The regulation of KIF3 motor complex by POPX2 phosphatase

Phang, Hui Qun

2011

Phang, H. Q. (2011). The regulation of KIF3 motor complex by POPX2 phosphatase. Doctoral thesis, Nanyang Technological University, Singapore.

<https://hdl.handle.net/10356/49045>

<https://doi.org/10.32657/10356/49045>

**THE REGULATION OF KIF3 MOTOR COMPLEX BY
POPX2 PHOSPHATASE**

PHANG HUI QUN

SCHOOL OF BIOLOGICAL SCIENCES

2011

**THE REGULATION OF KIF3 MOTOR COMPLEX BY
POPX2 PHOSPHATASE**

PHANG HUI QUN

SCHOOL OF BIOLOGICAL SCIENCES

A thesis submitted to the Nanyang Technological University in
fulfillment of the requirement for the degree of Doctor of Philosophy

2011

Acknowledgements

First, and foremost, I would like to express sincere gratitude to my supervisor Dr. Koh Cheng Gee for her invaluable support and direction which guided me through the process of the project. Many of the ideas in this thesis are attributable to her; without her patience and careful proofreading, this thesis would be impossible. Her kindness towards people and meticulousness about work gave me deep impression. I have learned a lot from the inspiring conversations with her.

I am utmost grateful for the financial support granted to me by the Nanyang Technological University during my PhD study

Many thanks go to past and present lab mates: Agatha Susila, Alison Cheng, Anna Chan, Heng Yi Wen, Hoon Jing Ling, Ou Sirong, Pritpal Singh, Tan Meihua, Wong Hong Yuen, Xie Yi and Zhang Song Jing. It is them who created a collaborating and conducive research environment in the lab. Particularly, I want to thank Heng Yi Wen and Tan Meihua for their constant help and encouragement in my research and life; Anna Chan for her stimulating ideas on experimental work; Xie Yi for teaching me basic mammalian cell culture and molecular cloning.

I would also like to convey deep appreciation for my thesis advisory committee, Dr. Andrew Tan and Prof. Mark Featherstone for their inspirational discussions and critiques during the annual committee meetings. Many thanks also go to Dr. Li Hoi Yeung for his ideas and advices on this project.

Last but not least I would like to extend my heartfelt gratitude to my family, who is always caring and supportive. Special thanks go to my husband who has taken great care of me when I was suffering from medical conditions and has given me faith to complete this PhD program.

Table of Contents

Acknowledgements.....	i
Table of Contents.....	iii
Lists of Figures	vi
Abbreviations.....	viii
Abstract	x
Chapter 1 Introduction.....	1
1.1 The cell cytoskeleton	1
1.2 Kinesin superfamily proteins	5
1.2.1 Kinesin II – the KIF3 heterotrimeric motor	6
1.3 N-cadherin and β -catenin	10
1.3.1 Structure of N-cadherin and β -catenin	11
1.3.2 Role of N-cadherin in cell-cell adhesions and cell migration	13
1.3.3 N-cadherin trafficking	15
1.4 Calcium/calmodulin-dependent protein kinase II (CaMKII)	18
1.4.1 Structure and autoregulation of CaMKII.....	18
1.4.2 Functions of CaMKII in the neuronal and non-neuronal cells.....	19
1.5 The Rho GTPases-mediated signalling pathways.....	21
1.5.1 Rho family small GTPases	21
1.5.2 Guanine nucleotide exchange factor PIX.....	23
1.5.3 Serine / Threonine protein kinase PAK.....	23
1.6 The POPX phosphatase.....	26
Chapter 2 Materials & methods	34
2.1 Materials.....	34
2.1.1 Chemicals	34
2.1.2 Medium for yeast culture	35
2.1.3 Tissue culture materials.....	35
2.1.4 Enzymes for cloning.....	35
2.1.5 Commercial Kits.....	36
2.1.6 Mammalian cell transfection reagents.....	36
2.1.7 Other materials	36

2.2 Bacterial strains, yeast strains and mammalian cell lines	37
2.3 Buffers	38
2.4 Plasmid Constructs	40
2.5 Primers.....	43
2.6 Antibodies.....	46
2.6.1 Primary antibodies.....	46
2.6.2 Secondary antibodies	46
2.7 Methods.....	47
2.7.1 Yeast Two-Hybrid Screen	47
2.7.2 X- β -Gal Assay.....	48
2.7.3 General tissue culture.....	48
2.7.4 Western Blot	49
2.7.5 Glutathione S-transferase (GST) pull-down assay	49
2.7.6 FLAG immunoprecipitation assay.....	50
2.7.7 Immunoprecipitation.....	50
2.7.8 Immunofluorescence	51
2.7.9 Time-lapse imaging of N-cadherin/Rab11a vesicles.....	52
2.7.10 Cell aggregation assay.....	52
2.7.11 RNA interference experiment	53
2.7.12 Expression and purification of recombinant proteins	53
2.7.13 <i>In vitro</i> kinase and phosphatase assays	54
2.7.14 Statistical analysis.....	54
Chapter 3 Results	55
3.1 Identification of POPX2-interacting proteins by yeast-two hybrid	55
3.2 Association of POPX2 with the KIF3 kinesin motor complex.....	59
3.2.1 Initial characterization of interaction between POPX and KAP3.....	59
3.2.2 Mapping of KAP3 interacting domain on POPX	60
3.2.3 Mapping of POPX2 interacting domain on KAP3.....	64
3.2.4 POPX2 binds to KAP3 and β 1-PIX with comparable efficiencies	67
3.2.5 POPX2 binds to KIF3 complex via interaction with KAP3.....	69
3.2.6 Interaction of endogenous KAP3 with GFP-POPX2 in the NIH3T3 stable line	72
3.2.7 POPX2 and KAP3 localized to vesicular structures	73
3.3 Localization & tracking of KIF3A in Ctrl and X2 cells	75
3.4 POPX2 inhibits N-cadherin-mediated cell-cell adhesions.....	77

3.4.1 Altered subcellular localization of N-cadherin and β -catenin in POPX2 overexpressing cells.....	77
3.4.2 POPX2 overexpression did not disrupt interaction between N-cadherin and KAP3..	79
3.4.3 POPX2 phosphatase activity is required for its inhibition on N-cadherin transport to cell periphery and membrane ruffles.....	80
3.4.5 POPX2 inhibits calcium-dependent cell-cell aggregation	84
3.4.6 KAP3 siRNAs failed to silence the <i>kap3</i> gene in NIH3T3 cells	86
3.5 POPX2 overexpression affects distribution of Rab11.....	87
3.6 POPX2 overexpression did not affect motility of Clathrin.....	90
3.7 Mutation of KIF3A on serine 690 perturbs transportation of N-cadherin and Rab11 to cell periphery	92
3.8 Phosphorylation of KIF3A serine-690 does not affect cargo binding.....	97
3.9 KIF3A interacts with calcium/calmodulin-dependent kinase II	99
3.10 KIF3A is regulated by CaMKII and POPX2 on serine-690	100
Chapter 4 Discussions	104
4.1 POPX2 interaction with the KIF3 motor complex	104
4.2 Tracking of the KIF3A motor subunit.....	108
4.3 POPX2 regulates N-cadherin and Rab11 trafficking.....	109
4.4 Phosphorylation of KIF3A serine-690 may contributes to motor function	113
4.5 KIF3A is a potential substrate of CaMKII and POPX2	116
4.6 Proposed model for the regulation of KIF3 motor function by POPX2	118
Chapter 5 Conclusions and future perspectives.....	120
References.....	123
Selected publications	134
Appendix	135

Lists of Figures

Figure 1 Structures of actin filaments and microtubules.....	5
Figure 2 Schematic of the KIF3motor.....	7
Figure 3 Structure and dynamics of cadherin.....	13
Figure 4 Architecture of adherens junction in fibroblast.....	15
Figure 5 Intracellular transport of N-cadherin vesicles.....	17
Figure 6 Domain structure of CaMKII α	19
Figure 7 The Rho GTPase conformation cycle.....	22
Figure 8 Structural domains of PAKs.....	25
Figure 9 Schematic representation of POPX1 and POPX2.....	28
Figure 10 Verification of cDNA inserts sizes.....	57
Figure 11 Re-transformation of library plasmid into AH109 containing the bait..... plasmid confirmed interactions between the bait and the putative binding partners	58
Figure 12 POPX2 interacts with full length KAP3 in mammalian cells.....	60
Figure 13 Association of KAP3 with POPX1.....	62
Figure 14 Mapping the KAP3 binding region in POPX2.....	63
Figure 15 Mapping of the regions in KAP3 required for binding to POPX2.....	65
Figure 16 Specific KAP3 armadillo repeats required for interaction with POPX2.....	66
Figure 17 Comparing the binding efficiencies of various POXP2-interacting..... Proteins	68
Figure 18 Reciprocal co-precipitations of POPX2, KAP3 and β 1-PIX.....	69
Figure 19 Direct interaction between KAP3 and POPX2.....	71
Figure 20 POPX2, KAP3 and KIF3A form a complex.....	71
Figure 21 Association of endogenous KAP3 with GFP-POPX2.....	73
Figure 22 Localization of POPX2 and KAP3 in NIH3T3 cell.....	74
Figure 23 Localization and tracking of KIF3A in NIH3T3 cells.....	76
Figure 24 Reduced N-cadherin and β -catenin localization at cell periphery and..... cell-cell contacts of cells overexpressing POPX2	78
Figure 25 Effect of POPX2 overexpression on the interaction between KAP3 and..... N-cadherin	79

Figure 26 The phosphatase-dead POPX2 mutant did not inhibit localization of81	N-cadherin and β -catenin to cell periphery and cell-cell contacts
Figure 27 Transportation of N-cadherin-GFP to cell periphery is impaired in.....83	POPX2-overexpressing cells
Figure 28 Cell-cell adhesion is inhibited in POPX2-overexpressing cells.....85	
Figure 29 Knockdown of kap3 in NIH3T3 cells using RNAi.....86	
Figure 30 Effects of POPX2 overexpression on Rab11 distribution.....88	
Figure 31 Transportation of dsRed-Rab11a to cell periphery is impaired in.....89	POPX2-overexpressing cells
Figure 32 POPX2 overexpression did not affect motility of clathrin-LCA vesicles.....91	
Figure 33 Serine-690 of KIF3A is a phosphorylation site.....92	
Figure 34 Overexpression of KIF3A-S690A mutant inhibit localization of.....94	N-cadherin to cell-cell contacts
Figure 35 Transportation of N-cadherin-GFP to cell periphery is impaired in.....95	S690A-overexpressing cells
Figure 36 Transportation of dsRed-Rab11a to cell periphery is impaired in.....96	S690A-overexpressing cells
Figure 37 Mutation of KIF3A serine-690 does not affect binding of KIF3A.....98	to KAP3
Figure 38 Interaction between KIF3A and CaMKII.....99	
Figure 39 <i>In vitro</i> phosphorylation of KIF3A by CaMKII.....101	
Figure 40 POPX2 dephosphorylates KIF3A on serine-690 <i>in vitro</i>102	
Figure 41 Interaction between the tail and motor domain of KIF3A.....103	
Figure 42 A schematic representing a possible mechanism by which POPX2.....119	negatively regulates the KIF3 motor complex

Abbreviations

ADP	Adenosine diphosphate
APC	Adenomatous Polyposis Coli
ARM	Armadillo
ATP	Adenosine triphosphate
c-Abl	Abelson murine leukemia viral oncogene
CaCl ₂	Calcium chloride
CaMKII	Calcium/calmodulin-dependent protein kinase II
CaMKIIpase	Calcium/calmodulin-dependent protein kinase II phosphatase
Cdc42	Cell-division cycle 42
Clathrin-LCA	Clathrin-Light chain A
ERK	Extracellular signal-regulated protein kinases
GFP	Green fluorescent protein
GSK3	Glycogen synthase kinase 3
GST	Glutathione S-transferase
HA	Haemagglutinin
HRP	Horseradish peroxidase
IP	Immunoprecipitation
KAP3	Kinesin-associated protein 3
LC-MS	Liquid chromatography–mass spectrometry
PAK	P21-activated kinase
PH domain	Pleckstrin homology domain
PIX	PAK-interacting exchange protein
POPX	Partner of PIX
PPM	Metal-dependent protein phosphatase

PPP	Phosphoprotein phosphatase
PTP	Protein tyrosine phosphatase
Rac1	Ras-related C3 botulinum toxin substrate 1

Abstract

The POPX2 serine/threonine phosphatase was first identified as a Ca^{2+} /calmodulin-dependent protein kinase phosphatase (CaMKPase). Later, the phosphatase was isolated as a binding partner of PIX, a guanine nucleotide exchange factor of the Rho GTPases. POPX2 has been shown to dephosphorylate and downregulate the activity of the Cdc42/Rac1-activated kinase, PAK1, through formation of POPX2-PIX-PAK1 trimeric complex. Recent studies have also demonstrated that POPX2 interacts with the mammalian Diaphanous (mDia) protein and this interaction reduced the ability of mDia to activate transcription mediated by the serum response factor (SRF). Furthermore, POPX2 has also been implicated in the regulation of breast cancer cell motility and invasiveness.

In this study, we undertake yeast-two hybrid analysis and identified KAP3, the non-motor subunit of the KIF3 heterotrimeric complex, as a novel POPX2-interacting partner. KIF3 is a microtubule plus end-directed molecular motor and regulates the intracellular transport of an extensive array of proteins. Interaction between KAP3 and POPX2 was verified by co-immunoprecipitation and localization experiments in the mammalian cells. *In vitro* binding assay further demonstrated direct interaction between the two proteins. POPX2 was also found to form a multimeric complex with KAP3 and KIF3A, the motor subunit of KIF3. Overexpression of POPX2, but not its catalytically inactive mutant, inhibited trafficking of N-cadherin, an established molecular cargo of KIF3, to intercellular junctions, resulting in decreased cell-cell adhesiveness. These results suggest novel functions of POPX2 in regulation of kinesin-mediated transport

and cell-cell adhesion. Furthermore, we found that the localization and trafficking of another KIF3 cargo, Rab11, were also disrupted by the overexpression of POPX2.

In addition, we have investigated the significance of phosphorylation on KIF3A serine-690. Mutation of KIF3A serine-690 to an alanine residue, to preclude phosphorylation, resulted in impaired KIF3-mediated cargo transport. The similarities between the effect of POPX2 and KIF3A-S690A overexpression on cargo transport, suggest a possibility that POPX2 is negatively regulating the KIF3 motor via dephosphorylation of KIF3A on serine-690. Moreover, through a phosphorylation motif search, we found that the serine-690 residue resides within a consensus sequence for CaMKII. This makes CaMKII a potential kinase for KIF3A. Using *in vitro* kinase and phosphatase assays, we demonstrated that CaMKII phosphorylates KIF3A specifically on serine-690 and POPX2 dephosphorylates this residue.

Chapter 1 Introduction

In this chapter we introduce the main topics and key proteins, which are of relevance to the research work reported in this thesis.

1.1 The cell cytoskeleton

Eukaryotic cells possess the ability to alter their shape and vary the degree of their mechanical interactions with the extracellular environment in response to extracellular signals. These dynamic processes are highly dependent on the reorganization of the cell's cytoskeleton. The cytoskeleton of a cell comprises three major protein filaments, namely the actin filaments, microtubules and intermediate filaments. Each type of filament has distinct mechanical characteristics and dynamics, but some fundamental properties are common to them. Actin filaments define the morphology of the cell's surface and are essential for whole-cell locomotion. Microtubules determine the positions of membrane-bound organelles and regulate intracellular protein transport. Intermediate filaments provide mechanical strength and resistance to shearing force.

Among the elaborate arrays of protein filaments that compose the cell cytoskeleton, the actin filaments are the most dynamic and robust. As shown in Figure 1A, filamentous actin (F-actin) is assembled from monomeric globular actin (G-actin) subunit via polymerization (Straub and Feuer, 1950). Each actin monomer has a molecular mass of about 43 kDa. At least six actin isoforms are expressed in mammalian cells, they are further classified into three classes (alpha, beta and gamma) according to their

isoelectric points. Typically, α -actin is highly expressed in muscles, while β -actin and γ -actin are more abundant in non-muscle cells. G-actin monomers are capable of binding ATP and ADP. During formation of F-actin, the ATP bound to G-actin is hydrolysed to ADP by the ATPase domain. Crystal structure of actin revealed that there are two major domains in each actin monomer and the nucleotide binding region resides within the cleft between the two domains (Kabsch et al., 1990). The actin monomers all orient with the cleft toward the same end of the filament. This is designated as the minus end (pointed end), while the other end of the filament is known as the plus end (barbed end). This gives rise to the actin filament polarity. Actin filaments constantly undergo net incorporation of ATP-actin monomers at the plus end and dissociation of ADP-actin monomers from the minus end. This dynamic process, known as treadmilling, is further regulated by other proteins such as actin nucleating factors and actin-depolymerization factors. Electron micrographs of actin filaments showed that each filament is composed of two strands of actin monomers twist around each other in a double helix, with a diameter of 5-9 nm and a loop of helix repeating every 37 nm.

Microtubules are built from polymers of α - and β -tubulin heterodimers that are arranged longitudinally to form protofilaments (Nogales et al., 1999). Typically, 13 protofilaments are bundled laterally into hollow cylindrical tube with a diameter of 25 nm (Figure 1B). Microtubules also exhibit structural and kinetic polarity. The plus ends are crowned by β -tubulin and assemble faster, while the minus ends are crowned by α -tubulin and assemble slower. Both assembly and disassembly can occur at the plus end, thus, allowing the microtubule to constantly switch between phases of growth and shrinkage. This process is termed dynamic instability. The transition from growth to shrinkage is called a catastrophe, and the switch back to growth is called a rescue

(Mitchison and Kirschner, 1984; Walker et al., 1988). During polymerization, both the α - and β -tubulin are bound to GTP. While the GTP bound to α -tubulin is stable, the GTP bound to β -tubulin may be hydrolyzed to GDP soon after assembly, leading to depolymerization. Hence, a growing microtubule has a cap of GTP-tubulin at its tip. To initiate microtubule polymerization, cells utilize a specialized organelle known as microtubule-organizing center (MTOC) to nucleate microtubules. Within cells, microtubules are organized according to their polarity. In fibroblasts, microtubules are arranged into radial arrays with the minus end near the cell center and the plus end towards the cell periphery. By contrast, epithelial cells have parallel microtubules with the plus end at the basal membrane and the minus end at the apical membrane.

To efficiently execute their respective functions, the cytoskeletal filaments utilize a large number of accessory proteins that connect the filaments to one another, as well as to other cellular components. The accessory proteins not only are necessary for the regulated synthesis of the cytoskeletal filaments but also play direct roles in cellular processes controlled by the cytoskeleton. The motor proteins are a major component of the accessory proteins; they transport organelles along the filaments or move the filaments themselves. Depending on the type of filament utilized for movement, the motor proteins are categorized into two main groups: 1) actin motors such as myosin move along actin filament. 2) Microtubule motors like kinesin and dynein move along microtubule. Cell adhesion molecules are considered important members of the accessory proteins. Interactions between the cytoskeletal filaments and cell adhesion molecules are crucial for cellular functions including cell-cell adhesion, cell-matrix interaction, cell migration, internalization and recycling of receptor molecules. Cell adhesion molecules constitute diverse groups of proteins that vary both structurally and

functionally. The cadherins and integrins are two of the most intensively studied families of adhesion molecules. While cadherins promote assembly of adherens junction, integrins mediate adhesion between the cell and the extracellular-matrix (ECM) and play important role in the spatiotemporal control of actin-based motility in mammalian cells. Ligands of integrin cytoplasmic tails are major components of signalling pathways involving cytoskeletal adaptor proteins (Critchley, 2004) as well as Rho GTPases and kinases that regulate F-actin crosslinking, actin treadmilling and *de novo* nucleation of actin filaments (Choi et al., 2008; Galbraith et al, 2007). In migrating cells, inhibition of actin polymerization by cytochalasin D prevents clustering of β 1-integrin at leading edge protrusions, thereby demonstrating the tight correlation between actin polymerization and integrin assembly (Galbraith et al, 2007). Likewise, a strong connection between actin polymerization and presentation of cadherin at cell adhesion sites has been proposed (Kovacs et al., 2002); cadherin-based adhesion could trigger an instructive effect on cells to mark sites for cortical actin assembly (see *section 1.3.2 and 1.3.3* for further discussions on the relationship between cytoskeleton dynamics and cadherin function and trafficking). In all, cell adhesion molecules and cytoskeletal elements are highly inter-dependable.

Figure 1. Structures of actin filaments and microtubules. (A) The actin filament is composed of monomers that wind around each other as two parallel strands of a helix, with a twist repeating every 37 nm. (B) α - and β -tubulin heterodimers are arranged longitudinally to form protofilaments. Staggered assembly of 13 protofilaments yields a hollow cylindrical filament.

