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THE IMPACT OF GENETIC VARIANTS IN KINESIN MOTOR PROTEINS AND THE LINK TO NEUROLOGICAL DISORDERS

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ABSTRACT

Axonal transport driven by kinesin motor proteins is essential for the function of neurons and the transport of vesicles and organelles toward the synaptic cleft. Kinesins hydrolyze ATP as they move in a stepping motion along microtubules, and defects in their function can result in neuron malfunction and neurological disorders. Non-conservative missense mutations in the motor domain of a specific axonal kinesin, KIF1A/UNC-104, have been shown to have adverse effects on the motor speed and the distance moved by the motor prior to dissociation from the microtubule. The goal of this study is to characterize the potential pathogenicity of missense variants of KIF1A/UNC-104. Several key variants have been identified in the literature as mutations that may have a direct causal relationship with neurodegenerative, neuromuscular, and other nervous system disorders. Three missense mutations, S69L, T99M, and A255V that have shown disease association were functionally examined through gliding and single molecule motility assays to assess the impact of the mutations. In single-molecule assays, wild type UNC-104 travelled an average of 2.40 μm at a velocity of 2.32 μm/s before dissociation, providing a baseline to assess mutants. The mutant kinesins were unable to achieve this rapid, processive motility and were shown to have defective tight-binding states while retaining their unique weak-binding functionality. To add to the number of mutants in the literature and to determine a larger set of variants that are associated with disease, the MyCode database of Geisinger Health System was employed. The database was queried to determine patients in the population who have mutations in the motor domain of several axonal kinesins beyond KIF1A/UNC104 that also have been linked to neurological disorders. Using the arguments of the disease association and the decrease in functionality, it is hoped that this study will identify novel pathogenic variants.

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Chapter 1

Introduction to Kinesin

Kinesin motor proteins are essential for intracellular transport, and they are especially important in neurons as they transport organelles toward the synapse. In humans, one axonal transport motor, KIF1A, may play key roles in neuron physiology, and defects in its function may result in the manifestation of neurological disorders [1]–[3].

1.1 Kinesin Overview

Kinesin motor proteins, a family of enzymatic molecular motors, are essential for the process of intracellular transport. Kinesins perform directed transport as they hydrolyze adenosine triphosphate (ATP) to move in a stepping motion across filamentous structures in the cytoplasm known as microtubules. To produce movement, kinesins must hydrolyze ATP and harness the chemical energy to produce force. Within the cell, they are able to pull cargoes such as mitochondria and vesicles to their necessary locations against opposing loads up to several piconewtons. They have many structural similarities with myosin, but most kinesin operate as dimers and have key differences in their mechanism of operation [4]. Many kinesins structurally consist of two catalytic motor domains that perform the hand-over-hand "stepping" motion, a stalk that associates with the cargo at the C-terminus, and a neck-linker region that connects the motor domains to the stalk [5]. Dimeric kinesin walk processively as the motor domains alternate between bound and unbound states in a coordinated manner. As one head remains bound tightly,

the tethered head is able to move to the next binding location on the microtubule. These "steps" measure 8.2 nm in length as the unbound head binds to an adjacent tubulin unit [6]. A single kinesin is capable of taking hundreds of steps before dissociating from the microtubule. Each step is coupled with the hydrolysis of one molecule of ATP, and recent research has investigated the hydrolysis cycle, especially to determine the duration and stage in which the heads are bound to the microtubule [6].

1.2 Physiological Relevance in Neurons

The transport capabilities of kinesin make them instrumental in circumstances where simple diffusion would be insufficient, especially in neurons where vesicles must be rapidly transported to the synapse. Active anterograde transport is required to move organelles along the axon, and several kinesin families facilitate this transport as they pull mitochondria and vesicles to the plus end of microtubules [7]. Without these motors, neurotransmitter would not be able to travel efficiently to the synaptic cleft and action potentials would be inhibited. In addition, the buildup of organelles at the proximal end of the neuron may be prevented by the healthy function of kinesins. Axonal jams caused by abnormal intracellular transport may result in the impairment of neuron function and cell death [8].

1.3 Characteristics of KIF1A/UNC-104

The KIF1A motor protein, known as UNC-104 in the C. elegans model, is a member of the kinesin-3 family and plays a key role in fast axonal transport. UNC-104 is an important transporter of synaptic vesicles along microtubules toward the synapse [9]. Interestingly, KIF1A

is capable of functioning as a monomeric kinesin and even shows processivity in the single-head state [10]. It remains weakly bound to the microtubule through the electrostatic interaction between the motor domain and the C-terminus of the tubulin subunit [11]. The mechanism of monomeric kinesin transport is not well understood, but the accepted model involves diffusion with a drift that is made possible through Loop 12, known as the K-Loop, that weakly tethers the motor to the microtubule [10]–[12]. In addition, the monomeric kinesin-3 motors may dimerize to produce processive motility. KIF1A/UNC-104 dimers have been shown to move in a "superprocessive" fashion and are capable of walking abnormally long distances along microtubules before dissociating [13].

Mechanically, KIF1A has a dynamic α-4 helix that plays an essential role in motor function through its melting and reformation. KIF1A also has a disordered neck-linker domain that distinguishes it from other kinesin families [14]. One theory of motility involves the rotational movement of the catalytic core that drives the docking of the neck-linker and the displacement of the motor toward the plus end of the microtubule [15].

1.4 Alignments and Key Functional Regions

Kinesins have key regions in their motor domains that perform the functions necessary for motility. Kinesin undergo nucleotide-dependent conformational changes in order to generate motion [16]. Through x-ray crystallography and investigation into the hydrolysis cycle, specific regions of importance have been identified, including the microtubule-binding region and the regions associated with the binding and hydrolysis of ATP [4], [10], [17]. Upon the binding of a nucleotide, three regions of the KIF1A motor domain structure undergo significant changes: the

switch I, switch II, and neck-linker. All three of these regions, shown by figure 1 in the crystal structure solved by Nitta et al., are thought to play major roles in the motility of KIF1A motors [10].

