The regulation of KIF3A motor subunit by phosphorylation

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NANYANG
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**THE REGULATION OF KIF3A MOTOR SUBUNIT
BY PHOSPHORYLATION**

CHEN KUNNING

SCHOOL OF BIOLOGICAL SCIENCES

2018

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Abbreviations

ADP	Adenosine diphosphate
APC	Adenomatous Polyposis Coli
ATP	Adenosine triphosphate
CaMKII	Calcium/calmodulin-dependent protein kinase II
CDK1	Cyclin-dependent kinase 1
CLSM	Confocal laser scanning microscope
Cryo-EM	Cryo-electron microscopy
ECFP	Enhanced cyan fluorescent protein
EYFP	Enhanced yellow fluorescent protein
FLIM	Fluorescence-life time imaging microscopy
FRET	Förster resonance energy transfer
GFP	Green fluorescent protein
GST	Glutathione-S transferase
HRP	Horseradish peroxidase
IP	Immunoprecipitation
ICK	Intestinal cell kinase
IFT	Intraflagellar transport
JNK	c-Jun N-terminal kinase
KAP3	Kinesin-associated protein 3
KIFs	Kinesin superfamily proteins

MBP	Maltose-binding protein
MD	Molecular dynamics
MT	Microtubule
NMR	Nuclear magnetic resonance
PAK	P21 activated kinase
PH domain	Pleckstrin homology domain
PIX	PAK-interacting exchange protein
PKA	Protein kinase A
POPX	Partner of PIX
SAXS	Small-angle X-ray scattering
siRNA	Small interfering RNA
SPR	Surface plasmon resonance

Abstract

The Kinesin-2 family member KIF3 forms a heterotrimeric complex comprising the motor subunits KIF3A, KIF3B and the cargo carrying subunit KAP3. N-cadherin and β -catenin are reported to be cargoes of KIF3 motor. Serine-689 residue at the C-terminal of KIF3A has been identified as a major phosphorylation site significant for the transport of cargoes. In an attempt to find out the effect of phosphorylation on KIF3A's function, serine-689 was mutated to alanine (S689A) to preclude phosphorylation, and to aspartic acid (S689D) to mimic continuous phosphorylation. In this study, we showed that overexpressing S689A mutant impaired the transportation of N-cadherin and β -catenin to the cell-cell junction, indicating that the phosphorylation status of serine-689 residue plays a crucial role in the intracellular KIF3-mediated cargo transport. We also identified that the interaction between the KIF3A motor and tail domain was relieved by Calcium/calmodulin-dependent protein kinase II (CaMKII) phosphorylation *in vitro* and *in vivo* by different assays. Our results also suggest that KIF3A's ATPase activity was inhibited by the motor-tail interaction. Phosphorylation on serine-689 residue relieves the motor-tail interaction to allow recovery of the motor's ATPase hydrolysis activity. Computational simulation of KIF3A motor-tail complex under different phosphorylation states also supports our observations.

Based on these findings, we hypothesize that KIF3A adopts a folded

conformation resulting in an autoinhibited state, and phosphorylation on the serine-689 residue at the C-terminal tail domain of KIF3A subunit will relieve this autoinhibition and induce a conformational change to expose the microtubule and ATP binding regions at the motor domain. The activated KIF3 motor can thus slide on microtubules to transport its cargoes. Detailed structural information is needed to explain KIF3A's mechanisms. In this study, a low-resolution structure of KIF3A's motor domain was reconstructed by Small-angle X-ray scattering (SAXS) data. The screening of the proper protein crystal growing conditions for X-ray crystallography is ongoing. Promising conditions for crystallization will be discussed.

Chapter 1 Introduction

1.1 Kinesin superfamily proteins

Intercellular transportation is an important process to designate large molecules, intracellular vesicles and organelles to the correct cellular locations where they are needed. The cytoskeletal molecular motors functioning in intercellular transportation are dynein, kinesin, and myosin superfamilies. There are two major groups of dynein superfamily, cytoplasmic dyneins and axonemal dyneins. Axonemal dynein regulates the microtubule sliding in the axonemes of cilia and flagella. Cytoplasmic dynein was found in animal and plant cells, acting as molecular motors for organelle transport and centrosome assembly (1). Dyneins hydrolyze ATP to generate movement along microtubules towards the minus-end (2), whereas kinesins (discussed in later sections) generally move along the microtubules in the plus-end direction. Myosin was originally identified as a motor protein in muscles controlling muscle contractions (3). To date, 35 different classes of myosins have been classified (4). Myosins functions in a variety of cellular activities, such as cytokinesis, muscle contraction and vesicle transportation. Similar to kinesins and dyneins, myosins have motor domains that hydrolyze ATP to generate the moving force. Myosin moves along actin filaments, while dynein and kinesin move on microtubules (1).

1.1.1 Classification and structures of kinesin superfamily proteins

To date, 45 kinesin superfamily members have been identified and classified into 15 families, which are termed kinesin 1 to kinesin 14B, based on phylogenetic analyses (Fig. 1.1a) (5). Kinesin family members share similar characteristics. They are composed of a motor domain which binds and moves along microtubule tracks by the energy released from ATP hydrolysis, a tail domain that binds to cargoes, and a coiled-coil stalk region that links the two domains. The motor domains are well conserved among different species while the tail domains and the stalk regions are highly variable (6). Based on the different positions of the motor domains on kinesins, kinesin families are also grouped into three types: N-kinesins have motor domains located at the amino-terminal region, C-kinesins have their motor domains located at the carboxyl-terminal region and M-kinesins' motor domains are located in the middle (Figure 1.1b) (7). It has been reported that N-kinesins move along microtubules in a plus-end direction while C-kinesins drive a minus-end directed movement. M-kinesins are responsible for microtubule depolymerization. Most of the kinesin families are N-kinesins. The only M-kinesin that has been found is KIF2A. KIFC2 and KIFC3 are the currently identified C-kinesins.

Figure 1.1 The phylogeny and structure of mammalian kinesin superfamily (5). (a) The kinesin superfamily (KIFs) is classified into 15 subfamilies. (b) The domain structures of some well-studied kinesins are illustrated. In general, kinesin is composed of a motor domain, a coiled-coil domain and a tail domain. Other gene specific domains such as the pleckstrin homology (PH) domain, the CAP-Gly domain and the WD40 repeats are also found in specific kinesins. The three groups of kinesins, N-kinesins with the motor domain located at the amino terminus, C-kinesins with the motor domain located at the carboxyl terminus and the M-kinesins with the motor domain located at the middle are also shown. aa, amino acids; PX, Phox homology.

1.1.2 Mechanisms of kinesin intracellular transport

Kinesin motors play important roles in intracellular transportation. Cargo transportation by kinesin motors in neurons, cilia and flagella are widely

studied in the past decades. Various cellular proteins, organelles and macromolecules are transported along cilia and flagella by intraflagellar transport (IFT). Kinesins have been reported to be essential for synapse generation. Kinesin-3 family KIF1A and KIF1B β motors play major roles in transporting synaptic vesicle precursors (8, 9). Kinesin-1 family member KIF5 motor has been reported to be responsible for the transport of several axonal and dendritic cargoes (10, 11). Kinesin-2 family motors KIF3 and KIF17 are found to be involved in IFT (12, 13). Due to the important roles played by kinesins in physiology, malfunctions of KIFs have been linked to pathogenesis of diseases. KIF3 motor complex has been reported to function as a tumor suppressor (14). KIF1A, KIF1B β , KIF5A, KIF2A, KIF17 and KIF21A are reported to be responsible for neuronal and brain development and functions, and related to nervous system diseases (15-20). Kinesin-1, KIF13B and KIF12 are found to play unexpected roles in the pathogenesis of metabolic diseases (21, 22), and KIF12 was implicated in type-2 diabetes (23).

In general, kinesins associate with their cargoes via their tail domains, but some specific kinesins such as KIF3 associates with its cargo through adaptor protein KAP3 (24). The regulation mechanisms of cargo transportation have been gradually revealed by recent studies. Kinesins are found to be phosphorylated and their functions can be regulated by phosphorylation. Two kinases, protein kinase A (PKA) and glycogen synthase kinase 3 (GSK-3) have been reported to inhibit the association of KIF5 and its cargoes (25, 26). It has

been found that when KIF5 was phosphorylated by c-Jun N-terminal kinase (JNK), the binding between KIF5 and microtubule became weaker (27). The Ca^{2+} /calmodulin-dependent protein kinase CaMKII has also been reported to release KIF17 from cargo binding (28). Rab GTPase has also been shown to be a regulator of kinesin-cargo association and dissociation. There have been reports showing that GTP-bound forms of Rab3, Rab5 and Rab6 can recruit specific kinesin motors for the transport of certain cargoes (29-31). Ca^{2+} signaling has also been reported to regulate kinesin activity in a way that when high Ca^{2+} was added, the motility of KIF5 was inhibited (32).

1.1.3 Kinesins regulated by autoinhibition mechanism

Kinesin's activity has been reported to be regulated in many ways. Common regulatory mechanisms include the control of expression and activation of kinesins during cell cycle by GTPases and kinases (33, 34). Another regulatory mechanism that has been widely studied is the autoinhibition mechanism (35). Autoinhibition has been found to be a general model for many kinesin families. In an autoinhibited state, kinesins form folded, compact conformations that cannot hydrolyze ATP or associate with cargoes or microtubules. When activated, kinesins are relieved from autoinhibition and transformed into active, extended configurations. Kinesin-1, kinesin-2 family members OSM-3 and KIF17, Kinesin-3 family member KIF1A and Kinesin-7 family member Cenp-E have all been reported to adopt autoinhibited conformations by

single-molecule analysis, biochemical assays, sedimentation assays and FRET (36-42). All reported models of autoinhibition involve the interaction of the motor domain with other regions of the kinesins leading to a conformation that blocks the motor's ATPase activity and microtubule binding. The tail peptide of Kinesin-1 has been reported to inhibit its motor domain's ATPase and motility properties (36, 43, 44). Direct interaction of C-terminal tail domain with the motor domain has also been observed in KIF17 and Cenp-E (40, 45). KIF1A and KIF13b (also known as GAKIN), on the other hand, use their internal segments such as FHA (forkhead-associated domain) and coiled-coil domains to interact with the motor domain (41, 42, 46).

Currently there are two models, cargo binding and phosphorylation, that explain the relieve of autoinhibition. Kinesin-1 stays in a folded, inactive conformation in cargo-free state. Cargo binding on Kinesin-1 monomer relieves the autoinhibition and activates its MT gliding motility (47). Cargo binding also activates KIF1A by driving the monomeric motors to form dimers, thereby activating its processive motility (48, 49). For KIF11 and Cenp-E, however, autoinhibition is reported to be relieved by phosphorylation. Phosphorylation of threonin-937 on KIF11 by CDK1 (cyclin-dependent kinase-1) activates the motor and increases its MT-binding efficiency (50). Phosphorylation by TTK (dual specificity protein kinase) or CDK1-cyclin B causes Cenp-E to unfold into an extended conformation and increase its processive motility along microtubules (40).

1.1.4 Approaches to studying kinesins

In recent years, many approaches have been applied to uncover the moving patterns of kinesin motors. Cargo transportations along microtubules in cilia or axons are tracked by immunostaining using fluorescence microscopy (51). The gliding motility assay has also been a good tool to visualize individual kinesin motor's movement (52). In the past 3 decades, 60 structures of kinesins from 9 kinesin families have been determined by X-ray crystallography and high-resolution cryo-electron microscopy (53). The structural details have provided us deeper understandings of kinesins, such as cargo binding, kinesin-MT binding, ATP hydrolysis mechanism, as well as the processive moving mechanisms of the kinesin motors. Using Cryo-EM, Kinesin-1 motor domain has been shown to adopt a docked conformation when binding to microtubules in the presence of ATP and undergo a conformational change in the presence of ADP or in the absence of nucleotide (54). X-ray crystallography studies have also revealed the conformational change between ATP and ADP bound states of kinesins. X-ray crystallography structure reconstructions help us to discover the roles of the highly conserved nucleotide binding motif switch-1 and microtubule binding motif switch-2 in the kinesin motors during conformational changes (55, 56). Several models describing kinesin's moving modes have also been proposed based on the findings from cryo-EM, X-ray crystallography, as well as the gliding motility assay. The most widely accepted model is the processive moving mode called

the “hand-over-hand” walking model (57, 58). In this model, the kinesin motor move along microtubules step by step with at least one motor domain attaching to the microtubule, while the other one moves to the next binding site. The energy for processive moving is generated by the hydrolysis of ATP. However, Ncd, a C-kinesin, adopts a “hopping” model, where its stalk/neck acts as a rigid lever to generate a power stroke for its movement (59, 60). In addition, KIF1A has been reported to move in a recessively biased diffusion way by single-molecule motility assay (61). In this study, they demonstrated that the highly positively charged K-loop of KIF1A binds to the highly negatively charged E-hook of tubulin. This binding functions as an anchor for the one-dimensional Brownian movement of single KIF1A on microtubules.

The studies of the kinesin structures have also helped us to understand their regulation mechanisms. The crystal structure of Kinesin-14 motor protein Kar3 (CaKar3) fused with a maltose-binding protein (MBP) showed the details of the MBP-Kar3 interaction (62). Single-molecule analysis combined with the crystal structure of Kinesin-1 motor domain in complex with the tail domain have revealed the autoinhibition mechanism of Kinesin-1 (63, 64). The structures of Kinesin-5 complex ligated with potential drug targets have helped to push anticancer drug development (65). With more detailed kinesin structures determined, we can unveil the whole picture of the kinesin superfamily in the future.

1.1.5 KIF3 heterotrimeric motor

KIF3 is a family member of the Kinesin-2 family. Unlike the homodimeric KIF17 (66), which is another member of Kinesin-2, KIF3 forms a heterotrimeric complex. The KIF3 heterotrimeric complex is composed of two motor subunits—KIF3A, KIF3B and a non-motor subunit, kinesin-associated protein KAP3 (8) (Fig. 1.2). Molecular structure analysis has shown that the globular motor domains of KIF3 motor complex is formed by the N-terminal 350 amino acids of the KIF3A/B, spanning about 10 nm. Amino acids 351-597 form the α -helical coiled-coil stalk region, with a length about 30 nm. The C-terminal tail domain (598-701 aa) associates with the 752-amino acid KAP3. KAP3 contains 9 armadillo (arm) repeats in the central region. The KIF3 heterotrimeric complex is estimated to be around 50 nm in length (67). Another KIF3 family member KIF3C is found to partly associate with KIF3A, but not KIF3B, and functions both in complex with KIF3A or independently from the KIF3 motor complex (68).

Figure 1.2 Schematic of the KIF3 motor. The heterotrimeric KIF3 complex comprises KIF3A, KIF3B motor subunits and KAP3 cargo binding subunit.

1.1.4.1 *Biophysiological functions of KIF3 motor*

Based on genetic approaches and the reconstitution of holoenzymes from recombinant subunits, Kinesin-2 motor has been identified in diverse organisms, including algae, nematodes, insects, echinoderms and vertebrates (69-72). Previous findings have shown that the KIF3 motor plays important roles in mitosis (73), cilia assembly and ciliogenesis (74, 75), human immunodeficiency virus (HIV) life cycle in primary macrophages (76) and neuroepithelium development (77). KIF3A has also been reported to regulate microtubule dynamics and orientation, and guide lumen formation in MDCK cells (78). KIF3B is mainly found in the nervous system (79) and has been identified to function in mitosis (73, 80). KIF3C is reported to be required for axon growth and regulate the microtubule cytoskeleton in axon growth cones (81).

A well-studied function of the KIF3 motor is the KIF3-driven anterograde IFT of different cellular complexes during cilium biogenesis. KIF3 motor is required to deliver the axoneme precursors to the axoneme tip and transport ciliary membrane-associated signaling complexes (82, 83). KIF3 motor serves significant functions in tumorigenesis as well. Several cancer-related proteins such as the PAR-3 polarity complex, N-cadherin, β -catenin and adenomatous polyposis coli (APC) have been identified as cargoes of KIF3 motor (84, 85). KIF3 cargoes such as N-cadherin and β -catenin have been shown to associate

with KIF3 motor by interacting with the cargo-binding subunit KAP3 (84). APC and β -catenin are transported and accumulated at the tips of membrane protrusions to regulate cell migration (86). KIF3A subunit is reported to interact with PAR-3, a cell polarity protein, to form a complex functioning in the establishment of neuronal polarity (13, 85).

It has been reported that loss of KIF3 leads to early development defects (87). There have been many studies showing that silencing or knocking out KIF3A or KIF3B motor subunits cause defects in ciliogenesis and motility (88), Hedgehog signaling (89, 90), left-right asymmetry in mouse development (91), axoneme formation and photoreceptor axoneme maintenance in mouse retina progenitors (92), as well as dental mesenchymal stem/precursor cell differentiation (93). Silencing *KIF3A* also accelerates renal cyst formation and the malignant potential of lung cancer cells (94, 95).

1.1.4.2 Stepping models of KIF3 motor

Studies have shown that Kinesin-2 is less processive along microtubules compared to Kinesin-1, and the processivity is load-dependent. Force-clamp measurements done by *Andreasson et al.* found that the run length of KIF3A/B is sharply reduced under load (96). By studying the homodimeric mutants KIF3A/A and KIF3B/B, the KIF3A/B stepping model can be deduced. In their model, Kinesin-2 strongly binds to microtubules in ATP or nucleotide-free

states, and detaches from microtubules in the ADP state. Interestingly, Kinesin-2 stays a longer time in a low-affinity state and occasionally slips backward, then rapidly reattaches to the microtubules and continues stepping. *Chen et al.* have characterized the entire kinetic cycles of the KIF3A homodimer by stopped-flow and steady-state kinetic experiments, as well as single-molecule and multi-motor motility assays (Fig. 1.3) (97). The hydrolysis cycle begins with the ADP-bound motors in solution (state 1). Then one motor domain binds to the microtubule in a weak binding state (state 2). Following the release of ADP, the motor-MT binding converts to a strong binding state (state 3). ATP binding (state 4) then triggers the neck linker docking, followed by ATP hydrolysis (state 5). Both motor domains then bind to the microtubules (state 6), following by the tethered head's ADP release (state 7). The rear head detachment (state 3) from state 7 completes the hydrolysis cycle and results in one 8-nm step. ATP binds to the trailing head before it detaches if front head gating is not present (state 8). In this study, they found out that in the weak binding state, KIF3 has a higher microtubule affinity than Kinesin-1 in the corresponding state. The less processivity of KIF3 might be because that it spends a larger fraction of its hydrolysis cycle in the weak binding state. To understand the conformational change in different nucleotide states, detailed structure information is needed.

