Engineered Cytoskeletal Arrays Reveal Mechanisms Of Membrane Transport And Tubulation

Betsy Buechler Mcintosh

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Engineered Cytoskeletal Arrays Reveal Mechanisms Of Membrane Transport And Tubulation

Abstract
Within the cell, cytoskeletal molecular motors transport and remodel membrane-bound cargos along microtubule and actin filament tracks. Typically, there are multiple actin and microtubule motors attached to the same cargo, which must coordinate to navigate a complex cytoskeletal environment and deliver their cargos to specific locations. We used an engineering, in vitro reconstitution, approach to investigate the interplay between a processive, microtubule-based motor, kinesin-1, and a non-processive, actin filament-based motor, Myo1c, in a simplified environment with increasing physiological complexity. First, we examined the interplay between purified motors attached to a membrane-coated bead at individual actin filament/microtubule intersections on the surface of a coverslip. We found that Myo1c is capable of initiating and terminating microtubule-based, kinesin-1-driven runs at actin filament/microtubule intersections. This ability of Myo1c to affect kinesin-1 motility at actin intersections is inhibited by the presence of nonmuscle tropomyosin Tm2 at the actin intersection. This suggests that tropomyosin may regulate Myo1c tethering of kinesin-1-driven cargo within cells by preventing termination of motility until reaching the highly dynamic actin just beneath the plasma membrane, sorting cargo to distinct subcellular domains. Next, we investigated the interplay between Myo1c and kinesin-1 on deformable giant unilamellar vesicles (GUVs) at physiologically relevant micropatterned arrays of sparse microtubules crossing dense actin filaments. We found that the lipid composition of GUVs regulates its frequency of tubulation along microtubules by kinesin-1 and actin filaments by Myo1c. GUVs containing a PtdIns(4,5)P2-rich lipid composition (PIP2-GUVs) tend to deform at actin/microtubule intersections along the microtubule, yet, the BAR domain protein endophilin is necessary for robust tubulation. Alternatively, in the presence of a physiological lipid mixture (LM-GUVs), kinesin-1 can readily tubulate Myo1c-tethered cargo at actin/microtubule intersections, with no significant change upon addition of endophilin. Myo1c can also transport both PIP2-GUVs and LM-GUVs along actin, yet significantly more deformation and tubulation occurs with LM-GUVs. In both cases, the presence of endophilin increases the frequency of tubulation along actin filaments by Myo1c. Overall, the ability of Myo1c and kinesin-1 to transport, sort, and deform vesicles along microtubules and actin filaments depends on the type of actin track, scaffolding-type membrane deformation-factors like endophilin, and the lipid composition of the vesicle.

Degree Type
Dissertation

Degree Name
Doctor of Philosophy (PhD)

Graduate Group
Cell & Molecular Biology

First Advisor
E. M. Ostap

Second Advisor
Erika L. Holzbaur

Keywords
actin filament, intracellular transport, kinesin, microtubule, myosin, tubulation
Subject Categories
Biophysics | Cell Biology | Molecular Biology

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ENGINEERED CYTOSKELETAL ARRAYS REVEAL MECHANISMS OF MEMBRANE TRANSPORT AND TUBULATION

Betsy Buechler McIntosh

A DISSERTATION

in

Cell and Molecular Biology

Presented to the Faculties of the University of Pennsylvania

in

Partial Fulfillment of the Requirements for the

Degree of Doctor of Philosophy

2017

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ENGINEERED CYTOSKELETAL ARRAYS REVEAL MECHANISMS OF MEMBRANE TRANSPORT AND TUBULATION

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I dedicate this Ph.D. dissertation to my husband, Logan McIntosh, my parents, Dennis and Lisa Buechler, and my sister, Jennifer Buechler. Thank you for unconditional support and love. I wouldn’t be here without you!
ACKNOWLEDGMENTS

By the very nature of my highly collaborative thesis work, this project would not have been possible without a great number of people. First, I would like to thank my co-advisors, Drs. Mike Ostap and Erika Holzbaur. I was very fortunate to be able to work directly with two such skilled and passionate researchers, who teamed-up to guide me through experimental challenges and successes, grants, papers, presentations, and the day-to-day nitty gritty of academic biomedical science. I have also appreciated and highly benefitted from the many opportunities for leadership roles, interactions with visiting speakers, and attendance to scientific meetings that you both provided. Mike, I appreciate your endless patience with my many interruptions of scientific questions big and small, and the fact that I have someone with whom I can share enthusiasm for snow and mountains. Erika, thank you for your endless energy and enthusiasm and for providing a strong, successful, female scientist role model. Mike and Erika, through your collegial interactions during our weekly meetings and the many journal clubs and seminars in between, I have learned endlessly about the rewards of successful collaboration, which I will to take with me in my future endeavors.

This dual mentorship gave me limitless access to incredibly intelligent and skilled researchers, and more tools and reagents than even I could manage to use. Thank you to all members of the Ostap and Holzbaur laboratories, past and present; you all positively impacted my project and graduate experience. In particular, I would like to express my gratitude to Michael Woody, Mara Olenick, Drs. Adam Hendricks, Sandy Maday, Swathi Ayloo, Meredith Wilson, Jake Lazarus, Mariko Tokito, Ionas Pyrpassopoulos, Michael Greenberg, Allison Zajac, Abbey Weith, Beth Feerer, Dan Safer, Tianming Lin, and Liqiong Chen for thoughtful conversation and technical expertise throughout my thesis work. Thank you also to Swathi, Greenberg, Woody, Abbey, and Beth for invigorating conversation about politics, the world, life, and other random topics (like the weather). I appreciate Lisa Davidson, Riley Payne, and Erin Masucci for enriching my science with mentorship opportunities, and to Erin for serious experimental assistance during this last scramble to finish my dissertation work. I would like to thank Eric Johnston and Brock Peterson from the
Singh Center for Nanotechnology for their help with the Excimer laser patterning. Additionally, I would like to acknowledge the scientific support and reagents I received from: Dr. Henry Shuman about optics and optical trapping; Dr. Yale Goldman and his lab members Drs. Lisa Lippert, Matt Caporizzo, and Jody Dantzig Brody; Dr. Katya Grischuk and her lab members Drs. Anatoly Zaytsev and Suvranta Tripathy; Dr. Roberto Dominguez and his lab members Dr. David Kast and Greg Rebowski; Dr. Tobias Baumgart and his lab members Jaclyn Robustelli and Dr. Zhiming Chen; and the many other participants of the Pennsylvania Muscle Institute. I cannot express how much I learned from each and every member during informal discussion, journal clubs, and seminars.

Thank you also to my thesis committee members Drs. Erfei Bi, Tanya Svitkina, Yale Goldman, and Roberto Dominguez for always pushing me to think harder about my research.

To my funding sources at the American Heart Association and National Institutes of Health, I greatly appreciate the opportunities that were afforded to me by your patronage. These organizations enable astounding training opportunities and life-saving and life-enriching scientific advances. I hope to continue to apply these experiences in the future.

I would like to extend a huge thank you to Ashley Douglass of the Pennsylvania Muscle Institute for managing the many annoying and essential administrative tasks that have made doing researcher efficient, timely, pleasant, and possible! Thank you for ordering the food, ordering reagents/supplies/chasing down suppliers, organizing everything, for the many chats, and all the support. I also appreciate the Physiology Department front office staff, and Roz Schorr and Dr. Kevin Foskett the essential scientific, networking, and community-building opportunities offered. Thank you to Kathy O'Connor-Cooley for organizing everything within the Cell Biology, Physiology, and Metabolism (CPM) subgroup and for helping keep me and all the other students on track and informed during graduate school. Also, thank you to my other CPM student and faculty members for the scientific support, advice, and opportunities to present my research work.

I would also like to acknowledge Michael Woody, Dr. Lisa Lippert, and Dr. Abbey Weith for the many scientific and non-scientific discussions over margaritas, without which I wouldn’t have made it through these projects. A most heartfelt thank you to Scott Ashley, Ariel Lefkovith, Joe
Zinski, Alan Tso, Winnie Chan, Brad Heron, Dr. Judite Costa, Dr. Viky Syrovatkina, Shawna and Boris Bayerman, Janet and Susan Rauscher, Dr. Roland Rivera Santiago and many, many others for wonderful conversation (scientific and otherwise) over games, laughter, and delicious food. You all made living in Philly during graduate school a wonderful adventure!

Last, but absolutely not least, I would like to thank my family. Thank you to my cats, Casanova and Merlin, for entertaining and loving me over the years, and for sitting by/on me while I wrote and edited my thesis. I am grateful to my husband, Logan McIntosh, for all your love, support, and companionship over these past 11.5 years. Thank you for encouraging me, and believing in me, laughing with me, listening to me drone on and on about my project and the challenges of graduate school, for listening to practice talks, and for reading papers (and this dissertation!). Thank you to my parents, Dennis and Lisa Buechler, for instilling a passion for learning and education, and the means and support to pursue them. Thank you to my sisters and best friends Jennifer Buechler, Renée Mulcare, and Danielle Hauf for your lifelong friendship and support. Thank you also to the many teachers and mentors who got me here, in particular, Dr. Jennifer DeLuca, for first exposing me to the wonders of cell biology and for encouraging me to pursue this Ph.D.
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“What I cannot create, I do not understand.”
~Richard Feynman
CHAPTER 1: Introduction

Within the cell, molecular motors transport cargo, such as protein and organelles, along microtubule and actin filament tracks. This specific and directional trafficking of components is essential for normal cellular homeostasis. Throughout transport, each cellular cargo is attached to multiple types of microtubule and actin filament-based molecular motors, which must coordinate to successfully transport and remodel membrane-bound cargo. Membrane-bound cargo are deformed and tubulated at many points during trafficking, for instance, during the sorting of protein and lipid components from one compartment to another at the trans Golgi or plasma membrane. This transport and remodeling can be regulated by the specific composition of molecular motors on a particular cargo, adaptors and regulators that directly bind the motors, the types of tracks these motors interact with, modifications to these tracks by additional proteins or post-translational modifications, and other cytosolic factors. A significant amount of research has been done to investigate the individual roles and modes of regulation of isolated components of these processes, yet, many questions remain pertaining to how individual components combine to produce the complex cellular physiology we observe. A couple of the unanswered questions in the field are (1) how do microtubule and actin filament-based molecular motors coordinate to transport, target, and sort cellular cargo, and (2) how do molecular motors coordinate to remodel membrane-bound cargo?

In this dissertation, I aim to address these two broad questions. In order to gain perspective on these questions as well as the following investigations undertaken during my thesis work, it is important to understand (1) the two types of cytoskeletal tracks
molecular motors transport cargo along: microtubules (Section I) and actin filaments (Section V); (2) the general properties of transporters along these two types of tracks: kinesin and dynein along microtubules (Section II), and myosin along actin filaments (Section VI); (3) the ways that microtubule and actin filament tracks are differentiated within the cell and how these modifications can either activate or inhibit specific types of molecular motors, by investigating post-translational modification, in the case of the microtubule “tubulin code” hypothesis (Section IV), and additional protein binding, in the case of actin filaments (Section VI); (4) specific properties concerning our chosen representative motors: kinesin-1, a processive, long distance, microtubule-based transporter (Section III), and myosin-Ic, a single-headed, non-processive actin filament-specific motor whose roles on trafficking cargo are largely unclear at the molecular level (Section VII); (5) once we have a basic understanding of individual motors, we need to explore how researchers have started increasing complexity of in vitro and cellular studies by investigating how teams of the same or differing motors work together (Section VIII); (6) in an effort to better understand membrane-bound cargo transport and remodeling, we need to explore the basic properties of cargo membranes and how they can be remodeled by motors and other membrane deformation factors such as BAR domain proteins (Section X). Finally, after describing my thesis work in Chapters 2 through 5, I will summarize and contextualize my work, and propose future directions in Chapter 6.
I. Microtubules

There are two broad classes of tracks along which cytoskeletal motors transport cargo within the cell: microtubules and actin filaments. As a generalization, microtubules are the “highways” along which long distance transport occurs, while actin filaments are the “city streets” that facilitate more localized transportation. Microtubules are composed of obligate α- and β-tubulin dimers that both bind GTP; however, only β-tubulin hydrolyzes GTP as the dimers polymerize into linear protofilaments (Wade, 2009). Typically 13 protofilaments bind laterally to form a cylindrical microtubule that is 25 nanometers in diameter (Desai and Mitchison, 1997; Tilney et al., 1973), yet, microtubules containing between 9 and 16 protofilaments have also been observed (Evans et al., 1985). Microtubules themselves are not static structures. Rather, they are dynamic tracks that undergo repeated cycles of depolymerization (catastrophe) and re-polymerization (rescue) in response to cellular cues (Desai and Mitchison, 1997; Mitchison, 1984). When the free tubulin concentration is above a certain point, the critical concentration, tubulin dimers add more rapidly to the microtubule plus-end (exposed β-tubulin end) than the minus-end (exposed α-tubulin end). Over time, β-tubulin stochastically hydrolyzes its GTP, causing a conformational change from a straight to a bent conformation. This bent conformation is restrained in the straight microtubule lattice by a so-called GTP-cap at the tip of the plus-end. The length and composition of this GTP-cap is still under debate (Howard and Hyman, 2009; Ohi and Zanic, 2016; Zakharov et al., 2015); however, it is thought that loss of this GTP-cap and/or accumulation of a certain number of defects in the polymerized lattice will stochastically cause protofilament catastrophe, where the bent tubulin dimer conformation causes the microtubule protofilaments to bend outward from the lattice. The energy from this depolymerization can be harnessed as a molecular machine, for example, during the segregation of chromosomes in the final stages of
mitosis (McIntosh et al., 2010). Although microtubule dynamics have been a topic of interest for decades, exactly how individual tubulin dimers add to growing protofilament tips, as well as whether and how tubulin replacement occurs within an already polymerized microtubule, are still hotly debated (Aumeier et al., 2016; Howard and Hyman, 2009; Ohi and Zanic, 2016; Zakharov et al., 2015).

A variety of proteins can directly affect microtubule growth and shrinkage. Although XMAP215 and many kinesins can independently bind to the growing plus-end of the microtubule, the plus-tip family of binding proteins is largely recruited by end binding (EB) protein binding specifically to GTP-bound β-tubulin at the growing tip (Bieling et al., 2007; Kumar and Wittmann, 2012; Maurer et al., 2012). EB proteins then recruit a cascade of other proteins in different cellular contexts, including CLIP-170, MCAK, MACF, STIM1, and CLASP2 to the dynamic plus-end (Bieling et al., 2008; Dixit et al., 2009; Honnappa et al., 2009; Montenegro Gouveia et al., 2010). At the minus-end, microtubules can be polymerized and stabilized by the gamma-tubulin ring complex (γTuRC), which remains tethered near the centrosome, often near the nucleus in the center of the cell (Kollman et al., 2011). Away from the centrosome, the CAMSAP/Nezha/Patronin family of proteins have been found to bind to and protect both static and dynamically growing microtubule minus-ends from motor-driven and stochastic depolymerization (Akhmanova and Hoogenraad, 2015; Baines et al., 2009; Goodwin and Vale, 2010; Meng et al., 2008). Molecular motors can also affect microtubule dynamics by increasing the chance for catastrophe (MCAK, Kip3), or enhancing both polymerization rate and catastrophe frequencies (KIF21B) (Ghiretti et al., 2016; Howard and Hyman, 2009; Muhia et al., 2016). For example, MCAK (kinesin-13) stabilizes the bent conformation of GDP-bound β-tubulin, thereby accelerating GTP-hydrolysis by β-tubulin (Kinoshita et al., 2006). Finally,
microtubule severing proteins such as katanin (McNally and Vale, 1993; Vale, 1991),
spastin (Solowska et al., 2008; White et al., 2007), and fidgetin (Mukherjee et al., 2012)
can break microtubules and generate free plus-ends for additional dynamic microtubule
growth (Bailey et al., 2016).

Non-mitotic somatic cell

Neuron

Dendrite

Myocyte

Microtubule Polarity

Figure 1.1 Microtubule polarity is organized within cells.

Non-mitotic somatic cells tend to be organized with their microtubule minus-ends clustered near the
nucleus, and their plus-ends arrayed out toward the cell cortex. Mammalian neurons have axons with
plus-end-out microtubules (plus-ends oriented toward the growth cone), and dendrites with mixed
microtubule polarity. Myocytes have mixed microtubule polarity in their centers and plus-end-out
microtubules on the ends.

Interphase microtubules are generally organized in a radial geometry within cells,
where stabilized minus-ends are concentrated in the center of the cell by the centrosome
in the peri-nuclear region, and highly dynamic plus-ends are oriented toward the cell periphery (Allen and Borisy, 1974; Figure 1.1). Specialized cell types such as neurons have even more organized microtubule-containing compartments. For example, in mammalian neurons, axonal microtubules are largely oriented plus-ends out toward the growth cone of the growing neurite, while dendritic microtubule polarity is mixed (Conde and Cáceres, 2009). Mature myocytes (muscle cells) lose their radial microtubule organization and instead have microtubules of mixed polarity running both parallel and perpendicular to contractile sarcomeric units (Tassin et al., 1985; Warren, 1974). In this case, microtubules are important for mechanically resisting heart contraction in addition to acting as highways for cargo transport (Robison et al., 2016).

II. Microtubule-based molecular motors

Microtubule-based molecular motors couple chemical energy released from adenosine triphosphate (ATP) hydrolysis to adenosine diphosphate (ADP) and inorganic phosphate into mechanical work and directional translocation along microtubule tracks. There are two classes of microtubule-based motors: dynein and kinesin. In general, dynein motors walk toward the minus-end of microtubules, whereas all known kinesin motors, with the exception of kinesin-14 family members, walk toward the plus-end (McDonald et al., 1990).

Cytoplasmic dynein is the predominant minus-end directed microtubule-based motor in metazoan organisms (Schiavo et al., 2013). The dynein molecular motor family is broadly composed of two groups: axonemal and cytoplasmic motors (Pfister et al., 2006). Axonemal dynein is responsible for powering the movement of cilia and flagella (Gibbons and Rowe, 1965; Karki and Holzbaur, 1999). Cytoplasmic dynein has two heavy chain isoforms: isoform-2, which is important for intraflagellar transport and cilia and
flagella assembly; and isoform-1, the more broadly expressed isoform involved in intracellular transport, microtubule tethering to the cortex, and centromeric separation during mitosis (Eshel et al., 1993; Lye et al., 1987; Paschal and Vallee, 1987; Paschal et al., 1987). Functional dynein is a multisubunit complex composed of two dynein heavy chains, and multiple intermediate, light intermediate, and light chains. The heavy chain is composed of a microtubule-binding domain, a ring containing six AAA domains (some of which can hydrolyze ATP), and a cargo-binding tail domain (Figure 1.2). Interestingly, dynein motors have their microtubule-binding domain and sites of ATP-hydrolysis spatially separated by a long coiled-coil stalk (Cianfrocco et al., 2015). Dynein is also associated with various regulatory proteins such as dynactin, lis1, and Nudel/NudE, which are thought to participate in activation, localization, and specific functioning (Bradshaw et al., 2013; Cianfrocco et al., 2015; Lam et al., 2010; Moughamian et al., 2013; Schroer and Sheetz, 1991; Shao et al., 2013).

Mammals contain 45 different kinesin superfamily (KIF) genes, which are generally classified into 15 kinesin families according to phylogenetic analyses (Hirokawa and Tanaka, 2015). These cytoskeletal motors are generally composed of a motor domain, which binds to microtubules and hydrolyzes ATP, a neck-linker domain, which transduces conformational changes caused by ATP hydrolysis along the motor, a coiled-coil stalk, and a cargo-binding tail region (Figure 1.2). The vast majority of kinesin motors are organized with their motor domain at the amino-terminus of the protein, however notable exceptions include KIF2A, a kinesin-13 family motor that has a central motor domain and depolymerizes microtubules, and KIFC2 and KIFC3, kinesin-14B family members which contain a carboxyl-terminal motor domain (Hirokawa et al., 2009; Miki et al., 2005). Kinesin family members show a wide range of cargo-binding properties, force-sensitivities, and
affinities for microtubule tracks containing different post-translational modifications and associated proteins. These differences have cumulatively adapted motors for specific types of physiological functions.

Figure 1.2 Kinesin-1 and cytoplasmic dynein structural features.

Kinesin-1 has a microtubule (MT)-binding domain and ATP hydrolysis domain in close proximity to the microtubule, while dynein’s MT-binding and ATP hydrolysis domains are spatially separated. Both motors can form homo dimers and often bind light chains, intermediate chains, and adaptors in their cargo-binding domains. Kinesin-1 walks toward the MT plus-end, while dynein walks toward the MT minus-end. Adapted from Spudich J. A., 2011.

Cytoskeletal motors attach to their cargo either by direct membrane binding or via a protein adaptor. For example, the kinesin-3 family member KIF1A/UNC-104 binds directly to Phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) in *Caenorhabditis elegans*, and KIF16B binds directly to PtdIns(3)P (Blatner et al., 2007; Klopfenstein, 2004). Kinesin-1 (KIF5B) has recently been shown in vitro to directly bind to PtdIns(4,5)P_2 reconstituted and purified vesicles with a binding site in its tail region (Du et al., 2016).
Protein adaptors facilitate motor interaction with membrane-bound cargo and/or other protein components. While some adaptors appear to bind only a single type of motor at a time, others are capable of binding multiple types of microtubule-based molecular motors, often simultaneously. For instance, centaurin-1α links a single KIF13B dimer to PtdIns(3)P in lipid membranes (Venkateswarlu, 2005). Alternatively, the adaptor huntingtin can bind directly to the dynein intermediate chain (Caviston et al., 2007), as well as to the kinesin-1 heavy chain (Twelvetrees et al., 2010), the kinesin-1 light chain or dynactin via huntingtin-associated protein (HAP1) (Engelender et al., 1997; Li et al., 1998; McGuire et al., 2006), and to myosin-VI via optineurin (Sahlender et al., 2005; Figure 1.3). Huntingtin can specifically recruit these motors to either Rab5-labeled early endosomes via its binding to HAP40 (Caviston et al., 2007), or to autophagosomes via its association with HAP1 and optineurin (Fu and Holzbaur, 2014; Wong and Holzbaur, 2014). There is some evidence that phosphorylation may be one way to regulate which motor(s) bind(s) at a particular time. This one example beautifully illustrates how a single adaptor can recruit multiple plus- and minus-end-directed microtubule motors, as well as coordinate with actin filament-based motors to transport diverse cellular cargo under different signaling circumstances (see Figure 1.3).
Huntingtin is a protein adaptor that can recruit various motors to different organelles under different cellular circumstances. Huntingtin is an adaptor protein that can directly bind to kinesin-1 heavy chain and dynein intermediate chain, as well as to myosin-VI, and kinesin-1 light chain and dynactin through other protein intermediates, optineurin and HAP1, respectively. Huntingtin can recruit motors to either Rab5 early endosomes or autophagosomes via HAP40 interactions to Rab5, or HAP1 and/or optineurin interactions with autophagosomes. This example illustrates how protein adaptors can recruit multiple types of motors to different cargos, often regulated by availability of adaptors and phosphorylation. Adapted from Fu and Holzbaur, 2014.

III. Kinesin-1

The kinesin-1 family is composed of three isoforms: KIF5A, KIF5B, and KIF5C. These isoforms are differentially expressed with KIF5A and KIF5C preferentially expressed in neurons, and KIF5B more generally expressed in other cell types (Hirokawa and Noda, 2008). Kinesin-1 motors are important for long distance transportation of cargo including membrane transporters like Glucose Transporter 4 (GLUT4) (Semiz et al., 2003), and organelles such as early endosomes (Loubéry et al., 2008), lysosomes (Hollenbeck and Swanson, 1990; Tanaka, 1998), autophagosomes (Maday et al., 2012), mitochondria
(Pilling et al., 2006; Tanaka, 1998), and the nucleus (Wilson and Holzbaur, 2012; Wilson and Holzbaur, 2015). In addition to transporting cargo, kinesin-1 has also been implicated in microtubule sliding to drive neurite protrusion (Lu et al., 2013). Based on these varied cellular roles, it's unsurprising that loss of kinesin-1 can result in a number of neurological and metabolic disorders (Hirokawa and Noda, 2008; Hirokawa and Tanaka, 2015).

Kinesin-1 forms a homodimer with two heavy chains dimerizing via a long coiled-coil tail, which can also associate with two light chains to form a heterotetramer (Bloom et al., 1988; Figure 1.2). The motor domains of kinesin-1 have ATPase activity stimulated by microtubule binding, where the ATP-bound state has the tightest motor-microtubule binding, and the release of ADP limits the hydrolysis cycle (Cross, 2004). These features structurally and kinetically predispose this motor for processive motility along the microtubule, which is characterized by consecutive stepping toward the microtubule plus-end. Kinesin-1 motors take 8 nm steps from tubulin dimer to tubulin dimer, typically walking in a straight line along a single protofilament (Ray et al., 1993; Svoboda and Block, 1994; Visscher et al., 1999). As the first microtubule plus-end-directed transport motor discovered, kinesin-1 is one of the most thoroughly studied molecular motors in both cellular and in vitro contexts. Using in vitro studies, the Block Lab showed that an individual kinesin-1 molecule is capable of walking against loads of up to 5 to 7 piconewtons (pN) before it is no longer capable of resisting the load to step along the microtubule and halts transport (Schnitzer et al., 2000; Svoboda and Block, 1994; Visscher et al., 1999). This force is referred to as the “stall force,” and it varies between motor types. When multiple kinesin-1 motors are bound to the same cargo, both the stall force and run length can increase, yet, the velocity does not increase. Rather, when two or more kinesin-1 are attached to a cargo and engaged with the microtubule surface, a decrease in velocity is
observed (Furuta et al., 2013; Jamison et al., 2012; Uppulury et al., 2013). Simulations in conjunction with \textit{in vitro} DNA scaffolding assays that regulate the number of motors attached to a cargo, suggest that even when multiple kinesin-1 are attached to a cargo, they are incapable of working together to increase velocity, or to proportionally increase the amount of force they can resist (Jamison et al., 2012). In other words, although the stall force increases upon the presence of more kinesin-1 motors on a cargo, the increase in force does not increase proportionally with the number of motors present. This may be because not all motors are engaged with the microtubule at any given time, and/or the kinesin-1 motors interfere with the motility of one another and their force-generation capability. On the other hand, Increasing numbers of dynein motors are capable of resisting proportionally increasing resistive force generated by an optical trap; dynein motors collectively and individually show a “catch-bond” behavior whereby within a certain range of forces, increasing force leads to longer attachment lifetimes on the microtubule (Mallik et al., 2013; Rai et al., 2013; Soppina et al., 2009). These studies have indicated that while kinesin-1 motors prefer to operate as individual motors while transporting cargo, dynein motors can operate cooperatively within cells. Interestingly, although it’s thought that only a couple of kinesin-1 motors are interacting with the microtubule at any given time, it is estimated that approximately 1-4 kinesin-1 motors may be found on an individual neuronal trafficking cargo (Hendricks et al., 2010). \textit{In vitro} studies based on fluorescent localization of kinesin motors free to diffuse around a lipid membrane-bound cargo corroborate this and indicate that 5-10 motors may be in the vicinity of the microtubule during transport (Herold et al., 2012). One explanation for these apparent discrepancies may be that not all kinesins in the area of the microtubule are in their active state.
The full length kinesin-1 heavy chain dimer typically exists in an autoinhibited state within cells and \textit{in vitro} where the motor is folded and the tail domain interacts with the motor domains, decreasing binding to microtubules (Cai et al., 2007; Coy et al., 1999; Friedman and Vale, 1999; Hirokawa et al., 1989; Stock et al., 1999; Verhey and Hammond, 2009). There is some evidence that the kinesin light chains may also inhibit the motor through direct binding (Verhey et al., 1998), or be autoinhibited themselves (Yip et al., 2016). This inhibition has been shown in cells (Blasius et al., 2007; Kawano et al., 2012) and \textit{in vitro} (Cho et al., 2009; Fu and Holzbaur, 2013) to be relieved by binding to adaptor proteins and/or cargo; however, kinesin-1 motors bound to adaptors may still be found in the autoinhibited state (Woźniak and Allan, 2006) and require additional activation, for instance, by phosphorylation (Fu and Holzbaur, 2013).

\section*{IV. Tubulin Code Hypothesis}

Another mechanism for regulating motor function is in differences in the type of microtubule track motors encounter. There are nine different α- and nine β-tubulin isoforms in mammals. Although many of these isoforms are differentially expressed across tissues, numerous isoforms can be found within an individual cell (Cleveland et al., 1978; Ludueña, 2013). Until recently, we were unable to express and purify individual tubulin isoforms, and had to rely on heterogeneous brain-purified tubulin consisting of many isoforms and isotypes (Banerjee et al., 1988; Panda et al., 1994; Schliwa, 1976; Sullivan and Cleveland, 1986). Newly developed expression and purification techniques have shown some differences in tubulin polymerization and stability, and it will be interesting to see if these results coincide with differing motor motility (Vemu et al., 2016; Yu et al., 2015). In the meantime, researchers have focused on the influence of variability in the tubulin carboxyl-terminal tail (CTT), a typically negatively-charged intrinsically disordered
peptide sequence that extends outward from the barrel of the microtubule, and thus, facilitates interactions with microtubules. In the absence of both the α-tubulin and β-tubulin CTT, kinesin-1 shows decreased velocity and run length at the single molecule level (Sirajuddin et al., 2014). Interestingly, when examined at an ensemble level and under load, kinesin-1 showed a higher stall force, increased binding time, but decreased velocity on microtubules with their CTT cleaved by subtilisin (Feizabadi, 2016). Sirajuddin et al., 2014, additionally found that kinesin-1 and kinesin-2 exhibit very different responses to distinct tubulin signatures, while dynein was largely insensitive; however, the largest changes in velocity and/or run length observed were by 2-fold increase or decrease, questioning the physiological relevance of these changes. It will be interesting to see if these differences in motility are supported by studies utilizing purified mammalian tubulin rather than these chimeric yeast core/human CTT tubulin isotypes.

