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UNIVERSITY OF CALIFORNIA,
IRVINE

Regulation of motor proteins by signaling kinases

DISSERTATION

submitted in partial satisfaction of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

in Biological Sciences

by

Dail Elisse Chapman

Dissertation Committee:
Professor Steven P. Gross, Chair
Professor Tom F. Schilling
Professor Diane K. O'Dowd
Professor Naomi S. Morrissette
Professor Todd C. Holmes

2018

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DEDICATION

To

my parents, my Kevin, and my friends

in recognition of their unwavering support.

I could not have done any of this without you.

"Failure is not an option"

-Gene Kranz, NASA Flight Director for the Apollo Missions

"All of our dreams can come true, if we have the courage to pursue them."

-Walt Disney

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Kevin. There is no one else in my life that has supported me in so many ways. Your scientific suggestions, moral compass, patience and compassion have been appreciated every day more than I can put into words.

CURRICULUM VITAE

Dail Elisse Chapman

EDUCATION

2013-2018 University of California Irvine PhD in Biological Sciences (4.0 cumulative gpa), mentor: Steven Gross

2009-2013 Scripps College Claremont, CA Bachelor of Arts in Biophysics, graduated *cum laude* (3.7 cumulative gpa)

2011 (Fall Semester) Danish Institute for Study Abroad through Scripps College

2009-2010 AISS (Accelerated Integrated Science Sequence; a competitive yearlong program for students with excellence in science, team-taught by three professors in Intro-Biology, Chemistry, and Physics)

2005-2009 Santa Fe Preparatory School, Santa Fe, New Mexico

HONORS AND AWARDS

2018	Edward Steinhaus Teaching Award for the Department of Developmental and Cell Biology
2017	Honoree in Recognition of Teaching Excellence and Service to the Academic Community, Presented by: UCI Center for Engaged Instruction
2015-2018	Graduate Assistance in Areas of National Need (GAANN) Fellowship (Teaching Training Grant through US Department of Education, covered stipend and fees for 3 years of graduate education)
2015-2016	Pedagogical Fellow (Center of Engaged Instruction; UCI); highly competitive training program in advanced pedagogy
2014	Honorable Mention for NSF Graduate Research Fellowship Program
2013	Senior honors thesis (recognized by Capstone Day; Scripps College)
2012	Associate Member of Sigma Xi (Scientific Honor Society)
2012	Bechtel Foundation Fellowship for Summer Research (Scripps College)
2010, 2011, 2012	Dean's List (Scripps College: Spring '10, Fall '10, Spring '11, Spring '12)
2011	AMGEN Scholars Program (Columbia University)
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RESEARCH EXPERIENCE

2014-2018 UCI, Developmental and Cell Biology, mentor: Steven Gross
Molecular Biology and Biophysics: cell and in vitro approached to study the molecular motor proteins, kinesins and dynein

2014: Apr-June UCI, Molecular Biochemistry and Biophysics Department, mentor: Thomas Poulos
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2012: June-Oct Keck Science Department of the Claremont Colleges, Physics Department, mentor: Paul Nerenberg.

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2011: Jun-Aug AMGEN Scholars Program at Columbia University, Biology Department, mentor: Lars Dietrich
Molecular Developmental Biology: The Effect of phenazines on *pseudomonas aeruginosa* biofilm thickness.

2010: Jun-Aug Keck Science Department of the Claremont Colleges, Physics Department, mentor: Steve Naftilan.
Astrophysics: Emission analysis to determine the spectral class of the binary star system, Epsilon Aurigae

TEACHING EXPERIENCE

2016, 2017 Instructor: Teacher's Assistant Professional Development Program at UCI (led a two-day workshop to train new TAs in Biological Sciences) I was invited in 2017 by the Center for Engaged Instruction to repeat my workshop

2016 Strategies for Scientific Success: Amplify your Teaching Career (led symposium

2014-2018 Teacher's Assistant at UCI (Dev and Cell Biology Lab-Winter 2018, DNA to Organisms TA-Fall 2016, Dev and Cell Biology Lab Head TA-Fall 2015, Dev and Cell Biology Lab-Spring 2015, Quantitative Biology-Fall 2014, Scientific Writing-Summer 2014, Winter 2015)

2014-2018 Mentoring Undergraduate Researchers (Bio199 program at UCI: I implemented a formal system in Steve Gross' lab in which I mentor undergraduates through their own projects in the lab, hold one-on-one research article discussions, and hone their presentation skills. A total of 13 students have been involved in the program; formers students hold research associate positions at UCI and one has accepted a research associate position Harvard Medical School)

2016 Strategies for Scientific Success: Bio199 and Beyond (led symposium for UCI undergraduates interested in research)

2012-2013 Physics Tutor at the Keck Science Department of the Claremont Colleges

PUBLICATIONS

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Shojania, F.M., Narayanareddy, B.R.J., Vadpey, O., Jun, Y., **Chapman, D**., Rosenfeld, S., and Gross, S.P. 2015. Microtubule C-Terminal Tails Can Change Characteristics of Motor Force Production. *Traffic*. doi: 10.1111/tra.12307

Chapman, D.E. Steck, J.K., and Nerenberg, S.P. 2014. Optimizing Protein-Protein van der Waals Interactions for the ff9x/ff12 Force Field. *J Chem Theor Comput.* 10(1): 273-81.

RESEARCH TECHNIQUES

Very Familiar

Tissue Culture (mammalian cells)
siRNA and overexpression in mammalian cells
Cell Proliferation Assays
Microscopy (inverted fluorescent, DIC, FLIM)
Western Blots (IR, film)
Recombinant Protein Purification
Protein-Protein Interactions (coIP, Cross-linking)
Gel Electrophoresis (agarose and SDS-PAGE)
Site-Directed Mutagenesis

Familiar

DNA Assays (RT-PCR)
Lipid droplet purification from mammalian cells
Motor tracking and analysis (force production and motion)
Optical Trapping *in vivo* and *in vitro* (to analyze motor forces)
Microtubule Gliding
Cloning
DNA isolations (bacterial and mammalian cells)
Tissue Fixing
Cryostat Cryosectioning

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ABSTRACT OF THE DISSERTATION

Regulation of Motor Proteins by Signaling Kinases

By

Dail Elisse Chapman

Doctor of Philosophy in Biological Sciences

University of California, Irvine, 2018

Professor Steven P. Gross, Chair

Movement is an essential part of life. On the cellular level, movement of intracellular components is essential for a cell's vitality. Most eukaryotic cells are too large to rely on diffusion for this, and thus depend on motor proteins. Motor proteins maintain eukaryotic cellular organization by actively transporting cargos and controlling positioning of subcellular structures on actin and microtubule networks. My research focuses on kinesins and dynein, the microtubule-walking motors. Together, these molecules move along cytoplasmic microtubule (MT) highways, allowing appropriate cargo positioning and delivery. While single-molecule cytoplasmic motor function is relatively well understood, there are major gaps in mechanistic understanding of kinesin and dynein regulation in cells. My research has detailed two signaling pathways: one likely regulating the kinesin motor Eg5, and the other regulating dynein. Specifically, my results show that the kinase CK2 activates Eg5 *in vitro* (Chapter 1). However, the role for CK2 in Eg5 regulation in cells is unclear. I also find that the kinase CDK5 along with 14-3-3ε and KIAA0528 promote the phosphorylation of the dynein co-factor NudEL, and that this phosphorylation is essential

for dynein force adaptation in cells (Chapter 2). Finally, I discuss the lab's most recent work in uncovering the cellular function of dynein force adaptation, and put this work into the context of the current state of the motors field (Chapter 3).

INTRODUCTION

Molecular motors play an important role in active transport of intracellular organelles within a cell. Without molecular motors, cells must resort to relying on cytoplasmic diffusion, and this would cause such catastrophic delays that much of life as we know it could not exist. For perspective, the diffusion constant of a small molecule in water is $D = 10^{-5}$ cm^2/sec. Theoretical calculations of the time it would take for a small molecule to diffuse 40 cm would take 80 million seconds, or 926 days[1]. However, should the small molecule encounter the viscosity of the cytoplasm (which is roughly 10X more than that of water), or other obstacles in the cell, or should the small molecule actually be a much bigger entity (like a vesicle), the travel time will take even longer. Molecular motors increase this rate immensely, and could actively transport the small molecule 40 cm in one day[2,3]. Just as intracellular transport is important for normal cellular function, abnormal intracellular transport can result in various diseases including cancer[4], neurodegenerative[5,6] and metabolic diseases[7–9]. Therefore, proper motor protein function must be tightly controlled by the cell.

There are three classes of molecular motors: myosins, kinesins, and dynein. Myosins move along smaller actin roads, while kinesins and dynein move along microtubule highways[10]. ATP hydrolysis drives these motors to undergo cyclic conformational changes that culminate in the motor stepping along its track[10]. Much of the biophysics underlying such molecular motor mechanics (including run length on the microtubule and force production by the motor), and details on associated co-factors have been well studied[5,11–16]. And through the amalgamation of previous work, we have a very thorough understanding of molecular motor function in various cell processes[17–25].

For example, many motors play critical roles in cell division. Specifically, motor proteins are important for spindle formation, chromosome alignment, and segregation[21]. By forming bipolar homotetramers, the kinesin protein, Eg5, (studied here) cross-links oppositely-directed microtubules and orchestrates proper formation of the mitotic spindle[26]. Mutations in Eg5 can cause mitotic arrest, inhibition of Eg5 decreases tumor proliferation[27], and because of this, Eg5 is currently under investigation as an anti-cancer target[27–30].

On the other hand, processes involving transportation from the cell periphery to the cell center involves another motor protein, dynein (dynein-1, MAP1C), studied here[11,31]. Dynein plays roles in vesicular, viral, chromosomal, and nuclear transport, and is essential for moving the nucleus in neuronal migration during cerebral development[15,32]. We recently reported that when experiencing opposition to motion, dynein-transported cargos can adapt their forces in order to increase group-dynein sustainment of force and ultimately overcome the opposition. This was studied by trapping lipid droplet cargos (moved by dynein) with an optical trap and determining the force and persistence time (the time over which the force is produced) at each lipid droplet's attempt to escape the trap[12]. This is one example of how motor regulation is dynamic and responsive to cellular signals.

Intracellular transport must be actively tuned by the cell, otherwise all motors would be active at all times, resulting in unnecessary motor tug-of-war, potential inappropriate local buildups of cargos, and ultimately inefficient transport. Intracellular transport is dynamically controlled in order to navigate the complex environment inside a cell and insure proper cell function[33]. For example, in the insulin-secretion pathway whose

alteration is implicated in diabetes, GLUT4-containing vesicles must be dynamically and appropriately internalized to control sugar uptake, a dynein-dependent process[22]. In fibroblasts, dynein is known to promote directed cell migration by maintaining the position of the nucleus in the moving cell (as fibroblast motion is temporarily linked to nuclear movement)[23]. In neurons, kinesin upregulation is important for the delivery of organelles along a developing axon[24]. For example, increased velocity of axonal mitochondria transport was found to be important for maintaining mitochondria distribution during elongation[25]. For any of these intracellular transport processes to occur correctly, transport must be highly regulated. While we know much of how motors work on the single molecule level, and we know many cellular functions of transport, we have an incomplete understanding for how intracellular transport is regulated. Nonetheless, some forms of regulation have already been described: auto-inhibition (as described above), interaction with other motors[34–36], and interaction with cofactors[37–41].

In vitro studies suggested that motor proteins were regulated predominantly by auto-inhibitory conformational changes mediated by a head-tail interaction, which is subsequently released when the motor is recruited to the cargo[42–46]. Yet in the context of a cell, where motors are frequently cargo-bound, there are many other sources of regulation to consider.

Cargo-bound motors are also regulated by signaling kinases such as glycogen synthase kinase 3 beta (GSK3β), c-Jun N-terminal kinase (JNK), and casein kinase 2 (CK2)[47]. GSK3β phosphorylation of kinesin light chain decreases kinesin-1 binding to cargo; similarly, JNK phosphorylation of kinesin-1 heavy chain decreases the motor's activity by inhibiting microtubule binding. Both of these result in inhibited fast axonal transport, such

deficiencies are implicated in Alzheimer's disease[47]. Our laboratory has identified a novel regulatory pathway for kinesin-family proteins[40], including the mitotic motor Eg5 (unpublished), where motors undergo intrinsic inactivation *independent of the tail* and are reactivated by CK2.

CK2 is a disease-relevant kinase that has been heavily studied due to its myriad essential roles in cell division, differentiation, and apoptosis[40,48,49]. Further, alterations in such functions are associated with several cancers, making changes in CK2 potentially relevant for cancerous progression. Indeed, CK2 overexpression is observed in many cancerous tumors[50]. However, CK2's role in motor function has not been significantly explored.

Although CK2 is a pleiotropic kinase with over 300 known substrates [48], CK2's kinase-independent roles have been minimally analyzed; our laboratory has identified that CK2 can reactivate Eg5 *in vitro* independent of its kinase activity (unpublished). Also, recent anticancer drugs in clinical trials that act as CK2 kinase inhibitors (by competitive binding to CK2's ATP-binding domain) have been largely ineffective[51]. And inhibitory drugs targeting Eg5 have had mixed results[52]. This suggests that the current knowledge of Eg5 inhibition is lacking, and the work presented here in Chapter 1 addresses this problem.

As evident by the previous discussion, molecular motors carry out a large range of functions. To carry out this plethora of roles, kinesins have evolved *with* increasingly diverse intracellular transport in the cell during eukaryotic evolution, (i.e. there is a different kinesin motor for different kinesin functions)[53]. For example, kinesin-1 delivers cargo to presynaptic regions of axons, a process vital for synaptic transmission; kinesin-2 is the main kinesin involved in intraflagellar transport[54]. There are also many mitotic

kinesins. CENP-E, for example, is involved in establishing the kinetochore[54]. While fourteen different kinesin families have evolved to fulfill each niche, a single dynein motor can carry out a similarly diverse set of intracellular transport functions by complexing with different cofactors[55]. Dynein cofactors including dynactin, LIS1, NudE (NDE1), and NudEL (NDEL1)[15] act as sources of regulation. Dynein forms two major complexes: dynein-dynactin-BicD or dynein-NudEL-LIS1[14,56–58]. Dynactin is important for increased processivity, and dynein-dynactin-BicD complexes can travel longer distances[16]. However, dynactin on its own does not bind well to dynein, and so the adaptor family proteins (BicDs, Rabs, Spindly, Hook) are needed to activate processive motion and define cargo specificity[16]. Since much of this has been studied *in vitro*, the next challenge for the molecular motors field is to understand how motors function and are regulated within a cellular context. The pathway identified in Chapter 2 is one progressive step within this new goal of the field.

More specifically, we do not understand the signaling pathways that induce dynein to complex with either dynactin-BicD2 or NudEL-LIS1, nor do we fully understand the capabilities of these complexes. Although there is a report stating that BicD2 can increase a single dynein's force production[59], it is known that the NudEL-LIS1 cofactors are important for enhanced group force function by improving additivity of single-motor forces[12]. And NudEL and LIS1 and are the important dynein cofactors involved in nuclear movement, a function necessitating high dynein force production[11]. Understanding the function of the dynein-NudEL-LIS1 is particularly important as mutations or deletions in the *LIS1* gene cause neurodevelopmental diseases such as lissencephaly and Miller-Dieker syndrome respectively[11,60,61]. To add even more complexity to the regulation of the dynein-NudEL-LIS1 core complex, there are other NudEL-interacting regulatory proteins[38,62]. For example,

the signaling kinase CDK5 phosphorylates NudEL in neurons[17,19]. However, there are conflicting studies reporting contrasting effects of phosphorylated NudEL (see Chapter 2: Introduction). Most recently, the rerouting of mis-sorted cargo in the axon initial segment (AIS) was found to be carried out by phosphoryled NudEL and dynein[18]. It has been assumed[63] that CDK5 may not be essential in non-neuronal cells as the main activators for CDK5—P35 and P39—are only present in neurons. And so non-neuronal roles for CDK5-mediated control of dynein remain largely unexplored. However, new evidence of roles for CDK5[64] in non-neuronal cell types support further investigation.

14-3-3ε is another protein known to regulate phospho-NudEL. If there is a phospho-regulated dynein-NudEL-LIS1 complex, it could be further modified by 14-3-3ε[62]. 14-3-3 is a family of scaffolding proteins that often protect phosphorylated sites. Clinically, 14-3-3ε deficiency results in worse phenotypes of many dynein-impaired neuronal diseases[65–68]. Therefore, many neurodegenerative and neuropsychiatric disease studies are targeting 14-3-3ε. Although dynein impairment characterizes many of these diseases, little is known about the function of 14-3-3ε in intracellular transport. Interestingly, 14-3-3ε supports normal dynein complex localization and activity by increasing the strength of interaction between dynein and phospho-NudEL[38]. Further, NudEL can be dephosphorylated by the phosphatase PP2A, and 14-3-3ε antagonizes PP2A by phosphor-protecting NudEL[65].

Our hypothesis is that the recently described force adaptation of cellular lipid droplets[12] occurs because CDK5 becomes activated (either by P35/P39 in neurons or some other cofactor/s in non-neuronal cells), phophorylates NudEL, thus increasing the affinity of NudEL for DIC (dynein intermediate chain), and through increased NudEL-DIC interactions, promotes dynein's utilization of the NudEL-LIS1 system. Further, we

hypothesize that these phosphorylation events are protected by 14-3-3ε. We use lipid droplets (LDs) as a model system because their motion and protein makeup is well understood, their motion plays a known role in metabolism[7], and most importantly, they are amenable to force measurements in cells[12]. While studied in LDs, we further hypothesize that this regulatory pathway is generally important for other dynein cargos, and thus test it in the context of lysosomes and mitochondria. It is possible that upon some trigger (likely the dynein "recognizing" a high-load), there is a switch from dynein-dynactin-BicD to dynein-NudEL-LIS1. Validation of this hypothesis would be the first evidence of dynein complex control within the cell.

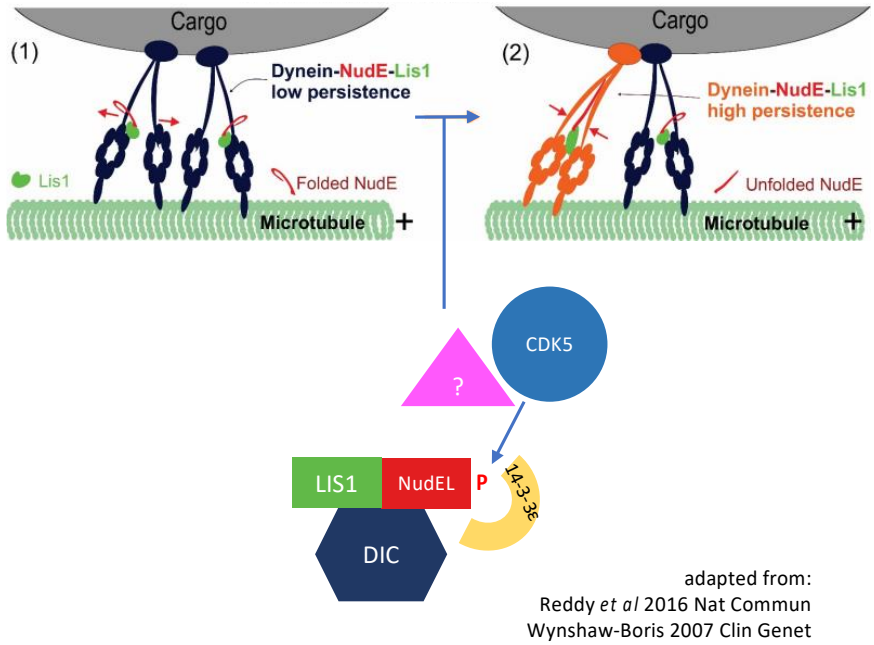


Figure 1. A model of dynein complex switching to high utilization of the NudEL-LIS1 system

In addition to providing insight into the first identified pathway for force regulation in cells, these studies have mechanistic implications in multiple diseases, where transport is likely to be important. CDK5 is implicated in Diabetes[64,69–72], neurodegenerative diseases[73], and cancer[74], and 14-3-3ε is a risk factor in schizophrenia[66].

Through these studies presented here, we have obtained a better understanding of molecular motor regulation *in vitro* and within the living cell. We suspect that these advances in basic biophysics will advance the understanding of the field and ultimately inform therapeutic approaches to multiple human diseases where molecular motors and their regulators are implicated.

