FUNCTIONAL CHARACTERIZATION OF A KAR3/VIK1-LIKE
KINESIN-14 HETERODIMER FROM THE FILAMENTOUS
MULTINUCLEATE FUNGUS ASHYA GOSYPII

by

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Abstract

Kinesins are motor proteins that convert chemical energy from ATP hydrolysis into mechanical energy used to generate force along microtubules, transporting organelles, vesicles and proteins within the cell. Kar3 kinesins are microtubule minus-end-directed motors with pleiotropic functions in mating and mitosis of budding and fission yeast. In *Saccharomyces cerevisiae*, Kar3 is multifunctionalized by two non-catalytic companion proteins, Vik1 and Cik1. A Kar3-like kinesin and a single Vik1/Cik1 ortholog are also expressed by the filamentous fungus *Ashbya gossypii*, which exhibits different nuclear movement challenges and unique microtubule dynamics from its yeast relatives. We hypothesized that these differences in *A. gossypii* physiology could translate into interesting and novel differences in its versions of Kar3 and Vik1/Cik1. Presented here is a structural and functional analysis of recombinantly expressed and purified forms of these motor proteins. Compared to the previously published *S. cerevisiae* Kar3 motor domain structure (*ScKar3MD*), *AgKar3MD* displays differences in the conformation of the ATPase pocket. Perhaps it is not surprising then that we observed the maximal microtubule-stimulated ATPase rate (*k_{cat}* of *AgKar3MD* to be approximately 3-fold slower than *ScKar3MD*, and that the affinity of *AgKar3MD* for microtubules (*K_{d,MT}* was lower than *ScKar3MD*. This may suggest that elements that compose the ATPase pocket and that participate in conformational changes required for efficient ATP hydrolysis or products release work differently for *AgKar3* and *ScKar3*. There are also subtle structural differences in the disposition of secondary structure elements in the small lobe (β1a, β1b, and β1c) at the edge of the motor domain of *AgKar3* that may reflect the enhanced microtubule-depolymerization activity we observed for this motor, or they could relate to its interactions with a different regulatory companion protein than its budding yeast counterpart. Although we were unable to gain experimentally determined high-resolution information on *AgVik1*, the results of Phyre2-based bioinformatics analyses may provide a structural explanation for the limited microtubule-binding activity we observed. These and other
fundamental differences in AgKar3/Vik1 could explain divergent functionalities from the ScKar3/Vik1 and ScKar3/Cik1 motor assemblies.
Co-Authorship

Portions of this thesis, particularly pertaining to the structural and functional data for AgKar3MD, and much of the interpretation of these data, are published in Duan, et al.[1]. Parts of the published data for which the work is shared with or contributed by the co-authors (Da Duan, Jillian Brenner, Darlene Davis and John S. Allingham) are referenced as “Duan, et al.[1]”, with an asterisk included beside the citation. Other parts of the published data are the sole work of the author of this thesis (Daniel J. Hnatchuk) and are referenced as “Duan, et al.[1]‡”, with a double-dagger included beside the citation.
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# Table of Contents

Abstract .................................................................................................................................. ii  
Co-Authorship .......................................................................................................................... iv  
Acknowledgements ................................................................................................................. v  
Table of Contents ...................................................................................................................... vi  
List of Figures ........................................................................................................................... vii  
List of Tables .............................................................................................................................. xi  
List of Abbreviations ................................................................................................................ xii

Chapter 1 Introduction ........................................................................................................... 1  
1.1 Motor Proteins ................................................................................................................... 1  
1.2 Kinesin interaction with the microtubule track ................................................................... 1  
1.3 Kinesin Structure ............................................................................................................. 5  
1.4 Kinesin Motor Proteins are Remarkable Examples of Diversity and Adaptation .......... 8  
  1.4.1 Kinesin-1: The Quintessential Kinesin Motor Protein ............................................... 9  
  1.4.2 Eg5 is a Homotetrameric Kinesin ................................................................................. 10  
  1.4.3 The Kinesin-13 MCAK and Kinesin-8 Kip3p are Microtubule Depolymerases .......... 10  
  1.4.4 The Kinesin-14 Ncd turns the Kinesin Superfamily on its Head ................................. 13  
  1.4.5 KCBP is a Plant Kinesin-14 Regulated by Calcium Concentration .......................... 14  
1.5 Kar3 Motors are Unusual Members of the Kinesin-14 Family ....................................... 17  
1.6 Orthologs of ScKar3 and ScVik1 are found in Ashbya gossypii ..................................... 20  
1.7 The Cell Cycle of Ashbya gossypii .................................................................................. 23  
1.8 Structural and Functional Characterization of AgKar3 and AgVik1 ............................ 29  
1.9 Specific Question Addressed in this Thesis ..................................................................... 33  

Chapter 2 Materials and Methods ........................................................................................ 34  
2.1 Cloning and Purification of Protein Constructs ............................................................... 34  
  2.1.1 Design of AgKar3 and AgVik1 Monomeric and Dimeric Constructs .......................... 34  
  2.1.2 Cloning and Purification of the Monomeric AgKar3 Construct ................................. 35  
  2.1.3 Cloning and Purification of the Monomeric AgVik1 Construct ................................. 36  
  2.1.4 Cloning of the AgKar3/Vik1 Dimeric Complex ........................................................ 38  
  2.1.5 Cloning and Purification of AgVik1-Maltose Binding Protein Fusion Constructs ...... 39  
2.2 Microtubule Preparation ................................................................................................. 41  
  2.2.1 Large Scale Preparation of High-Salt Tubulin from Bovine Brain Tissue ................. 41  
  2.2.2 Small-Scale Recycling of High-Salt Purified Tubulin .............................................. 42
List of Figures

Figure 1 – Mechnochemical Cycle of Kinesin-1 ................................................................. 2
Figure 2 – Quaternary Assembly of Kinesin-1 ........................................................................ 7
Figure 3 – Depolymerization Cycle of Kinesin-13 MCAK ..................................................... 11
Figure 4 – Mechnochemical Cycle of Ncd ........................................................................... 15
Figure 5 - Crystal Structures of Kinesin Regulatory Elements .................................................. 16
Figure 6 – Saccharomyces cerevisiae Kar3, Vik1 and Cik1 Knockout Studies ......................... 18
Figure 7 – Mechnochemical Cycle of Saccharomyces cerevisiae Kar3/Vik1 ........................ 19
Figure 8 - Morphology of Ashbya gossypii hyphae .............................................................. 22
Figure 9 – Cell Cycle of Saccharomyces cerevisiae ................................................................ 24
Figure 10 – Cell Cycle of Ashbya gossypii ........................................................................... 26
Figure 11 – Ashbya gossypii Kinesin Knockout Studies ....................................................... 28
Figure 12 – Crystal structure and nucleotide binding pocket of AgKar3MD .......................... 30
Figure 13 – Comparison of loop L1 and the small β-lobe in AgKar3MD and ScKar3MD ....... 32
Figure 14 – Multiple sequence alignment of Kar3 ortholog motor domains ......................... 51
Figure 15 – Structural Superposition of Sc and Ag Kar3 Motor Domains .............................. 53
Figure 16 – Multiple Sequence Alignment of Cik1 and Vik1 Motor Homology Domains ...... 54
Figure 17 – Crystal Structures of Neck Conformations of Candida glabrata Vik1 ............ 56
Figure 18 – Prediction of AgKar3 and AgVik1 Coiled-Coil Propensity .................................. 57
Figure 19 – Truncated AgKar3 and AgVik1 Construct Design ............................................. 58
Figure 20 – SDS-PAGE of AgKar3/Vik1 Co-purification ...................................................... 59
Figure 21 – Size Exclusion Chromatography Profile of AgKar3/Vik1 ................................. 61
Figure 22 – Circular Dichroism Spectra of AgKar3/Vik1 ...................................................... 62
Figure 23 – SDS-PAGE of AgKar3MD Purification .............................................................. 63
Figure 24 – SDS-PAGE of AgVik1 Purification .................................................................... 65
Figure 25 – Microscopy Still-Frames of the AgKar3/Vik1 Microtubule Motility Assay ......... 66
Figure 26 – AgKar3MD ATPase Kinetic Curves ................................................................. 68
Figure 27 – AgVik1 ATPase Kinetic Curves ........................................................................ 70
Figure 28 – AgKar3/Vik1 ATPase Kinetic Curves ............................................................... 71
Figure 29 – Representative SDS-PAGE of AgKar3MD and AgVik1 Microtubule-Binding .... 74
Figure 30 – AgKar3 and AgVik1 Microtubule-Binding Densitometry Curves ...................... 75
Figure 31 – AgKar3/Vik1 Microtubule Depolymerization Curves ......................................... 78
Figure 32 – SDS-PAGE of AgVik1-MBP Construct Purification ........................................... 81
Figure 33 – Size Exclusion Chromatography Profiles of AgVik1-MBP Constructs ............... 82

viii
List of Tables

Table 1 - Primers used to generate AgKar3 and AgVik1 constructs. .................................................. 40
Table 2 - Enzyme rate constants determined from the enzyme-coupled ATPase assay of Kar3 motors. .................................................................................................................. 69
Table 3 - Enzyme rate constants determined from the enzyme-coupled ATPase assay of Kar3/Vik1 and Kar3/Cik1 motors ........................................................................................................ 69
Table 4 - Microtubule binding constants of Kar3 motor domains. .......................................................... 76
Table 5 - Microtubule binding constants of Vik1 proteins. ....................................................................... 76
Table 6 - Microtubule binding constants of Kar3/Vik1 complexes. ......................................................... 76
# List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ACES</td>
<td>N-(2-Acetamido)-2-aminoethanesulfonic acid</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>Ag</td>
<td><em>Ashbya gossypii</em></td>
</tr>
<tr>
<td>AMPPNP</td>
<td>Adenylyl-imidodiphosphate (non-hydrolyzable ATP analog)</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>ATPase</td>
<td>Adenosine triphosphatase</td>
</tr>
<tr>
<td>BLAST</td>
<td>Basic Local Alignment Search Tool</td>
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<tr>
<td>BME</td>
<td>β-mercaptoethanol</td>
</tr>
<tr>
<td>Ca</td>
<td><em>Candida albicans</em></td>
</tr>
<tr>
<td>Cg</td>
<td><em>Candida glabrata</em></td>
</tr>
<tr>
<td>Cik1</td>
<td>Chromosome instability and karyogamy protein</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene glycol tetraacetic acid</td>
</tr>
<tr>
<td>GMPCPP</td>
<td>Guanosine-5’-[α,β)-methylene] triphosphate (slow-hydrolyzable GTP analog)</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosine diphosphate</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>His&lt;sub&gt;10&lt;/sub&gt;-tag</td>
<td>10x Polyhistidine tag</td>
</tr>
<tr>
<td>HMPB</td>
<td>High Molarity PIPES Buffer</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl-1-thio-β-D-galactopyranoside</td>
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<tr>
<td><em>K&lt;sub&gt;1/2,MT&lt;/sub&gt;</em></td>
<td>Dissociation constant (microtubule)</td>
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<td><em>k&lt;sub&gt;cat&lt;/sub&gt;</em></td>
<td>Turnover number</td>
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<td>KHC</td>
<td>Kinesin Heavy Chain</td>
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<td>Kl</td>
<td><em>Kluyveromyces lactis</em></td>
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<tr>
<td>KLC</td>
<td>Kinesin light chain</td>
</tr>
<tr>
<td>LB</td>
<td>Lysogeny broth</td>
</tr>
<tr>
<td>MBP</td>
<td>Maltose Binding Protein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>NAD&lt;sup&gt;+&lt;/sup&gt;</td>
<td>β-nicotinamide adenine dinucleotide (oxidized)</td>
</tr>
<tr>
<td>NADH</td>
<td>β-nicotinamide adenine dinucleotide (reduced)</td>
</tr>
<tr>
<td>Nc</td>
<td><em>Naumovozyma castelli</em></td>
</tr>
<tr>
<td>Ncd</td>
<td>Non-claret disjunction protein</td>
</tr>
<tr>
<td>Ni-NTA</td>
<td>Nickel-nitriloacetic acid</td>
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<td>PIPES</td>
<td>Piperazine-N,N'-bis(2-ethanesulfonic) acid</td>
</tr>
<tr>
<td>O/N</td>
<td>Overnight</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDB</td>
<td>Protein Data Bank</td>
</tr>
<tr>
<td>PEP</td>
<td>Phosphoenolpyruvate</td>
</tr>
<tr>
<td>RMSD</td>
<td>Root mean square deviation</td>
</tr>
<tr>
<td>Sc</td>
<td><em>Saccharomyces cerevisiae</em></td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate – Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>S/N</td>
<td>Supernatant</td>
</tr>
<tr>
<td>TCL</td>
<td>Total cell lysate</td>
</tr>
<tr>
<td>Vik1</td>
<td>Vegetative interaction with Kar3 protein</td>
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</table>
Chapter 1

Introduction

1.1 Motor Proteins

Movement is a fundamental characteristic of all forms of life. The bulk of controlled biological movement is powered by nano-sized protein machines known as “motor proteins”. The movement-promoting motor proteins in eukaryotes are myosin, dynein, and kinesin. These proteins traverse, or in some cases modulate the structure of, cytoskeletal protein tracks in the cytoplasm as a means to transport a wide variety of cargo and maintain an intracellular environment that is spatially and temporally differentiated [2,3]. Motor proteins accomplish this by converting the chemical energy from ATP hydrolysis into mechanical force using motor-specific mechanochemical cycles that are evolutionarily-tuned to couple ATP binding, hydrolysis and products release at their active site to a conformational change cycle in the rest of the motor (Figure 1) [4]. For this to occur, elements within the motor must ‘sense’ transitions in the active site and communicate information about these transitions to other elements that apply mechanical force and that interact with the track. The molecular details of how this communication occurs in most motor proteins are unknown, and may differ substantially between different classes of motors, as well as between different motor isoforms within a particular motor family. A goal of many motor proteins researchers is to understand the basic principles of motor design that facilitate coordination of track binding and force production in different motor protein classes.

1.2 Kinesin interaction with the microtubule track

The eukaryotic cytoskeleton is an intricate network of dynamic protein polymers that act
Figure 1 – Mechanochemical Cycle of Kinesin-1. (A) Two motor domains are bound to the microtubule, with an ATP-bound motor domain (light blue) closer to the microtubule plus-end, and an ADP-bound motor domain (dark blue) one tubulin heterodimer closer to the minus-end. (B) The dark blue motor domain detaches from the microtubule and swings towards the plus-end. (C) The light blue head turns over ATP to ADP and inorganic phosphate, while the dark blue motor domain exchanges ADP for ATP. (D) Inorganic phosphate is released from the light blue motor domain, which subsequently releases the microtubule and swings toward the plus-end to continue to cycle.
as structural scaffolding, molecular tethers, and intracellular transportation networks. There are three categories of cytoskeletal proteins – microtubules, actin microfilaments, and intermediate filaments – each with different properties suited to specific roles in eukaryotic cells. Intermediate filaments, including keratins, desmin, vimentin, and nuclear lamins, are involved in purely structural roles, including anchoring of organelles, tensile support of cell shape, and intercellular adhesion junctions. Microtubules and microfilaments also play major structural roles, but they are more dynamic and adaptable, due in part to the ability of their monomeric subunits to bind and turnover nucleotide triphosphates, but also due to a supporting cast of proteins that help organize them into more complex three-dimensional networks [5].

Microtubules are made up of polymerizing protein subunits: α/β-tubulin heterodimers (Figure 1). As with actin, tubulin monomers have a nucleotide-binding site, except that tubulin is specific for GTP rather than ATP. GTP-bound heterodimers favour association into linear protofilaments. These protofilaments then assemble into a microtubule: a hollow cylinder with a circumference of typically thirteen tubulin protofilaments. Due to the polar nature of the α/β heterodimers, the microtubule itself has a defined polarity (Figure 1). The end that originates near the nucleus at the microtubule organizing center (referred to as the spindle pole body in some fungi and algae) is known as the minus-end, and is typically capped by γ-tubulin to prevent it from growing or shrinking [6]. The faster growing plus-end extends out toward the cell membrane. These ends of microtubules experience phases of growth, stasis and shrinkage. This ‘dynamic instability’ is controlled by the hydrolysis of GTP by newly associated tubulin heterodimers at a rate slower than their association [7,8]. The discrepancy between these rates leads to the formation of a GTP-tubulin cap. If this cap is lost, due to an unavailability of free GTP-tubulin or through
another process, then the microtubule undergoes ‘catastrophe’, with protofilaments separating, and tubulin heterodimers dissociating from the end [9].

There are a vast assortment of microtubule-associated proteins (MAPs) and actin-binding proteins (ABPs) that allow a fine balance to be struck between the strength and dynamic capabilities of the cytoskeleton. Some MAPs and ABPs proteins mediate microfilament and microtubule length through stabilization, severing, or depolymerization [10]. Others add to the complexity of the three-dimensional matrix through crosslinking and branching. One family of MAPs, the plus-end tracking proteins (+TIPs), dynamically track the growing plus-ends of microtubules, carrying out important functions, including recruitment of other proteins to the cell cortex [11,12]. MAPs and ABPs allow for extrinsic control of cytoskeletal dynamics beyond simple equilibrium-based treadmilling, growth and catastrophe.

Motor proteins form an important and highly specialized subset of MAPs and ABPs given their ability to move along microtubules or actin filaments, pulling cellular cargo behind them. If we scale up to the macroscopic world, these proteins are akin to train engines pulling cargo along a set of rails. Kinesins and dyneins move along microtubules, while myosins move along actin filaments [13]. Some kinesins have even evolved the ability to tear up the track behind them, contributing to the dynamic nature of the cytoskeleton [5]. Much progress has been made in understanding the mechanics of these motors and functions they play in cell division, cell motility and intracellular trafficking, but the ways in which these kinesins are regulated to ensure proper temporal and spatial activity is less well understood. Recent work has revealed a number of intriguing molecular control mechanisms for kinesin inhibition, activation, cargo-binding and cellular localization. This thesis will highlight some of these discoveries in order to help provide
context for the research described in the chapters to follow on the *Ashbya gossypii* kinesin Kar3 and its associated regulatory protein Vik1.