1.2 Kinesin superfamily proteins

Proteins in the cell are transported along directional cytoskeletal filaments to designated locations by molecular motor proteins. Molecular motors bind to a polarized cytoskeletal filament and draw on the energy resulting from ATP hydrolysis to move progressively along it. Kinesins and dyneins are the two major molecular motor superfamilies that mediate intracellular cellular transport along microtubules. Kinesins transport molecular cargoes along microtubule tracks from the minus end to the plus end while dyneins drive minus-end directed transport. Kinesins are first discovered in the giant axon of the squid, where it transports membrane-bound organelles from the neuronal cell body to the axon terminal by ‘walking’ toward the plus end of

microtubules (Scholey et al., 1985). Subsequently, an enormous number of kinesin motor proteins are isolated from a variety of cell types. In 2004, a standard kinesin nomenclature was adopted based on phylogenetic analysis, classifying the kinesins into 15 subfamilies (Lawrence et al., 2004). Despite the diversity, kinesins share common fundamental structure in general. Majority of the kinesins are composed of three distinct domains: 1) conserved N-terminal globular motor domain which binds ATP and microtubule; 2) stalk region for dimerization with each other; 3) variable C-terminal tail domain which binds molecular cargoes or adapter proteins. Here, we will focus on the structure and physiological roles of the kinesin II heterotrimeric motor protein.

1.2.1 Kinesin II – the KIF3 heterotrimeric motor

1.2.1.1 Structure of the KIF3 complex

The kinesin II family consists of the following members: KIF3A, KIF3B, KIF3C and KIF17. While KIF17 is homodimeric (Setou et al., 2000), KIF3A forms heterodimeric complex with either KIF3B or KIF3C (Muresan et al., 1998; Yamazaki et al., 1995). Furthermore, KIF3A and KIF3B interact with the non-motor subunit, kinesin-associated protein KAP3 (KIFAP3) to form the KIF3 heterotrimeric motor (Yamazaki et al., 1995). Molecular structure analysis has showed that the N-terminal 350 amino acids of KIF3A/3B form the globular motor domains, spanning about 10 nm (Figure 2). The stalk region (amino acids 350-590) dimerizes into an α -helical coiled-coil structure which is about 30 nm across. The primary structure of the 752 amino acid KAP3 consists of a central ~459 residue region composed of 9 armadillo (arm) repeats. KIF3A, KIF3B and KAP3 interact with a stoichiometry of approximately 1:1:1 and the

heterotrimeric complex is estimated to be 50 nm in length. Using various deletion mutants of KIF3A/3B and KAP3, *Haraguchi et al* further demonstrated that the C-terminal regions of KIF3A/3B are critical for binding the adapter protein, KAP3. On the other hand, amino acids 528-614 of KAP3 are essential for interaction with KIF3A/3B. Sequence analysis of the KIF3A/3B proteins has suggested a putative hinge region within the coiled-coil domain (Lupas, Van Dyke, and Stock, 1991). This hinge region may be important for the folding of the KIF3 motor. In fact, both folded and extended forms of KIF3 have been observed in sea urchin and this conformational change was found to be salt-dependent (Wedaman et al., 1996). However, the physiological implications of the different forms of KIF3 motor have not been determined.

Figure 2. Schematic of the KIF3 motor. (A) Domain structure of the Kinesin II subfamilies. In general, kinesin II proteins comprise an N-terminal motor domain and a central coiled-coil domain. (B) Domain structure of KAP3. KAP3 is characterized by 9 armadillo repeats, each consisting of about 42 amino acids. (C) Domain organization of the heterotrimeric KIF3 complex. (D.G. Cole, 1999)

1.2.1.2 Functional roles of the KIF3 motor

The KIF3 heterotrimeric motor powers a myriad of intracellular transport events. Northern blot analysis revealed that KIF3 is ubiquitously expressed and is highly abundant in the brain (Yamazaki et al., 1995; Yamazaki et al., 1996). Hence, it is not surprising that the KIF3 motor is engaged in both axonal and conventional protein transport. In neuronal cells, newly synthesized proteins and membrane organelles are transported from the cell body to the axon tip along microtubules to regulate neuronal functions such as neurite elongation and polarization. Fodrin, a membrane-associated cytoskeletal protein, was found to interact with KAP3 in a yeast two hybrid binding assay (Takeda et al., 2000). Transportation of fodrin-associated vesicles by the KIF3 complex was demonstrated and this process was essential for neurite elongation. Direct interaction between the cell polarity protein Par3 and the C-terminal domain of KIF3A has also been reported. Both inhibition of Par3-KIF3A binding and expression of dominant-negative KIF3A, which lacks the motor domain, blocked accumulation of Par3 at axon tips, resulting in the disruption of neuronal polarity (Nishimura et al., 2004). Furthermore, in an attempt to study the *in vivo* functions of KIF3 motor, the *kap3* gene was conditionally knocked out in the mice to avoid midgestation lethality (Teng et al., 2005). The conditional knockout mice displayed tumour-like abnormal hypertrophy of the cerebral cortex. In these regions, the distribution of N-cadherin and β -catenin to the cell peripheral was markedly decreased. In an immortalized embryonic fibroblast cell line with the *kap3*-null genotype, the release of ectopically expressed N-cadherin-GFP from the Golgi apparatus was significantly impaired, resulting in a decreased level of N-cadherin-GFP at cell-cell contacts. Immunoprecipitation showed that N-cadherin, β -catenin and the KIF3 motor formed a complex and immunofluorescence staining

demonstrated the co-localization of N-cadherin and KAP3. In a more recent report, protein-phosphatase Dusp26 was identified as a novel regulator of the KIF3 motor (Tanuma et al., 2009). Dusp26 is recruited to the KIF3 motor mainly by interaction with KIF3A, and it regulates the activity of KIF3 motor via dephosphorylation of KAP3. Overexpression of Dusp6 enhanced localization of N-cadherin and β -catenin to cell periphery, thereby promoting cell-cell adhesion.

The KIF3 motor was also reported to interact with the tumour-suppressor protein adenomatous polyposis coli (APC) via the armadillo repeat regions of KAP3 (Jimbo et al., 2002). APC is involved in the degradation of β -catenin and it binds to and accumulates at the plus end of microtubules. KIF3 motor was found to be necessary for the transportation of APC and β -catenin along microtubules and is essential for accumulating APC in the tips of membrane protrusions in migrating epithelial cells.

On the other hand, the generation of *kif3a* and *kif3b* knockout mice has demonstrated that the KIF3 motor is essential in early development (Takeda et al., 1999). Null mutants for *kif3a* and *kif3b* did not survive past 12 days postcoitum and displayed severe structural defects. Strikingly, left-right asymmetry was abolished in these mouse embryos, which may be caused by the lack of nodal cilia.

The KIF3 motor has also been detected on early, late and recycling endosomes (Bananis et al., 2004; Imamura et al., 2003; Schonteich et al., 2008). Inhibition of KIF3 function by RNAi of KAP3 or expression of dominant negative KIF3A, altered the distribution of late endosomes and lysosomes. KIF3 motor regulates the trafficking of recycling endosomes by interaction with the Rab11-Rip11 protein complex (Schonteich et al., 2008). This interaction is required for the routing of internalized receptors.

Apart from its role in mediating intracellular protein transport, the KIF3 complex has also emerged as an important regulator of cell mitosis. In a yeast-two-hybrid screen, the Human Chromosome-associated Polypeptide (HCAP) was isolated as a binding partner of KAP3 (Shimizu et al., 1998). Both KAP3 and KIF3B were detected together with HCAP in the nuclear fractions of COS-7 cells. However, a direct role of KIF3 complex in regulating mitosis was not elucidated. Detailed characterization of KIF3 function in mammalian cell mitosis was only reported in 2006 by Haraguchi *et al.* The KIF3 complex was shown to be localized to the mitotic spindle and KAP3 was phosphorylated during mitosis. Furthermore, expression of a KIF3B mutant, which is capable of binding KIF3A but not KAP3, led to chromosome aneuploidy and abnormal spindle formation. In addition, KIF3B has been shown to interact with p0071 (plakophilin-4), independent of the KAP3 subunit (Keil et al., 2009). p0071 belongs to a subfamily of cell contact-associated proteins of the armadillo superfamily. It is localized to the midbody during cytokinesis and is essential for cell division. Knockdown of KIF3B interfered with the targeting of p0071 to the midbody and caused reduction of actin and phospho-myosin-light-chain accumulation at the midbody during cytokinesis. Interestingly, these results suggest that KIF3, which is commonly known to regulate microtubule-dependent protein transport processes, may also be implicated in actin organization during cytokinesis.

1.3 N-cadherin and β -catenin

Cadherins belong to a family of Ca^{2+} -dependent cell adhesion molecules (CAMs). They are the major molecules of cell-cell adhesion and play critical role in various cellular processes. The best-characterized classical cadherins constitute the E-, P-, and N-

cadherins. The three subtypes of cadherins are classified according to their predominant cellular distribution. E-cadherin is highly expressed in epithelial cells; P-cadherin is mainly expressed in placental cells; N-cadherin is the predominant cadherin in neuronal cells and fibroblasts.

1.3.1 Structure of N-cadherin and β -catenin

N-cadherin is a single-pass transmembrane glycoprotein of 130 kDa, first identified in 1986 by Hatta *et al.* It consists of an N-terminal extracellular region, a single transmembrane spanning domain, and a C-terminal cytoplasmic tail. Crystal structure of N-cadherin reveals that the extracellular region is characterized by five immunoglobulin (Ig)-like domains (Tamura *et al.*, 1998). Each ectodomain contains Ca^{2+} binding sites which are necessary for cadherin dimerization and cell-cell junction formation (Figure 3A). In the absence of extracellular Ca^{2+} , the N-cadherin ectodomains are flaccid and fail to form homodimers, eventually the protein will be degraded by proteolytic enzymes such as calpain (Jang *et al.*, 2009). As Ca^{2+} concentration increases, the ectodomains become more rigid and extended. When sufficient amount of Ca^{2+} is bound, the N-cadherin homodimer engages in homophilic interaction by binding N-cadherin homodimer expressed by neighbouring cells (Figure 3B). The cytoplasmic tail of N-cadherin is about 150 amino acids long and is the most highly conserved region (Nollet, Kools, and van Roy, 2000). In the absence of a binding partner, the N-cadherin cytoplasmic domain is unstructured (Huber *et al.*, 2001). This cytoplasmic region was found to bind the β -catenin.

β -catenin is a key cellular regulator that functions both as a transcriptional co-activator regulated by the Wnt signaling and as a vital link between the adhesion molecules and actin cytoskeleton. Full-length β -catenin contains 781 amino acid residues. It has an N-terminal region of about 150 amino acids, a 520-residues central domain composed of 12 armadillo repeats and a C-terminal region containing 100 residues. X-ray crystallographic analysis of the central armadillo domain of β -catenin demonstrated that the 12 Armadillo repeats form an elongated superhelical structure that creates a long, positively charged groove (Huber et al., 1997). β -catenin utilizes this groove as part of the binding site for many of its negatively charged ligands, such as the cadherins, the Axin/APC degradation complex, and the LEF/TCF transcription factors. The N- and C-terminus of β -catenin are highly unstructured regions that are involved in recruitment of essential co-factors. However, under certain conditions, the C-terminal tail may limit the availability of the β -catenin arm repeat region for cadherins or APC binding (Castano et al., 2002; Choi et al., 2006). Interactions between N-cadherin and β -catenin are highly regulated via phosphorylation. *In vitro* phosphorylation studies have shown that the Src kinase phosphorylates N-cadherin on the tyrosine residue 860 (Y860). This result was further verified by mass spectrometry analysis and mutagenesis studies. Phosphorylation of Y860 caused a significant reduction in affinity for β -catenin (Qi et al., 2006). Likewise, c-Abl phosphorylation of tyrosine residue 489 (Y489) decreases β -catenin binding to N-Cadherin and leads to nuclear translocation and transcriptional activation (Lilien and Balsamo, 2005).

Figure 3. Structure and dynamics of cadherin. (A) Molecular structure of a classical cadherin. Cadherin exists as homodimer predominantly. The extracellular region of cadherin is composed of a polypeptide folded into five cadherin repeats. Each repeat contains Ca^{2+} binding sites and it resembles an immunoglobulin (Ig) domain. (B) Regulation of N-cadherin dynamics by extracellular Ca^{2+} . When Ca^{2+} is eliminated, the extracellular domain of cadherin turns floppy and undergoes proteolysis. As the concentration of Ca^{2+} rises, the extracellular domain becomes rigid. When concentration of Ca^{2+} reaches beyond 1 mM, the cadherin homodimer binds to another homodimer on adjacent cells.

1.3.2 Role of N-cadherin in cell-cell adhesions and cell migration

Cell-cell adhesion is involved in many fundamental cellular activities, such as the organization of cells into tissues during embryonic development, cell growth, migration, and differentiation (Gumbiner, 1996; Takeichi, 1991). Moreover, cell-cell adhesion is often modified in cancer cells and during cell metastasis. This highly dynamic process relies on both the remodeling of actin cytoskeleton and the assembly and disassembly of adherens junction, where cadherins are the key players. In fibroblasts, N-cadherin is indirectly linked to the actin filaments via interaction with β -catenin and the actin-binding protein α -catenin (Figure 4). As mentioned in the earlier section, the dynamics of adherens junction involved phosphoregulation of cadherin and β -catenin. In addition, results from several studies have established a major role for actin re-organization in

cadherin contact formation and dynamics (Lambert et al., 2007). Formation of Rac1-dependent lamellipodia and stimulation of actin polymerization, through activation of the Arp2/3, are pivotal during the assembly and maturation of N-cadherin adhesion complex (Lambert et al., 2007). Alternatively, studies led by El Sayegh *et al* have shown that tyrosine phosphorylation of cortactin, an actin binding protein, was necessary for strengthening N-cadherin-mediated cell-cell adhesion (El Sayegh et al., 2005; El Sayegh et al., 2004). Intercellular adhesion has direct influence on membrane dynamics; cellular regions with tight cell-cell contacts have relatively low membrane dynamics. In contrast, dissolution of cadherin adhesion complex triggers an increase in membrane dynamics, which is characterized by active actin polymerization and branching. The reduction in cell-cell adhesion favours cell migration, consequently, cells whose cadherin function is downregulated are more motile and invasive (Tanuma et al., 2009; Teng et al., 2005).

Figure 4. Architecture of adherens junction in fibroblast. The cadherin is indirectly attached to the actin filaments by the anchor proteins α -catenin and β -catenin. Association of cadherin complexes with actin filaments is required for the formation and maintenance of adherens junction.

1.3.3 N-cadherin trafficking

In 1983, Geller and Lilien first reported that N-cadherin is delivered to the plasma membrane in a microtubule-dependent manner (Geller and Lilien, 1983). Later studies piloted by Rouviere's lab, which involved analyzing the transportation of a GFP-tagged N-cadherin construct in myoblasts and fibroblasts, demonstrated that N-cadherin trafficking is dependent on the microtubule network and the activity of the microtubule-associated kinesin motor (Mary et al., 2002). They have also observed that the N-cadherin shuttles between an intracellular and a plasma membrane-bound pool. Assembly of N-cadherin adhesion complex requires recruitment of the intracellular pool of N-cadherin to the plasma membrane by transportation along microtubules. Further studies on the regulation of N-cadherin trafficking have revealed a positive role of the

p120-catenin protein in transporting N-cadherin to cell surface (Chen et al., 2003). p120 binds to the cytoplasmic domain of N-cadherin and promotes insertion of N-cadherin into the plasma membrane via interaction with kinesin. Disruption of the association between p120 and N-cadherin causes delayed accumulation of N-cadherin at cell-cell contact. In the analysis of conditional knockout mouse for the *kap3* gene, Teng *et al* have provided evidences that the KIF3 motor is directly involved in transporting N-cadherin to cell periphery (Teng et al., 2005). N-cadherin forms a complex and co-localizes with the KIF3 motor *in vivo*. Localization of N-cadherin to cell periphery was disrupted in the KAP3-deficient cells. Their findings were further supported by Tanauma *et al*, who demonstrated that dephosphorylation of KAP3 by the protein phosphatase Dusp26, promoted distribution of N-cadherin to cell periphery. Finally, recent works on the neuronal Rab-GTPases-dependent endocytic pathway have revealed mechanistic insights on the regulation of N-cadherin trafficking. Suppression of the early endosome protein Rab5 caused N-cadherin to be retained at the cell surface, whereas downregulation of the recycling endosome protein Rab11 led to abnormal intracellular accumulation of N-cadherin (Kawauchi et al., 2010). Altogether, studies over the last decade have illustrated a highly multifaceted mechanism of the N-cadherin intracellular transportation (Figure 5). Nevertheless, detailed regulatory mechanism on the association and release of N-cadherin from the protein transport machinery requires further elucidation.

(A)

(B)

Figure 5. Intracellular transport of N-cadherin vesicles. (A) Illustration of N-cadherin-containing vesicles transported along microtubules by kinesin motor from the ER/golgi compartment to cell periphery. (B) Schematic of the Rab-dependent endocytic pathways. Rab5 and Rab11 are involved in the early endocytosis and recycling of the N-cadherin-containing vesicles, respectively.

1.4 Calcium/calmodulin-dependent protein kinase II (CaMKII)

1.4.1 Structure and autoregulation of CaMKII

The multifunctional Ca^{2+} /calmodulin-dependent protein kinase II is a serine/threonine kinase, pivotal to the regulation of many Ca^{2+} -dependent intracellular processes. CaMKII is ubiquitously expressed and is highly abundant in the brain, where it phosphorylates a large array of downstream targets, including itself. CaMKII is encoded by four genes (α , β , γ and δ) to yield over thirty different alternative splice variants that share similar basic architecture (Tombes et al., 2003). Structurally, the CaMKII holoenzyme is composed of a dodecamer of twelve kinase subunits (Hoelz et al., 2003; Kanaseki et al., 1991). Each kinase subunit consists of an N-terminal catalytic domain followed by a regulatory segment that binds to the catalytic domain and inhibits its activity (Figure 6). The C-terminal region contains the association domain, which is responsible for oligomerization. Between the regulatory and association domain lies a linker segment. The length of the linker varies among the isoforms and splice variants. Electron microscopy showed that the catalytic and regulatory domains branch out from a central core of assembled association domains in a flower-petal-like arrangement.

Similar to other kinases regulated by second messengers, CaMKII is also activated through the relief of the autoinhibitory domain from the catalytic region (Chen et al., 1994). The autoinhibitory domain retains CaMKII in an inactive state by precluding the catalytic domain from its substrate. The regulatory domain of CaMKII contains the Ca^{2+} /calmodulin binding site which partially overlaps with the autoinhibitory domain (Figure 6). Upon binding of Ca^{2+} /calmodulin, CaMKII undergoes autophosphorylation at Thr286 within the autoregulatory domain. Autophosphorylation of Thr286 renders

the CaMKII active via several steps: firstly, it markedly increases the affinity of the enzyme for Ca^{2+} /calmodulin in a process known as ' Ca^{2+} /calmodulin trapping'; secondly, phospho-Thr286 disables the autoinhibitory domain of the kinase so that the enzyme is temporally independent of its stimuli even when Ca^{2+} /calmodulin dissociates from the enzyme. This phenomenon is called autonomous activity. Lastly, it exposes a site on the molecule which allows it to bind to an anchoring protein.

Figure 6. Domain structure of CaMKIIα. The catalytic/regulatory are domains shaded in pink and the association domain in red. Expanded view of the domain structure highlights important features of the autoregulatory domain

1.4.2 Functions of CaMKII in the neuronal and non-neuronal cells

Since CaMKII is highly expressed in the brain, its function in the central nervous system has been intensively studied. In neurons, active CaMKII plays a significant role in the regulation of synaptic plasticity through the phosphoregulation of neurotransmitters (Wang, 2008). Furthermore, CaMKII activity is essential in the induction and maintenance of long-term potentiation (Lisman et al., 2002). Besides being an important signaling molecule in neuronal functions, CaMKII has also been found to modulate cytoskeletal dynamics, focal adhesion turnover and cell motility in

fibroblasts. Terasawa *et al* have reported that CaMKII phosphorylates the intermediate filament protein vimentin on Ser-82 in the rat fibroblast cell line 3Y1 (Terasawa et al., 1991). Suppression of CaMKII function in the NIH3T3 mouse fibroblasts significantly reduced lamellipodial dynamics, promoted cell spreading, enlarged paxillin-containing focal adhesions and blocked cell motility (Easley et al., 2008). On the contrary, overexpression of constitutively active CaMKII mutant reduced cell attachment, eliminated paxillin from focal adhesions and decreased the phospho-tyrosine levels of FAK and paxillin. These results indicate a novel role of CaMKII in regulating dynamic cellular activities through phosphorylation of cytoskeletal elements in non-neural cells.

