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The Physical Interactions of KLP-4 & The Effects of Microtubule Acetylation

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Introduction

The nervous system is controlled by two different subsystems, the central nervous system and the peripheral nervous system. The peripheral nervous system is responsible for detecting external stimuli and relaying sensory information to the central nervous system which then signals for the appropriate response. The three types of neurons that comprise this information pathway are sensory neurons, interneurons, and motor neurons. Chemical and electrical signals are transmitted between these neurons in order for information to pass quickly and efficiently through an organism's body. The electrical impulse, or action potential, that travels down the length of a presynaptic neuron is not capable of crossing the synaptic cleft (Van Spronsen and Hoogenraad 2010). In order for the signal to reach the postsynaptic neuron, molecules known as neurotransmitters must be transmitted across the synaptic cleft and between the adjacent neurons. Neurotransmitters are stored in presynaptic vesicles and are responsible for carrying the electrical signal across the synapse. Receptors on the postsynaptic membrane recognize these neurotransmitters and propagate the electric signal to the next neuron (Van Spronsen and Hoogenraad 2010).

Microtubules and Motor Proteins

The process of transporting neurotransmitters and other molecules throughout a neuron is reliant on the cytoskeletal structure of the neuron. The cytoskeleton is composed of three

different filaments: microfilaments, intermediate filaments, and microtubules (Malhotra and Shnitka 1996). Each filament plays an important role in maintaining the structure of different cell types, but in neuronal cells, microtubules hold a greater purpose. In addition to upholding neuronal structure, microtubules can induce morphological changes to the structure of a neuron during various stages of its development, and guide the transport of neuronal cargo down the length of the axon (Kapitein and Hoogenraad 2015). This cargo consists of proteins, organelles, and vesicles containing neurotransmitters. Microtubules are formed from heterodimers of alpha and beta tubulin subunits. These subunits bind to one another end-to-end, creating a linear protofilament which associate together via lateral interactions in order to form one, long, tubular filament. Some extraordinarily stable, long-lived microtubules have been discovered to undergo post translational modification in which the alpha tubulin subunits become acetylated (discussed in detail later). The resulting microtubule structure is polar in nature with a negative end oriented towards the dendrites and cell body of the neuron and a positive end oriented towards the axon terminal (Kapitein and Hoogenraad 2015).

Intracellular cargo is transported within a cell via motor proteins. These mobile proteins walk along the microtubule scaffolding found within cells and orient themselves based on the inherent polarity of the filaments in order to successfully deliver cargo to various locations. Common motor protein enrichment sites within a neuron include the major structural features: the axon, dendrites, and synapses. (Hirokawa et al. 2010). There are two types of microtubular motor proteins: dyneins and kinesins. Although both utilize the hydrolysis of ATP in order to move up and down microtubules, the two motor proteins are known to travel in opposite directions. Dyneins are capable of retrograde movement which describes movement from the

positive end of a microtubule, located at the axon terminal, towards the negative end, located at the cell body of a neuron. Conversely, kinesins are capable of anterograde movement which describes the movement from the negative end to the positive end of microtubules (Motil et al. 2007). Differences in a motor protein's direction of travel play an important role in dictating its function, including the identity of its transported cargo. When propagating signals, neurons must constantly resupply the axon terminal with the neurotransmitters and vesicle building materials lost during the exocytosis of neurotransmitters into the synapse. This requires a motor protein that can complete anterograde movement such that it can travel on microtubules spanning from the cell body where neurotransmitters and vesicle materials are produced, to the axon terminal where they are needed (i.e. negative end to positive end) (Motil et al. 2007). This is why kinesins, capable of anterograde movement, are thought to be responsible for the transport of these materials and the maintenance of neuron functionality.

Kinesin-3

There are approximately 50 different kinesin motor proteins that are subdivided into 15 separate superfamilies (and one orphan group) (Magaletta et al. 2019). All kinesins are known to contain three general domains: the motor head domain, stalk domain, and cargo binding domain. The motor head domain is highly conserved between all kinesins, which is why kinesins are distinguished from one another based on the structure and composition of their cargo binding domain. The highly conserved nature of the motor head domain results in very similar motor structures and movement mechanisms between most kinesins. Most move in an anterograde direction towards the plus end of the axon, but some have been observed to exhibit retrograde and even bidirectional movement patterns (Motil et al. 2007). Anterograde movement of a

kinesin is driven by ATP hydrolysis. The switch I and II motifs found within the motor head domain of a kinesin are capable of recognizing the gamma phosphate found on ATP, allowing for the ATP molecule to bind. Once bound, the motor protein completes ATP hydrolysis, resulting in a conformational shift within the motor head domain. This change in shape creates strain within the motor protein which is then relieved via the release of force. This force drives the movement of the kinesin and propels it forward along microtubule filaments so that it may deliver its cargo. This process is referred to as the power stroke of a motor protein and is dependent on the availability of ATP (Kull and Endow 2002).