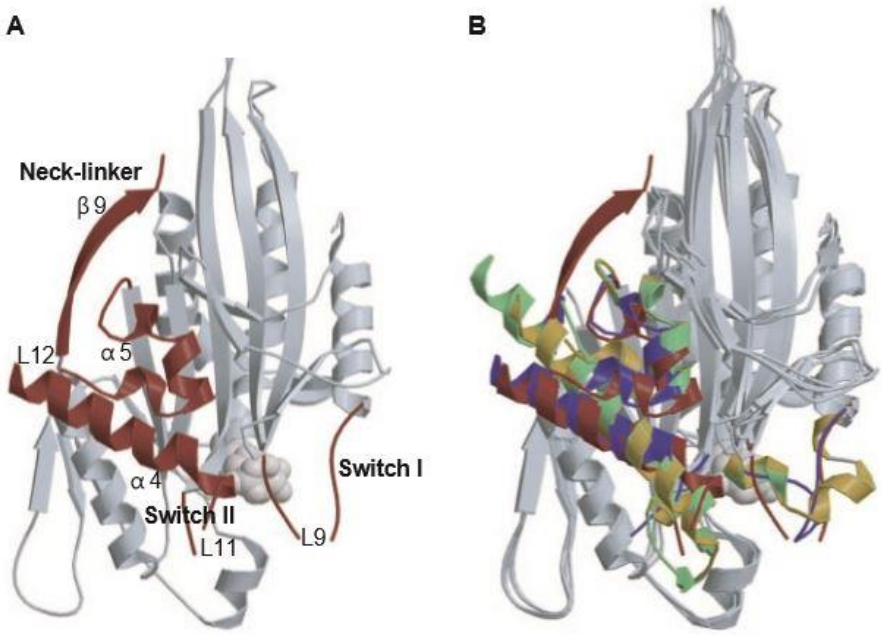

Figure 1. Crystal structures of KIF1A motor domain. (A) The AMP-PNP form of KIF1A, with the switch I, switch II, and neck-linker regions highlighted in red. (B) The superposition of the different forms of KIF1A when bound to nucleotide analogs. The AMP-PNP, ADP-AlFx, ADP-Vi, and ADP forms of KIF1A are shown in red, blue, green, and yellow, respectively [10]**.**

In addition to the 3D crystal structure, the locations of the mutations can be characterized by examining the protein alignment, reported by Sablin et al. and shown in figure 2 [4]. The locations of each residue along with the alpha helix, beta sheet, and loop structures of the protein are shown. The kinesin of interest in the alignment is MnKIF1B, which has 94% conserved identity and no gaps in the structural alignment with human KIF1A. The residues of interest in this study are Ser69, located at the L3/α1 interface, Thr99, located in the L4/N-1 region, and Ala255, located near the N-3 motif in the β7 region.

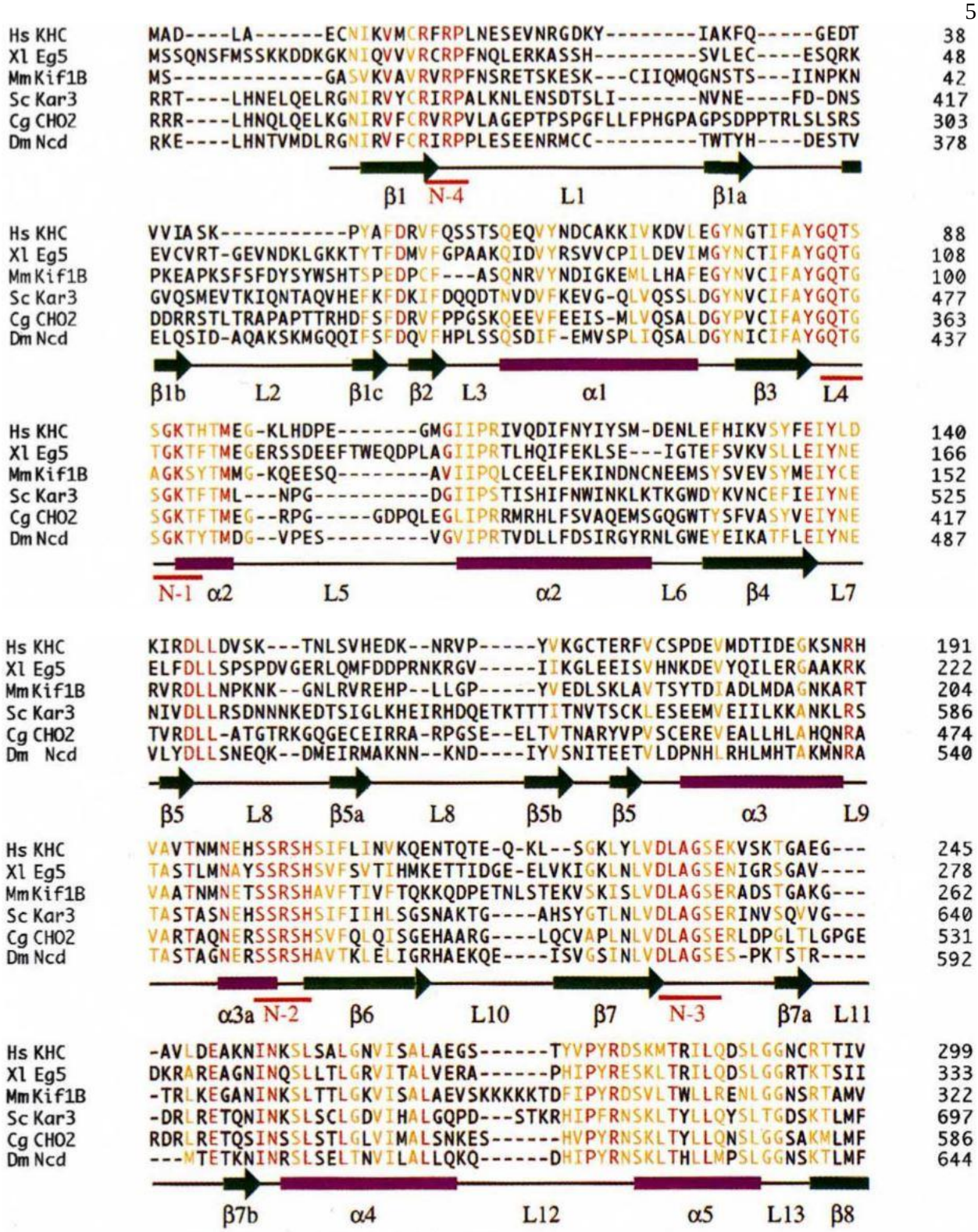

Figure 2. Protein sequence alignment of several kinesins. Residues that are absolutely conserved are shown in red and residues that are highly conserved are shown in orange. The locations of secondary structure elements and conserved motifs (N1-4) are shown [4]**.**

The switch I and switch II regions and the P-Loop, discussed in further detail in the following sections, are γ-phosphate sensing regions [14]. Kinesins are capable of sensing the presence of the γ-phosphate or lack thereof and transmitting this information through a cascade of conformational changes that culminate in force generation [18]. The communication between regions occurs through conformational changes that propagate from the nucleotide-binding pocket, through the switch I region, and to the switch II region to cause dramatic movement at the distal end of the kinesin motor [14].

1.4.1 P-Loop

The P-Loop is a component of the nucleotide-binding pocket and consists of a conserved structure (GxxxGKS/T) that is essential for nucleotide binding [16]. It is a highly conserved sequence shown in the L4/N-1 region in figure 2, and it is occasionally referred to as the ATP hydrolysis domain. Its role in the kinesin head domain is to stabilize the α and β phosphates of the bound nucleotide during the hydrolysis cycle between the events of ATP binding and ADP release [16], [19]. The chain of amino acids that compose the region is highly conserved in kinesin and does not significantly change conformation during hydrolysis [10]. As a result, it is expected to play a key role in ATP kinetics but less of a role in force transmission. A change in this region is expected to have an effect on the ability to use ATP as fuel and function effectively.