1.5 The Rho GTPases-mediated signalling pathways

1.5.1 Rho family small GTPases

The Rho GTPases are monomeric G proteins, which resemble the α -subunit of the trimeric G proteins. They form a subgroup under the Ras superfamily of 20-30 kD GTP-binding proteins. Phylogenetic studies have shown that these proteins are ubiquitously expressed across a wide spectrum of species, from yeast, *Drosophila*, mouse to human (Manser, 2005). The mammalian Rho GTPase family is further divided into eight distinct subfamilies: Rho (A, B, C isoforms), Rac (1, 2, 3 isoforms), Cdc42 (G25K, Cdc42Hs isoforms), RhoD, RhoG, TC10, Rnd and TTF. Like other members of the Ras superfamily, Rho GTPases function as molecular switches that cycle between two interconvertible states: the GDP-bound inactive state and the GTP-bound active state (Figure 7). The switch from inactive to active state is stimulated by proteins known as guanine nucleotide exchange factors (GEFs), which catalyze the exchange of GDP for GTP. The GTPase-activating proteins (GAPs), on the other hand, accelerate the hydrolysis of bound GTP, thereby subjecting the Rho GTPases to rapid inactivation. In addition to GEFs and GAPs, a third group of Rho GTPase regulators was discovered. In resting cells, the GDP-bound form of Rho GTPases is sequestered in the cytosol by forming a complex with the Rho guanine nucleotide dissociation inhibitory factors (GDIs). Upon stimulation with extracellular agonists, GDP-bound Rho GTPases are released from the GDIs and become tethered to the plasma membrane through their lipid tails. At the plasma membrane, GEFs catalyze the exchange of GDP for GTP, thus, producing the active form of Rho GTPases.

Figure 7. The Rho GTPase conformation cycle. Rho GTPases cycle between an active (GTP-bound) and an inactive (GDP-bound) conformation. The cycle is regulated by three groups of proteins: GEFs, GAPs and GDIs. GEFs catalyze the exchange of GDP for GTP, thereby, activating the GTPases. GAPs increase the intrinsic rate of GTP hydrolysis, leading to rapid inactivation. GDIs further regulate the GTPases by sequestering GDP-bound GTPases in the cytosol. Upon stimulation with extracellular agonists, the GTPases will be released from the GDIs and be translocated to the plasma membrane. All Rho GTPases possess a lipid tail which enables them to tether to the plasma membrane.

The founding members of Rho GTPases, Cdc42, Rac1 and RhoA, are key regulators of actin dynamics that lead to organized actin-based structures associated with the morphology and motility of cells. Cdc42 induces filopodia formation; Rac1 stimulates the production of lamellipodia and membrane ruffles, while RhoA promotes actin polymerization to produce actin stress fibers and focal adhesion complexes (Hall, 1998). Besides, an activation hierarchy seems to be present among the Rho GTPases; Kozma *et al* demonstrated that expression of Cdc42 leads to formation of filopodia followed by formation of lamellipodia and membrane ruffles (Kozma et al., 1995). Similar observations were made by Nobes and Hall, whereby activation of Cdc42 led to rapid activation of Rac1 (Nobes and Hall, 1995). Hence, a model of sequential activation has been proposed in which activation of Cdc42 triggers the activation of Rac1 and the subsequent activation of RhoA.

1.5.2 Guanine nucleotide exchange factor PIX

PIX (Pak-interactive exchange factor) / Cool (cloned-out of library) family proteins are GEFs for both Cdc42 and Rac, which are isolated as direct interactors of PAK (p21-activated kinase) (Bagrodia et al., 1998; Manser et al., 1998). Of the two isoforms, α -PIX is expressed primarily in hematopoietic cells and muscles, whereas β -PIX is ubiquitously expressed. PIX proteins belong to the Dbl-family exchange factors for Rho proteins, characterized by a Dbl homology (DH) domain in tandem with a Pleckstrin homology (PH) domain. The DH domain is important for interacting with the Rho family GTPases and stimulating GDP / GTP exchange, whereas the PH domain mediates the targeting of the protein to appropriate cellular localization (Hart et al., 1994; Zheng et al., 1996). Both α -PIX and β -PIX possess a GIT (G-protein-coupled receptor kinase-interacting targets)-binding domain that is important for the formation of focal complexes, and a coiled-coil motif that mediates dimerization and cytoskeletal organization (Zhao et al., 2000; Koh et al., 2001). α PIX is distinguished from β PIX by the presence of an additional calponin homology (CH) domain at the N-terminus. Both PIX isoforms display exchange activity towards Cdc42 and Rac1, which leads to the activation of PAK1 (Koh et al., 2001; Manser et al., 1998).

1.5.3 Serine / Threonine protein kinase PAK

The p21-activated kinases (PAKs) are one of the first identified Rho effectors. PAKs belong to the serine/threonine kinase family and play fundamental roles in a wide range of cellular processes including cell morphology, motility, apoptosis, hormone signaling and cancer progression (Arias-Romero and Chernoff, 2008). Based on the biochemical

and structural features, PAKs of higher eukaryotes have been classified into two groups: PAK1, PAK2 and PAK3 constitute the group I (conventional) family of PAK, whereas PAK4, PAK5 and PAK6 belong to group II (unconventional) PAK (Bokoch, 2003; Jaffer and Chernoff, 2002). PAK1 is highly expressed in the brain, muscle and spleen while PAK2 is ubiquitously expressed and PAK3 predominantly resides in the brain (Manser et al., 1994).

PAKs are composed of a C-terminal kinase domain and an N-terminal autoinhibitory region (Figure 8), which includes a binding site for activated Cdc42/Rac1 that is known as the Cdc42/Rac interactive binding (CRIB) domain (Lei et al., 2000). In resting cells, PAK1 exists as a homodimer through interactions between the autoinhibitory region of one monomer and the kinase domain of the second monomer, thereby confining the kinase in an inactive state. Binding of GTP-bound forms of Cdc42/Rac1 to the regulatory domain disrupts the autoinhibition and allows the kinase domain to undergo autophosphorylation. Recent evidences from the NMR spectroscopy and gel filtration experiments indicated the presence of an active PAK dimer (Pirrucello et al., 2006). This is contrary to previous findings whereby binding of an activated Cdc42/Rac1 displaces the dimeric kinase into monomers (Lei et al., 2000; Parrini et al., 2002).

A key event in the kinase activation process is the autophosphorylation of one PAK by another, so that maximal kinase activity is attained. To date, seven autophosphorylation sites have been identified in PAK1: Ser-21, Ser-57, Ser-144, Ser-149, Ser-198, Ser-203, and Thr-423. Of these, phosphorylation of the Thr423 residue appears to be the major event for its full activation and maintenance of the kinase in a catalytically competent state (Gatti et al., 1999; Yu et al., 1998; Zenke et al., 1999). Phosphorylation of Ser-21

and Ser-144 also contributes to kinase activation, whereas phosphorylation of Ser-198 and Ser-203 serves to down-regulate PIX–PAK interaction (Chong et al. 2001).

Figure 8. Structural domains of PAKs. The group 1 PAKs contain an N-terminal regulatory domain and a C-terminal catalytic domain. The PIX binding region is illustrated in pink.

PAK1 is stimulated by many upstream signaling pathways such as growth-factor receptors, cytokine receptors, integrin cell-adhesion complexes and G-protein-coupled receptors (Knaus et al., 1995; Price et al., 1998; Rosenberger and Kutsche, 2006). These signaling pathways activate GEFs, which in turn activate PAK1 by catalyzing the exchange of GDP for GTP on Cdc42/Rac1 and by targeting it to the cell periphery through direct binding to the fourth proline-rich domain in the N-terminus of PAK1 (Manser et al., 1998; Zhao et al., 2000). Being a predominantly cytoplasmic protein, membrane recruitment of PAK1 via SH3-containing adaptor proteins such as PIX, bring the PAK1 protein into close proximity with its upstream activators and downstream effectors. This facilitates the efficient transduction of intracellular signals. Additionally, PAK1 has been reported to be activated via distinct Rho GTPase independent mechanisms such as interaction with sphingolipids and indirect association of PAK1 with GIT1 through PIX (Bokoch et al., 1998; Loo et al., 2004).

During cancer cell metastasis, the primary tumor cells undergo major cytoskeleton remodeling to invade through tissue and the extracellular matrix (ECM) to distant sites. PAKs are often regarded as key regulators of the actin cytoskeleton and cell motility. Overexpression of constitutively active PAK1 mutant promotes disassembly of actin stress fibers, dissolution of focal adhesion complexes and enhances actomyosin contractility, which in turn stimulates cell migration (Sells et al., 1997). Accordingly, upregulation of PAK expression and activity have been correlated with carcinogenicity of various cancer cell lines including breast cancer, colon cancer, bladder cancer, ovarian cancer, brain cancer and T-cell lymphoma (Kumar et al., 2006).

1.6 The POPX phosphatase

Reversible protein phosphorylation plays crucial role in a vast variety of cellular activities. These processes are dependent on the highly regulated protein kinases and protein phosphatases, which catalyze the opposing activities of protein phosphorylation and dephosphorylation, respectively. Changes in the phosphorylation state of proteins on their serine, threonine and tyrosine residues dictate alteration in protein conformational and result in functional changes. Based on substrate specificity, eukaryotic protein phosphatases are categorized into three broad families: 1) protein tyrosine phosphatases (PTPs), which specifically dephosphorylate phosphotyrosines; 2) serine/threonine protein phosphatases, which dephosphorylate phosphoserine and phosphothreonine; 3) dual-specificity protein phosphatases (DSPs). The serine/threonine protein phosphatases are further divided into the phosphoprotein phosphatases (PPPs) and metal-dependent protein phosphatases (PPMs) family. PPPs

constitute the subfamily phosphatases PP1, PP2A and PP2B whereas PPM contains the single defining member, PP2C.

PP2C phosphatase activity requires the presence of $\text{Mn}^{2+}/\text{Mg}^{2+}$ metal cations and has been predominantly associated to signaling pathways that regulate cell growth and cellular stress response. As opposed to the PPPs family, where the phosphatase activities are regulated by inhibitory proteins or inhibitory subunits, the activity of the type 2C phosphatases is controlled by cell type-specific expression, post-translational modification, subcellular localization and degradation. Furthermore, despite being $\text{Mn}^{2+}/\text{Mg}^{2+}$ -dependent phosphatases, regulation of the PP2C phosphatase activities are not controlled by the metal cations, as intracellular $\text{Mn}^{2+}/\text{Mg}^{2+}$ concentration is relatively constant under physiological conditions.

In 2002, a pair of PP2C-like serine/threonine phosphatases known as POPX1 and POPX2 (Partner of PIX), were isolated as binding partners of both α PIX and β PIX in a yeast two-hybrid screen (Koh et al., 2002). The POPX1 and POPX2 phosphatase consists of 757 and 454 amino acids residues, respectively (Figure 9). POPX1 is highly expressed in the brain and testis while POPX2 is ubiquitously expressed in all the tissues studied, including lung, thymus, spleen, brain, uterus and pancreas (Kitani et al., 1999). *In vitro* studies showed that both POPX1 and POPX2 directly dephosphorylate activated PAK1 on the key phospho-residue, Thr423, and downregulate its kinase activity (Koh et al., 2002). Consequently, phenotypic effects downstream of PAK1 activation were inhibited. For instance, breakdown of actin stress fibers mediated by Cdc42 through PAK1 activation was blocked efficiently by overexpressing POPX.

Figure 9. Schematic representation of POPX1 and POPX2. Shaded area represents region of homology between POPX1 and POPX2 (Koh et al., 2002).

POPX2 is alternatively known as PPM1F, Ca^{2+} /Calmodulin-dependent protein kinase II phosphatase (CaMKII phosphatase) and hFEM-2. PPM1F and CaMKII phosphatase have shown to interact with CaMKII and specifically dephosphorylate CaMKII at Thr286 in rat brain and fibroblast cells (Harvey et al., 2004; Ishida et al., 1998). The functional significance of negative regulation of CaMKII activity by PPM1F was demonstrated in the NIH3T3 fibroblasts, where overexpression of PPM1F led to a decrease in the CaMKII-specific phosphorylation of the known substrate vimentin (Ser82). Functional analysis of CaMKII phosphatase *in vivo* has also been carried out. Downregulation of CaMKII phosphatase in the zebrafish embryos by antisense morpholino oligonucleotides resulted in severe morphological defects and widespread of apoptotic cell death (Sueyoshi et al., 2009). These findings strongly indicate an essential role of CaMKII phosphatase in the early development of zebrafish embryo. On the other hand, hFEM-2, the human orthologue of the *Caenorhabditis elegans* sex determining gene FEM-2, was reported to promote caspase-dependent apoptosis when overexpressed in mammalian cells. (Tan et al., 2001)

The *C.elegans* FEM-2 protein shares 28% amino acid identity with the human POPX2 (Tan et al., 2001). Recent studies using the *C.elegans* have identified FEM-2 to be a component of the CUL-2 ubiquitin ligase complex that contains FEM-1 as the substrate recognition subunit (CBC^{FEM-1}). The sex determination effector, TRA-1, was found to be the critical substrate of the CBC^{FEM-1}. Upon binding to FEM-1, TRA-1 would be targeted for ubiquitin-proteosomal degradation. FEM-1, FEM2 and FEM-3 proteins physically interacted with one another and with TRA-1. The ubiquitination of TRA-1 was enhanced in the presence of FEM-2 and FEM-3 compare to FEM-1 alone, suggesting that FEM-2 and FEM-3 acted as co-factors of the CBC^{FEM-1} complex. Although the role of the human FEM-2 protein (hFEM2) in regulating ubiquitin-mediated proteolysis has yet to be investigated, the interaction between hFEM2 and the human FEM-1 orthologue, FEM-1B, has been demonstrated. FEM-1B is a subunit of the human CBC complex, suggesting that hFEM2 might also function in the mammalian ubiquitin-proteosomal pathways. It would therefore be interesting to elucidate the mechanism on how hFEM2 enhances CBC^{FEM-1}-mediated ubiquitination in higher organisms.

Besides being a phosphatase that downregulates the activities of autophosphorylated kinases e.g PAK1 and CaMKII, the non-catalytic N-terminal domain of POPX2 also functions as a scaffolding protein that mediates serum response factor (SRF)-mediated transcription from the serum response element. (Xie et al., 2008). POPX2 is shown to bind the Diaphanous-related formin-1 (mDia1) *in vivo* and this interaction is enhanced by the presence of active RhoA. mDia1 was first discovered as a downstream effector of RhoA; binding of GTP-bound RhoA to mDia1 relieves the protein from its autoinhibited state and initiates actin nucleation and polymerization (Watanabe et al.,

1997). On the other hand, the activation of SRF-mediated transcription appeared to be regulated by the ratio of monomeric G-actin to F-actin in the cells (Sotiropoulos et al., 1999). When the level of G-actin is high, transcription from the SRE is inhibited. When RhoA is activated, it acts through mDia to induce stress fiber formation, thereby reducing the level of G-actin and initiates SRF-dependent transcription (Geneste, Copeland, and Treisman, 2002; Tominaga et al., 2000). Binding of POPX2 to mDia1 resulted in an inhibition on the SRF activity induced by active RhoA and mDia1. This negative regulation on SRF-mediated transcription is independent of the phosphatase activity of POPX2.

The role of POPX2 in the maintenance of actin stress fibers has also been examined. Studies showed that overexpression of POPX2 enables the cells to overcome the stress fiber loss induced by active PAK1 (Koh et al., 2002) or dominant negative mutant of mDia (Xie et al, 2008). To further verify the involvement of endogenous POPX2 in stabilizing stress fibers, siRNAs targeting to *POPX2* were introduced into HeLa cells. About 60-70% of cells containing the siRNA showed loss of stress fibers as opposed to 25% in the control cells (Xie et al, 2008). Since POPX2 is a negative regulator of PAK1, one would expect the stress fiber loss phenotype observed in the POPX2 knockdown cells to be a consequence of elevation of PAK activity. Nevertheless, a significant portion of cells (46%) without stress fibers can still be observed when plasmid encoding the PAK kinase inhibitory domain (KID) was co-transfected with the siRNA. Even though the proportion was not comparable to cells transfected with siRNA alone (60-70%), it is considerably higher than in the control cells (26%). These observations suggest that POPX2 regulates actin stress fiber via both PAK-dependent and PAK-independent pathways.

Recent studies from our lab have revealed a critical role for POPX2 in cancer cell motility and invasion (Susila et al., 2010). The protein level of POPX2 was analyzed across eight breast cancer cell lines with varying invasiveness. In the most invasive breast cancer cell line MDA-MB-231, POPX2 level was 7-8 fold higher than the non-invasive cell line MCF-7. At the transcript level, the amount of POPX2 in the immortalized normal breast epithelial cells MCF10A and in the non-invasive MCF-7 was similar. In contrast, the transcript level of POPX2 was found to be 4.8 times higher in the MDA-MB-231 cells than in MCF-7. Based on these findings, the relationship of POPX2 and breast cancer cell metastasis was then investigated using transwell chemotaxis assays. Silencing of POPX2 via siRNA in the MDA-MB-231 cells led to a decrease in cell motility and invasiveness. Immunofluorescence cell staining revealed loss of stress fibers and focal adhesions in the POPX2-knockdown cells. In addition, reduced β 1-integrin and RhoGDI levels were detected in these cells by western blot analysis. These observations suggest that the impairment of cell motility and invasiveness may be due to alterations in the actin cytoskeleton and cell attachment. The effect of POPX2 silencing on initial cell attachment was further examined in the nude mice by colonization/metastasis assay. Initial attachment of MDA-MB-231 cells onto the lungs was hampered by the downregulation of POPX2. β 1-integrin is a key player in mediating cell-matrix attachment and is also a SRF target gene. Thus, it is likely that POPX2 modulates cell attachment through the regulation of β 1-integrin via SRF-mediated transcription.

We have also found that NIH3T3 fibroblast cells that overexpressed POPX2 exhibited higher motility in scratch wound assay as compared to the wild type control cells. This

observation is consistent with earlier finding whereby POPX2 level is upregulated in the more motile and invasive breast cancer cell lines. To further elucidate the mechanism for the regulation of cell migration by POPX2, our lab has performed a LC-MS/MS-based phosphoproteomic study to explore the global effects of POPX2 overexpression and identify novel signaling pathways in which POPX2 exerts its cellular function (Singh et al., 2011). In this study, the phosphoproteome of control and POPX2 overexpressing NIH3T3 cells (X2 cells) was compared. Higher level of serine-21-phosphorylated Glycogen synthase kinase 3A (GSK3A) was identified in the X2 cells than in the control cells. GSK3A is a pivotal kinase that regulates many cellular processes, and phosphorylation at serine-21 renders the kinase inactive. Furthermore, studies have reported that GSK3A controls cell motility through regulation of the mitogen-activated protein kinases, ERKs. ERKs have been shown to promote cell migration; hence, inhibition of ERKs by activated GSK3A results in reduced cell motility. In accordance with this, we found higher levels of phospho-ERK (active) and higher levels of phospho-GSK3A (inactive) in the X2 cells by Western blot analysis. Consequently, the higher motility observed in the POPX2 overexpressing cells might be due the effects of active ERKs and inactive GSK3A. β -catenin, a known substrate of GSK3, is found to be degraded by the proteasome pathway when phosphorylated by GSK3 (Hart et al., 1998). Conversely, unphosphorylated β -catenin is translocated to the nucleus where it upregulates the expression of genes that promote cell motility (Iwai et al., 2010; Stein et al., 2006). Indeed, immunofluorescence staining has showed that accumulation of β -catenin in the nucleus is more pronounced in the X2 cells, which is consistent with the higher level of inactive GSK3A.

Aim of this study:

As shown in the previous section, POPX2 has emerged as a multifunctional protein participating in a wide variety of biological processes. However, the underlying molecular mechanisms pertaining to some of the cellular phenotypes observed in the POPX2 overexpressing and POPX2 knockdown cells remain obscure. Hence, the main objective of this study is to perform a systematic characterization of POPX2 using molecular and cellular approaches and search for its putative effectors that would provide clues on its cellular functions. As a first step towards elucidating the molecular regulation of POPX2 functions, identification of interacting partners was carried out via yeast-two hybrid screen. Further characterization and probing of the functional relationships between POPX2 and one of its interaction partners were also conducted after validation of the interactions.

We found POPX2 interacts with KAP3, which is a non-motor, cargo-carrying subunit of the kinesin II motor complex. Subsequently, we have done extensive work to determine how the POPX2 phosphatase can affect the trafficking of cargoes by the kinesin II motor. We have also postulated and tested how phosphorylation could affect the trafficking of cargoes. Finally, we have identified KIF3A, the critical motor subunit of KIF3 complex, as a novel substrate of CaMKII and POPX2.