One of the most studied groups of kinesins is the Kinesin-3 superfamily, consisting of 5 subfamilies: KIF1, KIF13, KIF14, KIF16, and KIF28 (Magaletta et al. 2019). This superfamily is known to transport cargo quickly over very long distances whilst demonstrating "superprocessivity" in which it remains bound to the microtubule for long periods of time before dissociating. These high levels of processivity are unmatched by other kinesin families and suggests that kinesin-3 proteins are the marathon runners of motor proteins (Soppina et al. 2014). It is understood that the superprocessivity observed in kinesin-3 proteins can be attributed to the interaction between the positively charged lysine-rich loop found within the kinesin-3 motor domain and the negatively charged glutamate-rich hook found on the alpha and beta tubulin subunits of microtubules (Siddiqui and Straube 2017). The interaction between these two charged regions allows for the motor protein to "stick" to the microtubule for much greater distances. This high level of processivity potentially explains why kinesin-3 motors are highly expressed in cells that require long distance trafficking, such as neurons, which can be meters in length (Magaletta et al. 2019).

Kinesin-3 proteins have an inactive and active state depending on their conformation. When they are not carrying cargo, kinesin-3 motors exist as monomers and exhibit autoinhibition (Soppina et al. 2014). In order to achieve autoinhibition, Kinesin-3 proteins must adopt a conformational change in which the protein folds back on itself and forms various interactions between its domains. The four conserved domains found in Kinesin-3 motor proteins are the motor domain (MD), neck coil domain (NC), coiled-coil domain (CC1), and forkhead-associated domain (FHA). Kinesin-3 motors autoinhibit themselves and prevent dimerization by creating self-interactions between the NC domain and at least one CC1 domain that is immediately adjacent to the MD (Soppina et al. 2014).

In order to achieve a dimerized, active conformation, the cargo domain must first bind to the appropriate cargo. Once the cargo is bound, interactions form between NC domains of two Kinesin-3 monomers in order to form a singular dimer (Soppina et al. 2014). This interaction not only aids in the dimerization process, but it has also been discovered to contribute to the overall superprocessivity of Kinesin-3 motor proteins (Yue et al. 2013). In addition to a monomeric state, inactive kinesin-3 motors can also exist in a constitutively dimerized state in which interactions between the tail domains prevents binding to microtubules. This dimer is only activated when cargo binds to the tail domains and breaks the inhibitory interaction, allowing for the kinesin to bind to microtubules (Siddiqui and Straube 2017).

The mechanism by which cargo binds and activates a kinesin depends on the conformation of the inactive kinesin monomer/dimer. Additionally, this mechanism can also be influenced by the identity of the cargo and where it needs to be transported to. Cargo adaptor proteins can also aid in the binding of cargo to kinesins with the purpose of orienting the direction of transport

within the neuron (Hoogenraad et al. 2016). When bound to motor proteins, adaptor proteins can interpret cues such as changes in Ca^{2+} levels, phosphorylation, or GTPase activity in order to control the movement of cargo on a local scale (Hoogenraad et al. 2016). In summary, adaptor proteins can facilitate specific responses to local environmental changes within the neuron, and aid with the overall efficiency of cargo transport.

C. elegans and klp-4

The gold standard model system for studying neuronal kinesins *in vivo* is the nematode *C. elegans*. The entirety of their nervous system, which is comprised of 302 cells, has been mapped as a result of thorough research (Ma et al. 2018). Three kinesins belonging to the Kinesin-3 superfamily have been found in *C. elegans*: *unc-104*/KIF1A, *klp-6*, and *klp-4*/KIF13A/KIF13B. KLP-4 in particular is highly expressed in *C. elegans* neurons, especially in the AVB interneuron which controls the majority of *C. elegans* locomotion (Rakowski et al. 2013). Therefore, any alterations or mutations affecting the functionality of *klp-4* can result in abnormal locomotive patterns in *C. elegans.*

This exact trend has been noted in the mutant strain RB2546, which contains the allele *klp-4* (*ok3537*). *C. elegans* possessing the *ok3537* allele exhibit a reduced number of spontaneous reversals in comparison to the wild type and have difficulty moving in reverse once they have bumped into an obstacle (Magaletta et al. 2019). Although this locomotive abnormality has been consistently observed in *klp-4*(*ok3537)* mutants, it has yet to be determined what changes on the molecular level are causing it. The *klp-4* (*ok3537*) allele contains a large, in-frame deletion in the cargo binding domain sequence (Magaletta et al. 2019). It is thought that this deletion could

have an effect on the physical interactions between the cargo binding domain and its natural cargo. If the deletion alters the ability for mutant KLP-4 to carry its usual cargo, or allows it to bind to a different cargo, this could explain why the worms are experiencing abnormal locomotive behavior.