Figure 1.3 KIF3A/A chemomechanical cycle model (97). KIF3A/A motor in solution (state 1) binds to microtubule (state 2), triggering ADP release (state 3). ATP binds to the tethered head (state 4) and ATP hydrolysis is triggered (state 5). Both heads bind to microtubule (state 6) and the tethered head releases ADP (state 7). The rear head detaches from the microtubule (state 3) or ATP binding to the tethered head (state 8) to complete the cycle.

1.1.4.3 KIF3A subunit

The KIF3 subunit KIF3A is composed of an N-terminal motor domain (1-350 aa), a C-terminal tail domain (598-701 aa) and a coiled-coil stalk region (351-597) linking the motor and tail domain (Fig 1.4). KIF3A's activity can be regulated by phosphorylation. Intestinal cell kinase (ICK) has been reported to be a KIF3A kinase and phosphorylation of KIF3A at threonine-674 residue is essential for proper ciliogenesis (98). Protein phosphatase Dusp26 binds KIF3A and dephosphorylates KAP3. Overexpression of Dusp26 increases the amount of N-cadherin and β -catenin transported to the cell periphery, thereby promoting cell-cell adhesion (99). Serine-690 residue on the C-terminal tail of

human KIF3A has been identified as a major phosphorylation site (100-102).

For the mouse KIF3A, the serine-689 is the equivalent of human serine-690.

Figure 1.4 KIF3 subunit KIF3A. KIF3A is composed of the N-terminal motor domain (human 1- 354 amino acids, mouse 1-350 amino acids), a coiled-coil stalk region and the C-terminal tail domain (human 601-702 amino acids, mouse 598-701 amino acids). Serine-690 of human KIF3A and serine-689 of mouse KIF3A is a major phosphorylation site.

1.1.4.4 Regulation of KIF3A by phosphorylation

Earlier studies from our laboratory suggested that phosphorylation status of KIF3A could regulate the function of KIF3 motor and that CaMKII and POPX2 are the kinase and phosphatase pair that is responsible for regulation of phosphorylation (103). Ca^{2+} /calmodulin-dependent protein kinases (CaMKs) are known to play key roles in the activity-dependent protein phosphorylation events during neuronal transmission, gene expression, synaptic plasticity and cytoskeletal remodeling. The catalytically active members of CaMKs that have

been known are CaMKI, CaMKII, CaMKIII, CaMKIV and CaMKK (104). The structure and regulatory mechanisms of the multifunctional CaMKII have been thoroughly studied. CaMKII is a serine/threonine kinase composed of an NH₂-terminal ATP-binding catalytic domain, a COOH-terminal self-association domain, a variable domain and a regulatory domain that contains the Ca²⁺/calmodulin (CaM)-binding domain and an auto-inhibitory domain (Fig. 1.5A) (105). Under basal state, the substrate binding pocket is occupied by the auto-inhibitory domain, making CaMKII stay in an autoinhibition inactive state. Binding of Ca²⁺/CaM to the regulatory domain then triggers a conformational change in which the auto-inhibitory domain is released from the substrate binding pocket and the Thr-286 residue on the regulatory domain is exposed. The POPX2 phosphatase is alternatively known as PPM1F, CaMKII phosphatase and hFEM-2. POPX2 is a serine/threonine phosphatase belonging to the PP2C family. It dephosphorylates and down-regulates the activities of PAK1 and CaMKII kinases (106, 107). CaMKII was reported to phosphorylate KIF3A on serine-690 residue. POPX2 phosphatase binds to the KIF3 complex by associating with KAP3 and inhibits the trafficking of N-cadherin and Rab11 through dephosphorylation of serine-690 of KIF3A (103). Mutations have been made on serine-690/689 residue to alanine (S690A/S689A) to preclude phosphorylation or to aspartic acid (S690D/S689D) to mimic continuous phosphorylation to study the effect of phosphorylation on serine-690 residue of human species or serine-689 residue of mouse species.

Figure 1.5 CaMKII structure and activation (105). (A) CaMKII holoenzyme and the domain structure. CaMKII monomer is consist of an NH₂-terminal catalytic domain, a regulatory domain that contains the Ca²⁺/CaM binding domain and Thr-286, and a COOH-terminal association domain. CaMKII holoenzyme is formed by twelve to fourteen of the monomers. (B) CaMKII activation. Binding of Ca²⁺/CaM to the regulatory domain triggers the conformation change and release CaMKII from the autoinhibited state. The catalytic domain and Thr-286 are exposed and the autophosphorylation of Thr-286 maintains CaMKII's catalytic activity in the absence of Ca²⁺/CaM, which is termed Ca²⁺/CaM-independent activity.

Different approaches have been applied to study the regulation mechanism of KIF3A in our study. In the following sections, we describe two major strategies, Förster resonance energy transfer (FRET) and small angle X-ray scattering (SAXS) to decipher the KIF3A motor.

1.2 Förster resonance energy transfer (FRET)

1.2.1 Principle of FRET

Förster resonance energy transfer (FRET) is a physical phenomenon that describes the energy transfer between two fluorophores (108). When two fluorophores are close enough (typically in the range of 1-10 nm), the electronic properties will be changed by each other and the transition dipole moment donor fluorophore will transfer excitations to the absorption transition dipole moment of the acceptor fluorophore via electronic coupling (109). The distance range required for energy to transfer between two fluorophores is dependent on their spectral characteristics, but generally is 10-100 Å. The energy transfer efficiency of intermolecular or intramolecular FRET can be measured by attaching fluorophores to specific sites of the molecule. The distance change over macromolecular length scales can thus be monitored by measuring the FRET efficiency (110). (Fig. 1.6).

The FRET donors and acceptors are pairs of fluorophores. The most commonly used FRET pairs are BFP/GFP and CFP/YFP. Blue fluorescent protein (BFP), cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) are all color variants of green fluorescent protein (GFP) (111). The fluorophores are fused to proteins separately, and the excitation at the absorbance wavelength of the donor fluorophore can cause the emission by the acceptor fluorophore due to FRET. The interaction of the two labeled

proteins can then be determined by detecting the change of FRET efficiency.

Figure 1.6 Principle of Förster resonance energy transfer (FRET) (112). A. The absorption of a photon (cyan arrow) causes the excitation of the acceptor from the ground state (S_0) to the first excited state (S_1). Donor decays from the excited state to the ground state via fluorescence (green arrow). FRET is caused by the transfer from the donor excited state to the acceptor non-radio-actively. The decay of the excited state of the acceptor to the ground state results in the emission of fluorescence (red arrow); B. FRET requires overlap (yellow area) between the donor emission (green line) and the acceptor absorption spectra (orange line); C. Relationship between donor-acceptor distance and the FRET efficiency.

FRET signals are obtained by using a confocal laser scanning fluorescence microscope (CLSM), or a microscope with fluorescence lifetime imaging (FLIM), and thereby make the measurements of FRET efficiency (113). The FRET technique has been widely applied to make measurement of the distance between interacting molecules or monitor the conformational change

of molecules. This technique has a major advantage in detecting and spatially resolving protein-protein interactions in living mammalian cells.

Three light microscopy approaches are commonly used to detect FRET (114). The first approach is termed the FLIM-FRET approach (115). In this approach, change of the fluorescence lifetime of the donor molecule caused by FRET interaction is measured by FLIM (fluorescence-lifetime imaging microscopy). The FLIM-FRET approach has the advantage that the FRET measurement will not be affected significantly by the protein concentrations or their diffusion rates. In addition, it can provide information about the mean separation distance of the interacting proteins and the fraction of proteins engaged in the interactions. The second approach is to measure FRET by sensitized emission (116). This approach can be performed by either a wide-field fluorescence microscope or a laser scanning confocal fluorescence microscope. FRET is measured by detecting the increased fluorescence of the acceptor by the excitation of the donor. This measurement is highly influenced by protein concentration, thus subsequent correction and normalization procedures are required. The last approach for measuring FRET is based on acceptor photobleaching (117). The principle is that when the acceptor fluorophore is photobleached, FRET is disrupted, resulting in the increase of the donor fluorescence signal. To perform this approach, the fluorescence signals of the donor before and after bleaching are monitored by a confocal laser scanning fluorescence microscope with the appropriate excitation and emission filter

sets and a high-intensity laser line to perform complete photobleaching. By detecting the fluorescence lifetime (τ_{FRET}) of the donor before photobleaching, and the donor's fluorescence lifetime after photobleaching (τ), which represents the non-interacting donor population, we can determine the E_{FRET} as followed,

$$E_{\text{FRET}} = 1 - \tau_{\text{FRET}}/\tau$$

This approach is more reliable and less complicated than the other two approaches to measure FRET at a predetermined location because only the fluorescence signal of the donor is measured. However, due to protein diffusion, it can only be applied once in a live cell and is not suitable for time-lapse FRET measurements or detailed FRET location mapping.

1.2.2 The application of FRET measurement on kinesin research

Our knowledge of kinesins including their ATPase kinetics, motility and their microtubule-binding properties have largely been obtained from electron microscopy and X-ray structural analysis. These studies have given us insights into the structure and enzymatic functions of kinesins. However, understanding kinesin behavior *in vivo* requires a direct visualization of kinesin processivity. Single-molecule technique can help us to better understand the dynamics in polymers and in other complex environments by directly observing the behavior of single motor protein (118-121). In addition to the single-molecule

technique, FRET can be used to study structural conformational change and the motility of kinesins in their natural environment. The mechanisms resulting in the motility of kinesins such as the hydrolysis of ATP in kinesin stepping (54, 122-131), the interactions of kinesin and microtubule tracks (132), the stepping along microtubules (133, 134) and autoregulation of kinesin activity (38, 135-137) have been studied using FRET approaches (112).

It has been known that conformational change of kinesins drives their motility, therefore the fast and transient structural transitions detected by FRET can provide deeper insight into the overall behaviors of kinesins. FRET has been applied to study the ATP hydrolysis mechanism of kinesins. The fluorescent nucleotide-analogue MANT-ATP or MANT-ADP containing the N-methylanthraniloyl chromophore were used to study the binding and releasing of nucleotides by the kinesin motors. For kinesins that contain tryptophans close to the nucleotide binding site (Kinesin-5 and Kinesin-7), the tryptophan-FRET approach, which detects the FRET between tryptophan and MANT-nucleotides by ensemble steady-state and stopped-flow FRET measurement, has been used to determine the kinetics of ATP-binding (138, 139). FRET technique has also been used to measure the distance changes between kinesin neck linkers and their catalytic cores in different microtubule or nucleotide binding states (54, 132). These studies revealed the connection between the kinesin conformation changes and processive motility.

The affinity between the kinesin motor domains and microtubules in different nucleotide-binding states also determines the kinesin stepping mechanism. The microtubule-bound and unbound states of single-motor or dimeric-motor in different nucleotide-binding states determined by FRET have been used to show the mechanochemical cycle and stepping mechanism of kinesin motor domains (140, 141).

FRET approach has also been used to study the regulation of intracellular transport activities of kinesins. Previous studies have shown that Kinesin-1 exists in two conformations. FRET-based MANT-ADP assays were used to reveal that the tail domain of Kinesin-1 binds to the motor domain when no cargo is bound, and the IAK-homology region in the tail domain inhibits ADP-release by the motor domain, thus inhibiting its activity (44, 142). The autoinhibited mechanism of KIF17, another member of the Kinesin-2 family, has also been studied by monitoring the FRET signals of the KIF17 FRET biosensors. The direct visualization of the extended and compact kinesin in living cells was monitored by detecting the FRET efficiency of mCherry-KIF17-EmGFP using FLIM (143).

1.2.2.1 FRET model for KIF3A autoregulation study

In our proposed study of the autoinhibited mechanism of KIF3, the biosensors with an EYFP fluorophore at the N-terminal and ECFP fluorophore at the

C-terminal of KIF3A were designed. Here we apply the enhanced yellow fluorescent protein (EYFP) and the enhanced cyan fluorescent protein (ECFP) as the FRET fluorophore pairs. When KIF3A is in an extended active state, the two fluorophores are too far away to cause FRET. If KIF3A is adopting an autoinhibited state, where the motor domain interacts with the tail domain, the excitation at the absorbance wavelength of ECFP (458 nm) causes the emission by EYFP (527 nm) (Fig. 1.7). The FRET efficiencies of KIF3A in live cells were determined by the photobleaching approach. We can further determine how phosphorylation status affects the activity of KIF3A by comparing the FRET signals collected from KIF3A wild type and the mutants S689A and S689D overexpressing cells.

Figure 1.7 Model of the KIF3A FRET constructs. When the motor and tail domains are not interacting, the excitation at 458 nm will only cause the emission of cyan at 475 nm. When KIF3A is in an autoinhibited state, EYFP and ECFP come close enough to cause FRET. The emission by EYFP (527 nm) is caused by the excitation at the absorbance wavelength of ECFP (458 nm).

1.3 Small Angle X-ray Scattering (SAXS)

Small angle X-ray scattering (SAXS) is a technique that provides low-resolution structural and physical information about biological macromolecules such as proteins in solid or liquid conditions (144). SAXS is a good tool to study protein folding and functions in solution. The conformations and the behaviors of protein monomers, assembling proteins, multi-domain proteins, as well as the protein-nucleotide complex can be characterized by SAXS.

In SAXS experimental setup, a highly collimated and monochromatic X-ray beam is scattered by the sample. The scattered intensity at very low-angles is recorded by a two-dimensional detector (Fig. 1.8). The beamstop in front of the detector absorbs the transmitted primary beam and the vacuumed flight path before and after the sample prevents the disruption from air. The one-dimensional scattering function $I(q)$ can therefore be integrated by the two-dimensional scattering pattern. We can then extract the information of the sample from Guinier, Fourier and Porod. The radius of gyration of the macromolecule is determined in the Guinier region. Fourier region provides the information regarding the general particle shape. The surface information, the Porod volume, as well as the molecular weight of the macromolecule are determined in the Porod region. The three-dimensional shape of the macromolecule can then be reconstructed from the scattering pattern. The *ab*

initio model delivers the details of the macromolecule structure (145-148).

Figure 1.8 Layout of the conventional SAXS setup (147). The X-ray beam is scattered by the sample, and the scattered intensity at angle θ is recorded by the detector behind the beamstop. q , scattering vector.

X-ray crystallography, nuclear magnetic resonance (NMR), electron microscopy (EM) and SAXS have provided extensive protein structural information at atomic resolution in the past few decades. Comparing to the SAXS experiment's low-resolution imaging, X-ray crystallography, NMR and EM can provide high-resolution protein structure details. However, each technique has its limitations. In the case of performing X-ray crystallography, a highly-organized crystal lattice is required, as the structure parameters need to be collected from specific orientations of the protein sample (149). However, the flexible regions inside the protein might lead to poor crystal quality or no crystal forming at all. The crystals of the multi-domain proteins or protein complexes are also hard to obtain. SAXS however, can provide the overall structural information of the protein in solution, even the ones with flexible regions and protein complexes, without complex sample preparation steps. Similar with SAXS, NMR also provides the protein information on their

dynamics and flexibility in solution. However, the protein molecular size NMR can analyze is limited below 50 kDa (150). Cryo-EM is also an efficient technique to obtain high-resolution protein structures. However, cryo-EM can only be applied on large-sized proteins and cannot be used to study the dynamic configuration changes of proteins. In these cases, SAXS can compensate the limitations of these techniques. By combining all these techniques, we can characterize the detailed structures, dynamics and interactions of protein monomers or complexes in different states (151).

SAXS has been widely utilized to study the structures of both biological and non-biological samples (152-154). The SAXS data can provide us the parameters of the proteins or nucleic acids in solution, and help us to understand their properties and dynamics. To understand the protein conformations or functions, SAXS has been applied to identify the conditions for the proteins in which they form a native conformation, and to study how the conditions affect their folding and denature pattern. The kinetics of the protein during the transition of folding and unfolding and the transient conformational change of the ligand-protein binding can be obtained and analyzed by SAXS experiment (155-157). Studies using the integration of SAXS and the molecular dynamic (MD) simulation provide us more detailed and accurate information of the conformations and dynamics of more complex protein structures in solution (158, 159).

1.4 Aim of study

It has been known that several kinesin families adopt autoinhibited states that can be relieved by phosphorylation. Serine-690 residue on the C-terminal tail domain of human KIF3A has been found to be a significant phosphorylation site responsible for its cellular activities (103). Based on previous studies, we hypothesize that KIF3A exists in an autoinhibited state when the serine-690 residue on its C-terminal is in a dephosphorylated state. Phosphorylation of serine-690 leads to relieving of autoinhibition. Our group has previously reported (103) that cargo trafficking by KIF3A can be regulated by phosphorylation using human KIF3A and its mutants with serine-690 residue on the C-terminal tail domain mutated to Alanine or Aspartic Acid. Apart from some experiments to confirm our previous findings, we have used mouse KIF3A in other experiments in this study. The reason we chose mouse KIF3A is that compare to human KIF3A, which has three variants, mouse KIF3A has higher sequence identity among its three variants. In this study, we wish to test the autoinhibition hypothesis using different biochemical and biophysical methods. We also propose to determine the 3-D structure of KIF3A which we believe will help us to understand how the KIF3 motor functions. Pull-down essays, *in-vitro* kinase assays and surface plasmon resonance (SPR) experiments were applied to determine KIF3A's motor-tail interaction under different phosphorylation status. We also used FRET microscopy to determine if there is indeed a motor-tail interaction leading to autoinhibition of KIF3A *in*

vivo. In order to understand the structure of KIF3A motor, we applied the small angle X-ray scattering technique to obtain low resolution structural and physical information of the KIF3A motor domain. We hope that the combination of biochemical and biophysical approaches will lead to a clearer picture on how KIF3A is regulated.