Chapter 2 Materials & methods

2.1 Materials

2.1.1 Chemicals

Acrylamide	Bio-Rad
Agarose	Bio-Rad
Ampicillin	USB
ABB (Ammonium bicarbonate)	Sigma
APS (Ammonium persulfate)	Bio-Rad
Bisacrylamide	Bio-Rad
Bromphenol blue	Sigma
BSA (Bovine serum albumin)	Sigma
Deoxynucleotides (dG/A/T/CTP)	Roche
DTT (Dithiothreitol)	Sigma
Ethidium bromide	Bio-Rad
Ethanol	Merck
IAA (Iodoacetic acid)	Sigma
Lyticase	Sigma
Methanol	Merck
Paraformaldehyde	Merck
PMSF (Phenylmethylsulfonyl fluoride)	Sigma
Poly-L-lysine	Sigma
Proteinase K	Promega
Ponceau S	Sigma
RNase A	Qiagen
SDS (Sodium dodecyl sulfate)	USB

Sodium Carbonate	Sigma
Sodium Thiosulphate	Merck
TEMED (N,N,N',N'-Tetramethylethylenediamine)	Bio-Rad
Triton X-100	Bio-Rad
Tween 20	Bio-Rad
X-β-Gal (5-bromo-4-chloro-3-indolyl-b –galactopyranoside)	Fermentas

2.1.2 Medium for yeast culture

YPD Medium	BD Biosciences
Minimal SD base	Clontech
DDO (SD/Leu-/Trp-)	BD Biosciences
TDO (SD/His-/Leu-/Trp-)	BD Biosciences
QDO (SD/Ade-/His-/Leu-/Trp-)	BD Biosciences
3-AT (3-amino-1, 2, 3-triazole)	BD Biosciences

2.1.3 Tissue culture materials

Dulbecco's modified Eagle's medium (DMEM)	Sigma
Minimum Essential Medium Eagle (MEM)	Sigma
Fetal Bovine Serum (FBS)	PAA
L-Glutamine	Gibco
Sodium bicarbonate	Merck
Trypsin	Sigma
Fast PES filter unit	Nalgene
Tissue culture dish	Corning/Nunc

2.1.4 Enzymes for cloning

Alkaline Phosphatase (CIP)	NEB
Expand High Fidelity Polymerase	Roche

Expand Long Template Polymerase	Roche
Ex Taq™ Polymerase	Takara
Restriction endonucleases	NEB
Kpn1	Roche
T4 DNA ligase	NEB

2.1.5 Commercial Kits

Matchmaker™ GAL4 Two-Hybrid System & Libraries	Clontech
QIAquick® Gel Extraction Kit	Qiagen
QIAquick® PCR Purification Kit	Qiagen
QIAquick® Spin Maxiprep Kit	Qiagen
QIAquick® Spin Miniprep Kit	Qiagen
ECL Western blotting detection reagents and analysis system	Amersham
QIAquick® Site-Directed Mutagenesis Kit	Stratagene

2.1.6 Mammalian cell transfection reagents

LipofectAMINE® 2000	Invitrogen
---------------------	------------

2.1.7 Other materials

Vectashield®	Vector labs
Glutathione Sepharose™ 4B beads	Amersham
Protein G Sepharose beads	Zymed
Anti-FLAG M2 agarose beads	Sigma

Mouse IgG	Sigma
Nitrocellulose membrane	Bio-Rad
Clear Blue X-Ray film	Thermo Scientific
Sterile filter 0.22 μm , cellulose acetate	Sartorius
Sterile filter 0.45 μm , cellulose acetate	Sartorius
Protease inhibitor cocktail tablet	Roche
PhosSTOP phosphatase inhibitor cocktail tablets	Roche

2.2 Bacterial strains, yeast strains and mammalian cell lines

DH5 α	Chemically competent	Invitrogen
KC8	Chemically competent	Clontech
AH109	Bait strain, MAT α , Ade ⁻ , His ⁻ Leu ⁻ , Trp ⁻	Clontech
Y187	Library host strain, MAT α ; Ade ⁻ , His ⁻ , Leu ⁻ , Trp ⁻	Clontech
COS-7	African green monkey kidney fibroblast	ATCC-CRL-1651
HeLa	Human cervix adenocarcinoma epithelial	ATCC-CCL-2
NIH3T3	Mouse embryonic fibroblast	ATCC-CRL-1658
NIH3T3-X2	Stable cell line overexpressing GFP-POPX2 and Neomycin resistance	
NIH3T3-GFP	Stable cell line infected with pBABE-GFP retroviral vector. Puromycin resistant.	
NIH3T3-GFP-POPX2	Stable cell line infected with pBABE-GFP-POPX2 retroviral vector. Puromycin resistant.	
NIH3T3-GFP-POPX2m	Stable cell line infected with pBABE-GFP-POPX2m retroviral vector. Puromycin resistant.	

NIH3T3-GFP-KIF3A	Stable cell line infected with pBABE-GFP-KIF3A retroviral vector. Puromycin resistant.
NIH3T3-GFP-KIF3A-S690A	Stable cell line infected with pBABE-GFP- KIF3A-S690A retroviral vector. Puromycin resistant.
NIH3T3-GFP-KIF3A-S690D	Stable cell line infected with pBABE-GFP- KIF3A-S690D retroviral vector. Puromycin resistant.

2.3 Buffers

DNA loading buffer	10 ml TE buffer 10 ml glycerol
Hank's balanced salts solution (HBS)	37 mM NaCl 5.4 mM KCl 0.34 mM Na ₂ HPO ₄ 5.6 mM Glucose 10 mM HEPES 2.0 mM CaCl ₂
Mammalian cell lysis buffer	20 mM Tris-HCL, pH 7.5 0.2 M NaCl 1 mM EGTA 1% Triton X-100 Protease inhibitor cocktail tablet (Roche) Phosphatase inhibitor cocktail tablet (Roche)

Nitrocellulose transfer buffer, 10 ×	30.3 g Tris base
	144 g glycine
	1000 ml water
Nitrocellulose transfer buffer 1 × buffer	50 ml 10× nitrocellulose transfer
	50 ml methanol
	400 ml water
Phosphate Buffered 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
SDS sample buffer, 6 ×	7 ml 4X Tris-Cl/SDS pH 6.8
	3 ml glycerol
	1 g SDS
	0.93 g dithiothreitol (DTT)
	1.5 mg bromophenol blue
SDS-PAGE electrophoresis buffer, 10 ×	30.2 g Tris base
	144 g glycine
	10 g SDS
	1000 ml water

TAE buffer, 50 ×	242 g Tris base, pH 8.0 57.1 ml acetic acid 100 ml 0.5 M EDTA top up with water to 1000 ml
TE buffer	1 ml 1 M Tris-Cl pH 8.0 0.2 ml 0.5 M EDTA 98.8 ml water
Trypsin stock solution	0.25% (w/v) trypsin 1 mM EDTA

2.4 Plasmid Constructs

pACT2	Primary vector; Gal4 activation domain; LEU2 expressing; Amp ^r
pACT- α -PIX	α -PIX full length; amino acids residue 1-772
pBABE-GFP	Primary vector; GFP tag; Amp ^r
pBABE-GFP-KIF3A	KIF3A full length; amino acid 1-702
pBABE-GFP-KIF3A-S690A	KIF3A full length; mutation S690A
pBABE-GFP-KIF3A-S690D	KIF3A full length; mutation S690D
pBABE-GFP-POPX2	POPX2 full length; amino acid residue 1-454
pBABE-GFP-POPX2m	POPX2 full length; mutation R326A, I338A
pBABE-mCherry	Primary vector; mCherry tag; Amp ^r

pBABE-mCherry-KIF3A	KIF3A full length; amino acid 1-702
pBABE-mCherry-KIF3A-S690A	KIF3A full length; mutation S690A
pBABE-mCherry-KIF3A-S690D	KIF3A full length; mutation S690D
pDsRed-Rab11a*	Rab11a full length; Kan ^r
pEGFP-C1-CaMKII α	CaMKII full length; addgene; Kan ^r
pEGFP-N1-N-cadherin	N-cadherin full length; addgene; Kan ^r
pGADT7-Rec	cDNA library vector; Gal4 activation domain; LEU2 expressing; Amp ^r
pGBKT7	Primary vector; Gal4 DNA binding domain; TRP1 expressing; Kan ^r
pGBKT7-POPX2	POPX2 full length; amino acid residue 1-454
pGEX-4T-1-KIF3A-Tail	KIF3A amino acids residue 601-702
pGEX-4T-1-S690A-Tail	KIF3A amino acids residue 601-701; mutation S690A
pGEX-6P-1-POXP2	POXP2 full length
pmCherry-Clathrin-LCA*	Clathrin-LCA full length; Kan ^r
pXJ-FLAG	Primary vector; FLAG tag; Amp ^r
pXJ-FLAG-KAP3	KAP3 full length; amino acids residue 1-792
pXJ-FLAG-KAP3-ARM1	KAP3 amino acids residue 156-239
pXJ-FLAG-KAP3-ARM2	KAP3 amino acids residue 286-410
pXJ-FLAG-KAP3-ARM3	KAP3 amino acids residue 449-614
pXJ-FLAG-KAP3-M1	KAP3 amino acids 156-792
pXJ-FLAG-KAP3-M2	KAP3 amino acids 284-792
pXJ-FLAG-KAP3-M3	KAP3 amino acids 449-792
pXJ-FLAG-KAP3-M4	KAP3 amino acids 1-250
pXJ-FLAG-KAP3-M5	KAP3 amino acids 1-555
pXJ-FLAG-KAP3-M6	KAP3 amino acids 615-792
pXJ-FLAG-KIF3A	KIF3A full length; amino acid 1-702
pXJ-FLAG-KIF3A-S690A	KIF3A full length; mutation S690A

pXJ-FLAG-KIF3A-S690D	KIF3A full length; mutation S690D
pXJ-FLAG-POPX2	POPX2 full length; amino acid residue 1-454
pXJ-GFP	Primary vector; GFP tag; Amp ^r
pXJ-GFP-POPX2	POPX2 full length; amino acid residue 1-454
pXJ-GFP-POPX2m	POPX2 full length; mutation R326A, I338A
pXJ-GST	Primary vector; GST tag; Amp ^r
pXJ-GST-POPX1	POPX1 full length; amino acid residue 1-757
pXJ-GST-POPX1-1-152	Amino acids residue 1-152
pXJ-GST-POPX1-1-269	Amino acids residue 1-269
pXJ-GST-POPX1-236-488	Amino acids residue 236-488
pXJ-GST-POPX1-236-757	Amino acids residue 236-757
pXJ-GST-POPX1-443-757	Amino acids residue 443-757
pXJ-GST-POPX2	POPX2 full length, amino acid residue 1-454
pXJ-GST-POPX2-1-191	Amino acid residue 1-191
pXJ-GST-POPX2-65-454	Amino acid residue 65-454
pXJ-GST-POPX2-128-454	Amino acid residue 128-454
pXJ-GST-POPX2-150-454	Amino acid residue 150-454
pXJ-GST-POPX2-194-454	Amino acid residue 194-454
pXJ-GST-POPX2-241-454	Amino acid residue 241-454
pXJ-mCherry-POPX2	POPX2 full length; amino acid residue 1-454
pXJ-mCherry-POPX2m	POPX2 full length; mutation R326A, I328A

* pDsRed-Rab11a and pmCherry-Clathrin-LCA are kind gifts from Dr. Low Boon Chuan and Dr. Lu Lei, respectively.

2.5 Primers

Name	Sequence
CaMKIIa	For: 5' GCCCGGGATCCAGTGCCAGGATGG 3' Rev: 5' TTAGGTACCATTCGGCGAAGCAAGAG 3'
KAP3	For: 5'TTACTCGAGATGCAAGGGGAGGACGCCAGATAC 3' Rev: 5'CGCGGTACCTCAAGATCCATAGCCATAGTAGTAAGG 3'
KAP3-ARM 1	For: 5' TATGGATCCATCCTGCAGCTTGCTCGAAATC 3' Rev: 5' TATCTCGAGCTTCTTGCCAAAGCTCATGTC 3'
KAP3-ARM 2	For: 5' CACGGATCCCTTCTGAATCTTGCTGAGGAT 3' Rev: 5' CCGCTGGAGTATGTGGTAAAGAACACACAT 3'
KAP3-ARM 3	For: 5' CACCTCGAGTGCATTAATCTTGCTGCTAAC 3' Rev: 5' GCGGGTACCGACATAAATTATCTGACACAC 3'
KAP3-M1	For: 5' GTACTCGAGATCCTGCAGCTTGCTCGAAATCCT 3' Rev: 5'CGCGGTACCTCAAGATCCATAGCCATAGTAGTAAGG 3'
KAP3-M2	For: 5' GGGCTCGAGCTTCTGAATCTTGCTGAGGATACT 3' Rev: 5'CGCGGTACCTCAAGATCCATAGCCATAGTAGTAAGG 3'

KAP3-M3	For: 5' CACCTCGAGTGCATTAATCTTGCTGCTAAC 3' Rev: 5' CGCGGTACCTCAAGATCCATAGCCATAGTAGTAAGG 3'
KAP3-M4	For: 5' TAAGGATCCCTGCCTCTCAAGATACCC 3' Rev: 5' ATACTCGAGGATGCCCACCCTCTAGAG 3'
KAP3-M5	For: 5' TTAGGATCCATGCAAGGGGAGGACGCCAGATAC 3' Rev: 5' CTACTCGAGCAAAGCAGAACCCCGAACTTTGTC3'
KAP3-M6	For: 5' GGGCTCGAGTTCTACCAGATGGTTTTCCACCAA 3' Rev: 5' CGCGGTACCTCAAGATCCATAGCCATAGTAGTAAGG 3'
KIF3A	For: 5' GGGGGATCCATGCCGATCAATAAATCAGAGAAG 3' Rev: 5' CCGCTCGAGAGGTATAAAGTTATCAATAATAAGC3'
KIF3A-S690A	For: 5' CAGGGAGAAGAAAGCGTGCTGCAAAGCCTGAAACT 3' Rev: 5' AGTTTCAGGCTTTGCAGCACGCTTTCTTCTCCCTG 3'
KIF3A-S690D	For: 5' CCAAAGACAGGGAGAAGAAAGCGTGATGCAAAGCCTGAAAC3' Rev: 5' GTTTCAGGCTTTGCATCACGCTTTCTTCTCCCTGTCTTTGG 3'
KIF3A-Tail	For: 5' ATAGGATCCGATTATCAGGAAATGATTGAA 3' Rev: 5' CCGCTCGAGAGGTATAAAGTTATCAATAATAAGC3'

KIF3A-Motor	For: 5' GGGGGATCCATGCCGATCAATAAATCAGAGAAG 3' Rev: 3' GGGCTCGAGTCTTCATTAATTCTAGCTTTATT 3'
POPX2-1-191	For: 5' AATGGATCCATGTCCTCTGGAGCCCCACA 3' Rev: 3' TAACTCGAGGCGGTTACAGGGTCAGACAA 3'
POPX2-65-454	For: 5' TATGGATCCTTTCTGGGCAGCAGGAAGGC 3' Rev: 5' TATCTCGAGCTAGCTTCTTGGTGGAGCCTG 3'
POPX2-125-454	For: 5' TCCGGATCCCTGGCACAGAGTTTCTTTAAC 3' Rev: 5' TATCTCGAGCTAGCTTCTTGGTGGAGCCTG 3'
POPX2-194-454	For: 5' CGCGGATCCTTTGCTGTGTTTGATGGTCAC Rev: 5' TATCTCGAGCTAGCTTCTTGGTGGAGCCTG 3'
POPX2-241-454	For: 5' ACCGGATCCATGTTTCTCAGGAAAGCCAAG3' Rev: 5' TATCTCGAGCTAGCTTCTTGGTGGAGCCTG 3'
PP2C α	For: 5' ACGGGATCCATGGGAGCATTTTTAGACAAG 3' Rev: 5' GCGCTCGAGAGTTTGATTGTGTTGAAGATT 3'

2.6 Antibodies

2.6.1 Primary antibodies

β -catenin	Rabbit, polyclonal	Sigma	C2206
FLAG	Mouse, monoclonal	Sigma	F3165
FLAG	Rabbit, polyclonal	Sigma	F7425
GFP	Rabbit, polyclonal	Invitrogen	11122
GST	Rabbit, polyclonal	Bethyl	A190-122A
HA	Mouse, monoclonal	Santa Cruz	7392
HA	Rabbit, polyclonal	Invitrogen	71-5500
KAP3	Mouse, monoclonal	Santa Cruz	74425
KAP3	Mouse, monoclonal	BD Biosciences	610637
N-cadherin	Mouse, monoclonal	BD Biosciences	610920
Phospho-Ser/Thr	Rabbit, polyclonal	ECM Biosciences	2551
POPX2	Rabbit, polyclonal	Self-raised	-

2.6.2 Secondary antibodies

Goat anti-mouse IgG, HRP conjugated	Dako
Goat anti-rabbit IgG, HRP conjugated	Dako
Goat anti-mouse IgG, Alexa Fluor 488	Molecular Probes
Goat anti-rabbit IgG, Alexa Fluor 488	Molecular Probes
Goat anti-mouse IgG, Alexa Fluor 546	Molecular Probes
Goat anti-rabbit IgG, Alexa Fluor 546	Molecular Probes

2.7 Methods

2.7.1 Yeast Two-Hybrid Screen

Yeast two-hybrid screening was performed via the yeast mating approach according to the manufacturer's product protocol for the Matchmaker yeast two-hybrid system. Briefly, full length POPX2, which was cloned into the pGBKT7 vector, was transformed into the AH109 yeast strain that expressed ADE2, HIS3 and lacZ reporter genes under the control of Gal4-responsive element. The POPX2 bait was used to screen the Matchmaker™ human brain cDNA library fused to the GAL4 DNA-activation domain (AD) of the pGADT7-Rec vector and the human heart cDNA library fused to the GAL4 DNA-activation domain (AD) of the pACT2 vector. The pretransformed Y187 library host strain and the AH109 bait strain were mixed and incubated for 24 hours at 30 °C. The diploid Y187- AH109 strain was plated onto synthetic medium deficient in histidine, leucine and tryptophan (TDO). 5mM 3-amino-1,2,4-triazole was included in the medium to eliminate false HIS3 positives. Colonies that grew on the TDO medium were transferred onto synthetic medium deficient in adenine, histidine, leucine and tryptophan (QDO) for more stringent selection. Colonies that grew successfully on the QDO medium were further assayed for their expression of the lacZ reporter gene via the X-β-gal assay. Library plasmids were recovered from the putative positive clones according to manufacturer's protocol (Clontech) and the size of the cDNA inserts were verified by both restriction enzyme digestion with HindIII and PCR amplification using primers (Matchmaker LD AD-Insert Screening Amplimer) provided by the manufacturer. PCR reactions were run for 5mins at 95°C, followed by 25 cycles of 1 min at 95°C, 1 min at 52°C, 4 mins at 72°C, and a final extension of 4 mins at 72°C. Identities of the cDNA inserts were then

revealed by sequencing. The library plasmids of the putative positive clones were re-transformed into AH109 containing the bait plasmid by the lithium acetate method described in the Clontech protocol and the transformants were plated onto synthetic medium deficient in leucine and tryptophan (DDO). pACT2- α PIX and empty pACT2 vector were individually transformed into AH109 containing the bait plasmid. Colonies expressing pACT2- α PIX were used as positive controls whereas colonies that express the empty pACT2 vector were used as negative controls. All colonies that grew on the DDO medium were replica plated and transferred onto TDO medium to ascertain the expression of HIS3 reporter gene. The X- β -gal assay was then repeated.

2.7.2 X- β -Gal Assay

Colony-lift filter assay (5-bromo-4-chloro-3-indolyl β -D-galactopyranoside as substrate) was performed in triplicates as described in the Clontech product protocol. The colonies were incubated in dark at 30 °C and were checked every 30 mins for colour development for up to 8 hours.

2.7.3 General tissue culture

Mammalian cells were grown in a humidified 95% air, 5% CO₂ incubator at 37 °C. NIH 3T3 and COS-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 4500 mg/L Glucose supplemented with 3.7g/L sodium bicarbonate and 10% fetal bovine serum (FBS). HeLa cells were cultured in Minimum Essential Medium Eagle (MEM) supplemented with 2.2g/L sodium bicarbonate, 0.292 g/L L-glutamine and 10% FBS.

2.7.4 Western Blot

Protein lysates were loaded on 8-12% SDS polyacrylamide gels. After electrophoresis, the proteins were transferred to nitrocellulose membranes. The membranes were blocked for one hour at room temperature with 2% BSA in TBS, and then incubated with the appropriate primary antibodies overnight at 4 °C. The membranes were washed 10 minutes for 3 times in TBS containing 0.05% Tween 20 and then incubated with a 1:4000 dilution of horseradish peroxidase-conjugated anti-mouse/rabbit monoclonal antibodies for one hour at room temperature. The membranes were washed 3 times for 10 minutes, and the bound antibodies were detected with the ECL detection system. Primary antibodies were diluted in 1:1000, unless otherwise stated. Anti-KAP3 was diluted in 1:200.

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

COS-7 cells were grown to 80-90% confluency on 35-mm tissue culture dish. Plasmid DNA was transfected into COS-7 cells using Lipofectamine 2000 Transfection Reagent according to the manufacturer's instruction. Protein lysates were homogenized in 300µl protein lysis buffer 24 hours post transfection. The lysates were clarified by centrifugation at 14, 000rpm for 15 min, 4°C. The supernatant was mixed with 50µl Glutathione Sepharose™ 4B (Amersham Biosciences) bead slurry at 4°C for 2 hours with constant rotation. Beads were washed twice with protein lysis buffer.

Bound protein was eluted in SDS sample buffer by heating at 100 °C for 10 mins and subjected to SDS-PAGE and western blotting.

2.7.6 FLAG immunoprecipitation assay

Cells were grown to 80-90% confluency on 35-mm tissue culture dish and were transfected with relevant plasmid DNA using Lipofectamine 2000 Transfection Reagent according to the manufacturer's instruction. Protein lysates were homogenized in 300µl protein lysis buffer 24 hours post transfection. The lysates were clarified by centrifugation at 14, 000rpm for 15 min, 4°C. The supernatant was mixed with 40µl of ANTI-FLAG® M2 Affinity Gel (Sigma) bead slurry at 4 °C for 2 hours with constant rotation. Beads were washed twice with protein lysis buffer. Bound protein was eluted in SDS sample buffer by heating at 100 °C for 10 mins and subjected to SDS-PAGE and western blotting.