CHAPTER 1: Characterizing Eg5 inhibition and regulation by CK2

Abstract

Eg5 is a kinesin that crosslinks oppositely oriented microtubules and ultimately establishes formations of the mitotic spindles during mitosis. The Gross lab recently discovered that Eg5 inactivated spontaneously (meaning it loses its ability to bind microtubules) simply due to aging, that this inactivation could be accelerated due to pharmacological inhibition of Eg5, and that both classes of inactivation could be reversed by treating the motors *in vitro* with CK2. We investigated whether this reactivation required CK2's kinase activity and found that it did not: reactivation of Eg5 was kinase-independent as a kinase-dead CK2 could not reactivate Eg5 motors. CK2's kinase-independent roles have been minimally analyzed. Recent drugs that act as CK2 kinase inhibitors tested for their effects on cancer have been found to be ineffective in clinical trials (by competitive binding to CK2's ATP-binding domain). And Eg5 inhibitory drugs have had mixed results. Importantly, CK2 is over-expressed in many cancers. So, it is possible that through aberrant CK2 activity, inactive Eg5 is becoming active, and increasing the proliferative state of these cells. Thus, we suggest that CK2 and Eg5's interaction likely counteracts these drugs' function as potential cancer treatments, explaining their poor performances in the clinic. In order to test this hypothesis, I further detailed the mechanism of reactivation *in vitro* and hoped to extend this finding *in vivo*.

Introduction

Many motors play critical roles in cell division. Mutations in the mitotic motor, Eg5, can cause mitotic arrest[27], and because of this role in spindle function, Eg5 is currently

under investigation as an anti-cancer target[27–30]. In order to develop a new inhibitory method, we must further understand the regulation of Eg5. As highlighted in the introduction, our laboratory has identified a novel regulatory pathway for kinesin-family proteins[40], including Eg5 (unpublished), where motors undergo intrinsic inactivation *independent of the tail* and are reactivated by CK2.

CK2 is a hetero-tetrameric protein composed of two catalytic subunits α and α', and two regulatory β subunits. There are few known functional differences between the α and α' subunits; often CK2 functions with two α subunits without α'[75]. The cellular levels of CK2α are downregulated in some neurodegenerative diseases[76], and overexpressed in many cancerous tumors[50]. Although the link between such diseases and transport is not well understood, our lab has characterized the reactivation of kinesin-family member proteins by CK2-holo *in vitro*. We began our CK2 regulatory studies with a truncation-mutant of kinesin-1 (K560: lacking the tail domain) because this is the most well-studied motor in the field, and because—since it lacks the tail—it cannot undergo tail-mediated auto-inhibition. Published data from our lab shows that when incubated for extended periods of time, both full-length endogenous kinesin-1 and K560 become incapable of binding MTs, which we refer to as "inactive"[40] (see Figure 1 in reference). Since the tail-less truncated construct is inactive, this process does not involve the kinesin-1 tail, suggesting a new method of inactivation previously unknown to the motor field[40] (see Figure 2 in reference). Importantly, the CK2 holo enzyme reactivates the inactive kinesin-1; and this occurs *in vitro* with only CK2-holo and K560 present. Furthermore, knocking down CK2α in cells reduced the forces powering lipid droplets transport[40] (see Figure 6 in reference), which is a process dependent on kinesin-1. Thus, CK2α contributes to kinesin function and

overall force production *in vivo*. Considering this and the highly conserved motor domain in the kinesin family, we hypothesized that CK2 regulates another kinesin-family member protein in cells, Eg5.

Eg5 is the mitotic motor responsible for the assembly and separation of the mitotic spindles[20]. Inhibition of Eg5 prevents a functional spindle from forming, after which normal chromosomal segregation cannot take place, a monoaster forms, and cell cycle checkpoint proteins will direct mitotic arrest[77]. Numerous small molecule anti-cancer drugs have targeted Eg5 to try to take advantage of this essential function.

As a signaling kinase, CK2 has over 300 substrates[48]. Its kinase ability is thus well characterized. However, about 10% of the 518 evolutionarily conserved human kinases lack one or more of the three amino acids necessary for a phosphotransfer, but still are active regulators[78]. Our lab has discovered a new and significant kinase-independent, non-canonical role for the signaling kinase CK2 by studying its regulation of kinesin-1 and Eg5. We mapped the phosphorylation site of kinesin-1, mutated it (S520A), and saw comparable reactivation by CK2[40] (see Figure 3f in reference). Furthermore, reactivation of kinesin-1 by CK2 occurred even in the presence of a known CK2-kinase inhibitor, TCBA, at a concentration where kinase activity was clearly obstructed. Thus, based on these results, we hypothesized that CK2's activation of Eg5 in cells is devoid of a phosphotransfer. This suggested that CK2 may act independently of its kinase ability to regulate Eg5, a finding which I investigate here in Chapter 1.

The assembly of the mitotic spindle is essential for cell division, and thus small molecule cancer drugs have often targeted it. One successful example is paclitaxel (Taxol), which binds to tubulin-β and stabilizes the mitotic spindle so that the spindles cannot

separate. This is arguably the one of the most successful anti-cancer drugs, and it is widely used to stabilize malignant tumors[79]. However, many patients have accrued resistance to the drug due to alteration of their microtubule dynamics, including changes in tubulin regulation, as well as point mutations in tubulin-β that inhibit binding of the drug[79]. Research has since sought out other targets to inhibit the mitotic spindle. Since the motor Eg5 is essential for mitotic spindle separation, there has been investigation of anti-Eg5 drugs. Monastrol, a first generation compound, and ispinesib, a derivative of monastrol showed promising results *in vitro*[80]. However, these drugs have failed in clinical trials[52]. While this might suggest that the approach is fundamentally flawed, our data suggests another possibility: that the monastrol-derived drugs function by increasing the kinetics of Eg5 inactivation. If so, the approach is faulty because the cells have a way to reverse the Eg5 inactivation via CK2, a protein that is upregulated in most cancers. Thus, there is a need for a drug that targets Eg5 through a different mechanism, either by blocking the Eg5-CK2 interaction, or by inactivating Eg5 by a non-endogenous pathway that cannot be easily reversed by CK2. One appealing candidate is a new natural product, Terpendole E, which was recently reported to inhibit Eg5[29]; Terpendole E binds to a different and currently unknown locus on Eg5, distinct from the other monastrol, and monastrol-derivative inhibitors. I investigate the effects of Terpendole E and its derivative small molecule inhibitor, Emindole SB, in this Chapter.

To summarize, I aimed to determine CK2 relevance to Eg5 function *in vivo* by: 1) detailing the mechanism of reactivation of Eg5 by CK2 *in vitro* via microtubule sedimentation experiments and microtubule gliding assays with purified Eg5 motors, and

2) extending these *in vitro* findings to the cell by analyzing cell proliferation in various formats of CK2 inhibition.

Results

CK2 subunits α and α' together reactivate Eg5 *in vitro*

In order to determine the activity of a sample of molecular motors, one can conduct a microtubule sedimentation experiment. Only active motors bind microtubules; so, to separate the active motors from the inactive motors, one can incubate the sample of motors with microtubules, and use high-speed centrifugation to pellet the microtubules along with the fraction of active motors. To determine if CK2 affected the ability of inactivated Eg5 to bind microtubules, I conducted microtubule sedimentation experiments with various subunits of the CK2 holo enzyme in order to determine which subunit(s) was/were responsible for the reactivation of Eg5. I found that the CK2 holo enzyme could reactivate Eg5 as evidenced by greater amounts of Eg5 detected in the microtubules fraction of the sedimentation experiment. Interestingly, a combination of CK2α and CK2α' were also able to reactivate Eg5 to a similar degree as the CK2 holo enzyme. This suggested that the regulatory subunit, CK2β, is not needed for Eg5 reactivation. However, when testing CK2-α or CK2-α' alone, they were not able to reactivate Eg5 (**Figure 1.1**), suggesting that CK2-α and CK2-α' work together to reactivate inactive Eg5 motors.

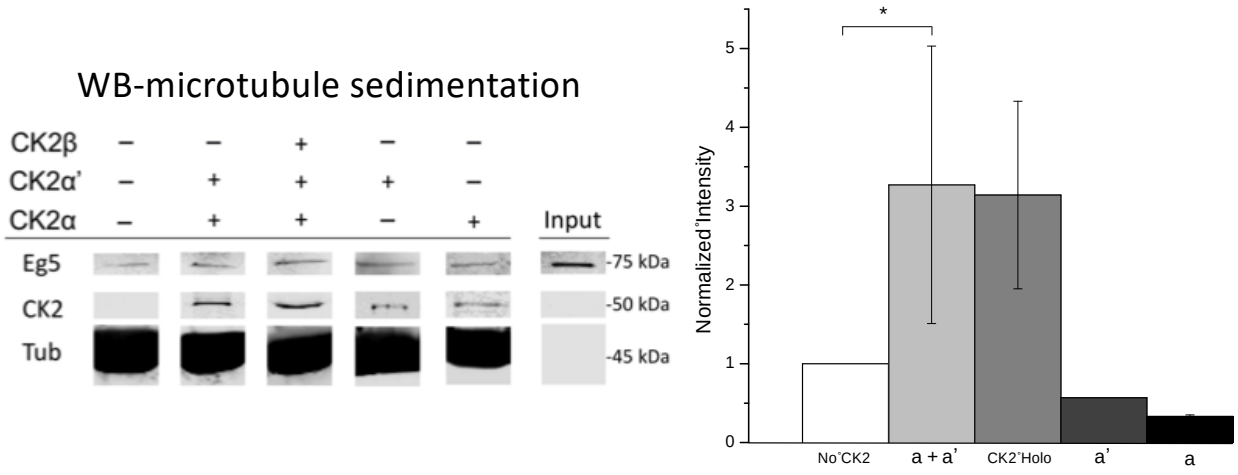


Figure 1.1. Increased co-sedimentation of Eg5 motors with microtubules with CK2
Purified Eg5 motors were inactivated on ice and then submitted to microtubule sedimentation experiments with various CK2 subunits. WB analysis (left) and quantification of WBs (right) shows of the microtubule sediment showed a significant increase in the amount of Eg5 in CK2-holo and CK2α + α' samples, but not in samples with no CK2, α, or α' alone.

To support this data, our collaborators in Steve Rosenfeld's lab at the Cleveland Clinic performed TMR (tetramethylrhodamine) dimerization experiments to look at the kinetics of Eg5 reactivation with the CK2 holo enzyme and CK2-α'. They used purified TMR-tagged recombinant Eg5 motors; when the Eg5 motor neck linker docks (i.e. the motor is in its inactive conformation), the TMR-fluorescence quenches. When the Eg5 motor is in its active form, the TMRs are too far apart, and they observe fluorescence. By measuring the amount of fluorescence in the presence of either CK2 holo enzyme or CK2α', they can determine the amount of active Eg5 motors. They found that the CK2 holo enzyme was able to reactive Eg5 (**Figure 1.2**), which supported our microtubule sedimentation experiments (**Figure 1.1**). To our surprise, our collaborators found that the presence of CK2-α' causes more Eg5 neck-linker undocking than the CK2 holo enzyme (**Figure 1.2**).

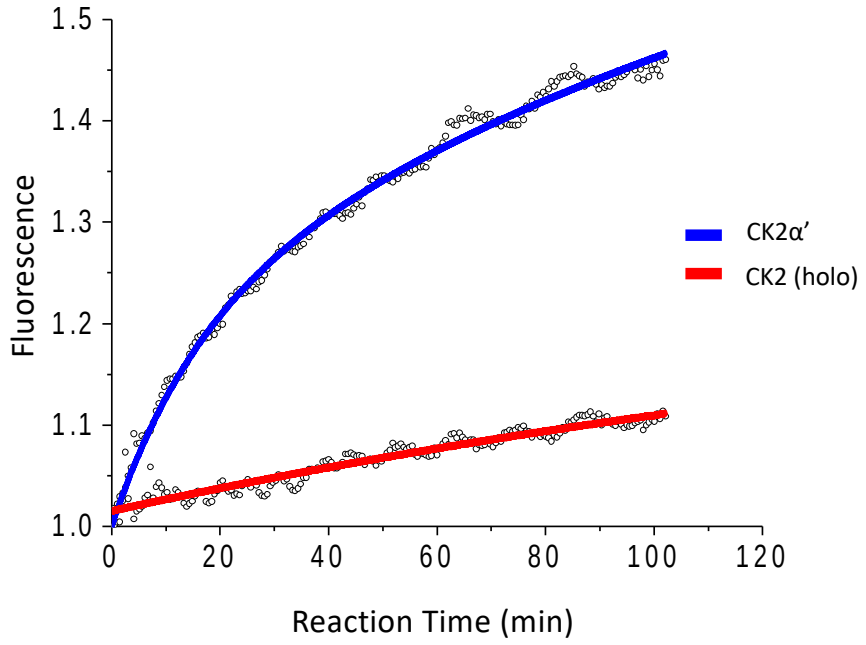


Figure 1.2. CK2α' acts differently than CK2 holo enzyme in Eg5 reactivation.
Eg5 labeled with TMR at sites engineered to detect NL docking via TMR dimerization, was thawed and diluted to 0.1μM and incubated at room temperature for 20 mins. After the 20 minute incubation, CK2 (NEB) or CK2-alpha (Millipore) was added to a final concentration of 0.4μM. Time-resolved fluorescent waveforms were acquired every 30 seconds for 100 minutes. Data is plotted as total fluorescence from samples containing CK2 (red) or CK2α' (blue).

Eg5 inhibitors have various efficacies *in vitro*

Now that we had a suggestion of the mechanism of action of CK2, I wanted to test different Eg5 inhibitors *in vitro* via microtubule gliding experiments. As a proof of principle, I first tested previously studied Eg5 inhibitors, monastrol and ispinesib, and indeed saw that increasing concentrations of these two inhibitors decreased the percentage of microtubules gliding along the microscope slide (**Figure 1.3a, b**). This suggests that as the concentration of the inhibitor increases, there are less active Eg5 motors able to bind and walk along microtubules. I then tested two members of a new class of inhibitors: Terpendole E and Emindole SB created by Sergi Pronin's lab at University of California Irvine. Findings from this experiment suggested that Terpendole E but not Emindole SB inhibited Eg5 significantly (**Figure 1.3c-f**). Unfortunately, no new developments in this project can be reported here.

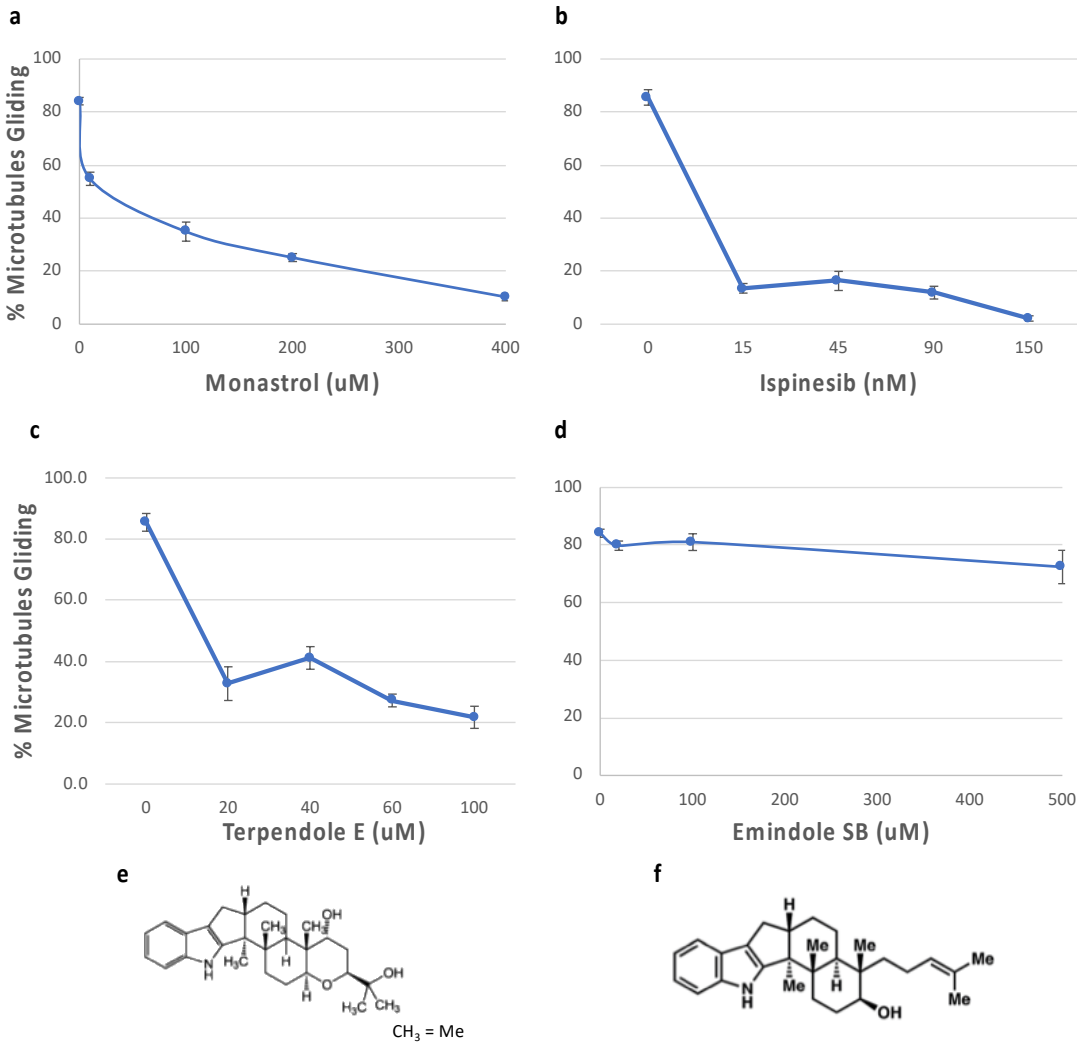


Figure 1.3. Eg5 inhibitors have different efficacies. Various concentration of four Eg5 inhibitors were tested in microtubule gliding experiments. Monastrol (**a**), ispinesib (**b**), Terpendole E (**c**) all showed significant Eg5 inhibition. However, Emindole SB (**d**) did not significantly inhibt Eg5. Chemical structures of Terpendole E (**e**) and Emindole SB (**f**) are shown. Quantified data represent mean ± s.e.m.

CK2 kinase activity is important for cell proliferation

I moved on to determine the cellular importance of CK2-α (and CK2-α') reactivation of Eg5 *in vitro*. Since Eg5 is necessary for spindle formation and cell proliferation[20,21], we conducted CK2-α knockdowns and cell proliferation assays in order to inhibit the ability of CK2-α to reactivate Eg5 *in vivo.* Indeed, cell proliferation was decreased in the CK2-α knockdown cells (**Figure 1.4**). Since we had determined that CK2-α can reactivate kinesin-

1 independent of its kinase ability, we wanted to repeat our cell proliferation assays with the CK2 kinase inhibitor, CX4945. If CK2 regulates Eg5 *in vivo*, and this regulation is independent of CK2's kinase activity (as seen in our *in vitro* data), I would expect to see a slightly increased proliferation rate in these cells as compared to the CK2-α knockdown cells. This is because in the cells treated with CX4945, CK2's ability to regulate Eg5 should still be intact. However, this is not what we observed. Treatment with CX4945 shows similar decrease in cell proliferation as the CK2-α knockdown cells as compared to the control cells which were treated with either transfection reagent or vehicle (DMSO) (**Figure 1.4**).