Chapter 2 Materials and Methods

2.1 Materials

2.1.1 Chemicals

Acetic Acid	Merck
Acrylamide	Bio-Rad
Agar	BD
Agarose	Bio-Rad
Ampicillin	USB
APS (Ammonium persulfate)	Bio-Rad
Bis-acrylamide	Bio-Rad
Bromphenol blue	Sigma
BSA (Bovine serum albumin)	Sigma
Calcium Chloride Dihydrate	Merck
Calyculin A	Millipore
Carbon Dioxide	Soxal
Deoxynucleotides (dG/A/T/CTP)	Roche
DMSO (Dimethyl Sulphoxide)	Sigma
1 Kb DNA Ladder	NEB
DTT (Dithiothreitol)	Sigma
EDTA	Sigma

EGTA	Sigma
Ethidium bromide	Bio-Rad
Ethanol	Merck
Glycerol	Affymetrix
Glycine	Bio-Rad
Hepes	Roche
Hydrochloric Acid Fuming 37%	Merck
Imidazole	Sigma
IPTG	USB
Isopropanol/ 2-propanol/IPA	Merck
Kanamycin Sulfate	Gibco
L-Glutathione reduced	Amersham
Lysozyme	Sigma
Magnesium Chloride hexahydrate	Merck
2-Mercaptoethanol	Sigma
Methanol	Merck
Nitrogen, Liquid	Soxal
N-Lauroylsarcosine (Sarkosyl)	Sigma
Paraformaldehyde	Merck
Peptone	BD
Pfu DNA Polymerase	Stratagene
Phosphatase inhibitor	Roche

PMSF (Phenylmethanesulfonyl Flouride)	Sigma
Polyethylene Glycol	Sigma
Ponceau S	Sigma
Precision Plus protein standard dual colour	Bio-Rad
Protease inhibitor	Roche
Restore PLUS Western Blot Stripping Buffer	Pierce
RNase A	Qiagen
SDS (Sodium Dodecyl Sulfate)	USB
disodium Hydrogen Phosphate Dihydrate	Merck
Sodium Chloride	USB
Sodium Dihydrogen Phosphate Dihydrate	Merck
TEMED (N,N,N',N'-Techamethylethylenediamine)	Bio-Rad
Tris [Base]	J.T.Baker
TritonX-100	Bio-Rad
Tryptone	BD
Tween 20	Sigma
Yeast Extract	BD

2.1.2 Tissue Culture Materials

LB Agar	BD
LB Broth	BD
IPTG	USB

2.1.3 Tissue Culture Materials

Dulbecco's modified Eagle's medium (DMEM)	Sigma
Fetal Bovine Serum	PAA
Sodium Bicarbonate	Merck
Fast PES Filter Units	Thermo
Trypsin	Gibco
Tissue Culture Dish	SPL/Thermo

2.1.4 Mammalian Cell Transfection Reagents

Lipofetamin ® 2000	Invitrogen
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2.1.5 Enzymes for cloning

EX Taq Polymerase	Takara
Restriction endonucleases	NEB
T4 DNA Ligase	NEB

2.1.6 Commercial Kits

Calmodulin-dependent Protein Kinase II reaction Kit	NEB
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ECL Western blotting detection reagents and analysis system	Amersham
Pierce™ Cell Surface Protein Isolation Kit	Thermo
QIAquick® Gel Extraction Kit	Qiagen
QIAquick® PCR Purification Kit	Qiagen
QIAquick® Spin Maxiprep Kit	Qiagen
QIAquick® Spin Miniprep Kit	Qiagen

2.1.7 Affinity binding matrix

Anti-FLAG M2 agarose beads	Sigma
Glutathione Sepharose™ 4B beads	Amersham
Ni Sepharose® 6Fast Flow	GE

2.1.8 Other Materials

Nitrocellulose membrane	Bio-Rad
Sterile filter 0.22 µm, cellulose acetate	Acrodisc
Sterile filter 0.45 µm, cellulose acetate	Acrodisc
Protease inhibitor cocktail tablet	Roche
PhosSTOP phosphatase inhibitor cocktail tablet	Roche
VDX48 Plate with sealant	Hampton
VDX Plate without sealant	Hampton

2.1.9 Bacterial stains and mammalian cell lines

BL21	Chemically competent	NEB
DH5 α	Chemically competent	Invitrogen
Rosetta TM (DE3)	Chemically competent	Merck
COS-7	African green monkey kidney fibroblast	ATCC
HeLa	Human Cervix adenocarcinoma epithelial	ATCC
HEK-293	Human embryonic kidney epithelial	ATCC

2.1.10 Buffers and Solutions

0.05% Coomassie Blue Staining Solution	400 mL	methanol
	100 mL	acetic acid
	500 mL	water
	0.5 g	coomassie brilliant blue
Bacterial Lysis Buffer	50 mM	Tris-HCl pH 7.5
	200 mM	NaCl
	10%	glycerol
	0.8 mM	DTT
	2 mM	Pefabloc
		DNase, RNase

Destaining Solution	400 mL	methanol
	100 mL	acetic acid
	500 mL	water
6X DNA Loading Dye	10 mL	TE buffer
	10 mL	glycerol
		Bromophenol blue
50 nM free $[Ca^{2+}]$ buffer	0.74 mM	$CaCl_2$
	1.13 mM	$MgCl_2$
	2 mM	EGTA
	20 mM	HEPES
	5 mM	KCl
	140 mM	NaCl
	10 mM	glucose
		pH 7.36

1.26 mM free $[Ca^{2+}]$ buffer

4.38 mM $CaCl_2$

1.13 mM $MgCl_2$

2 mM EGTA

20 mM HEPES

5 mM KCl

140 mM NaCl

10 mM glucose pH 7.36

10 nM Calyculin A

10 μ M Ionomycin

Mammalian Cell Lysis Buffer

25 mM Tris-HCl, pH7.4

100 mM NaCl

1% Triton X-100

Protease inhibitor

Phosphatase inhibitor

10X Nitrocellulose Transfer Buffer

30.3 g Tris base

144 g glycine

1000 mL water

10X Phosphate Buffer Saline (PBS)	13.7 mM NaCl
	2.7 mM KCl
	80.9 mM Na ₂ HPO ₄
	1.5 mM KH ₂ PO ₄
	add NaOH to pH 7.4
10X SDS/Electrophoresis Buffer	30.2 g Tris base
	144 g glycine
	10 g SDS
	1000 mL water
6X SDS/Sample Buffer	7 mL 4X Tris-Cl/SDS pH 6.8
	3 mL glycerol
	1 g SDS
	0.93 g DTT
	bromophenol blue
0.1 M Sodium Phosphate Buffer (NaP)	3.1 g NaH ₂ PO ₄ * H ₂ O
	10.9 g Na ₂ HPO ₄
	pH 6.0
	1 L H ₂ O

Superdex Column Washing Buffer	50 mM	Tris-HCl
	200 mM	NaCl
	10%	glycerol
	5 mM	DTT
	2 mM	CaCl ₂
	2 mM	MgCl ₂
	pH 7.5	
50X TAE Buffer	242 g	Tris base
	57.1 mL	acetic acid
	100 mL	0.5 M EDTA
	pH 8	
TBS	24.2 g	Tris base
	80 g	NaCl
	1000 mL	water
	pH 7.6	

2.1.11 Plasmid Constructs

Plasmid	Description
pECFP-C1	Primary vector; Neomycin selection; Kan ^r
pEYFP-N1	Primary vector; Neomycin selection; Kan ^r
pEYFP-ECFP	EYFP and ECFP fusion
pEYFP-KIF3A-Tail	Mus KIF3A tail domain; amino acid residue 598-701; N-terminal EYFP
pEYFP-S689A-Tail	Mus KIF3A amino acid residue 598-701; mutant S689A; N-terminal EYFP
pEYFP-S689D-Tail	Mus KIF3A amino acid residue 598-701; mutant S689D; N-terminal EYFP
pECFP-KIF3A-Motor	Mus KIF3A motor domain; amino acid residue 1-350; C-terminal ECFP
pEYFP-KIF3A-ECFP	Mus KIF3A full length; amino acid residue 1-701
pEYFP-S689A-ECFP	Mus KIF3A full length; mutation S689A
pEYFP-S689D-ECFP	Mus KIF3A full length; mutation S689D
pEYFP-KIF3A Δ Tail-ECFP	Mus KIF3A tail truncated; amino acid residue 1-600
pET30a_mod	Primary vector; 6xHis tag and S-tag at the N-terminal; Kan ^r
pET30a_mod-KIF3A	Mus KIF3A full length; amino acid residue 1-701
pET30a_mod-S689A	Mus KIF3A full length; mutation S689A
pET30a_mod-S689D	Mus KIF3A full length; mutation S689D
pET30a_mod-KIF3A-Motor	Mus KIF3A motor domain; amino acid residue 1-350
pET30a_mod-KIF3A-Tail	Mus KIF3A tail domain; amino acid residue

	598-701
pET30a_mod-S689A-Tail	Mus KIF3A amino acid residue 598-701; mutant S689A
pET30a_mod-S689D-Tail	KIF3A amino acid residue 598-701; mutant S689D
pET24b_mod-KIF3A-Tail	Mus KIF3A tail domain; amino acid residue 598-701
pET24b_mod-S689A-Tail	Mus KIF3A amino acid residue 598-701; mutant S689A
pET24b_mod-S689D-Tail	Mus KIF3A amino acid residue 598-701; mutant S689D
pET32b_mod-KIF3A-Motor	Mus KIF3A motor domain; amino acid residue 1-350
pGEX-6P-1	Primary vector; GST-tag at the N-terminal; Amp ^r
pGEX-6P-1-KIF3A-Tail	Mus KIF3A tail domain; amino acid residue 598-701
pGEX-6P-1-S689A-Tail	Mus KIF3A amino acid residue 598-701; mutant S689A
pGEX-6P-1-S689D-Tail	Mus KIF3A amino acid residue 598-701; mutant S689D
pXJ-GFP	Primary vector; GFP tag; Amp ^r
pXJ-GFP-KIF3A	Homo sapiens KIF3A full length; amino acid residue 1-702
pXJ-GFP-S690A	Homo sapiens KIF3A full length; mutation S690A
pXJ-GFP-S690D	Homo sapiens KIF3A full length; mutation S690D
pXJ-FLAG	Primary vector; FLAG tag; Amp ^r

pXJ-FLAG-CaMKIIa	CaMKIIa full length; amino acid residue 1-478
pXJ-FLAG-KIF3A	Homo sapiens KIF3A full length; amino acid residue 1-702
pXJ-FLAG-S690A	Homo sapiens KIF3A full length; mutation S690A
pXJ-FLAG-S690D	Homo sapiens KIF3A full length; mutation S690D
pXJ-FLAG-KIF3A-Motor	Homo sapiens KIF3A motor domain; amino acid residue 1-350
pXJ-FLAG-KIF3A-Motor	Mus KIF3A motor domain; amino acid residue 1-350
pXJ-GST	Primary vector; GST tag; Amp ^r
pXJ-GST-CaMKIIa	CaMKIIa full length; amino acid residue 1-478
pXJ-GST-POPX2	POPX2 full length; amino acid residue 1-454
pXJ-GST-KIF3A-Tail	Homo sapiens KIF3A tail domain; amino acid residue 598-702
pXJ-GST-S690A-Tail	Homo sapiens KIF3A amino acid residue 598-702; mutant S686A
pXJ-GST-S690D-Tail	Homo sapiens KIF3A amino acid residue 598-702; mutant S686D
pXJ-GST-KIF3A-Tail	Mus KIF3A tail domain; amino acid residue 598-701
pXJ-GST-S689A-Tail	Mus KIF3A amino acid residue 598-701; mutant S689A
pXJ-GST-S689D-Tail	Mus KIF3A amino acid residue 598-701; mutant S689D

2.1.12 Primers

Name	Sequence
EYFP	For: 5' CAACCGCTAGCGATGGTGAGCAAGGGCGAG 3' Rev: 5' GCTTGGATCCCGCTTGTACAGCTCGTCCAT 3'
EYFP-KIF3A	For: 5' CAACCGCTAGCGATGGTGAGCAAGGGCGAG 3' Rev: 5' GCTTGGATCCCGCTGAAGTAAAGAATCAATTAC 3'
EYFP- KIF3A △Tail	For: 5' CAACCGCTAGCGATGGTGAGCAAGGGCGAG 3' Rev: 5' GCTTGGATCCCGCTGAGGTATGAAGTTATCAAT 3'
EYFP-KIF3A-T ail	For: 5' CAA CCG AAT TCC GAT TAC CAG GAA ATG ATT GAA 3' Rev: 5' AAG TCT GGA TCC TTA CTG AAG TAA AGA ATC AAT 3'
Motor-ECFP	For: 5' CAA CCG GAA TTC ATG CCG ATC AAT AAA TCA GAG 3' Rev: 5' AAG CTT TGG ATC CCG TCT CGC TTT GTT TTT 3'
KIF3A	For: 5' CAACCGGATTCATGCCGATCAATAAGTCGGAG 3' Rev:5' AAGTCTGGATCCTTACTGAAGTAAAGAATCAAT 3'
KIF3A △Tail	For: 5' CAACCGAATTCCATGCCGATCAATAAGTCGGAG 3'

	Rev: 5' AATAATGGATCCCTGAGGTATGAAGTTATCAAT 3'
KIF3A-Motor	For: 5' CAACCGGAATTCATGCCGATCAATAAGTCGGAG 3' Rev: 5' AAGTCTGGATCCTCTCGCTTTGTTTTTAATGTT 3'
KIF3A-Tail	For: 5' CAACCGGAATTCGATTACCAGGAAATGAATGAA 3' Rev: 5' AAGTCTGGATCCTTACTGAAGTAAAGAATCAAT 3'
S689A	For: 5' GATGGGGCGAAGAAAGCGTGCTGCAAAGCCTGAGACC G 3' Rev: 5' CGGTCTCAGGCTTTGCAGCACGCTTTCTTCGCCCCATC 3'
S689D	For: 5' GCCAAAGATGGGGCGAAGAAAGCGTGATGCAAAGCCT GAGACC 3' Rev: 5' GGTCTCAGGCTTTGCATCACGCTTTCTTCGCCCCATCTT TGGC 3'

2.1.13 siRNA

Name	Sequence
KIF3A siRNA#1	Sense sequence: 5' CCCAUAAAUUUAAGUCCAUUCUGU 3' Anti-sense sequence: 5' ACAGAAUGGACUUUAAAUUUAUGGG 3'

KIF3A siRNA#1	<p>Sense sequence:</p> <p>5' UCAGUUAUCUUUGCCUGCGUGUAUA 3'</p> <p>Antisense sequence:</p> <p>5' UAUACACGCAGGCAAAGAUACUGA 3'</p>
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2.1.14 Antibodies

2.1.14.1 Primary Antibodies

Antibody	Description /Immunogen	Source	Catalog
Actin	Monoclonal, Mouse	Sigma-Aldrich	A2228
β -catenin	Polyclonal, Rabbit	Sigma-Aldrich	C2206
FLAG	Monoclonal, Mouse	Sigma-Aldrich	F3165
FLAG	Polyclonal, Rabbit	Sigma-Aldrich	F7425
GFP	Polyclonal, Rabbit	Abcam	Ab290
GST	Polyclonal, Rabbit	Bethyl	A190-122A
His	Polyclonal, Rabbit	Cell Signal	2365S
KIF3A	Monoclonal, Mouse	BD Biosciences	611508
N-cadherin	Monoclonal, Mouse	BD Biosciences	610920
Phosphoserine/threonine	Polyclonal, Rabbit	ECM Biosciences	PP2551
POPX2	Monoclonal, Mouse	Abcam	Ab56648

2.1.14.2 Secondary Antibodies

Antibody	Description /Immunogen	Source	Catalog
Anti-mouse IgG-HPR	Polyclonal, Goat	Dako Cytomation	P0447
Anti-rabbit IgG-HPR	Polyclonal, Goat	Dako Cytomation	P0448
Alex Fluor anti-mouse 546 IgG	Polyclonal, Goat	Molecular Probes	A11003
Alex Fluor anti-rabbit 546 IgG	Polyclonal, Goat	Molecular Probes	A11010

2.2 Methods

2.2.1 Cell culture

HeLa cells, U2OS cells, COS7 cells and HEK293 cells were maintained in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37 °C in 5% CO₂.

2.2.2 Transfection

20,000 cells and 100,000 cells were seeded in a 35 mm dish and 10 cm dish respectively 16-20 hours before transfection. 1 µg of plasmid DNA were transiently transfected into a 35 mm tissue culture dish with cells reaching around 80% confluence using LipofectamineTM 2000 (Invitrogen) according to the manufacturer's protocols. 8 µg of plasmid DNA were transfected into a 10

cm tissue culture dish with 80% confluence cells. For co-transfection, 1 µg of each plasmid DNA was transfected into a 35 mm dish with 80% confluence cells. Cells were fixed or collected 24-36 hours after transfection.

2.2.3 RNA interference experiment

Stealth siRNA (Invitrogen) targeting the 3' UTR of the *KIF3A* gene were used and the sequences are as follow: 5' CCCAUAAAUUUAAAGUCCAUUCUGU 3' (sense strand) and 5' UCAGUUAUCUUUGCCUGCGUGUAUA 3' (sense strand). 15,000 HeLa cells were plated on a 35 mm dish one day before transfection. The siRNAs were introduced into HeLa cells by mixing with the transfection complex containing the siRNA and Lipofectamine™ 2000 for 30 min. Transfected cells were harvested after 48 hours for Western blot analysis to determine the efficiency of the gene knockdown.

2.2.4 Immunofluorescence

Cells were fixed 24-36 hours after transfection by 4% paraformaldehyde (PFA) in PBS at room temperature for 10 minutes, followed by permeabilization using 0.2% Triton X-100 in PBS for 5 minutes. Cells were then incubated with primary antibody in 5% BSA/PBS (1:100 dilution) for 1 hour at room temperature. After washing 3 times with 0.1% Triton X-100 in PBS, cells were incubated with Alexa-conjugated secondary antibodies in 5% BSA/PBS (1:1000 dilution) at room temperature for 1 hour. Coverslips were dried and mounted using DAPI to show nuclei. Sample images were then captured and

analyzed using Axio Observer Z1 Microscope and MetaMorph software programme.