1.4.2 Switch I Region

The switch I region, which consists of Loop 9 in the kinesin structure, plays a key role in ATP hydrolysis and force transmission. The loop bends outward and effectively widens the nucleotide-binding pocket [14]. Residues in the switch I region may be responsible for the formation of a salt bridge with the switch II region and the stabilization a water molecule that is necessary for ATP hydrolysis [14], [19]. Without this salt bridge formed in part by the switch I region, the kinesin would not be able to function [20].

In the ADP bound state, the switch I region of KIF1A rearranges into a pseudo-β hairpin or is completely disordered, and this conformation is distinct from the structure of other kinesin [18]. Movements of the switch I region during the hydrolysis cycle in the presence of microtubules may disrupt Mg^{2+} coordination and result in faster ADP release, thus overcoming the rate limiting step and providing the activation of ATPase activity in the presence of microtubules [18]. In Loop 9, a loop-to-helix transition occurs that likely aids in the γ-phosphate release. Nitta et al. suggest that the γ-phosphate is efficiently released through a "back door," essential for fast ATP hydrolysis, formed by the highly conserved R216 of the switch I region and E253 of the switch II region. The R216 residue is shown in figure 3, and a mutation at this location has been shown to slow ATP hydrolysis [10], [18]. Other mutations in the switch I region, including R210A as found in drosophila, can reduce catalytic activity and inhibit microtubule binding [14], [19], [20].

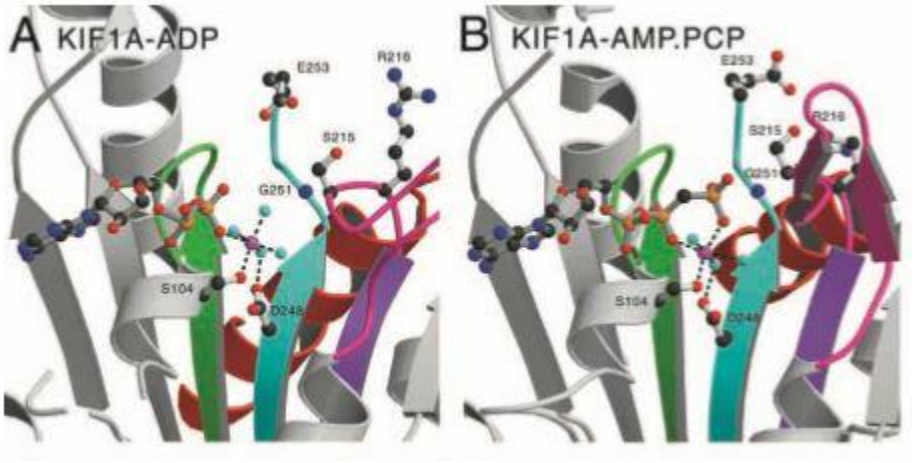

Figure 3. Coordination of the nucleotide and Mg^{2+} by essential elements in the KIF1A structure. (A) The nucleotide-binding site of KIF1A-ADP. The nucleotide is bound primarily by the P-Loop (green) and the switch II region (cyan). The switch I region (magenta) does not initially interact with the nucleotide. (B) The nucleotide-binding site of KIF1A-AMPPCP. The switch I loop forms a pseudo-β-hairpin structure and the R216 residue is unable to form the salt bridge with the E253 residue [18]**.**

1.4.3 Switch II Region

The switch II region includes Loop 11, the α-4 helix, Loop 12, the α-5 helix, and Loop 13. It contains several primary microtubule-binding elements of KIF1A, including Loop 11, the α-4 helix, and Loop 12 [10]. The α-4 helix, also known as the relay helix, is essential in the KIF1A motor as it undergoes significant rotational and translational changes based on the nucleotide state [16], [18]. Loop 11, the first structure in the switch II region, appears to play a significant role in transmitting the status of nucleotide phosphorylation to the microtubule-binding region. Mutations in this region interfere with the changing from high affinity to low affinity states, affect microtubule binding, and decrease catalytic activity [19]. Loop 11 fits tightly against the two outermost helices of tubulin, H11 and H12 [10], [18]. Thus, it is essential for microtubule binding, and mutations in this region could have an effect on microtubule affinity [10], [16].

In the kinesin-3 family, a significant portion of the Loop 12 domain is conserved across species. In kinesin-3, this loop has been called the K-Loop and is associated with weak microtubule binding that allows for diffusive motion. A sequence of several lysine residues is conserved in KIF1A human, KIF1A mouse, and UNC-104 C. elegans. Any disruption of this region would likely result in a decrease of the high processivity that is characteristic of many kinesin-3 motors.

Nitta et al. has suggested the alternated use of Loop 11 and Loop 12 to allow processive KIF1A motility. The Loop 11 and Loop 12 interactions with the microtubule have been proposed as the tight and weak bindings, respectively. The tight-binding state is given by Loop 11, but after phosphate release, the flexible Loop 12 extends down onto the tubulin C-terminus E-hook while Loop 11 is retracted. It has been shown that mutations in Loop 11 affect only the tight-binding state, and mutations in Loop 12 affect only the weak-binding state. This proposed mechanism explains the directed diffusion governed by weak-binding state and the processive motion of KIF1A by alternating the extended loops [10].

1.5 Axonal Kinesin Dysfunction

Defects in kinesin function have been shown to have drastic physiological effects. In 1996, Hurd and Saxton published a pioneering study that investigated the physiological effect of an in vivo knockout of the kinesin-3 gene. In the C. elegans model, the gene coding for kinesin-3 was entirely removed and the phenotype of the nematode was observed. From the observations, the researchers asserted that the lack of kinesin function caused posterior-ventral paralysis. The uncoordinated muscle movements of the mutant worms parallel the uncoordinated muscle

movements in humans with amyotrophic lateral sclerosis and other neurodegenerative disorders. The study also suggested that the kinesin knockout caused deficiencies in axonal transport that could lead to disease and would also lead to neurological disease in humans [1]. In another study, the inactivation of KIF1A in mice led to motor and sensory disturbances, and the mice died less than 24 hours after birth [21].

Recent studies have shown that entire knockout of kinesin genes may not be necessary to remove functionality of the protein. The disruption can also be caused by single amino acid changes that affect the shape and functionality of the protein. In order to characterize any change in functionality, kinesin velocity is used as the standard for assessing impaired function. A mutation examined by Wang and Brown, N256S, changed a single amino acid in the microtubule-binding portion of the motor domain of a kinesin-1 protein. This mutation is present in a population of humans that have been diagnosed with hereditary spastic paraplegia and other movement disorders. Wang and Brown showed that the mutation was detrimental to the transport frequency of the kinesin cargo [22]. Ebbing et al. published similar results to show that the same mutation caused decreased velocity. The article also investigated several other mutations, including K253N, which is located in the switch II region shown in figure 3. As expected, this mutation showed a decrease in catalytic rate, and this data furthers the argument that the mutations are detrimental to the kinesin function and have a possible link to disease [3].