Another major subject of interest in the motor proteins field is in the area of motor protein regulation. Myosin motors can be regulated by light chains, which are calmodulin, or calmodulin-like proteins that bind to the α-helical neck domain (light chain binding region) and enhance its rigidity. This rigidity allows it to act as lever arm that can amplify smaller movements within the head domains into larger movements within the tail of the molecule in order to produce displacement of the motor in relation to its track [14]. The activity of calmodulin light chains is dependent on Ca\(^{2+}\) concentration. Phosphorylation of light chains by myosin light chain kinases and phosphatases plays a role in myosin regulation [15].

Several kinesins are also regulated by light chains, some of which can differentially adapt the function of the tail domain in ways that determine the specificity and timing of its cargo interactions (Figure 1). In order to create a spatially differentiated cytoskeleton to help dictate the placement of cargo and cell polarity, post-translational modifications of the microtubule track can also influence kinesin affinity and motility [16]. Beyond these two main methods of regulation, some kinesins have adaptor proteins that play an active role in changing the activity of the complex. This thesis will focus on an interesting class of kinesin adaptor proteins, whose structural characteristics and functional properties are only beginning to be studied in detail.

### 1.3 Kinesin Structure

Kinesins are a large and diverse family of eukaryotic motor proteins that use energy derived from ATP hydrolysis to move along the surface of microtubule-based cytoskeletal filaments [17], or to regulate microtubule dynamics through controlled depolymerization of the
microtubule ends [18,19]. Motility and depolymerization are regulated by conformational changes in the kinesin motor domain (Figure 2), which are coupled to ATP turnover and product release at the nucleotide-binding site [19,20]. The nucleotide-binding site consists of conserved structural elements, which change conformation depending on whether the site is occupied by ATP, ADP·P_i, ADP or no nucleotide [21-23]. These conformational changes are felt throughout the motor domain, and control the affinity of the microtubule-binding site for the microtubule (Figure 1) [24-26]. Since most kinesin motors contain two subunits with active motor domains, conformational changes must also account for communication between the motor domains. This communication allows the subunits to act cooperatively in a finely tuned molecular walk.

The kinesin stalk region is largely responsible for kinesin dimerization, and acts as a rigid linker between the motor domain and cargo-binding tail domain (Figure 2). Dimerization and rigidity are both achieved through the formation of a coiled-coil motif, whereby alpha-helices from the two subunits wrap around each other into a right-handed helix [27]. Residues facing inward form a hydrophobic core and residues along the edge of this core form ionic interactions with the adjacent helix. Residues align in the correct configuration for coiled-coil formation when the primary sequence follows a conserved ‘heptad repeat’ pattern of seven residues. The residues of the heptad repeat are labeled \(a\)-\(b\)-\(c\)-\(d\)-\(e\)-\(f\)-\(g\). Positions \(a\) and \(d\) are regularly occupied by small to medium sized hydrophobic residues (70-75% of positions are leucine, isoleucine and valine), while positions \(e\) and \(g\) line up along the edge of the hydrophobic core and are usually charged residues [27]. Deviations from the heptad repeat pattern exist, and can result in ‘stutters’ in the coiled-coil motif that influence the stability and flexibility of the structure.

The kinesin tail domain is a globular region found in most kinesins, usually found at the C-terminus of the polypeptide. The tail domain is typically responsible for binding specifically to
Figure 2 – Quaternary Assembly of Kinesin-1 [17]. Kinesin heavy chain (KHC) consists of an N-terminal motor domain, a coiled-coil stalk with hinge regions that allow for flexibility, and a C-terminal tail domain. Kinesin light chain (KLC) associates with the KHC stalk, and contains TPR motifs that bind specifically to cargo. Figure adapted from Verhey et al., 2011.
cargo, which can include organelles, microtubules or protein complexes [28]. Some kinesins undergo autoinhibition in the absence of cargo, whereby the stalk folds in half, allowing the tail domain to bind and inactivate the motor domain [29]. Because tail domains adapt specifically to the functionality of each individual kinesin, it is the most variable region among kinesins. The tail domain is also the most structurally disordered region of kinesins, with the average tail domain predicted to be 71.8% disordered [30]. Intrinsic disorder is common among proteins, estimated to occur in forty or more sequential residues in 35-50% of all eukaryotic proteins [31,32]. Intrinsically disordered proteins are prevalent where structural flexibility, post-translational modifications, or high-specificity ligand binding are important factors [33], all of which are the case for kinesin tail domains.

1.4 Kinesin Motor Proteins are Remarkable Examples of Diversity and Adaptation

Kinesins have evolved into a large superfamily, with high variability across species and isoforms [34,35]. Variations in kinesin functionality are the result of differences in primary sequence, domain arrangement, and quaternary assembly [36]. All kinesins have a highly conserved ~350 residue globular motor domain responsible for microtubule binding, ATP hydrolysis, and force production [13,37]. Subtle structural differences between kinesins in this region can result in variations in the rate and equilibrium constants governing their mechanochemical cycles and in turn elicit unique motile and depolymerase properties and further separate their individual cellular roles [1]. The roots of kinesin functional diversity go beyond subtle differences in the motor domain. The arrangement of the stalk, structure of the tail domain association of light chains, and domain order of the kinesin can have drastic impacts on the activity of each kinesin motor.
1.4.1 Kinesin-1: The Quintessential Kinesin Motor Protein

Kinesin-1, initially recovered from giant squid axon and formerly known as conventional kinesin [38], is found almost ubiquitously in protozoa, fungi and animals [39]. Kinesin-1 moves processively, transiting the microtubule without detaching for several micrometers at a time. Processivity is achieved through a hand-over-hand mechanism of movement, whereby the motor domain of one subunit is always bound to the microtubule when the other motor domain is detached from the microtubule and swinging forward (Figure 1) [24,40,41]. The two motor domains alternate through this process to walk along the microtubule, allowing Kinesin-1 to transport cargo (organelles, nuclei, microtubules, or protein) efficiently between locations in the cell. The hand-over-hand mechanism requires communication between the motor domains to allow them to work together, coordinating their ATP turnover and microtubule binding state. Like most members of the Kinesin superfamily, Kinesin-1 moves towards the microtubule plus-end at the cell periphery.

Kinesin-1 is structured as a heterotetramer, made up of two heavy chains (KHC) and two light chains (KLC) [17]. Two identical KHC subunits form a parallel homodimeric complex, consisting of an N-terminal motor domain, neck, coiled-coil stalk and C-terminal globular tail domain (Figure 2). A KLC homodimer associates with the KHC homodimers along the coiled-coil stalk at the base of the tail domain to form the quaternary assembly. While the heterotetramer is the standard quaternary assembly of the Kinesin-1 motor, there is some evidence that the KHC homodimer can function independently of the KLCs [17], and fungal kinesin-1 has no associated light chains [42]. Hinge regions in the coiled-coil stalk allows multiple motors sufficient flexibility to work together when attached to the same cargo [43-45], and allows the motor to fold in half into
an autoinhibited state [39,46]. Autoinhibition occurs when Kinesin-1 is not bound to cargo, and allows the motor to function only when and where it is required by the cell.

1.4.2 Eg5 is a Homotetrameric Kinesin

Eg5 is a Kinesin-5 family motor involved in mitosis [47]. Eg5 is different from Kinesin-1; its subunits first form a parallel homodimer, and two homodimers subsequently assemble into a heterotetramer via an anti-parallel 4-helix coiled-coil. As such, the Eg5 tetramer has two motor domain subunits at either end of its stalk region, and can crosslink and move processively along adjacent microtubules [48,49], gliding them against each other. In this way, Eg5 is able to pull cellular components tethered to these microtubules towards each other [48]. While Eg5 proceeds through its mechanochemical cycle in much the same way as Kinesin-1, the way it initiates interaction with the microtubule is slightly different. Instead of binding the microtubule when the motor is ATP-bound, Eg5 must bind the microtubule in a nucleotide free state, and then undergo a conformational change to bind ATP [47]. In the world of kinesins, Eg5 represents a mild adaptation of the more orthodox Kinesin-1, but it emphasizes the often subtle relationship between kinesin structure and function.

1.4.3 The Kinesin-13 MCAK and Kinesin-8 Kip3p are Microtubule Depolymerases

MCAK is a Kinesin-13 family motor capable of depolymerizing microtubules (Figure 3) [5]. Microtubules are typically stabilized at the ends by a GTP-cap that counteracts the entropic favourability of microtubule depolymerization [50,51]. MCAK and the Kinesin-8 Kip3p are both able to depolymerize microtubules that are stabilized by GMP-CPP, a slow hydrolysable GTP-analog that simulates a strong GTP-cap [52]. MCAK does not move directionally along the microtubule lattice, but it is capable of rapidly targeting the microtubule ends [53]. Microtubule
Figure 3 – Depolymerization Cycle of Kinesin-13 MCAK [54]. (1) Nucleotide-free MCAK binds the microtubule end. (2) ATP-binding by MCAK causes a conformational change within MCAK, which stabilizes the MCAK-microtubule complex, pulling a protofilament away from the microtubule (Depolymerization transition state). (3) Depolymerization of the microtubule occurs, with an MCAK-tubulin heterodimer complex detaching from the microtubule end. ATP hydrolysis may occur before, during or after this step. (4) Release of ADP and P$_i$ products from MCAK allows release of the free tubulin heterodimer. Figure is adapted from Hertzer, et al., 2006.
end targeting is achieved by a ‘diffusion and capture’ mechanism whereby it associates weakly to the microtubule lattice, and diffuses along the microtubule until it locates the end. Unlike the highly controlled movement of motile kinesins, diffusion is a much faster process, requires no ATP turnover, and it is directionless, allowing MCAK to target both ends [53]. This mechanism is similar to the way restriction enzymes and transcription factors find their binding sites on DNA [5].

Energy from the MCAK ATP hydrolysis cycle is directed toward removal of GTP-tubulin heterodimers from the microtubule plus- and minus-ends [19]. MCAK initiates depolymerization in its ATP or ADP·Pᵢ state, binding to and stabilizing a bent conformation of the end of a microtubule protofilament. Bending of the protofilament away from the microtubule is a structural intermediate for microtubule depolymerization [55]. After the MCAK-tubulin heterodimer complex dissociates from the microtubule ADP and Pᵢ are released [19], followed by dissociation of MCAK from the tubulin. Upon removal of all of the GTP-tubulin from the microtubule end, the microtubule end is destabilized and will undergo catastrophe.

As opposed to the diffusional movement of MCAK, the yeast Kinesin-8 Kip3p moves towards the microtubule plus-end in an ATP-dependent manner, which it depolymerizes upon arrival [56,57]. Kip3p is an incredibly processive motor, moving up to 12 micrometers before falling off [56]. Most yeast microtubules are shorter than 10 micrometers, so any Kip3p that binds to a microtubule invariably ends up at the plus-end. The rate of depolymerization of Kip3p is dependent on the length of the microtubule, whereas microtubule length is of lesser consideration for the non-processive, diffusive movement of MCAK. It has been suggested by Howard and Hyman that evolution has adjusted the processivity of microtubule depolymerases to alter the level of microtubule depolymerization by a motor [5].
1.4.4 The Kinesin-14 Ncd turns the Kinesin Superfamily on its Head

The Kinesin-14 family of motors is defined by placement of the motor domain at the C-terminal end of the kinesin polypeptide, as opposed to the N-terminus. The first discovered and most well studied Kinesin-14 is the homodimeric non-claret disjunction protein (Ncd) from Drosophila melanogaster [58,59]. Ncd is involved in organization of the microtubule cytoskeleton during mitosis and meiosis [60-62]. Ncd has been shown to bind microtubules with both its motor and tail domain, crosslinking them, and sliding anti-parallel microtubules against each other, while locking parallel microtubules in place [63]. The discovery of Ncd as the first C-terminal motor brought along with it another surprising revelation: Ncd moves along the microtubule towards the minus-end [64].

Like Kinesin-1, Ncd has two active motor domains in which ATP hydrolysis is coupled to microtubule-binding state, and while Kinesin-1 uses both motor domains to ‘walk’ processively along the microtubule, Ncd is a non-processive motor [63], requiring multiple motors on a single cargo to achieve motility (Figure 4). Indeed, electron microscopy experiments indicate that only one Ncd motor domain binds to the microtubule [65-67], and a heterodimeric Ncd construct with one subunit intact and one subunit lacking a motor domain, has been shown to retain almost all of the motility of the wild-type [66]. Based on these studies, it is proposed that Ncd moves along the microtubule by incorporating a power stroke mechanism into its mechanochemical cycle rather than alternate motor domain stepping [66]. Interestingly, there is also evidence that the motor domains of Ncd are dependent on each other for motility [68], and that the nucleotide state of the unbound motor domain affects the microtubule binding affinity and nucleotide state of the microtubule bound motor domain [69,70]. A more recent study showed no microtubule-activated ATPase activity or motility when one of the motor domains had an inactivating mutation [61].
These findings show that parts of the kinesin motor assembly need not interact directly with the microtubule to influence the mechanochemical properties of the rest of the complex, and that understanding intersubunit communication is both important and worthwhile in the study of both processive and non-processive kinesin motors.

1.4.1 KCBP is a Plant Kinesin-14 Regulated by Calcium Concentration

Kinesin-like Calmodulin Binding Protein (KCBP) represents an adaptation of Kinesin-14 motors to the regulatory pathways of plants. Discovered originally in *Arabidopsis thaliana*, KCBP is involved in formation of bipolar spindles during nuclear envelope breakdown, the anaphase stage of mitosis, trichome morphogenesis, and bundling of microtubules [71,72]. The activity of KCBP is downregulated during metaphase and telophase to allow for unbundling of the microtubule cytoskeleton [73]. The microtubule dependent ATPase activity and microtubule binding affinity of KCBP is negatively regulated by both calmodulin and the KCBP-interacting Ca$^{2+}$-binding protein (KIC) [74,75]. Both regulators (adaptor proteins) are turned on in the presence of Ca$^{2+}$, but KIC requires 3-fold less concentration of Ca$^{2+}$ than calmodulin to fully inhibit KCBP activity. Recently, the X-ray crystal structure of KCBP bound to Ca$^{2+}$-KIC was solved, allowing the structural mechanism of regulation to be characterized [73]. Ca$^{2+}$-KIC and Ca$^{2+}$-calmodulin both act by binding to an α-helix in the ‘neck mimic’ region near the C-terminal motor domain of KCBP (Figure 5A) [73,76]. This interaction stabilizes the motor domain of KCBP in a conformation that is unable to bind microtubules [74,75], and this is fundamentally different from the Kinesin-1 and Ncd assemblies, and how they are regulated (Figure 5B,C).
Figure 4 – Mechanochemical Cycle of Ncd. (A) The Ncd motor domain binds to the microtubule and binds ATP. (B) The Ncd stalk undergoes a ‘power stroke’ mechanism in relation to the motor domains, moving the microtubule in relation to cargo bound by the tail domain. (C) ATP is turned over, and inorganic phosphate is released. (D) The Ncd motor domain detaches from the microtubule, and the stalk resets to its original position relative to the motor domains.
Figure 5 - Crystal Structures of Kinesin Regulatory Elements. (A) Structure of the Kinesin-like calmodulin binding protein (KCBP; grey) bound to its Ca\(^{2+}\)-activated inhibitor, KIC (magenta), which locks the neck mimic region in place (PDB: 3H4S). (B) Structure of the Kinesin-1 homodimer, with the motor domains and neck regions shown in grey (PDB: 2Y65). The autoinhibitory beta strand from the Kinesin-1 tail domain (blue) folds between the motor domains to crosslink them at a second location besides the neck. (C) Structure of the Ncd homodimer, in which one motor domain binds the microtubule and is motile (grey), and the other is necessary to direct its mechanochemical cycle (red) (PDB: 1N6M). (D) Structures of *Saccharomyces cerevisiae* Kar3 and Vik1 modeled into a hypothetical heterodimeric assembly, via alignment of the motor domains to the heterodimeric structure of Ncd (PDB: 3KAR, 2O0A). Only *Sc*Kar3 (grey) is enzymatically active, yet *Sc*Vik1 (green) is responsible for initial binding of the microtubule.
1.5 Kar3 Motors are Unusual Members of the Kinesin-14 Family

Kar3 kinesins possess a C-terminally positioned motor domain and exhibit microtubule minus-end directed motility, placing them within the Kinesin-14 family of motors [35]. In budding and fission yeasts, Kar3 kinesins play numerous roles in chromosome movement and shaping of the spindle pole bodies during mitosis and meiosis [77-79]. In the budding yeast *Saccharomyces cerevisiae*, the multifunctionality of Kar3 is mediated with the help of two non-catalytic accessory proteins, Vik1 and Cik1, which appear to form yet another type of motor assembly (Figure 5D) [80-82].

*Sc*Vik1 is expressed during periods of vegetative growth, and is involved in targeting Kar3 to the spindle pole bodies during mitosis (Figure 6) [82]. The crystal structure and accompanying functional studies on *Sc*Vik1 have given strong clues towards the mechanism with which it contributes to *Sc*Kar3 motility [83]. Briefly, *Sc*Vik1 lacks a nucleotide-binding site, but is actually able to bind microtubules tighter than *Sc*Kar3. Also, the *Sc*Kar3/Vik1 heterodimer binds microtubules in the ADP-state, meaning that *Sc*Vik1 makes first contact with the microtubule and can tether Kar3 to the microtubule lattice [83]. Based on these findings, and the fact the Kar3 and Vik1 are physically connected by a coiled-coil, it has been proposed that the *Sc*Kar3/Vik1 heterodimer is able to move non-processively along microtubules using a mechanism in which *Sc*Vik1 releases its grasp on the microtubule as a result of conformational strain within the coiled-coil that develops after ATP- and microtubule-binding in *Sc*Kar3 occurs (Figure 7). The specific molecular details of how strain development between Kar3 and Vik1 triggers release of Vik1 from the microtubule remains poorly understood [84].