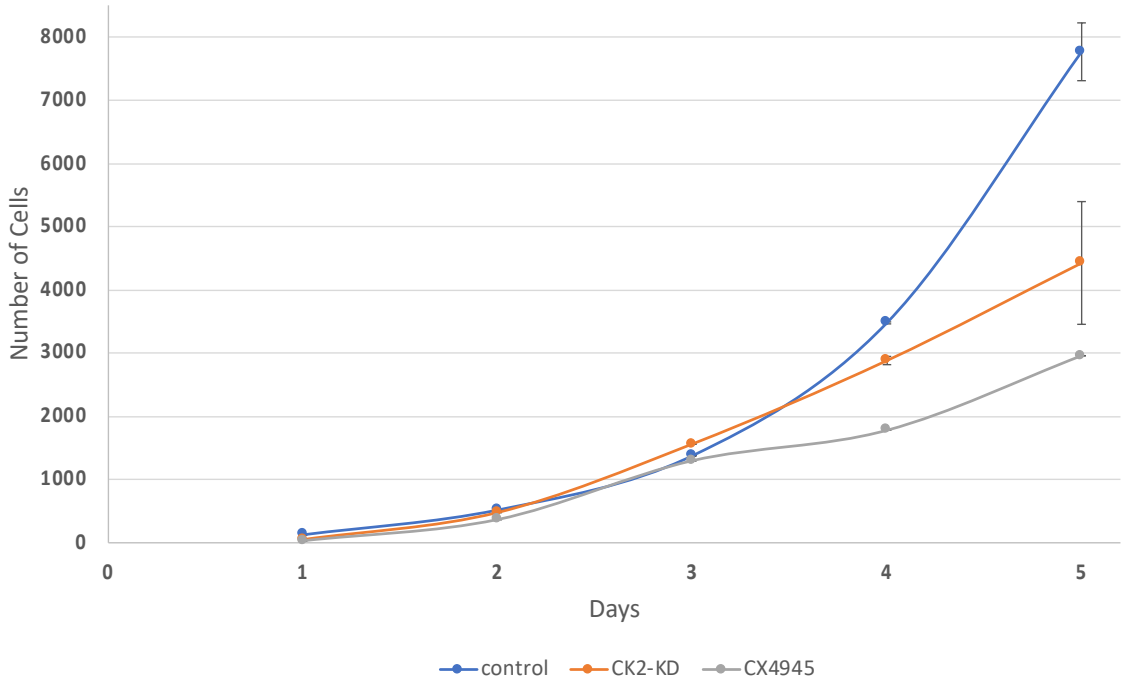


Figure 1.4. CK2 knockdown and kinase inhibition decrease cell proliferation. Knocking down CK2 decreases cell proliferation. Treatment with CK2 kinase inhibitor, CX4945, also shows a similar decrease in cell proliferation compared to the control.

Discussion

Upon starting my dissertation work, the lab had recently published a paper stating that CK2 could reactivate kinesin-1[41]. It was important to further study this reactivation by extending it to other kinesins, including Eg5, uncover the mechanism of reactivation, and determine if this motor control pathway was important in cells. To start, I determined that certain CK2 subunits can reactivate Eg5 motors *in vitro*. Specifically, CK2-α and CK2-α' together, but not CK2-α or CK2-α' alone, could reactivate Eg5 motors as evidenced by the increased amount of Eg5 motors in the microtubule sediment when CK2-α and CK2-α' were incubated together with Eg5 (**Figure 1.1**). This allowed us to determine which subunit was important and focus our knockdowns on that particular subunit *in vivo*. Interestingly, it also suggests that the catalytic subunit, CK2-α, is not enough to reactivate Eg5. Somehow, CK2-α' is needed. The kinetic experiments from our collaborators in Steve Rosenfeld's lab provided more information, suggesting that CK2-α' is responsible for Eg5 neck-linker undocking. Since in these experiments, amount of undocked motors is relative to the amount of fluorescence, incubation with CK2-α' caused more undocking than the CK2-holo enzyme (**Figure 1.2**). However, this must not be enough to fully reactivate the motor as there was no increase in amount of active motors found in the microtubule sedimentation samples containing CK2-α' alone. It is possible that there is a second conformational change for which CK2-α is needed. Therefore, it likely takes both CK2-α and CK2-α' to change the conformation of Eg5 into the completely active form[40,41]. However, our data are not detailed enough to prove this completely.

Since aberrant CK2 and Eg5 activity is characteristic of many cancers[50,81], and we had evidence suggesting a kinase-independent pathway that could function in

carcinogenesis, we wanted to study new inhibitors of Eg5 developed by Sergi Pronin's lab. Perhaps one of them functions to block the reactivation of Eg5 by CK2. To start, we tested Terpendole E and Emindole SB in microtubule gliding assays. Indeed, Terpendole E inhibited Eg5 sufficiently (**Figure 1.3c**); however Emindole SB did not shown inhibition (**Figure 1.3d**). Since Terpendole E was developed to interact with a different locus on Eg5, we considered it might possibly inhibit the reactivation by CK2 and had plans to further study this promising inhibitor. As Emindole SB was newly synthesized, our collaborators in the Pronin lab suggested that there might be impurities that were altering the efficacy of the small molecule inhibitor. While they worked to optimize these small molecules structures and synthesis, we worked to determine if the reactivation of Eg5 by CK2 is important in cells.

Since it is well known that both CK2 and Eg5 have important functions in cell proliferation[28–30,82], we decided to use cell proliferation as a read-out of the importance of this pathway. We thought that if we knocked down CK2-α, that would limit its ability to reactivate Eg5 (as well as its function in other cellular pathways), and that this would likely decrease cell proliferation. Indeed, this is what we saw in the CK2-α knockdown cells (**Figure 1.4**). We then hypothesized if Eg5 reactivation by CK2-α is an important pathway in cells, then inhibiting the kinase ability of CK2 (by treatment with the inhibitor, CX4945) would not show as much of a decrease in cell proliferation (compared to the control cells) since this pathway would remain intact. Yet, the cells treated with CX4945 show similarly limited cell proliferation to the CK2-α knockdown, suggesting that CK2's ability to reactivate Eg5 motors is likely not important to cell proliferation (**Figure 1.4**). Although we had characterized a possible interaction *in* vitro, our data suggested that this pathway is

possibly not important in cells (or it is important, but not for cell proliferation). Because we found evidence that the reactivation of Eg5 by CK2 is not important *in vivo*, we moved on to study the regulation of dynein.

Materials and Methods

Microtubule Sedimentation: Purified Eg5 (a gracious gift from the Rosenfeld Lab, 150nM) was incubated for 2 hours on ice in order to inactivate the motors. In the meantime, tubulin (6.6 mg/mL) was polymerized by incubating microtubules in microtubule growing buffer (100mM PIPES, 1mM $MgSO_4$, 2mM EGTA, 0.3mM GTP, 20μM Taxol) at 37C for 20 mins to make microtubules (MT). Then, the motors were incubated with assay buffer (5μM MT, 80mM PIPES, pH 6.9, 5mM potassium acetate, 4mM AMP-PNP (EMD)) with recombinant CK2 (holoenzyme or subunits as indicated, up to 250nM, holo-New England Biolabs, α/α' subunits Millipore) in 20μl reaction buffer (20mM tris–HCl pH 7.5, 50mM KCl, 10mM MgCl2, 0.5mM EGTA, plus 500μM ATP) at 30 °C for 40min (optimal for CK2 kinase activity). Reactions from which CK2 had been omitted served as controls.

In vitro CK2 re-activation. After motor inactivation, 10μl each of the kinesin samples are identically transferred to two sets of new Eppendorf tubes. For one set, 10μl CK2 reaction buffer (Millipore) was added. For the second set containing identical kinesin samples, 10μl CK2 (500nM) was added, followed by a 2 hour incubation at room temperature, then assayed for active motor fraction via microtubule pulldown.

Microtubule affinity pulldown. Kinesin with indicated amounts of CK2 holoenzyme or subunit and indicated incubation conditions were incubated with the polymerized microtubule-containing assay buffer for 15 min at room temperature (RT). The reaction

was centrifuged in a TLS55 rotor for 10min at 170,000g at 25 °C. The resulting microtubule pellet was dissolved in 30µl 1X reducing SDS sample buffer, separated by SDS–PAGE, and analyzed by immunoblot. Experiments were conducted in triplicate. Previously described[40].

TMR Dimerization Fluorescence Imaging: previously described[83].

Microtubule Gliding: clean slides were silanized by incubating slides in a 0.05% DSS solution in TCE for 1 hour. Slides were then transferred to various methanol baths and sonicated for 5, 10, 15, and 30 minutes. Slides were dried with clean nitrogen gas. Tubulin (30% rhodamine-tubulin (Cytoskeleton Inc, T331) and 70% nonfluorescent tubulin) was polymerized as described above, and kept in the dark during the polymerization incubation. A flow chamber was constructed with a microscope slide and a silanized slide separated by double-sided tape. Penta-His antibody (200µg/mL, Qiagen) was diluted to 20µg/mL in assay buffer (2.5mL antibody, 80mM PIPES, 4mM $MgSO_4$, 1mM EGTA) and flown through the chamber for 5 mins. The surface was then blocked with CDB5.5 solution (35mM PIPES, 5mM $MgSO_4$, 1mM EGTA, 0.5mM EDTA, plus 5.55mg/mL of casein) for 5 mins. Next, Eg5 motors were added in 80 mM PIPES (pH 6.9), 50mM potassium acetate, 4mM $MgSO_4$, 1mM DTT, 1mM EGTA, 10µM taxol, 1 mg/mL casein and incubated for 5 min to bind the motors specifically to the antibodies. Finally, 1µL of fluorescent MTs was added in 50µL of motility buffer with 0.6 mM ATP and an oxygen-scavenging system (250 µg/mL glucose oxidase, 30 µg/mL catalase, 4.5 mg/mL glucose). MT gliding was monitored using an inverted microscope (TE2000; Nikon) with a 100X objective (NA = 1.49). Fluorescence images were acquired at 11.8 frame/s (FPS) using an EMCCD camera (Quantem 512SC; Photometrics; 512 × 512 imaging pixels) and µ-Manager software; one pixel corresponded

to 60 nm. Movies were composed of approximately 500 images. Percent of gliding microtubules was calculated. Previously described[84].

Cell Proliferation: Mycoplasma-free COS-1 cells (obtained from ATCC) were grown in DMEM (Genesee) supplemented with 10% fetal bovine serum and 1% Pen-Strep at 37° C in 5% CO_2. 1000 cells were plated in each well of a 96-well tissue culture treated plate. A calibration curve of cells was plated with 500, 1000, 2000, 4000, and 8000 cells. HiPerfect (Qiagen) was used according to manufacturer's instructions. CK2α was knocked down by diluting each CK2 siRNA (Qiagen SI02660497 and SCBT sc-29918) to 300nM final concentration. Cells were incubated for 24 hours. Cell count was analyzed on a plate reader with the CCK-8 Kit (Sigma-Aldrich) according to manufacturer's instructions. The plate was read every 24 hours for 5 days. Absorbance values were used to calculate the number of cells in each well at each time point.

CHAPTER 2: Regulation of in vivo dynein force production by CDK5, 14-3-3ε, and KIAA0528

Abstract

Single-molecule cytoplasmic dynein function is relatively well understood, but there are major gaps in mechanistic understanding of dynein regulation in cells. We previously reported a novel mode of dynein regulation, force adaptation, where lipid droplets adapt to opposition to motion by increasing the duration and magnitude of force production, and found LIS1 and NudE/NudEL to be essential. Since adaptation reflects dynamically increasing utilization of NudE/NudEL-LIS1, we here hypothesize that CDK5-mediated NudEL phosphorylation controls utilization of NudEL, and through this, makes force adaptation possible. Here, we report that CDK5, the phospho-protector 14-3-3ε, and the newly studied CDK5 cofactor KIAA0528 are all essential for dynein force adaptation. We extend this in vivo regulatory pathway to lysosomes and mitochondria. Ultimately, we show that dynein force adaptation can control the severity of lysosomal tug-of-wars among other important intracellular transport functions involving high force.

Introduction

Cytoplasmic dynein (dynein-1, MAP1C) is essential for intracellular transport of organelles and other cargos towards the cell's nucleus[31,11]. Together with the opposite directed plus-end kinesin family of motors, these molecules move along cytoplasmic microtubule (MT) highways, allowing appropriate cargo positioning and delivery.

Dynein plays roles in vesicular, viral, chromosomal and nuclear transport, and is essential for neuronal migration during cerebral development[32,15]. Due to this diversity of

roles, it is highly regulated, frequently via regulatory cofactors including dynactin, LIS1, NudE (NDE1), and NudEL (NDEL1)[15]. Dynein forms two major complexes: with dynactin or with NudEL-LIS1[14,56–58]. Dynactin is important for increased processivity, and dynein-dynactin-BicD complexes can travel longer distances[16]. Deletions in the *LIS1* gene cause Miller-Dieker syndrome[61], presumably because its presence enhances additivity of single-motor forces[12], and thus facilitates dynein's high-load function, which is important for the nuclear migration[11,60] underlying neuronal migration; through utilization of the NudEL-LIS1 system, neurites can undergo cell migration: a leading extension of the soma, followed by the discontinuous movement of the largest organelle, the nucleus. As the nucleus progresses, the rest of the cell follows. It has been shown that LIS1 and dynein together are important for extension and soma movement[60]. Furthermore, NudEL tethers LIS1 to dynein, and helps regulate the dynein-LIS1 interaction. It is unclear how these two complexes (dynein-dynactin or dynein-NudEL-LIS1) coordinate to regulate dynein. They may or may not function simultaneously: they share an either-or interaction site on the dynein intermediate chain (DIC)[56], but LIS1 associates with moving dynein-dynactin-BicD2 complexes[39]. One model is that the two complexes multiplex, or trade off binding with dynein, but how this might be regulated is not understood.

In addition to the dynein-NudEL-LIS1 core complex, there are other NudEL-interacting proteins that provide further regulation[38,62]. In neurons, the signaling kinase CDK5 phosphorylates NudEL[19,17]. However, the mechanistic implications of this phosphorylation are controversial, with respect to the effect on microtubule dependent cargo transport in axons. Klinman *et al* suggest that NudEL phosphorylation by CDK5 increases dynein-NudEL-LIS1 affinity and locks dynein in a nucleotide-bound state that

decreases processive motion of various dynein cargos[17]. In contrast, Pandey *et al* suggest that CDK5 phosphorylation of NudEL leads to increased dynein activity by promoting a high affinity dynein-NudEL-LIS1 complex, which then increases transport by dynein[19]. Most recently, CDK5 phosphorylation of NudEL was found to be critical for rerouting mis-sorted dendritic cargo out of the axon initial segment (AIS), a dynein-dependent process[18]. Any role for CDK5-mediated control of dynein in non-neuronal cells is unknown. Because the main activators for CDK5—P35 and P39—are only present in neurons, it has been assumed[63] that CDK5 may not be important in non-neuronal cells. However, new evidence for pleiotropic non-neuronal roles for CDK5[64] supports a re-evaluation of this assumption.

Assuming a phospho-regulated dynein-NudEL-LIS1 complex, it could be further modified by 14-3-3ε[62], since clinically, many dynein-related neuronal diseases are made worse by 14-3-3ε impairment. For instance, decreased 14-3-3ε protein levels correlate with a worsened lissencephaly phenotype in LIS1-deficient patients[65]. Further, 14-3-3ε mRNA expression levels are decreased in the prefrontal cortex of schizophrenic and bipolar patients[66,67]. Lewy bodies, abnormal protein aggregates found in Parkinson's disease nerve cells, contain 14-3-3ε[68]. Thus, 14-3-3ε is an actively studied target in neurodegenerative and neuropsychiatric diseases. However, little is known about its function in intracellular transport. Interestingly, 14-3-3ε interacts strongly with phospho-NudEL to promote normal dynein complex localization and activity[38]. Finally, NudEL can be dephosphorylated by the phosphatase PP2A, and 14-3-3ε protects phospho-NudEL by sterically inhibiting PP2A access to the phosphorylation sites[65].

All of this leads to the model—relevant in both non-neuronal and neuronal cells—tested in this work. Our hypothesis is that the recently described force adaptation of

cellular lipid droplets[12] occurs because CDK5 becomes activated, phophorylates NudEL, thus increasing the affinity of NudEL for DIC, and through increased NudEL-DIC interactions, promotes dynein's utilization of the NudEL-LIS1 system. Further, we hypothesize that NudEL phosphorylation is protected by 14-3-3ε (**Figure 1**). We use lipid droplets (LDs) as a model system because their motion and protein makeup is well understood, their motion plays a known role in metabolism[7], and most importantly, they are amenable to force measurements in cells[12]. While studied in LDs, we further hypothesize that this regulatory pathway is generally important for other dynein cargos, and thus test it in the context of lysosomes and mitochondria.

In addition to providing insight into the first identified pathway for force regulation in cells, these studies have mechanistic implications in multiple diseases, where transport is likely to be important. CDK5 is implicated in Diabetes[64,69–72], neurodegenerative diseases[73] and cancer[74], and 14-3-3ε is an important risk factor in schizophrenia[66].

Results

Force adaptation in wildtype COS-1 cells

We previously showed that under load, both forces and persistence times (the time over which the force is maintained) in dynein-driven LD cargos increase at each attempt[12]; the increase is typically first apparent in attempt two or three, and statistically significant by attempt four or five (**Figure 2.1, Supplementary Figure 2.8**); the data here reflect new unpublished measurements, made concurrently with the other studies described in this manuscript. This trend occurs when measurements are made both in unperturbed

wildtype cells and in control cells treated with transfection reagent (**Supplementary Figure 2.1**).

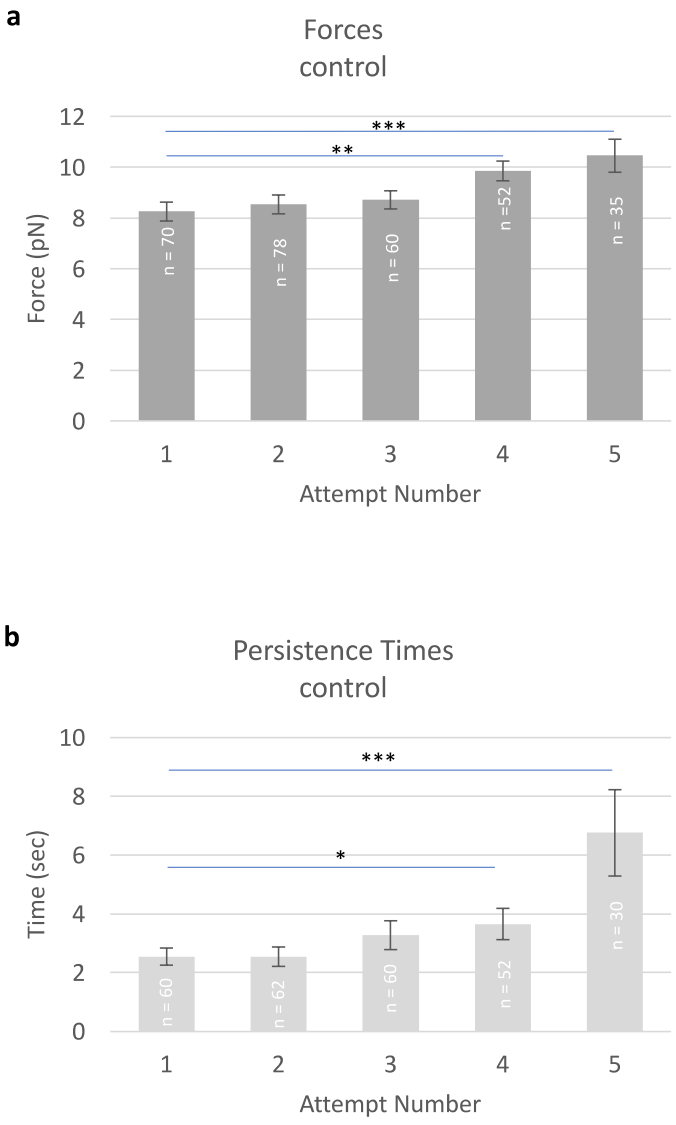


Figure 2.1. Minus-end forces and persistence times increase when dynein-driven LD cargo are under load
Average peak forces for minus-end moving LDs show slight increase in the control background (**a**). Average persistence times for dynein-driven LDs show significant increase in WT background (**b**). Quantified data represent the mean ± s.e.m. of n = 5 independent experiments. One-tailed t-test *P <0.05, **P < 0.01, ***P < 0.001

CDK5 is essential for dynein force adaptation

CDK5 phosphorylation of NudEL has been shown by three separate groups [63,37,85,86]. Thus, we explored the possibility that CDK5 phosphorylation of NudEL is important for

dynein force adaptation. We knocked down endogenous CDK5 (**Figure 2.2e**) and under such conditions found that dynein force production does not increase at each attempt as for the wildtype, but instead forces and persistence times decreased with subsequent attempts (**Figure 2.2a, b, Supplementary Figure 2.8**). The same phenotype was observed when we blocked CDK5 activity by overexpressing a previously characterized dominant-negative CDK5 construct (CDK5dn, containing an R33T mutation)[19] (**Figure 2.2c, d, f,** Supplementary Figure 2.5, Supplementary Figure 2.8). Neither CDK5 knockdown nor CDK5dn overexpression altered plus-end forces (Supplementary Figure 2.2). Thus, inhibiting CDK5's kinase activity coincides with alteration of dynein force adaptation in cells, where forces and force durations decrease rather than increase. After conducting immunolocalization experiments on purified LDs, we found that CDK5 was indeed present on the LDs, supporting this CDK5 regulatory function (**Figure 2.3**, **Supplementary Figure 2.6**).