Many neurodegenerative disorders are characterized by over-accumulations of specific proteins in neurons. Studies have shown that chemical compounds used to inhibit axonal transport cause abnormal protein accumulations and proximal axon enlargements that are characteristic of neurodegenerative disorders [23]. Mutations in motor proteins can also cause axonal jams and result in stranded synaptic vesicles within the neuron cell body. Neuronal

dysfunction such as these may be the cause of neurodegenerative disorders in humans [8]. The defects in axonal transport likely play a role in the manifestation of neurodegenerative disorders such as ALS, Alzheimer's disease, Parkinson's disease, and Huntington's disease [8], [24], [25]. In addition, defects in kinesin-driven axonal transport cause neuronal abnormalities such as those found in patients with hereditary spastic paraplegia and other neuropathies such as Charcot-Marie-Tooth Disease [3], [24], [25].

Chapter 2

Genetic Association Analysis

Specific genes can be studied in community and family populations as well as in case studies of individual patients. Occasionally, mutations, which are found in the genes that are passed down from generation to generation, can cause diseases and disorders in humans. In order to accurately diagnose and predict disease, current research in the field of genomics seeks to identify mutations that lead to diseases and disorders. Examples of the results from literature and the approach of further studies are outlined in the following sections. If a bioinformatics database is available, the information can be applied to execute genetic association tests and basic frequency analysis. Sophisticated phenome-wide association tests can be designed to show correlation between mutations and phenotypes. However, these association tests are beyond the scope of this paper, and simple frequency analysis can instead be used to show basic correlations and provide hypotheses for the relationship between a mutation and a disorder.

2.1 Previous Genetic Studies

Previous genetic studies present in literature have been used to show the relationship of kinesin mutations to several nervous system disorders. Following the study of two distinct families, Klebe et al. asserted that mutations in KIF1A are responsible for the manifestation of hereditary spastic paraplegia (HSP). This disorder is characterized by spastic behavior and uncontrolled muscle movements, especially in the extremities. The mutations that were studied were single missense mutations that changed the identity of a single amino acid in the protein sequence. A mutation found in one family, A255V, segregated with HSP in an association test

[26]. Ylikallio et al. contributes to the arguments by studying a different mutation in the KIF1A gene that likely causes HSP. This mutation, S69L, is located on the motor domain, specifically on the ATP-binding pocket. The disruption of the ATP-binding pocket based on the change from a polar amino acid to a nonpolar amino acid would likely interfere with the motor's coupling to ATP hydrolysis, inhibit the function of the overall motor, and therefore result in decreased axonal transport and neuron malfunction. The mutation segregates with the HSP phenotype through the use of MutationTester software, which analyzes a variant's disease causing potential [27]. The disorders of the central nervous system are more difficult to link to specific mutations because they are less thoroughly understood, but mutations in KIF1A have also been shown to have a relationship to several brain disorders. Several patients with kinesin variants who exhibit HSP have also been diagnosed with mild cerebellar atrophy and sensory disturbances [26]. While these genetic association studies are not as convincing as functional analysis of the kinesin, they provide hypotheses for mutations that have drastic impacts on the motility of kinesin.

One recent KIF1A study reports the clinical phenotype of several missense mutations in the motor domain. Several pediatric patients were shown to have nervous system disorders and KIF1A mutations, R216C and T99M. Two patients with the T99M mutation were diagnosed with microcephaly, global development delay, hyperreflexia, and movement disorders such as spastic paraparesis and myoclonic seizures. A different patient with an R216C mutation displayed similar phenotypes [2]. In a different case study, T99M was also found in a patient with an intellectual disability, peripheral spasticity, and cerebral atrophy [28]. The reported mutations and associated clinical data from literature provide a basis for functional analysis of KIF1A/UNC104.

2.2 Geisinger/Regeneron DiscovEHR Project

The Geisinger MyCode Community Health Initiative was developed to assist researchers in understanding the links between genetic mutations and diseases. To date, over 100,000 patients in the Geisinger Health System have consented to providing their genetic information and de-identified health records for research. The database of whole exome sequences not only provides avenues for personalized medicine such as earlier disease predictions and unique treatments, but also allows researchers to examine the genetic information and compare mutations to past diagnoses and phenotypes. Within the patient population, a subset of individuals possess mutations in the genes coding for axonal kinesin [29].

The Geisinger DiscovEHR project through Regeneron and Geisinger Health System provides a powerful tool for determining the pathological significance of mutations in the genes coding for kinesin motor proteins. Through this database, specific mutations can be linked to specific diseases and disorders. The phenotypes of all patients who have a specific mutation can be analyzed to determine any overrepresentation of a specific disease or class of disorders [30]. In combination with the functional analysis in the laboratory, this technique of association will be executed for several axonal kinesins in an attempt to strengthen the argument of disease association.

2.2.1 Geisinger Database: Methods

Using the genetic and phenotype information, mutations in the kinesin genes will be linked to neurological disorders. Initially, the database will be queried to determine all of the patients with genetic variants in the genes coding for axonal kinesin. The respective electronic

health records of the patients will be paired with the mutation information to show the diagnoses for each patient with a mutation. The database can show which diagnoses and disorders are overrepresented in the population. That is, if members of the mutant kinesin cohort display an abnormally high frequency of a particular disease or disorder, investigation will uncover the strong correlation. The relationship will be determined using basic methods of frequency analysis as compared to the overall population.

The initial step in the use of the Geisinger database is to design an appropriate query. The query that is in progress includes the KIF1A, KIF1B, KIF1C, KIF3A, KIF3B, KIF3C, and KIF5A genes because they code for important axonal kinesins. This study focuses on missense variants, and this type of mutation that only changes a single amino acid was specified. The results will not return synonymous mutations, which change a single base pair but do not affect the amino acid that is translated. Also excluded were frameshift and stop-gain mutations which would completely change the peptide sequence or prematurely truncate the protein. These mutations could show important information in genetic analysis because they would completely destroy the function of the protein and may result in predicted phenotypes, but they are not interesting in functional analysis except for the purpose of a negative control. The query was also designed to only identify mutations in the first 1,050 base pairs in the kinesin genes, which encompasses the motor domain of the protein that is estimated as the first 350 amino acids. The final essential specification of the query is the allele frequency of the mutation. This study used the lower allele frequency that corresponded to no less than 10 patients in the database who had a specific mutation. The lower limit of 10 patients was chosen because small population sizes cannot be used to draw conclusions about the overall population. Lower allele frequencies could be used to investigate case studies and extremely rare mutations, but this study does not focus on

case studies. Based on the prevalence of the neurological disorders of interest and the predicted penetrance of the mutations, the upper allele frequency was set to 0.003. This value was estimated based on the possible prevalence of neurodegenerative disorders in any given population. In order to perform rare variant analysis, this frequency cannot exceed 0.01, or 1% of the population.