*Sc*Cik1 is expressed during mating, and is involved in localization of *Sc*Kar3 to the spindle pole bodies and cytoplasmic microtubules of cells treated with mating pheromone (Figure 6) [79,81]. *Sc*Cik1 is also involved in some of the vegetative functions of *Sc*Kar3. Knockout
Figure 6 – *Saccharomyces cerevisiae* Kar3, Vik1 and Cik1 Knockout Studies [87]. Localization of Kar3 is shown in red, nuclei are shown in blue, and cytoplasm is shown yellow. Spindle pole bodies are represented as filled in ovals at the poles of the nuclei, and microtubules are shown as lines protruding from the spindle pole bodies. Cik1 knockouts indicate that it localizes Kar3 to the cytoplasmic microtubules during formation of the mating projection. Without Cik1, Kar3 is diffuse throughout the nucleus during mating. Vik1 knockouts indicate that it localizes Kar3 to the spindle pole bodies during mitosis. Without Vik1, Kar3 is localized to nuclear microtubules during mitosis. Figure adapted from Manning *et al.*, 2000.
Figure 7 – Mechanochemical Cycle of *Saccharomyces cerevisiae* Kar3/Vik1. (A) When the complex is detached from the microtubule, ScKar3 (orange) is ADP-bound. (B) ScVik1 (purple) binds the microtubule first. (C) ScKar3 binds the microtubule, and exchanges ADP for ATP. Both motors are bound to the microtubule simultaneously. (D) ScKar3 communicates to ScVik1 that it is bound to the microtubule, and ScVik1 releases the microtubule, swinging the stalk and cargo forward. (E) ScKar3 hydrolyzes ATP, and releases the microtubule and inorganic phosphate.
studies have shown that Cik1 is an effector of cytoplasmic microtubule and mitotic spindle length, and in mitotic checkpoint control [79,85,86]. In the absence of either of the accessory proteins, expressed ScKar3 is found diffuse throughout the nucleus, and mitotic defects are evident.

The ScKar3/Cik1 heterodimer acts as a depolymerase on microtubules stabilized by low molarity Taxol, but unlike MCAK and Kip3p, it is not strong enough to depolymerize GMP-CPP-stabilized microtubules. While ScKar3/Cik1 is a minus-end directed motor, it depolymerizes microtubules specifically at the plus-end of the microtubule [88], and not at the minus-end [89]. It is not clear if the motile and depolymerase activities of ScKar3/Cik1 are linked, or involve separate mechanisms within the motor. Recent studies have suggested that Vik1 can tether Kar3 to the microtubule, and that this complex abrogates binding of Kar3/Cik1 complexes to adjacent binding sites [90]. How this relates to the depolymerase activities of the motor remains unknown, and as of yet, there are no structures solved for Cik1, nor the Kar3/Cik1 complex.

In Schizosaccharomyces pombe, two Kar3-like proteins named Pkl1 and Klp2 are expressed, which localize to different cellular regions and have distinct functions during mitotic division [78-82,91,92]. ScVik1 and Cik1, and SpPkl1 and Klp2 represent two different ways that organisms can adjust a motor to different phases of the cell cycle and different locations within the cell. This raises the possibility that more unusual forms of the Kar3 motor and its accessory proteins exist in species related to Saccharomyces cerevisiae.

1.6 Orthologs of ScKar3 and ScVik1 are found in Ashbya gossypii

Beyond the budding yeast Saccharomyces cerevisiae (also known as baker’s or brewer’s yeast), orthologs of Kar3, Vik1, and Cik1 kinesins are found in several other members of the Ascomycota phylum of Fungi. While closely related genetically, there is a large amount of
biological diversity among Ascomycete species. Members of Ascomycota include the fission yeast *Schizosaccharomyces pombe*, the red bread mould *Neurospora crassa*, the *Aspergillus* genus comprising many common food and mildew moulds, the opportunistic human pathogens *Candida albicans* and *Candida glabrata*, and the cotton pathogen *Ashbya gossypii* which takes center stage in this story. Some of these ascomycete organisms have cytoskeletal structures and requirements for nuclear movement that are different from those of budding and fission yeasts [93,94]. This opens the door to the possibility that divergences in structure, motile properties, and mode of multifunctionalization may exist among Kar3 orthologs. The Kar3-like kinesin in the filamentous fungus *Ashbya gossypii* is particularly interesting in this regard. Although its genome is closely related to budding yeast [95], *A. gossypii* grows as very long multinucleated hyphae in which nuclei exhibit long-range migration toward the hyphal tip during hyphal extension, as well as extensive oscillations and intermittent bypassing of one another [96,97] (Figure 8). Accordingly, the microtubule cytoskeleton and nuclear movement activities of *A. gossypii* are strikingly different from budding and fission yeasts [94]. Although the specific roles played by the *A. gossypii* Kar3 in these events have not yet been determined, expression profiling using *A. gossypii* Affymetrix oligonucleotide chips showed that in slow and fast growing hyphae, which experience ongoing mitotic divisions, a single Kar3-like kinesin is expressed (R. Rischastch and P. Philippsen, Personal Communication) [95-98]. Alternatively, in spores and sporulating hyphae, which do not experience mitotic divisions, *AgKAR3* mRNA is virtually absent, and mitotic division defects are observed in *AgKAR3* deletion strains [99]. Based on these observations, this kinesin must play a role in nuclear movement and mitosis in *A. gossypii*. Another point of interest is that while *S. cerevisiae* includes both Vik1 and Cik1 accessory proteins, *A. gossypii* has only one ortholog of
Figure 8 - Morphology of *Ashbya gossypii* hyphae[101]. Dozens of nuclei (blue) are contained within a single cell of *A. gossypii*. Nuclear movement is restricted by microtubule tethers that are controlled by kinesin and dynein motors. As hyphae extend, septin rings (green) form at locations dependent on a cascade of spatial checkpoint regulators. F-actin (red/yellow) is polarized to growing hyphal tips and into concentrated rings at sites of septation. Septin and actin rings separate mycelia into compartments, and isolate senescent regions from new growth. Figure taken from DeMay, *et al.*, 2009.
these accessory proteins, which shares a slightly higher level of sequence identity with \textit{ScVik1} than \textit{ScCik1}. Henceforth, this protein is thus referred to as \textit{AgVik1}.

### 1.7 The Cell Cycle of \textit{Ashbya gossypii}

\textit{Ashbya gossypii} was initially described in 1926 because of its role as a pathogen, causing stigmatomycosis in cotton and citrus fruits [100]. It was originally designated as a yeast because the mycelia (a mass of threadlike branching hyphae) it forms on the skin of the fruits are quite similar to the pseudomycelia formed by \textit{Saccharomyces cerevisiae} on the surface of grapes. \textit{A. gossypii} requires insects to spread its spores or mycelial fragments, so the use of insecticides has largely eradicated its role in this disease. \textit{A. gossypii} is commonly used in the vitamin and food additive industry for its ability to overproduce riboflavin (Vitamin B$_2$) [102-104], a natural defense for the organism against ultraviolet light [105]. The \textit{A. gossypii} genome is relatively simple and efficient, and shares a high degree of synteny (conservation of gene order between two loci) with the \textit{S. cerevisiae} genome [95]. Together, these factors have led to the \textit{A. gossypii} genome becoming one of the most complete among eukaryotes.

The synteny of the \textit{S. cerevisiae} and \textit{A. gossypii} genomes aided in the discovery of the Kar3, and particularly Vik1 homologs. Kar3 is one of 6 kinesins found in \textit{S. cerevisiae} [106]; all 6 have \textit{A. gossypii} orthologs [98]. Approximately 95\% of all \textit{A. gossypii} genes have a homolog in \textit{S. cerevisiae}, indicating a high level of conservation of cell functions and molecular machinery [107]. At some point during its ancient evolution, an ancestral species of \textit{S. cerevisiae} underwent a genome duplication, leading to duplicate copies of genes that evolved into similar, but distinct proteins, including Vik1 and Cik1 [95,107,108]. This very same lack of genome duplication in the ancestors of \textit{A. gossypii} explains why we have only found a single Vik1/Cik1 homolog.
Figure 9 – Cell Cycle of *Saccharomyces cerevisiae* (Public Domain Image). Budding (1) events occur from both haploid and diploid cells, while $\alpha$ and $a$ haploid cells may mate (2) through conjugation and karyogamy (nuclear fusion) to form diploid cells. Diploid cells may sporulate (3) into 2 $\alpha$ and 2 $a$ spores, which mature into haploid cells in favourable environmental conditions.
Considering the high level of gene synteny and similarly, the life cycle of A. gossypii is strikingly different from that of S. cerevisiae. S. cerevisiae is the archetypal budding yeast, spending its time in both haploid and diploid forms (Figure 9). Haploid cells undergo vegetative growth and mitosis, and are averse to stressful environmental conditions. They are, however, capable of mating between cell types α and α to form a diploid cell. Mating occurs via the fusion of mating projections, induced by α and α pheromones. Diploid cells are generally heartier than haploid cells, and under stressful conditions, they will enter into meiosis and sporulate. Spores are haploid, and develop into mature haploid cells under favourable conditions.

A. gossypii, on the other hand, starts off life as needle shaped spores, known as ‘spikes’ that are conjoined at one end by narrow filaments (Figure 10). The spores germinate by growing outward isotropically with no defined polarity, resulting in a spherical germ cell. Eventually, the cell will switch to polar growth, forming a hyphal tip which septates from the germ cell, and will grow indefinitely in the presence of satisfactory conditions. A second hyphal tip will also form on the opposite side of the germ cell, and grow in the same manner as the first hypha. Once hyphae have partially matured, dichotomous Y-shaped branches form preferentially at the hyphal tip [109,110], and lateral (t-shaped) branches form at other points along the hyphae. After some time, mature hyphae will only branch in the Y-shaped manner to form a mature mycelium. A. gossypii spends most of its life cycle continuing to grow as a mature mycelium. Hyphal tubes fragment into separate, but adjoined, cells at septal sites, with up to dozens of nuclei encompassed in a single continuous cytoplasm. Septation in A. gossypii is homologous to cytokinesis in yeasts [111]. There are, however, significant differences in the positioning and construction of septa. In particular, the hyphae remain attached after sepatation, and the septa contain pores that allow controlled transport between cell compartments. In fact, even nuclei are able to cross the septal border in A. gossypii [97].
Figure 10 – Cell Cycle of *Ashbya gossypii* [115]. At the center of the figure, an *A. gossypii* colony is shown, with spores forming the ring of brighter colour at the center, and non-sporulating mature mycelia surrounding it. The yellow colour is the result of riboflavin production. *A. gossypii* progresses through growth stages of (a) an isotropic growth phase during germination, (b) germ tube formation, (c) bipolar branching pattern, (d) juvenile mycelium formation including Y-shaped and lateral branching, (e) exclusively Y-shaped tip branching (mature mycelia), and (f) sporulation (formation of ‘spikes’ conjoined by a narrow filament). Figure taken from Wendland *et al.*, 2005.
In *S. cerevisiae*, septation is initiated by Cdc42, which triggers the effector proteins Cla4 and Bni1 to assemble a ring of septin proteins at the bud site [112,113]. An *A. gossypii Δcla4* deletion mutant shows defects in hyphal growth and septation [110]. Timelapse recordings of immature *A. gossypii* mycelia indicate that Cla4 is involved in a tip-based process of positioning septa at regular intervals along the hyphal tube [114]. Septation is one example of an *A. gossypii* homolog of an *S. cerevisiae* protein carrying out a specialized role in the context of very different cell morphology. Ultimately, after formation of the mature and septated mycelium, one turn of the *A. gossypii* life cycle is completed when controlled sections of hyphae undergo sporulation to form new ‘spikes’.

The multinucleate nature of the *A. gossypii* hyphae brings with it the necessity to control and coordinate the timing of mitosis and precise location of nuclei within the cytoplasm. In *Aspergillus nidulans*, apical growth of the hyphae and mitosis have been shown to be independent of each other, which implies that there are different regulatory pathways controlling nuclear movement and mitosis in yeast-like and filamentous fungi [116]. It is known from knockout studies that *AgKar3* plays a role in mitosis, but it may also have a specific role in mediating nuclear movement through the cytoplasm, particularly because it is known that this is a microtubule-dependent process. In particular, *A. gossypii* kinesins Kip2 and Kip3, and the microtubule plus-end attachment protein Bik1 are involved in regulating nuclear oscillation and positioning within hyphae (Figure 11). Kip3 is a Kinesin-8 microtubule depolymerase that is required for microtubule-cortical interactions, normal spindle assembly and kinetochore interactions. Kip3 depolymerizes the microtubule plus-end, and *kip3Δ* knockouts cause microtubules that are too long, giving nuclei tethered to the cell cortex a longer leash and more flexibility of movement [96]. Kip2 moves toward the microtubule plus-end, delivering Bik1, which coordinates attachment of
The amplitude of nuclear oscillation and bypassing are dependent on microtubule length and the extent of attachment of microtubules to cell cortex associated proteins, mediated in part by the kinesins Kip2 and Kip3 and the microtubule plus-end marker Bik. (A) Visualization of microtubules within *Ashbya gossypii* hyphae, using anti-α-tubulin immunostaining microscopy (Bar = 5 μm). (B) Schematic representation of nuclei (circles) and microtubules (lines), and Bik1 (red dots) within an *A. gossypii* hyphae, with the extent of oscillations indicated by blue arrows. Figure adapted from Grava et al., 2010.
the microtubules at the cell cortex, and recruits dynein motors to pull the microtubules as they are depolymerized by Kip3. Both \textit{bik1Δ} and \textit{kip2Δ} knockouts cause microtubules to detach from the cell membrane, allowing nuclei to move freely by diffusion [96].

### 1.8 Structural and Functional Characterization of AgKar3 and AgVik1

The focus of this thesis is to further our understanding of kinesin assemblies like \textit{ScKar3/Vik1} by studying its orthologs in the filamentous fungus \textit{Ashbya gossypii}. In parallel with the studies described in Chapter 3, the X-ray structure of the motor domain region of \textit{AgKar3} was determined, which showed several novel characteristics in its nucleotide-binding pocket. Prior to the work of Duan \textit{et al.}[1]*, structural and functional information of purified forms of Kar3 kinesins had only been obtained for \textit{S. cerevisiae} Kar3 [23,83,117-119]. To gain a molecular and mechanistic description of the \textit{Ashbya gossypii} Kar3, its motor domain region (AgKar3MD) was cloned, expressed, and purified and its X-ray crystal structure was determined in the presence of MgADP [1]*. The structure showed a single molecule in the crystallographic asymmetric unit whose structure adopts a typical $\alpha/\beta$ kinesin motor domain fold (Figure 12A). Relative to the crystal structure of the \textit{S. cerevisiae} Kar3 motor domain (ScKar3MD), the most prominent differences in the structure of \textit{AgKar3MD} are observed in the small three-stranded $\beta$-sheet at the $N$-terminus of the motor domain ($\beta1a$, $\beta1b$, and $\beta1c$), within part of the microtubule-binding cluster (L8a, $\beta5a$, L8b, and $\beta5b$), and along nearly the entire length of helix $\alpha3$ upstream of the Switch I motif that composes the nucleotide-binding pocket.

Of these regions, the Switch I element of AgKar3MD shows the most disparity from ScKar3MD (Figure 12B,C). Together with Switch II, this region helps to organize catalytic water molecules and form a nucleotide $\gamma$-phosphate sensing mechanism in the nucleotide-binding pocket.
Figure 12 – Crystal structure and nucleotide binding pocket of AgKar3MD [1]. (A) The crystal structure of AgKar3MD is shown in ribbon representation. The secondary structure elements have been numbered consecutively from the N-terminus of the construct. Poor electron density existed for several loops (dashed lines), which were not built into the final model. Bound MgADP in the nucleotide-binding pocket is displayed in ball-and-stick form. The configuration of the ATP binding pockets of (B) ScKar3MD and (C) AgKar3MD. The interactions of Switch I and Switch II motif residues with the catalytic water molecules, Mg$^{2+}$, and the nucleotide are shown to illustrate the differences in the active site conformations of each structure. In AgKar3MD, the Switch I-Switch II salt bridge is broken and L9 and part of $\alpha$3 of Switch I are disordered (dashed lines). Figure is adapted from Duan et al., 2012.
of kinesins, myosins, and G-proteins[120-122]. Switch I includes helix α3, loop L9, and helix α3a. The Switch II “cluster” includes loop L11, helix α4, loop L12, helix α5, and loop L13. Previous studies have indicated that with ADP in the nucleotide-binding pocket, or when the initial ADP-kinesin collision complex forms, the Switch elements exhibit an “open” conformation. When kinesin binds microtubules, a “closing” of Switch I and Switch II occurs that promotes the hydrolysis of ATP [122,123]. In this process, a salt bridge forms between Switch I and Switch II to stabilize this “closed” state and provide an environment for an ordered network of waters between the salt bridge and the γ-phosphate of ATP. This is evident in the recent structure of Eg5 kinesin bound to MgAMPPNP (PDB: 3HQD) [122]. Following ATP hydrolysis, there is a structural rearrangement, particularly within Switch I (α3, L9, and α3a) that is associated with the loss of interactions between residues in these elements and the nucleotide. These rearrangements are in turn amplified in other elements of the protein involved in microtubule binding and motility [121].