2.2.5 FLAG Immunoprecipitation assay

COS7 cells were co-transfected with FLAG-KIF3A-Motor and GST-KIF3A-Tail in a 35 mm tissue culture dish. Cells were collected 24 hours after transfection. Protein lysates were homogenized in 200 μ L lysis buffer containing 25 mM Tris-Cl pH7.4, 100 mM NaCl, 0.5% Triton X-100 and 1 $\times$ cOmplete Protease Inhibitor (Roche). The lysates were then clarified by centrifugation at 13,200 rpm for 10 min. 40 μ L of ANTI-FLAG[®] M2 Affinity Gel (Sigma) bead slurry were added to the supernatant and mixed for 3 hours at 4 °C with constant rotation. Beads were then washed with washing buffer containing 25 mM Tris-Cl pH7.4, 500 mM NaCl for four times. The bound FLAG-tagged protein was eluted by SDS sample buffer by heating at 100 °C for 10 min and subjected to SDS-PAGE and western blot.

2.2.6 Glutathione S-transferase (GST) pull-down assay

COS7 cells were co-transfected with FLAG-KIF3A-Motor and GST-KIF3A-Tail in a 35 mm tissue culture dish. Cells were collected 24 hours after transfection. Protein lysates were homogenized in 200 μ L lysis buffer containing 25 mM Tris-Cl pH7.4, 100 mM NaCl, 0.5% Triton X-100 and 1 $\times$ cOmplete Protease Inhibitor (Roche). The lysates were then clarified by centrifugation at 13,200 rpm for 10 min. 50 μ L of Glutathione Sepharose[™] 4B (Amersham Biosciences)

bead slurry were added to the supernatant and mixed for 3 hours at 4 °C with constant rotation. Beads were then washed with washing buffer containing 25 mM Tris-Cl pH7.4, 500 mM NaCl for four times. The bound GST-tagged protein was eluted by SDS sample buffer by heating at 100 °C for 10 min and subjected to SDS-PAGE and western blot.

2.2.7 Western Blot

Samples were lysed in 1×SDS sample buffer containing 17.5 mM Tris-Cl pH6.8, 9% glycerol, 1% SDS, 2.5% β-mercaptoethanol and bromophenol blue. Equal amounts of protein sample were loaded on 8-15% SDS polyacrylamide gels. Proteins were then transferred to nitrocellulose membranes from SDS-PAGE gels. The membranes were incubated with primary antibodies (1:1000 dilution) in TBS containing 0.05% Tween 20 and 5% non-fat milk overnight at 4 °C. The membranes were incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibodies (1:4000 dilution) for one hour at room temperature. Signals were detected with Noovex[®] ECL (Invitrogen) detection system.

2.2.8 Cell Surface Protein Isolation Essay

The experiment was performed using Pierce[®] Cell Surface Protein Isolation Kit. Four 10 cm culture dishes of cells were transfected with plasmid DNA. Supply EZ-Link Sulfo-NHS-SS-Biotin solution to each dish of 90-95% confluent cells and gently agitate for 30 minutes at 4 °C. Add 500 µL of Quenching Solution to

each dish to quench the reaction and subsequently lysed the cells with lysis buffer. Labeled proteins were then isolated with 500 μ L of Immobilized NeutrAvidin Gel slurry. The bound proteins were released by incubating with SDS-PAGE sample buffer and subjected to SDS-PAGE and western blot.

2.2.9 Surface Plasmon Resonance (SPR)

Biacore 3000 (GE Healthcare) was used to perform the SPR experiment. Flow cell (Fc) 1-4 surfaces were activated by injecting a mixture of 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide(EDC)/N-hydroxysuccinimide (NHS) (1:1) for 7 min at the flow rate of 10 μ L/min. 30 μ g/mL Anti-GST antibody in 10 mM NaOAc, pH 5, was immobilized to the chips for 4 min. Run ethanolamine through the chips to deactivate the remaining active esters for 7 min. Inject 5 μ M of GST-KIF3A-Tail, GST-S689A-Tail and GST-S689D-Tail to Fc 2-4 for 3 min respectively, where Fc 1 acted as a reference. Following capture of tail proteins, 5 μ M recombinant GST was then injected at 10 μ L/min flow-rate to block any remaining anti-GST binding sites to prevent non-specific interactions. Concentration series of 3 μ M - 0.1875 μ M His-KIF3A-motor protein was injected over the chip surfaces with captured tail proteins. Regenerate the surfaces with 20 μ L Glycine-HCl pH 2.1 and 10 μ L 10 mM NaOH. The experiments were performed at 4 $^{\circ}$ C to keep the motor domain stable.

2.2.10 *In Vitro* Kinase Assay

FLAG-CaMKII was expressed in HEK293 cells and subjected to FLAG-immunoprecipitation. FLAG-CaMKII was subsequently eluted out by 100 µg/mL 3X Flag peptide. The autophosphorylation of CaMKII was performed by incubating the eluted FLAG-CaMKII for 10 min at 30 °C with 1.2 µM calmodulin, 2 mM CaCl₂ and kinase buffer containing 50 mM Tris-HCl, 10 mM MgCl₂, 0.1 mM EDTA, 2 mM DTT, 0.01% Brij 35, pH7.5, supplied with 200 µM ATP. Purified GST-KIF3A-tail was phosphorylated by incubating with autophosphorylated CaMKII supplied with 200 µM ATP for 30-45 min at 30 °C with constant shaking. Half of the reacted solutions were subjected to SDS-PAGE and western blot, and the phosphorylation level was checked by anti-Phosphoserine/threonine antibody (1:500). 50 µL of Glutathione SepharoseTM 4B (Amersham Biosciences) bead slurry were mixed with the other half of the reacted solutions, supplied with purified His-KIF3A-motor for 3 h at 4 °C. Beads were then washed with washing buffer containing 25 mM Tris-Cl pH7.4, 500 mM NaCl for four times at 4 °C. The bound protein was eluted by SDS sample buffer by heating at 100 °C for 10 min and subjected to SDS-PAGE and western blot. His-KIF3A-motor and GST-KIF3A-tail were detected by anti-His antibody (1:1000) and anti-GST antibody (1:1000).

2.2.11 KIF3A Phosphorylation by CaMKII in Cells

Plasmids pXJ-GST-CaMKII and pEYFP-KIF3A-ECFP was co-transfected in

U2-OS or HELA cells in two 35 mm dishes. Treat one of the dish with 50 μ M $[Ca^{2+}]$ (140 mM NaCl, 5 mM KCl, 10 mM glucose, 20 mM HEPES, 2 mM EGTA, 1.13 mM $MgSO_4$, 0.74 mM $CaCl_2$, pH 7.36) as control. Treat the other dish with 50 μ M $[Ca^{2+}]$ for 10 min and subsequently treat with 1.26 mM $[Ca^{2+}]$ (140 mM NaCl, 5 mM KCl, 10 mM glucose, 20 mM HEPES, 2 mM EGTA, 1.13 mM $MgSO_4$, 4.38 mM $CaCl_2$, pH7.36) supplied with 10 μ M ionomycin and 10 nM calyculin A. Perform the FRET experiment of the control and the phosphorylated samples immediately after the treatments.

2.2.12 Förster Resonance Energy Transfer

The fluorophore EYFP was cloned into vector pECFP-N1. Full length KIF3A and its mutants KIF3A-S689A and KIF3A-S689D, and a truncated construct KIF3A- Δ Tail (residues 1-590 aa) was cloned into pEYFP-ECFP. The motor domain (residue 1-350 aa) and the wild-type and mutants tail domains (residues 598-701 aa) of KIF3A were cloned into vector pECFP-N1 and pEYFP-C1 respectively. pEYFP-KIF3A-ECFP and the mutants were transfected in U2-OS cells. pEYFP-Tail domain of the wild-type and mutants were co-transfected with Motor-pECFP respectively in U2-OS. Zeiss LSM 710 Confocal Microscope (Carl Zeiss) was used to perform the acceptor photobleaching FRET experiment. The cells were observed under 63X oil immersion objective lens. EYFP was excited by the laser at the wavelength of 514 nm with the emission filter BP of 525-551 nm. The gains of both channels

were adjusted to make sure both channels reach saturation. ECFP was excited by the laser at the wavelength of 458 nm with the emission filter of 469-501 nm. A whole cell area was selected as the region of interest (ROI). The iteration was set at 120 and scan for 10 cycles. The unbleached samples were set as control by scanning the cells for 10 cycles without bleaching. The photobleaching of EYFP within the ROI was achieved by scanning the region 10 times using 514 argon laser line. For the bleached samples, photobleaching was applied between the 5th and 6th time points. The signals of ECFP within the ROI before and after EYFP bleaching were acquired. The intensity decrease of the EYFP channel shows the bleaching efficiency where the intensity increase of the ECFP channel indicates the FRET efficiency. FRET efficiency was determined by the formula,

$$E_{\text{FRET}} = \frac{I_6 - I_5}{I_6}$$

where I_5 and I_6 is the ECFP intensity of 5th and 6th time points.

2.2.13 Protein Expression and Purification

The motor domain of *Mus musculus* KIF3A (residues 1-350 aa) was cloned into a modified pET30a-X vector, yielding an N-terminal hexahistidine-tagged protein. The tail domain of KIF3A (residues 598-701 aa) was cloned into a modified pGEX-6p1 vector, yielding an N-terminal GST-tagged protein. The plasmids were transformed into *Escherichia coli* strain BL21. 1 liter of bacterial LB culture media with 50 mg/L Kanamycin was grown at 37 °C till A600

reached approximately 0.6. The bacterial was induced by 0.4 mM isopropyl β -D-thiogalactoside (IPTG) and incubated overnight at 160 rpm, 16 °C. Centrifuge the bacteria at 4000 rpm for 40 min and store in -80 °C refrigerator.

For the His-tagged motor domain purification, harvested bacteria were suspended in protein lysis buffer containing 50 mM Tris-Cl pH7.5, 200 mM NaCl, 10% glycerol, DNase, RNase, supplied with 0.8 mM DTT and 2 mM pefabloc and sonication (25-30% power, 1 min*3 times, with 2 min interruption). Clear lysate was loaded to Ni SepharoseTM 6 Fast Flow (GE Healthcare) bead slurry packed in an Econo-Pac[®] Chromatography Column (Bio-Rad) and mixed for 1 hour. The resin was then washed with protein lysis buffer containing 20 mM imidazole. The protein was eluted on a gradient from 50 to 500 mM imidazole. The eluted protein was then concentrated and injected into HiLoad 16/600 Superdex 200 column (GE Healthcare). Proteins were eluted out by 50 mM Tris-Cl pH7.5, 200 mM NaCl, 10% glycerol, 10 mM DTT, 2 mM MgCl₂, 2 mM CaCl₂.

For the purification of the GST-tagged tail domain, harvested bacteria were suspended in protein lysis buffer containing 50 mM Tris-Cl pH7.5, 200 mM NaCl, 1.5% Sarkosyl, 1 mM lysosome, supplied with 1 mM DTT and 1 mM PMSF and sonication (25-30% power, 1 min*3 times, with 2 min interruption). Clear lysate was loaded to Glutathione SepharoseTM 4B (Amersham Biosciences) bead slurry packed in an Econo-Pac[®] Chromatography Column

(Bio-Rad) and mixed for 3 hours. The resin was then washed with protein lysis buffer containing. The proteins were eluted with elution buffer containing 20 mM reduced L-glutathione.

2.2.14 ATPase Hydrolysis Assay

10 µg of the purified KIF3A-motor domain was added into 1 mL of the reaction solution containing ATP, phosphoenolpyruvate, pyruvate kinase, L-lactic acid dehydrogenase and NADH. The change at 340 nm, which is the absorbance wavelength of NADH, is recorded by Ultrospec™ 2100 pro UV/Visible Spectrophotometer (GE healthcare). Data were normalized and the ATPase activity was calculated,

$$\frac{\Delta OD (60 \text{ sec})}{6.22 \text{ act sec}(\Delta I)} = \text{ATPase}_{\text{spec. activity}}$$

$$\frac{\text{ATP hydrolyzed}}{\text{min} \cdot (\text{mg of protein})} \text{ or Units/mg}$$

One unit corresponds to 1 µmol of ATP hydrolyzed/min.

2.2.15 Small-angle X-Ray Scattering (SAXS)

SAXS experiments were performed by Bruker NanoStar equipped with MetalJet eXcillum X-ray source and VÅNTEC-2000 detector. To determine the protein structure in solution by SAXS, the purified protein solution was concentrated to different concentrations and the primary SAXS data were processed by the program package PRIMUS. The low angle regions of the protein scattering profiles were compared among different concentrations to

evaluate if the result is concentration-dependent. The forward intensity $I(q)$ and the radius of gyration R_g were then determined from Guinier approximation and the pair-distance distribution $p(r)$. At low angle ($q < 1/R_g$), $I(q)$ was calculated as followed,

$$I(q) = I(0)e^{\frac{-q^2 R_g^2}{3}}$$

A linear curve of the scattering intensity is also plotted as $\ln I(q)$ vs q^2 , and $I(0)$ and R_g can be derived from the curve. The pair-distance distribution $P(r)$, which describes all the distances between two electrons as a paired-set in a protein particle, can be determined as:

$$p(r) = \frac{r^2}{2\pi^2} \int q^2 I(q) \frac{\sin qr}{qr} dq$$

where r is the value of a distance vector.

The model of the protein was reconstructed by DAMMIF in a relatively low resolution. A collection of dummy atoms were generated by DAMMIF simulation according to the low angle data of SAXS, representing the particles of the protein model. In order to validate the reproducibility of the model, 20 independent reconstructed models were uploaded in the program DAMAVER, which can align the models to construct an averaged model. Every two models were compared and generated a normalized spatial discrepancy (NSD) value. The two models were considered similar if NSD value was close to one. By discarding the high NSD dispersion models, DAMMIF will generate an average model with a mean NSD over all model pairs.

2.2.16 Protein crystallization

Purified protein was concentrated to 4.5-12 mg/mL concentrations. For the coarse crystallization screening, the 48-well VDX48 Plate with sealan (Hampton Research) for hanging drop crystallization was applied. 1 μ L protein was mixed with 1 μ L precipitant on a coverslip, and the coverslip was sealed on a reservoir well containing 150 μ L precipitant. For the repeating and optimization conditions, the mix of 2 μ L protein and 2 μ L precipitant was placed on a well containing 500 μ L precipitant in a 24-well VDX Plate with sealan (Hampton Research). More than one drop of the protein-precipitant mix can be placed on one coverslip. During the set-up, the plates were kept on ice at all times. The plates were incubated at 10 °C for crystal growing.

2.2.17 Statistical analysis

FRET and ATPase assay graphs and statistics were done by GraphPad Prism 5.01 and Origin 8.6. Other analyses were done by Excel software (Microsoft office 2010). All experiments were performed independently for at least three times for statistical analysis. Significance was determined by two-way ANOVA for comparison between samples. P values < 0.05 were considered significant in all analyses.

Chapter 3 Results

3.1 Cargo transportation is impaired in cells overexpressing KIF3A mutant S690A

Serine-690 residue on the C-terminal of KIF3A has been reported to be a major phosphorylation site of KIF3 motor (103). In order to determine how phosphorylation status affects cargo transport by the KIF3 motor, two mutations on serine-690 residue were generated. Serine-690 was mutated to alanine (S690A) to preclude phosphorylation, or to aspartic acid (S690D) to mimic continuous phosphorylation. GFP-KIF3A-WT, GFP-KIF3A-S690A and GFP-KIF3A-S690D constructs were overexpressed in HeLa cells. Immunofluorescence staining of N-cadherin in the transfected HeLa cells showed that overexpressing KIF3A-S690D did not cause any observable changes of N-cadherin transported to the cell periphery and cell-cell junction compare to the wild type. However, in KIF3A-S690A overexpressing cells, there was an apparent reduction of N-cadherin at the cell periphery, which implies that KIF3A-S690A led to defective cargo transportation (Fig. 3.1A). We also performed the same experiment detecting the localization of β -catenin, another reported cargo of the KIF3 motor, in KIF3A-WT, KIF3A-S690A and KIF3A-S690D overexpressing cells. Because β -catenin binds to cadherin receptors near the cell-cell junction, so we also checked the localization of β -catenin at the cell periphery. Similarly, the amount of β -catenin at the cell

periphery was reduced in KIF3A-S690A overexpressing cells compared to wild-type and the KIF3A-S690D overexpressing cells (Fig. 3.1B).

To further compare the amount of N-cadherin/ β -catenin complex at the cell surface of KIF3A-WT, KIF3A-S690A and KIF3A-S690D overexpressing cells, we used a cell surface protein isolation approach to quantify the amount of N-cadherin and β -catenin at the cell surface. COS7 cells were first labeled with biotin. During this step, total surface proteins were biotinylated. The cells were subsequently lysed with a mild detergent and the labeled proteins were then isolated with the Streptavidin resins. The bound proteins were released by incubating with SDS-PAGE sample buffer and analyzed by western blotting. Although β -catenin is not expressed at the cell surface, it can be pulled-down together with N-cadherin as a complex. Therefore, in our experiment, we were detecting the β -catenin, which was targeted to the cell periphery through its binding to N-cadherin. Fig. 3.2 shows that the amount of β -catenin and N-cadherin targeted to the cell periphery of KIF3A-S690A overexpressing cells were significantly less than KIF3A-WT and KIF3A-S690D overexpressing cells. Previous data from our group also showed that the N-cadherin trafficking velocity was inhibited in KIF3A-S690A overexpressing cells but not the KIF3A-wild-type and KIF3A-S690D overexpressing cells (103). These results indicate that KIF3A-S690A led to defective cargo transportation.

Figure 3.1 Overexpression of KIF3A-S690A mutant inhibits localization of N-cadherin and β -catenin to cell-cell contacts. HeLa cells were transfected with pXJ-GFP-KIF3A, pXJ-GFP-S690A or pXJ-GFP-S690D. Representative images of HeLa cells were immunostained with (A) anti-N-cadherin and DAPI, (B) anti- β -catenin and DAPI. Scale bar: 20 μ m.