The query that was executed shows all of the variants that are found based on the specifications and all of the patient numbers for the patients who possess the mutations. In order to protect patient privacy, the information will have previously been de-identified and replaced by a patient number that represents a singular patient in Geisinger Health System. For each patient number, the corresponding electronic health records that show all diagnoses for the patient can be requested. This subset of patients who have missense variants in the motor domain of KIF1A was pulled from the MyCode database. The health records were checked for phenotypes of interest by searching for all cases of specific ICD-10 codes that identify each possible diagnosis. In this study, the diagnoses of interest were the neurodegenerative, neuromuscular, neuronal, and general nervous system disorders shown in Appendix A. The neurodegenerative disorders are weighted as the highest priority, and the general brain disorders are weighted as the lowest when characterizing pathogenicity.

Through this outlined process, the number of patients who have a specific mutation and have also been diagnosed with a specific phenotype will be identified. The frequency of disease in the cohort of patients who have a specific genetic variant will be compared to the frequency of the disease in the overall population. If the subset of patients have an abnormally high frequency of these diagnoses as compared to the general population, then they will be identified as possible

disease-causing variants. A test of proportion can be applied to show significance of the frequency difference:

$$z = \frac{\hat{p} - p_0}{\sqrt{\frac{p_0(1 - p_0)}{n}}}$$

Where $\hat{p}$ is the fraction of patients with a given mutation who have the phenotype of interest, p_0 is the fraction of the general population who have the phenotype of interest, and n is the number of patients in the sample with the given mutation. The value of z must be higher than 1.96 to reject the null hypothesis with a 95% confidence interval. However, a second confidence calculation must be performed because the sample size of patients who have the rare variant will likely be small. The margin of error for a sample proportion can be calculated:

$$Error = z\sqrt{\frac{\hat{p}(1 - \hat{p})}{n}}$$

Where z would be 1.96 for the 95% confidence interval. This error become negligible with a large sample size but must be accounted for in the rare variant analysis. Sophisticated statistical analysis is necessary to further prove the disease causation of a specific variant, but the simple test of proportion can generate a strong hypothesis for further statistical or functional studies.

2.2.2 Geisinger Database Current and Future Results

In the MyCode database, 902 unique variants are found across the KIF1A gene in the population of 52,000 patients. Of these variants, a number of them can be excluded because they

do not produce missense mutations. Further information must be obtained through an actual query of the Geisinger database.

Although the patient information is previously de-identified, the research collaborators at Geisinger Health System must submit many proposals and permission documents through administration before beginning projects or sharing data, and the entire process requires several months to complete. Currently, the frequency analysis has been performed for the KIF1A and other kinesin proteins that were requested, but the information sharing is not yet approved. The data may show a disease correlation for mutations in axonal kinesins, but the information is not yet available and will be reported in future research.

2.2.3 Assessing Pathologic Significance

In the case of kinesin, the diagnoses of interest will include nervous system, neuromuscular, neurodegenerative disorders such as hereditary spastic paraplegia and amyotrophic lateral sclerosis. Because on the essential nature of kinesin in neurons, kinesin defects would be expected to cause neuronal disorders, and higher penetrance variants will be more likely to be causal of the specific disorder. Genetic variants that have shown association to neurological disorders in literature or in the Geisinger patient population and are shown to have decreased function in vitro are declared to play key roles in the manifestation of disease [3], [22]. Although neurodegenerative disorders were the first disorders to be linked to the loss of kinesin function, current literature does not convincingly show the direct correlation between kinesin missense mutations and neurodegenerative disorders such as amyotrophic lateral sclerosis. These disorders are weakly linked to kinesin through several case studies, small patient populations,

and functional studies that do not elucidate the causation the disorder. Although research has been promising on the topic of neuromuscular disorders, further research is warranted to prove the link between kinesin mutations and neurodegenerative disorders.

In genetic studies, it is important to recognize that correlation does not prove causation. Many confounding variables may be present in correlation studies, so further methods must be applied to convincingly show causation. The functional analysis in the following chapter contributes to the argument for the disease-causing capabilities of a specific mutation based on the in vitro behavior of the variant kinesin.

Chapter 3

Functional Analysis and Pathogenic Significance

Because the Geisinger database results could not be obtained in a timely manner, mutations of interest were determined from available literature. The mutations of interest include those that have been associated with disease but have not been functionally tested and those that have been functionally tested in the laboratory through gliding assays. These variants are S69L, T99M, and A255V. The variant kinesins were expressed and functionally tested through gliding and single molecule assays in the Hancock laboratory. The results of the functional experiments can show the detrimental or benign impact of the mutation on the function of the protein. The previous genetic studies and the future Geisinger genetic data will be used to strengthen the arguments of the disease association of kinesin and show the important functional regions of the motor domain. The combination of previous studies, functional analysis, and Geisinger genetic analysis provides a clearer picture of the kinesin mechanism, shows the importance of kinesin in neurons, and ultimately shows the strong relationship between kinesins and disease.

3.1 Functional Analysis: Methods

Variant and wild type kinesins were tested through gliding assays and single molecule assays. The gliding assay data is useful for determining kinesin velocity and to providing the comparison to literature. The single molecule assays provide a more convincing method of

assessing the behavior of individual kinesin and can be used to measure velocity and run length prior to dissociation.

3.1.1 Gliding Assays

Gliding assays are effective methods of determining the velocity of a population of kinesins. In these assays, kinesins are attached to the cell surface by their C-terminus via an antibody. The surface is blocked with casein to prevent the motors themselves from attaching to the surface because kinesins absorbed to glass surfaces often lose functionality [31]. Through the antibody, the stalk of the motor is immobilized, and the motor domains are free to interact with microtubules in a flow cell. The free microtubules can associate with the motor domains and move across a population of kinesin. The microtubules, labeled with Cy5 dye, are observed to be "gliding" when imaged with an inverted microscope. The kinesin velocity can be roughly determined by measuring the gliding velocity of the microtubules. In excess ATP, the microtubules will glide as the functional kinesins continue to hydrolyze the nucleotide to generate force. In excess adenylyl-imidodiphosphate (AMP-PNP), a non-hydrolysable nucleotide, the kinesins will become locked in the tightly bound state and no microtubule motility will be observed. The kinesin can be tested using homogenous populations or populations of mixed motors. To determine the velocity of one specific type of motor, only one type of motor is utilized, but mixed motor assays can be a powerful tool for determining how the kinesin interact to gain a clearer picture of their function in vivo [32]. A representation of a gliding assay is shown in figure 4.

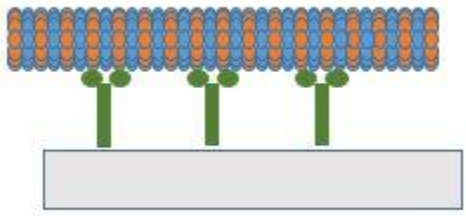

Figure 4. A representation of a gliding assay. The kinesin (green) attached to the glass surface through an antibody (not shown) are capable of moving the microtubule that is imaged (orange/blue) though the solution.