The structural features that distinguish the small three-stranded β-sheet (β1a, β1b, and β1c) at the edge of the motor domain of AgKar3 from ScKar3 are also of interest (Figure 13). The length of strand β1a is shorter for AgKar3, contributing to the closer approach of loop L1 to the nucleotide, whereas strand β1c is longer. The latter of these differences may translate into loop L2 being more protrusive from the small lobe than that of ScKar3, however, we can only speculate on its configuration, because there was not sufficient electron density to model this structure fully. Given that this region, specifically loop L2, has been shown to be a defining structural element of the microtubule-depolymerizing Kinesin-13s [124,125], this difference may represent yet another site engineered by evolution for functional divergence between ScKar3 and AgKar3.
Figure 13 – Comparison of loop L1 and the small β-lobe in AgKar3MD and ScKar3MD [1]*.
The molecule shown in gray ribbon representation is ScKar3MD. Note that the length of strand β1a is shorter for AgKar3MD, whereas strand β1c is longer. Although loop L2 is disordered, we speculate that it may protrude from the small lobe to a greater degree than that of ScKar3 because of the length and orientation of β1c. Also shown is the alanine (A591) in the bulge of loop L9 of ScKar3MD Switch I, which is replaced by an arginine in AgKar3.
1.9 Specific Question Addressed in this Thesis

Given the unusual morphological characteristics of \textit{A. gossypii}, the apparent existence of only one putative Kar3-associated protein (AgVik1), and the structural divergence of AgKar3 and ScKar3, a number of important questions have arisen about the molecular interactions and kinetic properties of these kinesin proteins: (1) Do AgKar3 and AgVik1 form a stable motor assembly? (2) Are their microtubule-binding and ATPase properties related to those of orthologous Kinesin-14 motors? (3) Do they possess novel activities that are unique or accessory to those of their budding yeast relatives?

To begin to address these questions, I have characterized the biochemical properties and microtubule interactions of these proteins. I also showed that AgKar3 forms a stable complex in solution with AgVik1. Although AgVik1 did not appear to bind directly to microtubules in my analyses, it did influence the microtubule-binding and ATPase activity of AgKar3. Moreover, I show AgKar3/Vik1 is a microtubule depolymerase, and have used molecular modeling approaches to attempt to explain the structural basis for these unique functional properties.
Chapter 2

Materials and Methods

2.1 Cloning and Purification of Protein Constructs

2.1.1 Design of AgKar3 and AgVik1 Monomeric and Dimeric Constructs

Full length sequences of Ashbya gossypii genomic DNA for Kar3 and Vik1 were found through homology searches using the protein basic local alignment search tool (BLAST) algorithm (NCBI, Bethesda, MD, USA), and through comparative searches for syntenic homologs between the Saccharomyces cerevisiae genome and the A. gossypii genome (Ashbya Genome Database [95,98]). Together, these searches uncovered hypothetical proteins AGR253Wp (AgKar3) and AGOS_AFL170C (AgVik1). Based on previous studies of ScKar3 and ScVik1 [83], and predictions from the COILS prediction server [126], the boundaries of the Motor (Homology) Domain (MD/MHD) and coiled-coil stalk region of each protein were determined. A construct of the AgKar3 motor domain region of the AgKar3 polypeptide, ‘AgKar3MD’ was created to study its activity and structure independently. Likewise, a construct of the AgVik1 motor homology domain and neck, ‘AgVik1 K^{356}-A^{633}’, was created for study of its activity, independent of AgKar3. Constructs of AgKar3 and AgVik1 containing both their motor (homology) domains, their neck, and a long section of the coiled-coil forming region of the stalk, named ‘AgKar3 Q^{244}-R^{709}’ and ‘AgVik1 Y^{260}-A^{633}’, were created such that they could form a coiled-coil-based complex, and the effect of intersubunit interaction on structure and function could be studied.
2.1.2 Cloning and Purification of the Monomeric AgKar3 Construct

‘AgKar3MD’ (residues L^{363}-R^{709}) was amplified from A. gossypii genomic DNA (ATCC No:10895) by polymerase chain reaction and ligated into the pET24d vector (Novagen®, Madison, WI, USA) using NcoI and NotI restriction sites and the primers shown in Table 1. This plasmid when expressed yields amino acid residues Met-Ala-Leu^{363}-Arg^{709} with a predicted molecular mass ($M_r$) of 39,859. The AgKar3MD containing vector was transformed into BL21 (DE3) Arginine Isoleucine Leucine (RIL) CodonPlus E. coli cells (Stratagene, La Jolla, CA, USA) and cells were grown in Luria Broth (LB) media supplemented with 50 μg/mL Kanamycin and 50 μg/mL Chloramphenicol to OD$_{600}$ ≈0.8 and induced with 1.0 mM isopropyl (beta)-D-1-thiogalactopyranoside (IPTG). After overnight induction at 20° C, cells were harvested by centrifugation and flash frozen in liquid nitrogen for storage.

Purification of AgKar3MD was a multi-step process, involving cell lysis, cation and anion exchange chromatography, and size exclusion chromatography. All purification was carried out at 4° C. Frozen cells were resuspended in Ion Lysis Buffer (10 mM HEPES pH 7.2 (NaOH), 20 mM NaCl, 2 mM MgCl$_2$, 1 mM EGTA, 1 mM DTT, 20 μM ATP, and 1 EDTA-free Complete® Protease Inhibitor tablet (Roche®, Basel, Switzerland)), and stirred at 4° C until thawed and homogenous. Cells were lysed with a macrotip-outfitted Misonix® (Farmingdale, NY, USA) sonicator at four 50 s pulses at power level 7, interspersed by 120 s rest periods. DNaseI restriction enzyme was added to the sample, to a final concentration of 50 μg/mL, and the cells were incubated on ice for 15 minutes with occasional mixing by inversion. Cell lysate was centrifuged in a JA-25.50 rotor (Beckmann-Coulter®, Brea, CA, USA), at 21,000 rpm for 30 minutes at 4° C. The supernatant fraction was pooled, and loaded onto an SP-Sephadex cation exchange column (GE Healthcare®, Uppsala, Sweden) pre-equilibrated with 10 column volumes (CV) of Ion Wash
Buffer (10 mM HEPES pH 7.2 (NaOH), 50 mM NaCl, 2 mM MgCl₂, 1 mM DTT, 20 μM ATP, and 1 EDTA-free Complete® Protease Inhibitor tablet (Roche®)). The column was washed with Wash buffer until the level of protein elution decreased to a baseline absorbance at 280 nm. A linear gradient of 20 CV Ion Wash buffer and Ion Elution buffer (10 mM HEPES pH 7.2 (NaOH), 600 mM NaCl, 2 mM MgCl₂, 1 mM DTT, 20 μM ATP, and 1 EDTA-free Complete® Protease Inhibitor tablet (Roche®)). Eluted protein, indicated by absorbance at 280 nm, was analyzed by SDS-PAGE, and fractions with bands at the molecular weight of AgKar3MD were pooled and dialyzed overnight at 4°C in 2 L Dialysis Buffer (10 mM HEPES pH 7.2 (NaOH), 50 mM NaCl, 2 mM MgCl₂, 1 mM EGTA, 1 mM DTT, 20 μM ATP, and 1 EDTA-free Complete® Protease Inhibitor tablet (Roche®)). The dialyzed protein was loaded onto an QAE-Sephadex anion exchange column (GE Healthcare®, Uppsala, Sweden) pre-equilibrated with 10 column volumes (CV) of Wash buffer, and washed and eluted in the same manner as with the cation exchange column. Eluted protein was again analyzed by SDS-PAGE, and AgKar3MD containing fractions of high purity were pooled and dialyzed in Modified Dialysis buffer (with 150 mM NaCl). Ion exchange purified protein was further purified by size exclusion chromatography. Protein was loaded onto a Hi-Load 26/60 Superdex 200 (S200) preparation-grade column (GE Healthcare®) pre-equilibrated and eluted with Modified Dialysis buffer. Protein containing fractions were analyzed by SDS-PAGE, and pure fractions were pooled. Pooled protein was concentrated with an Amicon Ultra 30 kDa nominal MWCO (molecular weight cut-off) Concentrator (Millipore®, Billerica, MA, USA), flash frozen in liquid nitrogen and stored at -80°C.

2.1.3 Cloning and Purification of the Monomeric AgVik1 Construct

‘AgVik1 K³⁵⁶-A⁶³³’ was amplified from A. gossypii genomic DNA (ATCC No: 10895) by polymerase chain reaction and ligated into the pET16b vector (Novagen®), using NdeI and BamHI
restriction sites and the primers shown in Table 1. A 10xHis-tag and a TEV protease digestion site (ENLYFQG), has been engineered into the vector, where the TEV protease cuts between the Gln and Gly residues. The plasmid when expressed yields amino acid residues Met-Gly-His$_{10}$-Ser-Ser-Gly-Arg-Glu-Asn-Leu-Tyr-Phe-Gln-Glu-His-Met-Lys$^{356}$-Ala$^{633}$ with a predicted molecular mass ($M_r$) of 34,346. The AgVik1 K$^{356}$-A$^{633}$ containing vector was transformed into BL21 (DE3) RIL CodonPlus E. coli cells (Stratagene®) and cells were grown in LB media supplemented with 50 μg/mL Ampicillin and 50 μg/mL Chloramphenicol to OD ~0.8 and induced with 1.0 mM IPTG. After overnight induction at 20° C, cells were harvested by centrifugation and flash frozen over liquid nitrogen.

Purification of AgVik1 K$^{356}$-A$^{633}$ was a multi-step process, involving cell lysis, nickel-affinity chromatography, optional cleavage of the His-tag, and a second run through a nickel-affinity column to remove the His-tagged TEV protease. Cell lysis by sonication and protein harvesting by centrifugation was conducted as described in section 2.1.2, but cells were resuspended in Ni$^{2+}$-Lysis buffer (10 mM NaPO$_4$ pH 7.2 (NaOH), 300 mM NaCl, 2 mM MgCl$_2$, 1 mM EGTA, 5 mM β-mercaptoethanol, and 1 EDTA-free Complete® Protease Inhibitor tablet (Roche®)). The supernatant was loaded onto a His-Pur® Ni-NTA® (nickel-nitroloacetic acid) resin column (Thermo Fisher Scientific®, Rockford, IL, USA), pre-equilibrated with 10 CV of Ni$^{2+}$-Lysis buffer. The column was washed with Wash buffer (10 mM NaPO$_4$ pH 7.2 (NaOH), 300 mM NaCl, 2 mM MgCl$_2$, 1 mM EGTA, 5 mM β-mercaptoethanol, 20 mM Imidazole, and 1 EDTA-free Complete® Protease Inhibitor tablet (Roche®)). The column was washed until the level of protein elution decreased to a baseline absorbance at 280 nm. A linear gradient of 20 CV Ni$^{2+}$-Wash buffer and Ni$^{2+}$-Elution buffer (10 mM NaPO$_4$ pH 7.2 (NaOH), 300 mM NaCl, 2 mM MgCl$_2$, 1 mM EGTA, 5 mM β-mercaptoethanol, 600 mM Imidazole, and 1 EDTA-free Complete®
Protease Inhibitor tablet (Roche®)). Eluted protein, indicated by absorbance at 280 nm, was analyzed by SDS-PAGE, and fractions with bands at the molecular weight of AgVik1 K365-A633 were pooled and dialyzed overnight at 4°C in 2 L Dialysis Buffer (10 mM HEPES pH 7.2 (NaOH), 50 mM NaCl, 2 mM MgCl₂, 1 mM EGTA, 1 mM DTT, and 1 EDTA-free Complete® Protease Inhibitor tablet (Roche®)). Optionally, the His-tag can be cleaved off the protein by incubating the protein with 50 μg/mL TEV protease at room temperature for 2 hours. The TEV protease is His-tagged, so a second run through the Ni-NTA column allows the cleaved AgVik1 K₃₅₆₋₆₃₃ to flow directly through the column, while TEV is retained. Concentration was conducted as described in section 2.1.2.

2.1.4 Cloning of the AgKar3/Vik1 Dimeric Complex

‘AgKar3 Q²⁴⁴-R⁷⁰⁹’, was amplified from A. gossypii genomic DNA (ATCC No:10895) by polymerase chain reaction and ligated into the pET24d vector (Novagen®) using NcoI and NotI restriction sites and the primers shown in Table 1. This plasmid when expressed yields amino acid residues Met-Ala-Gln²⁴⁴-Arg⁷⁰⁹ with a predicted molecular mass (Mᵣ) of 53,470. ‘AgVik1 Y²₆⁰-A⁶₃₃’ was amplified from A. gossypii genomic DNA (ATCC No: 10895) by polymerase chain reaction and ligated into the pET16b vector (Novagen®), using NdeI and BamHI restriction sites. A 10xHis-tag and a TEV protease digestion site (ENLYFQG), has been engineered into the vector, where the TEV protease cuts between the Gln and Gly residues. The plasmid when expressed yields amino acid residues Met-Gly-His₁₀-Ser-Ser-Gly-Arg-Glu-Asn-Leu-Tyr-Phe-Gln-Glu-His-Met-Tyr²₆⁰-Ala⁶₃₃ with a predicted molecular mass (Mᵣ) of 46,113. The AgKar3 Q²⁴⁴-R⁷⁰⁹ and AgVik1 Y²₆⁰-A⁶₃₃ containing vectors were cotransformed into BL21 (DE3) RIL CodonPlus E. coli cells (Stratagene®) and cells were grown in LB media supplemented with 50 μg/mL Ampicillin, 50 μg/mL Kanamycin, and 50 μg/mL Chloramphenicol to OD ~0.8 and induced with 0.5 mM
IPTG. After overnight induction at 16° C, cells were harvested by centrifugation and flash frozen over liquid nitrogen. Purification by nickel-affinity chromatography, and optional cleavage of the TEV site to remove the His-tag was conducted as described in section 2.1.3, except that all buffers included 20 μM ATP. Purification by size exclusion chromatography, concentration, and flash freezing were conducted as described in section 2.1.2, except that all buffers included 20 μM ATP.

2.1.5 Cloning and Purification of AgVik1-Maltose Binding Protein Fusion Constructs

Three MBP fusion constructs were created by ligating A. gossypii genomic DNA (ATCC No: 10895) by polymerase chain reaction into a pMal-MATa1 vector (Addgene, Cambridge, MA, USA) using BamHI and NdeI restriction sites and the primers shown in Table 1. The constructs yield amino acid residues [MBP(Met1-Lys362)-Ala-Ala-Gln-Thr-Asn-Ala-Ala-[‘AgVik1 L375-A633’ (Leu375-Ala633)] or ‘AgVik1 I368-A633’ (Ile368-Ala633) or ‘AgVik1 T353-A633’ (Thr353-Ala633)]-Lys-Leu, with predicted molecular masses (Mr) of 69,751, 70,580, and 72,502 respectively. Each of the fusion constructs was transformed separately into BL21 (DE3) RIL CodonPlus E. coli cells (Stratagene®) and cells were grown in LB media supplemented with 50 μg/mL Ampicillin, and 50 μg/mL Chloramphenicol to OD ~0.8 and induced with 1.0 mM IPTG. After overnight induction at 20° C, cells were harvested by centrifugation and flash frozen over liquid nitrogen.

Purification of each of the AgVik1-MBP fusion proteins was a multi-step process, involving cell lysis, amylose affinity chromatography, and size exclusion chromatography. Purification was conducted at 4° C. Cell lysis by sonication and protein harvesting by centrifugation was conducted as described in section 2.1.2, but cells were resuspended in Amylose Column buffer (10 mM NaPO₄ pH 7.2 (NaOH), 200 mM NaCl, 2 mM MgCl₂, 1 mM EGTA, 1 mM DTT, 5 mM β-mercaptoethanol, and 1 EDTA-free Complete® Protease Inhibitor tablet (Roche®)).
Table 1 - Primers used to generate AgKar3 and AgVik1 constructs.

<table>
<thead>
<tr>
<th>Primer sequence</th>
<th>Primer name</th>
<th>Restriction site (underlined in sequence)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCCATGGCTTCACCACCCACCGACGGAGAAGCAACGG</td>
<td>AgKar3 Full Length Forward</td>
<td>NcoI</td>
</tr>
<tr>
<td>GTGCAGGCCGTCACCGCTTTGCTATTTGAGGAGTACAC</td>
<td>AgKar3 Full Length Reverse</td>
<td>EagI/NorI</td>
</tr>
<tr>
<td>ATCCATGGCTGGGAGTATATTTCTAAGTTCA</td>
<td>AgKar3MD Forward</td>
<td>NcoI</td>
</tr>
<tr>
<td>CTATCATATAGGAACAGGACTTTTTTGCGGTTAAAGAGG</td>
<td>AgKar3 Q244-R709</td>
<td>NdeI</td>
</tr>
<tr>
<td>ATATACCGTCTGAAGGCTTTGCTGTGGTTTTCCGTTGACC</td>
<td>AgVik1 Full Length Reverse</td>
<td>HindIII</td>
</tr>
<tr>
<td>CTATCGTTGAAAATGAGGAGTACAGCTTTAGCGG</td>
<td>AgVik1 K356-A633 Forward</td>
<td>NdeI</td>
</tr>
<tr>
<td>CTATCGTTGAAACAGGACTTTTTGCAGGTTAAAGAGG</td>
<td>AgVik1 Y260I-A633 Forward</td>
<td>NdeI</td>
</tr>
<tr>
<td>CTATCGAGCGTCAGAGAAACATACGTATTTTC</td>
<td>AgVik1 L575-A633-MBP Forward</td>
<td>PstI</td>
</tr>
<tr>
<td>CTATCGAGCGATTGAAACACCATCGAGAAGACTC</td>
<td>AgVik1 I588-A633-MBP Forward</td>
<td>PstI</td>
</tr>
<tr>
<td>CTATCGAGCGACTTTGTATGATAAAATGGAGGATG</td>
<td>AgVik1 T597-A633-MBP Forward</td>
<td>PstI</td>
</tr>
</tbody>
</table>
The supernatant fraction of the cell lysate was loaded onto an Amylose resin column (New England Biolabs Canada®, Pickering, ON) pre-equilibrated with 10 CV of Column buffer. The column was washed with Column Buffer until the level of protein elution decreased to a baseline absorbance at 280 nm. The protein was then eluted with Elution buffer (10 mM NaPO₄ pH 7.2 (NaOH), 200 mM NaCl, 2 mM MgCl₂, 1 mM EGTA, 1 mM DTT, 5 mM β-mercaptoethanol, 10 mM Maltose, and 1 EDTA-free Complete® Protease Inhibitor tablet (Roche®)). Eluted protein, indicated by absorbance at 280 nm, was analyzed by SDS-PAGE, and fractions with bands at the molecular weight of the respective AgVik1-MBP fusion protein were pooled and dialyzed overnight at 4°C in 2 L Maltose Dialysis Buffer (10 mM HEPES pH 7.2 (NaOH), 50 mM NaCl, 2 mM MgCl₂, 1 mM EGTA, 1 mM DTT, 5 mM Maltose, and 1 EDTA-free Complete® Protease Inhibitor tablet (Roche®)). The Purification by size exclusion chromatography, concentration, and flash freezing were conducted as described in section 2.1.2, except that all buffers included 5 mM Maltose.