The structure of KLP-4 consists of three specific domains: a globular motor domain, a rod or stalk domain, and a C-terminal globular tail domain. The globular motor and C-terminal globular tail domain show many similarities to other kinesins within the Kinesin-3 superfamily. What makes KLP-4 unique is the composition of NC domain, which is located within the rod/stalk domain. In other kinesins, this area is typically high in alpha helical content, but this domain in KLP-4 is often broken up by frequent random coils, making the motor ultimately more hydrophilic. This divergence leads one to question how KLP-4 motors function in relation to other Kinesin-3 motor proteins, and makes it a good target for future research (Siddiqui 2002).

Microtubule Post Translational Modifications and Kinesin-3 Superprocessivity

Although the superprocessivity of kinesin-3 motor proteins, including KLP-4, has been mainly attributed to the interaction between the K-loop of the kinesin motor domain and the E-hook of the alpha and beta tubulin subunits (Siddiqui and Straube 2017), other hypotheses have been proposed in regards to the stability and longevity of the kinesin-microtubule interaction. One area of interest focuses on the relationship between the acetylation of alpha tubulin subunits and the stability of microtubules. Microtubules themselves undergo a wide variety of post translational modifications (PMTs), including glutamylation/deglutamylation, detyrosination, and acetylation, each of which can affect the organization and stability of the microtubule as a whole (Wloga and

Gaertig 2010). Of these PTMs, the acetylation of alpha tubulin has been associated with microtubule stability and has inspired the most research as it is unique amongst the other microtubule PTMs. The site of acetylation on alpha tubulin is found inside of the microtubule filament, otherwise known as the lumen, at the lysine 40 (K40) sites of the alpha tubulin subunits, and is the only PTM that is not found on the exterior of the microtubule (Janke and Montagnac 2017). This internal site indicates that the enzymes responsible for acetylating alpha tubulin must be capable of finding their way into the narrow microtubule lumen, which is only 15 nm in width (Janke and Montagnac 2017). The exact method by which alpha tubulin acetyltransferases enter the lumen of the microtubule is still unknown, but there are a few suspected hypotheses.

One possible method of access to the microtubule lumen could be through irregularities, or cracks, along the length of the microtubule itself. Microtubules polymerized *in vivo* have been discovered to develop defects in their surface lattice organization, which results in luminal openings large enough for alpha tubulin acetyltransferases to pass through (Chretien et al. 1992). This model is considered non-exclusive because it would require microtubules to accumulate numerous defects and cracks along its length in high quantities which is unlikely as it would result in structural instability. Additionally, recent research has indicated that microtubules are capable of self-repair. This is completed by either incorporating new tubulin to patch up any defects or realigning any protofilaments that had separated or slid past one another due to mechanical stress (Schaedel et al. 2015). With the capability to repair these microtubule cracks relatively efficiently, there is likely a second acetyltransferase entry site found on microtubules. This additional entry site can be found at each end of a microtubule. Unless bound to a tip-binding protein, the ends of microtubules are open to their surroundings and grant alpha tubulin acetyltransferases access to

the internal lumen cavity (Akhmanova and Steinmetz 2015). This hypothesis is supported by the discovery that some alpha tubulin acetyltransferases have shown a higher affinity for the open extremities of microtubules. This is most likely due to the large number of exposed luminal acetylation sites for the acetyltransferases to target (Coombes et al. 2016).

Alpha Tubulin Acetyltransferases

There are two major alpha tubulin acetyltransferases found within the model organism *C. elegans*: alpha tubulin N-acetyltransferase 1 (ATAT-1/MEC-17) encoded for by the gene *mec-17* and alpha tubulin N-acetyltransferase 2 (ATAT-2) encoded for by the gene *atat-2*. MEC-17 is the *C. elegans* ortholog to the human alpha tubulin acetyltransferase ATAT-1, demonstrating the same function and similar structure. ATAT-1 is a highly conserved alpha tubulin acetyltransferase between all organisms, which is why it is the focus of most microtubule acetylation studies. ATAT-1 has been known to bind to the external walls of microtubules, positioning themselves to enter the lumen via cracks along the length of the filament. Additionally, ATAT-1 shows a high affinity for the open ends of the microtubule, indicating that it can enter the microtubular lumen in multiple ways. Once inside the lumen ATAT-1 facilitates the acetylation of the K40 sites on the alpha tubulin subunits of the microtubule structure, potentially contributing to the stability of the overall filament.

