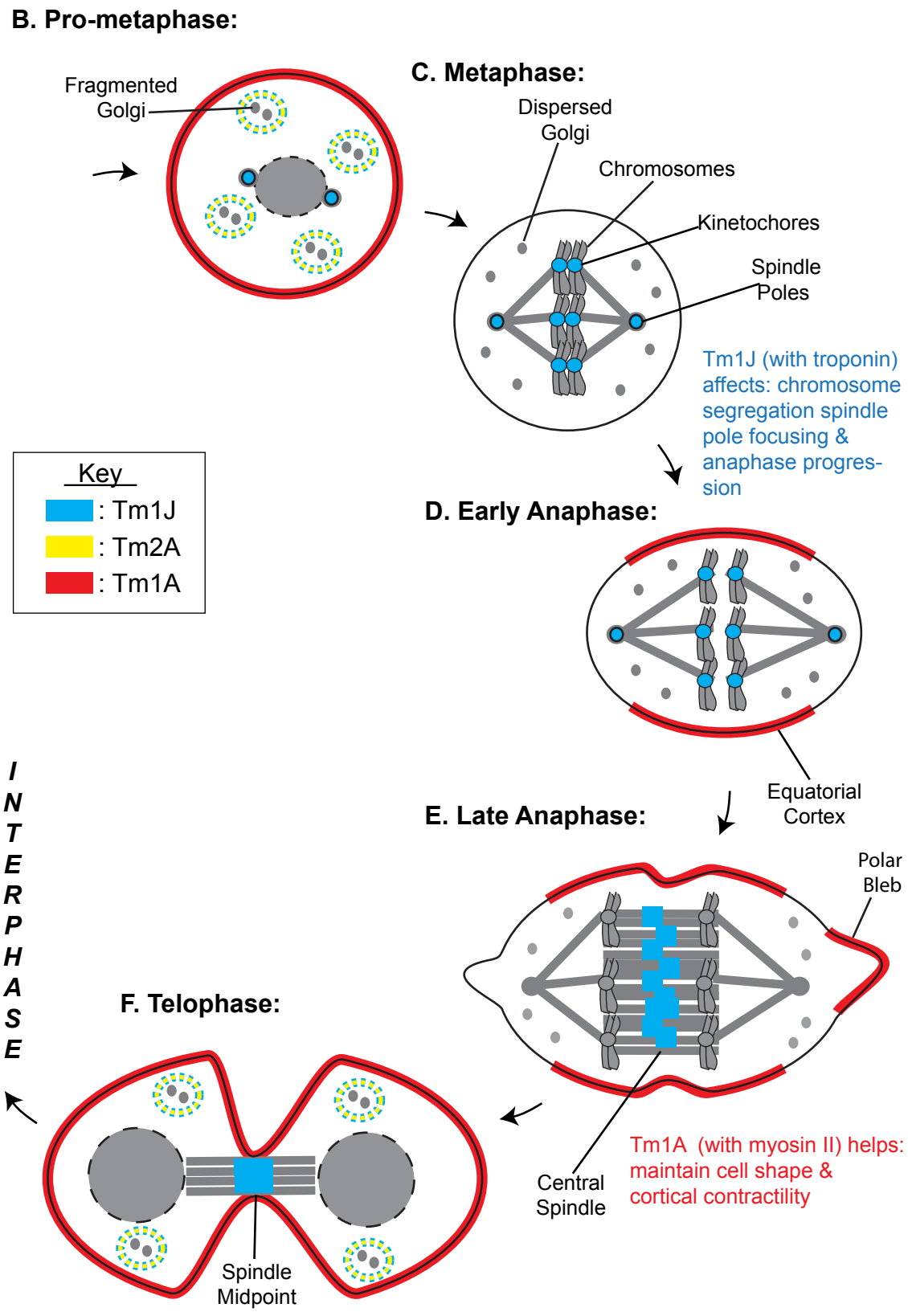

B. Pro-metaphase:
Fragmented Golgi
C. Metaphase:
Dispersed Golgi
Chromosomes
Kinetochores
Spindle Poles
Tm1J (with troponin) affects: chromosome segregation spindle pole focusing & anaphase progres-sion
Key
: Tm1J
: Tm2A
: Tm1A
D. Early Anaphase:
Equatorial Cortex
E. Late Anaphase:
Polar Bleb
Tm1A (with myosin II) helps: maintain cell shape & cortical contractility
Central Spindle
F. Telophase:
Spindle Midpoint
INTERPHASE

Figure 3-12. Model of TM isoform localizations and functions during the cell cycle.

Cartoon representation displaying Tm1A (red), Tm1J (blue), and Tm2A (yellow) localization and function in Drosophila S2 cells during various stages of the cell cycle. Cellular structures are labeled in the cartoon. (A) Late G2 phase/Prophase, Tm1J and Tm2A localize to the Golgi before the nuclear envelope breaks down (NEBD) at the onset of mitosis. A subset of Tm1J also localizes to the centrosomes. Tm1A remains at the cortex. (B) Pro-metaphase, soon after NEBD (dashed line around nucleus), Tm1J and Tm2A (and also Diaphanous) disassemble from ring-shaped structures surrounding the Golgi while Golgi ministacks fragment and disperse. Tm2A and Golgi remain scattered around the dividing cell during mitosis. (C) Metaphase, Tm1J remains at centrosomes at the spindle poles and localizes to kinetochores while chromosomes align at the metaphase plate. (D) Early anaphase, Tm1A and myosin II co-localize at the equatorial cortex but are absent from the cell poles. Tm1J remains at kinetochores and spindle poles as chromosomes begin to separate. (E) Late anaphase, as the cell elongates and intracellular pressure builds, Tm1A and myosin II co-localize specifically to retracting blebs at the cell poles to maintain cortical contractility and cell shape. Tm1J localization shifts from the centrosomes and kinetochores to the central spindle. (F) Telophase, Tm1A remains at the cortex as the cell completes cytokinesis. As central spindle microtubules bundle, Tm1J initially localizes to the midpoint, but as the nuclear envelope reforms, Tm1J, Tm2A, and Dia reassemble into ring-shaped structures surrounding the Golgi as the golgi ministacks also reform. During interphase, the Golgi-associated TM isoforms, Tm1J and Tm2A, function to influence G1/G2 cell cycle progression.

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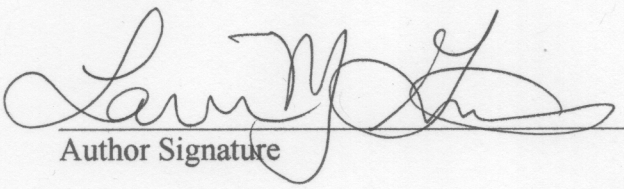

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