2.7.7 Immunoprecipitation

NIH 3T3 cells were washed with ice-cold PBS and harvested with 500µl/100mm dish of protein lysis buffer. The lysates were cleared at 14,000rpm for 10 mins at 4°C. The supernatant was pre-cleared with 100µl of Protein G sepharose for every 500 µl of lysates. After 30 mins incubation at 4 °C on an orbital shaker, the concentration of the

lysates was measured. The protein lysates were diluted to approximately 2 µg/µl with PBS to reduce the amount of detergent in the buffer. In total, 1mg of total protein was used for each immunoprecipitation reaction. 20 µg of antibodies was added to 1mg of protein lysate and the lysate-antibody mixture was incubated for 2 hours at 4 °C on an orbital shaker. Immunocomplexes were then captured by 100µl of Protein G sepharose bead slurry at 4 °C, overnight, on an orbital shaker. The sepharose beads were washed twice with 1ml protein lysis buffer. Bound immunocomplexes were eluted in SDS sample buffer by heating at 100 °C for 10 mins and subjected to SDS-PAGE and western blotting.

2.7.8 Immunofluorescence

Cells were plated on poly-L-lysine-coated glass coverslips and transfected using Lipofectamine 2000 Transfection Reagent according to manufacturer's protocol. Cells were fixed in 4% paraformaldehyde/PBS 24 hours post transfection. Then the cells were permeabilized in 0.2% Triton X-100/PBS and blocked with 10% FBS in 0.1% Triton X-100/PBS. Cells were incubated with primary antibody in 1% Triton X-100/PBS (1: 100 or 1:50 dilution) at 4 °C overnight, followed by washing for three times in 0.1% Triton X-100/PBS, 10 mins each time. Cells were then incubated with secondary antibody in 1% Triton X-100/PBS (1: 100 dilution) at R.T for 1 hour and washed as above. Coverslips were dried and mounted using Vectorshield with DAPI to show nuclei. Carl Zeiss axiovert microscope and Zeiss objective plan neo-fluar

100X/1.3 oil were used. The MetaMorph software programme was used to capture images using a Roper Scientific CoolSNAP CCD camera.

2.7.9 Time-lapse imaging of N-cadherin/Rab11a vesicles

Time-lapse epifluorescence microscopy was performed on Carl Zeiss axiovert microscope equipped with 100X Zeiss objective plan neo-fluar 100X/1.3 oil immersion objective, sample heater (37°C) and CO₂ incubation chamber. Images were captured with Roper Scientific CoolSNAP CCD camera. MetaMorph imaging software was used to analyze the images and 10 vesicles from each cell were tracked by the “Track Objects” function in the software. Data regarding the mean distances and velocities of the vesicles were logged to disk and treated with Excel software.

2.7.10 Cell aggregation assay

Cells were trypsinized in HBS, followed by two washes in HBS supplemented with 1% BSA and passed through a 27G needle thrice to obtain single cells. Cells were then diluted to 5×10^5 cell/ml in the same buffer and were incubated in 1.5 ml tubes at 37 °C with gentle rotation. After 30 mins, the number of cell particles was counted to determine the aggregation index $(N_0 - N_{30})/N_0$, where N_{30} is the total particle number after 30 min incubation and N_0 is the total particle number at the start of incubation. Images of cell particles on the hemocytometer were captured with phase-contrast microscopy.

2.7.11 RNA interference experiment

Stealth siRNA (Invitrogen) targeting different regions of the *kap3* gene were used and the sequences are as follow: 5' CAGCUACUGAGAGUUGCUCUCUACU 3' (sense strand) and 5' CAUAUAAGCAUGGAUGACCGCUUUA 3' (sense strand). The siRNAs were introduced into NIH3T3 cells via reverse transfection. Briefly, cells were trypsinized, resuspended in culture medium and mixed with the transfection complex containing the siRNA and Lipofectamine before plating on culture dish. Transfected cells were harvested after 48 hours for Western blot analysis to determine the efficiency of the gene knockdown.

2.7.12 Expression and purification of recombinant proteins

The cDNAs encoding full length POPX2, KIF3A-Tail (601-702) and KIF3A-S690A-Tail (601-702) were cloned into the pGEX-4T-1 vector. The expression of GST fusion proteins was induced in Rosetta competent cells by addition of 1 mM IPTG for 16 hours at 18 °C. Cell pellets were resuspended in STE buffer (10 mM Tris-HCL pH 8.0, 150 mM NaCl, 1 mM EDTA) supplemented with 1 mg/ml of lysozyme, 10 mM DTT and 1.5% of Sarkosyl. Samples were then sonicated at 30% amplitude and centrifuged at 14,000 rpm for 10 mins. Triton X-100 was added to the cleared samples to a final concentration of 2%. The soluble proteins were incubated with glutathione sepharose beads for 2 hours at 4 °C. The beads were washed twice with 1% Triton X-100 in PBS. GST fusion proteins were eluted with 40 mM of reduced L-glutathione (Sigma) at room temperature for 1 hour. Proteins were dialyzed against PBS and 10% glycerol.

2.7.13 *In vitro* kinase and phosphatase assays

FLAG-CaMKII was expressed in COS-7 cells and subjected to FLAG-immunoprecipitation. Activation of CaMKII was performed by adding kinase buffer (50 mM Tris pH 7.5, 10 mM MgCl₂, 5 mM MnCl₂, 1 mM dithiothreitol and 0.05% Triton X-100) supplemented with 1.2 μM calmodulin, 2 mM CaCl₂ and 200 μM ATP. The activation reaction was incubated at 30 °C for 10 mins. Kinase assays were performed by adding 4 μg of purified GST-fusion protein to the activated FLAG-CaMKII, in 30 μl of kinase buffer supplemented with 200 μM ATP. The kinase reaction (30 °C, 30 mins) was stopped by the addition of SDS sample buffer. For phosphatase assays, GST-KIF3A-Tail phosphorylated by CaMKII, was incubated with bacterially expressed POPX2 or CIP (New England Biolabs) for 30 mins at 30 °C. The reactions were stopped by the addition of SDS sample buffer.

2.7.14 Statistical analysis

Excel software (Microsoft office 2007) was used for all statistical analyses. All experiments (with relevance to statistical analysis) were performed independently for at least three times. Means of the measurement variable e.g. cell number or distance travelled, were computed into the Excel workbook to generate the standard deviations. Differences between various samples were analyzed by two-tailed, unpaired Student's t-tests. P values < 0.01 were considered highly significant, <0.05 significant in all analyses.

Chapter 3 Results

3.1 Identification of POPX2-interacting proteins by yeast-two hybrid

In an attempt to identify proteins interacting with POPX2, yeast two-hybrid screening of both the human brain and heart cDNA libraries was carried out, with full length POPX2 as bait. In total, 9×10^6 clones were screened from the human brain library and 2×10^6 clones were screened from the human heart library. Eventually, 5 colonies from the brain library and 12 colonies from the heart library survived the nutritional selection on the synthetic medium deficient in adenine, histidine, leucine and tryptophan (QDO). The cDNA library plasmids were then extracted from each of the clones and the sizes of the cDNA inserts were determined by both restriction enzyme digestion and PCR amplification (Figure 10). Identities of the cDNA clones were revealed by DNA sequencing and summarized in table 1. Two of the clones isolated from the human brain library corresponded to a truncated Kinesin-associated protein 3 (*KIFAP3*), alternatively known as KAP3. The cDNA had a 717-bases open reading frame which encodes a protein with a calculated *Mr* of 26, 290 Da. Five of the clones obtained from the human heart library corresponded to one of the immunoglobulin-like domain of Myosin Binding Protein C, cardiac (*MYBPC3*). The cDNA had a 576-bases open reading frame which encodes a protein with a calculated *Mr* of 21, 010 Da. The neighboring sequence of the first ATG was consistent with the Kozak consensus sequence of translation initiation start site. Partial cDNA sequence analysis revealed that the remaining clones encoded the leucine rich repeat containing 22 (*LRRC22*), SERTA domain containing 2 (*SERTAD2*), MAD2L1 binding protein (*MAD2L1BP*), triadin (*TRDN*), lactate dehydrogenase B (*LDHB*) and three unknown

genes with only partial EST sequences in the database (Table 1). All the clones were in truncated form. To ascertain the interactions between POPX2 and these putative positive clones, the cDNA library plasmids of these clones were re-transformed into AH109 yeast strain containing the bait plasmid. Nutritional selection and X- β -gal colony-lift filter assay were then carried out. α PIX, a known interacting partner of POPX2, was included in the re-transformation experiment as a positive control whereas an empty library plasmid vector was used as a negative control. Clones expressing KAP3 and MYBPC3 survived on the TDO selection medium and exhibited strong β -galactosidase activities. In contrast, growth of the remaining clones on the TDO medium was significantly suppressed and no significant β -galactosidase activities were detected within 8 hours (Figure 11). Since MYBPC3 is specifically expressed in cardiac tissue, while KAP3 is ubiquitously expressed, we have selected KAP3 for further analysis.

Figure 10. Verification of cDNA inserts sizes. The cDNA library plasmids of the 17 putative positive clones were subjected to (A) restriction enzyme digestion with HindIII and (B) PCR using primers flanking the cDNA inserts. Since the HindIII sites were located 555bp upstream and 313bp downstream of the insert, 868bp had to be subtracted to obtain the precise insert size. 5' and 3'primers for amplifying the inserts were located 177bp upstream and 111bp downstream of the inserts respectively, thus, 288bp have to be subtracted. From the brain cDNA library: Lane 1, insert size: ~0.9Kb; lane 2, insert size: ~0.9Kb; lane 3, insert size: ~1.07Kb; lane 4, insert size: ~1.35Kb; lane 5, insert size: ~2.5Kb. From the heart cDNA library: Lane 1, insert size: ~1.4Kb; lane 2, insert size: ~2.4Kb; lane 3, insert size: ~2.4Kb; lane 4, insert size: ~1.6Kb; lane 5, insert size: ~1.1Kb, lane 6, insert size: ~2.4Kb; lane 7, insert size: ~1.6Kb; lane 8, insert size: ~1.7Kb; lane 9, insert size: ~2.4Kb; lane 10, insert size: ~1.4Kb, lane 11, insert size: ~2.4Kb; lane 12, insert size: ~0.9Kb.

Figure 11. Re-transformation of library plasmid into AH109 containing the bait plasmid confirmed interactions between the bait and the putative binding partners. (A) Nutritional selection on TD0 medium. Other than MAD2BP and Triadin, all of the remaining putative positive clones survived on the TD0 medium. (B) X- β -Gal assay. Other than MAD2BP and Triadin, all of the remaining putative positive clones demonstrated strong β -galactosidase activities. α PIX served as the positive control whereas pACT served as the negative control. Results shown are representative of three independent experiments.

Type of Library	Clone no.	Gene symbol	Accession number	Size of cDNA obtained (kbp)	Schematics of selected clones (Black bar represents region isolated with POPX2)
Brain	1,2	<i>KIFAP3</i>	NM_014970	0.9	<p>KAP3</p>
	3	<i>LOC643329</i>	XR_018653	1.07	
	4	<i>LRRC22</i>	NM_001017924	1.35	
	5	<i>SERTAD2</i>	NM_014755	2.5	
	5	<i>SERTAD2</i>	NM_014755	2.5	
Heart	1	<i>MAD2L1BP</i>	NM_014755	1.4	<p>MYBPC3</p>
	2,3,6,9,11	<i>MYBPC3</i>	NM_000256	2.4	
	4	<i>Hypothetical protein</i>	NM_174921.1	1.6	
	5	<i>Hypothetical protein</i>	NM_174921.1	1.1	
	7	<i>TRDN</i>	NM_006073	1.6	
	8	<i>TRDN</i>	NM_006073	1.6	
	10	<i>LDHB</i>	NM_002300	1.4	
	12	<i>LOC23731</i>	NM_032012.3	0.9	

Table 1: Summary of putative interacting partners of POPX2 isolated in the yeast-two hybrid screen.

3.2 Association of POPX2 with the KIF3 kinesin motor complex

3.2.1 Initial characterization of interaction between POPX and KAP3

To verify the interaction between POPX2 and KAP3 in mammalian cells, full length GST-POPX2 or FLAG-POPX2 was co-expressed with full length FLAG-KAP3 or GST-KAP3, respectively in COS-7 cells. The bound proteins obtained by GST pull-down assay using glutathione beads indicated that there is interaction between POPX2 and KAP3 (Figure 12).

Figure 12. POPX2 interacts with full length KAP3 in mammalian cells. Full length FLAG-POPX2 was co-expressed with full length GST-KAP3 or GST alone in COS-7 cells (*left panel*). Full length FLAG-KAP3 was co-expressed with GST-POPX2 or GST alone in COS-7 cells (*right panel*). GST-fusion proteins were isolated on glutathione-Sepharose beads, and the bound protein complexes were eluted and separated by SDS-PAGE followed by Western blotting using anti-FLAG and anti-GST antibodies.

3.2.2 Mapping of KAP3 interacting domain on POPX

We next determined if KAP3 is capable of binding POPX1, the longer isoform of POPX. Full-length as well as deletion mutants of GST-POPX1 were transiently co-expressed with FLAG-KAP3 in COS-7 cells and precipitates were generated using glutathione beads. The presence of KAP3 was determined by Western blot analysis of bound proteins using anti-FLAG antibodies. Full-length POPX1 was found to co-precipitate with KAP3 (Figure 13). To identify the binding domain of POPX1, we tested interaction between KAP3 and various POPX1 deletion mutants. We examined the 1-488 mutant and found that this mutant was able to bind KAP3, indicating that

the C-terminal region of POPX1 is not essential in binding KAP3. We then proceed to determine if the N-terminal region of POPX1 is involved in binding KAP3. Two mutants spanning the N-terminal 1-152 and 1-269 residues were generated. KAP3 was found to interact with the 1-269 mutant but not the 1-152. In addition, a mutant containing residues 236-757 was also capable of binding KAP3, suggesting that amino acids 236-269 of POPX1 may be critical for its interaction with KAP3. Similar pull-down experiment was also performed with a series of POPX2 deletion mutants. KAP3 was found to interact with the mutant that contains the N-terminal 1-191 residues of POPX2 (Figure 14A). Sequential deletion of the N-terminal residues of POPX2 further revealed the importance of amino acids 150-191 for KAP3 binding. By combining the results of pull-down experiments using deletion mutants of POPX1 and POPX2, we have mapped the minimal domain required for KAP3 binding (Figure 14C). Since several residues within this domain are also conserved in the PP2C phosphatase, we tested if KAP3 is able to bind PP2C α . As shown in Figure 14B, KAP3 did not co-precipitate with GST-PP2C α , demonstrating the specificity of its interaction with POPX. In addition to deletion mutants, a full-length phosphatase-dead POPX2 (GST-POPX2m) was constructed by converting the highly conserved Arginine-326 and Isoleucine-328 to alanine residues. This mutant was capable of binding KAP3 (Figure 14A), suggesting that the phosphatase activity of POPX2 is not essential for KAP3 binding.

Figure 13. Association of KAP3 with POPX1. Plasmid encoding FLAG-KAP3 was co-transfected with full length GST-POPX1 or its truncation mutants (illustrated on the right panel). The lysates were subjected to GST pull-down assay and the precipitated proteins were resolved by SDS-PAGE followed by Western blot analysis with the indicated antibodies.

Figure 14. Mapping the KAP3 binding region in POPX2. (A) Plasmid encoding FLAG-KAP3 was co-transfected with full length GST-POPX2 or with the series of mutants depicted in the bottom panel. The lysates were subjected to GST-pull-down assay and precipitated proteins were separated by SDS-PAGE followed by Western blot analysis using the indicated antibodies. (B) COS-7 lysates expressing GST-PP2C and FLAG-KAP3 were subjected to GST pull-down assay and Western analysis to determine interaction between the two proteins. (C) Alignment between POPX1, POPX2 and PP2C. Boxed sequences indicate residues that are critical for binding KAP3. Filled circles denote identical residues across POPX1, POPX2 and PP2C and open circles represent identical residues between POPX1 and POPX2.

3.2.3 Mapping of POPX2 interacting domain on KAP3

To determine the regions of KAP3 responsible for its interaction with POPX2, various truncated constructs of KAP3 were generated (Figure 15). Each of the FLAG-tagged constructs was ectopically expressed with GST-POPX2 in COS-7 cells and subjected to GST pull-down assay. The two KAP3 mutants (M5, M6) lacking the Armadillo repeats, failed to co-precipitate with POPX2, suggesting that the Armadillo domains are essential in the interaction between KAP3 and POPX2. To identify the specific regions within the Arm repeats that bind POPX2, three deletion mutants containing various regions of the Arm repeats were tested for their capacity to bind POPX2. When ectopically expressed in COS-7 cells, mutant that contains only the Arm repeats 3-5 was unable to interact with POPX2 (Figure 16). Thus, Arm repeats 1-2 and 6-9 may be important for KAP3 interaction with POPX2.

GST-Pulldown: **Total lysate:**

Figure 15. Mapping of the regions in KAP3 required for binding to POPX2. KAP3 is composed of an N-terminal region, 9 Armadillo repeats and a C-terminal region. GST-POPX2 was transfected with various Flag-tagged KAP3 deletion constructs into COS-7 cells. Cell lysates were subjected to GST pull-down assay. The bound proteins were eluted and analyzed by SDS-PAGE and western blotting using anti-Flag antibodies to detect co-purification of KAP3 molecules. Mutant M4 represents the yeast two-hybrid clone isolated with POPX2.

Figure 16. Specific KAP3 armadillo repeats required for interaction with POPX2. FLAG-tagged constructs consisting of various regions of the KAP3 Armadillo repeats were tested for their ability to bind GST-POPX2 in the GST pull-down assay.

3.2.4 POPX2 binds to KAP3 and β 1-PIX with comparable efficiencies

In previous reports, POPX2 has been shown to interact with β 1-PIX (Koh et al, 2002) and mDia-DN, the dominant negative mutant of mDia (Xie et al, 2008). Here, we compare the efficiency of POPX2 binding to KAP3 to that of β 1-PIX and mDia-DN. GST-POPX2 was co-expressed with the various FLAG-tagged proteins and the lysates were subjected to FLAG-IP. The amount of immunoprecipitated GST-POPX2 protein was detected using anti-GST antibodies. POPX2 demonstrated the highest binding efficiency to mDia-DN, followed by β 1-PIX and KAP3, which bind POPX2 with comparable efficiencies (Figure 17). We next determined if KAP3 and β 1-PIX compete for the same binding site on POPX2. Reciprocal precipitations of GST-POPX2, FLAG-KAP3 and HA- β 1-PIX were performed. As expected, POPX2 interacted with KAP3 and β 1-PIX respectively while no interaction between KAP3 and β 1-PIX was observed (Figure 18). Moreover, KAP3, POPX2 and β 1-PIX formed a trimeric complex indicating that KAP3 and β 1-PIX bind to different regions of POPX2.

Figure 17. Comparing the binding efficiencies of various POXP2-interacting proteins. FLAG- β 1-PIX, FLAG-mDia, FLAG-KAP3 and FLAG-KIF3A were co-transfected with GST-POPX2 or GST into COS-7 cells. Lysates were subjected to FLAG-IP followed by immunoblotting with anti-FLAG and anti-GST antibodies. 10% of the lysates were also analysed with GST antibody to show the equal expression of GST-POPX2 with all FLAG-tagged proteins.

Figure 18. Reciprocal co-precipitations of POPX2, KAP3 and β1-PIX. Epitope-tagged proteins were co-expressed in COS-7 cells in double combinations or all together. Each protein was precipitated (noted at the top) and analyzed by western blotting for co-precipitated proteins. The last panels on the right are Western analysis of the total lysates.

3.2.5 POPX2 binds to KIF3 complex via interaction with KAP3

In the cells, a functional KIF3 motor complex exists as a heterotrimer, consisting of KIF3A-KIF3B-KAP3. Protein that binds to one component of the heterotrimer could usually co-immunoprecipitate with the other components. For example, the protein phosphatase Dusp26 was first identified as a binding partner of KIF3A in a yeast-two hybrid screen (Tanuma et al., 2009). Later, interaction between Dusp26 and KAP3 was also demonstrated in the mammalian cells. Having this in mind, we proceed to verify if POPX2 can be detected in the immunoprecipitate of KIF3A, the critical motor subunit of the KIF3 complex. Using FLAG-IP, we have demonstrated that FLAG-KIF3A co-immunoprecipitated with GST-POPX2 but not GST (Figure 17). Notably, the binding efficiency between POPX2 and KIF3A is somewhat lower than that of the binding between POPX2 and KAP3. This led us to wonder if POPX2 is binding directly to KIF3A or if the interaction is mediated through KAP3. To end this, bacterially expressed GST-POPX2 was generated and tested for its binding efficiency with KAP3 and KIF3A. As we failed to obtain bacterially expressed KAP3 and

KIF3A proteins (data not shown), we overexpressed FLAG-KAP3 and FLAG-KIF3A in the COS-7 cells, subjected the lysates to FLAG-IP and washed the immunoprecipitates with strong buffer (see Figure 19 for details) to eliminate weak interacting proteins. Equal amount of GST or GST-POPX2 was then added to the immunoprecipitates. GST-POPX2 but not GST interacted with FLAG-KAP3, demonstrating direct interaction between the two proteins. A very weak immunoreactive band of GST-POPX2 was detected in the immunoprecipitate of FLAG-KIF3A. Collectively, these results suggest that POPX2 binds to the KIF3 motor complex primarily through interaction with the KAP3 subunit. Indeed, both GST-POPX2 and HA-KIF3A co-immunoprecipitated with FLAG-KAP3, demonstrating the formation of the POPX2-KAP3-KIF3A trimeric complex (Figure 20). This also indicates that POPX2 and KIF3A do not compete for the same binding site on KAP3.