Figure 3.2 Amount of N-cadherin/β-catenin complex on the cell surface of KIF3A-S690A overexpressing cells are less than WT and KIF3A-S690D overexpressing cells. COS7 cells were transfected with pXJ-FLAG-KIF3A, pXJ-FLAG-S690A or pXJ-FLAG-S690D. Cell surface proteins were biotinylated and eluted. Represented cell surface proteins were analyzed by western blotting with (A) anti-β-catenin and (B) anti-N-cadherin. (C) Normalized amount of β-catenin and N-cadherin on cell surface. The bar chart was generated from two independent experiments.

In an attempt to further validate our findings, we designed two siRNAs targeting the 3' UTR of human *KIF3A*. In combination with KIF3A siRNA transfection, we also co-transfected pXJ-GFP, pXJ-GFP-KIF3A-WT, pXJ-GFP-KIF3A-S690A and pXJ-GFP-KIF3A-S690D into HeLa cells. 48 hours after the transfection, the cells were fixed and stained with N-cadherin antibody. In parallel, a set of HeLa cells transfected with siRNAs but not KIF3A constructs were lysed for western blotting analysis. Antibody against KIF3A showed that both KIF3A siRNAs can effectively knock down the endogenous KIF3A (Fig. 3.3B, D). Fig. 3.3 shows that upon the transfection of both KIF3A siRNAs, N-cadherin localization to the cell periphery was lost. When wild-type KIF3A and KIF3A-S690D were overexpressed together with KIF3A knockdown, N-cadherin could still localize to the cell periphery. However, when we overexpressed KIF3A-S690A with KIF3A knockdown, N-cadherin remained mis-localized. These observations imply that phosphorylation of serine-690 residue is critical for the transportation of N-cadherin to the cell periphery by KIF3A. Taken together, we validated and concluded that the phosphorylation status of the KIF3A serine-690 residue is important in regulating cargo transport.

Figure 3.3 Overexpression of wild-type KIF3A and KIF3A-S690D mutant rescued the loss of N-cadherin localization induced by KIF3A-Knockdown. HeLa cells were transfected with control siRNA against the luciferase gene (siLuc) or siRNA targeting KIF3A gene (siKIF3A#1 or siKIF3A#2). Control cells were co-transfected with pXJ-GFP. KIF3A-knockdown cells were co-transfected with pXJ-GFP, pXJ-GFP-KIF3A, pXJ-GFP-KIF3A-S690A or pXJ-GFP-KIF3A-S690D. (A, B) Representative images of HeLa cells transfected with siKIF3A#1 (A) and siKIF3A#2 (B) were immunostained with anti-N-cadherin and DAPI. Scale bar: 10 μ m. (C) Cells were harvested after 48 h after transfection of KIF3A siRNAs and endogenous KIF3A expression was detected by anti-KIF3A antibody.

3.2 Serine-689 residue is phosphorylated by CaMKII

CaMKII has been reported to be the kinase that phosphorylates KIF3A (human) at serine-690 residue. However, *Ichinose et al.* showed in their LC-MS/MS results that threonine-694 and serine-698 of the mouse KIF3A were the phosphorylation sites for CaMKII, while serine-689 (equivalent to human serine-690) is phosphorylated by PKA (160). Serine-689 (mouse) or serine-690 (human) residue is among the conserved amino acid sequence RKRS at the C-terminal tail domain of mouse or human KIF3A, respectively. This conserved sequence fits the consensus recognition sequence for CaMKII (RXXS/T) while neither threonine-694 nor serine-698 falls in the recognition motif of CaMKII (Fig. 3.5A).

For subsequent studies, we use the mouse KIF3A cDNA rather than the human KIF3A, therefore we generated mutant constructs based on mouse KIF3A. Since the serine-689 residue on the mouse KIF3A is conserved with the serine-690 residue of human KIF3A, we mutated serine-689 residue of mouse KIF3A to the phospho-mimic S689D or the phospho-dead S689A. The DNA constructs expressing motor and tail domains of KIF3A and the mutants were cloned into *pXJ* vectors for transfection, or into *pET* or *pGEX* vectors for protein purification.

The motor domain of mouse KIF3A (1-350 amino acids) was cloned into *pET-30a* vector. *pET-30a* vector contains an N-terminal hexahistidine-tag (His6)

(161). The expression of the proteins in BL21 cells was induced by 0.4 mM IPTG overnight at 16 °C. The cells were then lysed and sent for sonication subsequently. The protein was purified by binding to Ni Sepharose™ 6 Fast Flow (GE Healthcare) beads and eluted by elution buffer containing imidazole. His-KIF3A-motor was mainly eluted out from 100-200 mM imidazole (Fig. 3.4A). Further purification steps to get purer KIF3A-motor protein for protein structure study will be discussed in later section.

The wild-type, S689A and S689D tail domains of KIF3A (598-701 amino acids) were cloned into *pGEX-6P-1* vector containing an N-terminal GST-tag. The expression of the proteins in BL21 cells was induced by 0.4 mM IPTG overnight at 22 °C. The cells were then lysed with buffer and sent for sonication subsequently. The proteins were isolated with Glutathione Sepharose™ 4B beads (Amersham). Buffer exchange by PD-10 desalting column (GE Healthcare) was performed to remove imidazole from the purified His-KIF3A-motor, or L-glutathione from the purified GST, GST-KIF3A-tail, GST-S689A-tail, GST-S689D-tail proteins. SDS-PAGE gels staining showing the sizes of the purified proteins were shown in Fig. 3.4B.

Figure 3.4 KIF3A motor and tail domain purification. (A) SDS-PAGE gel showing the purified His-KIF3A-motor domain (45 kDa), GST-KIF3A-tail (37 kDa) and GST (24 kDa). (B) GST-KIF3A-WT-tail, GST-KIF3A-S689A-tail, GST-KIF3A-S689D-tail was purified by Glutathione Agarose beads.

In order to confirm the phosphorylation of CaMKII on serine-689 residue of KIF3A, we performed an *in vitro* kinase assay by incubating activated CaMKII with the purified tail proteins of KIF3A wild-type or the phospho-mutants. FLAG-CaMKII protein was purified from HEK293 cells and activated by CaCl_2 , CaM and ATP for 10 min. 5 μg of GST-KIF3A-tail of the wild-type, S689A-tail and S689D-tail were incubated with the activated CaMKII supplied with ATP for 45 min at 30 °C. We observed that only the wild-type KIF3A-tail is highly phosphorylated by CaMKII, while both the mutants were only slightly

phosphorylated (Fig. 3.5B).

Figure 3.5 *In-vitro* kinase essay showed that serine-689 residue of KIF3A is mainly phosphorylated by CaMKII. (A) The domain organization of the mouse KIF3A subunit. Serine-689 residue on the mouse KIF3A is conserved with serine-690 residue on the human-source KIF3A. The conserved CaMKII recognition sequence RXXS/R is marked by green characters and the serine residue is highlighted by red in both human and mouse sequences. (B) Activated CaMKII phosphorylates KIF3A tail domain. Lane 1, inactive FLAG-CaMKII + GST-WT-tail; lane 2, activated FLAG-CaMKII + GST-WT-tail; lane 3, inactive FLAG-CaMKII + GST-S689A-tail; lane 4, activated FLAG-CaMKII + GST-S689A-tail; lane 5, inactive FLAG-CaMKII + GST-S689D-tail; lane 6, activated FLAG-CaMKII + GST-S689D-tail; lane 7, inactive FLAG-CaMKII.

In order to confirm that CaMKII phosphorylates KIF3A at serine-689 residue *in vivo*, we applied the CaMKII inhibitor KN62 in the Ca^{2+} stimulated cells and detected the effect it caused to KIF3A phosphorylation. Since the endogenous autophosphorylated CaMKII level was too low to detect in cells, in order to see the difference in phosphorylation levels between control and KN62 treated cells, we first stimulated the cells by treating them with buffer containing higher concentration of Ca^{2+} (1.26 mM compared with 50 nM) supplemented with 10 μM ionomycin, which induces intracellular calcium stimulation, and 10 nM calyculin A, which is a PP1/PP2A (phosphatase) inhibitor. In this experiment, we stimulated the cells with high $[\text{Ca}^{2+}]$ buffer for 30 min as the CaMKII activated sample. For the inhibition sample, 10 μM of KN62 was added in cells before the Ca^{2+} stimulation (KN62 was also included during the stimulation). Cells treated with 10 μL of DMSO without further treatments were used as control. The cell lysates were subjected to immunoprecipitation by anti-KIF3A antibody and the phosphorylation levels of KIF3A and CaMKII were detected by anti-phospho serine/threonine and anti-phospho CaMKII (T286) antibodies.

We observed that KN-62 effectively reduced the autophosphorylation levels of CaMKII. We also found that there was a corresponding reduction in phosphorylated KIF3A when CaMKII was inactive suggesting that KIF3A was phosphorylated by CaMKII *in vivo* (Fig. 3.6A). In addition, we transfected the FLAG-KIF3A-WT and FLAG-KIF3A-S689A (phospho-dead mutant) in HeLa cells and treated the cells with Ca^{2+} stimulation and KN62. For

FLAG-KIF3A-WT overexpressing cells, Ca^{2+} stimulated sample showed higher FLAG-KIF3A phosphorylation level than the KN62 treated sample, which is consistent with the results obtained when we detected phosphorylation levels of endogenous KIF3A. However, no phospho-bands were observed for FLAG-KIF3A-S689A by Western analysis (Fig. 3.6B). These results suggest that CaMKII phosphorylates KIF3A at serine-689 residue *in vivo*. Taken together, we concluded that CaMKII is the kinase for KIF3A, and it mainly phosphorylates KIF3A at serine-689 residue.

(A)

(B)

Figure 3.6 Phosphorylation of KIF3A by CaMKII *In-vivo*. (A) For the activated sample, HeLa cells were treated with high $[Ca^{2+}]$ and 10 μ M ionomycin and 10 nM CA for 30 min. The inhibited sample was treated with 10 μ M of KN-62 for 30min, and subsequently treated with high $[Ca^{2+}]$ stimulation supplied with 10 μ M of KN-62 for 30 min. 1.5 mg total lysate was subjected to anti-KIF3A-IP overnight and followed by SDS-PAGE and western blot analysis. 100 μ g of total lysate was loaded as input. (B) HeLa cells were transfected with FLAG-KIF3A and FLAG-S689A respectively, and the cells were treated with the same methods described above. 1 mg of total lysate was incubated with FLAG-M2 beads overnight, followed by SDS-PAGE and western blot analysis. 100 μ g of total lysate was loaded as input.

3.3 Phosphorylation of KIF3A tail domain prevents motor-tail interaction

3.3.1 KIF3A motor domain interacts with wild-type and phospho-dead tail domains but not phospho-mimic tail domain

There are reports suggesting that some kinesin families adopt autoinhibited conformations, where the head domains interact with the tail domains to keep the kinesins from binding to cargoes or moving along microtubules (64, 143). In an attempt to find out whether KIF3 motor also adopts an autoinhibited mechanism and how phosphorylation might affect autoinhibition, we performed a FLAG-immunoprecipitation assay to test the interaction between KIF3A motor domain and the tail domain of KIF3A-WT, KIF3A-S689A and KIF3A-S689D. We co-transfected FLAG-tagged KIF3A-motor construct with GST-tagged KIF3A-WT-Tail, KIF3A-S689A-Tail and KIF3A-S689D-Tail constructs in HEK293 cells respectively. The cells were lysed 24h later and 500 µg of total lysates were then subjected to FLAG-IP. The results showed that FLAG-KIF3A-motor can pull down wild-type and phospho-dead GST-KIF3A-Tail but not the phospho-mimic GST-S689D-Tail (Fig. 3.7A).

We also tested the interaction between the purified His-KIF3A-motor and the GST-tagged tail domains of KIF3A-WT, KIF3A-S689A and KIF3A-S689D. 5 µg of the His-KIF3A-motor were mixed with 5 µg GST-tail domains and subjected to GST-pull down. The result shows that the tail domain of wild-type and the

phospho-dead mutant S689A binds to the motor domain, while there's no binding between the motor domain and S689D-tail (Fig. 3.7B). These results suggest that KIF3A motor domain can interact with the tail domain, and phosphorylation of serine-689 residue disrupts this interaction.

Figure 3.7 Interaction between the motor and tail domain of KIF3A. (A) FLAG-KIF3A-motor construct was co-transfected with GST, GST-KIF3A-Tail, GST-S689A-Tail and GST-S689D-Tail constructs in HEK293 cells. Total lysates were incubated with anti-FLAG M2 Affinity Gel and the eluents were detected by western blot using anti-FLAG or anti-GST. (B) Purified His-KIF3A-motor and purified GST-KIF3A-Tail, GST-S689A-Tail or GST-S689D-Tail mixture were incubated with Glutathione Sepharose™ 4B. GST-fusion proteins were captured and the co-precipitated complex was separated by SDS-PAGE and analyzed by western analysis using anti-His and anti-GST antibodies.

3.3.2 Detecting the interaction of KIF3A motor and tail by SPR approach

To further verify our hypothesis, we also applied the Surface Plasmon Resonance approach using the Biacore system to verify the interaction between KIF3A motor and tail domains. Surface plasmon resonance (SPR) is an optical phenomenon used to measure the interaction of biomolecules. Biacore instrument utilizes SPR to directly monitor the binding of the analyte to its interaction partner immobilized on a sensor chip (162). The binding of the analyte causes the change of the resonance oscillation stimulated by incident light. The refractive-index changes of the medium at the SPR sensing surfaces are then detected by the Biacore system (163). SPR has been a helpful tool for drug screening, medical diagnostic, and the detection and analysis of protein interacting partners.

To perform the experiment, first, we immobilize anti-GST on the gold sensor chips. Then a certain amount of GST-KIF3A-tail of the wild-type and mutants were captured on three different chips. Optimization was performed to make sure that the amounts of captured tail domains on all chips were approximately equal. We then block all the chips with GST protein. After this, we injected the lowest concentration of KIF3A-motor in a concentration series. The injection of the KIF3A-motor was performed at 4 °C. We then regenerated the chips with sodium acetate and repeat the steps for the other 4 concentrations of motor protein. The Biacore system was kindly operated by Mr. Jedrael Ng Zheng Ying

from Dr. Susana Geifman-Shochat's laboratory (School of Biological Sciences, Nanyang Technological University).

Our results (Fig. 3.8) showed that the interaction between KIF3A-motor and S689A-tail is stronger than the binding between the tail domains of WT, and S689D. The equilibrium constant KD value of KIF3A-motor and S689A-tail is $4.836e^{-8}$, indicating a relatively strong binding. The binding signals of the motor domain with the wild-type and S689D-tail are too weak to calculate the KD based on the experiment. From the profiles and the KD value we calculated, we observed that S689D tail domain showed the lowest affinity with the motor proteins, while S689A showed the highest, consistent with our co-immunoprecipitation and in vitro binding results.

Figure 3.8 SPR approach showed that the interaction between KIF3A-motor and

S689A-tail is stronger than WT-tail, and S689D-tail. Anti-GST was first immobilized on the gold sensor chips. GST-WT and mutant tails were then captured on immobilized on three different chips. The chips were blocked by GST protein. GST-tail domains were then captured by anti-GST. Different concentrations of KIF3A motor were then injected into the Biacore system. (A) GST-WT-tail and (B) GST-S689D-tail show low binding intensity with KIF3A-motor. (C) The interaction KD value between GST-S689A-tail and KIF3A-motor is calculated as above. The sensorgrams are presented.

3.3.3 Phosphorylation on serine-689 residue of KIF3A tail domain inhibits the motor-tail interaction

Since S689A and S689D only show how KIF3A is regulated under simulated phospho-states, we also want to see how real phosphorylation regulates KIF3A. The *in vitro* kinase assay was applied to check the interaction between motor and tail domain under different phosphorylation status. After 30min or 45min reaction time, the wild-type GST-KIF3A-tail was subjected to western-blot analysis to check the levels of phosphorylation. Phospho-serine/threonine antibody detection showed that the tail domain was effectively phosphorylated. We then subjected the phosphorylated GST-KIF3A-tail sample to GST-pull down together with 1:1 or 2:1 of His-KIF3A-motor to the GST-KIF3A-tail. We found that the motor can be pulled down by the non-phosphorylated tail domain but cannot interact with the tail domain that has been phosphorylated. When we increase the amount of the motor domain, there was still no interaction with the non-phosphorylated tail domain detected (Fig. 3.9). These results indicate that when KIF3A is not phosphorylated, it might adopt an autoinhibited state, and autoinhibition can be

released by phosphorylation at serine-689 residue.

Figure 3.9 *In-vitro* kinase assay showed that phosphorylation inhibits the KIF3A motor-tail interaction. His-motor can be pulled down by the non-phosphorylated tail domain but cannot interact with the phosphorylated tail domain. Left panels: total cell lysate. Right panels: GST-pull down. Lane 1, inactive FLAG-CaMKII + 5 μ g GST-WT-tail + 5 μ g His-motor; lane 2, activated FLAG-CaMKII + 5 μ g GST-WT-tail + 5 μ g His-motor; lane 3, activated FLAG-CaMKII + 5 μ g GST-WT-tail + 10 μ g His-motor.

3.3.4 Detecting the interaction of KIF3A motor and tail *in vivo* using FRET

Having demonstrated that the motor and tail of KIF3A interacts and phosphorylation relieves the interaction *in vitro*, we also wanted to find out whether KIF3A really adopts this mechanism *in vivo*. To study how phosphorylation status affects the autoinhibition of KIF3 in the cells, a FRET approach was adopted. FRET constructs with EYFP at the N-terminal and ECFP at the C-terminal were designed. pEYFP-ECFP was used as a positive control as the two fluorophores are close enough to cause FRET. pEYFP- Δ Tail-ECFP with the tail domain of KIF3A truncated acted as a negative control representing the extended KIF3A with no interaction between motor and tail domain (Fig. 3.10A). We propose that when KIF3A is in an

extended state, EYFP and ECFP are too far away to cause FRET. When the motor and tail domains interact with each other, the excitation at the absorbance wavelength of ECFP (458 nm) causes emission by EYFP (527 nm) and thereby FRET occurs.