In addition to changes in amino acid length and composition, tubulin can vary in its post-translational modifications under different cellular scenarios. Some of these modifications to the CTT include addition or removal of a tyrosine (Barra et al., 1973), (poly-)glutamylation (Eddé et al., 1990; Redeker et al., 1992), and glycylation (Redeker et al., 1994). The tubulin core can be modified by acetylation (L’Hernault and Rosenbaum, 1983; L’Hernault and Rosenbaum, 1985; Soppina et al., 2012; Szyk et al., 2014), polyamination (Song et al., 2013), palmitoylation (Caron et al., 2001), S-nitrosylation (Jaffrey et al., 2001), and phosphorylation (Eipper, 1972; Fourest-Lieuvin et al., 2006). These post-translational modifications tend to be spatially organized within different cell types and regions within an individual cell (Yu et al., 2015). Within neurons, axonal microtubules tend to be heavily acetylated and glutamylated, whereas dendritic microtubules are tyrosinated. On the other hand, somatic cells tend to have highly
tyrosinated interphase microtubules, with acetylated microtubules nearer to the nucleus (Yu et al., 2015). Again, molecular motors show preferential recruitment and activity along different post-translationally modified microtubule tracks. For instance, kinesin-1 is preferentially motile on stabilized acetylated microtubules rather than dynamic, EB-labeled, microtubules in Cos7 cells (Cai et al., 2009); however, kinesin-1 motors did not show increased velocity, run length, or run initiation on acetylated microtubules in a single molecule in vitro reconstitution assay (Kaul et al., 2014; Walter et al., 2012). Thus, the reason for the preferential motility of kinesin-1 on acetylated microtubules in vivo is still unclear. Cumulatively, this idea that the features of the microtubule track along which a motor walks may regulate its activity is called the “tubulin code” hypothesis, and is still a very active area of scientific inquiry.

V. Actin filaments

Actin filaments are another type of track along which molecular motors can transport cargo within cells. These tracks are formed from globular actin monomers, which polymerize into polarized filamentous actin, composed of two protofilaments with a 36 nm repeat, right-hand helical twist. Comparable to the case of microtubules, globular actin (G-actin) can spontaneously polymerize into filamentous actin (F-actin) in the presence of a critical concentration of monomers and physiological salt concentration. ATP binds nucleotide-free G-actin rapidly (De La Cruz and Pollard, 1995), and is hydrolyzed stochastically once the actin is in filamentous form. The rate-limiting step of actin polymerization is nucleation of dimers and trimers of G-actin subunits; however, in vivo, the G-actin concentration is orders-of-magnitude higher than the critical concentration for polymerization. Thus, actin filament polymerization is regulated in space and time by an army of actin binding proteins which function to nucleate, elongate, depolymerize,
stabilize, and sever these different actin structures (Pollard, 2016). These various categories of actin-binding proteins are all necessary for proper actin network functioning in cells. Collectively, actin-binding proteins specify different actin filament populations within the cell, comparable to the tubulin post-translational modifications discussed previously. Also like microtubules, there is a dynamic and rapidly growing end, and a more slowly growing end, in this case referred to as the “barbed” and “pointed” ends, respectively. This generates an asymmetrical filament with ATP-bound subunits at the newer end and ADP-bound or nucleotide-free subunits at the older end.

Two important types of actin filament tracks are individual filaments often found in branched networks, and bundles. Branched actin filaments are nucleated by the Arp2/3 complex. This nucleation is controlled precisely in space and time by a variety of nucleation-promoting factors (NPFs) localized to the plasma membrane and specific organelle membranes. These NPFs are themselves activated during specific signaling cascades. For instance, during endocytosis, the cell cycle regulated Cdc42 GTPase binds to Neural-Wiskott-Aldrich Syndrome Protein (N-WASP)-family proteins to release autoinhibition, often in coordination with phosphoinositide 4,5-bisphosphate binding (Kim et al., 2000; Prehoda et al., 2000; Rohatgi et al., 1999; Takenawa and Suetsugu, 2007). In another example, a different NPF called WASP and SCAR Homologue (WASH), has been more recently been found on endosomes where it stimulates Arp2/3 branched actin formation to control endosomal sorting, possibly via controlled vesicular scission, although the mechanism is still unknown (Derivery and Gautreau, 2010; Derivery et al., 2009; Gomez et al., 2012; Jia et al., 2010).

Another category of actin filament polymerization is stimulated by formins, a class of formin homology 2 (FH2) domain-containing proteins that interact with the barbed end
of an actin filament (Goode and Eck, 2007; Paul and Pollard, 2009). Mammals have 15 formin isoforms with partially overlapping functions. Broadly, formins nucleate and elongate unbranched actin filaments for contractile rings, stress fibers, and filopodia while associating with the plasma membrane, the endoplasmic reticulum, and many other sites of actin polymerization and elongation (Campellone and Welch, 2010; Higgs, 2004). Filopodia are highly dynamic needle-like protrusions that are densely packed with 10 to 30 actin filaments and thought to act as sensory and signaling organelles, for instance in the chemotactic growth of nerve growth cones (Mogilner and Rubinstein, 2005; Svitkina et al., 2003; Weck et al.; Zheng et al., 1996). Actin in filopodia have uniform polarity with the barbed-ends (rapidly growing ends) pointed out, away from the body of the cell. These actin bundles are polymerized by the formin mDia2 and stabilized by the parallel bundling protein fascin (Mellor, 2010; Pellegrin and Mellor, 2005; Peng et al., 2003; Small et al., 1978; Vignjevic et al., 2006). Alpha-actinin (α-actinin) is another actin bundling protein found to stabilize and crosslink cellular actin filaments. However, it does not bind specifically to parallel actin filaments and can also stabilize anti-parallel actin filament geometry; thus, α-actinin is unlikely to be essential for filopodial formation and stabilization (Vignjevic et al., 2006).

Actin binding proteins tend to work in teams to organize the cytoskeleton for specific physiological needs. For instance, WASP/WAVE activates Arp2/3-nucleated branched actin in conjunction with capping protein and ADF-cofilin to break down actin beneath the plasma membrane in clathrin-mediated endocytosis and lamellipodium formation (Pollard, 2016; Rotty et al., 2012). In other locations, Ena/VASP formins tend to work with fascin to form parallel-bundled filopodia (Goode and Eck, 2007; Mellor, 2010; Paul and Pollard, 2009). Meanwhile, in stress-fibers, tropomyosin (discussed in more
detail below) and α-actinin tend to co-localize (Michelot and Drubin, 2011). Recent work suggests that different actin bundlers self-sort based on local actin filament spacing and local actin filament parallel or anti-parallel orientation (Winkelman et al., 2016). This may help explain how these different actin filament domains form within cells. Each of these general actin filament architectures—branched, parallel bundled, and anti-parallel bundled—tend to recruit certain types of molecular motors, for instance, type I, type X, and type II myosins, respectively.

VI. Actin filament-based molecular motors

Myosins are molecular motors that hydrolyze ATP and interact with actin filament tracks. Humans express 38 myosin genes belonging to 12 classes (Berg et al., 2001; Masters et al., 2016). These cytoskeletal motors largely move in the direction of the barbed-actin filament end; however, myosin-VI motors are known to move toward pointed-end of the actin filament (Wells et al., 1999). Although some myosins like myosin-Va, -VI, and -X can perform the hand-over-hand motility we associate with transportation of cargo along cytoskeletal tracks (Yildiz et al., 2003), not all myosin motors are associated with cargo transport. Other cellular myosin roles include facilitating cell motility, cytokinesis, and cell shape maintenance. The most famous myosin role is for sarcomeric myosin-II (traditionally referred to as “conventional” myosin-II) in the contraction of muscle through interaction with actin filaments (Holmes, 1997; Huxley, 1969).

Interactions between myosin and actin are highly regulated by the type of actin filament track, whether it is bundled by fascin or α-actinin, or bound by tropomyosin along its length. For example, myosin-X is highly recruited to and motile along fascin-bundled actin, on which it has adapted as a transport motor along parallel actin bundles within filopodia (Berg and Cheney, 2002; Ropars et al., 2016). Myosin-V, on the other hand, is
activated by tropomyosin-coated actin filaments (Clayton et al., 2014; Hodges et al., 2012; Sckolnick et al., 2016).

Tropomyosin is a particularly interesting regulator of myosin transport because there are four genes in humans that are differentially spliced to generate over 40 isoforms (Gunning et al., 2005). In some cases, these isoforms are expressed only in specific tissues; however, many tropomyosin isoforms can be expressed within an individual cell to define specific actin filament populations and different regions of a specific cell (Lin et al., 1988; Percival et al., 2000; Schevzov et al., 1997). Even motors like myosin-V which prefer tropomyosin-coated actin filaments show different motile properties when encountering different tropomyosin isoforms (Sckolnick et al., 2016). In this study, researchers found that cargo-activated MyoVa preferentially interacted with Tpm3.1-actin, rather than Tpm1.8-actin or Tpm4.2-actin, which may explain the preferential recruitment of MyoVa to the dendritic protrusions and cortical actin of melanocytes. Other myosins like myosin-I family motors are inhibited from interacting with actin filaments by the presence of tropomyosin, and thus avoid these actin filament populations in cells and in vitro (Kee et al., 2015; McIntosh et al., 2015; Tang and Ostap, 2001).

VII. Myosin-Ic

Myosin-I molecular motors are a family of single-headed actin-filament based motors that have a wide range of roles within cells (McIntosh and Ostap, 2016). During my thesis work, I have focused on the Myo1c gene, Myo1c protein product (Gillespie et al., 2001), isoform due to its ubiquitous expression and proposed functions during intracellular transport, cargo sorting, and membrane deformation (see Chapter 2 and McIntosh and Ostap, 2016 for more information about myosin-I motors in general). Myo1c, like its other myosin-I family members, can directly facilitate actin filament-membrane
interactions. It accomplishes these feats by both binding to actin and hydrolyzing ATP with its motor domain, while simultaneously binding directly to PtdIns(4,5)P$_2$ in lipid membranes through a Pleckstrin homology (PH) domain in its tail (Hokanson and Ostap, 2006; Hokanson et al., 2006). The Myo1c tail binds dynamically to PtdIns(4,5)P$_2$, with an increased lifetime on membranes containing additional anionic phospholipids such as phosphatidylserine (PtdSer) (McKenna and Ostap, 2009).

Figure 1.4 Structural features of Myo1c.

This post-powerstroke structure of Myo1c was built using the structure of Myo1b motor domain (PDB 4L79) appended to the structure of the Myo1c light chain binding domain (LCBD) and TH1 domains (PDB 4R8G). Figure adapted from McIntosh and Ostap, 2016.

Between the motor domain and tail domain is a light chain binding domain that is typically stabilized by calmodulin and functions as a lever arm to transduce conformational changes generated by ATP hydrolysis to the cargo-bound tail domain (Lewis et al., 2012). In the presence of calcium as low as 100 μM the calmodulin closest to the Myo1c motor
domain dissociates from the motor, inhibiting Myo1c from productively interacting with actin filaments during a gliding filament assay (Manceva et al., 2007); however, actin filament gliding is restored when exogenous calmodulin is added in the presence of calcium. This differs from the calcium sensitivity of another short-tailed myosin-I isoform Myo1b, where calmodulin is unable to rescue sensitivity to calcium (Lewis et al., 2012). Thus, calcium regulation may be one way that Myo1c activity is modulated within the cell where physiologically relevant concentrations of calcium would decrease the free calmodulin level such that it would be unlikely to be stabilizing the Myo1c lever arm (Black et al., 2004).

Myo1c has been proposed to participate in a wide range of processes within cells including the establishment/maintenance of membrane tension (Nambiar et al., 2009), the stability of adherens junctions (Oh et al., 2013; Petzoldt et al., 2012; Tokuo and Coluccio, 2013), organization of actin at the immune synapse of B cells and in neuronal growth cones (Maravillas-Montero et al., 2011; Wang et al., 2003), compensatory endocytosis (Sokac et al., 2006), lamellar body exocytosis in alveolar cells (Kittelberger et al., 2016), inner ear and kidney collection duct mechanical adaptation (Batters et al., 2004; Gillespie et al., 1993; Holt et al., 2002; Lin et al., 2011; Wagner et al., 2005), endoplasmic reticulum sheet stabilization (Joensuu et al., 2014), chromosome movement and nuclear organization (Chuang et al., 2006; Kyselá et al., 2005; Percipalle et al., 2006; Pestic-Dragovich et al., 2000; Philimonenko et al., 2004), and a wide range of intracellular transport functions including cargo sorting, transport, and membrane deformation. Myo1c has been proposed to participate in the trafficking of a number of cargo including glucose transporter-4 (GLUT4) exocytosis (Boguslavsky et al., 2012; Bose et al., 2002; Bose et al., 2004), vascular endothelial growth factor-2 (VEGF2) (Tiwari et al., 2013), nephrin-like
protein-1 (Neph1) (Nakamori et al., 2006), and cholesterol-rich lipid raft recycling compartments toward the plasma membrane (Brandstaetter et al., 2012); however, it is unclear how Myo1c is participating in these processes at a molecular level: as a dock/tether, transporter, or effector of membrane deformation.

Myo1c is an interesting candidate for cargo sorting and membrane deformation due to its direct binding between actin and membranes. Additionally, while Myo1c is involved in the tubulation and transport of lipid raft recycling cargo, Myo1b is not involved in these processes; rather, Myo1b is important for the formation of post Golgi carriers from the trans Golgi of glycosylphosphatidylinositol-linked (GPI-linked) proteins, where Myo1c is not localized (Almeida et al., 2011). This suggests specific involvement of certain myosin-I motors during different membrane deformation and transport events. Additionally, this well-positions Myo1c for important cellular functions at the interface between processive, microtubule-based transport, and the actin cytoskeleton. Finally, Myo1c may be further regulated in certain regions of the cell by actin binding proteins such as tropomyosin, since Myo1b and Myo1a have been previously found to avoid tropomyosin-coated actin (Collins and Matsudaira, 1991; Fanning et al., 1994; Tang and Ostap, 2001); this is one avenue we investigated during my thesis work (see Chapter 3 and McIntosh et al., 2015).

VIII. Actin and Microtubule Motor-Coordinated Cargo Transport

Studies looking at motor localization and knockdown phenotypes have shown that multiple types of microtubule- and actin filament-based molecular motors coordinate to transport cargo from beginning to end. For instance, dynein and its co-activator dynactin, heterotrimeric kinesin-2, and myosin-Va are all involved in the transport of melanosomes to and from perinuclear storage compartments and localization beneath the plasma
membrane (Gross et al., 2002; Rogers and Gelfand, 1998; Tuma et al., 1998). In this case, the microtubule motors kinesin-2 and dynein are largely responsible for the long-distance transport of melanosomes from the cell center to the periphery along microtubules. Myosin-V is important for the navigation of the dense cortical actin network beneath the plasma membrane.

Another example of multi-motor actin and microtubule-based transport is that of GLUT-4 (glucose transporter) recycling toward the plasma membrane, which involves kinesin-1, myosin-Va, and myosin-Ic (Boguslavsky et al., 2012; Chen et al., 2007; Chen et al., 2012, 10; Huang et al., 2005; Semiz et al., 2003; Yip et al., 2008; Yoshizaki et al., 2007). These cargo are transported by kinesin-1 toward the plasma membrane, where at some point they switch to myosin-V-driven motility along actin filaments. It has been hypothesized that Myo1c is responsible for facilitating motility along actin filaments (Bose et al., 2002; Chen et al., 2007; Huang et al., 2005; Yip et al., 2008), tethering beneath the plasma membrane (Boguslavsky et al., 2012), and/or plasma membrane fusion (Bose et al., 2004; Toyoda et al., 2011). All three motors, kinesin-1, myosin-Va, and myosin-Ic are found on GLUT4 cargo at the same time, yet how these motors coordinate throughout cargo transport is largely unknown.

To investigate how different combinations of motors navigate an increasingly complex cytoskeleton, researchers began adding complexity to in vitro reconstitution assays. These “bottom up” investigations involve recombining individual, purified, components in increasing complexity to understand the minimum requirements necessary to recreate observed cellular physiology. In these assays, individual kinesin-1 motors tend to pass or detach at microtubule intersections, while when multiple motors are attached to a 1 μm bead, kinesin-only cargo tend to pass or switch, depending on whether the
encountered intersection is an “underpass” or “overpass” (Ross et al., 2008). In contrast, single dynein motors (coupled with their co-factor dynactin) have an equal probability of passing, pausing, switching, detaching, or reversing the direction of motility at microtubule intersections; meanwhile, beads coated with multiple dynein-dynactin motors overwhelmingly tend to pause at both “underpass” and “overpass” intersections (Ross et al., 2008). These results suggested that kinesin-1 and dynein motors respond differently to load at microtubule-microtubule intersections, and illustrate the catch-bond nature of dynein motility where it can remain tightly bound to microtubules under load, in comparison to kinesin-1 motility, which largely causes detachment under loads at or above its stall force.

Similar themes hold true for actin filament-based motors. Single molecules of myosin-Va can effectively navigate individual actin intersections, as well as Arp2/3 branched actin (Ali et al., 2007). When a single myosin-V and a single myosin-VI were linked together by a quantum dot, myosin-Va motility toward the actin barbed end dominated over myosin-VI motility toward the pointed end 79% of the time; however, the motor that won the “tug-of-war” sacrificed its normal stepping rate due to the resistive load of the losing motor, and the losing motor took backward steps along the track, likely increasing the overall processivity of the complex (Ali et al., 2011). In this case, both myosin-Va and myosin-VI have similar stall forces of between 1.5 to 3 pN (Altman et al., 2004; Kad et al., 2008; Mehta et al., 1999; Rock et al., 2001; Uemura et al., 2004), but these motors have different tendencies to take back-steps with the application of resistive load. Myosin-Va has a resistive load, or amount of force applied to an opposing motor, of 2.1 pN, while myosin-VI has a resistive load of 1.4 pN of force. Thus, myosin-Va likely
dominates motility because it is a heavier load than myosin-VI, preferentially enhancing myosin-Va over myosin-VI-directed motility (Ali et al., 2011).

Another tug-of-war scenario that has been widely investigated is that of kinesin-1 and dynein. To directly test whether DNA origami cargo containing kinesin-1 and dynein motors were stalled due to a tug-of-war balance of forces, the Reck-Peterson Lab used a photo cleavable tag to release kinesins, resulting in dynein-driven motility being initiated (the opposite result occurred when dynein was cleaved) (Derr et al., 2012). Interestingly, in an in vivo system, the investigation of bidirectional lipid droplet motility in Drosophila embryos, researchers found that stalled cargo were more likely to resume motion in the same direction rather than reverse direction after a pause (Leidel et al., 2012). These results suggest that only one type of motor is active at a time, likely regulated by activation/inactivation rather than recruitment, and indicate that looking solely at purified kinesin-1 and dynein motors without their physiological regulators may not be showing the complete picture. Other investigations into the motility of dynein and kinesin-driven cargo in cells looked at the motility of membrane-bead phagosome compartments along microtubules. One study concluded that dynein and kinesin-1 and/or kinesin-2 exist at near force balance, resulting in high bi-directional motility (Hendricks et al., 2012). Alternatively, another group found that dynein dominates kinesin for mostly minus-end-directed transport of phagosomes (Rai et al., 2013). It is unclear why these two studies have such substantially different results in very similar systems; however, they may indicate different regulators are at play in subtly different processes resulting in bidirectional or unidirectional motility.

Due to the single molecule stall forces of kinesin-1 (~5 pN) vs. mammalian cytoplasmic dynein (~1.5 pN), it has been estimated that 4-7 dynein motors would be
needed to engage 1 kinesin motor in a tug-of-war; however, some estimates have put the dynein:kinesin ratio on mouse axonal membrane-bound vesicles at \( \sim 1.5:1 \), or approximately 1-4 kinesin and 1-5 dynein motors per vesicle (Hendricks et al., 2010). Recent optical trapping studies have shown that when dynein is activated by both dynactin and bicaudal D2 (BicD2), a single motor is then capable of resisting 4.3 pN of optical trap-generated force (Belyy et al., 2016). This indicates that dynein would be more capable of successfully resisting kinesin-driven vesicle motility with BicD2 present; yet, more work needs to be done to further clarify this hypothesis, especially in relation to which dynein activators are relevant for distinct trafficking processes. These results suggest that molecular motor co-factors and binding partners can have extreme influence on their force-generating and transport properties within cells.

In the case of motors bound to different cytoskeletal track types, dynein-dynactin and myosin-V, or kinesin-2 and myosin-V, the relative force generation properties of the different types of motors are not the only important parameters for determining the likely outcome of a cytoskeletal intersection (Schroeder III et al., 2010; Schroeder III et al., 2012). In this case, the ability of the motors to remain attached to their cytoskeletal track under load is also an important factor. For instance, kinesin-1 is better able to resist loads than kinesin-2, so it is likely to win a head-to-head competition.

Cumulatively, these data paint a picture in which the force generating and resisting properties of motors in the context of their physiological binding partners determines the trafficking outcome; however, these studies only investigated combinations of processive motors. We know that non-processive motors, such as myosin-I, are found on trafficking cargo, yet how these motors interact with highly processive motors capable of rapidly stepping along a cytoskeletal track was largely unknown prior to this dissertation work.
IX. Cargo membranes and remodeling

Many motor-transported intracellular cargos are contained in lipid membrane-bound compartments. For example, translated proteins are sorted and packaged for exocytic release from the endoplasmic reticulum or trans Golgi toward the plasma membrane. Similarly, extracellular or membrane-bound proteins can be endocytosed from the plasma membrane into early endosome compartments. These compartments can fuse with recycling compartments that shuttle receptors and other components back to the plasma membrane. Alternatively, early endosomes can mature into late endosomes and lysosomes through a series of protein additions and removals. At the same time, the lipid and protein composition of these compartments is changing (Granger et al., 2014).

The plasma membrane of mammalian cells is enriched in sphingolipids and sterols such as sphingomyelin (SM) and cholesterol, respectively, which pack at a high density and provide mechanical support to the cell (van Meer et al., 2008; Figure 1.5). It is estimated that the plasma membrane contains 50-80% of the total cholesterol in the cell (Ikonen, 2008). Intermediate levels of cholesterol are found in the Golgi and endocytic compartments, with even less cholesterol in the endoplasmic reticulum. Sphingolipids and cholesterol tightly pack to form lipid microdomains/rafts in the plasma membrane (Rao and Mayor, 2014). These microdomains can serve to organize necessary components for signaling cascades, and are also important stiffened platforms through which the cell can transduce force from extracellular connections to the internal cytoskeleton (Anishkin and Kung, 2013).
Figure 1.5 Lipid and membrane composition varies across the cell.

Cellular membranes have different compositions with sterols and sphingolipids, saturated acyl chains, PtdSer, and electrostatic protein binding concentrated at the plasma membrane. On the other hand, unsaturated acyl chains and packing defect-mediated protein binding is concentrated at the endoplasmic reticulum. These compositional and property differences between compartments can be thought of as a gradient, on average, because of high levels of mixing between compartments; however, the membrane composition at individual cellular events is tightly regulated by kinases, phosphatases, lipases, and flippases. Additionally, phosphatidyl inositol lipids (boxed with blue dashed lines) are generally found on different membrane compartments, with some overlap between isoforms. Again, phosphatidylinositols are tightly regulated locally for specific cell biological events. Figure adapted from Bigay and Antonny, 2012; Di Paolo and De Camilli, 2006.
Especially for motors that bind directly to the membrane, these microdomains of highly organized lipids may be necessary for force generation. For example, in the case of Myo1c, molecular dynamic modeling has suggested that it is the motors at diffusion barriers within a membrane that are largely responsible for force generation (Pyrpassopoulos et al., 2016). Microdomains can also cluster motors. Recent work in the Mallik Lab has shown that dynein can take advantage of these stiffened, cholesterol-rich platforms in order to collectively generate more force, and dominate motility over plus-end directed kinesins during phagosome maturation (Rai et al., 2016). The movement of early phagosomes along microtubules is largely bidirectional with both kinesin and dynein motors active; however, as phagosomes mature, there is an increase in cholesterol recruitment to the membrane. This results in the clustering of cholesterol-associated proteins Rab7 and flotillin, and the downstream clustering of dynein into functional teams. As dynein motors are clustered into these force-generating teams, this results in minus-end-directed motility of maturing phagosomes, enhancing lysosome fusion in the perinuclear region. Interestingly, more ordered membranes do not always lead to better collective motor motility. In the case of myosin-Va bound to synthetic vesicular cargo, myosin-Va teams transport cargo at a faster velocity when attached to less ordered membranes composed of the highly fluid 1,2-dioleoyl (both acyl chains have one double bond) DOPC, rather than the highly ordered 1,2-dipalmitoyl (both acyl chains are saturated) DPPC lipid (Nelson et al., 2014). These results suggest that the fluidity and organization of lipids in the membrane may directly affect motor motility and cooperativity (Pathak and Mallik, 2016).

Decades of research in the field of lipid biology have shown that the types of lipid in a membrane directly determine the fluidity and deformability of that membrane, in
addition to the types of protein it recruits (Bigay and Antonny, 2012; McMahon and Boucrot, 2015). In general, lipid type may affect the curvature and fluidity of the membrane through a combination of the size and charge of the hydrophilic head group, and the length and level of saturation of the hydrocarbon acyl tails (Bigay and Antonny, 2012; Pathak and Mallik, 2016; Zimmerberg and Kozlov, 2006). Phosphatidylinositolides (PtdIns) contain a polar head group that can be modified by up to three phosphates at a time and exist in a gradient, with different phosphorylation combinations predominantly localized to different membrane-bound compartments (Di Paolo and De Camilli, 2006; Picas et al., 2016; Figure 1.5). For example, PtdIns(4,5)P2 and PtdIns(3,4,5)P3 are predominantly localized to the plasma membrane, PtdIns(3,4)P2 is mostly at the plasma membrane and on early endocytic compartments, and PtdIns(4)P is concentrated at the Golgi; however, these lipids can still be found in other locations. For instance, PtdIns(4,5)P2 has also been found on endocytic compartments, exocytic pre-fusion compartments, lysosomes, and the Golgi (Arneson et al., 1999; De Matteis and D’Angelo, 2007; Di Paolo and De Camilli, 2006; Sweeney et al., 2002; Yin and Janmey, 2003). PtdIns(4,5)P2 tends to accumulate in a subset of the sphingosine and cholesterol-rich rafts described above (Laux et al., 2000; Martin, 2001).

In addition to transporting cargo, molecular motors can also remodel the membranes to which they are bound. In cellular studies, tubulation of intracellular cargo is largely thought to be driven by processive, microtubule-based motors. For instance, tubular transport intermediates are generated during endoplasmic reticulum (ER) to Golgi transport, highly dependent on microtubules, and in many cases, dynein (Blum et al., 2000; Presley et al., 1997; Simpson et al., 2006). Additionally, kinesin is required for the post-Golgi transport toward the plasma membrane, although in the absence of
microtubules, the Golgi fragments and myosin-based transport can sufficiently rescue exocytosis (Bloom and Goldstein, 1998; Kreitzer et al., 2000; Polishchuk et al., 2003; Toomre et al., 1999). Kinesin-1 (and dynein) are also implicated in the extension of tubular lysosomes, and the recycling of lysosomal components from autolysosomes (Du et al., 2016; Hollenbeck and Swanson, 1990; Li et al., 2016). Finally, kinesin-1 has been found to drive dynamic tubulation of mitochondria to power mitochondrial network formation (Wang et al., 2015). Other studies have suggested that kinesin-1, kinesin-2 and dynein-dynactin are important for the scission, as well as the elongation, of tubulated cargo in the presence of over-expressed sorting nexins (SNX) (see Section X for further discussion of BAR proteins) (Hunt et al., 2013).

Interestingly, in many of these studies, actin filaments are necessary for tubulation to occur. For example, the Golgi is supported and organized by both actin and microtubule cytoskeletons and their effectors. Through drug-based actin and microtubule inhibition studies, Cobbold et al., 2004, found that both cytoskeletal systems are involved in the trafficking of a copper-dependent protein transporter to the cell surface. In another example, tubules emanating from recycling endosomes that are carrying the β2-adrenergic receptor toward lysosomes or the plasma membrane require actin stabilization, whereas transferrin-receptor containing recycling tubes are not coated with actin (Cao et al., 1999; Putheveedu et al., 2010). This actin is Arp2/3 nucleated and also shows a concentration of WASH and cortactin, which is important for tubule scission, but no N-WASP or WAVE2 (Putheveedu et al., 2010; Vistein and Putheveedu, 2014). Finally, the actin-coated tubules in these so-called “ASSERT” (Actin-Stabilized Sequence-dependent Recycling Tubule) domains were longer lasting than the non-actin coated transferrin receptor recycling tubules, and inhibition of actin resulted in a lower total number of
tubulation events. This suggests that the actin and/or actin binding proteins are directly involved in sorting and/or stabilizing different tubule populations. Myo1b and Myo1c have also been proposed to play a role in the tubulation or deformation of trans Golgi and lipid raft recycling cargo, respectively, although how these motors are facilitating this membrane remodeling at a molecular level is still largely unknown (Almeida et al., 2011; Brandstaetter et al., 2012).