2.2 Microtubule Preparation

2.2.1 Large Scale Preparation of High-Salt Tubulin from Bovine Brain Tissue

Tubulin was purified from bovine brains according to the method of Castoldi and Popov [127]. Bovine brains were acquired from a local abattoir the morning of the purification. Brains were weighed, and transferred to a high-powered Waring® blender kept at 4°C. Cold High-Molarity PIPES Buffer (HMPB: 1 M piperazine-N,N’-bis(2-ethanesulfonic acid) (PIPES) pH 6.9 (KOH), 1 mM MgCl₂, 20 mM ethylene glycol tetraacetic acid (EGTA)) was added to the brains at a concentration of 1 L/kgₜₐₜₐₜ. Brains were homogenized several times at 30 s intervals. The homogenous mixture was centrifuged in a JLA 16.250 rotor (Beckmann-Coulter®, Brea, CA,
USA) at 14,000 rpm for 69 minutes at 4° C. Supernatants were pooled, and added to an equal volume of 37° C HMPB buffer (1/3 final volume), an equal volume of 37° C glycerol (1/3 final volume), and 1.5 mM ATP and 0.5 mM GTP (with respect to the final volume). The mixture was then transferred to a 50° C water bath, with stirring, to rapidly increase the temperature of the supernatant to 37° C. Upon reaching 37° C, the mixture was transferred to a 37° C water bath and tubulin was polymerized over a 1 hour incubation. The polymerized mixture was then centrifuged in a JLA 16.250 rotor at 14,000 rpm for 157 minutes at 37° C. Resulting microtubule pellets were resuspended by dounce in a minimal volume of 4° C depolymerization buffer (DB; 50 mM 2-(N-morpholino)ethanesulfonic acid (MES) pH 6.6 (HCl), 1 mM CaCl₂), and depolymerized on ice for 30 minutes. Tubulin was then repolymerized in 37° C HMPB, glycerol, ATP and GTP, as above, and held in a 37° C water bath for 1 hour. The polymerized mixture was then centrifuged in a Ti45 Rotor (Beckmann-Coulter®) at 70,000 g (36, 088 rpm) for 30 minutes at 37° C. Pellets were resuspended by dounce in a minimal volume of 4° C BRB80 buffer A (80 mM PIPES pH 6.8 (KOH), 1 mM MgCl₂, 1 mM EGTA), and incubated on ice for 10 minutes. Depolymerized tubulin was then centrifuged in a Ti45 rotor at 104,000 g (29,950 rpm) for 30 minutes at 4° C. The depolymerized tubulin supernatant was collected. Concentration of the tubulin was measured by absorbance at 280 nm, using an extinction coefficient of ε = 115,000 L·mol⁻¹·cm⁻¹. 3 mL aliquots of High-Salt tubulin were flash frozen in liquid nitrogen, and stored at -80° C.

2.2.2 Small-Scale Recycling of High-Salt Purified Tubulin

Aliquots (3 mL) of High-Salt tubulin were rapidly thawed in a 37° C water bath. Immediately after aliquots were completely thawed, aliquots were transferred to ice, and combined into a single volume. A 1/5 volume of 4° C 5xBRB80 Buffer B (400 mM PIPES pH 6.8 (KOH), 5 mM MgCl₂, 5 mM EGTA), and 4 mM MgCl₂ and 1 mM GTP (with respect to the final volume)
was added to the tubulin, and mixed on ice for 5 minutes. Tubulin was then transferred to a 37°C water bath, and after a 2 minute incubation, glycerol was added to a final concentration of 33% (v/v), and mixed by light vortexing. The 37°C water bath incubation was continued for an additional 40 minutes to polymerize the tubulin. The polymerized tubulin was then layered over a 37°C ‘cushion’ of glycerol, and centrifuged for 45 minutes in a Ti70 rotor (Beckmann-Coulter®) at 43,300 rpm for 45 minutes. The supernatant was aspirated off, and the pellet interface was rinsed three times with 37°C IB buffer (50 mM potassium glutamate pH 7(KOH), 5 mM MgCl₂) and all liquid was aspirated completely, to remove all residual glycerol and GTP from the tubulin. The pellet was resuspended in 1 mL of 4°C IB buffer, and resuspended by douncing on ice. The depolymerized tubulin was incubated on ice for 30 minutes, and douncing every 2-3 minutes. The sample was then centrifuged at 90,000 rpm in a TLA100.2 rotor (Beckmann-Coulter®) at 4°C for 15 minutes. The depolymerized tubulin supernatant was collected. The concentration of the tubulin was determined as described above, and 20 μL aliquots of recycled tubulin were flash frozen in liquid nitrogen and stored at -80°C.

2.2.3 Preparation of Taxol-stabilized Microtubules

On the morning of tubulin-based experiments, 20 μL aliquots of recycled tubulin were thawed quickly until a thin layer of liquid formed around the outside of the tube, and then 2 mM MgCl₂, 1 mM GTP and 1.2 mM DTT were added. The tubulin was then incubated on ice for 20 minutes, and centrifuged at 14,000 rpm for 15 minutes at 4°C. The unpolymerized tubulin supernatant was then collected, and incubated in a 37°C water bath for 20 minutes. 380 μL of 37°C PME10 buffer (10 mM PIPES pH 6.9 (KOH), 5 mM MgCl₂, 1 mM EGTA, 1 mM DTT) with 40 μM Taxol was added and mixed into the polymerized tubulin, and the 37°C incubation was continued for 15 minutes. The taxol-stabilized polymerized microtubules were layered onto a 500
μL 40% (v/v) sucrose ‘cushion’, and centrifuged at 13,300 rpm for 45 minutes at room temperature. The sucrose cushion was removed by pipette, and the polymerized tubulin pellet was resuspended by gentle pipetting (with a cut off 200 μL pipette tip) in 150 μL 37° C A25(A) buffer (25 mM ACES pH 6.9 (KOH), 2 mM Mg-acetate, 2 mM EGTA, 2 mM EDTA, 1 mM BME) with 20 μM Taxol. Tubulin concentration was determined using absorbance, as described above. Taxol-stabilized polymerized tubulin was maintained at 25-30° C on a heating block for the duration of experimentation.

2.2.4 Preparation of X-Rhodamine-labeled Microtubules

X-Rhodamine-labelled tubulin from bovine brain was purchased from Cytoskeleton Inc. (Cat. # TL620M; Denver, CO, USA), and reconstituted to a final concentration of 10 mg/mL in 4° C BRB80 Buffer C (80 mM PIPES pH 6.9 (KOH), 0.5 mM EGTA, 2 mM MgCl₂) with 1 mM GTP. X-Rhodamine fluoresces at an excitation wavelength of 540-560 nm, and emission wavelength of 610-630 nm. Unlabelled bovine brain tubulin from the High-Salt purification was concentrated to 8 mg/mL in 4° C BRB80 buffer. 0.5 μL of X-Rhodamine tubulin was added to 3.5 μL unlabeled tubulin, with 4 μL 4° C Polymerization Mix (BRB80 buffer, 20% v/v DMSO, 2 mM GTP), and tubulin was incubated on ice for 15 minutes. The tubulin was incubated in a 37° C water bath for 30 minutes, after which 8 μL of Stabilization Mix (BRB80 buffer, 2 mM DTT, 40 μM Taxol) was added, and polymerization continued at 37° C for an additional 20 minutes. Taxol-stabilized polymerized tubulin was centrifuged at 13,300 rpm for 30 minutes at room temperature. The supernatant was removed by pipette, and the microtubule pellet was resuspended in 15μL of Stabilization Mix by gentle pipetting (with a cut-off 200 μL pipette tip). X-Rhodamine-labelled Microtubules were kept at room temperature, protected from light sources to prevent photobleaching for the duration of experiments.
2.2.5 Preparation of GMP-CPP Stabilized Microtubules

An aliquot of microtubules, as prepared in section 2.2.2, was diluted to a concentration of 20 μM into BRB80 buffer supplemented with 1 mM Guanosine-5’-[(α,β)-methylene] triphosphate (GMPCPP), and incubated on ice for 10 minutes. Microtubules were then incubated in a 37° C water bath for 30 minutes, and centrifuged at 13,000 rpm for 45 minutes at room temperature. The microtubule-containing pellet was then resuspended in 100 μL of BRB80 buffer with 1 mM GMPCPP, and incubated on ice for 20 minutes. Two additional 37° C water bath incubations, room temperature centrifugations, resuspensions and ice incubations were completed in the same manner as above. After the final incubation, the tubulin concentration was measured, as described above, and 2 μL aliquots of the GMPCPP-stabilized microtubules were made, flash frozen over liquid nitrogen, and stored at -80° C.

2.3 Measurement of steady-state ATPase activity

Steady-state kinetics of the monomeric and dimeric constructs were determined using an enzyme-coupled assay [128]. Microtubule concentration-dependent reactions were assembled in A25(B) Buffer (25 mM N-(2-Acetamido)-2-aminoethanesulfonic acid (ACES) pH 6.9 (KOH), 2 mM magnesium acetate, 2 mM EGTA, 0.1 mM EDTA, 1 mM BME), 1 mM MgATP, 2 mM phosphoenolpyruvate, 250 μM NADH, 60 μg/mL pyruvate kinase, 60 μg/mL lactate dehydrogenase, 0–8 μM microtubules, and 200 nM motor in a total volume of 150 μL. A subset of these reactions were supplemented with 25 mM potassium acetate for reasons of comparability with the previous radiolabel-based ATPase measurements of Mackey and Gilbert [129]. A second subset of these reactions were also carried out without potassium acetate because the concentration of microtubules required to achieve microtubule saturation in the presence of potassium salts (>8 μM) create light scattering artifacts that interfere with the optical signal used by the enzyme-
coupled system to measure ATP turnover. ATP concentration-dependent reactions were assembled in A25 buffer with 0–800 μM MgATP, and a saturating concentration of microtubules (determined by the results of the tubulin-dependent ATPase assays) in a total volume of 150 μL. The ΔA₃₄₀ nm/s was monitored for each reaction, and converted to units of Mₐₙ₃dh/s using ε₉ₐ₈₃₄₀ nm = 6220 M⁻¹ cm⁻¹. The ATPase rate per motor head in units of s⁻¹ was calculated by dividing the change in NADH concentration per second by the concentration of the motor. Microtubule and ATP concentration were plotted against ATPase rates, and fit to the quasi-steady-state approximation of the Michaelis-Menten equation (Equation 1) using SigmaPlot 11⁰.

**Equation 1**

\[
v_0 = \frac{V_{\text{max}}[S]}{K_m + [S]}
\]

Where \( v_0 \) is the initial velocity of the enzyme reaction, \( V_{\text{max}} \) is the maximum velocity of the enzyme reaction at saturating concentrations of substrate, \( K_m \) is the Michaelis constant, represented by Equation 2, and \([S]\) is the total substrate concentration.

**Equation 2**

\[
K_m = \frac{k_{-1} + k_{\text{cat}}}{k_1}
\]

Where \( K_m \) is the Michaelis constant, \( k_{-1} \) is the rate constant for substrate unbinding from the enzyme-substrate complex, \( k_{\text{cat}} \) is the turnover number (the rate constant for overall conversion of substrate to product), and \( k_1 \) is the rate constant for binding of the substrate to the enzyme to form the enzyme-substrate complex.
2.4 Measurement of Equilibrium Microtubule Binding Affinity

The equilibrium microtubule binding affinity assay requires Taxol-stabilized microtubules, prepared as described in section 2.2.3 on the morning of the experiment. The steady state microtubule binding affinities of AgKar3MD, AgKar3 Q244-R709/Vik1 Y260-A633, and AgVik1 K356-A633 were determined as described previously,[83] with the following modifications. Reactions of 100 μL with varying microtubule concentrations (0–5 μM tubulin) were incubated with 4 μM AgKar3MD motor for 30 minutes at room temperature, in ATPase buffer (20 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), 5 mM magnesium acetate, 0.1 mM EGTA, 0.1 mM EDTA, 25 mM potassium acetate, 1 mM DTT, 40 μM Taxol, pH 7.2) with either 1 μM magnesium adenylyl-imidodiphosphate (MgAMPPNP) or MgADP. Reaction mixtures were sedimented by centrifugation at 312,530×g in a TLA100 rotor for 15 minutes at room temperature. Supernatant and pellet fractions were analyzed by SDS-PAGE and visualized with Coomassie Brilliant Blue R-250. The band intensities for the pellet fractions were quantified by densitometry using ImageJ™. The amount of kinesin-microtubule complex was plotted as a function of microtubule concentration and fit to Equation 3 in SigmaPlot 11 ®.

Equation 3

\[
\frac{(MT \cdot E)}{(E)} = 0.5 \times \left( (E_0 + K_{d,MT} + MT_0) - \left( (E_0 + K_{d,MT} + MT_0)^2 - (4E_0MT_0) \right)^{1/2} \right)
\]

Where \( MT \cdot E \) is the amount of kinesin that sedimented with the microtubule in the pellet, \( E_0 \) is the total amount of kinesin, \( MT_0 \) is the total amount of microtubules, and \( K_{d,MT} \) is the dissociation constant.
2.5 Measurement of Kinesin Motility by Fluorescent Confocal Microscopy

The motility assay requires X-Rhodamine-labelled microtubules, prepared as described in section 2.2.4 on the morning of the experiment. Glass slides and cover slips were prepared by stirring them periodically in 20% (v/v) sulfuric acid for 2 hours, and soaking overnight, followed by quenching of the acid etching with two rinses each of 0.1 M NaOH and ddH₂O, and storage in 70% ethanol. Perfusion chambers were made by placing two short pieces of double-sided tape with approximately 3 mm spacing between an etched glass slide and cover slide, with an approximate chamber volume of 14 μL. In this arrangement, the glass slide forms the bottom of the chamber, the cover slip the top, and the tape blocking the sides, with openings on either end of the rectangular shaped chamber for inflow and outflow of solutions. Exchanges of approximately 14 μL of solution were passed through the perfusion chamber on each exchange, pipetting into the chamber at the inflow, and extracting excess solution from the other end by absorption with filter paper. First, the chamber was perfused with Anti-His₈ tag antibody, purchased from Millipore® (Billerica, MA, USA; Fisher Sci. Cat.# 05949MI), at a concentration of 25 μg/mL. The chamber was then perfused with 1 mg/mL Casein and incubated for 3 minutes. The chamber was washed twice with OSM-0 buffer (BRB80 buffer, 1.5 mM magnesium acetate, 0.1 mg/mL (w/v) casein, 200 μg/mL glucose oxidase, 175 μg/mL catalase, 25 mM glucose, 2 mM BME). Either the supernatant fraction of AgKar3 Q²⁴⁴-R⁷⁰⁹/Vik1 Y²⁶⁰-A⁶³³ total cell lysate, or AgKar3 Q²⁴⁴-R⁷⁰⁹/Vik1 Y²⁶⁰-A⁶³³ purified as described in section 2.1.4 and diluted to approximately 0.15 μM in OSM-0 buffer, was added into the perfusion chamber, and incubated for 5 minutes. Microtubules in OSM-1 buffer (OSM-0 buffer, 1.5 mM AMPPNP, 40 μM Taxol) were shredded by drawing in and out of a 30 gauge needle approximately 6 times, and added into the perfusion chamber for a 5 minute incubation. The perfusion chamber was washed twice with OSM-2 buffer (OSM-0 buffer, 1.5 mM
ATP, 40 μM Taxol, 0.3 mg/mL Phosphocreatine Kinase, 2 mM phosphocreatine). If the purified AgKar3MDC/Vik1MHDC was added as the motor, then a final wash was made with motor in OSM-2 buffer. Imaging of the slide was performed on a spinning disc confocal microscope, taking images for 15 minutes, at a rate of 1 image/s.

2.6 Measurement of the Depolymerization of Microtubules

The microtubule depolymerization assay requires GMPCPP-stabilized microtubules, prepared the morning of the experiment, as described in section 2.2.5. 1 μM GMPCPP stabilized microtubules was added into a total reaction volume of 100 μL with BRB80-DACK buffer (BRB80 buffer, 1 mM DTT, 1 mM ATP, 0.1 mg/mL Casein, 60 mM KCl), and 0-2 μM purified AgKar3 Q²⁴⁴-R⁷⁰⁹/Vik1 Y²⁶⁰-A⁶³³ motor, mixed well, and read immediately. Light-scattering was measured at room temperature using a fluorimeter, at excitation and emission wavelengths of 340 nm, and excitation and emission slit widths of 0.75 nm. Both reading and integration time were set at 1 s, and reactions were run for 2700 s in total. Readings were normalized by translating the entire curve, such that the reading at time = 0 was set to equal 250,000 counts per second (cps).
Chapter 3

Results

3.1 Analysis of the primary structures of AgKar3 and AgVik1

The *A. gossypii* genome encodes a single Kinesin-14 (AGOS_AGR253W), whose open reading frame gives a 709 amino acid long protein that shares 58% sequence similarity (using the BLOSUM62 matrix, and defining the following groups of amino acids as similar: Aromatic (FYW), Aliphatic (VIL), Positive (RKH), Negative (DE), Polar (NQ), and Small (ATS)), and 47% sequence identity with *Sc*Kar3 using Clustal W2 and SIAS. A syntenic homolog of the Vik1 protein is also present (AFL170C), which is 633 amino acids long and shares 35% similarity and 25% identity with *Sc*Vik1. The region of highest conservation for both proteins is the C-terminal portion, which is where the motor domain resides in members of the Kinesin-14 family. We performed a multiple sequence alignment of the motor domain of *Sc*Kar3 and AgKar3, as well as closely related Kar3 proteins from *Candida glabrata*, *Kluyveromyces lactis*, and *Naumovozyma castellii*, in order to determine areas of conservation and divergence that might relate to different mechanochemical properties, and perhaps physiological functions, of these motors (Figure 14).