14-3-3ε is essential for dynein force adaptation

We next examined 14-3-3ε, since in a neuronal context, it was previously reported to protect phospho-NudEL[62]. Consistent with a potential role in LD function, we found that 14-3-3ε was present on purified LDs (**Figure 2.3, Supplementary Figure 2.6**). When we knocked down 14-3-3ε (**Figure 2.4, Supplementary Figure 2.5**), the result was reminiscent of that observed for the CDK5 knockdown, but less severe: in contrast to wild-type behavior, neither forces nor persistence times increased at successive attempts (**Figure 2.4a, b, Supplementary Figure 2.8**), though they did not decrease as was the case for CDK5 knockdown. Again, no obvious effect was observed on plus-end force production (**Supplementary Figure 2.2**).

KIAA0528 is a CDK5 co-factor necessary for force adaptation in COS-1 cells

Since CDK5 activity is highly characterized in neuronal cell types, but less is known about its function in non-neuronal cells, we became interested in factors contributing to its non-neuronal activity. Additionally, as CDK5 is a known key regulator for the dynein motor[19,17,18], further characterization of CDK5 interactors could lead to novel dynein regulatory mechanisms. We searched the CDK5 interactome via recent proteomic analysis of CDK5-associated protein complexes, and prioritized candidates that are known to have microtubule-related cellular functions. This led us to KIAA0528 for multiple reasons: 1) it was identified as the top binding protein[87], 2) this interaction was validated with anti-KIAA and anti-CDK5 immunoprecipitations[87], and 3) KIAA0528 is involved in GLUT4 transport[8], a motor-dependent process relevant in diabetes. KIAA0528 is also potentially involved in cancer metastasis as it plays roles in cell proliferation and migration[88]. However, as a newly studied protein, its function is relatively unknown. As KIAA0528's interaction with CDK5 had been confirmed via multiple approaches[87], it seemed likely that if present, it might alter CDK5 function. We found KIAA0528 on purified LDs in COS-1 cells (**Figure 2.3**, **Supplementary Figure 2.6**), positioning it to contribute to LD transport. We then knocked down KIAA0528 (**Figure 2.5c, Supplementary Figure 2.5**) and conducted force measurement experiments in this background. The effect of loss of KIAA0528 was similar to that of 14-3-3ε: adaptation was eliminated, and neither forces nor persistence times changed (**Figure 2.5, Supplementary Figure 2.8**). As for the CDK5 and 14-3-3ε knockdown backgrounds, the perturbation of KIAA0528 function did not alter plus-end force production (**Supplementary Figure 2.2**).

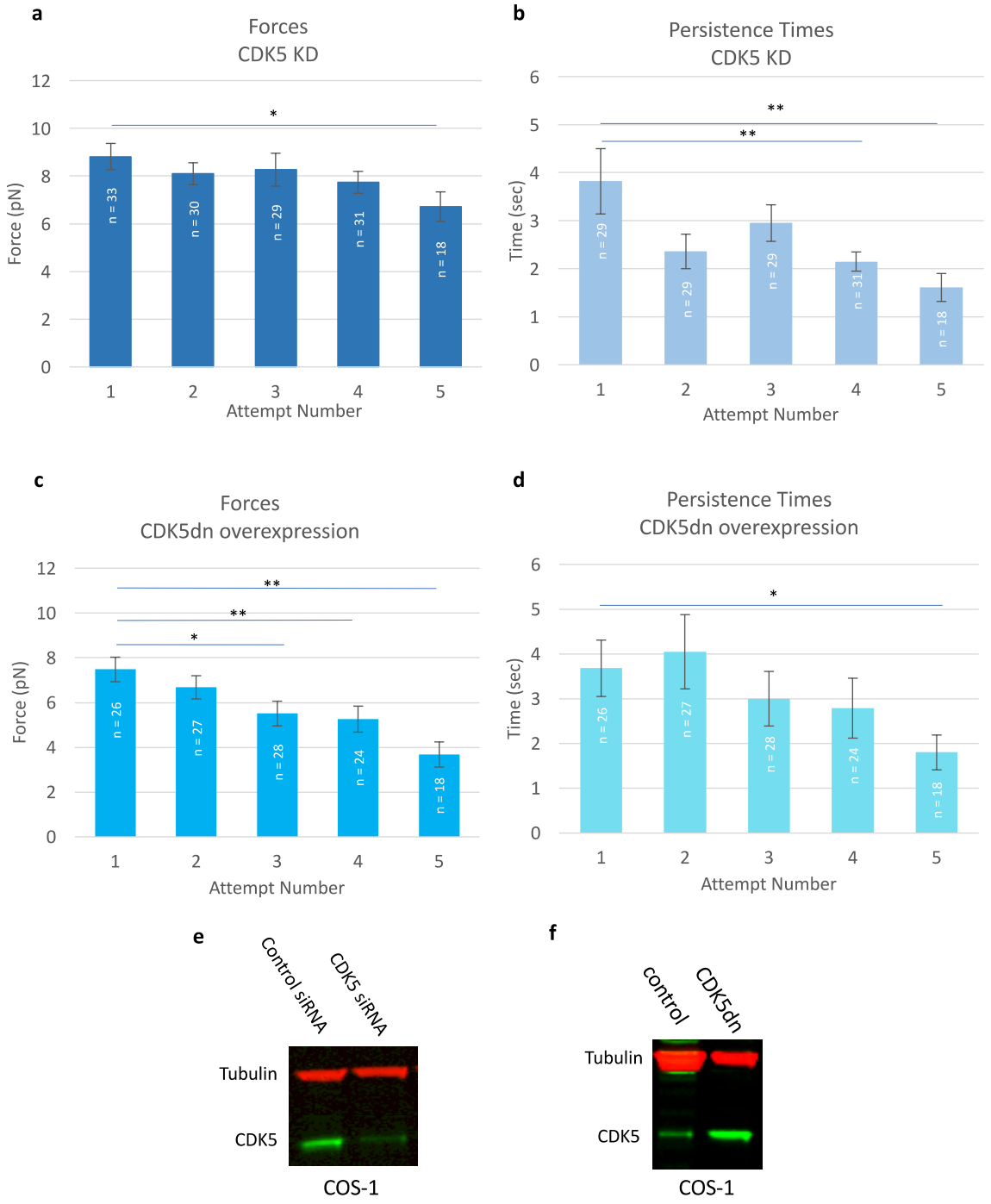


Figure 2.2. CDK5 is essential for dynein force adaptation in COS-1 cells

Average peak forces for minus-end moving LDs decrease at successive attempts in CDK5 knockdown background (**a**) and CDK5dn overexpression background (**c**). Average persistence times for dynein-driven LDs show a significant decrease in CDK5 knockdown background (**b**) and CDK5dn overexpression background (**d**). CDK5 knockdown was >90% effective (**e**), and CDK5dn overexpression was successful (over 15X increase in expression of control) (**f**). Quantified data represent the mean ± s.e.m. of n = 5 independent experiments. One-tailed t-test *P <0.05, **P < 0.01

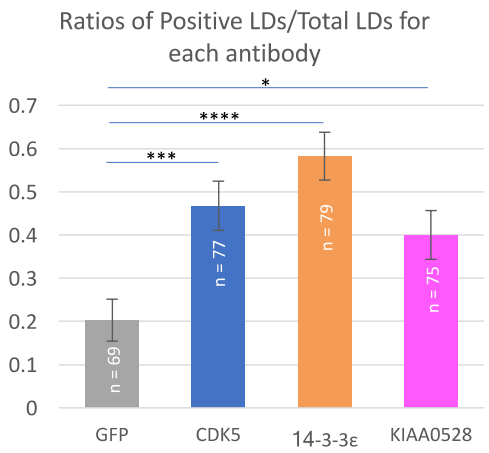


Figure 2.3. CDK5, 14-3-3ε, and KIAA0528 are found on lipid droplet (LD) cargo
Purified LDs were probed with antibodies against CDK5, 14-3-3ε, KIAA0528, and GFP (negative control) (**b**); shown are the percentage of LDs that had detectable immunofluorescence signal. Quantified data represent the mean ± s.e.m. of n = 2 independent experiments. Test of proportions *P <0.05, ***P < 0.001, ****P < 0.0001

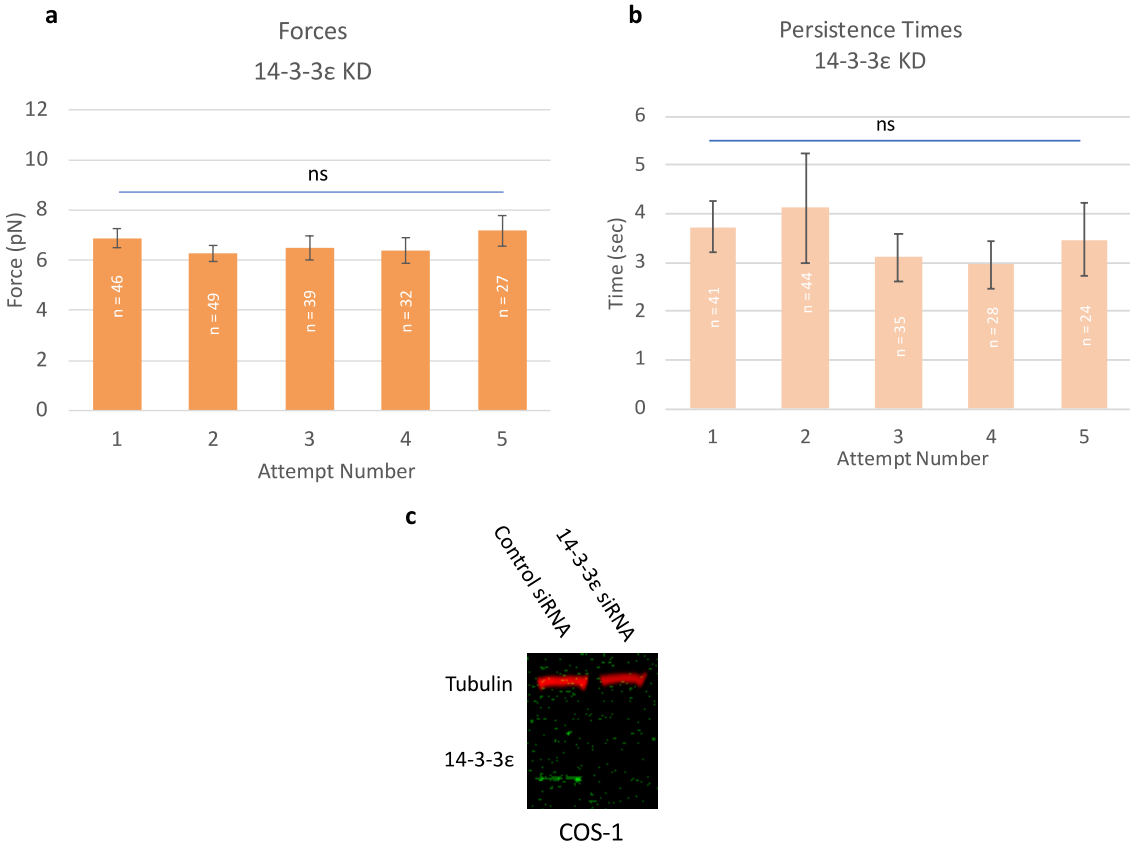


Figure 2.4. 14-3-3ε is essential for dynein force adaptation in COS-1 cells
Average minus-end peak forces (**a**) and persistence times (**b**) at each attempt do not increase in the 14-3-3ε knockdown background. 14-3-3ε knockdown was >80% effective (**c**) Quantified data represent the mean ± s.e.m. of n = 4 independent experiments. One-tailed t-tests results were not significant (ns)

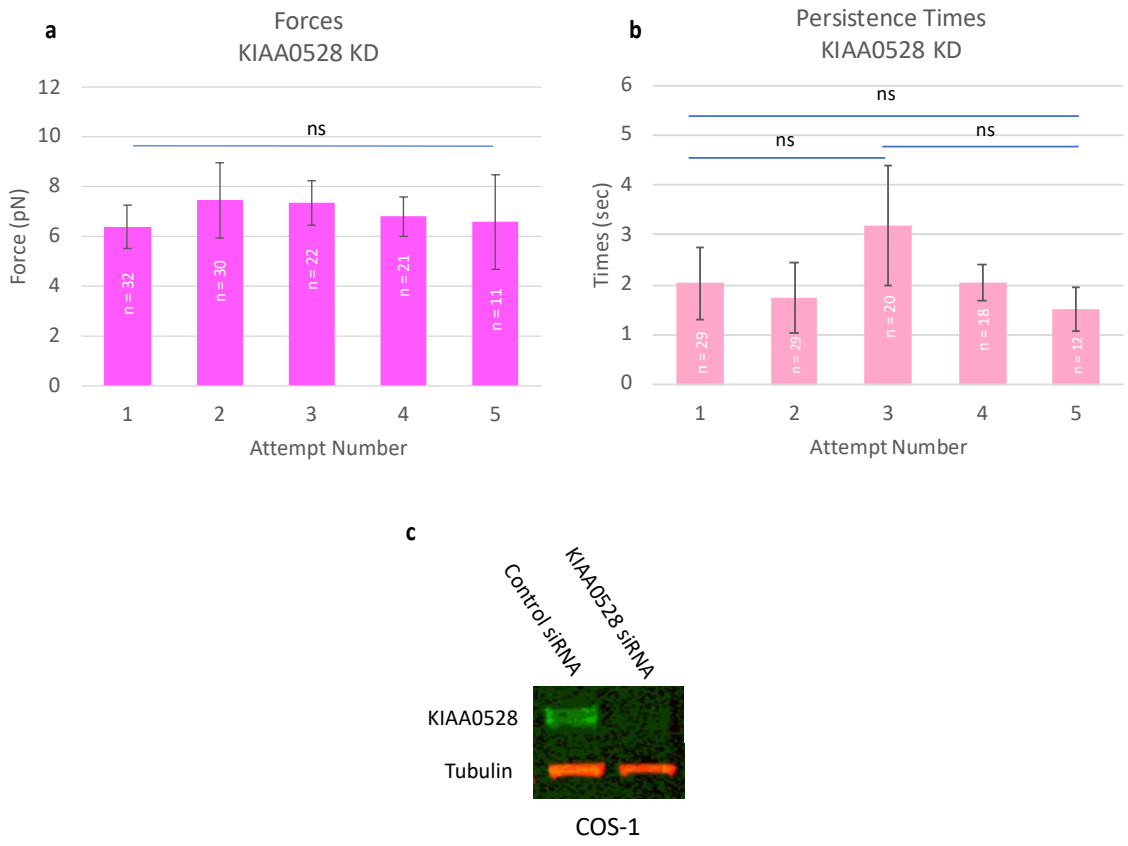


Figure 2.5. KIAA0528 is essential for dynein force adaptation in COS-1 cells
Neither average minus-end peak forces (**a**) nor persistence times (**b**) at each attempt increase in the KIAA0528 knockdown background. KIAA0528 knockdown was >99% effective (**c**) Quantified data represent the mean ± s.e.m. of n = 4 independent experiments. One-tailed t-tests results were not significant (ns)

Confirmation of the functional importance of NudEL phosphorylation.

NudEL and its sister protein NudE contain 55% identical protein sequence, but recent knockout mice of the respective proteins have suggested that the two might have slightly different functions[89,90]. Specifically, NudE KO mice are viable, but have smaller brains than control mice[89]; however NudEL KO mice are embryonic lethal[90]. If dynein is unable to adapt in the specific knockdown of NudEL, perhaps this suggests that force adaptation is in fact an essential dynein function. (However, we did not test force adaptation in a NudE knockdown background.) We previously reported[12] that dual knock-down of NudE and NudEL eliminated force adaptation. Here, we found that knocking down

NudEL alone also abolished force adaptation (KD in **Figure 2.6c, d, Supplementary Figure 2.8**); while we suspect that our NudEL knockdown is specific to NudEL as none of the four siRNA target sequences used were found in the NudE mRNA sequence, our western blot analysis of NudE levels remains inconclusive (data not shown). Thus, to confirm directly the importance of NudEL phosphorylation, we wanted to test for function in a background with modified NudEL phosphosites. Because CDK5 and 14-3-3ε are reported to affect NudEL phosphorylation in a neuronal context, and KIAA0528 is a CDK5-interacting protein potentially contributing to CDK5-mediated phosphorylation, we hypothesized that the overall impairment in force adaptation in all of these backgrounds reflects a loss of CDK5-mediated NudEL phosphorylation. The CDK5 phosphosites on NudEL had previously been determined[63] (and confirmed in multiple other publications[37,85,86]), so to directly assess the ramifications of such phosphorylation events, we carried out force measurement experiments after knocking down endogenous NudEL and replacing it with either NudEL-GFP-wild-type (KDandR-WT), NudEL-GFP-phospho-null (KDandR-null), or NudEL-GFP-phospho-mimetic (KDandR-mim) variants. Because NudEL dosage matters, the replacement constructs were expressed at levels comparable to endogenous NudEL expression (**Figure 2.6e, Supplementary Figure 2.5**), and all mutants were expressed at similar levels to each other.

Importantly, under KDandR-WT conditions, we found that both forces and persistence times increased at successive attempts, similar to the control condition (**Figure 2.6a, b, Supplementary Figure 2.8**). Since force adaptation was lost due to the NudEL KD (KD in **Figure 2.6c, d, Supplementary Figure 2.8**), this rescue showed not only that our rescue construct was indeed functional, but also that the previous loss of force adaptation

had indeed been due to loss of NudEL function (since the knockdown reagents used in both sets of experiments were identical, and the only difference was the presence or absence of the KD-resistant NudEL rescue construct). Under KDandR-null conditions, force adaptation was impaired: forces did not increase, and persistence times decreased significantly, similar to what occurred in the CDK5-KD background (KDandR-null in **Figure 2.6c, d, Supplementary Figure 2.8**). However, under KDandR-mim conditions, we partly rescued force adaptation: forces did not significantly increase, but persistence times increased by attempts 4 and 5 (KDandR-mim in **Figure 2.6c, d, Supplementary Figure 2.8**). Importantly, our data indicate that the phospho-null rescue construct is functional: replacing with the phospho-null mutant *decreases* persistence times relative to the KD alone. Further, combined, the results confirmed the importance of the phospho-sites, but modify our initial model that CDK5-mediated phosphorylation alone accounted for force adaptation (see discussion).

The CDK5 pathway is important for other dynein cargos

Having extensively characterized the role of CDK5, 14-3-3ε, and KIAA0528 in dynein force adaptation with LD cargos, we wondered whether this regulatory pathway was important for other dynein cargos. We thus examined lysosomes and mitochondria. First, we conducted knockdown experiments and visualized the cargos' spatial distribution in each case. Loss of NudEL affected the distribution of both cargos (**Figure 2.7, Supplementary Figure 2.7**), confirming that NudEL contributes to their positioning. Further, loss of CDK5 activity altered the distribution of both lysosomes and mitochondria in a manner quantitatively similar to the effects of loss of NudEL for both classes of cargos. Loss of 14-3-3ε also had a comparable effect to NudEL and CDK5 knockdowns (**Figure 2.7,**

Supplementary Figure 2.7). The lysosomal redistribution may reflect a feedback effect: in the wild-type, plus-end runs and minus-end runs are slightly longer than in the CDK5 knockdown background (**Supplementary Figure 2.3c, d**), possibly contributing to increased lysosomal dispersion. While we do not fully understand why there is a difference in the lysosome and mitochondrial distributions upon force adaptation impairment, the fact that there is, and that it is the same as what occurs for the NudEL knockdown, suggests that overall, NudEL utilization is being regulated by CDK5 and 14-3-3ε, and that this, among other things, contributes to positioning of multiple cargos. Intriguingly, there was a differential effect due to the loss of KIAA0528: its loss had no effect on lysosomes, but altered mitochondrial distributions similarly to CDK5 and 14-3-3ε loss. Thus, we concluded that as for the LDs, KIAA0528 is required for NudEL-CDK5-14-3-3ε utilization in the mitochondria, but the lysosomal NudEL-CDK5-14-3-3ε pathway functions without it.