In vitro studies have investigated whether cytoskeletal motors can pull membrane tubes from physiological and reconstituted membranes. Kinesin-1 was initially shown to be capable of pulling and deforming endoplasmic reticulum and lysosome membranes along microtubules with isolates from Xenopus egg cytosolic extract and squid axoplasm extract (Allan and Vale, 1994; Vale and Hotani, 1988). Additionally, kinesin-1 (KIF5B) can pull thin membrane tubes, having widths at or below the diffraction limit, from giant unilamellar vesicles when attached to the membrane via a bead intermediate (Roux et al., 2002). In similar studies also using constitutively active kinesin-1, motors can pull a membrane tube out of a giant unilamellar vesicle when attached to the membrane via a biotinylated lipid (Koster et al., 2003). More recently, it has been found that full length kinesin-1 can bind directly to PtdIns(4,5)P₂ on reconstituted membranes and purified mitochondria and autolysosomes to facilitate membrane tubulation (Du et al., 2016; Su et al., 2016; Wang et al., 2015). During these tubulation events, kinesin-1 is largely found at the tip of the growing tube (Leduc et al., 2004; Shaklee et al., 2010b). On the other hand, ncd, a non-processive kinesin-14 motor is also capable of generating tubes along microtubules; however in this case, the motors are found all along the tube/microtubule interface and the tubes are capable of dynamically growing and shrinking (Shaklee et al., 2008; Shaklee et al., 2010a). In all of these in vitro studies of kinesin-1-driven tubulation,
in order to deform the membrane, either non-specific vesicle attachment to the surface, a high microtubule density, and/or a non-physiologically high concentration of kinesin-1 motors were required in order to provide the resistive force against kinesin-1 motility to allow tubulation rather than simple vesicular transport. These conditions are largely non-physiological since it is thought that there are only 1-7 kinesin-1 motors per neuronal trafficking cargo (Hendricks et al., 2010), not micromolar concentrations, and tubulation events occur in many regions of the cell where there is not a high incidence of microtubule/microtubule overlap. Within cells, this tethering may be caused by motors of the opposite polarity (i.e. dynein to kinesin), myosins, or other tethering molecules, although this is largely unknown.

One example that implicates the tug-of-war between kinesin-1 (KIF5B) and dynein-dynactin in tubulation of cellular cargo occurs in the transport of matrix metalloprotease-1 (MMP) recycling in late endosomal cargo back toward the plasma membrane (Marchesin et al., 2015). In this system, MMP cargo are tubulated just beneath the plasma membrane by WASH and the exocyst complex in order to facilitate membrane fusion and effect extracellular matrix breakdown by metastatic breast cancer cells (Monteiro et al., 2013). Marchesin et al., 2015 found that kinesin-1 (KIF5B) and dynein-dynactin bind to MMP-1 recycling cargo via attachment to the adaptor proteins JIP3/4, which are themselves activated by Arf6. JIP4 requires WASH for association to these MMP-1 cargo, and all of these, kinesin-1, dynein-dynactin, and WASH are necessary for the formation of MMP-1 exocytic tubules and successful MMP release and activity. These results suggest that tubulation occurs due to the tug-of-war resistance generated between dynein-dynactin and kinesin-1. It also supports a role for WASH-nucleated Arp2/3 actin and other actin-binding proteins in either stabilizing or actively facilitating this membrane deformation and
tubulation. Interestingly, kinesin-2 (KIF3A) which is also found on these exocytic MMP-1 vesicles, can rescue peripheral distribution of MMP-1 cargo in the absence of kinesin-1, yet it is unable to rescue tubulation, suggesting that the roles for these two kinesins are non-redundant.

Myo1b has also been shown capable of tubulating giant unilamellar vesicles in vitro (Yamada et al., 2014). In this case, to generate membrane tubes, a high density of fascin-bundled actin was attached to the surface of the chamber. Fascin-bundled actin provides multiple parallel myosin binding sites, which is hypothesized to be necessary for this non-processive motor to coordinate and deform membranes. It is unknown whether other myosins can also undertake this membrane deformation.

X. Other membrane deformation factors

In addition to molecular motors, there are many other proteins thought to be responsible for generating and stabilizing curved membranes within the cell. One of these classes of molecules are called Bin-Amphiphysin-Rvs (BAR) domain proteins (Qualmann et al., 2011; Safari and Suetsugu, 2012; Suetsugu et al., 2014). These are a class of banana-shaped proteins that can directly bind to membranes to generate and stabilize membrane curvature. There are three general classes: F-BAR, I-BAR, and N-BAR proteins (Figure 1.6). F-BAR proteins, for example FCHo, have long, shallow BAR domains that bind to and stabilize positive membrane curvature of wider tubes from 60 to 100 nm in diameter (Frost et al., 2008; Qualmann et al., 2011). “Positive” membrane curvature is exemplified by endocytosis from the plasma membrane, or tubulation of endosomes within the cytosol. Alternatively, I-BAR or “inverted” BAR domain proteins like IRSp53 generate/stabilize negative membrane curvature, for instance the generation of filopodia, or the inward deformation necessary to generate multi-vesicular bodies, in 40 to
60 nm diameter tubes (Saarikangas et al., 2009). Both N-BAR and “classical” BAR domain proteins generate tubules from 20 to 60 nm wide, stabilizing positive membrane curvature. N-BAR proteins such as endophilin or amphiphysin have an N-terminal amphipathic helix that can directly insert into the membrane bilayer (Qualmann et al., 2011; Simunovic et al., 2015). This N-terminal helix was previously thought to generate membrane curvature, but rather seems to be important for stabilizing electrostatic binding and sensing membrane curvature for sorting to more highly curved membranes (Chen et al., 2016).

**Figure 1.6** There are three general types of BAR proteins: F-BARs (i.e. FCHo2), I-BARS (i.e. IRSp53), and N-BAR/classical BAR proteins (i.e. endophilin).

F-BAR proteins scaffold tubes toward the cell center (positive membrane curvature) of 60 – 100 nm in diameter. I-BARs (IRSp53) scaffold negatively-curved membranes of 40 – 60 nm in diameter, for instance, filopodia projecting out, away from the cell. N-BAR proteins, for instance endophilin, scaffold 20 – 60 nm diameter tubes with positive membrane curvature, such as in endocytosis. Figure adapted from Zhiming Chen of PDB structures: FCHo2 (2V0O), IRSp53 (1WDZ), and Endophilin (2C08).
Endophilins, in particular the neuronally expressed endophilin-A1, have been best studied in relation to endocytosis where they are in a cascade of membrane-binding proteins that successively induce higher curvature generation (Suetsugu et al., 2014). First in this cascade, F-BAR proteins like FCHo work with clathrin and its adaptors to deform the flat plasma membrane. Additional F-BAR protein FBP17 and classical BAR protein PACSIN2 are then recruited with actin nucleation-promoting factors (NPFs) like N-WASP. As the invagination deepens, N-BAR proteins amphiphysin, endophilin, and SNX9 work together with ARP2/3 actin to tighten the bud neck, ultimately recruiting dynamin and facilitating membrane scission. Thus, endophilin is a protein that can sense and stabilize highly curved membranes; however, it is still unclear whether BAR domain proteins generate these curved membranes within cells. This participation in endocytosis is especially marked in synaptic vesicle endocytosis where it directly interacts with dynamin to accomplish vesicle scission from the plasma membrane (Huttner and Schmidt, 2000; Ringstad et al., 1999; Schmidt et al., 1999). Endophilin-A1 has also been found to interact with ataxin-2, huntingtin, parkin, and LRRK2 suggesting neurodegenerative disease relevance (Matta et al., 2012; Ralser et al., 2005; Trempe et al., 2009). In addition to participating in endocytosis at the plasma membrane, endophilin-A1 has also been associated with trans Golgi, and early, late, and recycling endosomes upon epidermal growth factor (EGF) activation of ITCH ubiquitin ligase activity and recruitment to endosomal compartments containing ITCH (Angers et al., 2004). Other compartments include vesicular glutamate transporter type 1 exocytic cargo (Vinatier et al., 2006; Weston et al., 2011), phosphorylated Trk vesicles (Fu et al., 2011), and exocytic synaptic vesicles (Bai et al., 2010). The closely related endophilin-B isoforms localize to the endoplasmic reticulum and Golgi complex, consistent with a role in the early secretory pathway (Farsad et al., 2001; Huttner and Schmidt, 2002).
In vitro, endophilin is capable of both sensing and generating membrane tubes (Neumann and Schmid, 2013; Roux et al., 2006; Zhu et al., 2012). This tubulation is dependent on micromolar solution concentrations, whereas it is largely thought that the entire cellular concentration of endophilin is well below this range. Endophilin-A1 binds to membranes with some specificity to PtdIns(4,5)P$_2$; however, studies have shown that these molecules can more generally bind to anionic and curved membranes (Chen et al., 2016; Gallop et al., 2006). It is still unclear, however, how this BAR domain-mediated membrane deformation is accomplished in relation to molecular-motor-generated deformed and tubulated membranes observed within cells.

XI. Putting it all together

Both kinesin-1 and Myo1c are found on trafficking intracellular cargos of a variety of cell types, including exocytic GLUT4 and tubular-vesicular lipid raft recycling cargos (Boguslavsky et al., 2012; Brandstaetter et al., 2012; Semiz et al., 2003). These motors are both involved during the course of transport, with kinesin-1 undertaking the bulk of long distance microtubule based-transport. Although myosin-I motors are not likely to be directly transporting cargo along actin filament tracks over long distances, they may yet play large roles during the targeting, sorting, and localization of trafficked cargo. Both myosin-I and kinesin-1 can be regulated by the different types of actin filament and microtubule tracks in addition to many other forms of regulation. In addition to transporting membrane-bound cargo, kinesin-1 and myosin-I are also implicated in remodeling these same membranes. Here we investigate intracellular transport by increasing the complexity of in vitro reconstitution studies: (1) by interrogating how a processive microtubule-based motor and a non-processive actin filament-based motor interact at individual actin filament/microtubule intersections; (2) by varying the type of actin filament intersection
encountered; (3) by using motor-cargo attachment strategies that enable the free reorganization of motors around the cargo in response to the local cytoskeletal environment, and later the full deformation of the artificial cargo; (4) by investigating membrane deformation in an engineered cytoskeleton which mimics the cytoskeletal organization where cellular tubulation occurs—sparse microtubules encountering dense actin filaments; (5) by addition of BAR domain proteins to understand how these proteins influence motor-driven membrane deformation; (6) by varying the lipid composition of the synthetic, deformable, membrane-bound cargo. Ultimately, we aim to build complexity from the bottom-up to gain more insight into the minimal components necessary to reconstitute cellular physiology.
CHAPTER 2 : Myosin-I Molecular Motors at a Glance

This chapter is adapted from


We gratefully acknowledge Michael Woody, Dr. Michael Greenberg, and the rest of the Ostap laboratory for helpful discussions during preparation of the manuscript, as well as our funding from the National Institutes of Health: T32 AR053461 to B.B.M., P01 GM087253 to E.M.O., and R01 GM057247 to E.M.O.
I. Introduction

Myosin-I proteins were first discovered in lower eukaryotes (Pollard and Korn, 1973), but are recognized to be widely expressed. *Homo sapiens* and higher vertebrates express eight different myosin-I genes *Myo1a – Myo1h* with the corresponding proteins named Myo1a - Myo1h (Gillespie et al., 2001; Figures 2.1-2.3). Myosin-I molecular motors are comprised of a motor domain that binds to and interacts with actin in response to ATPase cycling, a light chain-binding domain (LCBD) that binds one to six calcium-sensitive calmodulin or calmodulin-like light chains and functions as a lever arm (Bähler et al., 1994; Köhler et al., 2005; Lin et al., 2005; Manceva et al., 2007; McConnell and Tyska, 2010; Ruppert et al., 1993; Sherr et al., 1993; Sielski et al., 2014; Stöffler and Bähler, 1998; Swanljung-Collins and Collins, 1991), and a tail domain (Figure 2.4). The tail domain is composed of a myosin-I family tail homology 1 (TH1) domain, which includes a Pleckstrin homology (PH) domain known to bind a variety of anionic phospholipids (Adams and Pollard, 1989; Doberstein and Pollard, 1992; Feeser et al., 2010; Hayden et al., 1990; Hokanson et al., 2006; Miyata et al., 1989). Myosin-I motors are generally classified into short-tailed (Myo1a, 1b, 1c, 1d, 1g, 1h) or long-tailed (Myo1e, 1f) groups based on the presence of additional glycine-rich (TH2) and SH3 (TH3) domains in the long-tailed isoforms. The eight isoforms evolved in pairs from four precursor motors, grouping Myo1e and Myo1f, Myo1d and Myo1g, Myo1a and Myo1b, and Myo1c and Myo1h (Figure 2.3).

The cellular localization of myosin-I isoforms depends both on the preference of their motor domains for different actin filament populations, as well as for specific anionic phospholipids found on different cellular membranes (Ruppert et al., 1995). *In vitro* and *in vivo* data suggests that myosin-I motors avoid tropomyosin-coated actin filaments (Collins
et al., 1990; Kee et al., 2015; McIntosh et al., 2015; Tang and Ostap, 2001) and instead prefer Arp2/3-nucleated (Almeida et al., 2011) and non-tropomyosin-coated cytoskeletal actin. Although Myo1a, Myo1b, Myo1c, and Myo1g have been found to preferentially bind to phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P$_2$), all characterized myosin-I isoforms can bind to some extent to anionic phospholipids (Adams and Pollard, 1989; Dart et al., 2012; Doberstein and Pollard, 1992; Feeser et al., 2010; Hayden et al., 1990; Hokanson and Ostap, 2006; Hokanson et al., 2006; Komaba and Coluccio, 2010; McKenna and Ostap, 2009; Miyata et al., 1989; Patino-Lopez et al., 2010). Thus, these preferences of the motor domains and tail domains result in the predominant localization of myosin-I to the plasma membrane, with additional binding to intracellular organelles (Figures 2.5-2.7). However, it is unclear how specific myosin-I isoforms establish their individual localizations. One hypothesis is that subcellular localizations are determined in part by protein-binding partners. Although individual binding partners have been discovered (see e.g., (Nakamori et al., 2006; Tang et al., 2007)), there is little evidence that these proteins indeed direct myosin-I localization or have any isoform specificity. In this review, we focus on metazoan myosin-I research with a particular emphasis on the molecular roles of the myosin-I isoforms in each case.
Box 1. Generation of statistically relevant evolutionary trees of the myosin-I molecular motor family in eukaryotes and Homo sapiens

The statistically relevant evolutionary histories shown on the poster (‘Phylogenetic analysis panel’) were inferred using the program MEGA6, by using the Maximum Likelihood method based on the JTT matrix-based model (Jones et al., 1992; Tamura et al., 2013). The trees with the highest log likelihood (left, $-20410.6144$; right, $-14298.3803$) are shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches, where values closer to 100% represent higher statistical confidence. Initial tree(s) for the heuristic search were obtained by applying the neighbor-joining method to a matrix of pairwise distances estimated using a JTT model. The trees are drawn to scale, with branch lengths measured in the number of substitutions per site. All positions containing gaps and missing data were eliminated. For the tree including multiple eukaryotic isoforms, the analysis involved 25 full-length amino acid sequences including all myosin-I isoforms from Homo sapiens (Hs) (MYO1A–MYO1H), Drosophila melanogaster (Dm) (Myo31DF, Myo61F), Caenorhabditis elegans (Ce) (HUM-1, HUM-5), Saccharomyces cerevisiae (Sc) (Myo3, Myo5), Schizosaccharomyces pombe (Sp) (Myo1), Dictyostelium discoideum (Dd) (MyoA–MyoF, MyoK) and Acanthamoeba castellani (Ac) (Myosin-IA, Myosin-IB, Myosin-IC). There were a total of 569 positions compared in the final dataset. For the Homo sapiens MYO1 tree, the analysis involved the eight full-length Homo sapiens myosin-I isoform amino acid sequences, comparing a total of 946 positions in the final data set. Sequences used in this comparison were MYO1A (GenBank NP_001242970.1), MYO1B motor a (GenBank NP_001123630.1), MYO1C isoform a (GenBank NP_001074248.1), MYO1D isoform 1 (GenBank NP_056009.1), MYO1E (GenBank AAH8392.1), MYO1F (GenBank NP_036467.2), MYO1G (GenBank NP_149043.2) and MYO1H (GenBank NP_001188784.1).

Figure 2.1 Generation of statistically relevant evolutionary trees of the myosin-I molecular motor family in eukaryotes and Homo sapiens. “Phylogenetic analysis panel” refers to Figure 2.3.
**Box 2. Myosin-I tissue expression and disease phenotypes**

Myosin-I isoforms show varying expression levels across different tissues (see supplemental table). Myo1b, Myo1c, and Myo1e are widely expressed and found in most cell types (Krendel et al., 2009; Ruppert et al., 1993; Sherr et al., 1993; Sielski et al., 2014; Skowron et al., 1998; Tyska et al., 2005). Loss of Myo1c or Myo1e is associated with kidney disease (Airf et al., 2011; Bi et al., 2013; Krendel et al., 2009; Mele et al., 2011; Wagner et al., 2005), in addition to deafness associated with Myo1c loss (Batters et al., 2004; Holt et al., 2002) and arteriosclerosis upon loss of Myo1e (Inouye et al., 2012). Mutations in Myo1a are also associated with hearing loss (Donaudy et al., 2003), although Myo1a is most highly expressed in the small intestines and colon where it is associated with colon cancer (Mazzolini et al., 2012, 2013; Skowron et al., 1998). Myo1d is highly expressed in the central and peripheral nervous system (Bähler et al., 1994; Benesh et al., 2012; Cahoy et al., 2008; Nielsen et al., 2006; Sherr et al., 1993), liver and small intestines (Bähler et al., 1994; Benesh et al., 2010), and has been linked to autism spectrum disorders (Stone et al., 2007). Myo1f and Myo1g are predominantly expressed in hematopoietic cells, where Myo1e is also found (Diakonova et al., 2002; Hao et al., 2008; Kim et al., 2006; Nebel et al., 2002; Oetzy et al., 2010; Wenzel et al., 2015). Myo1f is the third myosin-I isoform that has been associated with deafness (Baek et al., 2012; Chen et al., 2001) and also linked to acute monocytic leukemia (Taki et al., 2005). Myo1h is highly expressed in the testis, with considerably lower expression in adipocytes and the heart (Fishilevich et al., 2016), and has been implicated as a marker associated with the mandibular prognathism phenotype (Tassopoulou-Fishell et al., 2012). Although loss of myosin-I motors is associated with many disorders, there is evidence that partial rescue and overlapping functions by closely related myosin-I isoforms minimizes the observed cellular and whole-animal knockdown phenotypes (Tyska et al., 2005), consistent with well-documented examples in lower eukaryotes (Jung et al., 1996; Novak et al., 1995; Ostap and Pollard, 1996).

*Figure 2.2 Myosin-I tissue expression and disease phenotypes. “Supplemental Table” refers to Table 2.1.*
II. Myosin-I regulates membrane tension, cell adhesion, and actin architecture

The presence of both a lipid-binding region in the tail and actin-binding region in the motor domain equips myosin-I motors for cellular roles that link membranes and cytoskeleton. Seminal work with lower eukaryotes demonstrated a crucial role for myosin-I in cortical tension (Dai et al., 1999). All vertebrate myosin-I isoforms, except Myo1h, have been studied with regard to cellular membrane tension due to their contribution to membrane-cytoskeleton adhesion. For instance, the individual over-expression of Myo1a, Myo1b, Myo1c, Myo1d, or Myo1e causes an increase in the force required to pull a tether from the plasma membrane of NIH 3T3 fibroblasts using an optical tweezers, whereas over-expression of a membrane-binding, dominant-negative construct of Myo1a...
decreases the tether force required (Nambiar et al., 2009). This method of pulling on the plasma membrane probes the tension of the membrane as established by lipid and protein composition, as well as links to the cortical actin network (Gauthier et al., 2012). These data are supported by whole-animal studies. For example, the deletion of Myo1a in knockout mice leads to membrane herniation in intestinal epithelial cells due to this decreased membrane-actin attachment (Tyska et al., 2005), and the loss of Myo1b leads to increased plasma membrane blebbing, reduced cell movement directionality and net speed in the developing mesoderm of zebrafish embryos (Diz-Muñoz et al., 2010).

Figure 2.4 Structural and kinetic features of myosin-I molecular motors.
Figure 2.5 Localizations and functions of metazoan myosin-I isoforms.
Myo1g, which is highly expressed in T cells, has also been shown to have a role in maintenance of membrane tension, which is important for T cell migration and enhanced interaction between T cell and dendritic cells during lymph node surveillance (Gérard et al., 2014). Additionally, by utilizing an atomic force microscope to investigate cortical and membrane tension, researchers found that loss of Myo1g in B-lymphocytes leads to decreased cell stiffness due to a loss of cortical tension, again affecting cell adhesion, spreading, phagocytosis, and endocytosis (López-Ortega et al., 2016). Surprisingly, another study found that depletion of Myo1f did not result in decreased cortical tension of neutrophils, as measured by micropipette aspiration, which is the first example of a myosin-I motor that is not involved in membrane and/or cortical tension (Kim et al., 2006).
It is interesting that Myo1e, the other long-tailed myosin-I isoform, is required for plasma membrane tension, whereas the closely related Myo1f isoform is not involved in cortical tension; cortical tension is often one of the main determinants of plasma membrane tension generation and maintenance (Gauthier et al., 2012). The structural features that give rise to these functional differences between myosin-I isoforms are still unclear.

Figure 2.7 Proposed myosin-I functions (II): Myosin-Id, -le, -If, -Ig, and -Ih.

Myo1c, Myo1d, and Myo1e have been found to contribute to the stability of cell-cell adhesion at adherens junctions, and Myo1e has been found at focal adhesions (Bi et al., 2013; Gupta et al., 2013; Hegan et al., 2015; Oh et al., 2013; Petzoldt et al., 2012; Spéder et al., 2006; Stöffler and Bähler, 1998; Stöffler et al., 1995; Tokuo and Coluccio, 2013). Myo1c localizes to E-cadherin-rich areas in cell-cell contacts of Madin-Darby
canine kidney epithelial (MDCK) cells and contributes to the stability of these junctions, although it is unclear how Myo1c functionally establishes and/or maintains this stability (Tokuo and Coluccio, 2013). The Drosophila homolog of Myo1d, Myo31DF, has been shown to localize to adherens junctions, where it binds to both β-catenin and DE-cadherin (Petzoldt et al., 2012, 31; Spéder et al., 2006). Here, Myo31DF functions with its antagonist Myo61F, the Drosophila Myo1c homologue to pattern left/right visceral asymmetry (Petzoldt et al., 2012, 31; Spéder et al., 2006). Interestingly, mouse Myo1c has been shown to power asymmetric actin filament gliding in an in vitro motility assay, which has been provocatively suggested to be related to this organ-patterning asymmetry (Pyrpassopoulos et al., 2012). In rats, Myo1d function at adherens junctions influences either the establishment and/or maintenance of rotational planar cell polarity in ciliated tracheal and ependymal epithelial cells, but not all tissues that exhibit planar cell polarity (Hegan et al., 2015). Myo1e localizes to sites of actin polymerization and adhesion in lamellipodia, thereby influencing adhesion formation and actin dynamics through localization of its binding partners (Gupta et al., 2013; Stöffler et al., 1995). Myo1e also localizes to and regulates slit junctions, the highly specialized cell-cell contacts found in the glomerulus of kidney podocyte cells, suggesting why its absence results in kidney disease (Bi et al., 2013; Mele et al., 2011). Overall, it is still unclear whether these myosin-I-mediated adhesion functions can be separated from the roles of the same isoforms in the generation and maintenance of membrane and/or cortical tension.

The expression of Myo1a, Myo1c, Myo1d, and Myo1e have been shown to impact actin filament architecture. Myo1a knockdown results in mice with intestinal microvilli of irregular length (Tyska et al., 2005). Myo1d localizes to tips of microvilli, however it is unclear whether Myo1d directly influences the length, composition, and/or integrity of actin
filaments in this location (Benesh et al., 2010). Myo1c more directly regulates actin architecture by influencing cytoskeletal rearrangement in the neuronal growth cone (Wang et al., 2003), in B cells at the immunological synapse (Maravillas-Montero et al., 2011), and possibly by facilitating G-actin transport to the leading edge of migrating epithelial cells (Fan et al., 2012). Myo1e has been found to be a core component of cancer invadosomes, actin-rich adhesion structures important for degradation and invasion of extracellular matrix; here, Myo1e recruitment to newly forming invadosomes precedes that of actin and paxillin (Ouderkirk and Krendel, 2014). Finally, Myo1e has been proposed to be involved in clathrin-mediated endocytosis by recruiting the actin-polymerization factors Neural Wiskott-Aldrich syndrome Protein (N-WASP), WASP Interacting Protein (WIP), and WASP-binding protein (WIRE), as well as other clathrin-mediated endocytic proteins, such as synaptojanin-1 and dynamin (Cheng et al., 2012; Krendel et al., 2007). Thus, the ability of myosin-I motors to influence actin dynamics may be both direct, through binding to the motor domain and transport of G-actin, and indirect, by recruitment of factors that are involved in nucleation, polymerization, and stabilization of actin filaments.

III. Myosin-I and intracellular trafficking

In addition to affecting membrane tension, cell adhesion, and actin dynamics, Myo1a, Myo1b, Myo1c, Myo1e, and Myo1g have been found to participate in exocytosis, endocytosis, intracellular membrane trafficking, and nuclear organization. Myo1a is present on the cytoplasmic side of Golgi-derived vesicles where it may be operating as a transporter near the Golgi or within microvilli (Fath and Burgess, 1993; Fath et al., 1994; Kravtsov et al., 2012; Skowron et al., 1998). Myo1e has been proposed to have a role in the regulated secretion of cortical granules in Xenopus oocytes (Schietroma et al., 2007). Myo1c has been found to play a role in the exocytosis of VEGF2 (Tiwari et al., 2013), IκB
kinase (IKK) (Nakamori et al., 2006), and Neph1 (Arif et al., 2011), and in the recycling of lipid raft cargo toward the plasma membrane (Brandstaetter et al., 2012). In what may prove to be an entirely distinct role, Myo1c splice isoforms have been found in the nucleus where they are proposed to interact with RNA polymerase-I and -II and either directly or indirectly participate in signaling to enhance long-range chromosomal movement (Chuang et al., 2006; Kyselá et al., 2005; Percipalle et al., 2006; Pestic-Dragovich et al., 2000; Philimonenko et al., 2004).

Myo1b localizes to multivesicular sorting bodies, endosomes, and lysosomes (Almeida et al., 2011; Cordonnier et al., 2001; Raposo et al., 1999; Salas-Cortes et al., 2005). Myo1b has also been found to be involved in the sorting of Pmel17, which is important for melanosome maturation (Salas-Cortes et al., 2005). Myo1c is important for compensatory endocytosis in Xenopus oocytes, where it is thought to couple force-generating actin filaments to the plasma membrane (Sokac et al., 2006). Recently, both Myo1b and Myo1c have been found to play a role in actin coat compression where they are recruited to lamellar bodies of rat Alveolar type II cells after membrane fusion (Kittelberger et al., 2016). Interestingly, knock-down of Myo1b increases actin contraction rates, while loss of Myo1c decreases the rate of contraction of the lamellar body actin coat, thus suggesting that while Myo1c actively contracts the actin coat to expel surfactant proteins, Myo1b may act to slow contraction, fine-tuning the kinetics of this process. In addition to the effects of Myo1e on actin dynamics, Myo1e knock-down results in decreased early endosomal transport of transferrin toward the perinuclear region (Cheng et al., 2012). Both Myo1e and Myo1g (Dart et al., 2012; Swanson et al., 1999), but not Myo1f (Kim et al., 2006), have been found to have a role in phagocytosis and phagosome closure, again alluding to very different functions for the two closely-related long-tailed
myosin-I isoforms Myo1e and 1f. Although myosin-I motors have been repeatedly implicated in trafficking processes, conclusive cellular evidence of myosin-I-driven transport along actin filaments, rather than acting to sort or deform membranes, is still needed.

IV. Myosin-I is a molecular dock/tether

Both Myo1b and Myo1c have been suggested to function as anchors or tethers between membranes and/or other proteins, as well as actin filament tracks. For example, Myo1c has been proposed to facilitate docking of GLUT4-containing vesicles at the plasma membrane prior to fusion in response to insulin stimulation (Boguslavsky et al., 2012; Bose et al., 2002; Bose et al., 2004; Chen et al., 2007; Huang et al., 2005; Yip et al., 2008). In vitro work suggests that Myo1c is in fact capable of halting processive, microtubule-based transport at actin intersections, which not only implicates Myo1c in cargo docking, but also connects microtubule- and actin-based transport pathways (McIntosh et al., 2015). Additionally, in vitro and cellular studies have shown that cargo docking by Myo1c is regulated by the presence of nonmuscle tropomyosin, which might spatially regulate the location of cargo docking to tropomyosin-free filaments just beneath the plasma membrane (Kee et al., 2015; McIntosh et al., 2015). Myo1c has also been implicated in the mechanical adaptation of signaling in inner ear hair cells (Batters et al., 2004; Gillespie et al., 1993; Holt et al., 2002; Lin et al., 2011), as well as in the regulation of Na+ channel after antidiuretic hormone stimulation in the kidney collection ducts (Wagner et al., 2005). Myo1b has been proposed to tether amino acid transporters to the apical plasma membrane of kidney cells, thereby facilitating neutral amino acid transport across the membrane; however, more investigation is needed to differentiate this hypothesis from other potential roles such as facilitating membrane fusion of vesicles.
containing amino acid transporters, and/or mediating the transport and/or sorting of these cargo to the apical plasma membrane (Komaba and Coluccio, 2015). Similarly, Myo1a is important for the retention and/or localization of sucrose isomaltase in the intestinal brush border membrane, although it is again unclear whether it is the trafficking, sorting, or docking functions that are most important (Tyska and Mooseker, 2004).