The alignment in Figure 14 shows high levels of identity throughout the motor domain region between each fungal species. The nucleotide binding motifs – the phosphate binding P-loop (GQTXXGKS/T), Switch I (NXXSR), Switch II (DXXGXE), and base binding motif (RXRP) – are particularly well conserved. The most significant breaks in sequence conservation between AgKar3 and other Kar3 orthologs occur in the β1 lobe (β1a, β1b, β1c) at the edge of the motor domain, at loop L8 within the microtubule-binding region, and throughout the α3 helix, upstream of the Switch I motif. These differences in sequence translate into differences between the
Figure 14 – Multiple sequence alignment of Kar3 ortholog motor domains. Secondary structure elements are derived from the X-ray crystal structure of AgKar3MD (3T0Q). Microtubule- and nucleotide-binding motifs are indicated by green and orange bars, respectively. Candida glabrata Kar3 (NCBI Accession: XP_445628.1), Kluyveromyces lactis Kar3 (XP_452016.1), and Naumovozyma castelli Kar3 (XP_003677184) are included as the closest related homologs to AgKar3 (NP_986919.1) and ScKar3 (P17119). The alignment was performed using ClustalW2, and the shading was applied to identical residues using Jalview.
structures of AgKar3 and ScKar3 [1]*. In particular, a structural superposition of the two molecules shows that the regions of highest root mean squared deviation are found at areas within helix α3 and the adjacent loop L9, as well as at the loop regions on the top and bottom of the β1 lobe and along the top edge of the nucleotide binding cavity (loop L1) (Figure 15).

A multiple sequence alignment of AgVik1 against a selection of Vik1 and Cik1 orthologs from the related ascomycete fungi S. cerevisiae, Candida glabrata, and Candida albicans (Figure 16), all of which are studied in our lab, was also performed. Compared to Kar3 proteins, there is a very high level of divergence throughout the motor domain of all Cik1/Vik1 proteins. The most striking differences are found in the regions that, based on their homology in primary structure position to the microtubule binding elements in the motor domain of catalytic kinesins, may compose the microtubule-binding surface of the motor domain. These regions include loop L8a, loop L12 and the α4 helix. In ScVik1, the α4/L12 region contains primarily helix forming residues, however, the corresponding region in AgKar3 contains a high number of serine and threonine residues, as well as a number of helix-breaking glycine residues. This may indicate that their microtubule interactions differ. In catalytic kinesins, such as KIF1A, the α4 helix experiences a sizable rotation (~33°) during the transition from the ADP·P state to the ADP state, which results in a 45% decrease in microtubule binding [130]. While ScVik1 and CgVik1 do not have a nucleotide binding pocket, they both possess a well-structured α4 helix and a high affinity for microtubules [83] (Duan, et al., unpublished). In these proteins, the conformation and microtubule affinity is likely controlled through communication with their Kar3 binding partner.

Interestingly, the region exhibiting the most sequence conservation among Vik1 and Cik1 orthologs is concentrated at the point where the neck segment transitions into the motor domain (labeled as ‘neck’, the β1 lobe and helix α1) (Figure 16). This includes AgVik1 residues Ile372,
Figure 15 – Structural Superposition of Sc and Ag Kar3 Motor Domains [1]*. The coordinates for the α-carbons of AgKar3MD and ScKar3MD were superimposed and coloured according to the value of the root-mean-square deviation (RMSD) between equivalent residues as calculated by PyMol. Residues not used for superposition, and hence for RMSD calculation, are coloured yellow.
Figure 16 – Multiple Sequence Alignment of Cik1 and Vik1 Motor Homology Domains. Secondary structure elements are derived from the X-ray crystal structure of Sc.Vik1 (200A). A short C-terminal section of the neck is labeled with a and d residues of the heptad repeat, followed by the highly conserved 'pivot glycine', indicated by an asterisk (*). Putative microtubule-binding motifs are indicated by green bars. Homologs for Ashbya gossypii (Vik1: NP_985380.1) Saccharomyces cerevisiae (Vik1: NP_015070, Cik1: CAA87820.1) Candida glabrata (Vik1: XP_446826, Cik1: XP_449229) and Candida albicans (Vik1: orf_19.306) are included. The alignment was performed using ClustalW2, and the shading was applied to identical residues using Jalview.
Glu$^{374}$, Gly$^{377}$, Arg$^{380}$, and Ala$^{383}$. Recent studies in *Candida glabrata* Vik1 have shown this region to comprise a conformationally dynamic element that may alter its configuration in response to the catalytic cycle of Kar3 as it moves along microtubules (Figure 17) (Duan *et al.*, unpublished). These conformational changes are highly reminiscent of those observed in the *Drosophila* Kinesin-14 Ncd. Moreover, several of the residues that appear to stabilize each unique conformation of the neck module involve residues that are conserved in Vik1, Cik1, and many catalytic Kinesin-14 motors. In CgVik1 and Ncd, these residues form specific H-bonding, ionic, or van der Waals interactions between the neck and the nearby surface of the motor domain core.

Despite these intriguing similarities to catalytic kinesins, AgVik1 lacks the nucleotide binding motifs necessary to bind and turnover ATP, which is consistent with previous studies on ScVik1 [83]. Moreover, most of the segments of the motor domain that would harbour these elements (β1, L4, α4, β7, and L11) exhibit a high degree of sequence divergence between Vik1 orthologs, suggesting that as these proteins diverged from catalytic Kinesin-14 motors, conservation of regions that formerly composed the active site had little functional necessity.

### 3.2 AgKar3 forms a stable complex with AgVik1

COILS server [131] predictions show coiled-coil forming regions between residues 93-370 on AgKar3 and residues 90-384 on AgVik1 (Figure 18). Based on these predictions, we were able to identify the region through which these proteins could interact, and from this information we designed truncated versions of AgKar3 and AgVik1 for recombinant expression, co-purification and analysis of protein-protein interaction (Figure 19). AgKar3 Q$^{244}$-R$^{709}$ and AgVik1 Y$^{260}$-A$^{633}$, which contain 12 and 16 heptad repeats N-terminal to their respective C-terminal globular domain, were cloned into two different plasmids, and co-expressed in an *E. coli* expression system.
Figure 17 – Crystal Structures of Neck Conformations of *Candida glabrata* Vik1 (Personal Communication, Da Duan and John Allingham). (A and B) *Cg*Vik1 motor homology domain and neck, with the neck in two different positions. (C) *Cg*Vik1 motor homology domain, with half the number of the neck residues truncated from the N-terminal end, showing the neck in a disordered coil. *Cg*Vik1 and *Ag*Vik1 is 18.19% identical and 32.03% similar (ClustalW and SIAS). Colouring and was applied using PyMol.
Figure 18 – Prediction of AgKar3 and AgVik1 Coiled-Coil Propensity (COILS Prediction Server) [126]. The COILS server awards high probability scores for regions of query sequence that have high similarity to the heptad repeat. Our estimates of the boundaries of the tail, stalk and motor (homology) domains are indicated above each plot.
Figure 19 – Truncated AgKar3 and AgVik1 Construct Design. Representations for the full length sequences of AgKar3 and AgVik1 are shown above, with truncated constructs designed for recombinant co-expression and co-purification in a bacterial expression system. Constructs used exclusively for crystallography are outlined in red, whereas constructs used for crystallography as well as functional assays (and for which the purification gels and chromatograms are included in this thesis) are not outlined. The boundaries of coiled-coil forming regions (CC) were determined using the COILS prediction algorithm (Figure 18).
Figure 20 – SDS-PAGE of AgKar3/Vik1 Co-purification. Gels are stained by Coomassie Brilliant Blue. (A) Elution of AgKar3 Q244-R709/AgVik1 His10-Y260-A633 from Ni\textsuperscript{2+}-affinity resin. A 50 mL gradient from 50 mM imidazole to 600 mM imidazole was used to elute the protein. (B) Elution of AgKar3 Q244-R709/AgVik1 Y260-A633 from Ni\textsuperscript{2+}-affinity resin after cleavage of His\textsubscript{10}-tag with TEV protease. M = molecular weight marker, T = total cell lysate, S = lysate supernatant, W = column wash, S200 = size exclusion peak fraction. Pooled protein from each purification step is indicated by boxed lanes.
Using nickel affinity (Figure 20) and size exclusion chromatography (Figure 20B, Figure 21A), we show that AgKar3 and AgVik1 form a stable complex in solution whose ratio is approximately 1:1 based on densitometry analysis. The predicted size of the complex based on the primary sequences of the AgKar3 and AgVik1 polypeptides is 99,583, while the apparent mass calculated from the size exclusion molecular weight standards is 140,000 kDa. This discrepancy is likely due to the larger Stokes hydrodynamic radius of the kinesin molecule relative to its molecular weight. While molecular weight standards are generally spherical, the kinesin stalk makes it more rod-shaped, and will have a larger apparent volume in three-dimensional space, leading to elution from a size exclusion column earlier than would be expected for its molecular mass.

Circular dichroism (CD) spectroscopy measurements confirmed the stability of this complex, as the heterodimer maintained distinct α-helical and β-sheet character with little spectral movement at low temperatures (Figure 22). Even as the temperature was raised to 30° C, the heterodimer did not enter into a random coil state. Random coil folding would be expected if the coiled-coil were to unwind into separate α-helices, exposing the residues that make up its hydrophobic core to solvent, possibly leading to misfolding and aggregation.

In addition to the AgKar3/Vik1 complex, monomeric constructs comprising the C-terminal motor domain region of AgKar3 and the aligned region of AgVik1, which we refer to as ‘AgKar3MD’ and ‘AgVik1 K^{356-633},’ were created to study the effects of the subunits independently in the ATPase and microtubule binding assays described in sections 2.3 and 2.4. The purification of AgKar3MD by both cation and anion exchange chromatography removing the majority of impurities (Figure 23). Further polishing of AgKar3MD using size exclusion chromatography produced a single major peak containing AgKar3MD without visible contaminants by SDS-PAGE (Figure 23B). AgVik1 K^{356-633} was first purified by Ni^{2+}-affinity
Figure 21 – Size Exclusion Chromatography Profile of AgKar3/Vik1. (A) AgKar3 Q^{244}-R^{709}/AgVik1 Y^{260}-A^{633}, with standard molecular weight marker proteins. (B) The molecular weight standard curve used to estimate the protein size in the labeled peaks.
Figure 22 – Circular Dichroism Spectra of AgKar3/Vik1. (A) Temperature-dependent spectra of Ni²⁺-affinity and size exclusion purified AgKar3 Q²⁴⁴-R⁷⁰⁹/AgVik1 Y²⁶⁰-A⁶³³. (B) Expected theoretical spectra for selected secondary structural elements. Dotted lines indicate regions of the curve where data is less reliable due to high sample absorbance.
Figure 23 – SDS-PAGE of AgKar3MD Purification. (A) Cation exchange purification of AgKar3MD. (B) Anion exchange and size exclusion purification of AgKar3MD. Lanes containing molecular weight marker (M), total cell lysate (T), lysate supernatant (S), column wash (W), pooled cation exchange fractions (P), and a size exclusion chromatography peak fraction (S200) are labeled. In each case, a 60 mL gradient from 20 mM NaCl to 300 mM NaCl was used. Pooled protein from each purification step is indicated by boxed lanes.
chromatography via a His$_{10}$-tag that was recombinantly engineered onto the N-terminus. After initial purification, this tag was cleaved by TEV protease at the TEV-specific cut site, after which the His-tagged TEV protease was removed from the sample by a second round of Ni$^{2+}$-affinity chromatography, while the cleaved AgVik1 protein was collected and concentrated from the flow-through fraction (Figure 24).

### 3.3 AgKar3/Vik1 complexes exhibit microtubule-based motility

To examine the motor activities of our truncated AgKar3/Vik1 complexes, we utilized a microtubule-gliding assay which involves imaging purified X-rhodamine-labelled microtubules whose movement is driven by kinesin motors that are tethered to the glass surface by anti-His antibodies within a glass slide chamber. The heterodimeric AgKar3/Vik1 construct was designed with this in mind by virtue of a poly-histidine tag on the N-terminus of the Vik1 subunit and has a sufficiently long coiled-coil stalk region to allow for spatial separation between the glass slide and the microtubules. Although the Ni$^{2+}$-affinity and size exclusion purified AgKar3 Q$^{244}$-R$^{709}$/Vik1 Y$^{260}$-A$^{633}$ did not exhibit microtubule motility within our assay setup (Figure 25), the supernatant fraction of lysate from cells expressing these proteins, as per the methods of Heuston et al., 2010 [132], did display robust movement of the microtubules (Figure 25). The rate of movement we observed for the unpurified supernatant fraction of the AgKar3/Vik1 recombinant overexpression was similar to that of a positive control reaction containing purified Kar3/Vik1 motor from *Candida glabrata* (Figure 25). It should be noted at that, at this time, it cannot be said with certainty the AgKar3/Vik1 heterodimer is entirely responsible for the microtubule movement because AgKar3 homodimers may comprise a large portion of the supernatant material, which could exhibit motility as well. In a typical purification of the heterodimer, there is a large excess (approximately 2:1) of AgKar3 compared to AgVik1, most of which (whether in a free form
**Figure 24 – SDS-PAGE of AgVik1 Purification.** (A) Elution of AgVik1 His\textsubscript{10}-K\textsuperscript{356}-A\textsuperscript{633} from Ni\textsuperscript{2+}-affinity resin. (B) Elution of AgVik1 K\textsuperscript{356}-A\textsuperscript{633} from Ni\textsuperscript{2+}-affinity resin after cleavage of His\textsubscript{10}-tag by TEV protease. Molecular weight marker (M), total cell lysate (TCL), pooled eluted fractions (Pool), AgVik1 K\textsuperscript{356}-A\textsuperscript{633} with His\textsubscript{10}-tag (H), AgVik1 K\textsuperscript{356}-A\textsuperscript{633} with His\textsubscript{10}-tag cleaved off (C), and 600 mM Imidazole elution fraction (E) are labeled.
Figure 25 – Microscopy Still-Frames of the AgKar3/Vik1 Microtubule Motility Assay. Images of X-rhodamine-labeled microtubules were collected on a fluorescent confocal microscope. ‘Purified’ represents protein purified by Ni²⁺-affinity and size exclusion chromatography. ‘Lysate’ represents the supernatant fraction of the total cell lysate of cells expressing the protein. White stars indicate the positioning of one end of a single microtubule across timepoints in each different condition. Scaling is identical across all frames.
or homodimeric) is removed through the Ni\textsuperscript{2+}-affinity chromatography. Although the anti-His antibody is included in the gliding assay setup to select for the His\textsubscript{10}-tag of our AgVik1 Y\textsuperscript{260}A\textsuperscript{633} construct, it is possible that AgKar3 homodimers found in the supernatant are binding non-specifically to the etched glass chamber.

### 3.4 The ATPase activity of AgKar3 is influenced by AgVik1

Using an enzyme-coupled assay, we measured the steady-state ATPase kinetics of AgKar3MD, AgVik1 and AgKar3/Vik1 (Figure 26-28). The maximal microtubule-stimulated ATPase rate \((k_{cat})\) for AgKar3MD was approximately 3-fold slower than ScKar3MD (Table 2). This discrepancy in activity was observed in both the absence (0.17 \textit{versus} 0.42 s\textsuperscript{-1}) and presence (0.16 \textit{versus} 0.58 s\textsuperscript{-1}) of potassium acetate, which is often included in kinesin ATPase buffers to achieve optimal ionic strength for motor activity. Inclusion of potassium acetate was also useful for reasons of comparability of our enzyme-coupled assay data with the previous radiolabel-based ATPase measurements of ScKar3MD by Mackey and Gilbert.\[129\] As expected, the \(k_{cat}\) values for ScKar3MD in the presence of 50 mM potassium acetate were similar between these experimental systems (Table 2).

Duplication of these analyses in the absence of potassium acetate permitted us to extract more kinetic constants from our ATPase measurements. By lowering the ionic strength, the saturating microtubule concentration required for \(K_{M,ATP}\) determination decreased to a level at which light scattering artifacts from microtubules do not obscure the optical signal used by the enzyme-coupled system \[133\]. This relates to the fact that kinesin-microtubule binding is dominated by electrostatic interactions \[22\], and that moderate decreases in ionic strength can significantly increase their affinity for microtubules \[134,135\]. Indeed, the microtubule
Figure 26 – AgKar3MD ATPase Kinetic Curves [1]. Data was fit to the Michaelis-Menten equation (Equation 1). (A) 0 – 5 μM Tubulin-dependent reactions. (B) 0 – 800 μM ATP-dependent reaction. Error bars represent standard error of the mean (SEM) from three independent trials.
Table 2 - Enzyme rate constants determined from the enzyme-coupled ATPase assay of Kar3 motors.

<table>
<thead>
<tr>
<th></th>
<th>AgKar3MD[1]</th>
<th>ScKar3MD[1]</th>
<th>ScKar3MD[129]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 μM KAc</td>
<td>25 μM KAc</td>
<td>0 μM KAc</td>
</tr>
<tr>
<td>$K_{cat}$ (s$^{-1}$)</td>
<td>0.167 ± 0.006</td>
<td>0.16 ± 0.01</td>
<td>0.42 ± 0.01</td>
</tr>
<tr>
<td>$K_{1/2,MT}$ (μM)</td>
<td>0.08 ± 0.01</td>
<td>0.7 ± 0.2</td>
<td>0.26 ± 0.03</td>
</tr>
<tr>
<td>$K_{M,ATP}$ (μM)</td>
<td>1.6 ± 0.7</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

*Data is original to this thesis, or work published by the author of this thesis, using the Enzyme-Coupled ATPase assay; Data is from work by the cited authors (Mackey, et al., 2003), using the Radiolabeled ATPase assay; Data represents 3-4 independent experimental replicates.