The acceptor photobleaching method was conducted to detect KIF3A's motor-tail interaction in U2-OS and HeLa cells. To perform the experiment, the intensities of ECFP and EYFP were first adjusted to a proper range near saturation. Then the EYFP signal in the selected region was photobleached. The FRET efficiency can thus be calculated from the intensity change of ECFP. High FRET efficiencies represent that the two fluorophores are close to each other, indicating more interactions. FRET can be generated by the compact KIF3A, but not the extended active KIF3A. By comparing the FRET efficiencies of the cells transfected with the FRET constructs of KIF3A-WT, KIF3A-S689A and KIF3A-S689D, we can determine the level of extended and compact KIF3A under different phosphorylation levels. The FRET constructs were transfected into U2-OS cells and the photobleaching was performed using Zeiss 710 confocal microscope (Fig. 3.10B).

Figure 3.10 Expression test of the FRET constructs in U2-OS cells. U2-OS cells were transfected with pEYFP-ECFP (54 kDa), pEYFP-KIF3A-ECFP, pEYFP-S689A-ECFP, pEYFP-S689D-ECFP (107 kDa) and pEYFP-ΔTail-ECFP (95 kDa); KIF3A-motor-ECFP (65 kDa), pEYFP-KIF3A-tail, pEYFP-S689A-tail and pEYFP-S689D-tail (40 kDa). (A) The fluorescent proteins were detected by western blot using anti-GFP and anti-KIF3A. (B) To perform FRET experiment, cells expressing EYFP and ECFP were selected using confocal microscope. The EYFP and ECFP fluorescent intensity of selected cells were adjusted to a proper range. Scale bar: 10 μm.

3.3.4.1 FRET efficiency of full-length wild-type KIF3A was higher than the mutants

A graph that displays the FRET efficiency of the cells transfected with FRET constructs was generated by combining all the data from four independent experiments (Fig. 3.11A). The results showed that the phospho-dead mutant KIF3A-S689A samples had the lowest FRET efficiency while the wild-type had

the highest, which was contradictory to our hypothesis. As we do not know the exact binding sites of KIF3A motor and tail domain, we cannot figure out the exact configuration of the possible autoinhibited protein. There is a possibility that the motor and tail binding may result in a conformation that the two fluorophores are situated too far apart, so that FRET cannot be generated. However, for the extended protein, because the coiled-coil is flexible, there will be a certain percentage of tail domain bending near motor domain to let the two fluorophores get close enough to cause FRET (Fig. 3.11C).

(A)

(B)

(C)

Figure 3.11 FRET efficiency of WT and mutants EYFP-KIF3A-ECFP. (A) U2-OS cells were transfected with pEYFP-ECFP, pEYFP-KIF3A-ECFP, pEYFP-S689A-ECFP, pEYFP-S689D-ECFP or pEYFP- Δ Tail-ECFP respectively. Four independent experiments were performed and the graph is developed by combining the FRET efficiency of all the cells collected. The red dots represent the bleached samples. The unbleached samples were not shown in the graph. The number of cells analyzed (N) and statistical significance are indicated. (B) Representative images of U2-OS cells transfected with corresponding FRET constructs. Whole cell area was chosen as the ROI for FRET measurements. The signals of EYFP and ECFP pre- and post-bleach are presented from low to high intensity as color range from blue to red respectively. (C) The possible KIF3A motor and tail interacting configurations.

3.3.4.2 FRET efficiency of cells expressing both KIF3A-motor and S689A-tail was higher than that of cells expressing KIF3A-motor and wild-type or S689D

To rule out the possibility that the motor-tail interaction might cause the fluorophores to be far away from each other, we designed the FRET constructs expressing KIF3A-motor domain with ECFP at its C-terminal, and KIF3A-tail domains of the wild-type and mutants with EYFP at their N-terminals. We co-transfected the motor and tail domains into U2-OS cells and perform the photobleaching by Zeiss 710 confocal microscope. pEYFP and pECFP were co-transfected as the negative control and pEYFP-ECFP acts as positive control. The graph is generated by the data from three independent experiments (Fig. 3.12A). The average FRET efficiency of the cells co-transfected with KIF3A-motor-ECFP and EYFP-S689A-tail is higher than those of the cells transfected with wild-type or the phospho-mimic tails. This result supports our hypothesis that comparing to phospho-dead mutants, KIF3A might be more extended when phosphorylated at its C-terminal,

therefore preventing interaction between the motor and tail domains.

Figure 3.12 FRET efficiency of cells expressing KIF3A-motor-ECFP and EYFP-tail of KIF3A WT and mutants. U2-OS cells were transfected with pEYFP-ECFP or co-transfected with pEYFP and pECFP, pEYFP-KIF3A and motor-pECFP, pEYFP-S689A and motor-pECFP, pEYFP-S689D and motor-pECFP, respectively. Three independent experiments were performed and the graph is developed by combining the FRET efficiency of all the cells collected. The number of cells analyzed (N) and statistical significances are indicated. (B) Representative images of U2-OS cells transfected with corresponding FRET constructs. Whole cell area was chosen as the ROI for FRET measurements. The signals of EYFP and ECFP pre- and post-bleach are presented from low to high intensity as color range from blue to red respectively.

3.3.4.3 FRET efficiency of phosphorylated full-length KIF3A was lower than that of non-phosphorylated KIF3A

Since the S689A and S689D are only phospho-mimic mutants which cannot represent the true phosphorylated states of KIF3A, we also collected the FRET efficiencies of KIF3A under real phosphorylation situation by stimulating phosphorylation by cellular CaMKII. HeLa cells were co-transfected with GST-CaMKII and pEYFP-KIF3A-ECFP and cultured for 24 h. The control sample was treated with buffer whereas the experimental sample was treated with buffer containing higher concentration of Ca^{2+} and phosphatase inhibitor, as described in section 3.2.

To make sure that CaMKII's phosphorylation on KIF3A was active during the experiment, the phosphorylation levels of KIF3A after Ca^{2+} stimulation were first analyzed by western blotting. After 24h incubation, the cells expressing FLAG-CaMKII and FLAG-KIF3A were stimulated by the high $[\text{Ca}^{2+}]$ buffer. The cells were lysed after different time points and subjected to FLAG-IP. The western blot result shows that the phosphorylation levels of CaMKII and KIF3A

kept increasing within 1 h after stimulation (Fig. 3.13). So for the FRET experiment, we collect the data within one hour after the high $[Ca^{2+}]$ buffer treatment.

(A)

(B)

Figure 3.13 Phosphorylation levels of KIF3A and CaMKII at different time points after treating. HeLa cells co-transfected with FLAG-KIF3A and FLAG-CaMKII were treated with high $[Ca^{2+}]$ for 10, 20, 30, 45 and 60 min to trigger phosphorylation. The cells were then lysed and subjected to FLAG-IP (A) The FLAG M2 beads were boiled and sent for western blot. The phosphorylation level of KIF3A and CaMKII was detected by anti-phospho-serine/threonine antibody. (B) The graphs showing the intensity of CaMKII and KIF3A at each time point from the western-blotting were generated.

We co-transfected pEYFP-KIF3A-ECFP and GST-CaMKII into HeLa cells. After 24 h, we treated the control cells and experimental cells with the conditions mentioned above, and subjected one set of the cells (control and

experimental) for FRET, the other set (control and experimental) for western-blot to check the phosphorylation levels of KIF3A. For the western-blot samples, the Ca^{2+} /CaMKII stimulation was performed for 1 h, and phospho-serine/threonine antibody was used to detect the phosphorylation of total lysates. A graph is generated for the data obtained from three independent experiments (Fig. 3.14C). Our results showed that cells under Ca^{2+} /CaMKII stimulation have lower FRET efficiency compare to that of the control. Western-blot results confirmed that during the FRET experiment, more KIF3A was phosphorylated in the Ca^{2+} /CaMKII stimulated sample than the control sample. This experiment was repeated three times, although we only showed the western- blot results from the last two times (Fig. 3.14A and B). We also repeated this experiment in U2-OS cells, which have lower transfection efficiency than HeLa cells (Fig. 3.15C). The results are consistent with those performed in HeLa cells. The western-blot results from three independent repeats were shown in Fig 3.15A and B. We speculated that only a small portion of the highly overexpressed KIF3A was phosphorylated in our selected cells, therefore even the high concentration Ca^{2+} stimulated cells showed relatively high FRET efficiency. Even so, the p -value between the non-phosphorylated and phosphorylated HeLa and U2-OS cells ($p<0.05$) provides a strong evidence for conformational change between the folded and extended states. These experiments confirm that KIF3A might become extended when phosphorylated in the cell, leading to the loss of auto-inhibition.

Figure 3.14 FRET efficiency of non-phosphorylated and phosphorylated EYFP-KIF3A-ECFP expressed in HeLa cells. HeLa cells were transfected with pEYFP-ECFP as positive control, pEYFP- Δ Tail-ECFP as negative control, and co-transfected with GST-CaMKII and pEYFP-KIF3A-ECFP. Cells transfected with pEYFP-KIF3A-ECFP and CaMKII were expected to have higher levels of phosphorylated proteins. (A) Two independent experiments were performed and western-blot analyses of the phosphorylation levels in each experiment are shown. (B) Bar chart showing the phosphorylated KIF3A levels of each experiment. (C) The graph that shows the FRET efficiency of all samples was developed by combining the FRET efficiency of all the cells collected. The number of cells analyzed (N) and statistical significances are indicated. (D) Representative images of HeLa cells transfected with corresponding FRET constructs. Whole cell area was chosen as the ROI for FRET measurements. The signals of EYFP and ECFP pre- and post-bleach are presented from low to high intensity as color range from blue to red respectively.

(A)

(B)

(C)

(D)

Figure 3.15 FRET efficiency of non-phosphorylated and phosphorylated EYFP-KIF3A-ECFP expressed in U2-OS cells. U2-OS cells were transfected with pEYFP-ECFP as positive control, pEYFP- Δ Tail-ECFP as negative control, and co-transfected with GST-CaMKII and pEYFP-KIF3A-ECFP. Cells transfected with pEYFP-KIF3A-ECFP and CaMKII were expected to have higher phosphorylated protein levels. (A) Three independent experiments were performed and western-blot analyses of the phosphorylation levels in each experiment are shown. (B) Bar chart showing the phosphorylated KIF3A levels of each experiment. (C) The graph that shows the FRET efficiency of all samples was developed by combining the FRET efficiency of all the cells collected. The number of cells analyzed (N) and statistical significances are indicated. (D) Representative images of U2-OS cells transfected with corresponding FRET constructs. Whole cell area was chosen as the ROI for FRET measurements. The signals of EYFP and ECFP pre- and post-bleach are presented from low to high intensity as color range from blue to red respectively.

3.4 Purification of KIF3A-motor domain

Since we have demonstrated that KIF3A motor and tail domain tend to fold and interact with each other when the tail domain is not phosphorylated, in order to further illustrate how phosphorylation affects the autoinhibition status of KIF3A at the atomic level, we propose to determine the structures of KIF3A and its motor and tail domains.

The affinity purification steps were briefly summarized in section 3.2 (Fig. 3.16A). I found that purified His-KIF3A-motor was rather sensitive to high temperature. Stability test showed that the protein completely precipitated within 30 min at room temperature or at 18 °C. In order to improve the protein's stability, we did the optimization of induction conditions as well as the buffer conditions. We found out that glycerol and MgCl₂ helped to improve the motor proteins stability at room temperature (RT) (Fig. 3.17). Based on our

optimization processes, we determined the induction condition as 0.4 mM IPTG overnight at 16 °C, and the buffer condition as 50 mM Tris-HCl, pH7.5, 200 mM NaCl, 10% glycerol, 0.8 mM DTT, 2 mM pefabloc, 2 mM MgCl₂, 2 mM CaCl₂, DNase and RNase. All the purification steps were performed at low temperature (<10 °C). In order to remove the nucleotides binding to the protein, we then incubated the Ni-NTA beads eluted protein with 5 mM EDTA and 10 mM sodium phosphate, pH6.0 for 30 min. After the incubation, we concentrated the protein to 5 mL and injected into XK 16/100 superdex 200 pg (GE Healthcare) (Fig. 3.16A). The column was washed by washing buffer. KIF3A-motor was eluted out from 80 mL to 100 mL (Fig. 3.16C and D). We combined the proteins whose UV absorbance were 75% of the peak UV absorbance value and concentrated the protein to different concentrations according to the application later. Because the protein tended to precipitate after freeze-thaw step, it's preferable to use freshly purified proteins for each experiment.

Figure 3.16 Purification of KIF3A-motor domain. (A) The steps for purifying His-KIF3A-motor protein (45 kDa). (B) SDS-PAGE gel showing His-KIF3A-motor eluted with a gradient of imidazole concentration from 20-500 mM. (C) The profile of His-KIF3A-motor going through Superdex200-XK16 column. (D) SDS-PAGE gel showing the His-KIF3A-motor eluted from the Superdex200-XK16 column.

Figure 3.17 His-KIF3A-motor protein stability test. The supernatant of fresh purified protein and freeze-thaw protein after high-speed spinning was loaded to SDS-PAGE gels. (A) The SDS-PAGE staining gel of fresh and freeze-thaw His-KIF3A-motor with or without glycerol. Freeze-thaw protein supernatant sample had less protein than the fresh sample. Less protein precipitated in buffer containing 10% glycerol in 30 min at RT. (B) The SDS-PAGE staining gel of fresh and freeze-thaw His-KIF3A-motor with or without MgCl_2 . Freeze-thaw protein supernatant sample had much less protein than the fresh sample. More protein appeared in the supernatant in buffer containing 2 mM MgCl_2 in 20 min at RT.

3.5 ATPase activity of KIF3A-motor domain

3.5.1 ADP binding inhibits ATP hydrolysis activity of KIF3A-motor domain

KIF3 is a kinesin motor that can hydrolyze ATP and use the energy to travel along microtubules. The motor domain of KIF3 is the functioning part that catalyzes ATP hydrolysis. In order to determine whether the KIF3A-motor that we purified is active and whether nucleotide binding to the protein will affect its activity, we performed an ATPase hydrolysis assay. 10 µg of the protein was added to the reaction solution and the absorbance of NADH at 340 nm was recorded for 300 seconds. The ATPase hydrolysis activity of purified KIF3A-motor without removing the nucleotide, nucleotide-free KIF3A-motor and the nucleotide-free KIF3A-motor incubated with MgADP were tested and the results are shown in Fig. 3.18A. We found that after we removed the nucleotide from the protein, the activity increased. And the activity decreased when we incubated the protein with nucleotide again. The results indicate that the nucleotide occupies the position where KIF3A-motor actively catalyzes ATP, and by incubating KIF3A-motor with EDTA and 0.1 M sodium phosphate, we can effectively remove the nucleotide. It also suggests that KIF3A-motor is less active in ADP-binding state and more active in the empty state.

3.5.2 ATP hydrolysis activity of KIF3A-motor domain was inhibited by ATPase inhibitors

We also performed ATPase hydrolysis assays to test the inhibition of KIF3A-motor activity by ATPase inhibitors. ATPase inhibitors quercetin, resveratrol or NBD-Cl were incubated with KIF3A-motor, and the ATPase activities were assayed. All inhibitors tested inhibited KIF3A-motor activity, among which resveratrol and NBD-Cl showed more significant inhibition than quercetin (Fig. 3.18B). Previous studies have shown that these inhibitors inhibit F₀F₁-ATPase activity by targeting the F₁ domain (164). Therefore, we deduce that KIF3A-motor may share similar ATP catalytic core as F₁-ATPase where the inhibitors can bind to.

Figure 3.18 ATPase hydrolysis assay of KIF3A-motor. (A) The profile of UV absorbance at 340 nm of purified His-KIF3A motor domain (black), nucleotide-free His-KIF3A-motor domain (red) and His-KIF3A-motor domain incubated with MgADP (blue) in 5 min (left), and the ATP hydrolysis activity of the three samples (right). (B) The profile of UV absorbance at 340 nm of nucleotide-free His-KIF3A-motor domain (black), His-KIF3A-motor domain incubated with MgADP (green) and His-KIF3A-motor domain incubated with quercetin (red), resveratrol (blue) and NBD-Cl (pink) in 5 min (left), and the ATP hydrolysis activity of the five samples (right). The data of the graph are expressed as the mean of three replicates with error bars representing standard deviation. The statistical significances are indicated.

3.5.3 Tail binding inhibits ATP hydrolysis activity of KIF3A-motor domain

In order to check whether KIF3A-motor-tail interaction affects KIF3A-motor's activity, ATPase hydrolysis assay of the motor-tail mix was performed. We measured the ATPase activities of the KIF3A motor by incubating 10 μg His-KIF3A-motor with 20 μg purified GST-KIF3A-tail, GST-S689A-tail or

GST-S689D-tail for 1 h. The activity of GST-KIF3A-tail, GST-S689A-tail or GST-S689D-tail were measured as control. The results show that GST-S689D-tail did not inhibit the motor activity at all, while GST-KIF3A-tail and GST-S689A-tail inhibited the motor activity significantly (Fig. 3.19). Since we have shown that the interaction between wild-type or S689A-tail and KIF3A-motor was stronger than that of the S689D-tail (Fig. 3.7), our current observations from the ATPase assays suggest that motor-tail binding inhibits the motor's ATPase activity. Due to the dilution of the proteins in the reaction solution, there might only be a portion of the total tail protein that bound tightly to the motor protein. This can explain why the decrease of the ATPase activity caused by motor-tail interaction was not significant. Our results suggest that the motor-tail interaction affects the energy utilization of the motor domain. It is possible that the tail-binding site falls inside the motor's ATP catalytic core.

Figure 3.19 ATPase hydrolysis assay of the motor-tail mix of KIF3A. (A) The profile of UV absorbance at 340 nm of nucleotide-free His-KIF3A motor domain (black, line); His-KIF3A-motor domain incubated with GST-KIF3A-WT-tail (red, line), GST-KIF3A-S689A-tail (blue, line) and GST-KIF3A-S689D-tail (orange, line); GST-KIF3A-WT-tail (green, dashes), GST-KIF3A-S689A-tail (blue, dashes) and GST-KIF3A-S689D-tail (purple, dashes) in 5 min. (B) The ATP hydrolysis activity of the samples above. The data of the graph are expressed as the mean of three replicates with error bars representing standard deviation. The statistical significances are indicated.