Myo1b was initially proposed to be a tension-sensitive motor by kinetic experiments (Coluccio and Geeves, 1999), followed by direct evidence that showed low mechanical forces (~1 pN) slow ATP-dependent actin-detachment kinetics by nearly two-orders-of magnitude (Laakso et al., 2008; Lin et al., 2005). This range of forces is well within those expected to take place during intracellular transport and mechanotransduction (Gillespie and Cyr, 2004; Hendricks et al., 2012; Soppina et al., 2009). Recent structural and biophysical studies have shown that the tension-sensitivity of Myo1b is due in part to a structural element located within the N-terminus of the motor (Greenberg et al., 2015; Shuman et al., 2014). In contrast, despite similar biochemical properties and working stroke sizes, the ATP-dependent actin-detachment kinetics of Myo1c are largely independent of forces < 2 pN. Cellular studies have yet to clarify how the remarkable tension-sensitivity of Myo1b translates into distinct functional roles from Myo1c; however, the discrete cellular functions and localizations of Myo1b and Myo1c, despite similar tissue expression patterns, suggest a physiological relevance for these mechanical load-dependent differences.

V. Myosin-I powers membrane deformation

Since myosin-I isoforms link membranes to the actin cytoskeleton, they are ideally poised to provide tension, deform the plasma membrane, and participate in tubulation of organelle membranes. Indeed, Myo1a is instrumental for vesicular shedding off the tip of
microvilli in the intestines, which is important for membrane turnover, microvillar health, and antimicrobial hydrolase release into the intestinal lumen (McConnell and Tyska, 2007; McConnell et al., 2009). Myo1b is associated with a wide range of plasma membrane geometries, including cell protrusions (Komaba and Coluccio, 2010), lamellipodia, membrane ruffles, filopodia, and the cleavage furrow of dividing cells (Lewis and Bridgman, 1996; Ruppert et al., 1995; Tang and Ostap, 2001). However, it is still unclear how exactly Myo1b affects these plasma membrane geometries. One known cellular function for Myo1b in filopodia is in ephrin receptor-B2 (EphB2) signaling for cell-cell repulsion of Hek293 cells, which is important for tissue patterning and homeostasis (Prospéri et al., 2015). Nevertheless, more work is still required to elucidate the molecular functions and relevance of these Myo1b localizations.

Beyond facilitating plasma membrane deformations, Myo1b has been shown to participate in the formation of post-Golgi carriers from the trans-Golgi in conjunction with processive, microtubule-based motors (Almeida et al., 2011). Additionally, a recent study demonstrated that Myo1b alone can tubulate giant unilamellar vesicles along fascin-bundled actin in an in vitro reconstitution assay (Yamada et al., 2014). Similarly, Myo1c has been found to participate in the tubulation and recycling of glycosylphosphatidylinositol-anchored lipid raft-rich membrane components toward the plasma membrane (Brandstaetter et al., 2012), thereby influencing cholesterol-dependent lysosome/autophagosome fusion (Brandstaetter et al., 2014). Myo1d is involved in the fusion of organelle membranes, in particular, the fusion of early endosomes from the apical or basolateral membrane with recycling endosomes (Huber et al., 2000). Finally, Myo1c has been found to promote ER sheet stabilization over reticular patterning, likely by coupling actin dynamics to membrane geometry (Joensuu et al., 2014). In all of these
intracellular membrane deformations, however, it is unclear whether myosin-I affects membrane deformation directly, by producing the force required to deform the membrane, or by providing the resistive anchoring that is necessary for other motors to power the tubulation event. Further investigation is therefore needed to clarify how myosin-I motors interact with processive actin and microtubule-based motors, the local cytoskeleton, and other factors involved in membrane deformation such as Bin, Amphiphysin, and Rvs (BAR) domain proteins to induce membrane deformation within the cell.

VI. Conclusions

The myosin-I family of molecular motors is comprised of eight different isoforms that participate in a wide range of cell biological processes that requiring generation or regulation of membrane tension, formation of cell adhesions and changes in the actin architecture. Additionally, myosin-I motors affect intracellular trafficking, function as tension-sensitive docks/tethers, and power membrane deformation. More work is needed to understand how these myosin-I motors are targeted to their site of action (based on tail domain and motor domain preferences) and function to accomplish these distinct and varied cellular tasks. In order to fully understand the underlying physiology, a continued interdisciplinary approach is required to integrate the cell biological, biochemical, biophysical, and structural features of myosin-I molecular motors.
<table>
<thead>
<tr>
<th>Isoform</th>
<th># of IQ in LCBD</th>
<th>Domains in Tail</th>
<th>Intracellular Localization and Proposed Functions</th>
<th>High Tissue Expression</th>
<th>Associated Diseases</th>
</tr>
</thead>
</table>
| Myosin-Ia* | 3 (Swanljung-Collins and Collins, 1991) | PH domain (Hokanson et al., 2006; Tyska and Mooséker, 2002) | - Binds PtdIns(4,5)P₂ and anionic phospholipids (Hayden et al., 1990); membrane binding supplemented by IQ-region binding (Swanljung-Collins and Collins, 1994)  
- Motility (gliding filament assay) (Collins et al., 1990) and ATPase (Fanning et al., 1994) inhibited by nonmuscle tropomyosin-coated actin filaments  
- Localizes to Golgi-derived vesicles (Fath and Burgess, 1993; Fath et al., 1994)  
- Localizes to intestinal brush border, where it is essential for microvillar structure and organization; involved in membrane tension and apical membrane vesicle shedding from microvillus tip (McConnell and Tyska, 2007; Tyska et al., 2005) | Small intestines, colon (Skowron et al., 1998) | Non-syndromic and autosomal dominant hearing loss (Donaudy et al., 2003) |
| Myosin-Ib | 4-6* (Lin et al., 2005; Ruppe rt et al., 1993; Sherr et al., 1993) | PH domain (Hokanson et al., 2006) | - Binds PtdIns(4,5)P₂, PtdIns(3,4,5)P₃, and anionic phospholipids (Komaba and Coluccio, 2010)  
- Not found on nonmuscle tropomyosin actin filaments (Tang and Ostap, 2001)  
- Localizes to Arp2/3-nucleated actin filaments (Almeida et al., 2011)  
- Localizes to endosomes and lysosomes (Cordonnier et al., 2001; Raposo et al., 1999)  
- Influences transport from trans-Golgi network to endosomes; involved in tubulation (Almeida et al., 2011)  
- Found in cytosol, on the plasma membrane and endoplasmic reticulum-like microsomes, Golgi (Balish et al., 1999)  
- Found on lamellipodia, membrane ruffles, and filopodia (Komaba and Coluccio, 2010; Lewis and Bridgman, 1996; Prospéri et al., 2015; Ruppert et al., 1995; Tang and Ostap, 2001)  
- Localizes to multi-vesicular bodies (Salas-Cortes et al., 2005)  
- Localizes to kidney cell brush border apical membrane and stabilizes amino acid transporters (Komaba and Coluccio, 2015)  
- Involved in Pmel17 sorting into melanosomes (Salas-Cortes et al., 2005)  
- Effector of EphB2 signaling to control cell repulsion (Prospéri et al., 2015)  
- Localizes to lamellar bodies of Alveolar type II cells: involved in tethering lamellar body and/or slowing actin coat compression during exocytosis of surfactants (Kittelberger et al., 2016) | Lung, liver, heart, brain, and most everywhere else (Ruppert et al., 1993; Sherr et al., 1993, 19) | Lymph node metastasis of human head and neck squamous cell carcinoma (Ohmura et al., 2015) |
<p>| Myosin-Ic* | 3 (Manc eva et al., 2007; Sielski) | PH domain (Hokanson et al., 2006) | - Binds PtdIns(4,5)P₂, PtdIns(3,4,5)P₃, and anionic phospholipids (McKenna and Ostap, 2009) | Kidney, muscle, adipose, small intestine (Skowron et al., 2009) | Kidney disease (Arif et al., 2011; Wagner et al., 2005) |</p>
<table>
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<th>Domain Structure</th>
<th>Functions</th>
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<td>Pre-power-stroke motor domain structure (PDB:4BYF (Münnich et al., 2014))</td>
<td>Not found on nonmuscle tropomyosin actin filaments (Kee et al., 2015; McIntosh et al., 2015) Functions in GLUT4 cargo docking and transport beneath the plasma membrane (Boguslavsky et al., 2012; Bose et al., 2002; Bose et al., 2004; Chen et al., 2007; Huang et al., 2005; Toyoda et al., 2011; Yip et al., 2008) Involved in lipid raft recycling tubule deformation and transport (Brandstaetter et al., 2012), and found in lipid rafts in the plasma membrane; this also affects cell spreading, migration, and Salmonella invasion (Maravillas-Montero et al., 2011); cholesterol trafficking affects lysosome-autophagosome fusion (Brandstaetter et al., 2014) Localizes to podocyte membrane where it is involved in transport of Nep1 to podocyte membrane (Arlf et al., 2011); Involved in regulation of Na⁺ channel after ADH stimulation of collecting ducts (Wagner et al., 2005) Localizes to plasma membrane: mediates cell adhesion and spreading, stabilizes E-cadherin adherens junctions (Balish et al., 1999; Fan et al., 2012; Oh et al., 2013; Sokac and Bement, 2000; Tokuo and Coluccio, 2013) Localizes to lamellar bodies of Alveolar type II cells: involved in actin coat compression during exocytosis of surfactants (Kittelberger et al., 2016) Facilitates G-actin transport to the leading edge of migrating epithelial cells by binding G-actin and possibly facilitating plasma membrane ruffling (Fan et al., 2012) Involved in IKK transport toward plasma membrane, mediating TNF-α-induced downregulation of IRS-1 and glucose uptake (Nakamori et al., 2006) Involved in VEGF2 delivery to plasma membrane (Tiwari et al., 2013) Promotes ER sheet stabilization over reticular formation (Joensuu et al., 2014) Involved in B-cell cytoskeletal rearrangements at the immunological synapse; contributes to antigen presentation (Maravillas-Montero et al., 2011) Involved in neuronal growth cone cytoskeletal rearrangement, membrane protrusion, and compensatory endocytosis (Sokac et al., 2006; Wang et al., 2003) Involved in inner ear mechanical adaptation (Batters et al., 2004; Gillespie et al., 1993; Holt et al., 2002) Participates in establishment of left-right asymmetry during Drosophila development (Hozumi et al., 2006; Okumura et al., 2015; Petzoldt et al., 2012; Spéder et al., 2006) One splice isoform localizes to the nucleus: interacts with RNA polymerase I and II, may be involved in transcription and in helping direct long-range chromosomal movement</td>
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<td>LCBD and TH2 structure (PDB:4R8G (Lu et al., 2015))</td>
<td>and deafness (Batters et al., 2004; Holt et al., 2002)</td>
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<td>Myosin-Id*</td>
<td>PH domain (Hokanson et al., 2006)</td>
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<td>2 (Bähler et al., 1994; Köhler et al., 2005)</td>
<td>Both TH1 and IQ regions are needed for appropriate cellular targeting (Benesh et al., 2010)</td>
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<td>Localizes to intestinal brush border microvilli tips and basolateral membrane (Benesh et al., 2010)</td>
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<td>Involved in fusion of early endosomes (from apical or basolateral membrane) with recycling endosomes (Huber et al., 2000)</td>
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<td>Involved in plasma membrane tension (Namibiar et al., 2009)</td>
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<td>Myo31DF participates in establishment of left-right asymmetry during Drosophila development through interactions with β-catenin and DE-cadherin at adherens junctions; antagonized by Myo61F (Hozumi et al., 2006; Okumura et al., 2015; Petzoldt et al., 2012; Spéder et al., 2006)</td>
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<td>Involved in planar cell polarity of ciliated tracheal epithelial cells (Hegan et al., 2015)</td>
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<tr>
<td>Myosin-le</td>
<td>PH domain, TH2, and SH3 domain (Berg et al., 2001; Hokanson et al., 2006; Stöffler and Bähler, 1998)</td>
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<td>1 (Stöffler and Bähler, 1998)</td>
<td>Localizes to the plasma membrane</td>
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<td></td>
<td>Found at cell-cell contacts and adherens junctions (Bi et al., 2013; Stöffler et al., 1995; Stöffler et al., 1998, 3)</td>
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<td>Involved in regulated secretion (Schietroma et al., 2007), clathrin-mediated endocytosis (Cheng et al., 2012; Feesper et al., 2010; Krendel et al., 2007), and phagosome closure (Diakonova et al., 2002; Swanson et al., 1999),</td>
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<td>Involved in lamellipodial dynamics and adhesion formation (Gupta et al., 2013)</td>
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<td>Contributes to regulation of invadosome structure and dynamics (Ouderkirk and Krendel, 2014)</td>
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<td>Regulates TLR4-triggered macrophage spreading and antigen presentation (Wenzel et al., 2015)</td>
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<td>Protein</td>
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<td>Myosin-If</td>
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<td>Myosin-Ig</td>
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<td>Myosin-Ih</td>
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This chapter is adapted from


We thank Tianming Lin and Mariko Tokito for excellent technical assistance and Michael Greenberg and Serapion Pyrpassopoulos for help developing the experimental protocols. We also thank Michael Woody, Abbey Weith, Henry Shuman, and other members of the E.M.O. and E.L.F.H. labs as well as the Pennsylvania Muscle Institute for helpful suggestions and stimulating conversations. Finally, we thank Sarah Hitchcock-DeGregori for the cDNA encoding rat nonmuscle Tm2. This work was supported by an American Heart Association Predoctoral Fellowship 12PRE12030164 to B.B.M. and by NIH grants T32 AR053461 to B.B.M. and P01 GM087253 to E.L.F.H. and E.M.O.
I. Summary

Intracellular transport is largely driven by processive microtubule- and actin-based molecular motors. Non-processive motors have also been localized to trafficking cargos, but their roles are not well understood (Almeida et al., 2011; Boguslavsky et al., 2012; Bose et al., 2004; Brandstaetter et al., 2012; Santos-Argumedo et al., 2013; Tiwari et al., 2013; Yip et al., 2008). Myosin-Ic (Myo1c), a non-processive actin motor, functions in a variety of exocytic events, although the underlying mechanisms are not yet clear. To investigate the interplay between myosin-I and the canonical long distance transport motor kinesin-1, we attached both motor types to lipid membrane-coated bead (MCB) cargo, using an attachment strategy that allows motors to actively reorganize within the membrane in response to the local cytoskeletal environment. We compared the motility of kinesin-1-driven cargos in the absence and presence of Myo1c at engineered actin/microtubule intersections. We found that Myo1c significantly increases the frequency of kinesin-1-driven microtubule-based runs that begin at actin/microtubule intersections. Myo1c also regulates the termination of processive runs. Beads with both motors bound have a significantly higher probability of pausing at actin/microtubule intersections, remaining tethered for an average of 20 s, with some pauses lasting longer than 200 s. The actin-binding protein nonmuscle tropomyosin (Tm) provides spatially-specific regulation of interactions between myosin motors and actin filaments in vivo (Clayton et al., 2014; Fanning et al., 1994; Gunning et al., 2005; Ostap, 2008; Tang and Ostap, 2001); in the crossed-filament in vitro assay, we found that Tm2-actin abolishes Myo1c-specific effects on both run initiation and run termination. Together these observations suggest Myo1c is important for the selective initiation and termination of kinesin-driven runs along microtubules at specific actin filament populations within the cell (Figure 3.1).
Figure 3.1 Graphical abstract of publication.
II. Introduction

Membrane-bound cargos are transported throughout the cell by molecular motors that move along microtubules and actin filaments. This transport is essential for normal cellular function, as mutations in either the motors or their adaptors contribute to diseases including neurodegeneration (Millecamps and Julien, 2013) and sensory and metabolic disorders (Batters et al., 2004; Mele et al., 2011). Organelles and vesicles undergoing active transport in the cell typically bind multiple types of microtubule- and actin filament-specific motors (Holzbaur and Goldman, 2010). Most research in the field has focused on characterizing the cargo-associated motors that drive processive movement along cytoskeletal filaments (Ali et al., 2011; Holzbaur and Goldman, 2010). Non-processive motors, i.e. motors that take only a single step before detaching from their cytoskeletal track, also contribute to intracellular transport, yet their contributions to cargo dynamics during trafficking are not yet well defined (Almeida et al., 2011; Boguszewsky et al., 2012; Bose et al., 2004; Brandstaetter et al., 2012; Greenberg and Ostap, 2013; McConnell and Tyska, 2010; Santos-Argumedo et al., 2013; Tiwari et al., 2013, 2).

Myosin-I proteins are single-headed, non-processive molecular motors that facilitate a variety of dynamic actin-membrane interactions (Almeida et al., 2011; Bose et al., 2004; Brandstaetter et al., 2012; Greenberg and Ostap, 2013; Gupta et al., 2013; McConnell and Tyska, 2010). The widely expressed isoform, myosin-Ic (Myo1c), participates in exocytic trafficking (Brandstaetter et al., 2012), recycling of lipid raft cargos (Brandstaetter et al., 2012), and the final stages of GLUT4 transport beneath the plasma membrane (Boguszewsky et al., 2012; Bose et al., 2004; Yip et al., 2008), consistent with a possible role in cargo sorting to specific destinations (Boguszewsky et al., 2012; Brandstaetter et al., 2012). Throughout these transport events, Myo1c associates with
cargos that bind a range of processive microtubule and actin motors, including kinesin-1 and myosin-V (Boguslavsky et al., 2012; Chen et al., 2012; Ishikura and Klip, 2008; Semiz et al., 2003; Yoshizaki et al., 2007). It has been suggested that Myo1c acts as either a slow actin filament transporter (Brandstaetter et al., 2012; Tiwari et al., 2013) or a molecular tether (Almeida et al., 2011; Clayton et al., 2014; Tiwari et al., 2013) during these processes (Batters et al., 2004; Boguslavsky et al., 2012; Bose et al., 2004). Here, we use in vitro reconstitution assays to identify specific roles for Myo1c in both the initiation and the termination of long-distance kinesin-1-driven runs.

We examined the transport of synthetic membrane-bound cargos by directly observing fluorescently-tagged kinesin-1 in the presence and absence of Myo1c using engineered cytoskeletal intersections, in which coverslip-attached microtubules intersect with actin filament overpasses. We utilized a truncated, biotinylated, two-headed kinesin-1 construct and a biotinylated Myo1c construct truncated after the lever-arm domain (hereafter referred to as “kinesin-1” and “Myo1c” respectively, see Chapter 5). Motors were specifically attached to synthetic cargos via a NeutrAvidin intermediate to biotinylated lipids incorporated into a DOPC lipid bilayer, surrounding 1 μm silica beads (Pyrpapassopoulos et al., 2013). Biotin-mediated attachment to lipid membrane-coated beads (MCBs) permits control of the number of motors bound to the cargo by altering the mole-percent of biotin-PtdEth in the DOPC membrane (see Chapter 5 and Figure 3.2), and allows the diffusion of motors around the cargo in response to local changes in cytoskeletal filament geometry during transport.
The fluorescence intensity of kinesin-1-GFP increases linearly on 0.2% biotin-PtdEth membrane-coated beads (MCBs) through 250 nM kinesin-1-GFP. The fluorescence intensity of MCBs mixed increases linearly with increasing concentrations of kinesin-1-GFP; this relationship was used to estimate the maximum concentration of motors that can bind to 0.2% biotin-PtdEth MCBs. Error bars represent standard deviation over 100 beads from each of 3 chambers.

III. Results

In flow chambers containing actin filaments (AF) and microtubules (MT), MCBs bound to both kinesin-1 and Myo1c preferentially initiate processive runs on microtubules at AF/MT intersections (Figures 3.3B and 3.3C, Movie S1). In contrast, MCBs bound only to kinesin-1 show no preference for run initiation at intersections. Rather, kinesin-1-only MCBs stochastically initiate runs along the length of the microtubule (Figures 3.3A and 3.3C, Movie S1). When Myo1c is present, the distribution of landing distances from the nearest actin intersection is significantly different from randomly generated points in the same fields of view, whereas kinesin-only landing distances mimic the random points (Figure 3.3D). These observations suggest that Myo1c facilitates the initiation of a microtubule-based run by recruiting cargo preferentially to cytoskeletal intersections.
Figure 3.3 MCBs containing kinesin-1-only or kinesin-1 and Myo1c were observed as they initiated kinesin-driven motility along microtubules.

Events were scored by the distance between the location of initiated processive motility and the nearest actin filament intersection.

(A) A sample interaction showing that kinesin-1-only cargo stochastically initiate microtubule-based runs with respect to the nearest actin intersection. In this time series, microtubules are pseudo-colored green, while actin filaments are pseudo-colored purple. The MCB was monitored as it approached a microtubule immobilized on the surface, with the initiation event denoted as the location at which processive motility begins on the kymograph (labeled “0 s” in the time series). The blue arrowhead next to the kymograph indicates the actin filament intersection. Data acquired at 10 frames per second (fps). Scale bars represent 1 μm and 3 s. See Movie S1.

(B) Myo1c increases the frequency that kinesin-1-driven runs initiate at AF/MT intersections. This sample interaction shows a run beginning at an AF/MT intersection, “0 sec,” in which the center of the cargo initiates movement along the microtubule < 0.5 μm from the center of the intersection, in a time series and corresponding kymograph. The blue arrowhead next to the kymograph indicates the actin filament intersection. Scale bars represent 1 μm, 3 s. See Movie S1.

(C) Myo1c induces a significant increase in run initiation events at AF/MT intersections. Significantly more cargo initiation events are observed at AF/MT intersections when Myo1c is present on kinesin-1-driven cargo. Events are designated as beginning at an AF/MT intersection if the centroid of the MCB is ≤ 0.5 μm from the center of the intersection at the time processive motility along the microtubule is initiated by kinesin-1. Kinesin-1-only: n = 33 events from 2 chambers. Kinesin-1+Myo1c: n = 36 events from 2 chambers. Acquired at 10 fps. *** p ≤ 0.001 Kruskal-Wallis with Dunn’s multiple comparisons test.

(D) Cumulative frequency distribution showing the increased number of run initiation events occurring in proximity to the nearest AF intersection when Myo1c is bound to the MCB. While run initiation distances for kinesin-only cargo have the same distribution as randomly chosen points in the same fields of view, kinesin-1 and Myo1c cargo preferentially initiate runs at AF/MT intersections. *** p ≤ 0.001, ** p ≤ 0.01 Kruskal-Wallis with Dunn’s multiple comparisons test.
Next, we investigated the influence of Myo1c on kinesin-mediated processive motility at actin filament intersections encountered during motility along a microtubule. Kinesin-1-only MCBs tend to pass actin intersections, with only 33% of cargo pausing for greater than 0.5 s (Figure 3.4A and 3.4C, Movie S2). In contrast, 92% of MCBs bound to kinesin-1 and Myo1c paused at actin filament intersections (Figure 3.4B and 3.4C, Movie S2). The mean pause length was 20 s, with some pauses lasting longer than 200 s (Figure 3.4B). Kinesin cargo with or without Myo1c present tended to detach (without passing or pausing) at the actin intersection with similar low frequencies, 5% and 4% of the time, respectively (Figure 3.4C).
Figure 3.4 Myo1c halts kinesin-1-driven MCBs at engineered AF/MT intersections.

We observed the behavior of MCBs traveling along microtubules via kinesin-1-driven transport as they encountered actin filament intersections.

(A) MCBs with only kinesin-1-bound predominantly pass AF/MT intersections. Time series and kymograph showing a kinesin-only cargo passing an actin filament intersection. The blue arrowhead next to the kymograph denotes the actin intersection in the kymograph. Scale bars = 1 μm and 3 s; acquired at 2 fps. See Movie S2.

(B) MCBs with kinesin-1 and Myo1c primarily pause at actin filament intersections. Time series and kymographs depicting two example pauses. The top event illustrates a pause of average length, 20 s, while the bottom event shows a pause of >200 s. Note the change in time scale between frames in both time series. The blue arrowhead next to the kymograph denotes the AF intersection. Scale bars = 1 μm and 3 s. See Movie S2.

(C) Significantly more cargo pause at actin intersections when Myo1c is present (92% vs. 33%). A halt in motility along the microtubule at an actin intersection for > 0.5 s is denoted as a “pause” event. In contrast, kinesin-1-only MCBs tend to pass actin intersections (63% of the time). Both kinesin-only and kinesin and Myo1c cargo detach at actin intersections (without a pause or pass) at approximately the same frequency (~4%). Kinesin-1-only: n = 92 observed events from 7 chambers. Kinesin-1+Myo1c: n = 61 observed events from 5 chambers. Error bars: bootstrapped SD. **** p ≤ 0.0001 Kruskal-Wallis.
After a pause, MCBs continued motility along a microtubule an average of 73% of the time, or released from the intersection and diffused away an average of 27% of the time. This was not significantly altered by the presence of Myo1c on the MCB (Figure 3.5). Cumulatively, these *in vitro* data support the hypothesis that Myo1c can dock intracellular cargo at actin filament intersections within the cell. Following docking, MCB transport along actin filaments was not observed in our experiments. Myo1c has a 50-fold slower motility rate than kinesin-1 (Pyrpassopoulos et al., 2012; Walter et al., 2012), and is non-processive unless numerous motors are present (Greenberg and Ostap, 2013; Greenberg et al., 2012; Pyrpassopoulos et al., 2012), making it unlikely that we would observe substantial motility along, or recruitment to, actin filaments using these experimental parameters (see Discussion).

*Figure 3.5 MCB behavior after a pause.*

MCB behavior after a pause is not significantly different between kinesin-only and kinesin-1+Myo1c MCBs. Following a pause at an AF/MT or Tm2-AF/MT intersection, MCBs were scored for either detachment or continued motility along the microtubule. No significant differences were observed between cargos with or without Myo1c according to a Kruskal-Wallis significance test with Dunn's multiple comparisons. Error bars are bootstrapped SD.
To determine whether the observed cargo docking at actin intersections is specific to Myo1c, we tested the ability of the non-motor actin filament-binding protein, α-actinin, to stall cargo at AF/MT intersections. We added 200 nM of a biotinylated actin-binding domain construct of α-actinin (hereafter referred to as “α-actinin”) to kinesin-1-coated MCBs, the same concentration as was used for Myo1c. We found that in the presence of α-actinin, MCBs were sequestered to actin filaments, resulting in approximately 80% fewer microtubule-based runs (Figure 3.6A). Within 5 min of addition to the flow cell, 75% of kinesin-1 and α-actinin-coated cargo were stably bound to actin filaments (Figure 3.6B). In comparison, only 18% of kinesin-only, and 29% of kinesin and Myo1c-containing MCBs were bound to actin. Of the few kinesin-1 and α-actinin-containing MCBs that resulted in kinesin-driven motility, 63% of MCBs passed the actin intersection (Figure 3.6C), demonstrating that α-actinin is not able to dynamically tether MCBs to AF/MT intersections during processive kinesin-1-driven runs, unlike the observed results with Myo1c.
Figure 3.6 MCB behavior when coated with kinesin-1 + α-actinin.

(A) MCBs coated with kinesin-1 and α-actinin quickly become bound to actin on the surface, causing a significant decrease in the average number of observed events at AF/MT intersections per chamber. Kinesin-1-only n = 92 events over 7 chambers, kinesin-1+Myo1c n = 64 events over 5 chambers, kinesin-1+α-actinin n = 23 events from 10 chambers.

(B) MCBs with kinesin-1 and α-actinin bound become stably attached to actin filaments. Kinesin-1-only MCBs preferentially bind to microtubules and/or AF/MT intersections, while kinesin-1 and Myo1c-containing cargo bind to actin, microtubules, and AF/MT intersections. Kinesin-1-only n = 54 events, kinesin-1+Myo1c n = 48 events, kinesin-1+α-actinin n = 67 events from > 8 movies from > 3 chambers per condition.