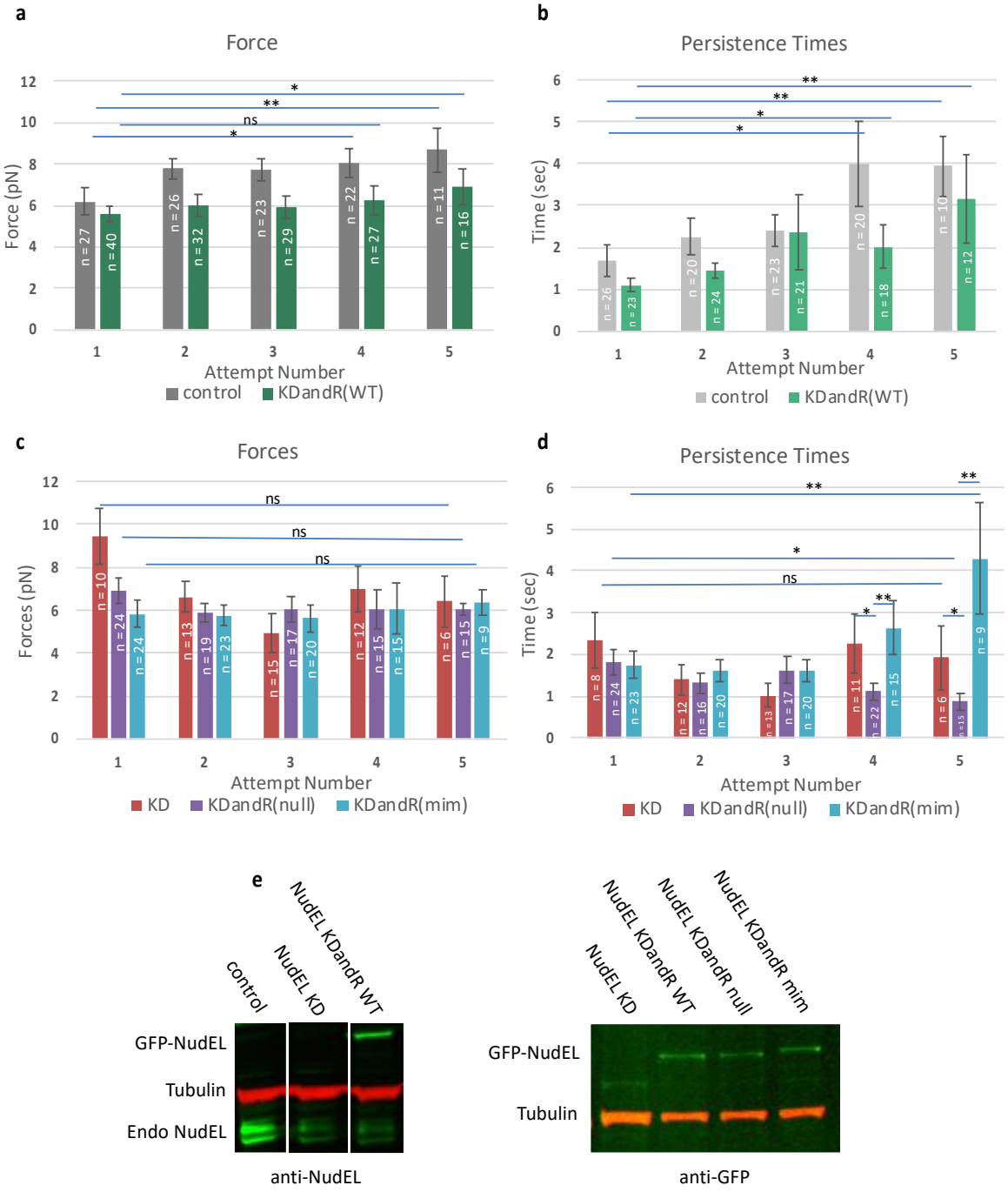


Figure 2.6. NudEL knockdown and replacement force profiles depends on NudEL phospho-state
Average minus-end peak forces (**a**) and persistence times (**b**) for control and NudEL knockdown and NudEL-WT replacement backgrounds. Average minus-end peak forces (**c**) and persistence times (**d**) for NudEL knockdown and NudEL knockdown and phospho-null and phospho-mimetic replacement backgrounds. Average endogenous NudEL (Endo NudEL) knockdown was 70%, average exogenous replacement (GFP-NudEL, band seen in NudEL KDandR WT lane) was 102% wildtype NudEL levels (left). WB probed for GFP found replacement with WT, null, and mimetic mutants resulted in similar expression levels (right). (**e**) Quantified data represent the mean ± s.e.m. of n ≥ 3 independent experiments. One-tailed t-tests *P <0.05, **P < 0.01

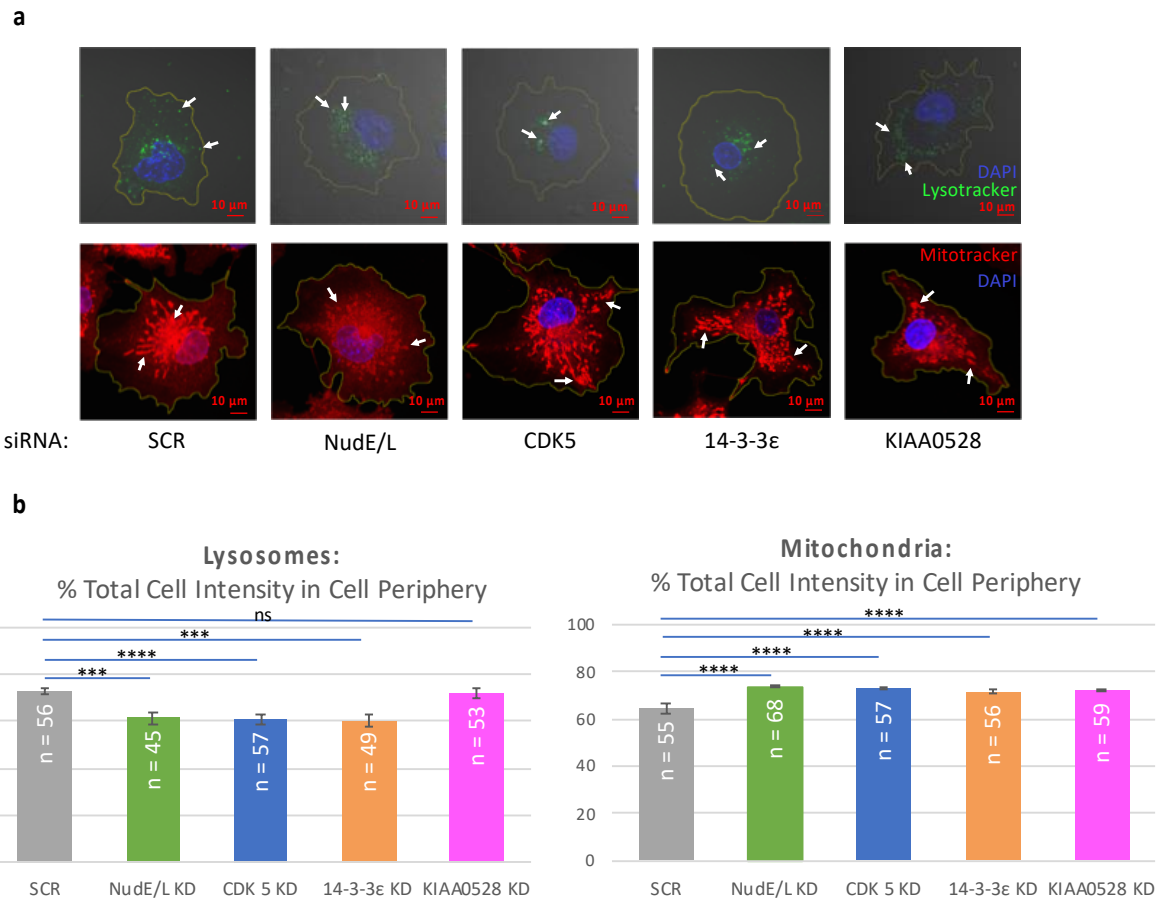


Figure 2.7. The CDK5-activated dynein-NudEL-LIS1 pathway affects lysosome and mitochondria distribution in live cells
Representative images of cells in various backgrounds stained with DAPI and either LysotrackerYellow or MitotrackerRed (**a**) and quantification of lysosome (left) or mitochondria (right) distribution (**b**) Quantified data represent the mean ± s.e.m. of n = 2 independent experiments. One-tailed t-tests ***P <0.001, ****P < 0.0001

The CDK5 pathway contributes to lysosomal tug-of-war

There is an ongoing controversy in the field about the extent to which opposite-polarity motors engage in a tug-of-war, and if so, the importance of such events, and how such tug-of-war events are regulated. We had previously published that such tug-of-war events do not appear important for LD motion[91]; but others reported that tug-of-war events do occur for endosome and phagosomes[92,93]. Since lysosomes are similar to endosomes and phagosomes, in that they are all membrane-bound and involved in cellular degradation, we reasoned that perhaps tug-of-war events between kinesin and dynein

occur here too. Because the NudEL-CDK5-14-3-3ε pathway improves dynein's average force production and especially the duration of the force production under load, one might expect that the magnitude/duration of tug-of-wars would be decreased when dynein's force production is impaired. If one assumes that the plus-end and minus-end motors are randomly placed on the cargos, tug-of-wars should lead to cargo deformations. Thus, to test this possibility, Dr. Babu Reddy developed a new quantitative approach to measure lysosomal deformation in the time-lapse images by using custom written MATLAB code for image processing (see methods). We found that we could indeed detect numerous elongated lysosomes, and that the extent of such deformations was decreased in the CDK5 knockdown background, consistent with a decrease in the magnitude of the tug-of-war events (**Figure 2.8, Supplementary Figure 2.4**). Thus, the data suggests that tug-of-war does occur between motors on the lysosomes, and that by tuning dynein's function, the CDK5 pathway can tune the severity of the tug-of-wars. In principle, this change in apparent stretching could be simply due to decreased dynein utilization, however this appears unlikely because initial forces (reflecting the number of engaged motors before adaptation) were not significantly decreased in LDs from control vs CDK5 knockdown LDs (compare attempt 1 forces in Figure 2.1a and Figure 2.2a and Supplementary Figure 2.1), particle tracking found no difference in the overall number of moving lysosomes (not shown), or in the pause durations (**Supplementary Figure 2.3a, b**). Run lengths do appear slightly shorter in the CDK5 knockdown (**Supplementary Figure 2.3c, d**) in both travel directions. Interestingly, although decreased CDK5 activity diminishes the magnitude of tug-of-war events (as judged by the decreased magnitude of deformations), this change in tug-of-war properties did not appear to alter directional switching probabilities

(**Supplementary Figure 2.3e**), perhaps suggesting that the termination of a tug-of-war is regulated by a switching complex rather than load-induced stochastic disengagement of the dynein or kinesin motors. In conclusion, our data suggests that tug-of-war occurs for dynein-driven lysosomes, that the magnitude of severity of the tug-of-wars can be controlled via the CDK5 pathway, and that effect of such tug-of-wars on the properties of overall lysosomal motion is quite subtle.

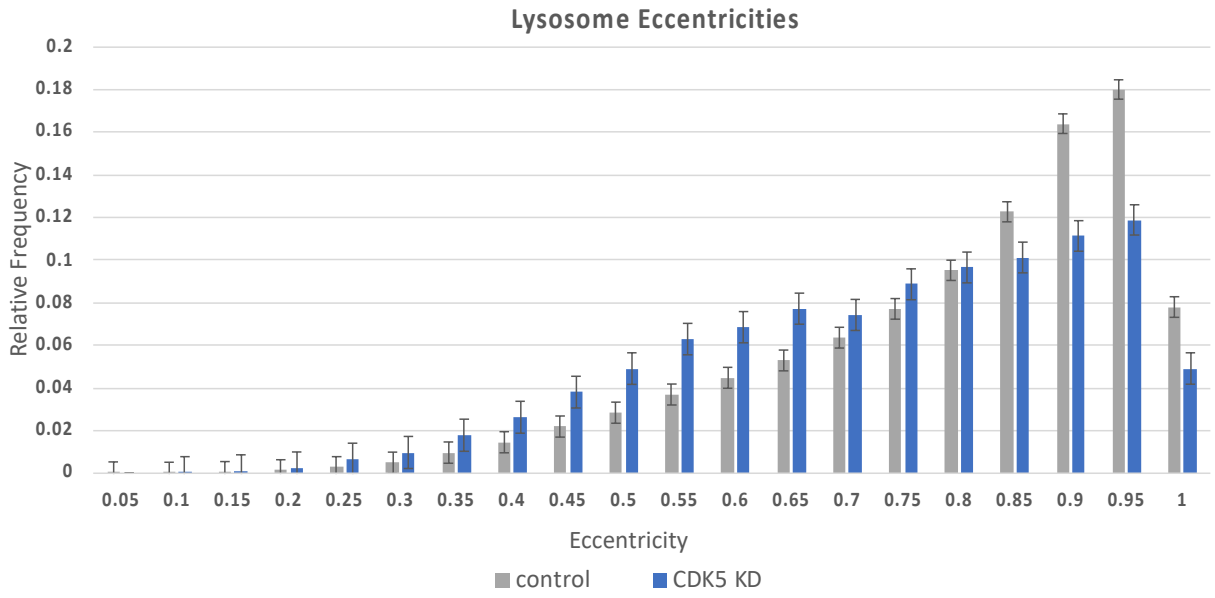


Figure 2.8. Lysosome deformation is reduced in CDK5 knockdown background
Histogram of lysosome eccentricities in control cells (grey) and CDK5 knockdown cells (blue). Eccentricity of 0 is a perfect circle, and eccentricity of 1 reflects highly elongated lysosomes; note the increased frequency of large eccentricities (grey bars at 0.9, 0.95, 1) in the control relative to the CDK5 knockdown. Distributions were determined to be very different (KS test, $p < 0.0001$) Quantified data represent n = 2 independent experiments

Discussion

Here, we investigated the regulation of dynein force production for LDs, mitochondria, and lysosomes; it is partly controlled by CDK5/14-3-3ε, with KIAA0528 also contributing to the first two. Overall, our data are consistent with a model where CDK5, 14-3-3ε, and KIAA0528 are constitutively present on the cargo (**Figure 2.3**, **Supplementary**

Figure 2.6). Dynein force adaptation occurs when the presence of an obstacle induces CDK5 activation, and thus NudEL phosphorylation (which increases the affinity of NudEL for DIC[86]). This increased interaction facilitates a dynein-NudEL-LIS1 complex, with subsequent dynein force adaptation (as previously described *in vitro*[11]). Our work shows the importance of NudEL phosphorylation and the proteins involved—CDK5, 14-3-3ε, and KIAA0528: without these proteins, dynein can no longer increase its duration of force production, nor adapt its maximal forces. Consistent with this, alteration of the NudEL CDK5 phosphorylation sites alters the magnitude and duration of ensemble dynein force production (**Figure 2.6**).

Our data suggest a two-step process for dynein force adaptation. First, our data show that force adaptation requires increased utilization of NudEL/LIS1; for such utilization to occur, NudEL must be phosphorylated at CDK5 phosphorylation sites, with 14-3-3ε, and KIAA0528 contributing to control of this pathway. Second, before engaging NudEL, dynein is likely working with a different cofactor ("Cofactor 1", likely dynactin); we believe that for successful force adaptation, Cofactor 1 must be released, in a CDK5-independent manner (**Figure 1**). Such a hypothesis is consistent with all of our data to date. First, we note that in the CDK5 knockdown, or CDK5dn overexpression backgrounds, adaptation leads to increasing impairment of force production with successive attempts. We hypothesize that this reflects increasing release of Cofactor 1, without the ability to concurrently bind NudEL (due to its lack of phosphorylation); since we have blocked CDK5 activity in these backgrounds, the Cofactor 1 release does not depend on CDK5 activity. Such a model is also consistent with the NudEL phospho-null and phospho-mimetic force measurements: we see increasing deterioration of duration of force production (in the

phospho-null case) and increasingly improved duration in the phospho-mimetic case, consistent with increasing release of Cofactor 1, paired with inability/ability to utilize NudEL phospho-null/phospho-mimetic. However, we do not see this decrease in forces and persistence times in the 14-3-3ε knockdown nor the KIAA0528 knockdown. One interpretation of the finding in the 14-3-3ε knockdown is that the key player CDK5 is still intact and can still phosphorylate NudEL, but the phosphorylation is more labile since 14-3-3ε levels are decreased enough that it cannot sufficiently protect the phosphorylations from the phosphatase PP2A. Similarly, in the KIAA0528 knockdown, we do not see any force adaptation, but there is no decrease in force or persistence times in subsequent attempts either. This might suggest another non-neuronal cofactor of CDK5 that is functionally redundant to KIAA0528; and so in the KIAA0528 knockdown, you still have enough phosphorylated NudEL that force and persistence times do not decrease and instead remain level.

Our hypothesis about modulation of Cofactor 1's involvement obviously raises questions about the role of dynactin in the force adaptation process. Dynactin (with BicD) is reported to improve dynein force production *in vitro* and *in vivo*[59]. If dynactin were playing a significant role here, one might have expected a significant drop in overall force production in the P150 KD background, which was not observed in our past study[12]. Instead, forces and persistence times adapted similarly to the wild-type[12] (Reddy *et al*, 2016 Figure 3a and c). Overall, if there were simple competition between binding NudEL and dynactin controlled predominantly by the DIC-dynactin interaction, we would have expected altered (perhaps improved) adaptation in the P150 KD background, which did not occur. Thus, we favor a hypothesis in which here, dynactin is not contributing significantly

to dynein force production, and that adaptation requires both release of dynactin, and increasing utilization of NudEL controlled by phosphorylation. We note that our published data suggest that dynactin is somehow involved in adaptation: while its loss did not alter the magnitude or duration of force production, it did alter the time between force production events[12] (Reddy *et al*, 2016 Figure 2h). Certainly, future work will need to investigate the control of dynactin utilization, and with this tool in hand, it may then be possible to unambiguously disentangle the coordinated sequence of events proposed above.

Having determined that CDK5 does control force production for lipid droplets, we then established that it was a generalized pathway contributing to positioning control of other dynein cargos (**Figure 2.7**). We analyzed its contribution to lysosomal motion in more detail and found that lysosomal motors appear to engage in a tug-of-war, and that by altering dynein force production, CDK5 can tune the severity of such tug-of-war events (**Figure 2.8**). Interesting, we found that in the CDK5 knockdown background, the outcome of motor tug of wars is not changed. Since you would expect that in this background kinesin might likely be stronger than dynein, and therefore more often win the war (as exemplified by the cargo ultimately moving in the plus-end direction), but this was not the case. There seemed to be no bias in the outcome of the tug of war in either the control or the CDK5 knockdown (**Supplementary Figure 2.3e**). We speculate that there is some other source of control acting as a traffic control for kinesins and dynein motors (that signals which motor to walk when) that we don't yet understand.

We also find that CDK5 knockdown results in slightly shorter plus-end and minus-end runs (**Supplementary Figure 2.3c, d**). This finding is quite subtle but might account

for differences seen in the lysosome distributions in the CDK5 knockdown (**Figure 2.7**). As there has been shown to be coupling between motor activity (when one knockdowns dynein activity, sometimes kinesin activity decreases too), and such phenomenon could explain this result. Another possible interpretation is that because CDK5 is important for MT orientation in neurons, perhaps it is playing a role in orienting MTs in our experiments. If MTs disoriented, it is plausible that this signals kinesin and dynein cargo to not proceed with motion, resulting in shorter run lengths in both directions.

Our findings have multiple implications. First, by directly measuring dynein cellular force production, and showing that CDK5 controls it in non-neuronal cells, we establish the CDK5 pathway as a validated mechano-control signaling pathway. Cells can thus locally increase ensemble dynein force production—one signature of which, for LDs, is force adaptation—as needed, via CDK5 activation.

Second, assuming that these roles are indeed carried over into neurons, our data provide mechanistic insight into neuronal function, and how alteration of these specific proteins could lead to neuronal impairment. For instance, 14-3-3ε is a schizophrenia risk factor, and our data suggest its loss will prevent dynein force production upregulation; thus, when evaluating candidate processes potentially altered by 14-3-3ε decrease, one may want to focus on those requiring high forces, such as large cargo transport, MT reorientation, etc. Similarly, CDK5 was recently shown to play an important role in proper microtubule orientation in the axon initial segment (AIS)[18]. This new role for CDK5 in facilitating high-force events may help explain why this process requires CDK5, as changing MT orientation likely necessitates high dynein force production.