Figure 19. Direct interaction between KAP3 and POPX2. COS-7 cell lysates transfected with FLAG-KAP3 or KIF3A were subjected to immunoprecipitation with anti-FLAG antibodies-conjugated agarose and the immunoprecipitates were washed stringently with strong buffer (500 mM NaCl, 20 mM Tris, 1 mM EGTA, 1% Triton-X 100 and 0.05% SDS). GST and GST-POPX2 were generated in the *E.coli* cells and 4 μ g of each of the purified recombinant protein was incubated with the immunoprecipitated FLAG-KAP3 or FLAG-KIF3A. The immunocomplexes were resolved by SDS-PAGE followed by Western blotting with the indicated antibodies.

Figure 20. POPX2, KAP3 and KIF3A form a complex. GST-POPX2, FLAG-KAP3 and HA-KIF3A were co-transfected into the COS-7 cells and lysates were subjected to FLAG immunoprecipitation. The immunocomplexes were resolved by SDS-PAGE followed by Western blotting with anti-GST, anti-FLAG and anti-HA antibodies, respectively.

3.2.6 Interaction of endogenous KAP3 with GFP-POPX2 in the NIH3T3 stable line

To further investigate the significance of POPX2 interaction with KAP3, we have made use of the NIH3T3 mouse fibroblast cells which stably overexpress human GFP-POPX2 (hereinafter known as X2 cells). The stable line was generated by transfecting the wild type parental NIH3T3 cells with the mammalian expression plasmid that contains the GFP-POPX2 cDNA and the geneticin resistant gene. Transfected cells were subjected to selection by G418 and cells that survived the selection were maintained as polyclonal line. Distribution of the GFP-POPX2 in the X2 cells is shown in Figure 21A. Majority of the X2 cells expressed moderately high level of GFP-POPX2 while a small number of the cells expressed the GFP-POPX2 protein at extremely low or at extremely high level. Under phase contrast imaging, the X2 cells appeared to be less clustered as compared to the parent NIH 3T3 cells (Ctrl). Since antibodies available can only recognize human but not mouse POPX2, our lab has used real-time quantitative PCR to estimate the amount of GFP-POPX2 and endogenous POPX2 mRNA transcripts (Singh et al., 2011). The transcript level of POPX2 was found to be 39 times higher in the X2 cells than that in the Ctrl cells. We then examined whether endogenous mouse KAP3 associates with the GFP-POPX2. After immunoprecipitation of the Ctrl and X2 cell lysates with anti-KAP3 antibody, respectively, and subsequent immunoblotting with anti-POPX2 antibody, KAP3 was found to co-immunoprecipitate with GFP-POPX2 (Figure 21B). Immunoprecipitation of the cell lysates with random mouse IgG failed to detect the presence of GFP-POPX2 in the immunoprecipitate.

Figure 21. Association of endogenous KAP3 with GFP-POPX2. (A) Phase contrast images of live NIH3T3 Ctrl and X2 cells. Expression and distribution of GFP-POPX2 in the X2 cells are shown in the bottom panel. Scale bar: 50 μ m. (B) Cell lysates prepared from Ctrl or X2 cells were immunoprecipitated with anti-KAP3 antibodies, resolved on SDS-PAGE gel and immunoblotted with the indicated antibodies. Mouse random IgG was used to demonstrate absence of non-specific binding.

3.2.7 POPX2 and KAP3 localized to vesicular structures

Initial attempt to examine co-localization of mCherry-POPX2 and GFP-KAP3 by confocal laser scanning microscopy failed to generate desirable images. This was due

to the low protein expressing cells being severely photo-bleached by the laser and the high expressing cells displaying co-localization throughout the cytoplasm. To resolve this, we cloned the mCherry-POPX2 cDNA into the mammalian expressing vector containing a truncated (weaken) CMV promoter, delta 2, and imaged the localization of mCherry-POPX2 and endogenous KAP3 using epi-fluorescence microscopy. A number of the mCherry-POPX2 vesicles were observed to localize to regions where endogenous KAP3 was found (Figure 22).

Figure 22. Localization of POPX2 and KAP3 in NIH3T3 cell. mCherry-POPX2 cDNA was expressed under the control of the delta 2 promoter in wild type NIH3T3 cells. Cells seeded on coverslips coated with poly-L-lysine were double-stained with anti-KAP3 and Dapi. Localization of mCherry-POPX2 and endogenous KAP3 to vesicles is shown in the enlarged box area. Scale bar: 20 μ m.

3.3 Localization & tracking of KIF3A in Ctrl and X2 cells

To examine the effect of POPX2 overexpression on the localization of KIF3 motor in the NIH3T3 cells, we stained for the endogenous KIF3A in the Ctrl and X2 cells. Overexpression of GFP-POPX2 resulted in a modest increase in perinuclear localization of the KIF3A (Figure 23; compare Ctrl and X2 cells). We then attempted to track the GFP-KIF3A protein in live cells, nevertheless, the protein appeared diffused and tracking of single vesicle was not possible. Consequently, we turned to studying the effects of POPX2 overexpression on the cargoes of KIF3 motor.

Figure 23. Localization and tracking of KIF3A in NIH3T3 cells. Ctrl and X2 cells were stained with anti-KIF3A antibodies to reveal the localization of endogenous KIF3A (left panel). Live imaging of NIH3T3 cells expressing GFP-KIF3A under the control of the delta 2 promoter (right panel). Scale bar: 20 μm .

3.4 POPX2 inhibits N-cadherin-mediated cell-cell adhesions

3.4.1 Altered subcellular localization of N-cadherin and β -catenin in POPX2 overexpressing cells

N-cadherin is a well-established molecular cargo of the KIF3 kinesin that binds to the KIF3 motor complex primarily through interaction with KAP3 (Teng et al., 2005). To investigate the effect of POPX2 on KIF3 cargo transport, we examined the localization of endogenous N-cadherin in the NIH3T3 stable cell line (X2), which over-expresses GFP-POPX2. Immunofluorescent staining revealed that N-cadherin is highly localized to cell periphery and cell-cell contacts in the parent NIH3T3 cells (ctrl). However, localization of N-cadherin to cell periphery and cell-cell contacts is greatly reduced in the GFP-POPX2 cells (Figure 24A). Similar results were obtained when the cells were immunostained for β -catenin (Figure 24B). The expression levels of N-cadherin and β -catenin in both the Ctrl and X2 cells were unaffected (Figure 24C).

Figure 24. Reduced N-cadherin and β -catenin localization at cell periphery and cell-cell contacts of cells overexpressing POPX2. Representative images of immunostaining of parent NIH3T3 cells (Ctrl) or NIH3T3 stable cell line overexpressing GFP-POPX2 (X2). Cells seeded on coverslips coated with poly-L-lysine were double-stained with anti-N-cadherin and Dapi (A), or anti- β -catenin and Dapi (B). Scale bar: 20 μ m. (C) Total protein level of N-cadherin and β -catenin in the Ctrl and X2 cells were analyzed by Western blotting with the indicated antibodies.

3.4.2 POPX2 overexpression did not disrupt interaction between N-cadherin and KAP3

To determine if overexpression of POPX2 perturbs interaction between KAP3 and N-cadherin, a co-immunoprecipitation assay was performed using lysates from the NIH3T3 cells stably transfected with GFP-POPX2. When N-cadherin was immunoprecipitated with an anti-N-cadherin antibody, the immunoreactive band of KAP3 was detected in the immunoprecipitate (Figure 25). N-cadherin was reciprocally co-immunoprecipitated with the KAP3 antibody, suggesting that POPX2 overexpression had no effect on the *in vivo* interaction between N-cadherin and KAP3.

Figure 25. Effect of POPX2 overexpression on the interaction between KAP3 and N-cadherin. Cell lysates of the parent NIH3T3 cells (Ctrl) and NIH3T3 stable cell line overexpressing GFP-POPX2 (X2) were subjected to immunoprecipitation with either anti-N-cadherin or anti-KAP3 antibodies. The immunoprecipitated complexes were eluted, resolved by SDS-PAGE and immunoblotted with the indicated antibodies.

3.4.3 POPX2 phosphatase activity is required for its inhibition on N-cadherin transport to cell periphery and membrane ruffles.

To ascertain that the phosphatase activity of POPX2 is required for the perturbation of N-cadherin localization, stable cell lines overexpressing GFP, GFP-POPX2 or GFP-POPX2m (phosphatase-dead mutant), were generated by retroviral infection of NIH3T3 cells. After drug selection, pools of cells were used for further analysis instead of single cell clones, so that the effects of clonal variation can be avoided. The cell lines used in this study were between passage numbers 4 to 10. Immunostaining of GFP and GFP-POPX2m cells showed prominent localization of N-cadherin and β -catenin at cell periphery and cell-cell contacts, while such localization was disrupted in the GFP-POPX2 cells (Figure 26A, B). Western blot analysis demonstrated that the expression level of both the N-cadherin and β -catenin were unaffected by the overexpression of GFP, GFP-POPX2 or GFP-POPX2m (Figure 26C). These results suggest that POPX2 overexpression perturbs the localization of N-cadherin to cell periphery and cell-cell contacts. Furthermore, the phosphatase activity of POPX2 appears to be essentially involved in the regulation of N-cadherin localization.

Figure 26. The phosphatase-dead POPX2 mutant did not inhibit localization of N-cadherin and β -catenin to cell periphery and cell-cell contacts. Representative images of immunostaining of NIH3T3 cells infected with retroviral vector encoding GFP, GFP-POPX2 or GFP-POPX2m. Cells seeded on poly-L-lysine-coated coverslips were double-stained with anti-N-cadherin antibodies and Dapi (A), or anti- β -catenin antibodies and Dapi (B). Scale bar: 20 μ m. (C) NIH3T3 cells infected with GFP, GFP-POPX2 or GFP-POPX2m were analyzed by Western blotting with antibodies shown on the left.

3.4.4 Impaired transportation of N-cadherin-eGFP in POPX2 overexpressing cells

Having found a decreased level of N-cadherin and β -catenin at the cell periphery and cell-cell contacts in POPX2 overexpressing cells, we next examine the *in vivo* dynamics of GFP-tagged N-cadherin using live cell imaging. N-cadherin-GFP was co-expressed with mCherry vector, mCherry-POPX2 or mCherry-POPX2m in NIH3T3 cells. Since previous studies have demonstrated that the dynamics of N-Cadherin vesicles is greatly reduced in fully contacting cells (Mary et al., 2002), we have selected only isolated or early contacting cells for imaging. Enormous centrifugal and centripetal movement (see *Discussions* for detailed definition) of the N-cadherin-GFP vesicles was observed in cells overexpressing mCherry vector and mCherry-POPX2m. Notably, various vesicles in these cells were seen to move in a straight and outward fashion (Figure 27A and movie 1). In contrast, motility of N-cadherin vesicles was severely hindered in the mCherry-POPX2 overexpressing cells and the vesicles were found to accumulate around the perinuclear region (Figure 27A and movie 1). After imaging, individual N-cadherin vesicle was tracked and analyzed using the Metamorph image analysis software. On average, the N-cadherin-GFP vesicles in mCherry-POPX2 overexpressing cells travelled shorter distance and moved at lower velocity (Figure 27B).

Figure 27. Transportation of N-cadherin-GFP to cell periphery is impaired in POPX2-overexpressing cells. (A) N-cadherin-GFP was co-transfected with mCherry, mCherry-POPX2 or mCherry-POPX2m in NIH3T3 cells. Motility of N-cadherin-GFP vesicles in live cell was tracked per 2s using the MetaMorph image analysis software programme. The experiment was repeated three times. 10 cells were randomly selected and 10 vesicles from each cell were tracked. Scale bar: 20 μm . (B) Measurement of mean distance and velocity of N-cadherin-GFP vesicles. (Mean $\pm$ S.D; ** $P < 0.01$, Student's t-test).

3.4.5 POPX2 inhibits calcium-dependent cell-cell aggregation

To further analyze the effect of POPX2 overexpression on N-cadherin-mediated intercellular adhesion, we assessed calcium-dependent cell-cell aggregation of the retroviral vector infected NIH3T3 cells using quantitative short-term aggregation assay. NIH3T3 cells overexpressing GFP or GFP-POPX2m formed large aggregates in the presence of 2 mM CaCl_2 while aggregate formation is severely impaired in cells overexpressing GFP-POPX2. When calcium-dependent adhesion is blocked by the addition of EGTA or in the absence of Ca^{2+} , the difference in aggregate formation between the various cell lines became non-significant, suggesting that POPX2 specifically impaired calcium-dependent cell-cell adhesion (Figure 28).

Figure 28. Cell-cell adhesion is inhibited in POPX2-overexpressing cells. (A) NIH3T3 cells infected with GFP. GFP-POPX2 or GFP-POPX2m vector were subjected to aggregation assay in the following conditions: 2 mM CaCl₂, 2mM CaCl₂ + 2 mM EGTA, no CaCl₂. Representative images of the assays taken under phase-contrast microscope are shown. Scale bar: 100 μm. (B) The level of cell aggregation was calculated by the aggregation index described by (Ozawa, Ringwald, and Kemler, 1990). Briefly, aggregation index = $(N_0/N_{30})/N_0$, where N_0 is the total particle number at the start of incubation and N_{30} is the total particle number after 30 min incubation. Data shown are averages of three independent assays.

3.4.6 KAP3 siRNAs failed to silence the *kap3* gene in NIH3T3 cells

In an attempt to validate the findings reported by Teng et al, in which the N-cadherin is transported by the KIF3 motor complex through interaction with KAP3, we designed two siRNAs targeting different regions of KAP3. Varying amounts of siRNA were transfected into the NIH3T3 cells and the knockdown efficiency was assessed by Western blot using anti-KAP3 antibodies. To our dismay, the siRNAs failed to downregulate the expression of *kap3*. As shown in the immunoblot, the total protein level of KAP3 in cells transfected with KAP3 siRNA is not significantly different from cells transfected with the control siRNA targeting the luciferase gene (Figure 29).

Figure 29. Knockdown of *kap3* in NIH3T3 cells using RNAi. NIH3T3 cells were transfected with control siRNA against the luciferase gene (Luc) or siRNA targeting *kap3* (K1 and K2). Cells were harvested 24 hours post-transfection and the lysates were resolved by SDS-PAGE followed by Western blotting to probe for the endogenous level of KAP3.

3.5 POPX2 overexpression affects distribution of Rab11

Apart from N-cadherin, the recycling endosome protein Rab11 has also been reported to be a molecular cargo of the KIF3 motor (Schonteich et al., 2008). Thus, to further demonstrate the negative regulation of POPX2 on the KIF3 motor, we have examined the distribution of Rab11 in the POPX2 overexpressing cells. In the majority of HeLa cells transfected with GFP vector or GFP-POPX2m, the dsRed-Rab11a vesicles were concentrated in the central region of the cell, with some around the periphery and the remainder scattered throughout the cell (Figure 30; *normal*). However, only less than 30% of cells expressing GFP-POPX2 exhibited this type of distribution. In about 50% of the cells, we observed that the dsRed-Rab11a vesicles had cleared from the periphery and clustered at the perinuclear region (Figure 30; *clustered*). In the most extreme cases, nearly all dsRed-Rab11a vesicles had accumulated in a tight perinuclear cluster (Figure 30; *highly clustered*). We then performed live cell imaging to better study the effect of POPX2 overexpression on the dynamics of Rab11 vesicles. In cells overexpressing the GFP vector and GFP-POPX2m, the dsRed-Rab11a vesicles were generally more motile and long, outward movement was observed in some vesicles (Figure 31A, B and movie 2). On the contrary, the dsRed-Rab11a vesicles in the GFP-POPX2 overexpressing cells were less dynamic and travelled shorter distances with lower velocities (Figure 31A, B and movie 2). These observations suggest that POPX2 overexpression perturbs cellular distribution of Rab11, possibly through regulation of the KIF3 motor.

Figure 30. Effects of POPX2 overexpression on Rab11 distribution. (A) dsRed-Rab11a was co-transfected with GFP, GFP-POPX2 or GFP-POPX2m in HeLa cells. After 24hours, cells were fixed and imaged by fluorescent microscopy. (B) Scoring of cells showing the three phenotypes (normal, clustered, highly clustered) depicted in (A). Data are averages of three independent experiments, $n = 50$. Scale bar: 20 μm .

Figure 31. Transportation of dsRed-Rab11a to cell periphery is impaired in POPX2-overexpressing cells. (A) dsRed-Rab11a was co-transfected with GFP, GFP-POPX2 or GFP-POPX2m in HeLa cells. Motility of dsRed-Rab11a vesicles in live cell was tracked per 3s using the MetaMorph image analysis software programme. The experiment was repeated three times. 8 cells were randomly selected and 10 vesicles from each cell were tracked. Scale bar: 20 μ m. (B) Measurement of mean distance and velocity of dsRed-Rab11a vesicles. (Mean $\pm$ S.D.; ** P < 0.01; Student's t-test).

3.6 POPX2 overexpression did not affect motility of Clathrin

Since Rab11 functions in the endocytotic pathways, we sought to confirm that the POPX2 phosphatase is acting specifically on Rab11 through regulation of the KIF3 motor, without affecting non-specific endosomal proteins. We have monitored the motility of clathrin-light chain A (LCA), a major protein that functions in vesicle formation during endocytosis. Imaging of live cells that co-expressed clathrin-LCA-mCherry and GFP-POPX2 demonstrated that the dynamics and localization of the clathrin-LCA vesicles were not perturbed by the overexpression of POPX2 (Figure 32 and movie 3).

Figure 32. POPX2 overexpression did not affect motility of clathrin-LCA vesicles. (A) Clathrin-LCA-mCherry was co-transfected with GFP, GFP-POPX2 or GFP-POPX2m in HeLa cells. Motility of clathrin-LCA-mCherry vesicles in live cell was tracked per 3s using the MetaMorph image analysis software programme. The experiment was repeated three times. 5 cells were randomly selected and 10 vesicles from each cell were tracked. Scale bar: 20 μm . (B) Measurement of mean distance and velocity of clathrin-LCA-mCherry vesicles. (Mean $\pm$ S.D.; Student's t-test).

3.7 Mutation of KIF3A on serine 690 perturbs transportation of N-cadherin and Rab11 to cell periphery

Various phosphoproteomics-based analysis have identified the serine-690 residue on the C-terminal tail of KIF3A as a major phosphorylation site (Ballif et al., 2004; Dephoure et al., 2008; Huttlin et al., 2010; Olsen et al., 2010; Pan et al., 2008; Tweedie-Cullen, Reck, and Mansuy, 2009; Wisniewski et al., 2010). This serine residue falls within the putative consensus sequence R-X-X-S for CaMKII and this sequence is found to be conserved among human, mouse and zebrafish KIF3A (Figure 33). Hence, to investigate the consequence of KIF3A-S690 phosphorylation, we generated KIF3A-S690 mutants and monitored their effects on KIF3 cargo transport. Serine-690 was mutated to alanine (S690A) to preclude phosphorylation, or to aspartic acid (S690D) to mimic constitutive phosphorylation.

Figure 33. Serine-690 of KIF3A is a phosphorylation site. Domain organization of KIF3A is shown in the upper panel. Serine 690 (S690) is located in the C-terminal tail of KIF3A. The alignment of 15 amino acid residues in the C-terminal tail of human, mouse and zebrafish is shown in the lower panel. The conserved putative consensus sequence for CaMKII is highlighted in yellow.

We first determined the consequence of mutating KIF3A-S690 on the distribution of N-cadherin. Stable cell lines, which overexpress GFP-KIF3A, GFP-S690A or GFP-S690D, were generated by retroviral infection of the NIH3T3 cells. After drug selection, the surviving cells were maintained as polyclonal lines. Cells between passage numbers 3 to 8 were used for subsequent analysis. Immunofluorescence staining of the endogenous N-cadherin showed that overexpression of the KIF3A-S690A mutant led to a decreased localization of N-cadherin to cell periphery and cell-cell junction (Figure 35). We then examined the effect of mutating KIF3A-S690 on the motility of N-cadherin-GFP vesicles. Long centrifugal movement of the N-cadherin-GFP vesicles were observed in NIH3T3 cells overexpressing mCherry-KIF3A or mCherry-S690D mutant, indicating that overexpression of these proteins did not cause significant change in the motility of N-cadherin vesicles (Figure 31 and movie 4). However, movement of the N-cadherin vesicles was impaired in cells overexpressing the mCherry-S690A mutant (Figure 35 and movie 4). We have noticed that this impairment occurred only in cells with very high expression of mCherry-S690A. Cells with low or moderate expression of mCherry-S690A did not show significant change in the motility of N-cadherin vesicles. This might be due to the basal activity of the endogenous wild type KIF3A. On the other hand, high expression of mCherry-KIF3A and mCherry-S690D did not have an apparent effect on the vesicular transport, indicating that the inhibition on N-cadherin trafficking in mCherry-S690A overexpressing cells is not due to off-target effect caused by high protein overexpression.