(C) MCBs coated with kinesin-1 and α-actinin tend to pass AF/MT intersections at a similar frequency as kinesin-1-only MCBs, rather than pause at AF/MT intersections like kinesin-1+Myo1c-coated cargo. Thus, the ability of Myo1c to halt kinesin-1-driven transport cannot be rescued by a different actin binding protein. Kinesin-1-only n = 92 events over 7 chambers, kinesin-1+Myo1c n = 64 events over 5 chambers, kinesin-1+α-actinin n = 23 events from 10 chambers.
Nonmuscle tropomyosins have been reported to activate (Chacko, 1981; Clayton et al., 2014; Umemoto et al., 1989), inhibit (Huckaba et al., 2006; Tang and Ostap, 2001), or not affect myosin motility and ATPase activities when bound to actin filaments in an isoform-specific manner (Collins and Matsudaira, 1991; Fanning et al., 1994; Ostap, 2008). We focused on nonmuscle tropomyosin-2 (Tm2, also known as Tpm1.6) because this isoform is generally found throughout the cytoplasm, but is specifically excluded from the leading edge of the cell and other areas of highly dynamic actin filament populations (Gunning et al., 2005; Lin et al., 1988; Percival et al., 2000). To test the effects of full-length Tm2 on Myo1c motility, we examined Tm2-actin gliding over a bed of Myo1c. In the absence of tropomyosin, Myo1c robustly powers actin filament motility with a velocity of 16 nm/s at room temperature (Figures 3.7A and 3.7B, Movie S3). In contrast, tropomyosin-bound actin filaments were not directionally driven, and only transiently interacted with the surface of the coverslip (Figures 3.7A and 3.7B, Movie S3). Even these transient interactions disappeared when methylcellulose, used to concentrate actin at the surface of the coverslip, was omitted from the assay buffer. Meanwhile, non-tropomyosin actin filaments were still capable of directionally gliding. Taken together, these results show that Tm2 inhibits force-generating Myo1c-actin filament interactions.
Figure 3.7 Nonmuscle Tm2 regulates Myo1c interactions at Tm2-AF/MT intersections.

(A) Actin filament gliding assays were performed in the presence or absence of Tm2. Representative images of the first frame, last frame, and maximum intensity projection from 5 min gliding assays in the presence and absence of Tm2, at 50 nM Myo1c. In the absence of Tm2, Myo1c powers continuous actin gliding. Tm2 disrupts gliding and only non-directional movement is observed. Magenta and cyan stars label two example actin filaments in each condition. Acquired at 0.5 fps. Scale bar = 1 μm. See Movie S3.

(B) Tm2 inhibits Myo1c actin filament gliding over a range of Myo1c concentrations (50 – 500 nM Myo1c). Actin filaments without Tm2 glide in directional tracks, whereas Tm2-actin interact transiently and non-directionally with Myo1c on the surface. n > 150 filaments per condition.

To determine if Tm2 can modulate the Myo1c-mediated effects of MCB behavior at AF/MT intersections, we added Tm2-coated actin to our in vitro crossed-filament assay. Strikingly, Tm2-actin inhibited Myo1c-specific cargo run initiation at Tm2-AF/MT intersections (Figure 3.8A). In contrast to our observations in the absence of Tm2, MCBs transported by kinesin-1 in the presence and absence of Myo1c initiate microtubule-based runs stochastically with respect to the nearest Tm2-actin intersection (Figure 3.8C). We also found that kinesin-1 MCBs with or without Myo1c are no more likely to land at MT/MT intersections than randomly chosen points in the same fields of view (Figure 3.8B). If run initiation at AF/MT intersections was solely due to increased motor-cytoskeleton binding sites at a particular point, we would expect to see a similar enhancement in initiation at MT/MT intersections. Thus, Myo1c recruitment of cargo to AF/MT intersections is a specific property of Myo1c coordinating with kinesin-1 to initiate microtubule-based cargo runs, and is regulated by the presence of Tm2.
Figure 3.8 Nonmuscle Tm2 inhibits Myo1c-derived kinesin-1-driven run initiation at Tm2-AF/MT intersections.

(A) Tm2-actin abolishes Myo1c-based cargo run initiation at Tm2-AF/MT intersections. Initiation of processive microtubule-based kinesin-1 motility was scored with respect to the nearest Tm2-actin intersection. Kinesin+Myo1c MCBs do not preferentially initiate runs at actin intersections when Tm2 is present. Acquired 10 fps; kinesin-1+Myo1c n = 37, kinesin-1-only n = 33, events from 27 fields-of-view from 2 chambers, each; Scale bar = 1μm. Error bars: bootstrapped SD. **** p ≤ 0.0001, *** p ≤ 0.001, ** p ≤ 0.01 Kruskal-Wallis with Dunn’s multiple comparison’s test.

(B) Run initiation is not enhanced at MT/MT intersections. Initiation of processive microtubule-based kinesin-1 motility was scored with respect to the nearest MT/MT intersection. Both kinesin-1-only and kinesin-1+Myo1c MCBs initiate runs at MT/MT intersections with the same frequency as randomly generated points in the same fields of view. Data acquired at 10 fps from Tm2-actin movies. Kinesin-1+Myo1c n = 41, kinesin-1-only n = 13 events from 2 chambers, each. Error bars: bootstrapped SD. Initiation event distances from MT/MT intersections between groups are not significantly different based on Kruskal-Wallis with Dunn’s multiple comparisons test.

(C) MCBs do not preferentially initiate runs at Tm2-AF/MT intersections. MCBs transported by kinesin-1 with or without Myo1c were quantitated for the distance between initiation of processive MT motility and the nearest Tm2-AF/MT intersection and scored as either landing at ( < 0.5 μm) or away from ( > 0.5 μm) the nearest Tm2-AF intersection. Neither kinesin-1-only, nor kinesin-1+Myo1c MCBs initiate runs at Tm2-AF/MT intersections more frequently than randomly generated points in the same fields of view. Thus, both kinesin and kinesin+Myo1c cargo stochastically initiate microtubule-based runs with respect to the nearest Tm2-AF/MT intersection. Data acquired at 10fps. Kinesin-1+Myo1c n = 41, kinesin-1-only n = 13 events from 2 chambers, each. Error bars: bootstrapped SD. Not significant (n.s.) via Kruskal-Wallis and Dunn’s multiple comparisons statistical tests.
Finally, we found that Tm2-actin robustly prevents Myo1c-induced pausing of MCBs at Tm2-AF/MT intersections (Figures 3.9A-C, Movie S4). In the absence of Tm2, 33% of kinesin-only cargo and 92% of kinesin and Myo1c MCBs pause at AF/MT intersections (Figures 3.4C, 3.9A). When Tm2-actin is present, rather than pausing at actin intersections, kinesin-1 cargo with Myo1c-bound pass Tm2-actin intersections with frequencies comparable to those observed for kinesin-1-only cargo (66%) (Figure 3.9A).
Figure 3.9 Nonmuscle Tm2 inhibits Myo1c-derived kinesin-1-driven run termination at Tm2-AF/MT intersections.

(A) Tm2-actin prevents Myo1c-based cargo pausing at AF/MT intersections. MCB behavior was observed at AF/MT or Tm2-AF/MT intersections and scored as: “detach,” meaning the cargo detached at the intersection without a pause > 0.5 s; “pass,” where the MCB passed the intersection without a pause > 0.5 s; or “pause” where MCBs paused for > 0.5 s (limited by 2 fps acquisition rate). The behavior of kinesin-1-only (red) and kinesin-1+Myo1c (blue) MCBs at AF/MT intersections was previously described in Figure 2. Bars with grey stripes illustrate bead behavior at Tm2-AF/MT intersections. Significantly fewer kinesin-1+Myo1c MCBs pause at Tm2-AF/MT than AF/MT intersections, replicating kinesin-1-only cargo behavior. Kinesin-1, n = 92; kinesin-1+Myo1c, n = 64; kinesin-1 (+ Tm2-actin), n = 37; kinesin-1 +Myo1c (+ Tm2-actin), n = 61 observed events in 5 chambers. Error bars: bootstrapped SD. **** p ≤ 0.0001 Kruskal-Wallis with Dunn’s multiple comparison’s test.

(B) Representative time series and kymograph showing that kinesin-1-only MCBs pass through actin intersections when Tm2 is present. Microtubules are pseudo colored green and Tm2-actin is pseudo colored pink; the blue arrowhead denotes the Tm2-actin intersection. Scale bars = 1 μm and 3 s; acquired at 2 fps. See Movie S4.

(C) Representative time series and kymograph showing that cargo with kinesin-1+Myo1c pass Tm2-actin intersections. When Tm2 is bound to actin filaments, kinesin-1+Myo1c cargo do not pause at AF/MT intersections. Scale bars = 1 μm and 3 s; acquired at 2 fps. See Movie S4.
IV. Discussion

Intracellular transport is largely driven by processive motors, such as kinesin-1, which are capable of transporting cargos over long distances within the cell. Previous studies have shown that when different types of processive motors compete at cytoskeletal intersections, both the number of motors bound and the biophysical properties of these motors and their cargo adaptors determine which motor type will dominate (Ali et al., 2011; Schroeder III et al., 2010; Schroeder III et al., 2012). Here, we use a similar strategy to investigate the role of non-processive motors in intracellular trafficking. Our observations suggest that non-processive motors such as Myo1c may be key in regulating the specificity of intracellular targeting of vesicular and organelle cargos.

Myosin-I motors participate in a variety of membrane-actin interactions, including dynamic membrane transformations (Almeida et al., 2011; Bose et al., 2002; Greenberg and Ostap, 2013; Gupta et al., 2013; McConnell and Tyska, 2010; Yip et al., 2008). For example, Myo1c has been hypothesized to participate in the recycling of lipid raft cargo toward the plasma membrane from peri-nuclear recycling tubes (Brandstaetter et al., 2012), and the last steps of GLUT4 trafficking toward the plasma membrane (Boguslavsky et al., 2012; Bose et al., 2002; Bose et al., 2004; Yip et al., 2008). The localization of Myo1c to these specific cargos suggests that myosin-I motors could be used to actively sort and target cargo to particular destinations within the cell.

Here we used an in vitro reconstitution assay to demonstrate that the non-processive motor Myo1c plays important roles at both the beginning and end of kinesin-driven long distance transport (Figure 3.10). Myo1c facilitates cargo run initiation by selectively recruiting cargo to cytoskeletal intersections, where kinesin-1 can rapidly begin to transport cargo along a microtubule (Figures 3.3 and 3.10). Strikingly, the Myo1c-
specific effects on cargo initiation and termination events are not reproduced by a different actin-binding protein, α-actinin, suggesting that the active motor activity of Myo1c, not just its actin binding capacity, is necessary for successful cargo tethering (Figure 3.6). Myo1c-specific cargo run initiation and termination is also abolished by the binding of Tm2 to actin filaments (Figures 3.8-3.10). This provides a mechanism to selectively regulate Myo1c activity, and thus cargo behavior in the cell, in a spatially controlled manner.

We note that while Myo1c motors induce the preferential binding of membrane-bound cargos at AF/MT intersections, these cargos do not become stably tethered at these junctions. This is likely due to the robust motor activity of kinesin-1 motors, as cargo that initiate runs at AF/MT intersections typically clear the intersection within 500 ms. In contrast, when cargo moving along a microtubule encounter an actin filament, Myo1c motors have over a second to bind to the actin intersection and form a more stable interaction. Further, kinesin-1 motors are not adept at navigating roadblocks (Figure 3.4) (Dixit et al., 2008), so the physical obstruction of the actin intersection likely helps facilitate Myo1c-domination of the processive/non-processive motor interaction during run termination.
Figure 3.10 Myo1c affects both the initiation and termination of kinesin-1-driven runs.

Myo1c facilitates kinesin-1 run initiation at AF/MT, but not Tm2-AF/MT intersections, and specifically delivers cargo to non-Tm2-AF intersections to terminate long-distance MT-based transport.

We know that multiple kinesins are driving the motility of the MCBs along microtubules, since the run lengths observed under our assay conditions (> 5 µm) exceed those of single kinesin-1 motors (~1 µm; (Walter et al., 2012)). Myo1c is a non-processive motor that interacts only transiently with actin filaments, so the engagement of many Myo1c motors are likely to be necessary to stall a cargo at an AF/MT intersection over tens to hundreds of seconds (Greenberg and Ostap, 2013; Greenberg et al., 2012). However, constraints dictated by the bead, motors, and cytoskeleton geometry limit the number of motors available to bind at the AF/MT intersection, so we predict that no more than 6 Myo1c and 11 kinesin-1 motors are capable of interacting at any given time (Figure 3.11). We estimate that only a few Myo1c motors are necessary to effectively stall kinesin-1 motility at AF/MT intersections.
Figure 3.11 There are an estimated 11 kinesin-1 and 6 Myo1c motors available to interact with the microtubule and actin filament, respectively, at an AF/MT intersection.

This cartoon of the MCB and motors at the AF/MT intersection is drawn to scale, and illustrates how we calculated the maximum number of motors interacting with the actin filament and/or microtubule at cytoskeletal intersections in our experiments. The estimated maximum number of motors that are able to interact with the actin and/or microtubule at the AF/MT intersection (N) is limited by the size of the bead (r = 1 μm), the length of the motor (d), the maximum length of cytoskeleton that motors attached to the bead are able to reach (S), the motor surface density on the bead (C), and the width of the cytoskeleton interaction surface (W). We used the calculations as in Walcott et al., 2009:
N = CWS

For Kinesin-1: We estimated the length of truncated kinesin-1 to be 20 nm (Kerssemakers et al., 2006). \( S = 2 \sqrt{2rd - d^2} = 0.28 \, \mu m \), which we divided by 0.008 \, \mu m because there is one kinesin binding site every 8 nm along the microtubule. By this calculation, there are 34 potential kinesin binding sites on this length of microtubule. \( W \), the width of interaction along the microtubule is affected by the curvature of the microtubule, radius 12 nm, and results in a 0.0179 \, \mu m interacting length. Based on the concentration of motors mixed with the beads, and the assumption that the motors, not the motor binding sites, are limiting, we determined a kinesin surface density on the bead of 2,018 motors \times \text{bead}^{-1} \times \mu \text{m}^{-2}. \) When these values are used, we find \( N = 12 \) motors/bead that can interact with the microtubule at a time. Since the actin intersection is on top of the microtubule, this will block at least one kinesin binding site, so we estimate that a maximum of 11 kinesin-1 motors can interact with the microtubule at the intersection at a time.

For Myo1c: We estimated the length of truncated Myo1c to be 25 nm (Shuman et al., 2014). For this motor, \( S = 0.31 \, \mu m \), which we divide by 0.036 \, \mu m since there is one myosin binding site every rotation of the actin helix (36 nm). This estimates 8 to 9 available Myo1c binding sites within reach at the actin intersection. The surface density of Myo1c on the MCBs is 10,000 motors \times \text{bead}^{-1} \times \mu \text{m}^{-2}, \) and the width of the interacting surface of the actin filament is 7 nm. These values result in an estimate of 6 Myo1c motors that can interact with the actin filament at any given time.

Myosin-V, kinesin-1, and Myo1c have all been localized to the insulin-responsive membrane compartment that contains GLUT4 glucose transporters. Actin-based transport has been proposed to play an active role in the transport of this compartment to the plasma membrane in adipocytes and muscle cells; yet, how GLUT4-containing membranes are transferred from kinesin-1-dependent microtubule transport to actin-based transport in the cell periphery, and the role of Myo1c during this process, are poorly understood (Chen et al., 2012; Ishikura and Klip, 2008; Semiz et al., 2003; Yoshizaki et al., 2007). The data presented here support the previously proposed role for Myo1c as a motor necessary for docking or tethering GLUT4-containing vesicles, trapping these vesicles in the cortical actin network before GLUT4 vesicle fusion (Boguslavsky et al., 2012; Bose et al., 2004). Specifically, we now show that the intrinsic properties of kinesin-1 and Myo1c motors allow for cargo docking at actin filament intersections without any further regulators. Cumulatively, these results support a model in which kinesin motors drive long distance transport of GLUT4 cargo toward the cell periphery along microtubules. At the periphery, Myo1c halts microtubule-based transport, docking cargo at AF/MT intersections until they
complete their transport to, and fusion with, the plasma membrane upon insulin-stimulated myosin-V transport. This finding is consistent with the observation of GLUT4 vesicle docking adjacent to microtubules prior to fusion with the plasma membrane (Dawicki-McKenna et al., 2012). Given its slow motility and non-processive nature, our data do not support a model in which Myo1c actively transports cargos along actin filaments in this geometry. Instead, our data support a model in which Myo1c is required as a dynamic tether between microtubule- and actin-based transport regimes.

Tropomyosin has been shown to both positively (Chacko, 1981; Clayton et al., 2014; Umemoto et al., 1989) and negatively (Huckaba et al., 2006; Tang and Ostap, 2001) regulate motor motility within the cell (Collins and Matsudaira, 1991; Fanning et al., 1994; Lord, 2011; Ostap, 2008). For instance, S. cerevisiae myosin-V is selectively activated by tropomyosin-coated actin populations (Clayton et al., 2014). Alternatively, Myo1b and Myo1a preferentially localize to tropomyosin-free actin populations and show inhibited actin gliding of tropomyosin-coated filaments (Collins and Matsudaira, 1991; Fanning et al., 1994; Tang and Ostap, 2001). Here, we find that Myo1c activity is inhibited by Tm2-actin in vitro in both gliding and crossed-filament assays. Tm2 inhibits Myo1c-mediated cargo run initiation, as well as processive run termination, at actin intersections. Since Tm2 is located just behind the leading edge of the cell, prevention of Myo1c-mediated cargo docking would promote cargo passage through the dense cortical actin network, enhancing delivery to the plasma membrane for exocytic fusion. These results strengthen the argument that Myo1c is an important factor in cargo sorting, providing an underlying mechanism by which Myo1c-induced cargo run initiation and run termination occurs preferentially when encountering specific actin filament populations, such as the highly dynamic actin filament populations near the peri-nuclear region and at exocytic zones just
beneath the plasma membrane (Figure 3.10) (Gunning et al., 2005; Vindin and Gunning, 2013).

There are a variety of actin-binding and microtubule-associated proteins and post-translational modifications that can modify the ability of motors to interact with specific cytoskeletal populations. While actin filament binding proteins may specify distinct subcellular domains, microtubules can be modified post-translationally to form differentially-localized cytoskeletal populations within the cell that may lead to either activation or inhibition of cargo transport (Sirajuddin et al., 2014). Cumulatively, these observations suggest that the specific complement of processive and non-processive molecular motors bound to an intracellular cargo specifies delivery to subcellular domains via motor-specific deciphering of the cytoskeletal "code."

V. Conclusion

Our in vitro reconstitution approach allowed us, for the first time, to directly observe the interactions between specific non-processive and processive molecular motors on a physiologically relevant, yet simplified, cargo in unambiguous cytoskeletal environments. Our results suggest Myo1c-bound cargo can be loaded on to microtubules for kinesin-driven long distance transport and later docked, or tethered, in peripheral actin-rich regions to facilitate the initiation and termination of long distance transport. Additionally, we find that both run initiation and run termination are regulated by the actin-binding protein tropomyosin, providing a mechanism to regulate the localized delivery of cargos to regions of tropomyosin-free actin filaments within the cell. Thus, the tropomyosin regulation of Myo1c may permit localized regulation of cargo behavior without direct cargo or motor modification, enabling effective sorting of exocytic cargo.
CHAPTER 4: Molecular Motor, BAR Domain, and Lipid-Driven Changes in Vesicle Morphology along Engineered Cytoskeletal Networks

This chapter is in preparation for Molecular Cell
(anticipated submission date: January 2017):

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We thank Tianming Lin, Liqiong Chen, Mariko Tokito, and Dan Safer for excellent technical assistance. Additionally, we thank Erin Masucci for experimental aid, and Michael Woody, Michael Greenberg, Henry Shuman, and other members of the E.M.O., E.L.F.H., and Yale Goldman labs as well as the Pennsylvania Muscle Institute for helpful suggestions and stimulating conversations. We also appreciate the assistance and expertise of Eric Johnston and Brock Peterson from the Singh Center for Nanotechnology for help establishing the micropatterned coverslips protocol. Finally, we thank Tobias Baumgart and his laboratory, in particular Zhiming Chen and Jaclyn Robustelli, for expressed and purified endophilin protein and BAR protein expertise. This work was supported by NIH grants T32 AR053461 to B.B.M. and P01 GM087253 to E.L.F.H. and E.M.O.
I. Summary

Intracellular vesicles are both transported and remodeled by microtubule- and actin-based motors; yet, it is unclear how these motors coordinate to deform and tubulate membranous cargos. Here we use micropatterned cytoskeletal networks with sparse microtubules spanning dense bands of actin filaments to model the intracellular environment. We test the hypothesis that Myo1c-tethering of kinesin-1-driven cargo at actin filament/microtubule intersections provides the resistance necessary for efficient tubulation of giant unilamellar vesicles (GUVs) by kinesin-1 motors. We find that the interplay between deformation and tubulation at actin/microtubule intersections can be regulated by both the membrane lipid composition and the presence of the BAR-domain protein endophilin. Unexpectedly, in addition to tethering microtubule-transported cargo, Myo1c correspondingly transports, deforms, and tubulates GUVs along actin filaments in a lipid composition and endophilin-sensitive manner. Cumulatively, this suggests that membrane composition directly influences the interplay between transport and tubulation of membrane-bound cargo by cytoskeletal motors.
II. Introduction

Molecular motors transport intracellular membrane-bound cargo along microtubule and actin filament tracks. However, in addition to transporting cargo, cytoskeletal motors also remodel their bound membranes. Typically, membrane remodeling is discussed in the context of plasma membrane invagination, extension, and ruffling; yet, internal membrane-bound compartments can also change shape and deform. For example, membrane vesicles are deformed and tubulated to transfer proteins between compartments and to generate new vesicular compartments. Interestingly, much of this vesicle tubulation and sorting occurs in regions of the cell with high actin filament/microtubule overlap (Cao et al., 1999; Cobbold et al., 2004; Puthenveedu et al., 2010). Within cells, it is largely thought that bulk tubulation occurs along microtubules pulled by kinesin and dynein motors (Bloom and Goldstein, 1998; Blum et al., 2000; Kreitzer et al., 2000; Polishchuk et al., 2003; Presley et al., 1997; Simpson et al., 2006; Toomre et al., 1999). For example, kinesin-1 (Kif5B) is important for the extension of tubular lysosomes (Hollenbeck and Swanson, 1990), the recycling of lysosomal components from autolysosomes (Du et al., 2016; Li et al., 2016), and the dynamic tubulation of mitochondria to power mitochondrial network formation (Wang et al., 2015). Kinesin-1 is also found on early, late, and recycling endosomes and likely important for their tubulation and sorting (Hunt et al., 2013).

Although microtubule-based motors are important for elongation of membrane tubes and transport of intracellular cargo, actin-based motors are also involved. For example, Myo1b is involved in the formation of post-Golgi carriers from the trans Golgi, and Myo1c is important for the tubulation and transport of lipid raft recycling components (Almeida et al., 2011; Brandstaetter et al., 2012; McIntosh and Ostap, 2016). Both kinesin-
1 and Myo1c can directly bind to phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P₂) and are involved in the trafficking of GLUT4 and lipid raft exocytic recycling cargo (Boguslavsky et al., 2012; Du et al., 2016; Hokanson and Ostap, 2006; Hokanson et al., 2006; Semiz et al., 2003). We previously investigated the interplay between Myo1c and kinesin-1 on beads coated by a membrane bilayer that had a limited ability to deform, and found that Myo1c is capable of halting processive, kinesin-1-driven, microtubule-based transport at individual actin filament intersections (McIntosh et al., 2015). We hypothesized that this ability of Myo1c to tether kinesin-1-driven cargo at actin intersections could provide a mechanism for resisting kinesin-1 transport of membrane cargo, allowing for the tubulation of this membrane along microtubules.

*In vitro* studies have shown that under specific circumstances, kinesin-1 and a myosin-I isoform, Myo1b, are capable of tubulating giant unilamellar vesicles (GUVs). Robust kinesin-1-driven tubulation was achieved in these experiments by artificially tethering GUVs to the surface of the coverslip via a biotinylated lipid to streptavidin interaction or by using a high density of microtubules on the coverslip, and allowing kinesins to process in multiple directions (Du et al., 2016; Koster et al., 2003; Su et al., 2016; Wang et al., 2015). This GUV tethering provides the mechanical resistance necessary for tubulation, rather than GUV transport. Alternatively, Myo1b, a non-processive, single-headed myosin-I motor, was only capable of tubulation on membranes containing a high concentration of PtdIns(4,5)P₂ and a meshwork of parallel fascin-bundled actin on the surface of the coverslip (Yamada et al., 2014). It was previously unknown whether Myo1c can tubulate membranes, and further, how a microtubule-based processive motor and an actin filament-based non-processive motor would interact to transport and deform vesicles.
Cytoskeletal motors are not the only cellular factors proposed to directly facilitate membrane deformation and tubulation. For instance, Bin/Amphiphysin/Rvs domain (BAR domain) proteins are banana-shaped proteins that bind, stabilize, and generate membrane tubulation *in vivo* and *in vitro* (Bai et al., 2010; Farsad et al., 2001; Gallop et al., 2006; Zhu et al., 2012). Endophilin-A1 is an N-BAR protein that has an N-terminal helix which directly inserts into membranes, enhancing electrostatic interactions between the protein and membrane (Capraro et al., 2013; Chen et al., 2016). Endophilin has been most widely characterized in clathrin-mediated endocytosis, but it has also been found to play a role in endocytic and exocytic trafficking (Angers et al., 2004; Bai et al., 2010; Suetsugu et al., 2014; Vinatier et al., 2006; Weston et al., 2011). *In vitro*, the N-terminal domain of endophilin-A1 (endophilin N-BAR) binds to and stabilizes highly curved membranes when present at concentrations in the nanomolar range; alternatively, the same protein can generate membrane curvature and tubulate giant unilamellar vesicular membranes at micromolar concentrations (Neumann and Schmid, 2013; Roux et al., 2006; Zhu et al., 2012). It is not well understood how this scaffolding-type of membrane deformation relates to cytoskeletal motor-driven membrane deformation and tubulation within cells.

Here we use deformable GUVs to investigate how two motors associated with membrane deformation, Myo1c and kinesin-1, interact in an *in vitro* reconstitution assay. Additionally, we added further physiological complexity by patterning a cytoskeletal network in which sparse microtubules intersect with a meshwork of actin filaments, which mimics the cellular environment where vesicle tubulation often occurs. We also investigated how the lipid composition of the membrane bilayer and the presence of BAR-domain proteins affect kinesin-1 and Myo1c-driven changes in membrane shape. We found that when attached to PtdIns(4,5)P₂-rich GUVs (PIP₂-GUVs), Myo1c and kinesin-1
can deform GUVs along microtubules at actin intersections; however, the addition of the membrane deformation factor endophilin is needed for robust PIP$_2$-GUV tubulation. In contrast, we find that kinesin-1 and Myo1c alone readily tubulate GUVs that have a composition that mimics the inner leaflet of the plasma membrane and late exocytic compartments. Moreover, this physiological lipid mixture (LM-GUVs) resulted in dynamic deformation and tubulation along actin filaments by Myo1c. These results implicate membrane composition as a regulator of the interplay between transport and tubulation by microtubule and actin filament-based molecular motors and BAR domain proteins.

III. Results

Kinesin-1 and Myo1c transport PtdIns(4,5)P$_2$-rich GUVs (PIP$_2$-GUVs) along microtubules and actin filaments

Our goal is to investigate membrane deformation and tubulation in vitro in a physiologically relevant cytoskeletal environment. We adapted a laser-patterning technique to construct arrays of sparse microtubules crossing dense actin filaments attached to the surface of a coverslip (Figure 4.1A) (Azioune et al., 2010; Boujemaa-Paterski et al., 2014; Portran et al., 2013; Reymann et al., 2014). NeutrAvidin was adsorbed to laser-patterned oxidized stripes on the surface of a PEG-silane-coated coverslip that was constructed into a flow chamber. Taxol-stabilized, biotinylated microtubules (MTs) were introduced into the chamber by flowing the solution perpendicular to the stripe pattern, which was then followed by the introduction of biotinylated phalloidin-stabilized filamentous actin (AF; see Chapter 5 for details). A mixture of constitutively active kinesin-1 (KIF5B, K560), full-length Myo1c, GUVs, and an ATP-containing activation buffer were perfused into the flow cell for observation by TIRF microscopy.
PIP2-GUVs contained 20.5% phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P2), 2.5% DGS-NTA(Ni), 0.5% Rho-PtdEth, and 76.5% PtdCho (Table 4.1). Myo1c attached to GUVs via its physiological attachment to PtdIns(4,5)P2, and His-tagged kinesin-1 bound to the NTA(Ni)-functionalized lipid. We used a kinesin-1 concentration (10 nM) that is below the concentration required for spontaneous tubulation, since it has been found that kinesin-1 tends to be found on intracellular cargo in low motor numbers (Hendricks et al., 2010).

We found PIP2-GUVs to pause at AF/MT intersections only 30% of the time in the presence of kinesin-1-only, yet transport is halted 100% of the time at AF/MT intersections in the presence of kinesin-1 and Myo1c (Figure 4.1B). These results are consistent with similar experiments in which Myo1c was attached to membrane-coated beads via a biotin-NeutrAvidin-lipid complex (McIntosh et al., 2015; Chapter 3). As found previously, we observed an increase in the percentage of kinesin-1-driven MT-based runs initiated at AF intersections in the presence of Myo1c. Surprisingly, 24% of all kinesin-1-driven runs in the presence of both kinesin-1 and Myo1c initiate MT-based transport by first moving along an AF and then switching to a MT (Figures 4.1C, 4.3A, Movie S5). This is the first \textit{in vitro} demonstration of vesicular cargo transport by Myo1c. Distance versus time kymographs (Figure 4.1D) show that Myo1c-driven motility is characterized by long periods of slow motility and pauses, interspersed with short periods of faster motility. This motility is easily distinguishable from the ~100-fold faster kinesin-1-driven, MT-based transport. Figure 4.1D (orange line) shows an example motile event that switched from 13 nm/s Myo1c-driven AF-based transport, to 408 nm/s kinesin-1-driven MT-based transport. We find that Myo1c-driven cargo are transported along AFs at an average of 14 nm/s, with motility typically continuing to the end of the AF. Additionally, we found that this full length
Myo1c construct can transport membrane-coated beads in a lipid membrane composed of 2% PtdIns(4,5)P$_2$ and 98% PtdCho in an ATP-dependent manner (Figure 4.3B, Movie S6). This suggests that an ensemble of Myo1c motors can transport even simplified membrane-bound cargo along individual AF tracks at a physiological PtdIns(4,5)P$_2$ concentration.