3.1.2 Single Molecule Assays

Single molecule assays are effective methods of determining how singular kinesin motors would behave in the presence of microtubules. The assays can be used to determine important characteristics such as velocity and run length. In this assay, microtubules are fixed to a coverslip using rigor kinesin motors, and the kinesins of interest, labeled with a fluorophore, are added to the flow cell in excess ATP. The assays are then imaged using Total Internal Reflection Fluorescence (TIRF) microscopy. In TIRF microscopy, an excitation light source of a laser is totally internally reflected at the air-glass interface. When this occurs, an evanescent wave is generated to excite the fluorophore on the glass surface. Because the evanescent wave decays exponentially, only molecules that are within a short distance of the surface are sufficiently excited, and background signal from free fluorophores within the flow cell is limited [33]. In order to accomplish effective imaging, the molecules of interest must be properly labeled. Kinesins can be labeled using quantum dots or green fluorescent protein (GFP) to accurately assess motility [34]. In the same manner as the gliding assays, ANP-PNP will lock the healthy kinesins in the tightly bound state. A representation of a single molecule assay is shown in figure 5. The kinesin, labeled with GFP, can be imaged in the single molecule assay to visualize its

motion and behavior. Once the images are recorded, they are processed using ImageJ, an open source image processing program developed by the National Institutes of Health. This programs is capable of translating image data to information of significance such as particle velocity and kinesin run length. The data can be obtained through a kymograph and no plug-in is required.

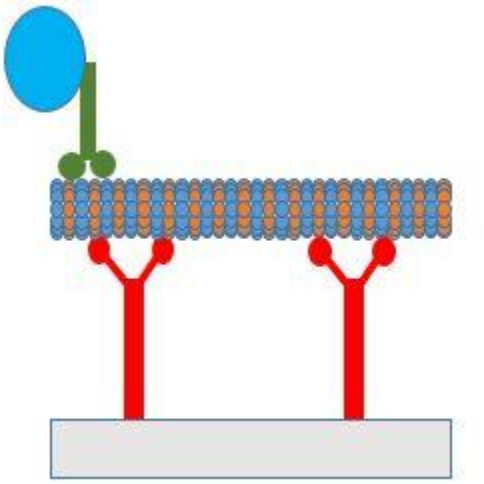

Figure 5. A representation of a single molecule assay. Rigor motors (red) are absorbed to the glass surface and microtubules (blue/orange) are immobilized on their stationary motor domains. The kinesins (green) are labeled with Q-dots or GFP and imaged as they bind and move freely along the microtubule prior to dissociation.

3.2 Functional Analysis: Results

3.2.1 Gliding Assay Results

The control experiment for the gliding assay of the UNC-104 protein was performed and the microtubule velocity was determined using ImageJ software. The UNC-104 gliding assay velocity was determined to be 3.0 ± 0.3 μm/s from the measurement of 42 microtubules in a

relatively normal distribution as shown in figure 6. In mutant kinesin assays, no gliding was observed in the S69L, T99M, or A255V mutants because the microtubules did not bind to the motors. In the presence of AMP-PNP, the microtubules locked to the kinesin surface in the wild type assay but did not bind in the mutant assays.

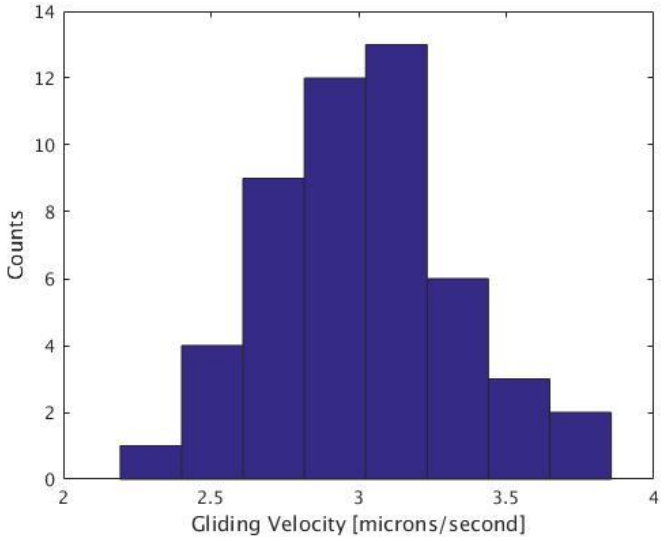

Figure 6. Velocity distribution for wild type UNC-104 gliding assays

3.2.2 Single Molecule Assays

Because gliding assays gave no motility, the variant kinesins were assessed with single molecule assays to determine the effect of the mutation on velocity and run length. The control experiment for single molecule assays was run using the wild type UNC-104 protein. The UNC-104 single molecule velocity was determined to be an average of 2.32 ± 0.09 μm/s in a normal distribution as shown in figure 7. The mean run length of the kinesin before dissociation was determined to be to be 2.40 ± 0.28 μm in an exponential distribution as shown in figure 8.

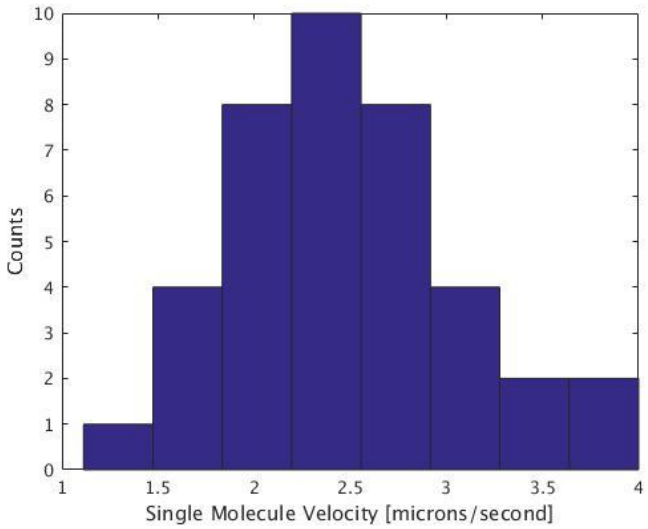

Figure 7. Velocity distribution of wild type UNC-104 as determined by single molecule assays

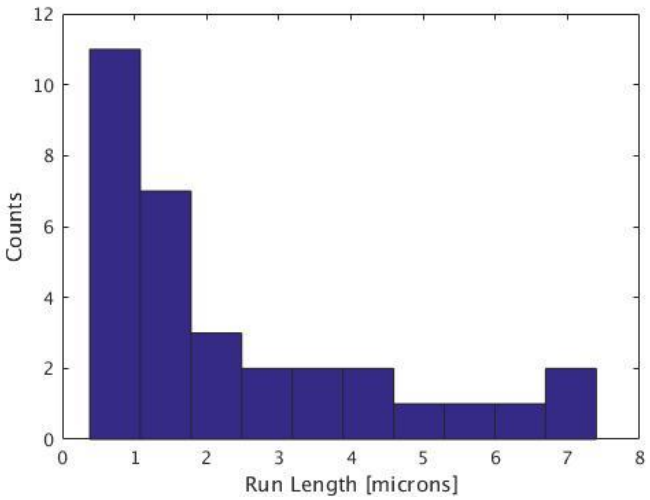

Figure 8. Distribution of wild type UNC-104 run length prior to dissociation as determined by single molecule assays

The velocity of the UNC-104 wild type and mutants are shown in figure 9. The variant kinesin produced a low rate of transient motility events when compared the wild type kinesin that showed frequent, processive motility.