Third, the importance of upregulating dynein force production may vary locally. Cells typically have regional variations in the densities of organelles and cytoskeletal structures. For example, in COS-1 cells, the perinuclear region has higher densities than the cell periphery. Assuming cargos can engage multiple MTs simultaneously, these differences in cytoskeletal architecture may mean that force adaptation becomes important as dynein cargos approach the nucleus. In axons, dynein and its cargo may encounter blocks such as protein aggregates or stalled organelles, and there are regions that appear to have higher organelle and cytoskeletal density, including axonal branch points and AISs. In AISs, "actin patches" are thought to halt vesicles carrying dendritic proteins[94], which may involve force adaptation allowing dynein to overpower kinesin plus-end motors in these patches. Local initiation of dynein force adaptation as motors encounter high density cytoplasm may thus be a critical role for the CDK5/14-3-3ε pathway.

Fourth, our data provide an intriguing framework for interpreting a previous report, which found that increasing neuronal CDK5 activity (via expression of its activator, P25) increased the number of stalled lysosomes[17]. Since we found that lysosomal-bound motors engage in moderate tug-of-war under normal conditions, and that the severity of those tug-of-war events is controlled by CDK5 activity, it seems likely that tuning CDK5 activity could control dynein properties to avoid severe tug-of-war. This allows lysosomes to avoid concomitant dramatic stalling, which has been reported to occur in unregulated competition between kinesin and yeast dynein[95] (working with NudEL and LIS1, cytoplasmic dynein functions more like yeast dynein (in the sense that it has a slow detachment under load). Therefore, one interpretation of the above-mentioned neuronal experiments suggests that increased CDK5 activity results in slower dynein detachment

under load, and even more severe tug-of-wars between lysosomal motors, resulting in increased numbers of stalled lysosomes. Further, this neuronal observation that increasing CDK5 activity leads to stalls—which we interpret as unresolved tug-of-war—provides an intriguing model for why control of CDK5 activity is so important, why its alteration may easily lead to problems, and why local force adaptation may be needed. If CDK5 is aberrantly active, dynein is too powerful, and competitions between dynein and kinesin cannot be well controlled. Thus, we hypothesize that dynein-mediated transport is tuned—via CDK5 activity/14-3-3ε levels and localization—to provide a moderately robust transport system with the ability to overcome typical obstacles to motion. We hypothesize that the role of force adaptation, then, is to prevent dynein from being too strong, so that tug-of-war typically resolves without aggravated traffic jams; it is only after repeated stalls (indicating a significant barrier) that maximum adaptation engages, subsequently allowing for increased dynein performance.

Fifth, for bi-directionally moving cargos, there is typically tight coupling between plus-end and minus-end motor activity[96,97]: when one up- or down-regulates one set of motors, there is usually feedback (though unknown mechanisms) resulting in concomitant changes in the opposite-direction. Interestingly, CDK5 modulation of dynein force production appears to predominantly bypass such feedback: while the slight change in minus-end travel distances in the lysosomes is matched by plus-end changes (Supplementary Figure 3c, d), there is no such matching of effects on force production. Because the underlying mechanism contributing to force feedback matching opposite directions is unknown, we do not understand why such feedback fails here. However, this decoupling may be functionally useful allowing for selective directional control. In the wild-

type force adaptation, minus-end forces and durations increase with attempt number, but plus-end ones stay constant. In the CDK5 knockdown/dominant-negative overexpression backgrounds, minus-end forces and durations decrease, but plus-end forces and durations remain unchanged (**Supplementary Figure 2.2**). Thus, at least for the cargos/cells considered here, the CDK5 pathway appears to almost entirely affect only minus-end motor activity, and allows for selective directional force production control.

Finally, our findings may have implications for diabetes. LD biology is linked to insulin resistance[98], and their transport directly affects metabolism[7], so alteration in their trafficking by changes in force adaptation could potentially impact metabolic disease. Insulin signaling can regulate dynein to influence lysosome motility in both neurons and non-neuronal cells[99]. This involves inhibition of GSK-3β, a kinase whose dysregulation is linked to both metabolic and neurological disorders[100]. GSK-3β phosphorylation of dynein reduces its interaction with NudEL[99]. CDK5 can inhibit GSK-3β, but to our knowledge the opposite has not yet been reported[101]. Nonetheless, it will be interesting to determine if GSK-3β modulates force adaptation, and if so, if this occurs through altered NudEL phosphorylation by CDK5. Dynein is also required for internalization of adipocyte GLUT4 receptors in low insulin conditions[22]. Dynein force adaptation could also modulate vesicle internalization. If that is the case, alterations in force adaptation mechanisms could lead to defective glucose transport. Interestingly, KIAA0528 may be involved in GLUT4 insertion into the plasma membrane, although this is not yet completely understood[8,102].

In summary, we found that NudEL phosphorylation control by the CDK5/14-3-3ε pathway directly regulates dynein force production in cells. It is required for the dynamic up-regulation of dynein force underlying the previously discovered LD force adaptation,

but also plays a general role for other cargos such as lysosomes and mitochondria, contributing to control of their subcellular localization. Further, the differential effects of KIAA0528 (affecting LDs and mitochondria, but not lysosomes) suggest cargo-specificity could in part be controlled by cargo-specific CDK5-interacting regulatory proteins like KIAA0528. Importantly, at least for lysosomes, the CDK5 pathway is able to tune dynein's force production capability, and ultimately modulate motor tug-of-war severity; this likely seems relevant for various cellular cargos. Future work will undoubtedly explore ramifications of control of tug-of-war severity, the role and regulation of dynactin in this process, and how cargo-specific proteins like KIAA0528 mechanistically contribute to regulation.

Materials and Methods

Cell Culture and transfections. Mycoplasma-free COS-1 cells (obtained from ATCC) were grown in DMEM (Genesee) supplemented with 10% fetal bovine serum and 1% Pen-Strep at 37° C in 5% CO_2. Gene expression knockdown for CDK5, 14-3-3ε, KIAA0528, NudE, and NudEL were completed by transient transfection using commercially available siRNAs. siRNA for the control (sc-37007), CDK5 (sc-29263), 14-3-3ε (sc-29588), and KIAA0528 (sc-95830) siRNAs were obtained from Santa Cruz Biotechnology. NudE (SI00655858, SI4374356, SI4341386, and SI05147835) and NudEL (SI03246600, SI03246936, SI04264379, and SI04321191) siRNAs were obtained from Qiagen.

For the CDK5, 14-3-3ε, KIAA0528, NudE, and NudEL knockdowns, HiPerfect reagent (Qiagen) was used following the manufacturer's instructions. CDK5 and KIAA0528 knockdowns were achieved by using a final concentration of 33nM siRNA; 14-3-3ε

knockdown was achieved by using a final concentration of 66nM siRNA; NudE and NudEL knockdowns were achieved by using a final concentration of 5nM of each siRNA. (Control transfections used the same final concentration of scrambled siRNA as the target siRNA.)

For the CDK5dn overexpression and NudEL replacement, Lipofectamine-2000 (ThermoFisher Scientific) was used following manufacturer's instructions. 2 μg of DNA was used for the overexpression and replacement experiments.

Transient knockdowns were incubated for 48 hours before force measurements, WB, IP, and tracking experiments were carried out. Transient overexpression or replacement experiments were incubated for 24 hours before force measurements, WB, and tracking experiments were carried out. NudEL replacement expression was induced with 20 nM doxycycline 18 hours before force measurements, WB, fluorescence, and tracking experiments were carried out.

Expression vectors. The CMV-myc-CDK5dn, pEGFP-C1-NudEL(WT) and phospho-null mutant (1-5A) expression vectors were previously described[19].

Mutagenesis and Cloning. The NudEL-WT plasmid was mutagenized into a phospho-mimetic mutant using site-directed mutagenesis via Multi-Site-Directed Mutagenesis QuikChange Lightning (Agilent) in two rounds of mutagenesis. In the first round, F1-F3 primers were used; in the second round, P4-P5 primers were used (**Table 2.1**). CDK5 phosphosites on NudEL 1-5 were replaced with aspartic acid.

Table 2.1. Oligos used in phospho-mutagenesis

Oligo Name	DNA Sequence 5'-3'
GFP-NudEL_S197D_F1	GCA GAG GGA GTT AGT GGA TCG GTA CCA AAA CCA TTT GG
GFP-NudEL_T219D_F2	CAG TCT AGA GTT GGA GAG TCA GGA GCC GAC TTT CTA
GFP-NudEL_S242D_F3	GTT CCT TTG CCA ACA GGG TCA GCT GGC AAA GAA AGT GAT GC
GFP_NudEL1-5D_mutagen2_P4	CCT AGC AGA GGG ATC TAG TGG ATC GGT ACC
GFP_NudEL1-5D_mutagen2_P5	GGT ATA GCT TCC GGA TCA GGA AAA GTG TTC TCC GTT CC

The three NudEL plasmids (WT, 1-5A, and 1-5D) were further mutagenized to achieve RNAi-resistance. Two silent mutations were introduced in the middle of each of the four siRNA target sequences of the NudEL mRNA sequence (for a total of eight mutations/construct). The NudEL plasmids were mutagenized using site-directed mutagenesis via Multi-Site-Directed Mutagenesis QuikChange Lightning (Agilent) in two rounds of mutagenesis. F1-F3 primers (**Table 2.1**) were used in the first round; and then F4-F6 primers (**Table 2.1**) were used in the second round.

Table 2.1. Oligos used in RNAi-resistance mutagenesis

Oligo Name	DNA Sequence 5'-3'
NudEL-RNAiResistant_F1	GGA GAA AGA GCA CCA ATA TGC ACA GAG C
NudEL-RNAiResistant_F2	CTC AGT GTT AGA GGA TGA TTT AAG TCA GAC TCG CGC CAT TAA GG
NudEL-RNAiResistant_F3	CAG TGG AAG TTG AGC AAA GGC TAA ACC
NudEL-RNAiResistant_F4	GGA GAA GCT AGA ACA CCA ATA TGC ACA GAG C
NudEL-RNAiResistant_F5	CTC AGT GTT AGA GGA CGA TTT AAG TCA GAC ACG CGC CAT TAA GG
NudEL-RNAiResistant_F6	CAC TGG AAG ACT TTG AGC AGA GGC TAA ACC

The three RNAi-resistant NudEL constructs were then subcloned into the expression pDONOR201 vector via Gateway Technology (Invitrogen). These were then recombined into the gateway-compatible doxycycline-inducible destination vector and contained a C-terminal-S-FLAG-SBP (SFB) tag[103].

Table 2.2. Oligos used to insert NudEL into pDONOR201

Oligo Name	DNA Sequence 5'-3'
GFP-NudEL_RNAiR4_F	GGG GAC AAG TTT GTA CAA AAA AGC AGG CTC CAT GGT GAG CAA GGG CGA GGA GCT GTT C
GFP-NudEL_RNAiR4_BP_Rc	GGG GAC CAC TTT GTA CAA GAA AGC TGG GTT TCA CAC ACT GAG AGG CAG CAT ACC CGG

Immunoblotting. Cells were washed with PBS and lysed using an ice cold 1% NP-40 buffer containing a 1X protease inhibitor cocktail (Roche) and the supernatant was collected. Proteins from the lysate were denatured using an SDS buffer and incubating at 70 °C for 10 min. The proteins were then separated in a 4-12% Bis-tris gel (Life Technologies) and transferred to a nitrocellulose membrane through a wet transfer method. The nitrocellulose was blocked with 5% non-fat milk in Tris buffered saline (TBS: blocking buffer) for 45 min at room temperature. Immunoblotting was completed with respective antibodies and blots were visualized with infrared detection on the Odyssey (Licor). Primary antibodies were diluted in blocking buffer with Tween20 at various concentrations (1:200-1:1000 v/v). Secondary antibodies were diluted in blocking buffer with Tween20 at 1:10000 v/v. Antibodies were purchased from: Cell Signaling Technologies 2506s (CDK5) and 9635s (14-3-3ε), Bethyl A301-469A-M (KIAA0528), ABClonal A5776 (NudEL), Bioss bs-5522R (pSer231NudEL), Developmental Studies Hybridoma Bank E7-s (Tubulin-α), Life Technologies (goat anti-mouse IgG 680), and Li-cor (goat anti-rabbit IgG 800). Data shown here are representative of 2-3 separate experiments. Quantification was completed in Licor's ImageStudio. Since there is potential cross-reactivity in the anti-NudEL WB shown in Figure 6e, only the bottom green band was considered for quantification (the top green band is likely NudE).

Force measurements in cells. Previously described[12,104].

Lysosome Distribution and Particle Tracking. After 48 hours of incubation with transfection reagents, COS-1 cells were stained with NucBlue Live ReadyProbes Reagent (Life Technologies) and either 200 nM LysoTracker-Yellow or 200 nM MitoTracker-Red (Thermo Fisher Scientific) and incubated for 30 min before visualization. Live COS-1 cells

were visualized at University of California Irvine Optical Biology Core on the Zeiss LSM700 confocal laser scanning microscope. At least thirty cells were imaged. The cell perimeter, nuclear perimeter, and respective intensities were measured for each fluorescent image using ImageJ. The perinuclear region was defined as 20% of the cytoplasm surrounding the nucleus. The cell periphery was defined at the other 80% of the cytoplasm. The intensity of the cell periphery was defined as the total cell intensity minus the intensity of the perinuclear region. The percent total cell intensity in the cell periphery was defined as (the intensity of the cell periphery/total cell intensity)*100 (previously described[105]).

Lipid Droplet Immunofluorescence. Lipid droplets (LDs) were purified using a sucrose gradient as previously described[12]. Purified LDs were incubated overnight with primary antibodies to a final concentration of 1 μg/ml in the buffer (80mM PIPES, 1mM $MgCl_2$, 2mM EGTA, 1mg/ml casein) followed by 1 hour incubation with secondary antibody (0.02 mg/ml) in the same buffer described above. Fluorescence imaging was carried out using EMCCD camera (quantEM 512, Photometrics) and a 488 nm excitation laser in semi-TIRF mode. Fluorescence signal integration time was fixed at 0.5 sec.

Eccentricity and Run Distances of Lysosomes. Cells were incubated with 200nM of Lysotracker red DND-99 (Life Technologies) for 30 min in the medium used for tissue culture. A sample chamber similar to the one adopted for force measurements was used to image the cells. Images were acquired in semi-TIRF mode at 10 fps using 568nm excitation laser and the quantEM 512 camera. Spatial resolution of the acquired images in the above setup is 60nm/pixel.

Eccentricity of lysosomes in each movie frame was determined using custom Matlab code for image processing. One hundred consecutive images for each cell (18 cells in each

condition from two experimental replicates) were used for this analysis, because the deformations could be dynamic, and we wanted to catch them if they occurred; the distributions shown thus reflect instantaneous multiple determinations of each lysosome's eccentricity. If a specific lysosome was visible in all 100 consecutive frames, and it were circular in the first fifty frames, and very stretched in the next fifty, it would have yielded 50 eccentricity measurements that were 0, and 50 that were 1, all included in the histogram. Binary images were generated from the raw images, and the eccentricity and areas of individual lysosomes were measured (after appropriate filtering to reduce the background). Eccentricity values range from 0 to 1, with 0 corresponding to a perfect circle and 1 representing highly deformed objects. Only lysosomes with areas in the range of 8-200 square pixels were considered.

Particle tracking was carried out by custom softwares LVcorr and Marathon. LVcorr was used to generate long tracks (track length ~100 sec and 90 tracks from 18 cells in each condition) from the videos recorded above. Using tracks files from LVcorr as input, the parsing software Marathon was used to extract the pauses and run distances of lysosomes moving linearly in kinesin and dynein directions.

Statistics. All graphs are mean and s.e.m. Statistical significance was determined by Student's t-test, test of proportions, Kolmogorov–Smirnov, and Wilcoxon sign-ranked t-test. *P <0.05, **P < 0.01, ***P < 0.001, ****P<0.0001

CHAPTER 3: Conclusion

Overview

The mechanism of regulation of dynein force adaptation was characterized in Chapter 2. While detailed in the motion of lipid droplets, we expanded our study to other dynein cargos including mitochondria and lysosomes in order to determine if this regulatory pathway was important for multiple classes of cargo. We found that CDK5 knockdown altered the distribution of lysosomes and mitochondria. To further study this pathway in the context of lysosomes, we knocked down other proteins involved in regulating dynein force adaptation and conducted our lysosome particle tracking and eccentricity analyses. We find that knocking down 14-3-3ε, but not KIAA0528, resulted in a similar phenotype in lysosome motion (increased motion with decrease levels of the pathway protein) and surprisingly the opposite phenotype in lysosome shape. In the 14-3-3ε knockdown, lysosomes are more elliptical, which was in direct contrast to the eccentricity phenotype in the CDK5 knockdown background (where lysosomes were found to be more spherical). This Chapter also details future directions aiming to determine the importance and cellular function of dynein force adaptation currently being explored by the lab and speculate on possible biological roles for dynein force adaptation.

Conclusion

The regulation of molecular motors remains elusive. However, I have presented here a number of discoveries we have made during my time in the lab. We have discovered that *in vitro*, the signaling kinase CK2 reactivates the mitotic kinesin Eg5, although the

importance of this regulation *in vivo* is unclear. We suspected it might be important for cell proliferation, but this is not the case (**Figure 1.4**).

For most retrograde intracellular transport, cells rely on the molecular motor dynein (MAP1C, dynein-1)[11,31]. Proper dynein function is essential to eukaryotic cellular vitality, and therefore it is important to understand the mechanisms and regulation of dynein function. As stated, dynein can form two main complexes: dynein-dynactin-BicD or dynein-NudEL-LIS1[14,16,56–58]. While dynein-dynactin-BicD is important for long travel distances[16], high processivity, dynein-NudEL-LIS1 is used when high force production is required[12]. Chapter 2 showed how the utilization of NudEL-LIS1 complex is regulated by CDK5 and detailed other proteins involved in this kinase signaling pathway. Specifically, we proposed that when dynein experiences an opposition to motion, CDK5 becomes activated possibly with the help of KIAA0528, phosphorylates NudEL, these phosphosites are protected (from dephosphorylation by the phosphatase PP2A) by 14-3-3ε, and once NudEL is phosphorylated, the interactions between dynein and NudEL are increased, and ultimately dynein can adapt its forces.

We found that variations of this pathway exist for multiple dynein cargos. CDK5, 14-3-3ε, and KIAA0528 are all essential for dynein force adaptation in the lipid droplet system (**Figures 2.2, 2.4, and 2.5**). However, in simply looking at differences in distribution of lysosomes, only knocking down CDK5 or knocking down 14-3-3ε show different distribution phenotypes. The KIAA0528 knockdown showed similar lysosome distribution as the control cells (**Figure 2.7**). For mitochondria, knockdown of CDK5, 14-3-3ε, or KIAA0528 show different distribution phenotypes than the control (**Figure 2.7**).

To further characterize the effect of this phosphorylation pathway on lysosome motion, we conducted lysosome particle tracking and eccentricity analysis in the CDK5 knockdown background. We found that while overall motion was not dramatically different between the control and the CDK5 knockdown, there was a significant difference in lysosome eccentricity (**Figure 2.8**). We speculated that this might be due to a difference in motor tug-of-war. Opposing molecular motors like dynein and kinesin can engage in tug-of-war, but the implications of cargo-specific tug-of-war in cells is less understood[91–93]. We suggested that in the CDK5 knockdown cells, dynein is not able to adapt its forces and therefore kinesin outcompetes dynein in lysosomal tug-of-wars resulting in less elliptical lysosomes in the knockdown cells. This advocates for the role of dynein force adaptation as a check point: to keep dynein from becoming too strong.

The recent continuation of this study shows interesting lysosomal motion and shape analysis results for 14-3-3ε knockdown and the KIAA0528 knockdown backgrounds. In particular, knocking down 14-3-3ε resulted in overabundance of lysosomal motion within the cell (similar to the increased motion in the CDK5 knockdown background in Chapter 2, data not shown). However, when we analyzed lysosomal eccentricity, we found that lysosomes were more elongated in the 14-3-3ε knockdown background than in the control (**Figure 3.1**). This was the opposite trend seen in the CDK5 knockdown background where the lysosomes were more spherical than the control (**Figure 2.8**). This suggests that there is interference from another 14-3-3ε pathway involving molecular motors. It is known that 14-3-3ε creates a bridge between dynein and kinesin-73 that ultimately mediates spindle localization[38]. One possible explanation of these surprising eccentricity results is that when this 14-3-3ε-dynein-kinesin-37 complex is formed, one motor is inactivated and the other

remains active. If 14-3-3ε is not there to form the complex, and ultimately regulate the activity of each motor, then each class of motor remains continually active, likely engages in tug-of-war events with oppositely directed motors, and this ultimately results in elongated lysosomes. In the KIAA0528 knockdown, we find that the overall motion (data not shown) and eccentricity distribution (**Figure 3.1**) is similar to that of the control. This is expected since we found that knocking down KIAA0528 did not change lysosomal distributions in Chapter 2 (**Figure 2.7**).