<table>
<thead>
<tr>
<th>Type of Lipid</th>
<th>PIP$_2$-GUV (%)</th>
<th>Biotin-PIP$_2$-GUV (%)</th>
<th>LM-GUV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rho-PtdEth</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>DGS-NTA(Ni)</td>
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<td>2.5</td>
<td>2.5</td>
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<tr>
<td>PtdIns(4,5)P$_2$</td>
<td>20.5</td>
<td>20.5</td>
<td>3.0</td>
</tr>
<tr>
<td>PtdCho</td>
<td>76.5</td>
<td>66.5</td>
<td>18.4</td>
</tr>
<tr>
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<td>--</td>
<td>--</td>
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</tr>
<tr>
<td>PtdSer</td>
<td>--</td>
<td>--</td>
<td>18.4</td>
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<tr>
<td>PtdIns</td>
<td>--</td>
<td>--</td>
<td>9.2</td>
</tr>
<tr>
<td>Sphingomyelin</td>
<td>--</td>
<td>--</td>
<td>4.6</td>
</tr>
<tr>
<td>Biotin-PtdEth</td>
<td>--</td>
<td>10</td>
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Figure 4.1 Kinesin-1 and Myo1c transport PIP2-GUVs along microtubule (MT) and actin filament (AF) tracks.

(A) The motility of PIP2-GUVs by kinesin-1 and Myo1c was observed on patterned cytoskeletal arrays composed of sparse MTs (green) crossing beneath dense AF (purple) regions. Scale bar = 10 µm.

(B) Graph and corresponding illustrations of the fraction of PIP2-GUVs motile along MTs containing kinesin-1-only or kinesin-1+Myo1c that pass or pause at AF intersections. Total number of events observed at AF/MT intersections from 17 (kinesin-1-only) and 16 (kinesin-1+Myo1c) different PIP2-GUV preparations is written above each bar. Kruskal-Wallis significance test p ≤ 0.0001.

(C) Graph and corresponding illustrations of the fraction of PIP2-GUVs motile along MTs that initiate their MT-based motility at AF intersections. See also Figure 4.3A, Movie S5.

(D) PtdIns(4,5)P2-bound Myo1c can transport GUVs along AFs. Distance vs. time graph showing example trajectories of Myo1c-driven PIP2-GUV motility along AF during 600 s acquisitions taken at 1 frame/second. The velocity of each constant-velocity segment is written in nm/s next to each track in grey. See also Movie S6.

(E) Graph showing the time-weighted velocities of constant-velocity segments of Myo1c-driven PIP2-GUV motility. n = 109 vesicles from 17 (kinesin-1-only) and 16 (kinesin-1+Myo1c) different GUV preparations.
Myo1c and kinesin-1 deform PIP2-GUVs along the MT at AF/MT intersections

We hypothesized that the ability of Myo1c to halt kinesin-1-driven transport at AF/MT intersections could provide the tethering resistive-force necessary for kinesin-1 to effectively deform or tubulate a GUV along the MT. Thus, we measured the deformation of GUVs as they interacted with an AF during MT-driven transport in the presence of 10 nM kinesin-1 and the absence and presence of 200 nM Myo1c. PIP2-GUV shape was quantified by measuring the length of the GUV along the MT as it changed shape over time, expressed as a ratio of its longest and shortest dimensions (termed $\beta$; Figure 4.2D). Kinesin-1-only PIP2-GUVs were largely non-deforming at AF/MT intersections with a mean of $\beta = 1.12 \pm 0.12$ (Figure 4.2A, 4.2D, Movie S7). We defined PIP2-GUV deformation as a $\beta$ value that is two standard deviations from the mean of kinesin-1-only membrane shape changes ($\beta \geq 1.36$). In the presence of kinesin-1 and Myo1c, there is a significant increase in the fraction of deformed PIP2-GUVs observed at AF/MT intersections, with 57% of all GUVs at AF/MT intersections deforming in the presence of both motor types, in contrast to 5% of kinesin-1-only PIP2-GUVs (Figures 4.2B, 4.2D, Movie S7). Tubulation events along MTs, as defined by the formation of membrane extensions with a diffraction-limited width (Figure 4.3C), were rare (3% of events), although deformation and tubulation of the same GUV were observed (Figure 4.2C, Movie S8). In this example, the PIP2-GUV was seen to translocate along an AF to an AF/MT intersection, were it deformed upon MT-based transport initiation, and then tubulated as it interaction with a second AF/MT intersection.
Figure 4.2 Kinesin-1 and Myo1c together deform PIP2-GUVs at AF/MT intersections along MTs.

(A) PIP2-GUVs with only kinesin-1-bound are largely non-deforming as they encounter AF intersections. Time series and kymograph showing a kinesin-1-transported GUV with unchanging shape in relation to the MT as it passes 3 AF/MT intersections and pauses at 1 AF/MT intersection. The shortest and longest width of the GUV over time in relation to the MT (as measured from a kymograph drawn along the length of the microtubule) is illustrated by the magenta lines below the kymograph, with the corresponding lengths in μm, and the β-ratio. AF intersections are indicated by purple arrows above kymograph. Scale bar = 1 μm, 5 s. See also Movie S7.

(B) PIP2-GUVs with both kinesin-1+Myo1c tend to deform at AF/MT intersections. Time series and kymograph showing an example deformation event when a PIP2-GUV is coated with both kinesin-1+Myo1c. Scale bar = 5 μm, 25 s (note scale differences between figures). See also Movie S7.

(C) PIP2-GUVs with both kinesin-1+Myo1c rarely tubulate at AF/MT intersections. Time series and kymograph showing an example tubulation event where β ≥ 1.36 and a tube of diffraction-limited width is pulled along a MT from a PIP2-GUV coated with both kinesin-1+Myo1c. Scale bar = 5 μm, 25 s. See also Figure 4.3C, Movie S8.

(D) There is significantly more GUV deformation in the presence of both kinesin-1+Myo1c than kinesin-1-alone (p ≤ 0.0001 via Kruskal-Wallis). Graph illustrating the β-ratio of PIP2-GUV deformation along the MT while at an AF/MT intersection. The threshold for “deformed” GUVs β ≥ 1.36 is illustrated by the line to the right. See also Movie S6.
Figure 4.3 PIP₂-GUV transportation along AF and MT, and deformation at AF/MT intersection.

(A) Example time series and kymograph of a PIP₂-GUV switching from AF-based to MT-based motility. Different modes of motility are indicated along the kymograph. Scale bars = 5 μm and 20 sec.

(B) Membrane-Coated Beads containing 2% PtdIns(4,5)P₂ in a PtdCho background are transported by Myo1c along AF in the presence (left) and absence (right) of 1 mM ATP. These temporally color-coded hyperstacks show Myo1c-driven motility in the presence of ATP where the membrane-coated bead initiated at the arrow and its motility ended at the asterisk. Membrane-coated beads specifically stuck to AF in the absence of ATP, but did not show motility. Scale bar = 1 μm. See also Movie S6.

(C) Tubulation events were separated from deformation events by looking at the thickness of the membrane deformation along the MT. These examples show a kymograph drawn perpendicular to the MT axis, along the dashed line, in the case of a deformation event and a tubulation event by the same vesicle (See Figure 4.2C for entire time series and kymograph drawn along the microtubule). Tubulation events are categorized as membrane elongations of diffraction-limited width. Scale bars = 1 μm, 5 sec.
Myo1c and endophilin coordinate to enhance kinesin-1-driven tubulation of PIP$_2$-GUVs along MTs

In addition to cytoskeletal motors, BAR-domain proteins are associated with membrane deformation and tubulation. Thus, we hypothesized that incidences of cytoskeletal-motor-driven tubulation of PIP$_2$-GUV could be increased by the addition the membrane-binding region of the N-BAR protein, endophilin. Since cellular concentrations of endophilin are thought to be in the nanomolar regime, we examined the effects of 8 and 80 nM N-BAR on membrane remodeling by kinesin-1 in the absence or presence of Myo1c AF/MT intersections. These concentrations of endophilin are not sufficient to spontaneously induce tubulation (Zhu et al., 2012).

Addition of endophilin N-BAR led to a significant increase in PIP$_2$-GUV tubulation along MTs at AF/MT intersections, but only when 10 nM kinesin-1 and 200 nM Myo1c were present (Figure 4.4). PIP$_2$-GUVs in the presence of kinesin-1-only are largely non-deforming (95%), even with the addition of 8 and 80 nM endophilin (both 96% non-deforming); however, the addition of both 200 nM Myo1c and endophilin resulted in a concentration-dependent increase in PIP$_2$-GUV tubulation along the MT at AF/MT intersections (Figures 4.4A, 4.4B, 4.5C, Movie S9). Tubulation occurred from PIP$_2$-GUVs with a range of diameters (0.61 – 3 µm; Figures 4.4A, 4.5C).

Endophilin significantly altered a number of tubulation parameters. We quantitated the deformation along the MT at the AF/MT intersection ($\beta$) in the presence of endophilin (Figure 4.5B), and determined the absolute membrane elongation length (longest minus shortest) (Figure 4.4C). In the presence of endophilin, kinesin-1 pulled tubes as long as 40 to 45 µm along MTs (e.g., Figure 4.4A, Movie S9). Interestingly, the tubulation length
was rarely limited by the length of the MT since the kinesin molecules pulling the tube could switch between MTs (Figure 4.4A, Movie S9). Finally, the presence of endophilin resulted in faster velocities of tube growth, with speeds approaching those of single molecule kinesin-1 (0.5 – 0.7 µm/s; Figure 4.4D) (Thorn et al., 2000). Finally, although both Myo1c and endophilin were necessary for enhanced kinesin-1-driven tubulation, actin filament pelleting assays suggest that under these experimental concentrations and buffer conditions endophilin and Myo1c do not directly interact (Figure 4.5A).

To determine if a non-motor anchor is able to mimic the effect of Myo1c on tubulation, we added 10% biotinylated lipid to the PIP$_2$-GUV mixture (biotin-PIP$_2$-GUVs, Table 4.1) so that it would tether to the NeutrAvidin stripes already patterned on the surface of the coverslip in the absence of Myo1c and AF (Figure 4.5D). We found that the lipid-tethered PIP$_2$-GUVs pause at NeutrAvidin stripes for an average of 64 seconds, which suggests this attachment is a more robust tether than the non-specific pausing of kinesin-1-only GUVs at AF/MT intersections (average of 32 seconds), yet, a weaker tether than dynamic Myo1c binding to AF, which pauses GUVs for an average of 131 seconds (Figure 4.5E). We found that these biotin-PIP$_2$-GUVs do not deform as frequently as those containing both kinesin-1 and Myo1c at AF intersections (7% vs. 57%), and yet, they tubulate more frequently (15% vs. 3%) (Figure 4.4B). These results suggest that although cargo tethering is essential for providing resistance for kinesin-1 to pull against and tubulate the membrane, this is likely not the only function for Myo1c in membrane deformation and tubulation.
Figure 4.4 Together Myo1c and endophilin promote PIP$_2$-GUV kinesin-1-driven tubulation at AF/MT intersections.

(A) PIP$_2$-GUVs show enhanced frequency of tubulation along MTs, at AF/MT intersections, in the presence of endophilin and Myo1c. Example time series and kymograph for a PIP$_2$-GUV tubulation event that occurs once encounters the AF stripe during its MT-based motility. AF intersections are indicated by purple arrows above kymograph. White arrows in time series indicate the tip of the elongating tube. Scale bar = 10 μm in distance, 50 s in time. See also Figure 4.5C, Movie S9.

(B) Kinesin-1-only PIP$_2$-GUVs remain non-deforming even in the presence of endophilin, while those containing both kinesin-1+Myo1c show a concentration-dependent increase in the fraction of tubulation events upon addition of endophilin. Graph showing percentage of all events at AF/MT intersections that are non-deform (dark purple), deformed (medium purple), or tubulated (light purple) at AF/MT intersections. The number of observed events at AF/MT intersections is written above each bar from ≥ 6 different GUV preps and ≥ 6 experimental chambers.

(C) There are significantly longer kinesin-1-driven PIP$_2$-GUV tubulation events in the presence of endophilin and Myo1c. Box plot showing the net tube growth (longest – shortest GUV length over time) along the MT while at the AF intersection. * p ≤ 0.05, **** p ≤ 0.0001. See also Figure 4.5B.

(D) The velocities (μm/s) of individual PIP$_2$-GUV tubulation events as they are elongated along MTs by kinesin-1 is plotted for every tubulation event observed. There is an increase in the tubulation velocity with the addition of endophilin.
Figure 4.5 Further exploration of Myo1c- and endophilin-enhanced PIP<sub>2</sub>-GUV deformation and tubulation.

(A) This coomassie gel, shown in normal and inverted contrast, demonstrates that endophilin does not co-sediment with Myo1c on AF under the conditions used in this assay. Myo1c was mixed with calmodulin, F-actin, and varying concentrations of endophilin in the BRB80 buffer used in the GUV assays. Endophilin is not visible in the pellet below 10 µg (1.42 µM), which is vastly higher than the
8 and 80 nM concentrations used in the reconstitution assay. Additionally, there was not a reciprocal decrease in endophilin in the pellet upon Myo1c ATP-release from AF. Thus, Myo1c and endophilin are not directly interacting to enhance kinesin-1-driven GUV tubulation.

(B) The fold change in GUV length (β) is significantly higher in the presence of both endophilin and Myo1c than with kinesin-1 alone. Box plot showing the fold increase in tube growth along the MT while at the AF intersection. Data included from previous figures for comparative purposes: kinesin-1-only + 0 nM endophilin and kinesin-1+Myo1c + 0nM endophilin data from Figure 4.2D.

(C) Example kymograph and before/after panels for a small PIP$_2$-GUV tubulating in the presence of kinesin-1+Myo1c and 80 nM endophilin. The shortest and longest width of the GUV in relation to the MT over time are illustrated by the magenta lines below, with the corresponding lengths in μm. This vesicle is determined to be tubulating because β ≥1.36 and there is diffraction-limited membrane tube growth. Purple arrows on top of kymograph indicate AF intersection, and white arrows in time series indicate leading edge of tube. Scale bars = 1 μm, 5 s.

(D) Experimental design for biotin-PIP$_2$-GUV experiments. 10% biotin-PtdEth lipid was added to PIP$_2$-GUVs. This biotinylated lipid was then able to interact with the NeutrAvidin patterned on the coverslip (indicated by cyan arrowheads) with only MTs, no AF present. Example cytoskeletal patterning of AF and MTs (left) and MTs-only (right).

(E) Box plot of pause lengths of PIP$_2$-GUVs plotted as the log of time in seconds. Number of pauses observed is listed above each box plot.

**A physiological lipid mixture (LM-GUVs) promotes tubulation along both AFs and MTs by Myo1c and kinesin-1**

The mole-percentage (20.5%) of PtdIns(4,5)P$_2$ used in the PIP$_2$-GUVs above was chosen to allow comparison with previous myosin-I studies (Yamada et al., 2014) and to mimic a PtdIns(4,5)P$_2$-rich microdomain proposed to exist in cellular membranes (Levental and Veatch, 2016; Yin and Janmey, 2003). We next investigated the deformation of GUVs composed of a lipid mixture (LM-GUVs) that better mimics the inner leaflet of the plasma membrane and membranes found in vesicles of the late exocytic pathway (Corbin et al., 2004; McKenna and Ostap, 2009). This mixture contains 43.4% PtdEth, 18.4% PtdSer, 18.4% PtdCho, 9.2% PtdIns, 4.6% sphingomyelin, 3% PtdIns(4,5)P$_2$, and the same 2.5% DGS-NTA(Ni) lipid, and 0.5% rhodamine-PtdEth as in the PIP$_2$-GUVs (Table 4.1). In contrast to PIP$_2$-GUVs, LM-GUVs in the presence of kinesin-1 and Myo1c tubulate along MTs at 52% of all encountered AF/MT intersections (Figure 4.6A). Additionally, the
presence of endophilin does not observably increase the incidences of deformation or tubulation of LM-GUVs of this lipid composition along MTs (Figure 4.6A).

In addition to deformation and tubulation at AF/MT intersections along MTs, we observed substantial deformation and tubulation of LM-GUVs along AFs away from MT intersections. Notably, we identified two separate populations of vesicles of LM-GUVs, those that were spherical when they encountered the cytoskeleton on the surface of the coverslip, and those that were irregularly shaped and morphologically dynamic (Figure 4.6B, 4.6F, 4.6G). In the absence or presence of 80 nM endophilin, LM-GUVs were 47% and 31% non-deforming, 27% and 19% deformed along AF, respectively (Figure 4.6B). We also observed an increase in the percentage of spherical LM-GUVs tubulating along AF, from 3% to 7%, with the addition of 80 nM endophilin, with 31 total tubulation events observed under each condition across all chambers (Movies S10, S11). In one particularly interesting example, a LM-GUV lands in an AF-rich area, tubulates along an AF, and then switches tube growth from Myo1c-driven AF-based elongation, to kinesin-1-driven MT-based elongation (Movie S11). Most striking, however, was the increase in the percentage of morphologically dynamic LM-GUV tubulation events from 23% to 43% upon addition of 80 nM endophilin (Figures 4.6B, D-G, Movie S10). In contrast, PIP$_2$-GUVs were only spherical and observed to be 88% and 64% non-deforming, with an increase in the percentage of deforming cargo from 12% to 33% and the appearance of 3% tubulation with the addition of 80 nM endophilin (Figure 4.6B, 4.6D, 4.6E). When measuring the β-factor deformation along AFs, we included only the spherical GUVs and saw a significant increase in the deformation of LM-GUVs compared to PIP$_2$-GUVs (Figure 4.6C). Interestingly, we consistently saw half as many AF-based transport, deformation, and tubulation events of LM-GUVs per field of view in the presence of endophilin—75 events
for kinesin-1+Myo1c LM-GUVs, and only 35 events for kinesin-1+Myo1c+80nM endophilin LM-GUVs over 4 different fields-of-view and 3 vesicle preps. Thus, the lipid composition can significantly affect membrane deformation along both AFs and MTs.

Figure 4.6 A physiological lipid mixture (LM-GUVs) promotes tubulation along both AFs and MTs by Myo1c and kinesin-1.

(A) There is significantly more tubulation by kinesin-1+Myo1c LM-GUVs than PIP2-GUVs. 0 nM and 80 nM endophilin data was taken from Figure 4.4B for comparison purposes. Total number of vesicle events at AF/MT intersections listed above each column. * p ≤ 0.05, *** p ≤ 0.001.

(B) Every AF-based transport event in 3-4 fields-of-view from at least 2 different GUV preps was recorded and analyzed for deformation and tubulation. LM-GUVs showed two different populations,
spherical and morphologically dynamic GUVs. Total number of vesicle events along AF from these fields-of-view are listed above each column. *** p ≤ 0.001, **** p ≤ 0.0001

(C) Spherical LM-GUVs are significantly more deformed along AF than PIP$_2$-GUVs. Vesicles were measured for β-factor deformation, with morphologically dynamic LM-GUVs excluded. All vesicles are in the presence of both kinesin-1+Myo1c. The 0 nM endophilin PIP$_2$-GUV data is the same set used in Figure 4.2D, provided for comparison purposes. Statistical testing by Kruskal-Wallis with Dunn’s multiple comparisons with all significantly different pairings indicated; all others were determined to be not significantly different; *** p ≤ 0.001, **** p ≤ 0.0001.

(D) PIP$_2$-GUVs with both kinesin-1+Myo1c and 0 nM endophilin are largely non-deforming as they are transported along AF by Myo1c. Sample transport event in which the first and last frame of motility are shown with the corresponding kymograph. The shortest and longest width of the GUV as determined by a kymograph drawn along the AF are illustrated by the magenta lines beside, with the corresponding lengths in μm, and β-ratio < 1.36. Scale bar = 1 μm, 20 s.

(E) PIP$_2$-GUVs with both kinesin-1+Myo1c and 80 nM endophilin show increased deformation and tubulation as they are transported along AF by Myo1c. Sample transport and deformation event in which β ≥ 1.36, but the membrane elongation is not diffraction-limited. Scale bar = 1 μm, 20 s.

(F) LM-GUVs in the absence of endophilin were more deformed and tubulated than PIP$_2$-GUVs, and showed the occurrence of morphologically dynamic, as well as spherical GUVs. Example tubulation event in the absence of endophilin with the corresponding kymograph. Scale bar = 1 μm, 20 s; note kymographs in 4.6D-F have the same label in distance and time, but are scaled differently to improve visual comparison. See also Movie S10.

(G) LM-GUVs in the presence of 80 nM endophilin were largely morphologically dynamic and tubulated. These examples show the underlying cytoskeleton, the GUV shape immediately prior to landing on AF, and the GUV morphology after tubulation of two events: one with 0 nM endophilin, and one with 80 nM endophilin. Scale bar = 2 μm. See also Movie S11.

IV. Discussion

Microtubule-based tubulation

Kinesin-1 has been found to participate in the intracellular elongation of lipid tubes along MT tracks (Bloom and Goldstein, 1998; Du et al., 2016; Hollenbeck and Swanson, 1990; Kreitzer et al., 2000; Li et al., 2016; Polishchuk et al., 2003; Toomre et al., 1999; Wang et al., 2015). Other microtubule-based motors such as KIF13A, KIF16b, and dynein-dynactin have also been implicated in these processes (Blum et al., 2000; Delevoye et al., 2014; Presley et al., 1997; Simpson et al., 2006). For tubulation to occur, however, these processive, MT-based motors must have something to pull against, to provide the resistance necessary to deform or tubulate a membrane-bound compartment. Previous
studies have used either attachment between biotinylated-lipids and streptavidin on the surface of the coverslip or performed experiments with a very high concentration of microtubules attached to the chamber surface in a dense, random, lawn where often high concentrations of kinesin motors can walk along tracks in opposite directions to generate the opposing forces required for tubulation (Du et al., 2016; Koster et al., 2003; Roux et al., 2002; Su et al., 2016; Wang et al., 2015). These experimental tools have been used to show that processive kinesin-1 and non-processive ncd (kinesin-13) motors are capable of pulling tubes from giant unilamellar vesicular and mitochondrial membranes in in vitro reconstitution assays (Leduc et al., 2004; Shaklee et al., 2008; Shaklee et al., 2010a; Shaklee et al., 2010b).

Here we used micropatterning to mimic the intersection of MTs with dense regions of AFs to begin to understand how different motor families cooperate to power changes in membrane morphology (Figure 4.1A). We found that Myo1c can anchor kinesin-1-driven cargo at AF/MT intersections, resulting in membrane deformation and tubulation along the MT (Figure 4.1B, 4.2D). The level of membrane deformation and tubulation is highly sensitive to the lipid composition of GUVs and the presence of Myo1c, with tubulation more likely in the presence of endophilin or physiological lipid mixtures (Figures 4.2 and 4.6, Summarized in Figure 4.7). Notably, although tubulation was enhanced via the simple presence of a static anchor (Figure 4.4B), PIP2-GUV deformation and tubulation were substantially more common in the presence of the active motor, Myo1c. This finding suggests that Myo1c may be actively deforming the GUV membrane to aid deformation and tubulation.

Kinesin-1-driven tubulation of Myo1c-tethered GUVs along MTs is differentially stimulated by endophilin based on lipid composition; we noted a significant increase in
tubulation of PIP$_2$-GUVs, but no significant difference in the tubulation of LM-GUVs upon endophilin addition. We hypothesize that endophilin is necessary to enhance tubulation of PIP$_2$-GUVs because it is capable of sensing and stabilizing transient membrane curvature generated by the two types of motors, increasing the probability of kinesin-1 pulling a thin membrane tube along the MT. The relative increase in tubulation velocities upon addition of endophilin supports this hypothesis (Figure 4.4D). In contrast, the irregular and dynamic shapes of LM-GUVs in the absence of cytoskeletal interactions suggests a higher deformability of these vesicles, which may explain the lack of additional tubulation induced by endophilin (Figure 4.6A). Alternatively, we may see no significant increase in the percentage of tubulated LM-GUVs upon addition of endophilin due to competition between endophilin and Myo1c for binding to the membrane. Myo1c binds specifically to PtdIns(4,5)P$_2$, with increased association times with the membrane with the presence of PtdSer in the membrane (McKenna and Ostap, 2009). Endophilin, as well, can bind specifically to PtdIns(4,5)P$_2$, but is less selective for this particular anionic phospholipid, and can bind well to only PtdSer in membranes (Chen et al., 2016; Gallop et al., 2006). While it appears that PIP$_2$-GUVs have sufficient binding sites for both Myo1c and endophilin, we consistently see half as many AF-based transport, deformation, and tubulation events of LM-GUVs per field of view in the presence of endophilin.

**Actin filament-based tubulation**

It has been previously found that Myo1b is important for the formation of post-Golgi carriers from the trans Golgi (Almeida et al., 2011). *In vitro*, Myo1b is capable of tubulating PtdIns(4,5)P$_2$-rich GUVs along only fascin-bundled AF, but not unbundled, AF (Yamada et al., 2014). Myo1c has also been implicated in cellular tubulation and membrane deformation events, but it was not previously known whether Myo1c is capable of
tubulating membranes on its own (Brandstaetter et al., 2012). Additionally, although Myo1c has been suggested to be a cargo transporter by single molecule mechanics investigations, and can power the gliding of AF on a supported lipid bilayer containing PtdIns(4,5)P$_2$, it was not known whether Myo1c could transport membranous cargo via its direct link to PtdIns(4,5)P$_2$ in a fluid lipid bilayer (Greenberg et al., 2012; Pyrpassopoulos et al., 2012; Pyrpassopoulos et al., 2016). Here we show that on PIP$_2$-GUVs, Myo1c robustly transports GUVs along individual and densely patterned AF. When endophilin is added, we see an increase in the fraction of deformed PIP$_2$-GUVs, as well as tubulated PIP$_2$-GUVs, along AF. Interestingly, by varying the lipid composition to a more physiological composition (LM-GUVs), we see very different AF-based GUV transport and tubulation behaviors. In these membranes, Myo1c shows similar percentages of non-deformation, deformation, and tubulation as PIP$_2$-GUVs with endophilin present to enable various deformation and tubulation events; however, no additional membrane deformation factor (endophilin) is needed to see populations of tubulated and deformed LM-GUVs transported along AF.

In contrast to the unchanging tubulation of LM-GUVs along MTs in the absence and presence endophilin, Myo1c-based tubulation along AF shows an increased percentage of tubulation in the presence of endophilin. The most striking finding, however, was the presence of tubulating vesicles in LM-GUV preps that were already morphologically dynamic before engaging with the cytoskeleton. The varied morphology of these non-spherical GUVs suggests increased deformability of the membrane, which may promote tubulation along AFs. It is these morphologically dynamic GUVs that show enhanced tubulation along AF in the presence of endophilin, suggesting that in this case,
endophilin binds to and stabilizes already curved membranes, causing further deformation that Myo1c can take advantage of to pull along AF tracks.

**Lipid composition affects transport, deformation, and tubulation, of actin filament and microtubule-based cargo**

Varying lipid composition by using cholesterol or acyl chain variability can affect vesicular cargo transport by molecular motors along MTs or AFs. For instance, addition of cholesterol to phagocytic membranes during maturation of the organelle can cause clustering of dynein-dynactin motors and increased cooperation between dynein motors, leading to increased minus-end-directed motility and further phagosome maturation (Pathak and Mallik, 2016; Rai et al., 2016). Alternatively, myosin-V motors bound to synthetic liposomes show increased velocity in fluid DOPC (1,2-dioleyl, two mono-unsaturated acyl chains), rather than viscous DPPC (1,2-dipalymityl, two saturated acyl chains) membranes (Nelson et al., 2014). Here we show that varying lipid head group composition, while still using the 1,2-dioleyl (i.e. DOPC) acyl chains preferred by *in vitro* researchers for their bilayer stability, can alter the ability of both MT and AF-based motors kinesin-1 and Myo1c to deform and tubulate membranes along their respective tracks. We chose a lipid composition that mimics that found on the inner leaflet of the plasma membrane and/or late exocytic cargo (Bigay and Antonny, 2012; Corbin et al., 2004; van Meer et al., 2008); however, we did not include cholesterol in our mixture. Without either saturated acyl chains and/or cholesterol, it is unlikely that our physiological lipid mixture (LM-GUVs) forms microdomains at room temperature (Bakht et al., 2007). Further experimentation needs to be done to investigate the roles of cholesterol and acyl chain saturation in motor-driven membrane deformation, as it has been found that
polyunsaturated phospholipids can lead to increased tubulation and scission by endophilin and dynamin (Pinot et al., 2014).