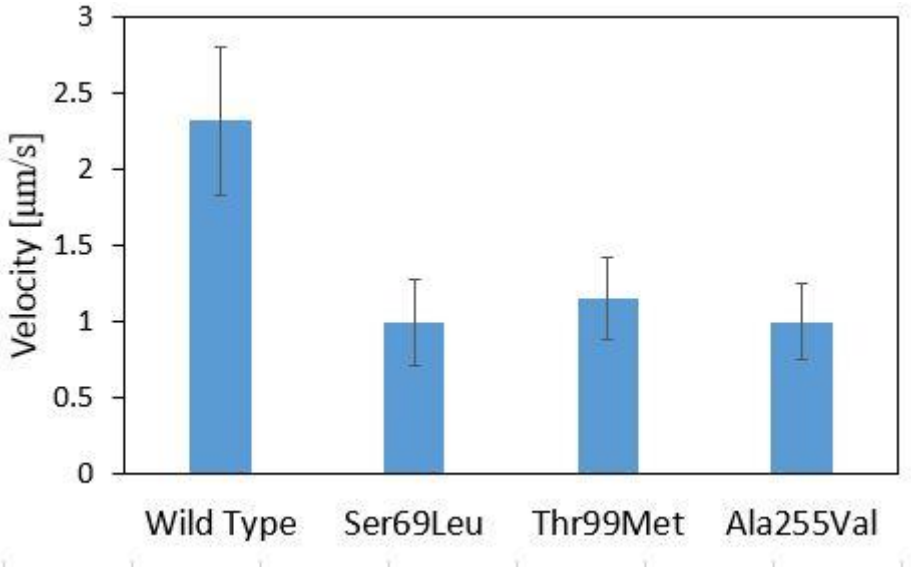

Figure 9. The velocities of the wild type and mutant UNC-104 in single molecule assays. The error bars display the standard deviation. The wild type velocity is significantly larger than the mutant velocities.

A summary of the velocity and run length of the wild type and mutant motors is shown in table 1. Each of the mutant motors produced short run lengths, and the wild type motor produced significantly longer runs prior to dissociation.

Table 1. Summary of average gliding velocity and run lengths for the chosen mutant kinesin, where n is the number of events observed. The velocity and run length are reported as the mean ± the standard error of the mean.

Mutation	Velocity (µm/s)	Run Length (µm)	n
Wild Type	2.32 ± 0.09	2.40 ± 0.28	39
S69L	0.99 ± 0.07	0.73 ± 0.10	14
T99M	1.24 ± 0.04	0.63 ± 0.08	12
A255V	1.08 ± 0.04	0.60 ± 0.12	11

The variant kinesin assays produced rare, short motility events that are reported in table 1 but may not be representative of processive kinesin motion. The majority of these short, rapid events lasted less than a second, and the low number of events may not have produced an accurate average velocity.

In order to assess the tight-binding functionality of the mutants, AMP-PNP single molecule assays were performed. In AMP-PNP, healthy kinesins will bind tightly to the microtubule and will not dissociate because they cannot hydrolyze the nucleotide. The results of the AMP-PNP single molecule assays are shown in figure 10.

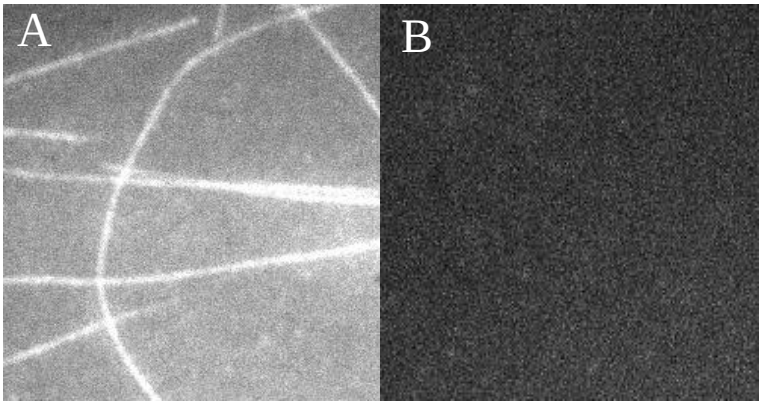

Figure 10. Single molecule assays with AMP-PNP instead of ATP for (A) wild type UNC-104 and (B) mutant UNC-104. Only the labeled kinesins are imaged.

In excess AMP-PNP, the wild type UNC-104 tightly bound to the microtubules, showed no motility, and did not dissociate. In figure 10A, the kinesins are shown to fully decorate the microtubules in their tight-binding state. However, the variant UNC-104 motors did not bind to the microtubules in the presence of AMP-PNP. No tight binding is observed in figure 10B, and these results lead to the determination that the mutations interfere with the tight-binding state of UNC-104.

3.3 Determination of Mutation Significance

The UNC-104 motor is essential in fast axonal transport, and it is observed to travel at high velocities in vitro. Nieh et al. reported binding without gliding for T99M mutants and slow gliding for A255V mutants, but the results of this study showed no binding for either mutant. The inconsistency could be explained by the difference in experimental techniques because the Nieh et al. study used only the motor domain rather than an entire kinesin construct [2]. The S69L mutation was not previously studied in functional assays, but it also resulted in no binding or gliding in this study.

The impacts of the mutation can be assessed through analysis of the locations of the missense mutation in the motor domain. The S69L mutation is located in the nucleotide-binding pocket, and the change from a polar to a nonpolar amino acid may disrupt the structure and prevent nucleotide binding. If nucleotide binding was inhibited, the motor would be unable to hydrolyze ATP and walk along the microtubule. The T99M mutation is located in the P-Loop of the KIF1A/UNC-104 motor domain. Threonine and methionine have distinct functionalities, and the change may disrupt the functionality of the highly conserved P-Loop. The region is conserved across families, and changes in this region could affect ATP kinetics and therefore change the overall function of the motor. The A255V mutation is found in the switch II region of KIF1A/UNC-104, and this mutation may also have an effect on ATP binding. The mutation may disrupt the essential E253 residue or sterically hinder the binding of nucleotide with the branched carbon chain of the new valine residue.

The mutations prevented motility in the gliding assays and drastically reduced run lengths and velocities in the single molecule assays. The reduced velocity and short run length may be the result of the completely inhibited tight-binding state. However, the weak-binding state

appears to retain functionality. This weak-binding state is unique to the kinesin-3 family, and the weak-binding state may explain the transient single molecule assay events. The positively charged K-Loop of UNC-104 may cause rare, diffusive motility events. If the variant UNC-104 motors diffuse near the microtubules on the surface, they may feel a temporary electrostatic force between the K-Loop and the microtubule. In wild type kinesin, the weak-binding state alternates with the tight-binding state to produce processive motion [10]. However, because the mutations likely prevent ATP binding or result in protein misfolding, the tight-binding state is prevented, and the UNC-104 motors dissociate rapidly. In addition, the diffusive motion of the weak-binding state is not as efficient as directed transport of the kinesin stepping motion. This suggests that KIF1A/UNC-104 motors rely more heavily on the tight-binding state than the weak-binding state for rapid, processive motion. The diffusive motion can be characterized by the simple diffusion coefficient calculation shown below using the event time and distance travelled in the mutant single molecule assays.

$$D = \frac{x^2}{2t} = 0.18\ \mu m^2/s$$

Okada et al. measured the diffusion coefficient of monomeric KIF1A as 0.044 $\mu m^2/s$ by fitting a mean square displacement plot with an equation for biased Brownian movement [12]. The results from this study are similar, and the motion of the mutant UNC-104 motors resembled diffusion rather than processive motility. The mechanism of the diffusive motion of KIF1A/UNC-104 has not been entirely solved, and it is unknown how the motor could achieve the diffusion velocities shown in this study of around 1.0 $\mu m^2/s$. However, the results are reasonable for this fast axonal transport motor.