Table 3 - Enzyme rate constants determined from the enzyme-coupled ATPase assay of Kar3/Vik1 and Kar3/Cik1 motors.

<table>
<thead>
<tr>
<th></th>
<th>AgKar3/Vik1</th>
<th>ScKar3/Vik1[83]</th>
<th>ScKar3/Cik1[83]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 μM KAc</td>
<td>25 μM KAc</td>
<td>50 μM KAc</td>
</tr>
<tr>
<td>$K_{cat}$ (s$^{-1}$)</td>
<td>0.30 ± 0.01</td>
<td>0.29 ± 0.02</td>
<td>3.7 ± 0.0</td>
</tr>
<tr>
<td>$K_{1/2,MT}$ (μM)</td>
<td>0.8 ± 0.1</td>
<td>1.5 ± 0.3</td>
<td>1.7 ± 0.3</td>
</tr>
<tr>
<td>$K_{M,ATP}$ (μM)</td>
<td>14 ± 1</td>
<td>–</td>
<td>15.0 ± 0.7</td>
</tr>
</tbody>
</table>

*Data is original to this thesis, or work published by the author of this thesis, using the Enzyme-Coupled ATPase assay; Data is from work by the cited authors (Allingham, et al., 2007), using the Radiolabeled ATPase assay; Data represents 3 independent experimental replicates.
Figure 27 – AgVik1 ATPase Kinetic Curves. Data were collected for AgVik1 K$^{356}\text{-A}^{633}$, and fit to the Michaelis-Menten equation (Equation 1). (A) Tubulin-dependent reactions. (B) ATP-dependent reactions. Error bars represent standard error of the mean (SEM) from three independent trials. Curve fits were not statistically significant.
Figure 28 – AgKar3/Vik1 ATPase Kinetic Curves. Data were collected for AgKar3 Q^{244-270}/AgVik1 Y^{260-286}/A^{633}, and fit to the Michaelis-Menten equation (Equation 1). (A) Tubulin-dependent reactions. (B) ATP-dependent reactions. Error bars represent standard error of the mean (SEM) from three independent trials.
concentration required for half-maximal ATPase stimulation ($K_{1/2,MT}$) was over 8-fold lower in the absence of potassium acetate than with 25 mM added (0.08 μM versus 0.7 μM, respectively; Table 2). These values, along with the $K_{M,ATP}$ constant for AgKar3MD (1.6 μM), were significantly lower than those determined for ScKar3MD. Since our ATPase measurements indicated a fairly low $K_{M,ATP}$ for AgKar3MD, it is unlikely that defective ATP binding is responsible for the slow turnover. Instead, elements that compose the ATPase pocket and participate in conformational changes required for efficient ATP hydrolysis or products release may be different between AgKar3 and ScKar3. Indeed, subtle differences were observed in the conformation of the ATPase pocket of AgKar3 relative to ScKar3 (Figure 15). At this time we do not fully understand how these differences relate to the unique ATPase kinetics of AgKar3.

As expected, AgVik1 K$^{356}$-A$^{633}$ exhibited minimal ATPase activity compared to AgKar3MD, to the point that it could not be differentiated from background ATP hydrolysis (Figure 27). This is consistent with the lack of nucleotide binding motifs in the primary sequences of both AgVik1 and ScVik1 (Figure 16) [83]. AgVik1 does, however, appear to influence the enzyme activity of AgKar3 in the context of the AgKar3/Vik1 complex (Figure 28). The $k_{cat}$ for the complex was double that of the AgKar3 monomer (0.3 s$^{-1}$ versus 0.167s$^{-1}$) (Table 2 and Table 3), indicating that AgVik1 accelerates ATP turnover in AgKar3. Oddly though, the increase in $K_{M,ATP}$ between the monomer and heterodimer from 1.6 μM to 14 μM indicates that the presence of AgVik1 causes a decrease in the nucleotide-binding and/or turnover efficiency of AgKar3. The $K_{1/2,MT}$ also experienced a slight upshift between the monomer and heterodimer (0.7 μM versus 1.5 μM, respectively), suggesting that the presence of AgVik1 created a greater demand for microtubules in order to stimulate the ATPase activity of AgKar3. However, this difference is small enough to suggest that a Vik1-induced conformational change in the AgKar3 microtubule-
binding region may be the cause of this stimulated ATPase, as opposed to an effect in which Vik1 forms a direct microtubule interaction as was demonstrated to be the case for ScVik1 [83]. Indeed, the $K_{1/2,MT}$ is almost 3-fold lower for ScKar3/Vik1 (1.7 versus 4.2 μM) and the increase in $k_{cat}$ when AgVik1 was bound to AgKar3 was nowhere near the previously observed difference in the maximal rate between the ScKar3 monomer (0.49 s$^{-1}$) and ScKar3/Vik1 heterodimer (3.7 s$^{-1}$) [83].

### 3.5 AgVik1 displays limited microtubule binding compared to AgKar3

In order to gain a clearer understanding of the effects of AgVik1 on AgKar3, a more direct analysis of the microtubule-binding interactions of AgKar3/Vik1 motors, as well as the individual AgKar3 and AgVik1 components, was performed. For these analyses, each kinesin construct was incubated with increasing concentrations of taxol-stabilized microtubules and the amount of microtubule-bound motor protein was measured by SDS-PAGE of the supernatant and pellet fractions after microtubule sedimentation by centrifugation (Figure 29). In the presence of excess ADP (1 mM), the affinity of AgKar3MD for microtubules ($K_{d,MT}$) was approximately 6-fold lower than previously measured for ScKar3MD by Allingham, et al. (1.2 versus 0.22 μM, respectively) [83]. Like other kinesins, addition of the ATP analog AMPPNP (1 mM) dramatically increased the microtubule affinity of AgKar3MD ($K_{d}$ = 0.35 μM), however, this value was still slightly lower than that of ScKar3MD ($K_{d}$ = 0.12 μM)[83](Figure 30A, Table 4). Also, microtubule binding by AgKar3 only achieved a saturation level of approximately 50%, even at 1.25-fold excess of tubulin dimer binding sites, while ScKar3 typically reached 100% at this amount of tubulin.

Unlike AgKar3MD, minimal microtubule binding was observed for the AgVik1 motor homology domain construct (Figure 30B, Table 5). This is in direct contrast to ScVik1, which binds microtubules tighter than ScKar3 ($K_{d}$ = 0.04 μM, 76% bound) [83]. It is also reflective of the
Figure 29 – Representative SDS-PAGE of AgKar3MD and AgVik1 Microtubule-Binding. Pellet and supernatant fractions are shown. (A) Kar3MD. (B) AgVik1 K356-A633. (C) AgKar3 Q244-R709/A633. AgVik1 Y260-A633. Molecular weight marker (MW) and supernatant (S/N) lanes are labeled. Reactions contained 0 – 5 μM tubulin and 4 μM kinesin.
Figure 30 – AgKar3 and AgVik1 Microtubule-Binding Densitometry Curves. (A) AgKar3MD. (B) AgVik1 K356A633. (C) AgKar3 Q244R269/AgVik1 Y266A633. Data is fit to Equation 3, and error bars represent the standard error of the mean (SEM).
### Table 4 - Microtubule binding constants of Kar3 motor domains.

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>AgKar3MD [1]</th>
<th>ScKar3MD [83]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AMPPNP</td>
<td>ADP</td>
</tr>
<tr>
<td>K&lt;sub&gt;d,MT&lt;/sub&gt; (μM)</td>
<td>0.35 ± 0.02</td>
<td>1.2 ± 0.7</td>
</tr>
<tr>
<td>% bound</td>
<td>50 ± 2</td>
<td>50 ± 9</td>
</tr>
</tbody>
</table>

<sup>a</sup>Original work of the author of this thesis. <sup>b</sup>Allingham <i>et al.</i>, 2007.

### Table 5 - Microtubule binding constants of Vik1 proteins.

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>AgVik1MHD</th>
<th>ScVik1[83]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AMPPNP</td>
<td>ADP</td>
</tr>
<tr>
<td>K&lt;sub&gt;d,MT&lt;/sub&gt; (μM)</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td>% bound</td>
<td>7 ± 3</td>
<td>6 ± 2</td>
</tr>
</tbody>
</table>

N.S.: Not significant (p > 0.05) <sup>a</sup>Original work of the author of this thesis. <sup>b</sup>Allingham, <i>et al.</i>, 2007.

### Table 6 - Microtubule binding constants of Kar3/Vik1 complexes.

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>AgKar3/Vik1</th>
<th>ScKar3/Vik1[83]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AMPPNP</td>
<td>ADP</td>
</tr>
<tr>
<td>K&lt;sub&gt;d,MT&lt;/sub&gt; (μM)</td>
<td>0.5 ± 0.2</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>% bound</td>
<td>100 ± 10</td>
<td>97 ± 7</td>
</tr>
</tbody>
</table>

<sup>a</sup>Original work of the author of this thesis. <sup>b</sup>Allingham <i>et al.</i>, 2007.
from the ATPase assay, which indicated that AgVik1 actually increases the amount of microtubules required for AgKar3 to turnover ATP at a half-maximal rate. This suggests that, although AgVik1 binds microtubules very weakly, it has an effect on the microtubule binding of AgKar3 within the AgKar3/Vik1 complex.

Relative to the individual subunits, the AgKar3/Vik1 complex exhibited particularly interesting microtubule-binding properties. Its overall level of microtubule binding at saturating microtubule concentrations was double that of AgKar3MD. Also, while the AgKar3MD bound microtubules much tighter with AMPPNP than with ADP, the AgKar3/Vik1 complex bound microtubules at approximately the same affinity in the presence of either nucleotide ($K_d = 0.5 \pm 0.2 \mu\text{M in AMPPNP versus } 0.3 \pm 0.1 \mu\text{M in ADP}$). This is different from the behavior of the ScKar3/Vik1 complex relative to ScKar3MD, whereby the ScKar3MD binds microtubules tighter in AMPPNP ($K_d = 0.12 \mu\text{M}$), and the ScKar3/Vik1 complex binds microtubules tighter with ADP present ($K_d = 0.038 \mu\text{M}$). In that case, ScVik1 subunits are predicted to form the majority of the microtubule-binding interactions, while the ScKar3 subunit is merely tethered to the microtubule by ScVik1.

3.6 AgKar3/Vik1 is a microtubule depolymerase

As described in Chapter 1, some kinesins are able to utilize energy derived from ATP turnover to catalyze depolymerization of the microtubule [19]. Because microtubule polymers scatter more light than microtubule subunits ($\alpha/\beta$-tubulin dimers) at 340 nm, we used light scattering to monitor the kinesin-dependent depolymerization of microtubules (Figure 31). In this assay, GMPCPP-stabilized microtubules are rapidly diluted into the reaction mixture containing kinesin and ATP. Although there is some dilution-based depolymerization observed, there is no concern of this event removing the GTP-capped region of the microtubules before the kinesin can
Figure 31 – AgKar3/Vik1 Microtubule Depolymerization Curves. Concentrations indicate the amount of purified AgKar3 Q^{244-709}/AgVik1 Y^{260-633} complex. Units of light scattering are in counts per second (cps).
induce depolymerization because of the presence of the slow hydrolyzing GTP analog GMPCPP. Thus, active depolymerization of the microtubule contributed by the addition of kinesin will result in an enhanced decrease in light scattering signal beyond that of the 0 μM kinesin baseline. Our results indicate that the AgKar3/Vik1 heterodimer is capable of depolymerizing microtubules in a concentration-dependent manner (Figure 31). Both the initial rate of depolymerization and the total magnitude of microtubule depolymerization are increased as higher concentrations of kinesin are included in the reaction.

3.7 AgVik1 is structurally unique from ScVik1

Before the X-ray crystal structure of the ScVik1 motor homology domain was determined, there was little basis with which to understand how it was able to influence the microtubule-binding and catalytic properties of ScKar3 because it shared no significant sequence similarity with any other known protein. Once it became apparent that ScVik1 possessed a kinesin-like structure, and was likely a descendent of an ancient Kinesin-14, more constraints could be placed on models developed to predict its mode of operation as part of the ScKar3/Vik1 motor assembly (Figure 7). In order to gain a molecular description of AgVik1, many attempts were made to crystallize a variety of different constructs that included the C-terminal globular region of the protein (Figure 19). Although the expression level and solubility of these constructs was of an acceptable degree for protein crystal screening, no crystals were obtained.

An additional strategy to produce crystals involved appending a Maltose Binding Protein (MBP) tag to the N-terminus of AgVik1 constructs (L^{375}-A^{633} and T^{353}-A^{633}). MBP is a well characterized protein that is commonly used as a fusion partner to improve the yield [136], and has also proven useful for improving structure determination of proteins that present particular
challenges to crystal formation or phase determination [137,138]. In these MBP-Vik1 constructs, a short three-alanine linker was included between MBP and Vik1 to reduce the conformational heterogeneity of the fusion tag in the hopes of improving the chances of crystallization (Figure 32A) [139]. The expression and purity of the MBP-AgVik1 fusion proteins were confirmed by SDS-PAGE analysis (Figure 32B,C), and size exclusion chromatography (Figure 33).

Interestingly, the longer of the two constructs, MBP-AgVik1 T^{353}\text{-}A^{633}, appears in two states (nominally 183 kDa and 64 kDa), which correspond roughly to dimeric and monomeric states (146 kDa and 73 kDa). The formation of a dimer is surprising as the construct contains very little of the predicted coiled-coil forming region of the AgVik1 polypeptide. Unfortunately, no crystals were obtained for either of these fusion constructs.

As an alternative to experimental structural determination, we attempted homology modeling of AgVik1 using the ScVik1MHD structure as a template. The Protein Homology/analogy Recognition Engine V 2.0 (Phyre²) allows users to submit a primary polypeptide sequence, which uses newly developed profile-to-profile matching algorithms to predict the structure of the folded protein based on that of homologous proteins of known three-dimensional structure [140]. For our model, the query sequence was the AgVik1 L^{375}\text{-}A^{633} construct, using the ‘intensive mode’ algorithm. The primary hit for sequence homology was ScVik1 (PDB: 2O0A), and the model relied heavily on this structural template. Phyre² reported a confidence in the model for 233/259 residues of >90% accuracy. According to Kelley and Steinberg, 2009 a confidence match over 90% indicates that the overall fold of the model is almost certainly correct, and that the central core of the model will tend to be accurate [140]. Since the aligned regions of ScVik1 and AgVik1 have greater than 20% sequence identity (26% identity and
Figure 32 – SDS-PAGE of AgVik1-MBP Construct Purification. (A) Construct design of AgVik1-MBP fusion proteins, including full length MBP and a polyalanine linker. (B) MBP-AgVik1 L375-A633 and (C) MBP-AgVik1 T353-A633 constructs were purified by amylose affinity and size exclusion chromatography. Lanes containing molecular weight marker (M), total cell lysate (T), lysate supernatant (S) are labeled. Size exclusion fractions are labeled by the nominal molecular weight of the peak from which they were taken, as they are annotated in Figure 33.
Figure 33 – Size Exclusion Chromatography Profiles of AgVik1-MBP Constructs. (A) MBP-AgVik1 L<sup>375</sup>-A<sup>633</sup> and (B) MBP-AgVik1 T<sup>353</sup>-A<sup>633</sup>, and (C) the molecular weight standard curve used to estimate the protein size in the labeled peaks. Check protein sizes.
38% similarity), peripheral regions of the modeled protein structure will deviate from the true structure minimally [140].

Looking at the Phyre² structure-based alignment and secondary structure predictions, ScVik1 and AgVik1 share high similarity in terms of conservation of secondary structure motifs and overall fold, but show considerable divergence within the putative microtubule-binding regions of the proteins (Figure 34). The predicted 3D model shows that the β5 strand and the α4 and α6 helix of AgVik1 are largely unstructured, or fluctuate between different secondary structure characteristics (i.e. change from α-helix to β-strand or random coil) (Figure 35). Additionally, if we compare representations of the electrostatic surface potentials of the 3D models of the two proteins, we see that this region of AgVik1 has a very different surface charge landscape than ScVik1 (Figure 35C and D). As previously noted, there is a stretch of serine and threonine residues within α4 and loop L12 of AgVik1, several of which align to charged amino acids in ScVik1. These residues may be sites of phosphorylation in AgVik1, which would change their charge and size characteristics in a way that could allow them to resemble some of the acidic residues in the corresponding region in ScVik1. Based on this possibility, a recombinant expression system may not be ideal for analyzing the true nature of AgVik1-microtubule interactions.