3.6 KIF3A-motor domain structure model

As the KIF3A-motor domain structure has not been reported, in order to get some ideas of the overall structure of KIF3A motor, we performed structure modeling on Swiss-Model using KIF3B and Kinesin-1 as templates (Fig. 3.20A). The sequence identity between KIF3A and KIF3B or Kinesin-1 motor domain are 68.44% and 46%, respectively. The overlap of the two models shows high similarity (Fig. 3.20B), suggesting that both models can represent a probable KIF3A-motor domain structure.

To explain the molecular mechanism of KIF3A's autoinhibition, the 3-D structure of the motor-tail complex is needed. The attempts to get KIF3A's 3-D structure will be discussed in later sections. Here we showed the computational simulation of the interaction between KIF3A motor and tail domains. The computational simulation was performed by Dr. Dilraj Lama from Dr. Chandra Verma's group at the Bioinformatics Institute of A* STAR.

Among all the kinesins that adopt the autoinhibition mechanism, Kinesin-1 is the only protein whose structure was solved as motor-tail complex with high resolution (64). The 3-D structure of the motor-tail complex has unveiled the autoinhibition mechanism of Kinesin-1. KIF3A motor domain shares 46% of the sequence identity with the motor domain of Kinesin-1 (Fig. 3.20B). The tail peptide containing the serine-689 residue that is critical for KIF3A's motor-tail interaction has a conserved AKP sequence with Kinesin-1 tail peptide applied

in the reported motor-tail complex structure determination (Fig. 3.21A). Based on the above reasons, the 3-D structure of Kinesin-1 motor-tail complex (PDB: 2Y65) was used as the template to simulate KIF3A's motor-tail interaction. The mouse KIF3A tail peptide ⁶⁸⁶RKRSAPETV⁶⁹⁵ was fit into the interface between the KIF3A motor domain dimer. The KIF3A tail peptide existed as an extended, linear conformation similar to the Kinesin-1 tail peptide (Fig. 3.21B). Serine-689 residue (red spheres) falls in a hydrophobic pocket formed by phenylalanine-143, leucine-144 and valine-145 on the motor domain (Fig. 3.21C). When serine was mutated to alanine, the hydrophobic force was enhanced and the interaction became stronger (Fig. 3.21D). The simulation of the complex's conformational change showed that serine-689 or alanine-689 maintained high affinity with the hydrophobic pocket over time, and the tail peptide stayed in the interface of the motor domain dimer the whole time (Fig. 3.21C, D). However, when a phosphate group (PO_3^{2-}) was added to serine-689, the hydrophobic interaction was broken. We observed from the simulation that the tail peptide became more flexible and kept coming in and out of the motor domain dimer interface (Fig. 3.21E), suggesting that the interaction between the tail peptide and the motor domain was not stable under the phosphorylated state. The simulation results support our observations that KIF3A motor domain interacts with the tail domain, and phosphorylation on serine-689 residue release the interaction.

Since the switch domains that are critical for the ATP, ADP binding was located

at the opposite side of KIF3A's motor-tail interacting interface (Fig. 3.21B), the regulation of the ATPase activity by autoinhibition should not be the one with direct blocking of the ATP binding core by the tail domain. A possible model is that the motor-tail interaction causes the conformational change of the motor domain, triggering the switch domains to have a higher affinity towards ADP, hence, the ATP intake is blocked. A high-resolution crystal structure of the motor-tail complex can help us to better decipher the autoinhibition mechanism of KIF3A in the future.

Swiss-Model		
Template PDB	Kinesin-like protein KIF3B (3B6U)	Kinesin heavy chain (2Y5W)
Sequence identity%	68.44	46
Sequence number	12-350	13-347

(B)

Figure 3.20 KIF3A-motor models. (A) The models of KIF3-motor domain predicted by Swiss-Model using KIF3B (red) and Kinesin-1 motor domain (green) as templates. (B) Overlapping of the two predicted models.

(A)

KIF3A	Mm	686	RKRS	AKP	ETV	695
	Hs	687	RKRS	AKP	ETV	696
	Rn	687	RKRS	AKP	ETV	696
	Pp	657	RKRS	AKP	ETV	666
Kinesin-1 Dm		939	QAQI	AKP	IRS	948

(B)

(C)

(D)

(E)

Figure 3.21 Simulation of the motor-tail interaction based on KIF3A model. (A) Sequence alignment of KIF3A isoforms (Mm, *Mus Musculus*; Hs, *Homo sapiens*; Rn, *Rattus norvegicus*; Pp, *Proechiopsis prolifica*) and Kinesin-1 (Dm, *Drosophila melanogaster*). Amino acids “AKP” are conserved between KIF3A and Kinesin-1. (B) Simulation of the interaction between mouse KIF3A peptide 686-695 (yellow sticks) and its motor dimer (green cartoon) using Kinesin-1 motor domain dimer (PDB:2Y65) as template. Serine-689 residue is presented as red spheres. ADP (orange sticks), Mg^{2+} ion (blue sphere), switch I (purple cartoon) and switch II (cyan cartoon) were also shown in the model. (C,D,E) Left: Stereoplots showing the interacting interfaces between the motor domain’s hydrophobic pocket (green sticks) and the peptides (white sticks) of the WT (A), S689A (B) and phosphorylated (C) tail domains. Serine-689 residue is highlighted with red color; Alanine-689 is purple and serine-689 PO_3^{2-} is shown as yellow sticks. Right: Simulations of the conformational changes of the motor-tail complex during a certain time period in the native state in solution. Motor domain dimer is presented as grey cartoon; tail peptides are shown as red sticks. Atom colors: C: backbone color; H: white; N: blue; O: red.

3.7 SAXS experiment to determine the low-resolution structure of KIF3A-motor

SAXS has been widely used to determine the structure of proteins in low-resolution, providing information on the parameters of proteins without getting a crystalline sample. In order to find out the structural information of KIF3A-motor, SAXS was performed. His-tagged KIF3A-motor domain was purified and concentrated to 4 concentrations from 1.1 mg/mL to 5.5 mg/mL. Data were collected every minute for 30 min by Bruker NanoStar equipped with MetalJet Excillum X-ray source and VÅNTEC-2000 detector. Buffer scattering was subtracted from the collected protein data. The protein samples were kept at 288.15 K during data collection. The protein’s overall structure parameters were detected from the protein solution by SAXS. The SAXS data collection was kindly helped by Dr. Saw Wuan Geok from Dr. Gerhard Grüber’s

laboratory (School of Biological Sciences, Nanyang Technological University).

The structure parameters $I(0)$, R_g and $D_{\max}$ value increased with the increase of the protein concentration, although not significantly. The derived R_g and $D_{\max}$ value for KIF3A-motor were 2.76 ± 0.03 to 3.00 ± 0.002 nm and 8.1 ± 0.8 to 9.7 ± 1.0 nm. The molecular mass determined by SAXS data also increased slightly with the protein concentration (Table 3.1), suggesting that there might be protein aggregation at higher concentration.

Data collection parameters				
Instrument (source & detector)	Bruker NanoStar equipped with MetalJet eXcillum X-ray source and VÅNTEC-2000 detector			
Beam geometry	100 μm slit			
Wavelength (nm)	0.134			
q range (nm ⁻¹)	0.16 – 4			
Exposure time (min)	30 (30 frames x 1 min)			
Protein sample	KIF3A motor domain			
Concentration range (mg mL ⁻¹)	1.1	2.0	4.6	5.4
Temperature (K)	288.15			
Structural parameters				
$I(0)$ (arbitrary unit) (from Guinier)	10.27 ± 0.30	10.60 ± 0.17	10.86 ± 0.09	11.43 ± 0.10

R_g (nm) (from Guinier)	2.61 ± 0.11	2.75 ± 0.07	2.83 ± 0.03	2.96 ± 0.04
$I(0)$ (arbitrary unit) [from $P(r)$]	10.3 ± 0.17	10.7 ± 0.11	10.8 ± 0.06	11.3 ± 0.06
R_g (nm) [from $P(r)$]	2.76 ± 0.03	2.86 ± 0.02	2.84 ± 0.01	3.00 ± 0.02
D_{max} (nm)	8.1 ± 0.8	8.7 ± 0.9	8.9 ± 0.9	9.7 ± 1.0
Porod volume estimate (nm ³)	~71.5	~78.2	~82.2	~81.7
DAMMIF excluded volume (nm ³)	~91.5	~99.8	~102.0	~105.0
Dry volume from sequence (nm ³) ‡	~52.8	~52.8	~52.8	~52.8

Molecular mass determination

Calculated monomeric MM (kDa) [from sequence*]	~43.6	~43.6	~43.6	~43.6
Molecular mass MM (kDa) [from Porod volume]	46.5 ± 5	50.8 ± 5	53.4 ± 5	53.1 ± 5
Molecular mass MM (kDa) [from excluded volume]	45.8 ± 5	49.9 ± 5	50.8 ± 5	52.7 ± 5
No. of monomer	1.1	1.1	1.2	1.2

Software employed

Primary data reduction	BRUKER SAS
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Data processing	PRIMUS
Ab initio analysis	DAMMIF
Validation and averaging	DAMAVR
3D graphics representations	PyMOL

Table 3.1 Structural parameters for His-KIF3A-motor domain.

No significant difference was observed among all the concentrations in the $P(r)$ function (Fig. 3.22D), suggesting that there was little change in the protein's conformation with concentration change. From the Guinier plots, we found that the protein showed linearity in all concentrations (Fig. 3.22C), indicating that no protein aggregation had occurred. The number of monomers in samples of different concentration was 1.1 to 1.2, which suggests that most of the protein present as monomers in the solution. The low-resolution protein structures of three concentrations that were *ab initio* reconstructed from the scattering patterns using DAMMIF program were shown in Fig. 3.20. The NSD value was calculated between 20 independent reconstructed models using DAMAVR. All three protein shapes have high similarity and fit well with the predicted protein model (Fig. 3.23D). Concentration-dependent effect of the protein structure was not observed, which indicates that the protein's properties should not differ in all other experiments when the concentration is different.

Figure 3.22 SAXS scattering patterns and analysis of His-KIF3A-motor domain.

(A) SAXS scattering profile of His-KIF3A-motor domain at 1.1 mg/mL (red), 2.0 mg/mL (green), 4.6 mg/mL (blue) and 5.5 mg/mL (pink). (B) The scattering profile of the four concentrations fits well when the scattering vector amplitude (q) is below 1 nm^{-1} . (C) The Guinier plots of His-KIF3A-motor domain in four concentrations all showed linearity, indicating no protein aggregation. (D) $P(r)$ functions of His-KIF3A-motor domains in four concentrations.

Figure 3.23 *Ab initio* reconstruction modeling for KIF3A-motor domain. The *Ab initio* reconstruction structure of KIF3A-motor domain at concentration (A) 1.1 mg/mL, with the NSD value 0.776 ± 0.111 ; (B) 2.0 mg/mL, with the NSD value 0.622 ± 0.039 ; (C) 4.6 mg/mL, with the NSD value 0.590 ± 0.043 . (D) Superimposition of the predicted KIF3A-motor model and the reconstruction structure.

3.8 Crystallization screening of KIF3A-motor domain

In order to get detailed structural information of KIF3A-motor domain, high-resolution protein structure is needed. NMR and cryo-EM are not applicable for KIF3A-motor domain because of its molecular weight. Here we introduce the X-ray crystallography approach to solve the structure of KIF3A-motor domain. In the attempt to determine the 3D structure by X-ray

crystallography, six steps are needed: step one, purification of the target protein; step two, searching of the initial crystallization conditions by coarse screening; step three, optimization of the crystallization conditions to obtain better crystal quality; step four, collection of the X-ray diffraction data; step five, reconstruction of the 3D model; step six, detailed analysis of the refined model (165). Protein's purity and oligomeric homogeneity are strictly required to obtain crystals. For KIF3A-motor protein, we performed several optimizations to obtain pure protein, and the oligomeric homogeneity was supported by the SAXS data.

The protocol of KIF3A-motor protein purification has been expatiated in the previous section. After purification, the protein sample was concentrated to 5, 7, 8, 10 or 12 mg/mL. The protein solutions in different concentrations were mixed with the precipitants from the screening kits for crystal growing using hanging drop vapor diffusion method and incubated at 10 °C. In the hanging drop method, equal volumes (0.5-10 μ L) of the protein sample and the buffer containing precipitant are mixed on a coverslip, and the coverslip is placed on a reservoir (well) containing the same buffer with precipitant (100-1000 μ L) (Fig. 3.24B). The vacuum grease then seals the coverslip and the rim of the well, preventing the evaporation of the droplet (166). Because protein solution dilutes the precipitant in the droplet, water will evaporate from the drop to the solution in the reservoir to maintain the vapor equilibration. In this way, the original unsaturated soluble protein is concentrated to saturation, inducing

protein crystal forming. Theoretically, when the protein sample is concentrated to the labile zone (the lowest level of supersaturation), protein crystal nucleus is formed and the crystal grows fast, causing the decrease of the protein concentration. The protein concentration decreases to the metastable zone, where the crystal grows slowly (Fig. 3.24A). In order to obtain the crystals of KIF3A-motor, we tried to mix different concentrations of protein samples with a series of precipitant conditions in 20 screening kits (Table 3.2).

Figure 3.24 Schemes of protein solubility curve and the hanging drop method. (A) Protein solubility curve. The figure was taken from *Krauss et al., 2013* (165); (B) The scheme of the hanging drop method.

The results from several conditions were shown in Fig. 3.25. We observed crystal forming in the following conditions: Precipitant Synergy #8, Cryo II #11, Cryo I #47 and Wizard III #42. However, when we picked out the nicely formed crystals and tried to break them, we found out that all the crystals had high rigidity and were hard to break. Because protein crystals always appear as elastic and soft, the rigid crystals have high possibility to be formed by salts. In addition, when the droplet was exposed to air and went through quick evaporation, more similar crystals formed. Based on these observations, we conclude that all the crystals we obtained are salt crystals. Same salt crystals were found when we repeated these conditions, and no protein crystals were observed. Among all the other conditions, the ones which showed phase separations are very promising, since the proteins are very likely to be concentrated to saturation in phase separation. We repeated all the conditions showing phase separation with different concentrations, however, we did not obtain positive results.

Since we could not find the initial crystallization conditions by coarse screening, we could not proceed further. Further optimizations and screenings are ongoing. We are also collaborating with the Protein Purification Platform (PPP) of NTU to design new constructs for KIF3A-motor domain in order to get a more stable protein. This experiment is ongoing.

Screening Kits	Conditions	Cat No.	Company
Crystal Screen I	1-50	HR2-110	Hampton Research
Crystal Screen II	1-48	HR2-112	Hampton Research
Index 1-48	1-48	HR2-144	Hampton Research
Index 49-96	49-96	HR2-144	Hampton Research
PEG/Ion Screening	1-48	HR2-126	Hampton Research
PEG/Ion 2 Screening	1-48	HR2-098	Hampton Research
SaltRx 1-48	1-48	HR2-108	Hampton Research
SaltRx 49-96	49-96	HR2-108	Hampton Research
Grid Screen-Ammonium Sulfate	1-24	HR2-211	Hampton Research
Grid Screen-MPD	1-24	HR2-215	Hampton Research
Grid Screen-PEG 6000	1-24	HR2-213	Hampton Research
Cryo I	1-48	1009536	Emerald Biosystems
Cryo II	1-48	1009537	Emerald Biosystems
Precipitant Synergy	1-64	1009539	Emerald Biosystems
Wizard I	1-48	1009530	Emerald Biosystems
Wizard II	1-48	1009531	Emerald Biosystems
Wizard III	1-48	1009533	Emerald Biosystems
Wizard IV	1-48	1009534	Emerald Biosystems
Morpheus 1-48	1-48	MDI-46	Molecular Dimensions
Morpheus 49-96	49-96	MDI-46	Molecular Dimensions

Table 3.2 Crystallization Screen Kits

Figure 3.25 Screening of KIF3A-motor protein crystallization conditions. (A) Steps for the crystallization of KIF3A-motor. (B) Selected results from the crystallization screening. a, clear drop; b, heavy precipitate; c, granular precipitate; d, microcrystalline precipitate; e and f, phase separation; g-j, well-shaped single crystals.

Chapter 4 Discussion

In this project, we studied the effects of phosphorylation on the KIF3A subunit of the heterotrimeric KIF3 motor complex. We confirmed that Serine-689 residue at the C-terminal tail domain of mouse KIF3A is a major phosphorylation site that regulates the activity of KIF3A. The cargo-trafficking activity of KIF3A was found to be inhibited when S689 (mouse) or S690 (human) was not phosphorylated. We also verified the interaction between KIF3A's motor and tail domains by different approaches. These experiments provided evidence that the motor domain interacts with the non-phosphorylated tail domain, possibly resulted in an autoinhibited state. Phosphorylation of S689 residue relieves the autoinhibition. We further characterized the motor domain by measuring its ATPase activity. A computational simulation of KIF3A motor-tail complex also supports our findings that phosphorylation of S689 residue disrupts the motor-tail interaction. In addition, we also obtained low-resolution structural information using SAXS.

4.1 Phosphorylation affects the activity of KIF3A

The phosphorylation status of serine-690 residue on the C-terminal of human KIF3A has been shown to be regulated by POPX2 and CaMKII phosphatase-kinase pair (167). Our immunofluorescence results showed that overexpressing KIF3A-S690A with the mutation from serine to alanine to

preclude phosphorylation inhibited the transportation of N-cadherin and β -catenin to the cell-cell contact. The localization of N-cadherin and β -catenin was not impaired in KIF3A wild-type and S690D (the mutation of serine to aspartic acid to mimic continuous phosphorylation) overexpressing cells (Fig. 3.1). We also performed the cell surface protein isolation assay to confirm that much less N-cadherin was localized to the cell surface in KIF3A-S690A overexpressing cells (Fig. 3.2). In KIF3A knockdown cells, cell surface N-cadherin was significantly decreased. Overexpressing KIF3A wild-type and S690D, but not S690A, effectively rescued the N-cadherin localization to the cell periphery (Fig. 3.3). These results indicate that the phosphorylation status of serine-690 residue plays a crucial role in the intracellular KIF3A-mediated cargo transport.