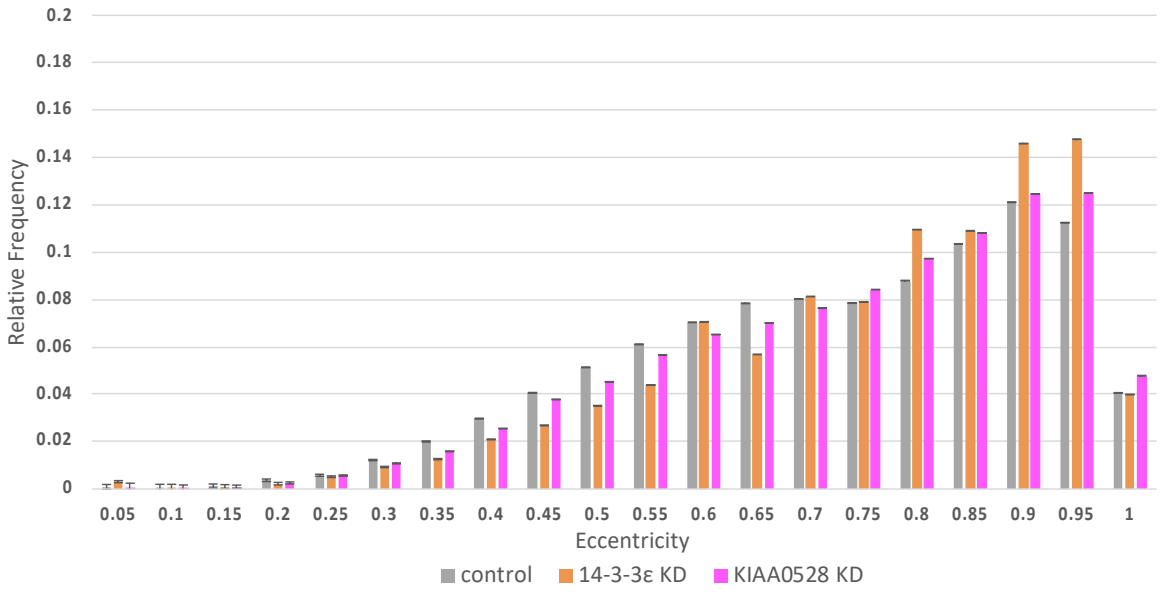


Figure 3.1. Lysosome eccentricity differences as a result of 14-3-3ε knockdown or KIAA0528 knockdown
Histogram of lysosome eccentricities in control cells (grey), 14-3-3ε knockdown cells (orange), and KIAA0528 knockdown cells (pink). Eccentricity of 0 is a perfect circle, and eccentricity of 1 reflects highly elongated lysosomes; Quantified data represent n = 2 independent experiments

Possible Cell Functions of Dynein Force Adaptation

We have characterized the CDK5 pathway controlling dynein force adaptation and generalized its importance for many dynein cargos. However, we have only begun to explore the possible cellular function of dynein force adaptation. There are many cellular

locations that could elicit dynein force adaptation. For example, dynein cargo may encounter more opposition to motion in crowded areas of the cell: like the perinuclear region where there is a large concentration of organelles. It could be that this is where increased utilization of the NudEL-LIS1 system is important for dynein to move its cargo through a concentrated area. Another similar location would be in the axon initiation segment in neurons. This is a major hub for dendritic cargo sorting, and therefore also quite concentrated. Maintaining dynein motors in a high force state may be important for dynein to navigate cargo through these segments.

Similarly, there are many cellular processes in which dynein force adaptation is essential. For example, it has been shown that when LIS1 is inhibited large (but not small) axonal cargo becomes stuck[106]. It seems likely, then, that dynein uses the NudEL-LIS1 system to move largo cargo up and down narrow axons. It would be interesting to rescue stuck cargo in a LIS1 knockdown with a photo-activatable P25 (the CDK5 activator) construct. Then one could locally activate CDK5 and ultimately increase utilization of the NudEL-LIS1 system locally to see if that resolves the molecular traffic jams.

Dynein is also known to be important for GLUT-4 internalization[88,107] and has been shown to transport endosomes in HeLa cells[108]. One can imagine that dynein needs to maintain a high force persistence state by utilizing the NudEL-LIS1 system in order to pull early endosomes in towards the center of the cell. To study if this is the case, we plan to first knockdown CDK5 in RAW cells (a macrophage cell line). We will then induce these cells to endocytose beads in the control and CDK5 knockdown backgrounds and perform force measurements and particle tracking analysis of the endocytosed beads in order to determine if this cellular process utilizes dynein force adaptation. Given that this is a

dynein-driven pathway, and one that likely involved high force production, it is likely for endocytosis to necessitate dynein force adaptation.

Regulation of Transport

My work here has focused on understanding the regulation of both kinesin, specifically Eg5, and dynein motors. While many labs are working to understand the cellular processes that these motors are involved in, we have focused on understanding how these motors become activated or inactivated in order to carry out their biological functions. We suggest that motors are frequently in their inactive state, and upon certain signal transduction (i.e. for Eg5: activation by the signaling kinase CK2, or for dynein: phosphorylation of NudEL by the signaling kinase CDK5), the motor (or the motor function, in the case of dynein force adaptation) is switched on at the proper space/time. Since many motors are found on the vesicles they transport[109], this theory is plausible.

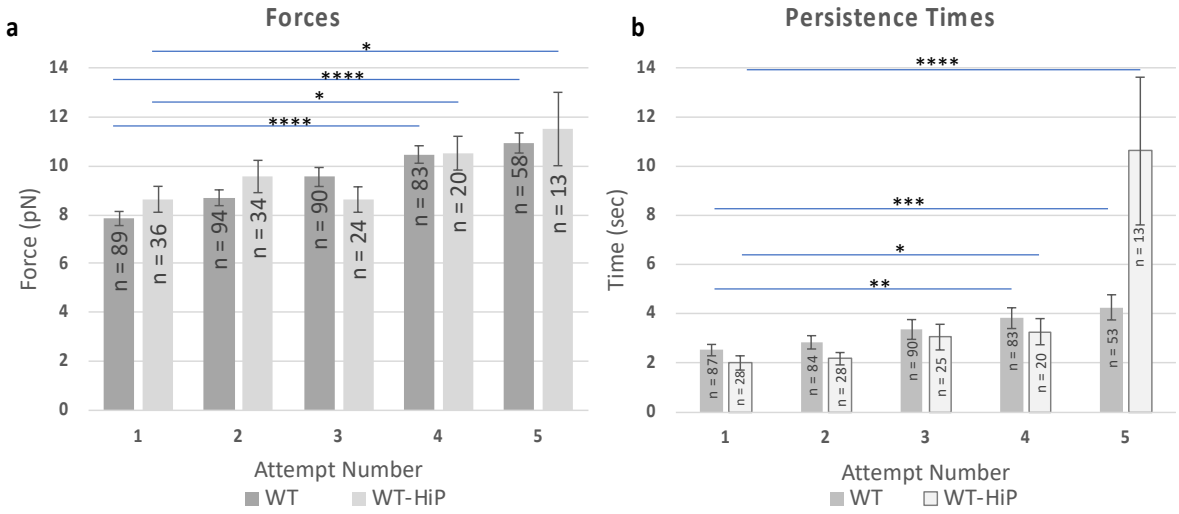
However, more work will undoubtedly need to be done to confirm this theory. Specifically, while we have considered the effect of other proteins on motor regulation in this work, the organization of motors on the cargo itself can play a regulatory role as well. This has recently been studied by our group and others[109–111]. For example, Monte Carlo simulations suggest that motors cluster together to carry large cargo along extensive distances (tens of microns) within the cell, which requires more engaged motors[111]. Furthermore, motors must navigate a complex 3D matrix of microtubules within the cytoskeleton of the cytoplasm. Our collaborators suggest computationally that the cytoskeleton itself also plays a regulatory role for molecular motors[112]. However, such studies are in their infancy and with the recent advances in high resolution microscopy,

will likely expand to *in vivo* studies in the near future. I expect that in cells, the regulation of transport happens collectively, as an effort between the motors, their cofactors, their cargos, and the obstacles that the cell cytoplasm introduces. As a field, we will gain an abundance of knowledge about the interplay of all these factors that come together to successfully regulate molecular motors in the context of a cell.

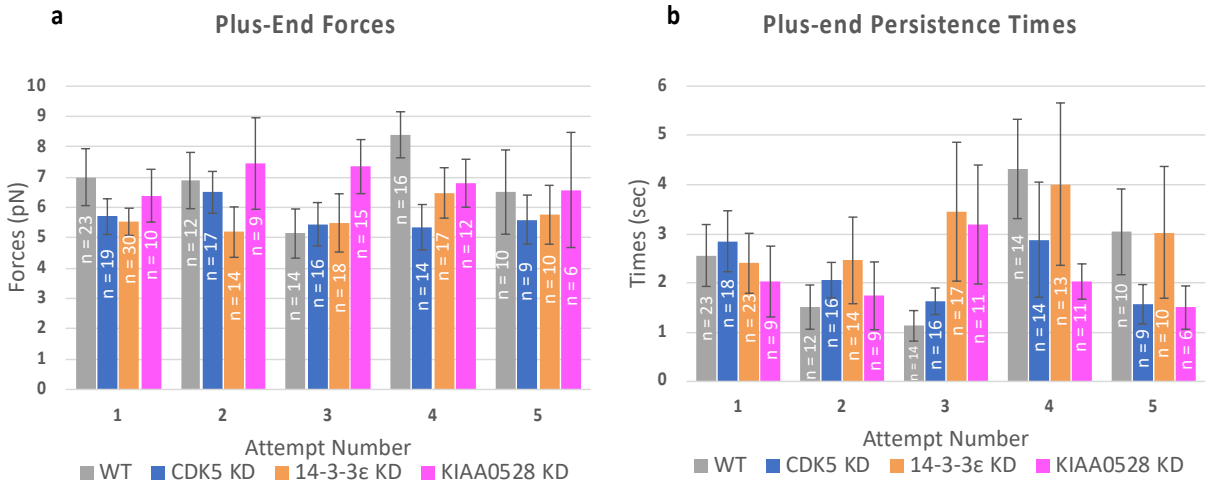
Perspectives

Overall, this works provides a basis of signaling regulation for two different classes of motors: the mitotic kinesin, Eg5, and the retrograde motor, cytoplasmic dynein. Although the work in Chapter 1 did not lead to interesting findings in cell biology, it provided more detail on the regulation of Eg5 *in vitro*. The work in Chapter 2, however, was much more successful. I expect that this work will provide a strong foundation for future studies of the biological importance of dynein force adaptation. The lab is already exploring one possible application of force adaptation in endocytosis by conducting endocytosis assays in RAW cells (a mouse macrophage cell line) in control and CDK5 knockdown backgrounds. I hypothesize that when dynein force adaptation is abolished (through knockdown of CDK5), the lab will see defects in endocytosis. Such studies will showcase the importance for dynein force adaptation in cell biology and reveal a new area of study for future molecular motor research.

SUPPLEMENTARY FIGURES

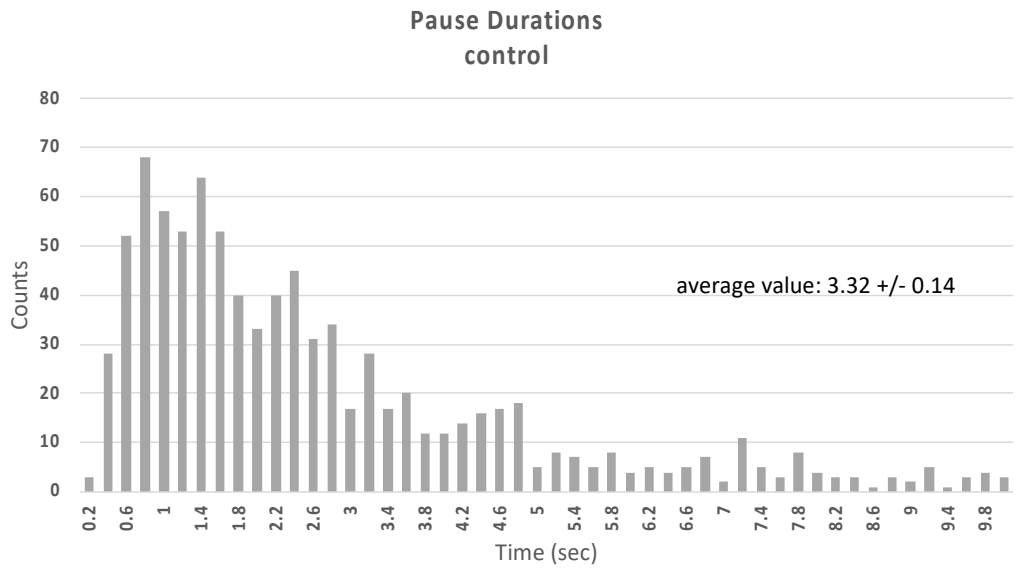


Supplementary Figure 2.1. Transfection reagent (control) does not affect dynein force adaptation
Average minus-end peak forces (**a**) and persistence times (**b**) were similar between wildtype cells (darker grey in each panel) and wildtype cells treated with HiPerfect (lighter grey in each panel). Quantified data represent the mean ± s.e.m. of n ≥ 5 independent experiments. * P <0.05, ** P <0.01, ***P <0.001, ****P < 0.0001

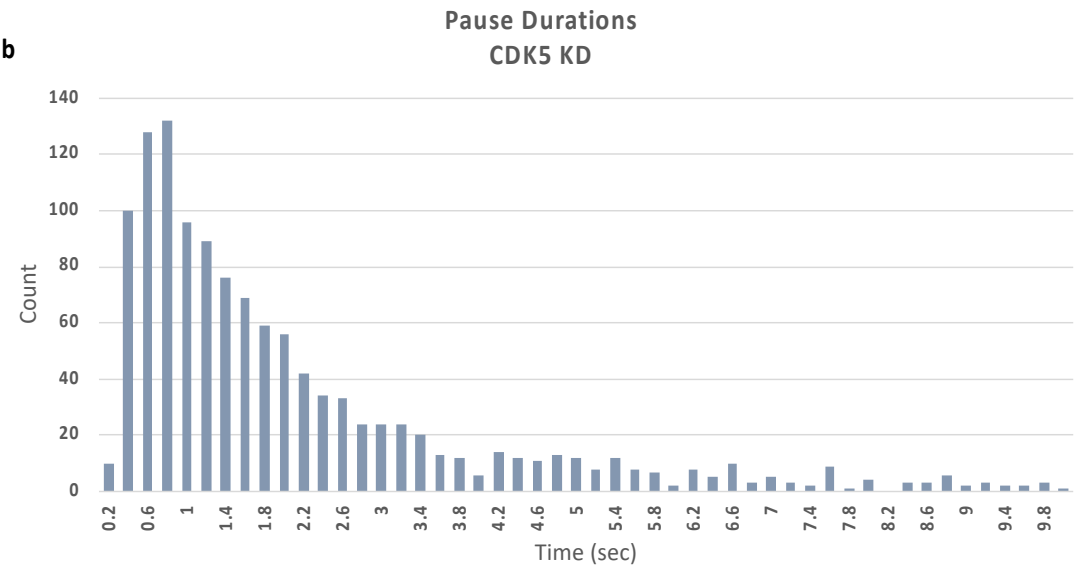


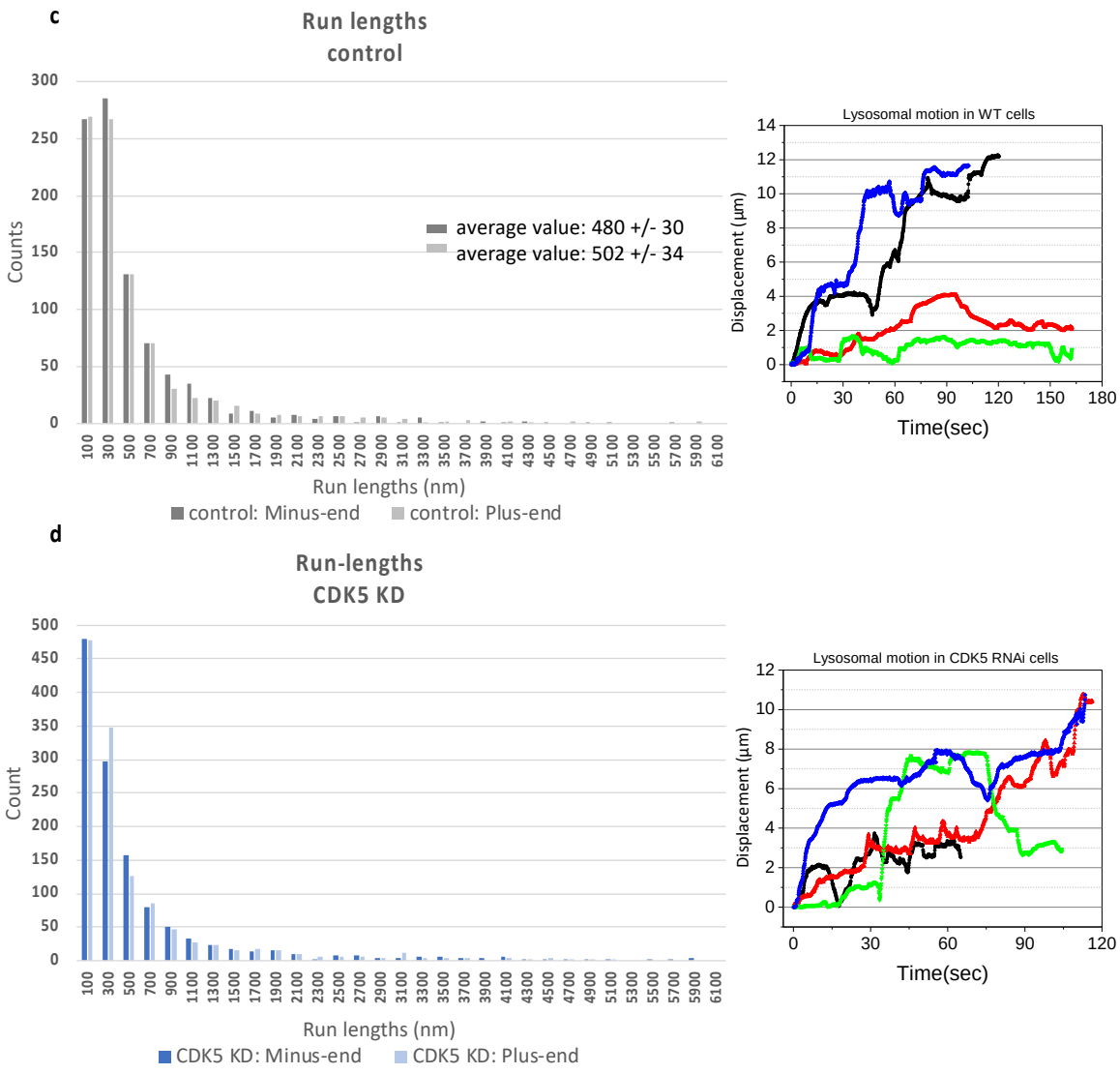
Supplementary Figure 2.2. Plus-end forces and persistence times show no force adaptation under any conditions
Average peak forces (**a**) and persistence times (**b**) from plus-end moving LD cargo do not increase at each attempt in WT, CDK5 knockdown, CDK5dn overexpression, 14-3-3ε knockdown, and KIAA0528 knockdown backgrounds. Quantified data represent the mean ± s.e.m. of n ≥ 4 independent experiments. Force and persistence times values at each attempt were compared via ANOVA within each sample. All p-values > 0.05