ATAT-2 is a paralog of ATAT-1 and demonstrates a similar function, but has also been found to play an important role in the maintenance of synaptic stability (Borgen et al. 2019). ATAT-2 regulates the maintenance of presynaptic boutons, otherwise known as the axon terminals, where vesicle enclosed neurotransmitters are produced and stored. Without the presence

of ATAT-2, these presynaptic boutons destabilize and retract from their appropriate presynaptic positions (Borgen et al. 2019). This process reduces the number of functional presynaptic boutons present and therefore limits the ability of the neuron to properly propagate a signal. Stable synapse maintenance is imperative for maintaining neuronal circuitry and allowing for plasticity within an organism (Borgen et al. 2019).

Dynamic versus Stable Microtubules and Motor Proteins

Most microtubules are naturally dynamic. They can grow, shrink, and reorganize themselves into specialized structures within a cell (Janke and Montagnac 2017). This dynamic activity is advantageous for cells that are constantly undergoing change, such as growth or replication, but in regards to neuronal cells, an axon comprised of dynamic microtubules may hinder the functionality of the neuron. Motor proteins, such as KLP-4, must remain associated with and travel long distances along axonal microtubules in order to successfully deliver their cargo (Soppina et al. 2014). Dynamic microtubules undergo constant dissociation and reassociation of their alpha and beta subunits during the reorganization process, which could potentially affect the efficiency of a motor protein's movement across the filament (Horio et al. 2014). This is why stabilized microtubules are thought to increase the superprocessivity of Kinesin-3 motor proteins, including KLP-4. Traveling along a stabilized microtubule would greatly improve the efficiency of kinesin motor proteins, and increase their ability to deliver cargo over long distances in a timely manner. With all this in mind, the question still remains: do acetylated K40 alpha tubulin sites result in microtubule stability, or does this PTM accumulate on already stable, long-lived microtubules?

Evidence of αK40 Acetylation Causing Microtubule Stability

Long-lived microtubules serve as highway systems for motor proteins, dyneins and kinesins alike, and frequently experience large amounts of mechanical stress. The power stroke of a motor protein, the process by which motor proteins propel themselves forward, imposes torque upon the microtubule that it is traveling on, causing it to buckle and bend in response (Portan et al. 2017). If the microtubule is stiff and brittle, this kind of mechanical stress can cause breakages along the microtubule and inhibit the functionality of the filament. Interestingly enough, recent research has discovered that the acetylation of αK40 sites can help microtubules cope with mechanical stress imposed by motor proteins.

Acetylated microtubules subjected to repetitive bending have been noted to recover much more quickly and with minimal breakages in comparison to unacetylated microtubules (Eshun-Wilson et al. 2019). Additionally, areas along the acetylated microtubule already containing defects and cracks did not worsen or become larger upon bending, which is a typical consequence of bending unacetylated microtubules. Based on this gathered data it was determined that the acetylation of αK40 sites not only allows for stabilization of microtubules, but it also increases the flexural rigidity of the microtubule as a whole (Portan et al. 2017). This increase in flexibility is possible through acetylation because it causes a shift in the conformational landscape of the αK40 loop, ultimately reducing the number of conformations it can assume (Eshun-Wilson et al. 2019). This reduction in loop mobility results in the weakening of alpha-alpha subunit lateral interactions between microtubule protofilaments. Although the protofilaments still remain associated with one another to form a stable microtubule, the weakened interactions between them allows for

protofilaments to slide past on another during mechanical stress, such as bending under the force of a motor protein, and prevent breakages (Portan et al. 2017).

This resilience is best exemplified by the touch receptor neurons found along the longitudinal axis of *C. elegans*. As nematodes navigate through their environment, their bodies move in a sinusoidal fashion, causing these touch receptors neurons to be repeatedly bent (Portran et al. 2017). Studies have shown that the removal of the αK40 acetyltransferase MEC-17/ATAT-1 dedicated to these neurons causes a large number of breakages along their axons, which can only be rescued after the nematode is paralyzed and the microtubules have had time to recover from axonal degeneration (Portran et al. 2017). Through the acetylation of αK40 sites, microtubules experience an increase in flexural rigidity and a resistance to fatigue generated by mechanical stress, therefore limiting the aging of long-lived microtubules.