Although the dominant negative effect of S690A overexpression was significant, the trafficking of N-cadherin and β -catenin was not totally inhibited in S690A overexpressing cells. The reason might be that the cargoes can also be transported by the endogenous KIF3 motor whose activity was not totally suppressed by the overexpression, or by other kinesins that can also act as carriers of these cargoes. Serine-181, serine-663 and serine-681 have been identified as the phosphorylation sites of KIF3B and phosphorylation on serine-663 was shown to inhibit the localization of IFT particles (102, 168). It might be possible that the activation of KIF3 motor requires phosphorylation regulation on both motor subunits, and precluding the phosphorylation of

KIF3A subunit alone does not totally suppress the KIF3 motor activity. Although understanding the mechanism that regulates KIF3 heterotrimeric functions requires further studies of all three subunits, here we focus mainly on KIF3A subunit. The regulation mechanism that explains why phosphorylation affects KIF3A's cargo transportation is interesting to be explored further.

4.2 Phosphorylation can relieve autoinhibited KIF3A

Autoinhibition has been found to be a general model of regulation for many kinesin families. In an autoinhibited state, kinesins form folded, compact conformations that cannot hydrolyze ATP or associate with cargoes or microtubules. When activated, kinesins are relieved from autoinhibition and transformed into active, extended configurations. Kinesin-1, Kinesin-2 family members OSM-3 and KIF17, Kinesin-3 family member KIF1A and Kinesin-7 family member Cenp-E have all been reported to adopt autoinhibited conformations. Based on our findings that KIF3A's activity is affected by its phosphorylation state, we proposed that KIF3A also adopts an autoinhibition state. Phosphorylation activates KIF3A by relieving the autoinhibition.

4.2.1 Serine-689 is the major phosphorylation site regulating KIF3A motor-tail interaction

In our previous study, CaMKII has been identified to be the kinase

phosphorylating KIF3A (human) at serine-690 residue, and cargo transportation of KIF3A was inhibited by CaMKII inhibitors KN62, KN93 and AIP (103). These results suggest that CaMKII affects KIF3A's intracellular transportation activity by the phosphorylation on serine-690 residue. The mechanism of how KIF3A's activity is regulated by phosphorylation was unknown. Here we confirmed that serine-689 residue of mouse-KIF3A was mainly phosphorylated by CaMKII using *in vitro* kinase assay (Fig. 3.5) and the *in vivo* CaMKII stimulation and inhibition experiments (Fig.3.6). Since serine-689 is the key residue, which is phosphorylated and can affect cargo transport, we validated and confirmed that phosphorylation on serine-689 residue by CaMKII relieves the autoinhibited configuration of KIF3A.

We have taken different approaches in our current study. We first phosphorylated KIF3A-tail protein by incubating them with activated CaMKII, and mixed the KIF3A-motor domain protein with non-phosphorylated or phosphorylated KIF3A-tail. The interaction between the motor and tail domains was detected by pull-down assays combined with western-blotting (Fig 3.9). Our results showed the interaction between KIF3A-motor and the non-phosphorylated tail but not the phosphorylated tail, suggesting that CaMKII phosphorylation could relieve the motor-tail association. This result was further supported by the FLAG-immunoprecipitation assay of the cell lysates from HEK293 cells overexpressing motor and tail domains. GST-KIF3A-WT-tail and GST-KIF3A-S689A-tail were significantly pulled-down

by FLAG-KIF3A-motor, while little GST-KIF3A-S689D-tail protein was pulled-down (Fig 3.7). The interaction between purified motor and tail domains were also detected by GST pull-down and SPR using the Biacore system. In the GST-pull down experiment, only the GST-KIF3A-S689A-tail can effectively pull down the motor domain (Fig. 3.8). SPR experiments also showed a relatively strong interaction between KIF3A-S689A-tail and KIF3A-motor with the KD value $4.836e^{-8}$. The interaction between the KIF3A-motor and the wild-type or the phospho-mimic KIF3A-tail was too weak to be detected in both experiments. These results imply that serine-689 residue is the major phosphorylation site regulating KIF3A's motor-tail interaction, and phosphorylation relieves the interaction between motor and tail domains.

4.2.2 KIF3A goes through a conformational change when getting phosphorylated *in vivo*

The *in vitro* experiments detecting KIF3A's motor-tail interaction have revealed the autoinhibition mechanism regulated by phosphorylation. Nevertheless, how KIF3A's activity is regulated *in vivo* is still not answered. To further verify that autoinhibition is observed in live cells, we designed biosensors of KIF3A to monitor its activity in cells using Zeiss 710 confocal microscope to detect FRET. The cells co-transfected with EYFP-KIF3A-S689A-tail and KIF3A-motor-ECFP biosensors showed higher FRET efficiency than the cells co-transfected with donor KIF3A-motor-ECFP and acceptor EYFP-KIF3A-WT-tail or EYFP-KIF3A-S689D-tail biosensors (Fig. 3.12). Our FRET results indicate that

compared to the wild-type and phospho-mimic tail domains, more phospho-dead tail interacted with KIF3A-motor domain in living cells, which was consistent with the *in vitro* experiments. Full-length KIF3A biosensors with EYFP fused to the N-terminal and ECFP at the C-terminal were also generated to mimic a more real *in vivo* situation (Fig. 3.14 and 3.15). The phosphorylation of KIF3A was triggered by the stimulation of CaMKII's activity. The average FRET efficiency of cells expressing more phosphorylated KIF3A was significantly lower than the control, suggesting more KIF3A existed as extended conformation in this condition. The FRET results together with the *in vitro* biochemical results provide us the evidence supporting that phosphorylation on serine-689 residue of KIFA motor subunit triggers a conformational change from a folded configuration to an extended and active configuration.

4.2.3 ATP hydrolysis activity of KIF3A is inhibited by autoinhibition

Kinesins are ATPases that hydrolyze ATP to generate the stepping along microtubules. The ATPase activity of the NH₂-terminal motor domain is essential for kinesin MT-dependent intracellular transportation. There have been reports showing that the compact conformation of a full-length *Drosophila* kinesin heavy chain formed by the interaction of its motor and tail domains inhibited its ATPase activity (38). The ATPase activity of Kinesin-1 was found to increase 2.4-fold after removing the C-terminal tail (169).

In an attempt to find out whether the autoinhibition of KIF3A motor subunit affects its activity, we performed ATPase hydrolysis assays using motor and tail domains. The interaction between KIF3A-S689A-tail and KIF3A-motor inhibited the ATPase hydrolysis activity of KIF3A-motor domain (Fig. 3.19). This finding suggests a possibility that the folding of the tail domain into the motor domain interferes KIF3A's ATPase activity.

The switch regions that are conserved among kinesins have been shown to play critical roles in the conformational change among different nucleotide-binding states (56, 170, 171). The conserved serine residue from switch I (**S**SRSH) was observed to interact with Mg-ATP at the active site. ADP was found binding to switch II and the P-loop. The conformational change of the switch regions affects kinesin's ATP binding, ADP releasing as well as MT-association. The conserved amino acids of the switch regions are highlighted in the KIF3A-motor protein model which was predicted using Kinesin-1 motor domain as the template (Fig. 4.1). The ATPase catalytic core is most possibly located around this area. Our ATPase hydrolysis results showed that KIF3A-motor activity decreased when pre-incubated with Mg-ADP (Fig. 3.16A), which indicates that ADP might compete with ATP at the same catalytic core. When ADP occupies in the switch regions, it either blocks the active binding site of ATP or triggers the conformational change of the binding site. We also found that the F1-ATPase inhibitors resveratrol, quercetin and NBD-Cl effectively inhibited KIF3A-motor's activity (Fig. 3.16B). Since these

inhibitors formed hydrophobic interactions with F1-ATPase in the hydrophobic binding pockets of its three catalytic core sites (172-174), we hypothesize that KIF3A-motor also shares similar hydrophobic binding pocket in its catalytic core site, where the inhibitors can bind and thus block ATP intake.

Figure 4.1 The switch regions of KIF3A. The conserved side chains of switch I SSRSH and switch II DLAGSE are shown in the predicted KIF3A-motor domain model in a Mg²⁺-ADP binding form. The helical part of switch I is shown in red color and the switch II loop is shown in yellow color. Mg²⁺ is presented as blue sphere and ADP is shown as orange sticks. The conserved S218 from switch I and D250 from switch II that might interact with Mg-ATP or ADP are also shown in the model.

The 3D structure of Kinesin-1's motor-tail complex has been reported and it provides us evidence to explain how autoinhibition affects the ATPase activity of Kinesin-1 (64). In this study, the authors found that the tail peptide binds to the motor domain on the opposite side of the nucleotide-binding site. Instead of directly blocking the nucleotide binding pocket, the Kinesin-1 tail peptide

binds to the motor dimer and induces a conformational change in the motor domain, hiding the switch regions from the nucleotide. KIF3A motor domain shares 46% of the sequence identity with Kinesin-1, and the tail peptide containing the serine-689 residue that's critical for KIF3A's motor-tail interaction has a conserved AKP sequence with the Kinesin-1 tail peptide applied in the reported work (Fig. 3.21A). Based on the above reasons, the computational simulation of KIF3A tail peptide 686-695 in complex with the KIF3A motor dimer using Kinesin-1 as template can represent the KIF3A motor-tail interaction. In this simulation, serine-689 residue of KIF3A tail peptide falls into a hydrophobic pocket formed by phenylalanine-143, leucine-144 and valine-145 at the motor domain (Fig. 3.21C). The simulation of the complex's conformational change in native state in solution showed that serine-689 or alanine-689 maintained high affinity with the hydrophobic pocket over time, and the tail peptide stays in the interface of the motor domain dimer the whole time (Fig. 3.21C, D). However, when a phosphate group (PO_3^{2-}) was added to serine-689, the hydrophobic interaction was broken and the interaction between the tail peptide and the motor domain was no longer stable. The simulation results support our observations that KIF3A motor domain interacts with the tail domain, and phosphorylation on serine-689 residue release the interaction. Based on the simulation result, we speculate that KIF3A might share the same regulatory mechanism of autoinhibition as Kinesin-1. We propose a possible model to explain how autoinhibition disrupts

KIF3A-motor ATPase activity: in the inactive state, the tail domain folds into the hydrophobic pocket of the motor domain, and the motor and tail interact via hydrophobic forces. This interaction causes the conformational change of the switch regions, resulting in higher affinity towards ADP, thus inhibit ATP intake. Phosphorylation on serine-689 residue on the tail domain breaks the hydrophobic affinity between motor and tail, thus relieving the motor-tail interaction. When the folded conformation is relieved, the catalytic core is once again free for ATP binding, resulting in the activation of KIF3A ATPase activity. Since this simulation was based on KIF3A's conserved AKP sequence with the Kinesin-1 tail peptide, further experiments to check whether mutation of AKP affects KIF3A's motor-tail interaction can be performed to verify the confidence of the simulation.

In another study of Kinesin-1's autoinhibition mechanism, *Dietrich et al.* identified a direct cross-linking between a conserved sequence of Kinesin-1's tail domain and its motor domain's switch I region by mass spectrometry analysis (175). We do not rule out the possibility that KIF3A's tail domain interacts directly with the motor's catalytic core and blocks the ATP binding site.

4.3 The autoinhibition model of KIF3 motor complex

KIF3 motor complex associates with its cargoes by the non-motor subunit

KAP3. Previous studies from our group showed that the interaction between KAP3 and N-cadherin was not affected by phosphorylation. The phosphorylation state on serine-690 residue doesn't affect the KIF3A-tail-KAP3 interaction either (103). In combination with our results, we conclude that phosphorylation state of KIF3A affects its ATPase hydrolysis activity rather than the cargo binding ability. Previous report also showed that the *in vitro* KIF3A-microtubule binding was impaired when the phosphorylation on serine-690 residue was precluded (167). Switch II loop/helix have been reported to interact with microtubules (176-179), which implies that the MT-binding site might also locate in the switch regions. The conformational change induced by phosphorylation might release both the ATP binding site and the MT-binding site. These findings suggest that phosphorylation regulates both the KIF3A's MT-binding and the force generation for MT-gliding.

Based on these findings, we propose an autoinhibition model for the KIF3 motor (Fig. 4.2). We hypothesize that when KIF3 stays in the autoinhibited state in which the motor domain interacts with the tail domain to form a folded conformation, the microtubule and the ATP binding domains are blocked by the folded configuration to prevent the motor-MT interaction and ATP hydrolysis. Phosphorylation on the serine-689 residue at the C-terminal tail domain of KIF3A subunit will relieve the autoinhibition and induce a conformational change to expose the MT and ATP binding domains. The extended KIF3 can then bind to the microtubule and actively move along microtubules to transport

cargoes driven by the energy from the ATP hydrolysis. When the transportation is done, serine-689 residue is dephosphorylated by POPX2 phosphatase and the KIF3 complex will return to the inactive autoinhibited state.

Figure 4.2 Proposed autoinhibition model of KIF3. When KIF3 is in an autoinhibited state, the motor and tail domains interact with each other and prohibit it from binding to microtubule. Phosphorylation might relieve KIF3 from the autoinhibition status. The active KIF3 motor can then bind to microtubule properly and transport cargoes along the microtubule.

In addition, the coiled-coil linker domain may also play a role in forming and regulating autoinhibition. *Hammond et al.* suggested that KIF17's coiled-coil and its motor domains formed an ionic interaction in the autoinhibited state (45). A report from *Kaan et al.* also indicates a double lock down autoinhibition mechanism, where the motor-tail interaction was coupled with the cross-linking between the neck linker and the motor domain to inhibit ADP release (64).

Although many have reported that the coiled-coil was not involved in most kinesin autoinhibition, it will be interesting to verify whether the coiled-coil plays a part in KIF3A autoinhibition.

The activity of KIF3 complex is dependent on the coordination between KIF3A and KIF3B subunits. How KIF3 complex is regulated in cells might be very complicated and involves all the subunits. No report has shown whether KIF3B adopts an autoinhibited conformation. *Liang et al.* reported that phosphorylation on serine-663 residue of KIF3B disrupts its cargo-binding activity (168). KAP3 might also regulate KIF3's activity in many ways. In addition to its cargo-binding role, some kinases or phosphatase regulate KIF3A or KIF3B via the interaction with KAP3 (180). Besides serine-689 residue, there might be other phosphorylation sites that are essential for the regulation of KIF3A's activity. There are still so many questions to be answered if we want to fully understand the how the KIF3 motor complex is regulated. Further experiments can be designed to determine how the three subunits which made up the KIF3A complex cooperate to allow proper function of the KIF3 motor.

4.4 The 3D structure of KIF3A is required for detailed information

Structural data on the conformation of KIF3A motor in active and inactive

states can help us better understand the autoinhibition mode of KIF3A motor at the atomic level. We have already obtained the parameters and a low-resolution reconstruction structure of the KIF3A-motor domain by Small-angle X-ray scattering (SAXS) (Fig. 3.20). The purity and oligomeric homogeneity of the protein have also been confirmed (Fig. 3.14 and Table 3.1). We are still in the process of screening for proper protein crystals for X-ray crystallography. In order to get the detailed information of the interaction between the motor and tail domain of KIF3A, we also plan to determine the structure of the N-terminal motor domain in complex with the C-terminal tail domain. However, for GST-KIF3A-tail protein, we failed to purify the tail protein after deletion of the GST-tag. For the His-KIF3A-tail protein, only little protein can be collected after gel filtration step. We are still working on the optimization of the purification conditions for the tail protein.

Chapter 5 Conclusion and future work

Using immunoprecipitation, surface plasmon resonance and FRET, we have demonstrated that the motor domain and the tail domain of KIF3A interact with each other when the tail domain is in a non-phosphorylated state. We have also proposed an autoinhibition model for KIF3A. The main amino acid residue which is important in this phospho-regulation has been identified to be serine-690 in human KIF3A or serine-689 in mouse KIF3A. For future work, we plan to obtain the structure of the KIF3A motor domain in complex with the tail domains from WT, S689A or S689D constructs.

We have not determined the exact binding motifs on the KIF3A motor domain and tail domain which are responsible for the interaction between these two domains. The amino acids AKP before serine-689 residue might be important for KIF3A's motor-tail binding. Mutation of these amino acids to check their effects on KIF3A's motor-tail interaction can help to identify the amino acid residues important for autoinhibition.

As the KIF3 kinesin is made up of KIF3A and KIF3B heterodimer together with KAP3, we also intend to broaden our study to investigate if phosphorylation also regulates KIF3B. The consensus phosphorylation motif for CaMKII is not found at the C-terminal of KIF3B. It would be interesting to determine if KIF3B also adopts an autoinhibited model or how the conformation of KIF3A can affect KIFB.

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Appendix

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Figure S1. KIF3A motor-tail interaction. Purified His-KIF3A-motor and purified GST-KIF3A-Tail, GST-S689A-Tail or GST-S689D-Tail mixture were incubated with Glutathione SepharoseTM 4B. GST-fusion proteins were captured and the co-precipitated complex was separated by SDS-PAGE and analyzed by western analysis using anti-His and anti-GST antibodies.

Figure S2. ATPase hydrolysis assay of the motor-tail mix (1:1 in mass) of KIF3A.

(A) The profile of UV absorbance at 340 nm of nucleotide-free His-KIF3A motor domain (black, line); His-KIF3A-motor domain incubated with GST-KIF3A-WT-tail (red, line), GST-KIF3A-S689A-tail (blue, line) and GST-KIF3A-S689D-tail (orange, line); GST-KIF3A-WT-tail (green, dashes), GST-KIF3A-S689A-tail (blue, dashes) and GST-KIF3A-S689D-tail (purple, dashes) in 5 min. (B) The ATP hydrolysis activity of the samples above. The data of the graph are expressed as the mean of three replicates with error bars representing standard deviation. The statistical significances are indicated.