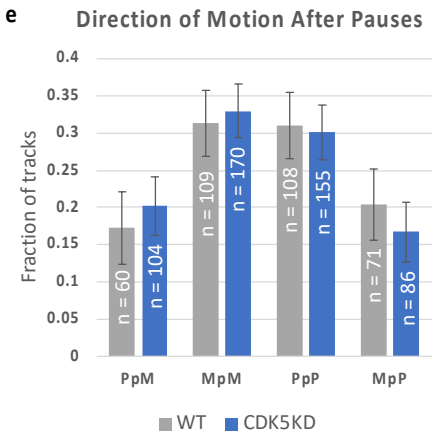
a


Pause Durations
control
Counts
Time (sec)
average value: 3.32 +/- 0.14

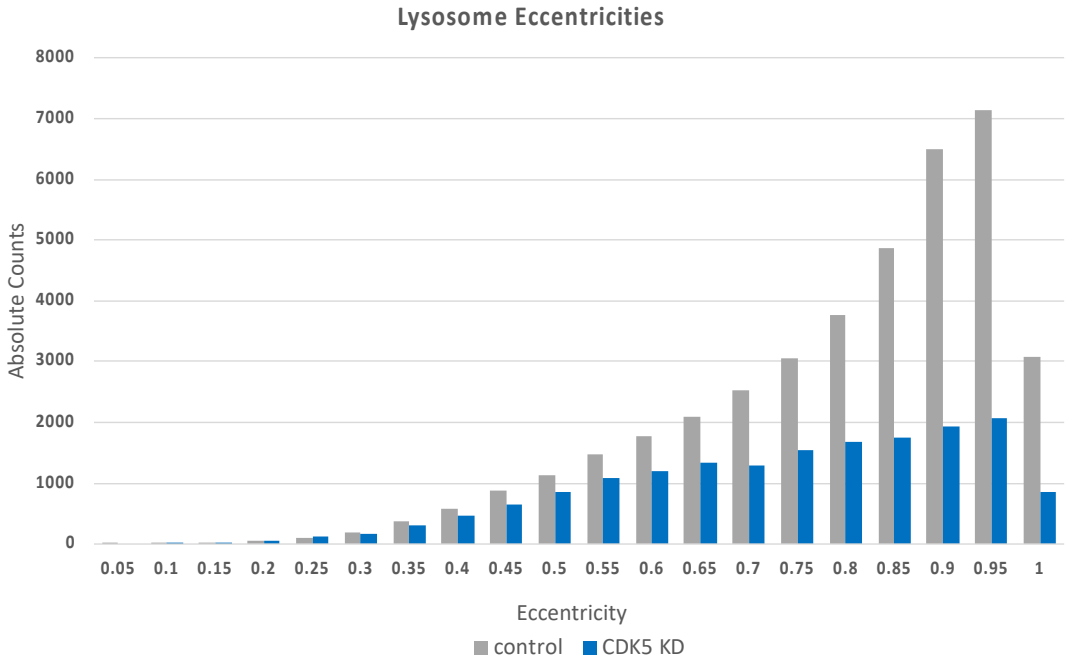
b


Pause Durations
CDK5 KD
Count
Time (sec)

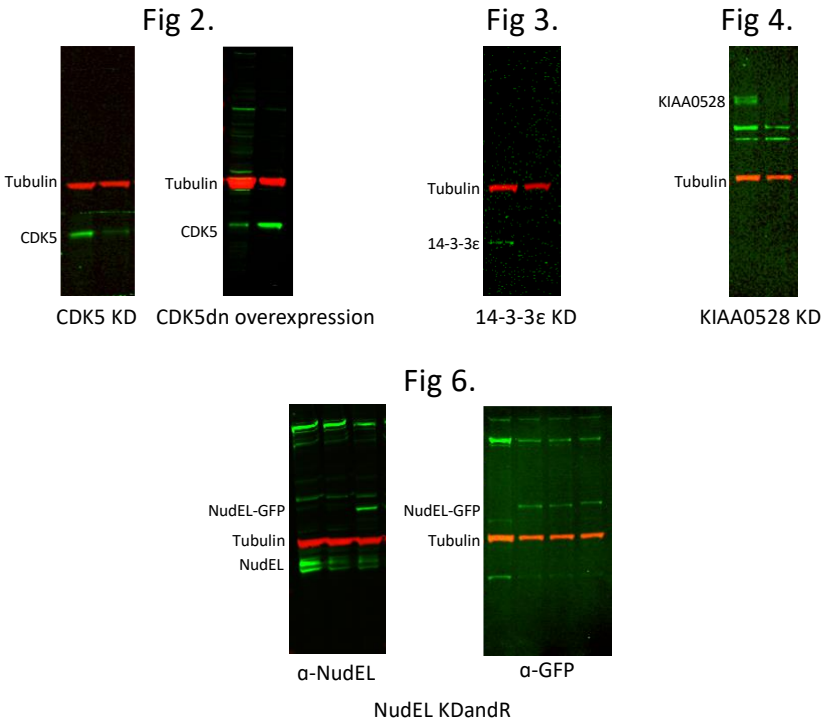

c
Run lengths
control
Counts
average value: 480 +/- 30
average value: 502 +/- 34
Run lengths (nm)
control: Minus-end
control: Plus-end
Lysosomal motion in WT cells
Displacement (µm)
Time(sec)
d
Run-lengths
CDK5 KD
Count
Run lengths (nm)
CDK5 KD: Minus-end
CDK5 KD: Plus-end
Lysosomal motion in CDK5 RNAi cells
Displacement (µm)
Time(sec)



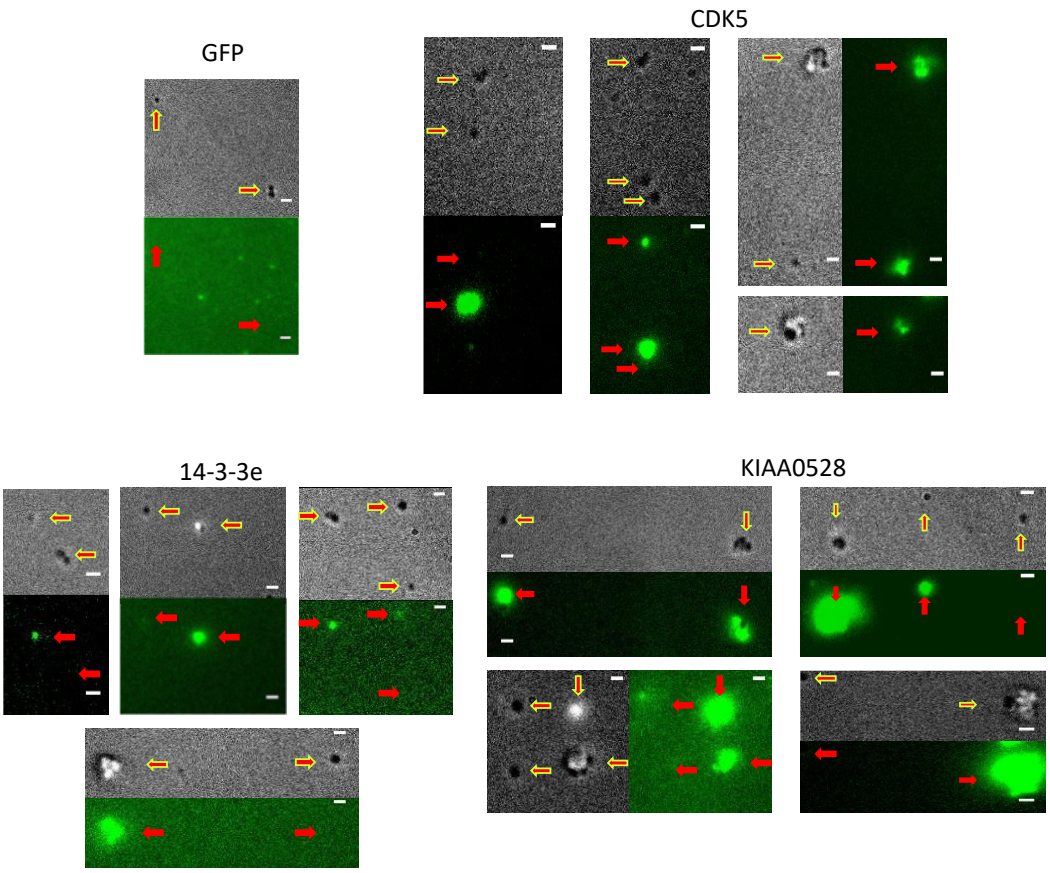
Supplementary Figure 2.3. Most aspects of lysosomal motion are not altered by changes in Tug-of-war severity
Particle tracking analysis of lysosomes shows no pause duration profile differences between control cells (**a**) and CDK5 knockdown cells (**b**), a small run length difference between control (four samples traces are included) (**c**) and CDK5 knockdown (four samples traces are included) (**d**) but no effect on the relative difference between plus-end run lengths and minus-end run lengths, and no significant differences in directional changes after pausing (**e**). PpM: lysosome moving towards the plus-end of MT, pauses, then moves towards the minus-end of MT,
MpM: lysosome moving towards the minus-end of MT, pauses, then moves towards the minus-end of MT,
PpP: lysosome moving towards the plus-end of MT, pauses, then moves towards the plus-end of MT,
MpP: lysosome moving towards the minus-end of MT, pauses, then moves towards the plus-end of MT
n = 2 independent experiments for a-d; n = 1 for e



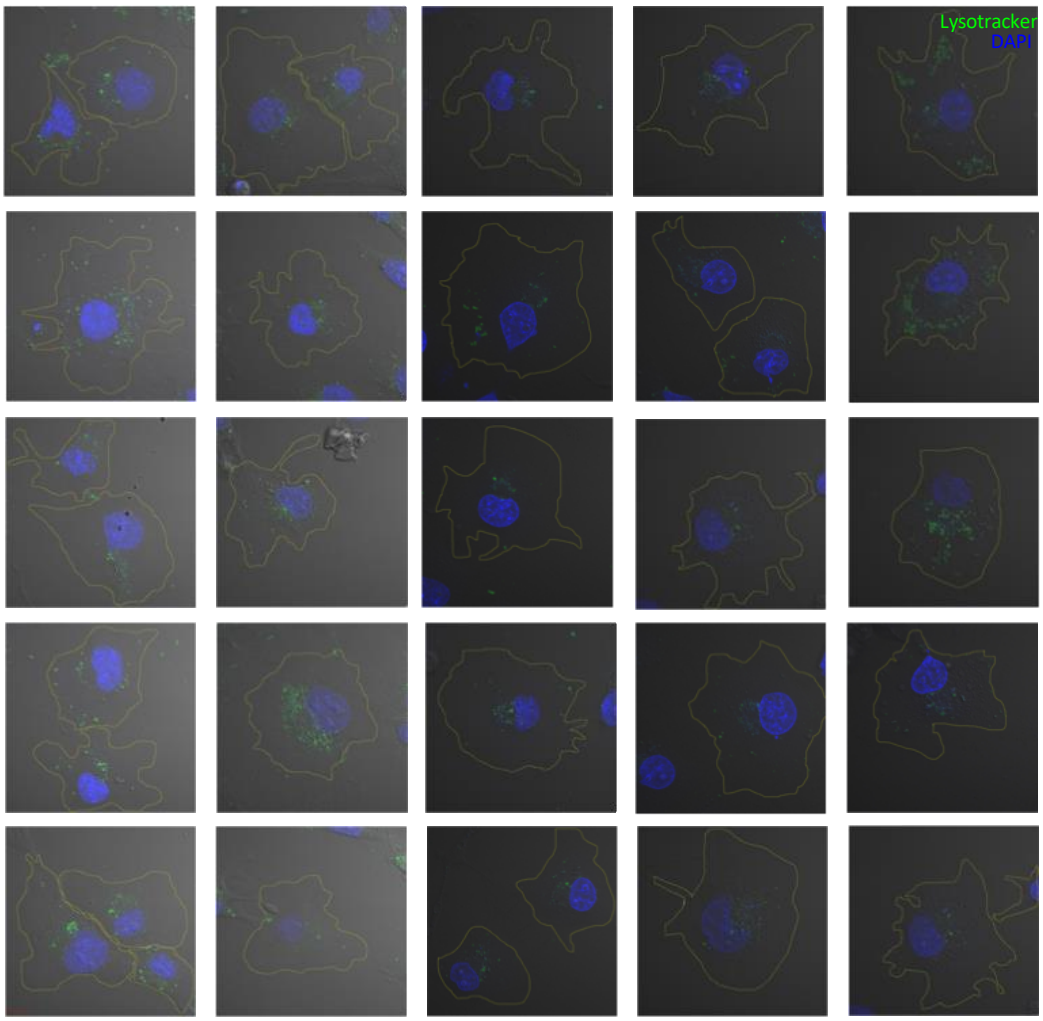
Supplementary Figure 2.4. Absolute counts of lysosome eccentricities from Figure 8 also show a difference in distribution
(Wilcoxon Sign-Ranked t-test p value 0.00014)

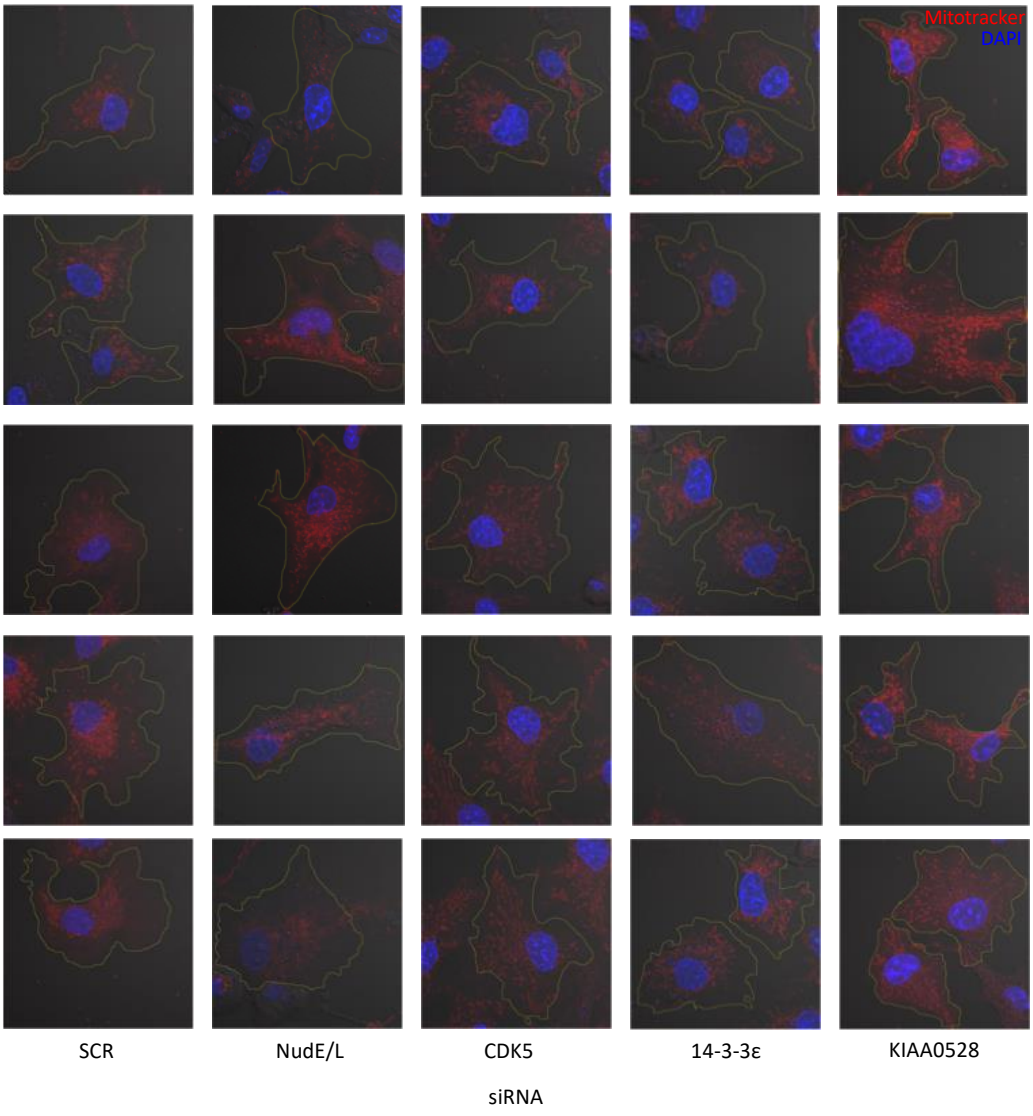


Supplementary Figure 2.5. Full WBs presented in main figures

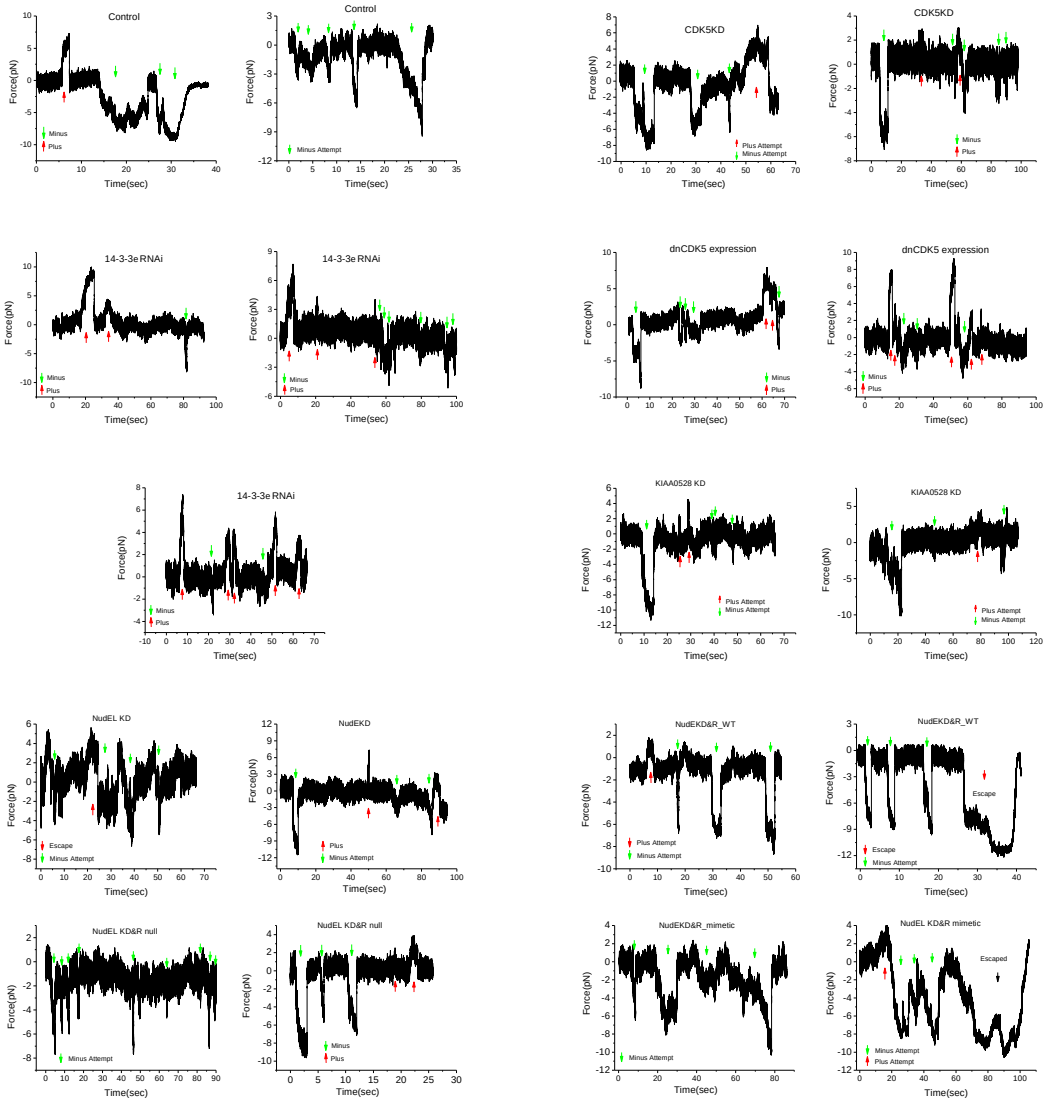


Supplementary Figure 2.6. Representative LDs from immunofluorescence (Figure 2.5)


Lysotracker
DAPI
SCR
NudE/L
CDK5
14-3-3ε
KIAA0528
siRNA



Supplementary Figure 2.7. Representative images from Lysotracker and Mitotracker experiments (Figure 2.7)



Supplementary Figure 2.8. Sample traces of force measurement experiments

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