The AMP-PNP assay did not result in tight binding of the motor, and this further supports the theory of protein denaturation and the possibility that the observed velocities and run lengths of the mutants are based solely upon the weak-binding state of UNC-104. The missense mutations appear to destabilize the protein structures in a manner that prevents tight microtubule binding. However, despite the loss of tight-binding functionality, the mutations do not significantly interfere with the weak-binding state.

The variant kinesins may lose their tight-binding functionality because the mutation causes misfolding and denaturing of the entire motor domain. While this interpretation should not be ruled out, it is unlikely that an A255V mutation would have drastic effects on the entire structure of the protein. The results can also be interpreted as the loss of protein function due to prolonged lack of nucleotide. The structures of kinesins are stabilized in the nucleotide-bound state [14], and kinesins are often stored in the presence of nucleotide to prevent denaturation. The missense mutations in this study are located in regions of the KIF1A/UNC-104 motor domain that are important in nucleotide binding. If the mutations inhibit nucleotide binding, the instability of the kinesins in the prolonged nucleotide-free state may result in damage to the motor. Whether due to entire misfolding of the head domain or loss of function from inhibited nucleotide stabilization, the mutations inhibited tight binding but did not affect the electrostatic interaction of the K-Loop. The results suggest that the functionality of the K-Loop may not be entirely nucleotide-dependent.

The S69L variant in KIF1A has been shown to have an association with hereditary spastic paraplegia [27]. The functional assay that has been performed elucidates the mechanism of disease causation. The mutation reduces the run length and velocity of the motor protein, which likely causes abnormalities in neurons in vivo. The function of KIF1A/UNC-104 is

essential for healthy neuron function, and the mutation likely results in slowed transport of organelles in motor neurons that eventually leads to damage and plays a role in hereditary spastic paraplegia.

In the studies of several pediatric patients, the T99M mutation has been shown to be associated with severe nervous system disorders and movement disorders. The mutation is present in patients with microcephaly, global development delay, hyperreflexia, spastic paraparesis, myoclonic seizures, intellectual disability, peripheral spasticity, and cerebral atrophy. This clinical phenotype association, in combination with the functional data showing the loss of tight-binding function of KIF1A/UNC-104 due to the mutation, shows that the T99M mutation may have a causal relationship to neurological disorders. A larger population size is needed to assert the causal relationship, but the mutation and loss of kinesin function appears to play a role.

In the study of a single family of Palestinian descent, the A255V mutation segregated with hereditary spastic paraplegia in a genetic association test. An abnormally high proportion of members of the family were diagnosed with progressive spastic gait and peripheral neuropathy [26]. Although the mutation appears to be relatively biochemically conservative, it occurs near the essential E253 residue in the switch I region of KIF1A/UNC-104. The mutation likely causes misfolding from the inhibition of nucleotide binding [26], and this hypothesis is supported by the functional data that shows the decrease in both velocity and run length of the motor. Based on the genetic studies and functional analysis, the mutation is determined to play a role in the manifestation of disease.

Another mutation that was considered as a candidate for expression was R216C, a highly conserved residue in the switch I region. It is shown in figure 3 and plays a key role in γ-

phosphate release. However, this mutation has been previously shown to result in no gliding, and the charge loss of the residue change would likely have a drastic impact on the KIF1A structure and ATPase function [10]. Therefore, it was deemed too harsh of a mutation to express in this study, but it will be examined in future studies to determine if the mutant produces any motility in a single molecule assay.

Chapter 4

Conclusions and Future Directions

Based on previous genetic analysis studies, the S69L, T99M, and A255V mutations in KIF1A/UNC-104 were shown to have a correlation with hereditary spastic paraplegia and other disorders of the nervous system. The associations of the clinical phenotypes with the mutations led to the functional analysis to determine the consequences of the mutation in vitro. The variant kinesins lack the ability to bind tightly to microtubules, yet they retain their weak-binding functionality to produce diffusive motion. The mutations ultimately result in defective motors that cannot processively walk on microtubules. As a result, the mutations have been identified as pathogenic variants and seem to play a role in the manifestation of neurological disorders.

Future research should draw conclusions from larger patient populations and include statistical techniques for rare variant analysis to enhance the arguments of pathogenicity. Once received, the data from Geisinger Health System will be analyzed to determine if a set of patients with specific mutations show an overrepresentation of the nervous system disorders of interest. If the statistical test of proportion shows significance, the mutations should be expressed in the Hancock laboratory and tested to determine the impact of the mutation.

Further functional analysis should include assays to determine the enzymatic activity of the mutant KIF1A/UNC-104 motors. These experiments will help to determine the effects of the mutation on ATP binding. In addition, the use of gold nanoparticles rather than GFP labeling could produce higher resolution and more insight into the tight and weak-binding states of KIF1A/UNC-104. Finally, mixed motor assays could be performed with a mixture of wild type and mutant kinesins to simulate the heterozygous nature of most KIF1A mutations found in humans.

Appendix A

Clinical Phenotypes of Interest

Class of Disorders	Disease/disorder	ICD-10 Code
Neurodegenerative Disorders	Alzheimer's disease	G30
	Parkinson's disease	G20, G21.19
	Huntington's disease	G10
	Amyotrophic lateral sclerosis	G12.21
	Dementia	F01.50, F01.51, F03.90
	Prion disease	A81.9
Neuromuscular Disorders	Hereditary spastic paraplegia	G11.4
	Abnormal gait	R26.9
	Restless leg syndrome	G25.81
	Tremor/spasm	R25.1, R25.0, R25.2
	SC ataxia	G11.8
Other Neuronal Disorders	Multiple Sclerosis	G35
	Neuropathy	G60.3, G60.9, G62.9, G90.09
	Neuritis	M79.2, G58.7, M54.17
	Bell's Palsy	G51.0
	Neuralgia	M58.1, M79.2, B02.02
General Brain Disorders	Depression	F32.4, F32.5, F32.9, F33.1, F33.3, F33.41, F43.21
	Migraine	G43.009, G43.109, G43.719, G43.809, G43.909, G43.919
	Epilepsy	G40.209, G40.209, G40.309, G40.401, G40.909
	Bipolar disorder	F31.30, F31.32, F31.9

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