Endophilin is an N-BAR domain protein that binds electrostatically and through the insertion of its N-terminal helix into membranes in the cell such as the plasma membrane and late exo/endocytic membranes (Chen et al., 2016). Although it shows a preference for binding to PtdIns(4,5)P$_2$, it can also associate with PtdSer and other anionic phospholipids in the membrane (Chen et al., 2016; Gallop et al., 2006). The ability of endophilin to tubulate GUVs in vitro when in the micromolar protein range has been difficult to connect to cellular observations where the protein concentration is thought to be significantly lower. Here we show that low nanomolar concentrations of endophilin N-BAR can enhance kinesin-1-driven tubulation in PIP$_2$-GUVs, and Myo1c-driven tubulation in LM-GUVs. It is unclear, however, whether these low concentrations would be sufficient in the context of a full length, autoinhibited construct (Chen et al., 2014). Although Myo1c is not involved in endocytosis, Myo1e, a long-tailed myosin-I isoform, participates in clathrin-mediated endocytosis, along with endophilin, raising the question whether Myo1e can aid in endophilin activation of membrane invagination through the generation of membrane deformation (Cheng et al., 2012; Krendel et al., 2007). Further experiments need to be done to test this hypothesis. It is also unclear how direct AF-based membrane deformation and tubulation of membranes relates to motor and BAR-domain protein-driven remodeling.
**Motility at AF/MT Intersections:**

Transportation along MT → Pause → Deformation at AF/MT → Tubulation at AF/MT

Myo1c

Endophilin on PIP2-GUVs or Lipid Mixture

**Motility along AF:**

Transportation along AF → Deformation along AF → Tubulation along AF

Endophilin on PIP2-GUVs or Lipid Mixture

Lipid Mixture +/− Endophilin

**Figure 4.7** Transportation, deformation, and tubulation by kinesin-1 and Myo1c along MT and AF tracks is mediated by lipid composition and endophilin.
V. Conclusion

Here we show that both Myo1c and kinesin-1 are capable of transporting, deforming, and tubulating GUV membranes along their respective cytoskeletal tracks in an *in vitro* reconstitution assay. Kinesin-1-driven tubulation is enabled by Myo1c-tethering to AF/MT intersections, connecting the AF and MT cytoskeletal regimes during tubulation. These experiments support the hypothesis that non-processive single-headed motors such as Myo1b and Myo1c can directly participate in membrane deformation, both alone, and in conjunction with powerful and processive, MT-based transporters like kinesin-1. These results also show that Myo1c can act as a capable, albeit slow, vesicle transporter along single AFs, and that deformation of AF-transported cargo by Myo1c is highly dependent upon the presence of BAR-domain stabilization of curved membranes and/or the lipid composition of these transported membranes. Finally, we show that in some membrane compositions, but not all, endophilin can enhance kinesin-1-driven tubulation of Myo1c-tethered cargo. Overall, we suggest that it is important not only to consider the interplay between MT and AF-based molecular motors in cargo transport and tubulation, but also to investigate the roles of membrane lipid composition and additional membrane deformation factors to understand the complexity of observed physiological phenomena.
CHAPTER 5 : Methods and Materials

I. Proteins and Reagents

Chapter 3:

We appended a biotinylatable AviTag sequence (Schatz, 1993) to the C-terminus of GFP-labeled, truncated human kinesin-1 heavy chain construct (amino acids 1-560; Pierce and Vale, 1998). The protein was expressed in E. coli, purified, and biotinylated (Pierce and Vale, 1998); reported concentrations refer to K560 heavy chain monomer concentrations, although kinesin-1 readily dimerizes in solution. A truncated mouse Myo1c construct spanning the motor domain, regulatory domain with three IQ motifs, a C-terminal FLAG tag for purification, and a C-terminal AviTag for site-specific biotinylation was baculovirus-expressed and purified from SF9 cells (Lin et al., 2011; Manceva et al., 2007). The α-actinin-biotin construct attached to MCBs with kinesin-1 was assembled by ligating the actin-binding domain of a halotag-α-actinin construct (Greenberg et al., 2012) to a halo-ligand-biotin construct (Promega) at 4 °C for 3 hours. Following halo-tag ligation, α-actinin-biotin was run over Nap5 and Nap10 buffer exchange columns (GE Healthcare Life Sciences) and then aliquotted and stored in liquid nitrogen. Biotinylation efficiency was compared to Myo1c at equimolar concentrations using the Pierce High Sensitivity Streptavidin-HRP reagent. Actin was purified from rabbit skeletal muscle (Spudich and Watt, 1971), polymerized at a concentration of 1 μM and stabilized with 1.1 μM rhodamine phalloidin (Invitrogen) in MB (50 mM Mops pH 7.0, 125 mM KCl, 5 mM EGTA, 5 mM MgCl₂, and 1 mM DTT). Vertebrate calmodulin (CaM) was prepared as described (Putkey et al., 1985). Microtubules were assembled from bovine-brain tubulin, TRITC tubulin (Cytoskeleton), and HiLyte 488 tubulin (Cytoskeleton) at 45 μM tubulin dimer with a ratio...
of 50:2:1.5 of unlabeled tubulin dimer:TRITC:HiLyte488 and stabilized with 40 μM Taxol (Cytoskeleton) in BRB80 (80 mM K-PIPES, 1 mM MgCl₂, 1 mM EGTA, pH 7.5).

A Rat TPM1 (variant 2) tropomyosin (Tm2) construct in the pET11d vector between Ncol and Bam Sites (Singh and Hitchcock-DeGregori, 2003) was mutated twice via a QuikChange protocol (Agilent): (1) the amino acid sequence was from rat to human, (2) an acetylation-mimetic amino acid sequence (MASM) was cloned into the N-terminus. Tm2 was purified from BL21 pLysS E. coli by the method of Hitchcock-DeGregori and Heald, 1987, with modifications. The lysis buffer contained 20% sucrose, 50 mM Tris (pH 7.5), 10 mM EDTA, 1 mM DTT, 1 mM PMSF, 10 μg/mL leupeptin, and 10 μg/mL aprotinin. Pellets from two liters of cell culture were resuspended in lysis buffer and incubated on ice for 90 min. The sample was then sonicated 6-times at 30% amplitude for 15 s at a time. 12 mL of 5 M NaCl were added and then sonication was repeated. Following sonication, the sample was pelleted at 16 krpm for 20 min and the supernatant was heated to 90 °C in a water bath for 5 min. Following at 40 min incubation on ice, the samples were pelleted at 16 krpm for 15 min, and the supernatant was adjusted to pH 4.4 by gradually adding 50% acetic acid. Again, the sample was pelleted at 16 krpm for 15 min and then the pellet was resuspended in 5 mL resuspension buffer (0.1 M KCl, 25 mM Tris, 1 mM DTT, and 1 mM NaN₃). Next, 1 M Tris was added drop-wise until a pH of 8.0 was reached. 140 mg ammonium sulfate was added per mL of solution. Following a 20 min spin at 4 °C, the sample was pelleted at 16 krpm for 15 min. The ammonium sulfate precipitation and spin were repeated, this time adding 258 mg ammonium sulfate per mL of supernatant to a final concentration of 2.6 M. The pellet was resuspended in 5 mL resuspension buffer and dialyzed in 2 L resuspension buffer overnight. For further purification, we used the FPLC MonoQ column chromatography and dialysis. The day of the experiment, Tm2 was
reduced at 56 °C for 5 min in 50 mM DTT and pelleted at 60 krpm for 30 min to remove aggregates. Tm2-actin was polymerized with 1 μm G-actin in tropomyosin buffer (TropoB: 500 mM NaCl, 50 mM Tris HCl pH 7.5, 10 mM MgCl₂, and 2.5 mM DTT) supplemented with 1 mM MgATP and 1.3 μM rhodamine phalloidin.

Chapter 4:

We expressed and purified a human kinesin-1 (KIF5B) construct from E. coli, which contains the first 560 amino acids of the heavy chain sequence with a 6x-His tag and GFP added to the C-terminus (Pierce and Vale, 1998). In this work, the concentration of kinesin-1 refers to the amount of heavy chain dimer in solution. This construct was attached to lipid membranes through its His-tag to a NTA(Ni)-functionalized lipid (see below for more information). A full length mouse Myo1c isoform C (construct pLT16) was baculovirus expressed and purified from SF9 cells as described in Pyrpassopoulos et al., 2012. Rat endophilin A1 N-BAR was kindly cloned and purified by the laboratory of Tobias Baumgart at the University of Pennsylvania with residues 1-247 and an E241C substitution for fluorescent labeling, and purified as described (Capraro et al., 2013; Chen et al., 2014). Note, all endophilin concentrations reported are the monomer concentration, yet, endophilin N-BAR exists as a dimer in solution (Chen et al., 2014). Human platelet G-actin (>99% pure) was purchased from Cytoskeleton, Inc. (Cat # APHL99-A), polymerized at a concentration of 2 μM and stabilized with either 1.33 μM Alexa Fluor 488 phalloidin (Life Technologies, Cat # A12379) and 0.57 μM biotin-xx phalloidin (Life Technologies, Cat # B7474) or 0.57 μM Alexa Fluor 488 phalloidin, 0.57 μM biotin-xx phalloidin, and 0.76 μM unlabeled phalloidin (Thermo Fisher Scientific, Cat # P3457) in MB (50 mM MOPS pH 7.0, 125 mM KCl, 5 mM EGTA, 5 mM MgCl₂, and 1 mM DTT). Vertebrate calmodulin (CaM) was expressed and purified as described (Putkey et al., 1985). Microtubules were
assembled from 92% bovine brain tubulin, 5% HiLyte 647 porcine brain tubulin (Cytoskeleton Inc., Cat # TL2670M), and 3% biotin-labeled porcine brain tubulin (Cytoskeleton Inc., Cat # T333P) at 50 µM tubulin dimer, and stabilized with 40 µM paclitaxel (Cytoskeleton Inc., Cat # TXD01) in BRB80 (80 mM K-PIPES, 1 mM MgCl₂, 1mM EGTA, at pH 6.8). Apyrase VII from potatoes was used for endophilin and Myo1c actin pelleting assays (Sigma Aldrich, Cat # A6535).

**PIP₂-GUVs** were formed as described below (Table 4.1) following previous work by Yamada et al., 2014, and composed of 20.5% porcine brain L-α-phosphatidylinositol-4,5-bisphosphate (referred to as PtdIns(4,5)P₂) (Avanti Polar Lipids, Cat # 840046X), 2.5% 18:1 1,2-dioleoyl-sn-glycero-3-[[N-(5-amino-1-carboxypentyl)iminodiacetic acid]succinyl] (nickel salt) (referred to as DGS-NTA(Ni)) (Avanti Polar Lipids, Cat # 790404C), 0.5% 18:1 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt) (referred to as Rho-PtdEth) (Avanti Polar Lipids, Cat # 810150C), and 76.5% 18:1 (Δ9-Cis) 1,2-dioleoyl-sn-glycero-3-phosphocholine (Referred to as PtdCho) (Avanti Polar Lipids, Cat # 851375C). Lipid Mix GUVs (LM-GUVs) were composed of 43.3% 18:1 (Δ9-Cis) 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (referred to as PtdEth) (Avanti Polar Lipids, Cat # 850725C), 18.4% PtdCho, 18.4% 18:1 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (referred to as PtdSer) (Avanti Polar Lipids, Cat # 840035C), 9.2% 18:1 1,2-dioleoyl-sn-glycero-3-phospho-(1'-myo-inositol) (ammonium salt) (referred to as PtdIns) (Avanti Polar Lipids, Cat # 850149P), 4.6% porcine brain sphingomyelin (Avanti Polar Lipids, Cat # 860062C), 3.0% PtdIns(4,5)P₂, 2.5% DGS-NTA(Ni), and 0.5% Rho-PtdEth.

Patterned coverslips were coated with 30 kDa mPEG-silane (Creative PEGWorks, Cat # PSB-2014), and blocked with Poly(L-lysine)-graft-poly(ethylene glycol) co-polymer
PLL-PEG was hydrated to 1 mg/mL in 10 mM HEPES (pH 7.4) and stored at 4 °C for up to 1 week. NeutrAvidin was ordered from Fisher Scientific (Cat # PI-31000); creatine phosphokinase from Rabbit muscle (C3755), phosphocreatine disodium salt hydrate (P7936), glucose oxidase from *Aspergillus niger* type VII (G2133), and catalase from bovine liver (C100) were ordered from Sigma-Aldrich.

II. Lipid Cargo

*Chapter 3—Preparation of Lipid Membrane Coated Beads (MCBs):*

Lipid membrane-coated beads (MCBs) were prepared as described, with modifications (Galneder et al., 2001; Pyrpassopoulos et al., 2013). A mixture of 99.8% DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine) (Avanti) and 0.2% biotin-PtdEth (1-oleoyl-2-(12-biotinyl(aminododecanoyl))-sn-glycero-3-phosphoethanolamine) (Avanti) were dried in a rotary evaporator under vacuum for 30 min at room temperature, and 2 mL HNa100 (100 mM NaCl, 10 mM Hepes, 1 mM EGTA, and 1 mM DTT) were added to the lipid film and vortexed. Resuspended lipids were subjected to 5 freeze-thaw cycles, in which the lipids were first frozen in an isopropanol-dry-ice bath followed by thawing while swirling by hand in a 37 °C water bath. Lipids and silica beads (1.0 ± 0.05 μm, Polysciences) were mixed with 600 μL of extruded lipids and 10 μL freshly washed beads. The bead-lipid mixture was incubated overnight at room temperature, washed three times with 1 mL HNa100, and then resuspended in to 200 μL in HNa100. Resuspended beads were blocked in 10 mg/mL casein, washed three times in HNa100, and mixed with 20 μM NeutrAvidin (Invitrogen). After three washes in BRB80, beads were resuspended in 600 μL BRB80 and stored on ice for up to two days. NeutrAvidin-blocked lipid beads were mixed with 20 nM (final solution concentration) of kinesin-1, incubated on ice for 10 min,
and then mixed with 4 μM CaM and either 200 nM (final solution concentration) Myo1c (diluted in BRB80 and 10 μM calmodulin), biotin (where biotin serves as a control on “kinesin-only beads” to modulate kinesin-1 concentration), or α-actinin-biotin. To aid in bead visualization and block the remaining biotin binding sites, 1 μL of 1 mM Atto-550 biotin (Sigma) was added to the beads and incubated on ice for 2 min. The beads were washed three times and then diluted 1:10 in the final buffer flowed into the chamber (see below).

Chapter 4—Preparation of Lipid Membrane Coated Beads (MCBs):

Silica beads 0.54 μm in diameter (Bangs Laboratories, Inc) were coated with membranes containing 2% PtdIns(4,5)P₂ and 98% PtdCho. PtdCho was mixed with 2 mol % of PtdIns(4,5)P₂ in a 50-mL round-bottom flask. The solution was thermally equilibrated in a water bath at 35°C for 5 min and dried rapidly (~1 min) in a rotary evaporator. The lipid film was kept under hard vacuum for at least another 30 min. The lipid film was dissolved in 2 mL of HNa100 (2.5 mM total lipid concentration) and multilamellar vesicles were formed by vortexing the flask for 2 min. Lipid solution was subjected to 4 freeze-thaw cycles using dry ice and isopropanol (Fischer) mix for freezing and water bath at 37 ºC for thawing. Using a mini lipid extruder (Avanti Polar Lipids, Inc.) lipid solution was extruded at room temperature (RT) 11 times through 30 nm pores (polycarbonate membranes, Whatman) to form small unilamellar vesicles (SUVs). 40 μL of silica beads solution 9.83% solid (Bangs Laboratories, Inc) were washed as follows: (1) 1 mL methanol (Fisher), (2) 1 mL 1N KOH water bath sonication for 15 min, (3) 7 times with 1 mL of distilled H₂O. All washings were done on a benchtop centrifuge for 2 min, 735 g, at RT (Centrifuge 5415C, Eppendorf). After the final washing beads were brought to their initial volume (~40 μL) and were mixed with 500 - 600 μL of SUVs. The mix of beads and SUVs was vortexed briefly.
at max speed and was let overnight at RT. Next day beads were washed 3 times with 1 mL HNa100 (2 min, 735 xg, at RT) to a final volume of ~ 500 µL. Right before bead motility assays 100 µL of lipid coated beads were transferred to motility buffer (60 mM MOPS, pH 7.0, 25 mM KCl, 1 mM EGTA, 1 mM MgCl₂).

Chapter 3—In vitro Membrane-Coated Bead Motility Assays:

Microscope coverslips were fashioned into an observation chamber (~20 µL volume) to allow the introduction of solutions via perpendicular flow (denoted as x- and y-directions) (Schroeder III et al., 2010; Schroeder III et al., 2012). A 18x18 mm (No. 1.5) coverslip was placed over four 0.0075 inch-thick Mylar shims (McMaster-Carr) on a 24 x 40 mm (No. 1.5) coverslip and sealed with UV-curable Norland-65 glue. Microtubules were bound to the coverslip using a polyclonal anti-β tubulin III antibody (Sigma or AbD Serotec), while actin filaments were bound to the coverslip using a truncated construct of the actin-binding domain of α-actinin (Greenberg et al., 2012). The 24 x 40 mm coverslip was coated with 0.01% colloidin diluted in amyl acetate (Electron Microscopy Sciences). Before introduction of experimental solutions, chambers were assembled and cured on a UV lamp for 30 min. Assembled chambers were used within 2 days.

For each experiment, 20 µL of β-tubulin antibody (1:10 to 1:50 dilution depending on the age of the antibody) followed by 20 µL of 20 µM α-actinin (both diluted in BRB80) was flowed into the chamber in the x-direction (5 min incubation each). The chamber was blocked with 40 µL 1 mg/ml BSA (Fisher Scientific), 10 mM DTT, and 10 µM Taxol (Cytoskeleton). 100 µL of microtubules (diluted 1:100 or 1:200 from stock) were flowed in the x-direction and incubated for 5 min. The chamber was washed with 4 - 5 chamber volumes of wash buffer (WB: 1 mg/ml BSA, 10 mM DTT, 10 µM Taxol, and 10 µM CaM), and the x-direction was sealed using vacuum grease. Phalloidin-stabilized actin filaments
(10 nM diluted in BRB80) were flowed into the chamber in the y-direction in 3 - 6, 100 µL volumes (depending on desired density of intersections) with 1 min incubation between volumes. The chamber was washed with 4 - 5 chamber volumes of WB. Finally, MCBs were flowed into the chamber diluted 1:5 in WB supplemented with 2.4 mM MgATP, 10 mM phosphocreatine (Sigma), 0.45 mg/mL creatine phosphokinase (Sigma), 3 mg/mL glucose, 100 µg/mL glucose oxidase (Sigma), and 40 µg/mL catalase (Sigma). We assume that sequential addition of microtubules followed by actin resulted in intersections with the actin filaments on top of the microtubules (Schroeder III et al., 2010; Schroeder III et al., 2012).

Tm2-actin was prepared by pre-incubating the proteins for 10 min at room temperature. Chambers for experiments performed in the presence of Tm2 were prepared as above, except 50 µL of 10 nM Tm2-actin and 5 µM Tm2 were flowed into the chamber 4 – 6 times with 1 min incubations between flows. After washing 4-times with WB, one chamber volume of 5 µM Tm2 in WB was flowed into the chamber. The final bead solution also contained 5 µM Tm2.

Chapter 4—In vitro Membrane-Coated Bead Motility Assays:

Glass coverslips 22 X 40-1.5 mm (12-544.B, Fisher Scientific) were coated with 4 µL of 0.1% solution of Nitrocellulose in Amyl Acetate (Electron Microscopy Sciences) and dried under a fume hood (~ 15 min). The Nitrocellulose coated coverslips were used to construct standard motility chambers (S.J. Kron, J.A. Spudich, PNAS, 83 (1986), pp. 6272–6276) using double-sided tape and vacuum grease. Solutions were added to the chamber in the following sequence: (1) 20 µM actin-binding domain of α-actinin for 10 min, (2) 1 mg/ml casein for 3 min (3), 50 nM filamentous actin stabilized with Rhodamine
Phalloidin in 1 mg/ml casein for 5 min, (4) washed with 1 mg/ml Casein, (5) mix of lipid-coated beads with 200 nM full length Myo1c, 10 µM Calmodulin, 1 mg/ml Casein, 1 mM MgATP, 20 µM DTT, 5 mg/ml glucose, 192 U/ml glucose oxidase, 48 µg/ml catalase. All protein and reagent solutions for each step were prepared in motility buffer (60 mM MOPS, pH 7.0, 25 mM KCl, 1 mM EGTA, 1mM MgCl$_2$). The open ends of the chamber was sealed with vacuum grease and it was placed on an inverted microscope (Leica, 100 x oil objective). Movies (S6) were recorded under simultaneous epifluorescence and transmission light illumination at RT.

Chapter 4—Preparation of Giant Unilamellar Vesicles (GUVs):

GUVs were prepared by mixing the lipids (described above) to 0.973 mM in chloroform, warming to 37 °C, thinly spreading on 37 °C-warmed Indium-Tin-Oxide (ITO) slides, and then drying in a vacuum desiccator for 2 hours or overnight. Next, lipid-coated slides were sandwiched with 300 mM sucrose in diH$_2$O and electroformed for 2.5 hours at 4 volts peak-to-peak and 5 Hz at 60 °C (Capraro et al., 2013; Chen et al., 2015). GUVs were used for no more than 48 hours at room temperature.

Chapter 4—In vitro reconstitution assay with GUVs:

Flow cells were assembled from patterned coverslips and cleaned glass slides formed into a chamber with double-stick tape to form 3 flow cells per coverslip with the pattern running perpendicular to flow cells. Each flow cell was treated as follows: (1) 25 µL of 0.25 mg/mL NeutrAvidin was incubated for 5 minutes; (2) 40 µL of casein wash buffer (30 mM DTT, 20 µM taxol, 15 µM CaM, and 1 mg/mL filtered casein diluted in pH 7.5 BRB80); (3) 20 µL of 0.1 mg/mL PLL-PEG block diluted in 10 mM HEPES pH 7.4 for 1 minute; (4) 40 µL casein block (30 mM DTT, 20 µM taxol in 5 mg/mL filtered casein
diluted in pH 7.5 BRB80) for 5 minutes; (5) 20 µL 1:100 taxol-stabilized microtubules with 3 x 30 second flows rapidly flowed into chamber (or to desired density on surface); (6) 3 x 20 µL washes with casein wash; (7) 20 µL of 1:10 phalloidin-stabilized F-actin incubated for 1 minute (or to desired density on surface); (8) 3 x 20 µL flows of casein wash buffer; (9) 10 or 20 µL of GUV flow mixture, depending on desired vesicle density in chamber. GUV flow: 10 nM kinesin-1 (dimer concentration) +/- 200 nM Myo1c +/- 8 or 80 nM endophilin (monomer concentration), 3.7 µL diH2O (to bring solution osmolarity to approximately 450 mOsm, which is slightly hypertonic to ~ 400mOsm electroformed GUV solution), 10 µL GUVs, and raised to 50 µL in Assay Buffer mixed (2.15 mM MgATP, 10 mM phosphocreatine, 0.45 mg/mL creatine phosphokinase, 1.1 mg/mL glucose, 2.37 mM DTT, 15 µM CaM, 1 mg/mL filtered casein, diluted in BRB80 pH 7.5, and mixed with glucose-oxidase-catalase before being mixed with motors and GUVs and flowed into the chamber).

III. Other Assays

Gliding Assays (Chapter 3):

Myo1c gliding assays were performed as previously described (Lin et al., 2005). In Tm2-AF gliding assays, 5 µM Tm2 was included in the final buffer.

Myo1c and endophilin co-pelleting assay (Chapter 4):

Polymerized 10 µM Human platelet G-actin was mixed 1:1:1.1 with unlabeled phalloidin in MB at room temperature for 1 hour, tapped gently to mix. Samples were mixed to a final concentration of: 2 µM F-actin; 20 µg (481.9 µM) Myo1c; 0 µg, 2 µg (283 nM), 5 µg (708.5 nM), 10 µg (1416.9 nM), or 20 µg (2833.9 µM) endophilin; 15 µM calmodulin; 0.05 units/mL apyrase VII or 1 mM MgATP; and BRB80 pH 7.5. Final reaction volume was
250 µL. All components were mixed in ultracentrifuge tubes and incubated at 4 °C for 30 minutes. Pelleted in Beckman TLA120.2 at 100 krpm for 20 minutes. Supernatant was separated from pellet, mixed with 5x sample buffer, and run on coomassie gel. The amount of endophilin in the pellet was compared with Myo1c in the presence or absence of MgATP at a range of endophilin concentrations.

*Preparation of patterned coverslips:*

Coverslip washing, coating, and patterning was adapted from Azioune et al., 2010; Boujemaa-Paterski et al., 2014; Portran et al., 2013; Reymann et al., 2014. 24 x 40 mm #1.5 coverslips were rinsed with 100% ethanol, wiped with a kimwipe, rinsed with running milliQ water for 15 minutes, and then dried in a filtered-air cell culture hood. Next, coverslips were bath-sonicated for 30 minutes at room temperature in acetone, rinsed with ethanol, and then incubated in 100% ethanol at room temperature for 10 minutes. Coverslips were then rinsed under running milliQ water for 10 minutes, and incubated in 2% Hellmanex III detergent for 2 hours or overnight. Finally, coverslips were vigorously rinsed with running milliQ water for 30 minutes and then dried in the filtered-air hood. For best results, coverslips were coated in PEG-silane and patterned within a couple of days. To coat with PEG-silane, coverslips were first plasma cleaned for 2 minutes, and then incubated in 1 mg/mL PEG-silane resuspended in 110-proof (55%) ethanol and 10 µL concentrated hydrochloric acid, diluted in diH₂O. Coverslips were incubated on a room temperature gentle shaker, covered from light, overnight. The next morning, coverslips were rinsed 3 times in 100% ethanol, and then bath sonicated 3 x 5 minutes in 100% ethanol at room temperature, before being rinsed twice with diH₂O, and dried in filtered air hood.
Coverslips were patterned by using an IX-255 MicroX Excimer Laser at 193 nm in low fluence, RVA mode. A 7 mJ fiducial line was drawn perpendicular to the parallel pattern used in the cytoskeletal assay. Parallel lines were made with a 5 mJ laser intensity at highest attenuation (45°), an approximately 3.2 µm diameter, 1 burst-per-spot, 100 Hz laser rep-rate, with approximately 20 µm spacing between lines, and 20 lines per pattern (two patterns per coverslip). Coverslips were stored at room temperature in an amber-colored jar with desiccant and used for up to two weeks or until non-specific sticking on surface caused ablation of cytoskeletal patterning.

IV. Microscopy

Chapter 3:

For Chapter 3, all microscopy was performed at 22 °C on an inverted epifluorescence microscope (Leica) with a mercury lamp and filters centered at 560 nm and 488 nm emission. Data for Figures 3.3 and 3.7-3.9 were acquired at 488 nm using a 200 ms exposure, 2x2 bin, at 0.5 fps (frames per second) for 5 min. Data for Figure 3.4 was acquired using the Metamorph (Molecular Devices) “streaming” feature at 488 nm and 10 fps for 1 min. For each field of view, images were taken before and after every movie at both 560 nm and 488 nm wavelengths to verify which intersections were composed of actin and microtubules (both cytoskeleton types can be seen at 560 nm, but only microtubules and GFP-kinesin on the beads can be visualized at 488 nm). In the acquired movies, the beads were visualized by kinesin-1-GFP fluorescence on the bead.

Chapter 4:

Microscopy was performed at 22 °C on two different inverted TIRF microscopes: (1) a dual-view Leica TIRF microscope with 488 nm Melles Griot 43 series ion laser and
633 nm Optoengine lasers, Photometrics Evolve EMCCD camera, and Metamorph (Molecular Devices) imaging software. We used this setup for all PIP₂-GUV experiments, and acquired images at 1 frame per second for 10 minutes. (2) Alternatively, a PerkinElmer Nikon Eclipse Ti TIRF with 488 nm, 561 nm, and 640 nm lasers and Volocity image acquisition software was employed. LM-GUV movies (and one round of PIP₂-GUV experiments with 80 nM endophilin to control for microscope variability—none found) were acquired with 488 nm and 640 nm lasers acquiring at 2 frames/minute, and a 561 nm laser acquiring at 2 frames/second.

Experiments containing membrane-coated beads were imaged on a Leica epifluorescence microscope using a 100x oil objective. Movies were recorded under simultaneous epifluorescence and transmission light illumination at RT with an acquisition rate of 2 seconds/frame, and a movie of 20 minutes.

Image Analysis:

Chapter 3:

Landing: Cytoskeleton filaments were sparsely coated on the coverslip surface so that fewer than 30 AF/MT intersections were present per field of view. Processive microtubule-based MCB runs were selected for kymograph analysis along the microtubule. Landing distances from the nearest AF (Figures 3.3, 3.8C) or MT (Figure 3.8D) intersection were quantitated by measuring the distance between the centroid of the intensity of the bead in the first frame of processive motility, and the centroid of mass of the actin intersection. Landing events were scored as initiating at the AF/MT intersection if they occurred within 0.5 μm from the nearest actin intersection. It was necessary to increase our image acquisition to 10 fps in order to capture these events, as kinesin-1
motility is approximately 1 μm/s and many MCBs that landed at the AF/MT intersection would be farther than 0.5 μm from the intersection from one frame to the next. We used a random number generator (random.org) to find random x and y points in the same fields of view from the quantitated movies. If a 1 μm circle at the randomly generated x/y points overlapped with a microtubule with a corresponding actin filament intersection, we measured the distance between the center of the 1 μm circle over the microtubule and the center of the nearest actin intersection. These randomly generated points serve to normalize for the density of AF/MT (or MT/MT) intersections in a given field of view.