C. elegans Acetyltransferase Mutants

Since discovering the *mec-17* and *atat-2* genes in *C. elegans*, knockout/null strains have been produced with the purpose of identifying the function and necessity of each encoded alpha tubulin acetyltransferase. Strain RB1696 is a *mec-17* (*ok2109*) mutant in which the entire *mec-17* gene has been knocked out (Neumann and Hilliard 2015). The consequences of producing this null allele is the complete absence of MEC-17 acetyltransferase from the animal. Research has found that without MEC-17, microtubule networks become destabilized and there is a resulting 2-fold increase in the number of dynamic, growing microtubules, making motor protein travel and transport far more difficult (Neumann and Hilliard 2015).

The strain RB1869 is an *atat-2* (*ok2415*) mutant in which a large deletion has been performed, removing almost 60% of the *atat-2* transcript (Neumann and Hilliard 2015). This allele has proven to be less detrimental to the stability of microtubules than the *mec-17* null allele. Although ATAT-2 may not be functioning, as long as MEC-17 is still present, microtubules are still being acetylated and stabilized (Neumann and Hilliard 2015).

Crossing Acetyltransferase Mutants with KLP-4 Mutant

Based on prior research we hypothesize that the *klp-4* (*ok3537*) allele results in a constitutively active mutant KLP-4 motor protein. The molecular consequence of this hyperactivity is suspected to be an increase in aggregation and disorganization of the interneuron synapses that KLP-4 is associated with (Magaletta et al. 2019). Additionally, via verbal communication with K. Verhey, we have also discovered that there is a drastic increase in the alpha tubulin acetylation of the microtubules that mutant KLP-4 is traveling on *in vitro*. Seeing as this level of acetylation is not typical in the presence of wild type KLP-4, this trend suggests that there is some form of signaling occurring between motor proteins such as KLP-4 and the microtubule "highway" that they are associated with. The hyperacetylation of microtubules paves an extremely stable path for motor proteins to travel on, and we suspect this excess stability allows for constitutively active KLP-4 to further exacerbate synaptic disorganization. The resulting phenotype in *C. elegans* with this *klp-4* (*ok3537*) allele consists of reduced spontaneous reversals, and impaired ability to move backwards.

In an attempt to support our hypotheses *in vivo*, we plan to cross the RB2546 strain (*klp-4* (*ok3537*)) of *C. elegans* with both mutant acetyltransferase strains RB1696 (*mec-17* (*ok2109*)) and

RB1869 (*atat-2* (*ok2415*)). In theory, crossing a KLP-4 mutant with a MEC-17 mutant would cause for a decrease in microtubule stability since the MEC-17 acetyltransferase is knocked out and no longer acetylating alpha tubulin. This destabilization of axonal microtubules would make it difficult for constitutively active KLP-4 to travel to the axon terminals and cause synaptic disorganization. Additionally, crossing a KLP-4 mutant with an ATAT-2 mutant should result in a decrease in synaptic stability since ATAT-2 is no longer maintaining presynaptic boutons. Mutant KLP-4 creates synaptic disorganization by randomly aggregating axonal terminals, and if ATAT-2 is not maintaining these same axonal terminal boutons, mutant KLP-4 would have difficulty causing disorganization. In conclusion, if our previously mentioned hypotheses are correct, the offspring of these crosses should experience a rescued phenotype and be capable of normal movement.

Aim and Goals

Although KLP-4 has been identified as a neuronal kinesin in *C. elegans* it has yet to be determined exactly what cargo it binds to and transports throughout the neuron. The first research question I am trying to answer is: What proteins physically interact with the cargo binding domain of KLP-4 and how does the *ok3537* mutation within this domain affect its ability to interact with certain cargo proteins and transport it throughout neurons?

Additionally, we suspect that acetylation of microtubules occurs in excess in the presence of mutant KLP-4 motor proteins, leading me to the second research question that I will address: Is there a relationship between the state of a motor protein, inactive or active, and the acetylation frequency of the microtubule it is traveling on?

Materials and Methods

C. elegans strains:

Caenorhabditis elegans strains were obtained from the *Caenorhabditis* Genetics Center (CGC, University of Minnesota-Twin Cities), funded by the NIH Office of Research Infrastructure Programs (P40 OD010440). *C. elegans* mutant strains RB2546 (*klp-4* (*ok3537*)), RB1696 (*mec-17* (*ok2109*)), and RB1869 (*atat-2* (*ok2415*)) was provided by the *C. elegans* Gene Knockout Consortium (Oklahoma Medical Research Foundation, Oklahoma City, OK). The EG9615 strain was as kind gift from Erik Jorgensen (University of Utah).

sgRNA Design and Construction

Oligonucleotides encoding crRNAs were designed in the program "A Plasmid Editor" (ApE) to match the 3' end of *klp-4.* All crRNA oligos contain BsaI overhangs (Top: TAGG, Bottom: AAAC) at the 5' ends of both strands to facilitate ligation into DR274 or pJNP32. crRNA sequences:

Top (JP329): 5' TAGGTAAGTTGACATGTACTATTG 3'

Bottom (JP330): 5' AAACCAATAGTACATGTCAACTTA 3'

Both the top and bottom oligonucleotides were resuspended to a concentration of 100 µM. Each oligo was diluted in annealing buffer (10 mM Tris, 1 mM EDTA, 50 mM NaCl, pH 8), to a concentration of 10 µM. Complementary oligos were annealed via heating the mixture to 95 ˚C and cooling by 1 ˚C /min until reaching room temperature. The resulting double stranded

oligos (JP329/JP330 Annealed) were phosphorylated via T4 polynucleotide kinase treatment (NEB).

Plasmid DR274 (pDR274) containing a Kanamycin resistance gene was digested using a BsaI restriction digest and treated with Antarctic phosphatase (NEB). The digested vector was purified via PCR purification. Appropriate ratios of diluted and phosphorylated JP329/JP330 Annealed oligos (7.8 fmol/μL) were ligated into pDR274 using quick DNA ligase (NEB).

Newly ligated pDR274 vectors were transformed into competent bacterial cells and cultivated on three different kanamycin treated plate depending on the molar ratio between the vector and the insert (1:0; 1:1; 1:3). After overnight incubation at 37˚C, four colonies were picked from the most promising plate (1:1) and used to inoculate four tubes of LB treated with kanamycin. Cultures were left to incubate over night at 37˚C and then a miniprep was conducted for each culture. The cultures that produced the highest concentration of DNA were sent for sequencing and verified a successful plasmid (pJNP43) (Figure 1).

sgRNA Production

Plasmid pJNP43 was linearized using a DraI restriction digest and the DNA was purified via PCR purification kit. The linearized vectors underwent *in vitro* transcription via the HiScribe T7 transcription kit (NEB) and the resulting sgRNA product was treated with RNAse free DNAse and purified via PCR Purification kit. Bleach agarose gel electrophoresis was conducted in order to verify successful *in vitro* transcription of sgRNA (Figure 2).

HDR Template Design and Construction

ApE was used to design left (JP351) and right (JP352) oligo arms of homology for the 3' end of *klp-4*. The nearest PAM sequence immediately downstream of the endogenous stop codon in *klp-4* was identified and utilized for targeting of Cas9. A homology directed repair template containing a Myc tag sequence was included at the 3' end of each arm of homology. Homology arms were also adjusted to remove the endogenous stop codon and ensure the Myc tag remained in frame with the *klp-4* coding sequence. Arms of Homology:

Left (JP351):

5'CCATTTCAGGAGACCTGGTGGATCTCAAAATCAACGAACAAAAACTCATCTCAG AAGAGG 3'

Right (JP352):

5'CGATTTTAAAGCAAAATATAAGTTGACATGTACTATTGCAACATCGTATTTACAG ATCCTCTTCTGAGATGAGTTTTTGTTC 3'

JP351 and JP352 oligos were resuspended to 100 µM. Oligonucleotides were resuspended in annealing buffer (10 mM Tris, 1 mM EDTA, 50 mM NaCl, pH 8), to a concentration of 10 µM annealed as above. The resulting double stranded oligos (JP351/JP352 Annealed) were extended and amplified via PCR. PCR product was purified using a PCR purification kit.

Injecting EG9615

An injection mixture containing the GFP co-injection marker pJNP9 (*unc-119* 5' UTR::*unc-119*::*unc-119*3' UTR) (30 ng/µL), myc homology directed repair template

(JP351/JP352 Annealed) (50 ng/µL), and sgRNA (from JNP43) (20 ng/µL) was injected into the syncytial gonad of young adult hermaphroditic *C. elegans* of the strain EG9615 (*pie-1*::Cas9::*unc-54* UTR). Injections were conducted using a Tritech MINJ-1000 complete injection system. Worms were given enough time to recover and for the injected materials to be incorporated into eggs. After 1-2 days, newly hatched worms were examined under a fluorescent microscope in search of GFP, which indicates a successful injection.

RB2546 (klp-4(ok3537)) Crosses

To induce production of males, strains (RB1696, RB1869) were heat shocked and allowed to lay eggs. After three days, F_1 male progeny were isolated for male propagation. To set up P_0 crosses, RB2546 hermaphrodites were mixed on NGM mating plates in a 1:4 hermaphrodites to males ratio. Once daily, all living adult P_0 worms were transferred to new NGM plates, leaving behind all new F_1 progeny. Cross was potentially successful if the F_1 progeny displayed 1:1 hermaphrodite to male segregation ratios. Following a potentially successful mating, multiple F_1 hermaphrodites (RB2546/RB1696 and RB2546/RB1869) were picked at random and transferred to new seeded plates and allowed to produce for F_2 progeny. Twenty F_2 worms were transferred to their own individual seeded plate. Allowed each worm to self-fertilize, and essentially clone themselves producing F_3 progeny.