Next, we proceeded to determine if overexpression of the KIF3A-S690A mutant perturbs motility of Rab11. Again, using live cell imaging, we observed that the dsRed-Rab11 vesicles were less motile and accumulated more around perinuclear

region in cells that overexpress GFP-S690A (Figure 36 and movie 5). Taken together, our results suggest that the phosphorylation status of the KIF3A serine-690 residue might be critical in regulating cargo transport.

Figure 34. Overexpression of KIF3A-S690A mutant inhibit localization of N-cadherin to cell-cell contacts. Representative images of immunostaining of NIH3T3 cells infected with retroviral vector encoding GFP, GFP-S690A or GFP-S690D. Cells seeded on poly-L-lysine-coated coverslips were double-stained with anti-N-cadherin antibodies and Dapi. Scale bar: 20 μ m.

Figure 35. Transportation of N-cadherin-GFP to cell periphery is impaired in S690A-overexpressing cells. (A) N-cadherin-GFP was co-transfected with mCherry-KIF3A, mCherry-S690A or mCherry-S690D in NIH3T3 cells. Motility of N-cadherin-GFP vesicles in live cell was tracked per 2s using the MetaMorph image analysis software programme. The experiment was repeated three times. 5 cells were randomly selected and 10 vesicles from each cell were tracked. Scale bar: 20 μm . (B) Measurement of mean distance and velocity of N-cadherin-GFP vesicles. (Mean $\pm$ S.D.; ** $P < 0.01$, Student's t-test).

Figure 36. Transportation of dsRed-Rab11a to cell periphery is impaired in S690A-overexpressing cells. (A) dsRed-Rab11a was co-transfected with GFP-KIF3A, GFP-S690A or GFP-S690D in HeLa cells. Motility of dsRed-Rab11a vesicles in live cell was tracked per 3s using the MetaMorph image analysis software programme. The experiment was repeated three times. 5 cells were randomly selected and 10 vesicles from each cell were tracked. Scale bar: 20 μm . (B) Measurement of mean distance and velocity of dsRed-Rab11a vesicles. (Mean $\pm$ S.D.; * $P < 0.05$, Student's t-test).

3.8 Phosphorylation of KIF3A serine-690 does not affect cargo binding

Previous studies have reported that phosphorylation of serine residues in the C-terminal tail of kinesin motor modulates interaction between the cargoes and the kinesin motor complex.(Guillaud, Wong, and Hirokawa, 2008; Ichimura et al., 2002; Vagnoni et al., 2011). Thus, we sought to elucidate if mutation of serine-690 of KIF3A affects binding of molecular cargo. Since N-cadherin associates with the KIF3A motor primarily through the KAP3 subunit, we determined the binding efficiency between KAP3 and the various KIF3A mutants. GST-KAP3 was co-expressed with the various FLAG-tagged KIF3A constructs and the cell lysates were subjected to both GST pull-down assay and FLAG immunoprecipitation. Wild type KIF3A, KIF3A-S690A and KIF3A-S690D interacted equally well with GST-KAP3 in these assays and no interaction was observed between FLAG-KIF3A and the control GST (Figure 37). The lack of effect of mutating KIF3A serine-690 on KAP3 binding implies that the inhibition on cargo transport is not due to modulation of binding efficiency.

Figure 37. Mutation of KIF3A serine-690 does not affect binding of KIF3A to KAP3. GST-KAP3 was co-transfected with FLAG-KIF3A, FLAG-S690A or FLAG-S690D. KAP3 was isolated using glutathione-sepharose beads (left; upper panel) while KIF3A was immunoprecipitated with anti-FLAG antibodies (left; lower panel). The bound proteins were eluted and resolved by SDS-PAGE and immunoblotted with the indicated antibodies. FLAG-KIF3A and GST vector were also co-transfected in the cells to demonstrate specificities of the pull-down assay and immunoprecipitation.

3.9 KIF3A interacts with calcium/calmodulin-dependent kinase II

Given the fact that serine-690 of KIF3A lies within the putative consensus sequence for CaMKII, it is tempting to speculate that KIF3A is a substrate of CaMKII. To support this hypothesis, we first tested if KIF3A physically interacts with CaMKII. FLAG-KIF3A was co-expressed with GFP-CaMKII in the COS-7 cells and immunoprecipitates were generated using anti-FLAG antibodies. POPX2, a known binding partner of CaMKII, was included in the immunoprecipitation assay as a positive control. GFP-CaMKII was found to co-IP with FLAG-KIF3A, and no signal for GFP was detected from cells transfected with FLAG-KIF3A and GFP-vector, demonstrating the specificity of the immunoprecipitations (Figure 38).

Figure 38. Interaction between KIF3A and CaMKII. FLAG-KIF3A was co-transfected with GFP or GFP-CaMKII into COS-7 cells. FLAG-POPX2 was co-transfected with GFP-CaMKII and acts as a positive control. Cell lysates were subjected to FLAG immunoprecipitation using anti-FLAG antibodies. The immunoprecipitates were eluted and separated by SDS-PAGE followed by Western blot analysis using the indicated antibodies.

3.10 KIF3A is regulated by CaMKII and POPX2 on serine-690

We then determined if KIF3A is substrate of CaMKII. To test this, bacterially expressed GST-KIF3A-Tail was incubated with active CaMKII, inactive CaMKII and active PAK1, respectively. Only active CaMKII could potentially phosphorylate KIF3A-Tail, as evidenced by the presence of the immunoreactive band that represents phospho-GST-KIF3A-Tail (Figure 39B, C; lane3). The S690A-Tail mutant, on the other hand, was not phosphorylated by the active CaMKII, indicating that the kinase phosphorylates KIF3A specifically on the serine-690 residue. To verify if KIF3A is a phosphoprotein *in vivo*, full-length FLAG-KIF3A was expressed in the COS-7 cells and the lysates were subjected to FLAG-IP followed by immunoblotting with anti-phospho-Ser/Thr antibodies. Indeed, a significant amount of phosphorylated FLAG-KIF3A was detected (Figure 39D). However, we noted that the FLAG-S690A mutant was phosphorylated too, suggesting that additional phosphosites, apart from serine-690, might be present in KIF3A.

Having known that KIF3A is phosphorylated by CaMKII on serine-690, we next assessed if the POPX2 phosphatase is likely to dephosphorylate this residue *in vitro*. Bacterially expressed GST-POPX2 or alkaline phosphatase (CIP), which serves as a random phosphatase control, was incubated with the phosphorylated GST-KIF3A-Tail. POPX2, but not CIP, significantly reduced the amount of phospho-GST-KIF3A-Tail, thus, making KIF3A a potential substrate of POPX2 (Figure 40).

Figure 39. *In vitro* phosphorylation of KIF3A by CaMKII. The tail domains of wild type KIF3A and S690A mutant, as depicted in (A), were expressed as recombinant GST fusion proteins in the *E. coli* cells. Lysates of COS-7 cells transfected with plasmid encoding active FLAG-PAK1, and FLAG-CaMKII were immunoprecipitated with anti-FLAG-conjugated agarose. CaMKII activation assay was then performed by incubating the immunoprecipitated FLAG-CaMKII with kinase buffer supplemented with 1.2 μ M calmodulin, 2 mM CaCl_2 and 200 μ M ATP at 30 $^\circ\text{C}$ for 10 mins. For FLAG-PAK1 and inactive FLAG-CaMKII samples, the immunocomplexes were incubated with kinase buffer alone. 4 μ g of GST fusion proteins were added to the active FLAG-PAK1, inactive FLAG-CaMKII and active FLAG-CaMKII, respectively and were subjected to *in vitro* kinase assay. The proteins were resolved on SDS-PAGE and analyzed by Western blotting with the indicated antibodies (C). The Ponceau S staining of the blot is shown in (B). Lane 1: active FLAG-PAK1 + GST-KIF3A-Tail; 2: inactive FLAG-CaMKII + GST-KIF3A; 3: active CaMKII + GST-KIF3A-Tail; 4: active FLAG-CaMKII + GST-S690A-Tail. (D) Plasmids encoding FLAG-S690A, FLAG-S690D and FLAG-KIF3A were transfected into COS-7 cells, respectively. FLAG fusion proteins were immunoprecipitated with anti-FLAG agarose and the immunocomplexes were resolved on SDS-PAGE gel and analyzed by Western blotting with the indicated antibodies.

Figure 40. POPX2 dephosphorylates KIF3A on serine-690 *in vitro*. CaMKII phosphorylated GST-KIF3A-Tail (4 μ g) was incubated with 4 μ g of bacterially expressed GST-POPX2 or CIP at 30 $^{\circ}$ C for 30 mins. The reaction mixtures were then resolved by SDS-PAGE and subjected to Western blotting using the indicated antibodies. Lane 1: unphosphorylated GST-KIF3A-Tail alone (negative control); 2: phosphorylated GST-KIF3A-Tail alone (positive control); 3: phosphorylated GST-KIF3A-Tail + GST-POPX2; 4: phosphorylated GST-KIF3A-Tail + CIP.

3.11 The tail domain of KIF3A interacts with the motor domain

The KIF3 motor has been shown to assume a folded conformation, where the tail domain interacts with the motor domain and thus, keeping the kinesin motor in an autoinhibited state (Wedaman et al., 1996). However, no biochemical-based evidence has been reported to verify this observation. Therefore, we set out to determine whether the tail domain and motor domain of KIF3A could physically interact in binding assay. GST-tagged KIF3A tail domain was co-expressed with FLAG-tagged KIF3A motor domain and the presence of FLAG-KIF3A-motor in the precipitate of GST-KIF3A-Tail was detected by anti-FLAG antibodies. We found that the FLAG-KIF3A-Motor domain binds to GST-KIF3A-Tail domain but not to the GST control. Furthermore, we showed in our earlier experiments that mutation of the KIF3A serine-690 residue to alanine appears to hamper the kinesin cargo transport function. Based on this observation, we hypothesized that the serine-690 of KIF3A is critical in

regulating the autoinhibition state of the kinesin. To verify our hypothesis, we tested if the interaction between the tail and motor domain of KIF3A is affected by mutation of this residue. To our surprise, both the S690A and S690D mutants bind equally well to the FLAG-KIF3A-motor domain. These results indicate that the tail domain indeed interacts directly with the motor domain but the serine-690 residue may not be critical in regulating this interaction.

Figure 41. Interaction between the tail and motor domain of KIF3A. Plasmid encoding FLAG-KIF3A-Motor was co-transfected with GST-KIF3A-Tail, GST-S690A-Tail, GST-S690D-Tail or GST in COS-7 cells. The lysates were subjected to GST pull-down assay and precipitated proteins were separated by SDS-PAGE gel followed by Western analysis using the indicated antibodies.

Chapter 4 Discussions

In the present work, we unveil a novel role for POPX2 in the regulation of KIF3 motor function. Herein we have identified KAP3 as an interacting protein of POPX2 and upregulation of POPX2 led to inhibition of KIF3-mediated intracellular transport. The phosphatase activity of POPX2 was found to be essential for this inhibitory role. In addition, we demonstrated that phosphorylation of the serine-690 residue in tail domain of KIF3A is critical to the KIF3 cargo transport function and CaMKII is found to phosphorylate this site.

4.1 POPX2 interaction with the KIF3 motor complex

KAP3, the accessory subunit of the KIF3 motor complex, has been reported to bind a vast variety of proteins (Jimbo et al., 2002; Lukong and Richard, 2008; Takeda et al., 2000; Tanuma et al., 2009; Teng et al., 2005). While some of these proteins are direct molecular cargoes of the KIF3 motor (fodrin, N-cadherin and APC), others appeared to be involved in regulating the KIF3 motor function (Dusp26 and PTK6). Here, we showed that KAP3 physically interacts with POPX2. Initial interaction was identified using the yeast-two hybrid system (Table 1) and subsequently, this interaction was verified by co-expression of the two proteins in the mammalian cells followed by GST pull-down assay (Figure 12). Endogenous KAP3 was found to co-immunoprecipitate with GFP-POPX2 in cell lysate of the POPX2 overexpressing-NIH3T3 stable cell line using anti-KAP3 antibody (Figure

21B). Hence, it is likely that POPX2 and KAP3 form a complex *in vivo*. Our data from the non-mammalian cell system (yeast-two hybrid) and the endogenous IP suggest that KAP3 is a *bona fide* POPX2 interacting protein.

KAP3 was also found to interact with POPX1, the longer isoform of POPX (Figure 13). Deletion mutant analysis of POPX1 revealed that the KAP3 binding domain falls within amino acid residue 236-269. Accordingly, deletion mutant analysis of POPX2 was carried out (Figure 14A). The KAP3 interacting region was mapped to amino acid residue 150-194 of POPX2. By combining the results obtained from the deletion mutant studies of both POPX1 and POPX2, we mapped the KAP3 interacting domain to a region of 34 amino acid residues (Figure 14C). This region resides within the N-terminal portion of the catalytic domain and is highly conserved between POPX1 and POPX2, with sequence identity of 59% (20 residues). Such interaction is not uncommon as CaMKII binds POPX2 on the C-terminal region of the catalytic domain (Harvey et al., 2004). In addition, 5 residues within the KAP binding region are also conserved in the PP2C α phosphatase. However, GST pull-down assay showed that there is no interaction between KAP3 and PP2C α (Figure 14B), suggesting that these residues are not essential for KAP3 binding and the interaction between KAP3 and POPX is specific. Further mutational analysis (site-directed mutagenesis) of the remaining 15 conserved residues would enable us to identify specific POPX residues that are critical for binding KAP3.

The domains on KAP3 responsible for POPX2 binding were mapped. Various KAP3 truncated mutants were tested for their ability to interact with POPX2 using GST pull-down assay (Figure 15). The armadillo (Arm) repeat regions

were found to be essential for the association between KAP3 and POPX2. The Arm repeats is well known to mediate protein-protein interaction, therefore, it is no surprise that KAP3 binds to POPX2 via this domain. Further dissection of the Arm repeat regions showed that POPX2 binds to ARM repeat region 1 and 3 but not region 2 (Figure 16). This is consistent with previous reports which showed that only part of the ARM repeats of KAP3 is required for interaction with its binding partner (Jimbo et al., 2002; Takeda et al., 2000).

The efficiency of binding of KAP3, β 1-PIX and mDia-DN to POPX2 was compared (Figure 17). In previous study, mDia-DN (residue 261-571), which contains the FH3 domain, was identified as a strong binding partner of POPX2 (Xie et al., 2008). In our experiment, mDia-DN also binds POPX2 with the highest efficiency. This may be due to the 'open' conformation of mDia-DN, which allows POPX2 to access the binding site easily. KAP3 and β 1-PIX bind POPX2 with comparable efficiencies. β 1-PIX binds POPX2 on the region spanning amino acids 1-191 (Koh et al, 2002) and this region overlap with the KAP3 binding domain (residue 160-193). Reciprocal precipitations of POPX2, KAP3 and β 1-PIX were performed to determine whether KAP3 and β 1-PIX compete for the same POPX2 binding site. Western analysis showed that POPX2 binds KAP3 and β 1-PIX respectively, and no interaction between KAP3 and β 1-PIX was observed. Furthermore, KAP3, POPX2 and β 1-PIX formed a complex, suggesting that KAP3 and β 1-PIX do not bind to the same region on POPX2. Even though a KAP3-POPX2- β 1-PIX trimeric complex was detected in the pull-down assay, it is uncertain if such protein complex exists physiologically. Reciprocal immunoprecipitation of the endogenous proteins would be required to determine if they form a complex *in vivo*.

The KIF3 motor complex is a heterotrimer consisting of two distinct but related motor subunits (KIF3A, KIF3B) and the non-motor subunit KAP3. KIF3A is generally considered as the essential motor subunit of the complex for the reason that heterodimers between KIF3A and KIF3B or KIF3C are found, but homodimers or heterodimers between KIF3B and KIF3C are not observed. In this study, we have determined if POPX2 also binds KIF3A (Figure 17, 19). When co-expressed in mammalian cells, POPX2 was detected in the immunoprecipitate of KIF3A. However, the efficiency of the interaction between KIF3A and POPX2 is lower compared to the interaction between KAP3 and POPX2. This implies that KIF3A may not be binding directly to POPX2. To better dissect the interactions between POPX2 and the KIF3 complex, *in vitro* binding assay was performed. KAP3 bound efficiently to bacterially expressed POPX2 while binding between KIF3A and the bacterially expressed POPX2 was poor, indicating that the interaction between KAP3 and POPX2 is direct.

We have shown in earlier experiments that POPX2 binds KAP3 on the Arm region 1 (residue 156-239) and 3 (residue 449-614). Sequence alignment of the various Arm domains in KAP3 revealed that several residues are conserved between the Arm 1 and Arm 3 regions. This may explain why POPX2 is able to interact with both the Arm domains.

KIF3A was reported to bind KAP3 on residues 528-614 (Haraguchi et al., 2006). To address whether KIF3A and POPX2 compete for the same binding site on KAP3, we co-expressed GST-POPX2, FLAG-KAP3 and HA-KIF3A in the COS-7 cells and subjected the lysates to FLAG-IP. We showed that the

three proteins formed a multimeric complex (Figure 20), thus implying that POPX2 and KIF3A bind to different regions of KAP3.

4.2 Tracking of the KIF3A motor subunit

Initial attempts to access the effects of POPX2 overexpression on KIF3 motor activity was carried out by examining the motility of the KIF3A motor itself. Imaging of live cells expressing fluorescent-tagged KIF3A protein was performed. However, we failed to obtain desirable results, as the KIF3A protein appeared diffused and tracking of single particle proved to be impossible (Figure 23). One might question whether addition of a fluorescent tag to the N-terminal region of KIF3A perturbs the motor function. However, immunofluorescence staining of NIH3T3 cells has demonstrated that N-terminal tagged GFP-KIF3B is able to localize to microtubules (Haraguchi et al., 2006). By inference, we believed that GFP-KIF3A also has the ability to bind microtubules. Furthermore, KIF3A/A homodimers have been shown to bind microtubules *in vitro*, and the homodimers exhibited processivity, which is defined as the ability to travel many steps along the filament track before dissociating (Howard et al., 1989; Zhang et al., 2004). Therefore, we propose that limitations in imaging techniques are probably the main reasons behind the failure of the experiments. Tracking of single kinesin particle in the cytosol of live cells is a challenging process due to cellular auto-fluorescence and freely diffusing fluorescence molecules. Nevertheless, successful tracking of single kinesin-I particle has been described (Cai et al, 2007). To overcome the

limitations of conventional imaging of fluorescent proteins, the authors have made the following improvements: 1) a constitutively active version of the kinesin heavy chain (KHC) was utilized. 2) The KHC was tagged with a three-tandem version of the fluorescent protein Citrine, a brighter and more photostable version of EYFP, which effectively reduced photo-bleaching. 3) Images were acquired using total internal reflection fluorescence microscopy (TIRFM). The shallow evanescent wave-excitation dramatically decreased the background and made imaging of individual fluorescent spots in the live cells possible.

As we were unable to access the dynamics of the KIF3A motor subunit efficiently in this study, we took an indirect approach by evaluating the effects of POPX2 overexpression on the trafficking of KIF3 cargoes: N-cadherin and Rab11. These proteins were expressed as defined vesicular structures in both fixed and live cell samples, thus, imaging and tracking of these molecules were less demanding.

4.3 POPX2 regulates N-cadherin and Rab11 trafficking

We have examined the localization and dynamics of N-cadherin in both fixed and live cells, respectively. Immunofluorescence staining revealed that localization of the endogenous N-cadherin to cell periphery and cell-cell contacts was reduced in POPX2 overexpressing cells (Figure 24, 26). Detailed studies on the intracellular trafficking of N-cadherin done by Rouviere and

colleagues have indicated a link between cell-cell contact formation and N-cadherin dynamics (Mary et al., 2002). The authors defined movement from the Golgi region to the plasma membrane as “centrifugal” and movement from the plasma membrane to the Golgi as “centripetal”. They reported that in isolated cells, vast centripetal and centrifugal movement of N-cadherin-GFP vesicles was observed. When cells establish early contacts, the proportion of centrifugal flow tends to rise while centripetal flow diminishes. Recruitment of N-cadherin from the cytoplasm to plasma membrane is almost absent in fully contacting cells where strong localization of N-cadherin is seen at intercellular junctions. In our study, tracking of single N-cadherin-GFP vesicles in isolated and early contacting cells, showed that trafficking of N-cadherin to cell periphery is significantly inhibited by the overexpression of mCherry-POPX2 (Figure 27). By contrast, massive centrifugal and centripetal motions were observed in cells overexpressing the mCherry vector. Vesicles were also seen to move in straight, outward manner, corresponding to KIF3-mediated anterograde transport. Our results were comparable to those described by the Rouviere’s group (Mary et al., 2002). Furthermore, by utilizing the POPX2 phosphatase-dead mutant, POPX2m, we found that the phosphatase activity of POPX2 is indispensable for the inhibition on N-cadherin trafficking. The POPX2m mutant harbors two single-residue mutations at R326A and I328A, which render it phosphatase-inactive (Koh et al., 2002).