**Run Termination Behavior:** Run termination events at AF/MT (or Tm2-AF/MT) intersections were quantitated as “detached,” “passed,” or “paused” by viewing kymographs of these runs in relation to AF intersections. In “detached” events, MCBs terminated their run (ceased processive motility) at the AF/MT intersection without a pause of any length detectable. The shortest pause detectable by our acquisition parameters was 0.5 s. “Passed” events were ones in which MCBs passed the AF intersection via processive motility without pausing. Although both actin and microtubules could be seen in the rhodamine channel, microtubules were doubled labeled with HyLite488. Only intersections in which a single actin filament crossed a single microtubule were analyzed. Bundled filaments were detected by increased fluorescence along the length of the AF or MT, irregular shape, or bidirectional kinesin motility, and were not analyzed.

**Gliding Assays:** Directionally gliding actin filaments were identified by overlaying the first and last frame on the maximum intensity projection of a movie acquired at 0.5 fps for 10 min. In directionally gliding filaments, the first and last frame overlapped with the maximum intensity projection in a continuous line. Filaments were scored as not gliding if they were stuck in place for the duration of the movie, or if the first and last frames did not
overlap with the max projection into a continuous line. Over 150 filaments were analyzed per condition, with 3 fields of view per chamber, and one chamber per Myo1c concentration. This experiment was repeated on two separate days.

**Stably Bound MCBs:** MCBs were described as being “stably bound” to either actin filaments, microtubules, or AF/MT intersections if the cargo was seen to land during the course of the movie onto a clearly-differentiable actin filament and/or microtubule and remained at that same position until the cytoskeleton was imaged following the conclusion of the 5 minute acquisition (Figure 3.6). At least 50 stably bound MCBs were observed for each condition, with significantly more actin-bound MCBs when containing α-actinin than with either kinesin-1-alone or kinesin-1 and Myo1c. The “average number of recorded events per chamber” (Figure 3.6) was determined by averaging the number of measured “detach,” “pass,” or “pause” events over the number of experimental chambers observed.

**Chapter 4:**

Events at actin/microtubule intersections were observed for the distance between microtubule-based run initiation and the nearest actin intersection (i.e. did the event “land” at the actin/microtubule intersection or switch from actin- to microtubule-based transport?), and the deformation of the vesicle while at the actin/microtubule intersection. Deformation was measured by drawing a 1-pixel-wide kymograph along the length of the microtubule and then measuring the longest and shortest lengths of the GUV over time, along the microtubule while at the actin intersection. The threshold for “deformed” GUVs was set at 2 standard deviations from the mean of kinesin-1-only longest-divided-by-shortest GUV length changes (designated as β) along the microtubule while at the actin intersection (β ≥ 1.36). Thus, GUVs with β ≥ 1.36 at an AF/MT intersection were classified as a
“deformation” event, unless a membrane extension of diffraction-limited width was pulled along the microtubule, in which case it was classified as a “tubulation” event. Deformation and tubulation were also assessed for the absolute length of the membrane growth along the microtubule by subtracting the shortest length from the longest length along the microtubule while at the actin intersection (as determined by a kymograph drawn along the length of the microtubule). Tube elongation velocity was measured for each tubulation event observed, including each tubulation event for every GUV with observed tubulation, whereas the analysis for the longest/shortest or longest-shortest analysis only included the longest tubulation event by a GUV at an individual AF/MT intersection. Morphologically dynamic GUVs were not analyzed for their shape change at actin/microtubule intersections in order to isolate kinesin-1-driven membrane shape changes along the microtubule. GUVs were also classified as “passing” or “pausing” at actin/microtubule intersections when already in the process of microtubule-based transport. A “pause” is classified as a halt in transport visible by kymograph, thus, a pause ≥ 2 seconds in length.

Actin-based transport, deformation, and tubulation were quantitated as a fraction of all events in at least 3 fields-of-view across at least 3 different vesicle preps. Actin-based deformation and tubulation were quantitated similarly to deformation and tubulation at actin/microtubule intersections, by drawing a line along the length of the actin and then measuring the length of the vesicle at its longest and shortest length during the course of transport. The thresholds for deformation and tubulation were the same used at actin/microtubule intersections, where deformation was classified as β ≥ 1.36, and a tubulation event is a membrane extension of a diffraction-limited width. Morphologically dynamic GUVs were designated by observing the GUV shape as it approached the cytoskeleton within the TIRF field. If the GUV showed any hint of having a non-spherical
shape (i.e. membrane blebs, extensions, tubes, etc.), then it was classified as “morphologically dynamic,” rather than “spherical.” Myo1c-driven GUV velocity was quantitated by using a kymograph drawn along the actin-based transport path and measuring the velocity of constant-velocity segments during GUV transport. We normalized the velocity of each segment by the length of time spent at that velocity to obtain the average velocity of 0.014 µm/s (Figure 4.2E).

V. Statistical Analysis

We used the Kruskal-Wallis test with Dunn’s multiple comparison post-hoc to compare multiple non-parametric variables. In many cases, bootstrapping was used to resample the data and generate standard deviation error bars. Box plots illustrate the 25th and 75th percentiles of the data with a central line at the median, and the whiskers extending to the minimum and maximum points.
I. Summary

We investigated how actin and microtubule-based molecular motors coordinate to sort, deform, transport, and target cellular cargo to their specific destinations in space and time. We approached these investigations by examining the interplay between a canonical microtubule-associated, processive motor, kinesin-1, and a non-processive, actin filament-based motor, Myo1c. We selected these two motors because they allow us to investigate the interplay between processive and non-processive motors, as well as microtubule and actin filament-based transport. Additionally, these motors are both found at locations in the cell where cargo sorting and membrane deformation often occur (Boguslavsky et al., 2012; Brandstaetter et al., 2012; Hunt et al., 2013; Semiz et al., 2003). We took advantage of in vitro reconstitution techniques in which we observed the behavior of purified motors on artificial cargo at engineered cytoskeletal intersections. These techniques allow us to investigate the minimal components necessary to recreate observed cellular physiology, and avoids the limitations of cytoskeleton and motor resolution, as well as biological redundancy, that confound traditional cell biological imaging studies.

We initially observed the behavior of kinesin-1 and Myo1c on lipid membrane-coated beads (MCBs) at individual actin filament/microtubule intersections (Chapter 3). These MCB cargo allowed for the reorganization of motors around the cargo in response to the local cytoskeletal environment, without adding the additional complication of membrane deformation. Here we found that Myo1c can initiate and terminate kinesin-1-
driven microtubule-based transport at individual actin filament/microtubule intersections. Furthermore, these Myo1c-based effects could not be replicated by the actin-binding domain of α-actinin rather than Myo1c, and were abolished in the presence of nonmuscle tropomyosin-2 (Tm2/Tpm1.6). These results suggest that one mechanism for cargo sorting and delivery to specific subcellular locations may be in the affinity of motors for specific types of tracks; in other words, Myo1c ignores tropomyosin-coated actin filaments, preferentially terminating kinesin-1-driven microtubule-based transport at the highly dynamic actin just beneath the plasma membrane in preparation for cargo fusion.

We went on to characterize the interplay between Myo1c and kinesin-1 on deformable giant unilamellar vesicles (GUVs) at micropatterned arrays of actin and microtubules that replicate physiological cytoskeletal organization (Chapter 4). In canonical somatic cells such as fibroblasts, the cytoskeleton is organized such that microtubules are largely radially oriented outward from the centrosome toward the plasma membrane, resulting in relatively little microtubule-microtubule overlap (Bartolini and Gundersen, 2006). In contrast, actin filaments are found densely populated at ER and trans golgi exit sites (Carreno et al., 2004; Cobbold et al., 2004; Egea et al., 2015; Gurel et al., 2014) and beneath the plasma membrane where endosomal recycling often occurs (Cao et al., 1999; Puthenveedu et al., 2010), resulting in high AF/MT overlap in these regions of intracellular sorting and remodeling.

We found that the ability of Myo1c to halt kinesin-1-driven transport of PtdIns(4,5)P<sub>2</sub>-rich cargo (PIP<sub>2</sub>-GUVs) at actin/microtubule intersections provides a mechanism for tethering the cargo, and enables kinesin-1-driven deformation along microtubules. Myo1c likely plays a direct role in this deformation, since the use of a biotin-NeutrAvidin linkage to halt kinesin-1-driven transport was unable to replicate the frequency
of deformed PIP$_2$-GUVs. Addition of endophilin to PIP$_2$-GUV experiments containing both kinesin-1 and Myo1c resulted in a significantly increased frequency of tubulation along microtubules at actin/microtubule intersections. We also found that the full length Myo1c construct binding to PtdIns(4,5)P$_2$ in GUV membranes is capable of transporting, and even deforming, vesicular cargo along actin filaments. Endophilin enhanced the deformation and tubulation of PIP$_2$-GUVs by Myo1c along actin filaments. Interestingly, when we switched the lipid composition to a more physiological lipid mixture (LM-GUVs), we saw robust tubulation along microtubules by kinesin-1 and Myo1c at actin/microtubule intersections. In this case, addition of endophilin did not enhance kinesin-1-driven tubulation, but strongly enhanced Myo1c-driven tubulation along actin filaments, particularly in the morphologically dynamic LM-GUV population. These results suggest that lipid composition can differentially regulate deformation and tubulation by kinesin-1, Myo1c, and endophilin. In the following sections, I will further explore the implications of these results, as well as propose potential avenues for future investigation.

II. Kinesin-1 and Myo1c on cellular compartments

Both kinesin-1 and Myo1c are found on GLUT4-containing exocytic cargo, which have been described as “tubulovesicular” cargo, suggesting that they undergo shape change during transport. GLUT4 transmembrane glucose transporters are stored in perinuclear recycling and storage compartments until they are transported to the plasma membrane upon insulin stimulation. These vesicles are transported along microtubules from the perinuclear region to the cortical region of the cell just beneath the plasma membrane (Semiz et al., 2003). At this point, actin-based transport takes over in the form of Myo1c and myosin-V molecular motors (Boguslavsky et al., 2012; Chen et al., 2012; Ishikura and Klip, 2008). Before our investigations, it was hypothesized that Myo1c is
acting as either a dock/tether, undertaking slow, actin-based transport, or facilitating plasma membrane fusion (Boguslavsky et al., 2012; Bose et al., 2002; Bose et al., 2004; Yip et al., 2008); however, it was unclear how Myo1c was functioning at a molecular level, particularly in relation to the kinesin-1-driven transport along microtubules.

We initially investigated the interplay between kinesin-1 and Myo1c in a minimalist environment where motors attached to a membrane-coated bead were observed as they interacted with an individual actin filament/microtubule intersection. Our investigations support the hypothesis that Myo1c can act as a cargo dock/tether, halting kinesin-1-driven transport beneath the plasma membrane where highly dynamic, non-tropomyosin-coated actin is found. This idea that tropomyosin-regulation of Myo1c may affect GLUT4 transport to the plasma membrane was supported by in vivo work by the Gunning lab (Kee et al., 2015). They found that over-expression of Tpm3.1 (Tm5NM1) caused an increase in glucose uptake in insulin-responsive muscle and adipose tissues. With Tpm3.1 over-expression, increased GLUT4 was localized to the membrane. Since Tpm3.1 also inhibits Myo1c interaction with actin filaments in an in vitro gliding filament assay, the authors hypothesized that increased tropomyosin-coated actin prevented Myo1c cargo docking, and promoted GLUT4 flux to the plasma membrane, resulting in increased glucose uptake (Kee et al., 2015). Although it has been previously hypothesized that low and high molecular weight tropomyosins (as designated by alternative splicing) may differentially regulate myosin interactions, Tpm3.1 is considered a low molecular weight tropomyosin isoform, while Tpm1.6 is considered a high molecular weight tropomyosin, and both inhibit Myo1c functioning (Gunning et al., 2005; Kee et al., 2015; McIntosh et al., 2015). It is still unknown whether Myo1c will productively interact with any tropomyosin isoform, yet
elucidation of different tropomyosin isoform localizations and interacting proteins is an active area of research for many groups.

Our investigations looking at interaction between kinesin-1 and Myo1c on fully deformable giant unilamellar vesicles (GUVs) support these initial conclusions. Myo1c attached to GUVs using the physiological interaction between the PH domain in its tail and PtdIns(4,5)P_2, halted kinesin-1-driven GUVs even more robustly than MCBs at actin filament intersections. Interestingly, we also found that 60% of all GUVs deformed along the microtubule at actin intersections. This deformation depends on free microtubules available for the kinesin to interact with on the other side of the actin intersection. It would be very interesting to look at where GLUT4 cargo are docked beneath the plasma membrane in relation to microtubule ends. It was found by a previous student in the Ostap lab that GLUT4 cargo tend to fuse to the plasma membrane when near, but not on, a microtubule (Dawicki-McKenna et al., 2012). This suggests that there is a switch from microtubule-based to actin filament-based transport. Interestingly, we never saw vesicle or membrane-coated bead motility switch from microtubules to actin, only actin to microtubules. Perhaps Myo1c halts kinesin-1-driven transport at actin intersections, tethering the cargo until the microtubule undergoes catastrophe, leaving the vesicle behind. Another hypothesis is that deformation of the vesicle at actin/microtubule intersections may be important for activation of fusion machinery. For example, during the exocytosis of matrix metalloproteases (MMPs), the deformation of exocytic vesicles by dynein-dynactin, kinesin-1, and WASH actin-based remodeling is essential for appropriate exocytosis and membrane fusion (Marchesin et al., 2015; Monteiro et al., 2013). It would be very interesting to see which, if any, myosins are involved in this MMP exocytic process, providing a nice model system for probing the complexity of tubulation/deformation of a
cargo by microtubule opposing force motors, actin-based membrane deformation, and/or myosin effectors.

It is still unclear how myosin-V fits in to this model where kinesin-1 transports vesicles to the cell periphery and where Myo1c then tethers, and perhaps facilitates deformation or tubulation of vesicles along microtubules. One hypothesis is that Myo1c acts to halt and tether kinesin-1-driven transport at actin/microtubule intersections, where myosin-V can then initiate actin-based transport and perhaps facilitate membrane fusion. This switch from kinesin-1-driven microtubule-based transport, to tethering at actin/microtubule intersections by Myo1c, to actin-based transport by myosin-V, could be directly tested using the same in vitro reconstitution methods utilized in this thesis work. Interestingly, myosin-V is a processive, actin-based motor that prefers to walk along tropomyosin-coated actin (Clayton et al., 2014; Sckolnick et al., 2016), and it is unclear how this preference for tropomyosin-coated actin spatially relates to the avoidance of tropomyosin-coated actin by Myo1c during the final stages of GLUT4 transport.

Another interesting phenomenon discovered in the membrane-coated bead and GUV studies was the propensity for kinesin-1-driven cargo to initiate microtubule-based runs at actin filament intersections in the presence of Myo1c. It is largely unknown how microtubule-based transport is initiated, particularly in the perinuclear region. In the periphery, for instance at the distant growth cones of neurons, studies have shown that dynein-dynactin loading onto microtubule plus-ends is aided by a range of accessory factors such as end-binding (EB) proteins and CLIP-170 (Moughamian et al., 2013; Nirschl et al., 2016). The cell center is dense with microtubules, actin, intermediate filaments, organelles, etc., and it is very difficult to resolve using traditional fluorescence microscopy techniques. It is known that branched, Arp2/3 actin is often found at sites of membrane
deformation, such as the trans Golgi network, so it’s possible that this is one site where actin-based transport can lead to microtubule-transport initiation (Almeida et al., 2011). Another organelle where both Myo1c and kinesin-1 are found are lipid raft recycling cargo, and Myo1c may be important for facilitating microtubule loading here, as well. It would be interesting to probe these phenomena further using super resolution microscopy techniques, for instance light sheet microscopy, which can be used to image thin layers through a cell at high time and spatial resolution.

III. Lipid composition investigations

Another conclusion from our experiments is that the lipid head group composition of a vesicle can drastically alter the frequency with which it is deformed or tubulated at actin filament/microtubule intersections or along actin alone. We found that GUVs composed of a high percentage of PtdIns(4,5)P₂ in a PtdCho background are significantly less deformable/tubulated at actin/microtubule intersections and during actin-based motility of Myo1c than GUVs composed of a more physiological lipid mixture with PtdEth, PtdSer, PtdCho, PtdIns, Sphingomyelin, and PtdIns(4,5)P₂. This is very interesting because the physiological lipid mixture is modeled after an inner plasma membrane/late exocytic membrane lipid head group composition. However, one of the most striking features of the plasma membrane is the high concentration of cholesterol. Thus, we would like to test the deformation/tubulation of GUVs with a higher percentage of cholesterol in the membrane in addition to varying the lipid head group composition. One hypothesis is that as lipid composition changes over time during exocytic or endocytic transport, this could be one mechanism for controlling the deformation/tubulation of vesicular cargo in space and time. This hypothesis could be tested by investigating the deformability of membranes of different head group composition in the same reconstitution assay.
employed in Chapter 4. This system would enable us to probe how tubulation by both a
processive, and a non-processive motor is effected by membrane composition. However,
lipid head group variability is only one way in which different lipids bring different features
to particular membranes. Other important factors to consider are clustering or the level of
(dis)orderedness, and acyl chain variability.

*In vitro* reconstitution studies tend to be accomplished by using DOPC (1,2-
dioleoyl-sn-glycero-3-phosphocholine) or other 1,2-dioleoyl lipids, which contain two
unsaturated hydrocarbon chains that each have a single double bond. Interestingly,
dioleoyl lipids compose a very small portion of the plasma membrane, trans Golgi, and
endosomal compartments, where a higher percentage of saturated acyl chains are found
in these regions (Bigay and Antonny, 2012; Levental and Veatch, 2016; van Meer et al.,
2008). Rather, these dioleoyl lipids are used because of their high fluidity and stability in
a planar bilayer and during electroformation of giant unilamellar vesicles and in extruded
liposomes. Most cellular lipids have one saturated and one unsaturated hydrocarbon tail,
for example POPC (1-palmitoyl-2-oleyl-sn-glycero-3-phosphocholine) (Levental et al.,
2016). These 1-palmitoyl-2-oleyl lipids may start to form ordered regions or microdomains
at room temperature, particularly in a mixture of PtdEth and PtdSer; however,
microdomains rapidly form in the presence of cholesterol (Bakht et al., 2007).
Polyunsaturated lipids are also found in cells, particularly at sites of high endo- and
exocytosis like synaptic vesicles and neuronal synaptic terminals, where up to 80% of
phospholipids contain at least one polyunsaturated acyl chain (Marszalek and Lodish,
2005; Takamori et al., 2006). Through *in vitro* reconstitution assays, direct perturbation of
cells, and molecular dynamic modeling, Pinot et al., 2014 found that these polyunsaturated
lipids dramatically increase the ability of membrane curvature sensing and stabilizing proteins endophilin and dynamin to bind, deform, and undergo membrane scission.

IV. **BAR protein investigations**

We were surprised to see differences in the effect of endophilin on PtdIns(4,5)P$_2$-rich GUVs in comparison to LM-GUVs. We found that PIP$_2$-GUV tubulation along microtubules and actin is highly dependent on the presence of endophilin, whereas LM-GUVs can deform and tubulate along both microtubules and actin filaments in the absence of endophilin. These conclusions raise questions as to whether the differences are purely due to the competitive binding of Myo1c and endophilin to the GUV membrane, or whether the lipid head group composition can directly regulate endophilin binding. We could differentiate between these two possibilities by modulating the concentrations of Myo1c and endophilin relative to one another. We would hypothesize that if Myo1c is outcompeting endophilin for binding, reducing the Myo1c concentration relative to endophilin will increase membrane deformation observed at actin/microtubule intersections. Alternatively, we could directly investigate endophilin binding to GUVs of different lipid headgroup composition by using the micropipette aspiration technique which can measure membrane curvature generation relative to local protein density (Zhu et al., 2012).

It would also be interesting to probe how different types of BAR domain proteins can affect the deformation of actin filament and/or microtubule-transported cargo. Endophilin is an N-BAR protein which inserts an N-terminal helix directly into the membrane during its binding and stabilization of curved membranes. Would an F-BAR or traditional BAR domain protein also be able to enhance deformation and tubulation? With the exception of endocytosis, it is largely unknown which BAR domain proteins are involved in which specific membrane deformation processes, particularly in relation to
endosome and organelle tubulation where sorting nexins are also implicated (van Weering et al., 2012a; van Weering et al., 2012b). Our micropatterned cytoskeletal arrays could be easily used to systematically probe the deformability of different BAR domain proteins in relation to different membrane composition, cytoskeletal geometries, and molecular motors to answer these questions. Further, it would add additional complexity to have physiological binding strategies for the processive motors to the vesicle membranes, for instance, to probe the effect of full-length kinesin-1 binding GUVs through its proposed binding to PtdIns(4,5)P$_2$, rather than via a biotin-NeutrAvidin or His-NTA(Ni) interaction in a constitutively active protein. We would hypothesize that the dynamic binding of kinesin-1 to membranes would add autoregulatory dynamics that may expose additional effects upon vesicle transport and tubulation; for instance, we observed that Myo1c dynamic binding to its physiological membrane attachment revealed the ability for Myo1c motors to transport, deform, and tubulate membrane-bound cargo along actin filaments.

Another outstanding question is, when and how is membrane scission regulated? Dynamin is largely thought to be responsible for membrane scission within cells, particularly during endocytosis; however, endophilin also appears to be instrumental for this membrane scission (Daumke et al., 2014; Neumann and Schmid, 2013; Renard et al., 2015; Roux et al., 2006; Takei et al., 1999). I am particularly curious whether we can use Myosin-Ie, endophilin, dynamin, and perhaps other BAR domain proteins in the endocytic signaling cascade to reproduce membrane deformation tubulation, and scission \textit{in vitro}. Myo1e is known to participate in endocytosis, but whether it is simply acting in signaling cascades to help recruit dynamin and synaptojanin (and possibly endophilin), or whether it is important for membrane deformation is still unknown (Cheng et al., 2012; Krendel et
Using in vitro reconstitution tools such as the ones developed during this thesis work could provide valuable insight into these cellular processes.

V. Tubulation by actin filaments

Finally, in addition to being deformed by BAR domain scaffolds and actin filament and microtubule-based molecular motors, cellular membranes have also been found to undergo deformation, tubulation, and scission directly mediated by actin filament polymerization. In many cases, this actin polymerization is thought to be initiated by recruitment of Arp2/3 nucleation promotion factors such as WASP to sites of membrane tubulation or fission. Interestingly, many BAR domain proteins have additional domains that can recruit these Arp2/3 activators (Suetsugu, 2009; Takano et al., 2008). It is still a mystery how induced actin polymerization interacts with motor-driven and scaffold-driven (BAR protein) membrane curvature generation, stabilization, and fission. Here, we could use full-length BAR proteins that can directly recruit actin filament nucleation and polymerization-promoting factors, mixed with reconstituted membranes, and even with different molecular motors to better understand the interplay between these various types of generating membrane deformation and tubulation of intracellular transport cargo.

VI. Final thoughts

Although many research labs around the world have investigated the transport and deformation of intracellular cargo by microtubule motors, actin motors, BAR proteins, or membrane properties, very little work has been done connecting between these regimes. Within cells, these transport and deformation processes require both cytoskeleton types, as well as a host of accessory proteins. During this thesis work, we have explored the interplay between these disciplines: processive and non-processive motors, microtubule and actin-based transport, non-deforming and deformable membrane-bound cargo, varied
cytoskeletal geometries and compositions, BAR proteins, and lipid membrane composition. We examined these components in a systematic fashion in which we explored which motor, cytoskeletal, and membrane components, when mixed together, recreate observed cellular physiology. There are a wide range of scientific questions still available to pursue using many of the same in vitro reconstitution techniques utilized throughout this project to increasingly add complexity and expand our understanding of cellular physiology.
**APPENDIX: SUPPLEMENTAL VIDEO LEGENDS**

**Movie S1: Myo1c initiates kinesin-1-driven runs at engineered AF/MT intersections.**

Lipid membrane-coated beads (MCBs) containing kinesin-1-only or kinesin-1+Myo1c were observed as they initiated kinesin-driven motility along microtubules. The movie on the left shows an example run of a kinesin-1-only MCB initiating a microtubule-based run stochastically in relation to the nearest actin filament intersection (See Figure 3.3A). The movie on the right shows a sample initiation event of a kinesin-1+Myo1c bead that begins at an AF/MT intersection (See Figure 3.3B). The movie is played back in real time (2 fps). Scale bar = 1 μm. Timestamp labels landing event at “0 s.”

**Movie S2: Myo1c halts kinesin-1-driven MCBs at engineered AF/MT intersections.**

We observed the behavior of MCBs traveling along microtubules via kinesin-1-driven transport as they encountered actin filament intersections. The movie on the left shows a sample kinesin-only MCB that passes the AF/MT intersection (See Figure 3.4A). The center and right movies show sample kinesin-1+Myo1c events that pause at the AF/MT intersection for 20 s (center) and 220 s (right), respectively (See Figure 3.4B). The movie is played back at 3x real time (6 fps). Scale bar = 1 μm.

**Movie S3: Nonmuscle Tm2 inhibits Myo1c-driven actin gliding.**

Actin filament gliding assays were performed with 50 nM Myo1c in the presence or absence of Tm2 and assessed for directional gliding. The movie on the right shows a sample non-Tm2 gliding assay with directionally gliding actin filaments, while the movie on the right shows a sample Tm2-actin gliding assay with transient Myo1c-actin
interactions (See Figures 3.7A and 3.7B). The movie is played back at 40x real time (20 fps). Scale bar = 1 μm.

**Movie S4: Nonmuscle Tm2 regulates Myo1c interactions at Tm2-AF/MT intersections.**

We observed the behavior of kinesin-1 MCBs in the presence or absence of Myo1c at they approached Tm2-AF/MT intersections. Both kinesin-1-only (movie on left) and kinesin-1+Myo1c MCBs (right) pass Tm2-AF/MT intersections. The movie is played back at 3x real time (6 fps). Scale bar = 1 μm.

**Movie S5: PIP$_2$-GUVs can switch from AF- to MT-based transport.**

PIP$_2$-GUVs containing both kinesin-1 and Myo1c can switch from AF-based transport to MT-based transport, as indicated by t = 0 s in time stamp. Cytoskeleton shown at the beginning of the movie with AF in purple and MT in green. During the movie, AF is shown in grey and GUV is shown in magenta. Scale bar = 1 μm. Played back at 10x real time.

**Movie S6: Myo1c can transport 2% PtdIns(4,5)P$_2$-containing membrane-coated beads along AF in the presence (left), but not absence (right) of ATP.**

During the movie, AF is shown in grey (fluorescence) and 0.5 μm membrane-coated beads are white (DIC). Scale bar = 1 μm. Played back at 50x real time.
Movie S7: Kinesin-1-only PIP₂-GUVs do not deform, while PIP₂-GUVs containing both kinesin-1 and Myo1c deform at AF/MT intersections.

Kinesin-1-only PIP₂-GUVs are largely non-deforming as at AF/MT intersections (top movie). PIP₂-GUVs containing both kinesin-1 and Myo1c (bottom movie) deform at AF/MT intersections, vesicle of interest indicated by arrow. Cytoskeleton shown at the beginning of the movie with AF in purple and MT in green. During the movie, AF is shown in grey and GUV is shown in magenta. Scale bar = 1 μm. Played back at 5x real time.

Movie S8: PIP₂-GUVs containing kinesin-1 and Myo1c rarely tubulate at AF/MT intersections.

Example of a PIP₂-GUV landing on an AF, undergoing AF-based transport, switching to MT-based transport and deforming along MT, then tubulating at second AF intersection along the MT. Cytoskeleton shown at the beginning of the movie with AF in purple and MT in green. During the movie, AF is shown in grey and GUV is shown in magenta. Scale bar = 1 μm. Played back at 5x real time.

Movie S9: In the presence of 8 nM endophilin, PIP₂-GUVs containing kinesin-1 and Myo1c readily tubulate at AF/MT intersections.

Example tubulation of GUV at AF/MT intersections in the presence of 8 nM endophilin. PIP₂-GUV is motile along MT until encountering AF patterned on the coverslip, at which point transport halts and MT-based tubulation is initiated. Cytoskeleton shown at the beginning of the movie with AF in purple and MT in green. During the movie, AF is shown in grey and GUV is shown in magenta. Grey fluorescence on growing tube is a combination of fluorescence from Rhodamine-PtdEth and GFP-kinesin-1. Scale bar = 5 μm. Played back at 10x real time.
**Movie S10: Tubulation of LM-GUVs along AFs.**

Both spherical (left) and morphologically dynamic (right) LM-GUVs tubulate along AF, even in the absence of endophilin. Cytoskeleton shown at the beginning of the movie with AF in purple and MT in green. During the movie, MTs are shown in grey and the GUV is shown in magenta. Scale bar = 1 μm. Played back at 20x real time.

**Movie S11: Tubulation of LM-GUVs along AFs and MTs in the presence of 80 nM endophilin.**

Tubulation of a spherical LM-GUV along AF by Myo1c (left) is more common in the presence of 80 nM endophilin. (Right) Example tubulation event that switched from tubulation along AFs to tubulation along MTs. Cytoskeleton shown at the beginning of the movie with AF in purple and MT in green. During the movie, MTs are shown in grey and the GUV is shown in magenta. Scale bar = 1 μm. Played back at 20x real time.