Single Worm Lysis and Genotyping

Once a large quantity of F_3 progeny are produced, a single F_2 parent animal was picked from the plate and single worm lysis was completed using lysis buffer (10 mmol/LTris, 50 mmol/L KCl, 1.5 mmol/L MgCl2, pH 8.3 with 1 mg/mL Proteinase K (NEB)) to extract genomic DNA.

The positive controls consisted of lysed RB2546, RB1696, and RB1869 worms. The negative control consisted of lysed N2 worms. Lysed worms were freeze cracked twice before being placed in the worm lysis protocol (65ºC for 60 minutes, 95 ºC for 30 minutes). Isolated genomic DNA was then used for genotyping PCR reactions using the following primer sets:

JP50	CACTGGGAGCTTCTATTTTCTGTG	mec-17(ok2109) GT forward 1
JP51	CAATGAAATTCTGAAAGCAATGTATCTC	mec-17(ok2109) GT reverse external 1
JP52	GAGGTGAATTGAATTATCATTTCATTC	Ce mec-17(ok2109) GT reverse internal 1
JP380	AAAACGATATTGGGCTGTGG	Ce atat-2(ok2415) GT outer left
JP381	GCATCGGTTTTCAGTGGAAT	Ce atat-2(ok2415) GT outer right
JP382	AGATTGCCTGGGTGAAATGT	Ce atat-2(ok2415) GT inner left
JP383	AGCCTATGCACAGAGCTGGT	Ce atat-2(ok2415) GT inner right

Future Directions

It has still yet to be determined whether the 200/1 del/in mutation found in *klp-4(ok3537)* has an effect on the physical interaction between the kinesins' cargo binding domain and the cargo it is carrying. In order to determine physical *in vivo* interactors of KLP-4, successful injections of both EG9615 and a RB2546 mutant strain must be completed. Large volumes of injected strains will be grown, lysed, and undergo a Myc-pulldown assay. The proteins pulled down will be run on an SDS-page, and any unknown proteins will be sent to be identified via mass spectrometry.

Additionally, it would be beneficial to determine the importance of each domain of the *klp-4* gene. By conducting a series of serial truncations of *klp-4* and monitoring the effects, one

can identify which portions of the gene are essential, and which are not. There are four different targeted truncation sites: at the end of the FHA region, in-between the CC and FHA regions, in-between the motor domain and the CC regions, and within the first exon, which effectively knocks out the entire gene. The consequence of these massive deletions will be the alteration or removal of the cargo binding domain itself within the KLP-4 protein. A mCherry sequence will be inserted at each truncation site which allowing for the now mutated KLP-4 protein to be visually located within *C. elegans.* It is necessary to visualize the KLP-4 protein *in vivo* because it allows one to see where each truncation localizes within *C. elegans* neurons. Localization provides clues as to how the kinesin's ability to function is affected by each sequential truncation.

Furthermore, the effects of crossing the *C. elegans* strain RB2546 (*klp-4* (*ok3537*)) with both RB1696 (*mec-17* (*ok2109*)) and RB1869 (*atat-2* (*ok2415*)) has yet to be discovered. To determine whether or not such crosses can rescue the abnormal locomotive phenotype associated with mutant KLP-4 one could employ the use of the drug taxol on the crossed progeny. Taxol is a known microtubule stabilizer, and once administered to the cross offspring, it should fill the same role as the now null MEC-17 and ATAT-2 acetyltransferases. A taxol treatment would be expected to cause a return in synapse disorganization and inhibit the ability for *C. elegans* to move in reverse once more, effectively reversing the effects of the cross.

Our long-term goals consist of using our novel data to better help the medical community's understanding of disease states associated with mutant kinesins and alpha tubulin acetyltransferases. KLP-4 is very similar to the human paralogs KIF13A and KIF13B which are kinesins responsible for the transport of vesicles throughout different cell types (Nakagawa et al. 2000). There are multiple disease states associated with KIF13A and KIF13B mutants including

spastic paraplegia, deafness, and corneal dystrophy. Using our understanding of both the identity of KLP-4's cargo and the physical interactions occurring in the cargo binding domain we could potentially provide some insight as to how these disease states arise and potential future therapies.