The neck docking regions of the ScVik1 and AgVik1 motor homology domains also diverge significantly in terms of their distribution of sites for electrostatic and hydrophobic interactions (Figure 36). While the ScVik1 surface is dominated by patches of positive and no charge, the AgVik1 surface is overwhelmingly negative. This highlights the possibility that the conformational dynamics of AgVik1 may differ from ScVik1 and CgVik1 and may thus communicate differently with AgKar3 during the motile cycle of the AgKar3/Vik1 motor complex.
Figure 34 – Phyre² Secondary Structure Prediction of AgVik1. ScVik1 was used as the structural template. Microtubule binding regions highlighted in Figure 35 are indicated by green bars below the sequence. Insertions and deletions relative to the template are indicated in yellow and red, respectively.
Figure 35 – Structural Model of AgVik1 Putative Microtubule-Binding Regions. Cartoon representations of the putative microtubule binding regions of ScVik1 (PDB: 2O0A) (A) and AgVik1 (Phyre² model) (B) are coloured in green. The electrostatic surface representations of the same ScVik1 (C) and AgVik1 (D) structures are coloured by charge, where red represents negative, blue is positive, and white is neutral. Charges were assigned using the vacuum electrostatics protein contact potential (local) tool in PyMol®. Putative microtubule binding regions and labels for elements of secondary structure were assigned using the multiple sequence alignment in Figure 16.
Figure 36 – Structural Model of AgVik1 Neck Docking Electrostatics. ScVik1 without (A) and with (C) the neck shown. AgVik1 (Phyre² model) without (B) and with (E) the neck shown. The neck was also modeled as an electrostatic surface, and rotated 180° axially to show the surface that interacts with the main body of the motor homology domain (D). The neck region of ScVik1 is shown in cyan, and is modeled into its likely position in AgVik1 through alignment of the two structures.
Chapter 4

Discussion

4.1 Rationale for studying AgKar3 and AgVik1

Despite being closely related to *S. cerevisiae* and other budding ascomycete yeasts, *Ashbya gossypii* never reproduces by budding, but instead grows exclusively as hyphae containing linear arrays of multiple nuclei that can divide asynchronously within the same cytoplasm [115]. In order to maintain a uniform distribution of nuclei in this environment, nuclei must migrate over long distances toward the growing hyphal tip and divide within the hyphae. In *S. cerevisiae* on the other hand, positioning of the nucleus at the mother-daughter bud neck is the only major nuclear movement needed to prepare this fungus for cytokinesis. Therefore, the requirements for orchestrating nuclear dynamics in *A. gossypii* are relatively complex compared with some of its evolutionary cousins. This makes it an attractive source of microtubule-associated motor proteins that may be uniquely adapted to orchestrate mitotic events under different cell shape and polarity constraints from budding yeast. Genetic studies, combined with the high degree of gene synteny between *A. gossypii* and budding yeast, have allowed identification of cytoskeletal molecular motors that are involved in *A. gossypii* nuclear dynamics [141,142]. As described in Chapter 1 of this thesis, Philippsen *et al.* have recently taken advantage of this to study evolutionary diversity in the physiological functions of the microtubule-based motor dynein and the kinesin motors Kip2 (Kinesin-7) and Kip3 (Kinesin-8) in *A. gossypii* compared to *S. cerevisiae* [143,144]. We have taken advantage of this information in a different way to clone, purify, and obtain the first detailed structural and functional description of motor proteins from *A. gossypii*. 
4.2 AgKar3 and AgVik1 exhibit activities that are distinct from their budding yeast counterparts

While the structure of the AgKar3 motor domain exhibits a number of commonalities to S. cerevisiae Kar3, a novel conformation of the nucleotide-binding pocket was observed. The critical salt bridge between Switch I and Switch II is unformed, and the C-terminus of α3, L9, and part of helix α3a of the Switch I region are unstructured. While this certainly does not rule out the possibility that AgKar3 can form states of its active site that are similar to ScKar3, and vice versa, the replacement of an alanine in the bulging L9 loop between α3 and α3a of ScKar3 with a bulky arginine in AgKar3 leads us to speculate that the conformation of this region, and as a result the dynamic properties of Switch I in general, may differ between these motors. Given that the size of the nucleotid-binding pocket is determined critically by the position of Switch I [145], such a difference could account for the discrepancies we observe in the ATPase rate and equilibrium microtubule-binding constants of AgKar3MD and ScKar3MD.

Additional or alternative explanations for the lower ATPase activity observed for AgKar3MD could relate to the differences in the amino acid sequences and structures of loop L1, or the microtubule binding elements L8a, β5a, L8b, β5b, and L12. Indeed, noticeable changes in the arrangement of both of these regions can be seen from superposition of the two structures (Figure 15). The narrowing of the cleft between loop L1 and the ADP ribose as a consequence of restricted torsional freedom near the RxRP motif may be particularly relevant in this regard.

AgVik1 differs prominently from ScVik1 in microtubule binding affinity, and has a different effect on AgKar3 in the context of the heterodimer. While ScVik1 binds microtubules very tightly ($K_d = 0.04 \mu M$), and has a dominant influence on the microtubule binding ability of the ADP-bound form of ScKar3/Vik1, our monomeric AgVik1 construct displays negligible
microtubule-binding. Although we can infer from this that AgVik1 cannot contribute directly to microtubule-binding within the AgKar3/Vik1 complex, the AgVik1 subunit does appear to influence the microtubule-binding affinity of the complex in the ADP-bound state. Indeed, as opposed to AgKar3MD, the AgKar3/Vik1 complex is indifferent to the nucleotide state of the Kar3 subunit ($K_d = 0.4 \, \mu M$), and binds with almost the same affinity as the AMPPNP-bound AgKar3MD monomer ($K_d = 0.35 \, \mu M$).

A number of scenarios can be envisioned to explain these results. One such possibility is that while AgVik1 does not bind microtubules directly, it constrains AgKar3 in a yet to be defined way that helps it maintain contact with the microtubule for a longer period of time. This may allow for AgKar3 to complete an action that requires additional time, such as a power stroke or deformation of the microtubule end to facilitate depolymerization. Another possibility is that complexation of AgVik1 with AgKar3 helps AgVik1 achieve a conformation that is capable of microtubule interaction. Our AgVik1 monomer construct may also be unsuitable for microtubule binding because it is missing a critical element that helps mediate its microtubule interactions. Indeed, we do not know how any of the Vik1 proteins bind microtubules, and thus it is uncertain what components of their structure are most important. The microtubule-binding regions of the Vik1 proteins may vary depending on the particular flavour of the Vik1 protein. Perhaps a section of the neck, beyond the part we used for the AgVik1 K$^{356-633}$ construct is involved in key microtubule interactions. It may also be possible that AgVik1 and/or the microtubule require some form of post-translational modification to achieve authentic microtubule binding. There are several putative phosphorylation sites at serines and threonines along AgVik1 helix α4 that may require yeast kinases that are not found in our E. coli expression system. Lastly, we do not know the extent
to which our bovine brain microtubules differ from those expressed in *Ashbya gossypii*, or if these differences are significant enough to have a meaningful impact on the function of *Ag*Kar3/Vik1.

Although *Ag*Vik1 does not turn over ATP, the Kar3/Vik1 heterodimer has almost double the ATPase rate of the Kar3 monomer (0.30 s\(^{-1}\) *versus* 0.167 s\(^{-1}\)). These rates are quite low compared to the *S. cerevisiae* motors, particularly considering the drastic increase in activity between the *Sc*Kar3 monomer and *Sc*Kar3/Vik1 heterodimer (0.49 s\(^{-1}\) *versus* 3.7 s\(^{-1}\)). As well, the *K*\(_{M,ATP}\) of the *Ag*Kar3 monomer is approximately 9-fold lower than the *Ag*Kar3/Vik1 heterodimer, corroborating the indifference of the heterodimer to the presence of ADP or AMPPNP in the microtubule-binding assays. Moreover, comparing the *K*\(_{1/2,MT}\) of the *Ag* and *Sc* motors also indicates that while the *Sc*Kar3/Vik1 heterodimer requires less microtubules to reach half-maximal activity (1.7 μM) than the *Sc*Kar3 monomer (4.2 μM), the *Ag*Kar3/Vik1 heterodimer actually requires more (1.5 μM) than the *Ag*Kar3 monomer (0.7 μM). This is reminiscent of the ATPase cycle of MCAK, where the motor is unable to release the products of ATP hydrolysis until the tubulin heterodimer has been released from the microtubule end. Because MCAK moves along the microtubule via ATP-independent lattice diffusion, and it is only active at the end of the microtubule [53], there are a limited number of sites where ATP hydrolysis can occur. Perhaps the *Ag*Kar3MD requires less microtubules to become activated because it is able to use the whole microtubule, while the *Ag*Kar3/Vik1 complex requires more microtubules because it is only activated at the ends of the microtubules.

### 4.3 What is the function of *Ag*Vik1 in the motile cycle of *Ag*Kar3/Vik1?

Recent studies on *Sc*Kar3/Vik1 have led to the proposal that the *Sc*Vik1 subunit binds the microtubule initially, at a point when the *Sc*Kar3 subunit is ADP-bound, but then releases from the
microtubule once ScKar3 communicates that it has bound both ATP and the microtubule [83]. ScKar3 is then able to turn over ATP, and detach from the microtubule. Our data do not support the use of this type of model for AgKar3/Vik1 and demand a different form of explanation. This is because a kinesin motor that has the same affinity for the microtubule, whether ADP- or ATP-bound, cannot move in the conventional manner, and yet AgKar3/Vik1 appears to possess microtubule-based motility. The difficulty in understanding how this is possible revolves around the lack of definitive information on the conditions that would release the AgKar3/Vik1 motor from the microtubule. There are, however, some functional parallels that can be drawn to the Kinesin-13 microtubule depolymerase MCAK that may fill in part of the story. MCAK binds the microtubule ends in either the ATP or ADP·Pᵢ state, and releases the tubulin heterodimer in the nucleotide-free state after the depolymerization reaction has completed [5,19,55]. Perhaps AgKar3/Vik1 follows some of these mechanistic principles more closely than it does those of ScKar3/Vik1.

4.4 The AgKar3/Vik1 complex is a robust microtubule depolymerase

Ashbya gossypii Kar3MD and Kar3/Vik1 both have lower ATPase rates compared to their Saccharomyces cerevisiae counterparts. Lower ATPase rates are loosely linked to kinesins that act as microtubule depolymerases. The Kinesin-13 MCAK has a relatively low ATPase rate until it finds the end of the microtubule and begins depolymerizing it and hydrolyzing ATP very rapidly. Considering that our ATPase rates were determined using our 40 μM Taxol-stabilized microtubules, they may not reflect the true ATPase activity of the AgKar3/Vik1 complex because the high concentration of Taxol hinders depolymerization, and therefore the ATP hydrolysis cycle. Our AgKar3/Vik1 protein has shown depolymerization of 1 μM GMPCPP-stabilized microtubules, and stabilization with the GTP analog may better simulate the native state of the microtubule. We
can infer from its ability to remove GMPCPP-tubulin that AgKar3/Vik1 is capable of removing the GTP-cap of microtubules in vivo, just as MCAK has been shown to do.

ScKar3/Cik1 is also a microtubule depolymerase [88,90], while ScKar3/Vik1 exhibits negligible depolymerase activity [83]. We show that AgKar3/Vik1 possesses MT depolymerization activity that is more comparable to the robust depolymerase activity of MCAK than to the depolymerase activity of ScKar3/Cik1. ScKar3/Cik1 is incapable of depolymerizing microtubules stabilized by 1 mM GMP-CPP and 40 μM Taxol, and can only depolymerize microtubules that are weakly stabilized by low-molarity (3 μM) Taxol.

Both Saccharomyces cerevisiae and Ashbya gossypii require kinesins that function in the mitotic spindle assembly, and ScKar3/Vik1 [82,87] and at least AgKar3 (in vivo expression and localization of AgVik1 is unknown) [99] are involved. ScKar3 is also involved in nuclear movement during the process of karyogamy (nuclear fusion) after it pairs with ScCik1 [88]. ScCik1 both cross-links and slides microtubules past each other, and depolymerizes them at the plus-end to achieve this nuclear movement [82,87,88]. It is possible that AgKar3’s multifunctionality is achieved by complexing in two different ways: (1) through formation of an AgKar3 homodimer or (2) through formation of an AgKar3/Vik1 heterodimer. It could be that AgKar3 homodimers are responsible for the movement we see in the motility assay involving the lysate of AgKar3/Vik1 overexpressing cells, possibly moving in a manner similar to Ncd. If that is the case, AgVik1 may be responsible for adapting AgKar3 to depolymerase activity. The possibility also exists that it could have both motility and depolymerase activity, similar to ScKar3/Cik1.

A. gossypii presents challenges to its kinesins that are different from that of S. cerevisiae. Multiple nuclei divide asynchronously, and mitosis must be coordinated meticulously to avoid over- or underpopulation of nuclei. S. cerevisiae mating involves nuclear movement and fusion,
but this takes place over the scale of two fusing yeast cells. *A. gossypii* nuclear movement requires kinesins and dyneins to move and depolymerize microtubules across the extended length of the hyphae, and throughout the mature stage of mycelium growth. Perhaps the *Ag*Kar3/Vik1 motor has adapted to this task by reducing the amount of ATP it uses per second, thus improving the efficiency of its motility and depolymerase functions. While karyogamy requires microtubules to be shortened as nuclei are pulled together, the sustained nuclear movement within *A. gossypii* would require the depolymerase activity of its kinesins to be balanced by phases of microtubule growth, such that nuclei would not be pulled too close to the cell cortex. Indeed, stunning videos (Amy Gladfelter Lab, Dartmouth University) of *A. gossypii* growth, nuclear movement, and mitosis show that *A. gossypii* motor proteins are up to the task [146,147].

4.5 Caveat

Although we believe our results legitimately represent the enzymology and biophysics of our *E. coli* expressed motor proteins, we cannot ignore the fact that our recombinant system may not allow for an accurate depiction of the authentic activity of *Ag*Vik1. There are putative phosphorylation sites along loop 12 and helix α4 in the motor homology domain of *Ag*Vik1, which potentially require yeast kinases to perform the modification. As well, there are a high level of conserved residues in the neck region, between *Sc*Vik1 and *Ag*Vik1. This level of conservation indicates that a rotation mechanism governs the neck position and intersubunit communication, much like what is hypothesized in the *S. cerevisiae* model [83], and this rotation would have to relate to microtubule binding. To address these concerns, our constructs would ideally have to be cloned into and expressed by a yeast expression system, such as *Pichia pastoris*, to simulate the native post-translational modifications of the *Ashbya gossypii* protein. An easier method would
involve site-directed mutagenesis of our existing recombinant constructs to change the serine and threonine residues over to larger, charged residues.

4.6 Conclusions

There are several questions surrounding AgVik1 that should be addressed in order to fully understand its function within the AgKar3/Vik1 complex. Phosphorylation of putative kinase sites within the AgVik1 motor homology domain may cause a gain of function in microtubule-binding activity. Expression of AgVik1 in a yeast system such as Pichia pastoris would closely simulate the post-translational modifications native to A. gossypii. Alternatively, site-directed mutagenesis could be used to simulate phosphorylated serine and threonine residues by replacing them with negatively charged residues. Repeating the microtubule-binding and ATPase assays with these modified constructs would determine if phosphorylation is a necessary component in the regulation of AgVik1. It is also possible that the bovine microtubules used in our studies have a phosphorylation status distinct from A. gossypii microtubules.

We must also consider that there may be accessory proteins other than AgVik1 that associate with and alter the activity of AgKar3. AgVik1 was discovered through a search of syntenic homologs, since standard sequence homology searches failed to turn up the protein, and other potential AgKar3-associated proteins may be hiding deep within the A. gossypii proteome. As bioinformatics techniques progress, and high throughput screening methods improve, another Vik1/Cik1-like protein or something more similar to the KCBP-interacting protein (KIC) may be uncovered.

We have attempted to crystallize AgVik1 in a multitude of conditions, including with a Maltose Binding Protein tag, and yet the protein has proven to be recalcitrant to crystal formation.
ScCik1 has presented a similar predicament to crystallographers for many years now, and based on the significant level of sequence similarity between AgVik1 and ScCik1, it is reasonable to conclude that there is a common cause of poor crystallizability among the Cik1-like Kar3 accessory proteins. We speculate that these proteins are at least partially intrinsically disordered, much like the majority of kinesin tail domains [30]. The current dogma is that proteins fold to gain function, but it may be more fluid than that. Keith Dunker, a pioneer in the field of intrinsically disordered proteins, posits that intrinsic disorder is more common than biochemists and structural biologists tend to believe. Bioinformatics software produced by his research group predicts that about 40% of human proteins are intrinsically disordered in at least one segment of 30 or more amino acids, and approximately 25% are completely disordered [32]. Indeed, we have attempted to purify Candida glabrata Cik1, and were hindered by overexpression of inclusion bodies and unsuccessful attempts at protein refolding, suggesting severe levels of intrinsic disorder. While AgVik1 is at least stable enough to remain in solution, even moderate levels of intrinsic disorder along the surface of the protein would make crystallization all but impossible without drastic attempts to stabilize the protein through mutations and chemical modifications. Future studies that apply circular dichroism spectroscopy to AgVik1 may prove useful to determine the extent of disorder within the AgVik1 motor homology domain.

An NMR solution structure of the AgVik1 motor homology domain, which is near to the current size limitations of NMR, may provide a means to better understand the structural nature of this protein, but to this point no such structures of kinesins have been determined by this method. Intrinsic disorder of AgVik1 may be reduced or eliminated in vivo through post-translational modification, or perhaps upon binding to its AgKar3 partner, much like the DNA-binding protein CREB which is disordered until it binds its partner CBP [148], and thus co-crystallization of
AgKar3 with AgVik1 will be attempted in the future. However, it should be noted that crystallization of the AgKar3/Vik1 complex presents its own challenges of large size and flexibility of the long stalk region in relation to the motor domain.

In summary, *A. gossypii* has the specific challenges of coordinating asynchronous mitotic events within a single cytoplasm, and moving nuclei in an ordered fashion during extension of the hyphal tip. The AgKar3/Vik1 motor assembly may be a new form of Kinesin-14, whose structural and mechanistic properties add to the repertoire of kinesin isoforms, and highlights the possibility for more surprises in the realm of molecular motors. At this time, we do not know if the structural and mechanistic differences we observe reflect physiological differences in the function of this protein in mitosis and nuclear movement in *Ashbya gossypii* relative to budding yeast because its specific roles in these events await characterization. By comparing multiple structures of these types of kinesins from other fungal forms, and carefully analyzing their mechanochemical properties and physiological functions, it should be feasible to identify patterns of conserved interactions and structural properties that are crucial for specific transport challenges.
References


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104. DeMay, B. S., Meseroll, R. A., Occhipinti, P. & Gladfelter, A. S. Regulation of distinct septin rings in a single cell by Elm1p and Gin4p kinases. Mol Biol Cell 20, 2311-2326 (2009).