Additionally, mutant ATAT-1 and ATAT-2 have also been found to produce multiple disease states. Mutations resulting in the under expression of *atat-1*, therefore reducing the levels of αK40 acetylation, can cause defective axonal transportation, which is characteristic of Huntington's disease and Parkinson's disease (Eshun-Wilson et al. 2019). Mutations resulting in the over expression of *atat-1*, therefore increasing the levels of αK40 acetylation, have been found to promote cell-cell aggregation, migration, and tumor reattachment in aggressive metastatic breast cancers (Eshun-Wilson et al. 2019). Mutant ATAT-2 has been linked to neurodegenerative diseases associated with synapse instability, including Alzheimer's and Parkinson's disease (Borgen et al. 2019). Each of these diseases have a significant presence in our society and impact people in every walk of life. The battle against neurodegenerative diseases and breast cancer could benefit from understanding the connection between kinesin superprocessivity and microtubule acetylation.

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Figures

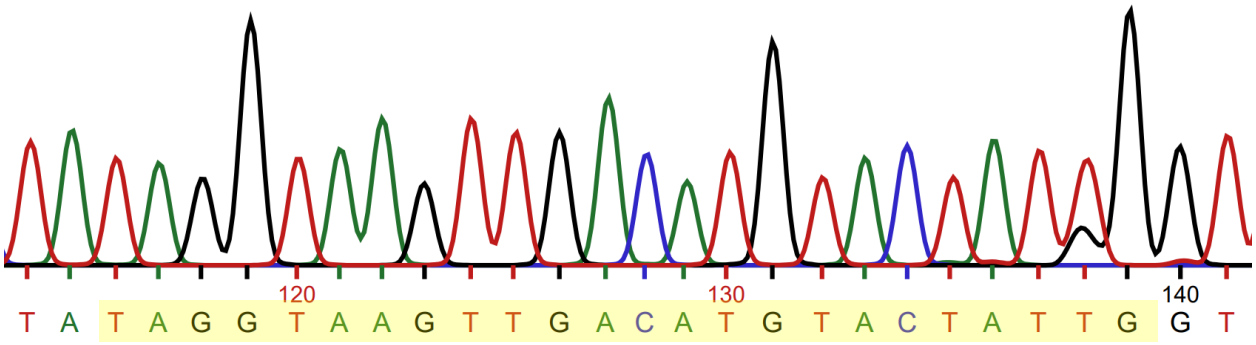

Figure 1. Sanger sequencing shows the successful cloning of the *klp-4* crRNA sequence into the pDR274 vector. The *klp-4* crRNA sequence containing BsaI overhangs were designed using ApE, cloned into pDR274, purified, and sent for sanger sequencing. The crRNA sequence was verified as indicated by the highlighted band.

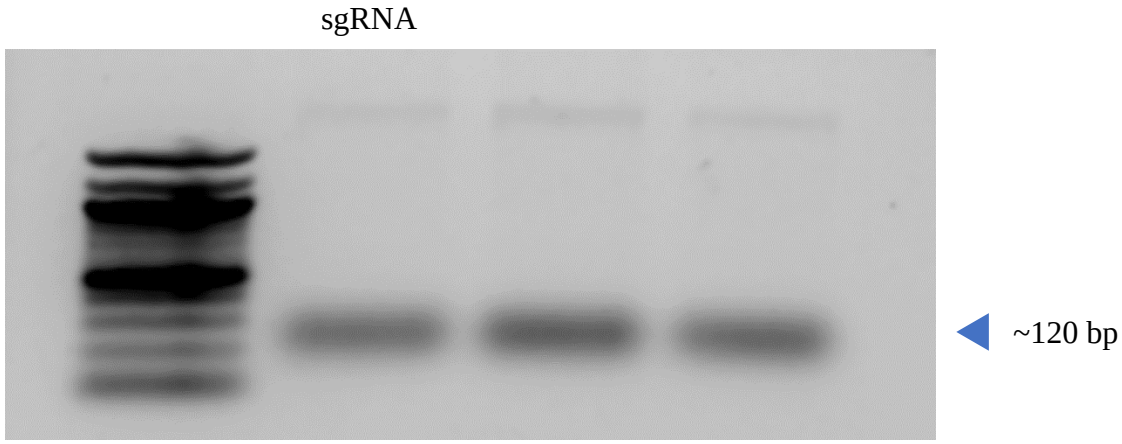

Figure 2. Bleach agarose gel electrophoresis confrims successful *in vitro* transcription of sgRNA. To create sgRNA transcripts, *in vitro* run off transcription of pJNP43 was performed via the HiScribe T7 Transcription kit (NEB). The sgRNA transcripts were purified via PCR Purification kit and a bleach denaturing agarose gel was conducted to show a band at ~120 bp as indicated by the blue marker. The band was compared to a 100 bp ladder.