Our observations on the effect of POPX2 overexpression on N-cadherin trafficking were similar to the phenotypes reported in the KAP3^{-/-} knockout cells; the authors have shown that localization of N-cadherin to cell periphery was disrupted in the KAP3-depleted cells (Teng et al., 2005). Their findings

support our hypothesis where POPX2 exerts negative effect on the KIF3 motor complex, which ultimately led to the disruption of N-cadherin subcellular localization. Using cell-cell aggregation assay, we further showed that the POPX2 overexpressing cells were unable to form large cell aggregates (Figure 28), demonstrating a physiological consequence of the loss of N-cadherin accumulation at intercellular junctions. Our finding might also be pertinent to the migration properties of POPX2 overexpressing fibroblasts. We have shown in earlier publication that NIH3T3 cells which overexpress GFP-POPX2 (X2 cells) migrated faster in the scratch-wound assay (Singh et al., 2011). Interestingly, we have also noted that while migration of the control NIH3T3 cells was maintained as a collective wavefront, the X2 cells migrated in a more random and isolated manner (Hoon and Koh, personal communication; Singh and Koh, personal communication). Similar results were reported in a recent study which demonstrated that downregulation of N-cadherin at cell-cell contacts influenced NIH3T3 cell migration when presented with geometric constrains (Mills et al., 2011). NIH3T3 cells that lost the ability to form N-cadherin-mediated intercellular junctions, no longer move as a collective wavefront, instead, they moved irregularly and independently from one another. This switch in migration characteristic enables the cells to migrate faster in the smaller channels.

To further verify if the effects of POPX2 overexpression on N-cadherin are mediated via direct interaction with KAP3, we have attempted to knockdown endogenous KAP3 in the NIH3T3 with siRNAs (Figure 29). Nevertheless, the siRNA failed to downregulate KAP3 to a significant level. Additional optimization of the siRNA transfection protocol is necessary. Since the

transfection efficiency of the NIH3T3 cells using liposomal method is relatively low (~40-50%), electroporation or viral infection could be used. Alternatively, new siRNA targeting to other regions of *kap3* could be constructed. Otherwise, a KAP3 dominant-negative mutant that binds potently to POPX2 but not to the KIF3A/B motors, could be overexpressed in the X2 cells to counteract the effect of POPX2 on the KIF3 complex.

The effect of POPX2 overexpression on KIF3 cargo binding was studied. We found that overexpression of POPX2 did not disrupt binding between KAP3 and N-cadherin (Figure 25) nor did it disrupt binding between KIF3A and KAP3 (Figure 20). Hence, we hypothesized that POPX2 influences the localization of N-cadherin by acting directly on the motor.

To further substantiate the negative influence of POPX2 on KIF3 motor function, we have examined the subcellular localization and dynamics of an alternative cargo of KIF3: Rab11a. Rab11 belongs to the family of small Ras-like GTPases. It is a protein marker for recycling endosomes and plays important role in regulating membrane trafficking from the endosomal recycling compartment to the plasma membrane. Rab11a, Rab11b and Rab11c are the three major members of the Rab11 GTPases. Like other Rab proteins, Rab11 exerts its function via interactions with the Rab11 family interacting proteins (FIPs). There are five members of this protein family, namely RCP (Rab-coupling protein), FIP2, FIP3, FIP4 and Rip11. One distinct function of the FIPs is to link the Rab11 GTPases to molecular motors of the cell. While FIP3 links Rab11a to the minus-end directed dynein motor (Horgan et al., 2010), Rip11 links Rab11a to the plus-end directed KIF3 motor (Schonteich et

al., 2008). Rab11a-Rip11 forms a complex with the KIF3 motor mainly through interaction with KIF3A and KIF3B. Although accumulation of some dsRed-Rab11a vesicles was seen around perinuclear region in the control cells, a significant increase in clustering of Rab11a vesicles in the perinuclear region was observed when we overexpressed POPX2 (Figure 30). We interpreted this result as a consequence of impaired trafficking of the Rab11 vesicles to the plasma membrane. Indeed, when visualized under time-lapse microscopy, most of the dsRed-Rab11a vesicles in the POPX2 overexpressing cells exhibited low motility and failed to translocate from the perinuclear region to the cell periphery (Figure 31). Again, the POPX2 phosphatase activity proved to be essential as overexpression of the phosphatase-dead mutant had little effect on Rab11 motility. The POPX2 function on trafficking reveals high degree of specificity for Rab11 because trafficking of another endosomal protein, the clathrin-LCA, was not perturbed by the overexpression of POPX2 (Figure 32). Collectively, these results suggest that POPX2 overexpression affects N-cadherin and Rab11 localization via negative regulation of the KIF3 complex.

4.4 Phosphorylation of KIF3A serine-690 may contribute to motor function

The mechanisms by which kinesins transport their molecular cargoes to specific subcellular locations and how this transport is regulated are not clearly understood. Generally, one tier of regulation involves modulation of the binding of kinesins to microtubule tracks; phosphorylation of kinesin-1 (serine-176) in the motor domain has been shown to reduce the ability of kinesin to

bind microtubules and inhibit cargo transport (Morfini et al., 2009). A second tier of control involves regulation of cargo-motor association. Phosphorylation of residues in the C-terminal tail region of kinesins has been shown to either promote or inhibit cargo binding. For instance, phosphorylation of kinesin light chain 2 on serine-575 stimulates binding of the 14-3-3 cargo protein (Ichimura et al., 2002), whereas phosphorylation of KIF17 on serine-1029 disrupts binding of Mint1 to the tail domain of the motor (Guillaud, Wong, and Hirokawa, 2008). Finally, a fundamental control of kinesin function comes from autoinhibition. Soluble kinesins are found to assume a folded, inactive conformation, where the C-terminal region interacts with the motor domain, leading to inhibition of ATPase activity and the ability to bind microtubule (Friedman and Vale, 1999; Stock et al., 1999). This interaction is thought to be relieved by high ionic strength or binding of molecular cargoes, which generates an extended, active conformation. In both Kinesin-I and Kinesin-II, an increase in salt concentration has been shown to induce a conformational change from folded to extended (Hackney et al., 1992; Wedaman et al., 1996). On the other hand, binding of cargo could also result in activation of Kinesin-I and KIF17 (Blasius et al., 2007; Hammond et al., 2010). Nonetheless, cargo binding may not be the complete answer to motor activation. Kinesin-I motor that are attached to membrane cargo can still be inactive (Wozniak and Allan, 2006). Instead, phosphorylation has been shown to play important role in relieving kinesin autoinhibition. In the first instance, phosphorylation of threonine-937 of KIF11 (Eg5) in the inhibitory C-terminal tail increases the efficiency of microtubule binding (Cahu et al., 2008). In the second example, phosphorylation of the inhibitory C-terminal tail of Kinesin-7 causes the motor

to unfold and acquire higher processivity along microtubules (Espeut et al., 2008). In the current study, we demonstrated that preclusion of phosphorylation on KIF3A serine-690 impaired the localization and trafficking of N-cadherin and Rab11 (Figure 34-36). This mutation, however, does not significantly affect cargo-motor binding (Figure 37). Having this in mind, and given the fact that the serine-690 residue resides at the C-terminal tail domain and that the KIF3 complex has been reported to adopt a folded conformation (Wedaman et al., 1996), our data suggest that phosphorylation of KIF3A on serine-690 may be important for relieving autoinhibition. We further show in a binding assay that the tail domain of KIF3A indeed interacts with the motor domain (Figure 41). Unexpectedly, mutation of the serine-690 residue to an aspartic acid fails to eliminate the interaction. One possible explanation is that truncation of the KIF3A protein exposed residues that are normally inaccessible, leading to non-regulated binding. Another reason could be the involvement of the hinge region. The KIF17 motor, a member of the kinesin II family, is reported to be autoinhibited by interaction of the motor domain with both the tail domain and coiled-coil region (Hammond et al., 2010). With regards to the inhibitory tail domain (residue 847-1039), residues that contribute to the direct interaction with the motor domain were not identified. In the case of the inhibitory coiled-coil segment (residue 739-846), glycine-754 and residues spanning 762-772 are involved in the autoinhibition. Based on these findings, it is reasonable to speculate that the both the coiled-coil region (residue 355-600) and the tail domain (residue 601-702) of KIF3A may be required for autoinhibition of the KIF3 motor.

4.5 KIF3A is a potential substrate of CaMKII and POPX2

In the search for potential kinase that is responsible for phosphorylating KIF3A on serine-690, we have identified CaMKII as a promising candidate. The minimal recognition motif for phosphorylation by CaMKII is RXXS/T where X can be any amino acid residue (Pearson et al., 1985). As shown in Figure 33, the amino acid sequence RKRS in the C-terminal tail of KIF3A fits the consensus sequence for CaMKII. To verify if KIF3A is a genuine substrate of CaMKII, we generated the recombinant GST-KIF3A-Tail protein in the *E.coli* cells and subjected it to *in vitro* kinase assay. Since site-specific phospho-antibodies for KIF3A were not available, we detected phospho-immunoreactive bands using anti-phospho-Ser/Thr antibody. We first tested the sensitivity and specificity of the antibody by subjecting the active and inactive versions of CaMKII to detection by the anti-phospho-Ser/Thr antibody. Activated CaMKII undergoes dramatic autophosphorylation, thus, serving as a good control. As illustrated by the Ponceau S staining and Western blotting of the total CaMKII protein, the activated CaMKII band showed an upward mobility shift on SDS-PAGE (Figure 39B, C). Correspondingly, the anti-phospho-Ser/Thr antibody detected a strong immunoreactive band in the activated CaMKII sample, while no signal was detected for the inactive CaMKII sample. We then used the antibody to detect for immunoreactive band of phospho-GSt-KIF3A-Tail. Active CaMKII potently phosphorylates GST-KIF3A-Tail, while active PAK1 (phosphorylated PAK was detected, Figure 39C), which serves as a random kinase control, failed to phosphorylate GST-KIF3A-Tail (Figure 39C). No phosphorylation was observed in the inactive CaMKII or the GST-S690A-Tail

samples, demonstrating that active CaMKII phosphorylates KIF3A specifically on the serine-690 residue. This finding implicates the involvement of calcium in the regulation of kinesin-mediated transport.

We have also verified that KIF3A is phosphorylated *in vivo* by overexpressing full-length FLAG-KIF3A in COS-7 cells and subjecting the lysates to FLAG-IP and Western analysis using anti-phospho-Ser/Thr antibodies. In consistent with the phosphoproteomics-based data that report the presence of phospho-KIF3A in cells under physiological conditions (Ballif et al., 2004; Dephoure et al., 2008; Huttlin et al., 2010; Olsen et al., 2010; Pan et al., 2008; Tweedie-Cullen, Reck, and Mansuy, 2009; Wisniewski et al., 2010), we have detected a significant amount of phosphorylated FLAG-KIF3A in the COS-7 cells. Notable phosphorylation was also observed in the FLAG-KIF3A-S690A mutant, indicating that alternative phosphorylation sites were present and other serine/threonine kinases might be acting on KIF3A. Protein sequence analysis of KIF3A reveals that additional CaMKII motifs (RXXS) are present. One motif lies in the motor domain and the other in the coiled-coil domain. It would be interesting to verify if CaMKII phosphorylates KIF3A at these sites too. Furthermore, *in vitro* phosphatase assay has shown that the POPX2 phosphatase is able to directly dephosphorylate the CaMKII-phosphorylated GST-KIF3A-Tail (Figure 40). This finding supports earlier experiments that suggest the requirement of POPX2 phosphatase activity in regulation of KIF3 cargo transport (Figure 26, 27, 31).

4.6 Proposed model for the regulation of KIF3 motor function by POPX2

Kinesin motors are found to cycle between active and inactive form; phosphorylation and dephosphorylation represent a key mechanism for regulation of motor activity (Verhey and Rapoport, 2001). In this study, we demonstrated the possible involvement of both kinase and phosphatase in regulation of kinesin function. We speculate that interaction with and phosphorylation by CaMKII may be one of the mechanisms for relieving autoinhibition of the KIF3 motor. On the contrary, dephosphorylation of KIF3A by POPX2, confines the motor in the autoinhibited state (Figure 42).

One remarkable feature of the KIF3 kinesin that distinguish it from other kinesins, is the presence of two different motor domains, KIF3A and KIF3B. The physiological significance of having two different motor subunits is still unclear. Phosphoproteomics analysis has indicated that the mouse KIF3B is phosphorylated on serine-181 and serine-681 (Huttlin et al., 2010; Wisniewski et al., 2010). Serine-181 is located in the motor domain while serine-681 resides at the tail domain. Whether POPX2 regulates phosphorylation of these two sites remains uncertain. In addition, consensus sequence for CaMKII is not found within these sites. However, it might be possible that phosphorylation of both KIF3A and KIF3B is required for full activation of KIF3 motor activity. Consequently, inhibition of merely one phosphorylation site might not totally abrogate the motor function. This may partly explain why the KIF3A-S690A mutant is able to reduce but not fully inhibit the trafficking of the KIF3 cargoes.

Moreover, interactions of the hinge regions with the motor domains may also contribute to the autoinhibition of the kinesin complex.

Figure 42. A schematic representing a possible mechanism by which POPX2 negatively regulates the KIF3 motor complex. The KIF3 motor exists as a heterotrimer consisting of two motor subunits, KIF3A and KIF3B, and a third non-motor subunit, KAP3. We propose that phosphorylation on KIF3A serine-690 by CaMKII is necessary for activation of KIF3 motor function. Even though KIF3B is shown to be phosphorylated in the motor domain and C-terminal tail, the functional significance of these phospho-sites remain obscured. On the other hand, overexpression of POPX2 might preclude phosphorylation of KIF3A on serine-690, and this might be one of the mechanisms to retain the motor in the inactive state. The illustrations shown are not drawn to scale.

Chapter 5 Conclusions and future perspectives

Studies over the last decade have established a pivotal role for the POPX2 phosphatase in the control of various cellular processes. Through regulation of the Rho-GTPase-mediated signaling pathways, POPX2 exerts an effect on the actin cytoskeleton (Koh et al., 2002; Xie et al., 2008). The phosphatase has also been shown to contribute to the motility and invasiveness of breast cancer cells (Susila et al., 2010). Using the zebra fish model, an essential function of POPX2 in embryonic development has also been established (Sueyoshi et al., 2009). Recent phosphoproteomic-based analysis has also demonstrated the involvement of POPX2 in the regulation of the master kinase GSK3, which in turn modulates extracellular signal-regulated kinase and cell motility (Singh et al., 2011). The data presented in this thesis further characterized the physiological roles of POPX2. Using the yeast-two hybrid assay, KAP3, the accessory subunit of the KIF3 motor complex, was identified as a binding partner of POPX2. This interaction was further validated in mammalian cells, and POPX2 was shown to form a multimeric complex consisting of KAP3 and KIF3A.

Results of biochemical and functional analysis provided preliminary evidences for POPX2 as a negative regulator of the KIF3 motor complex. Overexpression of POPX2 has led to mislocalization of the KIF3 cargoes; a likely consequence of impaired intracellular transport. KIF3A has previously been identified as a phosphoprotein by mass spectrometry-based experiments. In this study, we

further confirmed the presence of phospho-KIF3A by biochemical method and have indicated an implication of the phosphorylation. Here, we show that preclusion of phosphorylation on KIF3A serine-690, resulted in disruption of KIF3 cargo transport. The defects in cargo trafficking of KIF3A-S690A mutant are similar to those observed in POPX2 overexpressing cells. We proposed that phosphorylation of this site might contribute, to some extent, to the relief of autoinhibition on KIF3 motor. Lastly, we have showed that CaMKII phosphorylates KIF3A on serine-690 and POPX2 is able to dephosphorylate this site.

In summary, findings from our present study not only deepen our knowledge on the biological functions of the POPX2 phosphatase, they also provide new mechanistic insights into the regulation of the KIF3 motor. POPX2 is likely to be a regulator of both actin and microtubule-mediated transport. Although our initial work provided a putative model for POPX2 in the regulation of KIF3 motor activity, additional work is required to truly elucidate the mechanism of regulation.

We have shown in this study that exogenous POPX2, KAP3 and KIF3A form a trimeric protein complex; however, a reciprocal immunoprecipitation of the respective endogenous proteins would be necessary to ascertain the existence of the protein complex under physiological conditions.

Even though we have demonstrated that overexpression of POPX2 perturbs the localization of the KIF3 molecular cargoes, N-cadherin and Rab11, it offers only indirect evidence that POPX2 is regulating the KIF3 motor function. Single particle tracking of KIF3A would allow a more direct monitoring of the

effects of POPX2 on the KIF3 motor. *In vitro* microtubule binding assay can also be carried out with purified KIF3 proteins to determine if CaMKII or POPX2 affect the interaction between the kinesin motor and the microtubules.

The mode of autoinhibition of the KIF3 motor complex is not fully understood. Results from the present study seem to suggest that the KIF3 motor might be autoinhibited through interaction of the motor domain with the coiled-coil and tail domains. Further biochemical studies should be performed to confirm the involvement of the coiled-coil region. Structural data on the conformation of active and inactive KIF3 motor complex will greatly facilitate our understanding on the mode of autoinhibition. Therefore, it would be essential to solve the X-ray crystallographic structure of the KIF3 motor so that the protein complex can be analyzed at the atomic level.

Finally, we have focused our analysis on the more important motor subunit (KIF3A) of the KIF3 complex. Nevertheless, the role of KIF3B in the general regulation of the motor complex should also be elucidated. Whether KIF3B binds POPX, whether it undergoes phospho-regulation, what are the kinases and phosphatases involved and how it contributes to the autoinhibition of the motor complex, are issues that need to be addressed.

References

- Arias-Romero, L. E., and Chernoff, J. (2008). A tale of two Paks. *Biology of the cell / under the auspices of the European Cell Biology Organization* **100**(2), 97-108.
- Bagrodia, S., Taylor, S. J., Jordon, K. A., Van Aelst, L., and Cerione, R. A. (1998). A novel regulator of p21-activated kinases. *The Journal of biological chemistry* **273**(37), 23633-6.
- Ballif, B. A., Villen, J., Beausoleil, S. A., Schwartz, D., and Gygi, S. P. (2004). Phosphoproteomic analysis of the developing mouse brain. *Molecular & cellular proteomics : MCP* **3**(11), 1093-101.
- Bananis, E., Nath, S., Gordon, K., Satir, P., Stockert, R. J., Murray, J. W., and Wolkoff, A. W. (2004). Microtubule-dependent movement of late endocytic vesicles in vitro: requirements for Dynein and Kinesin. *Molecular biology of the cell* **15**(8), 3688-97.
- Blasius, T. L., Cai, D., Jih, G. T., Toret, C. P., and Verhey, K. J. (2007). Two binding partners cooperate to activate the molecular motor Kinesin-1. *The Journal of cell biology* **176**(1), 11-7.
- Bokoch, G. M. (2003). Biology of the p21-activated kinases. *Annual review of biochemistry* **72**, 743-81.
- Bokoch, G. M., Reilly, A. M., Daniels, R. H., King, C. C., Olivera, A., Spiegel, S., and Knaus, U. G. (1998). A GTPase-independent mechanism of p21-activated kinase activation. Regulation by sphingosine and other biologically active lipids. *The Journal of biological chemistry* **273**(14), 8137-44.
- Cahu, J., Olichon, A., Hentrich, C., Schek, H., Drinjakovic, J., Zhang, C., Doherty-Kirby, A., Lajoie, G., and Surrey, T. (2008). Phosphorylation by Cdk1 increases the binding of Eg5 to microtubules in vitro and in Xenopus egg extract spindles. *PloS one* **3**(12), e3936.
- Cai, D., Verhey, K.J., Meyhofer, E., 2007. Tracking single Kinesin molecules in the cytoplasm of mammalian cells. *Biophysical journal* **92**, 4137-4144.
- Castano, J., Raurell, I., Piedra, J. A., Miravet, S., Dunach, M., and Garcia de Herreros, A. (2002). Beta-catenin N- and C-terminal tails modulate the coordinated binding of adherens junction proteins to beta-catenin. *The Journal of biological chemistry* **277**(35), 31541-50.
- Chen, W., Lah, M., Robinson, P. J., and Kemp, B. E. (1994). Phosphorylation of phospholamban in aortic smooth muscle cells and heart by calcium/calmodulin-dependent protein kinase II. *Cellular signalling* **6**(6), 617-30.

- Chen, X., Kojima, S., Borisy, G. G., and Green, K. J. (2003). p120 catenin associates with kinesin and facilitates the transport of cadherin-catenin complexes to intercellular junctions. *The Journal of cell biology* **163**, 547-57.
- Choi, C. K., Vicente-Manzanares, M., Zareno, J., Whitmore, L. A., Mogilner, A., and Horwitz, A. R. (2008). Actin and alpha-actinin orchestrate the assembly and maturation of nascent adhesions in a myosin II motor-independent manner. *Nature cell biology* **10**(9), 1039-50.
- Choi, H. J., Huber, A. H., and Weis, W. I. (2006). Thermodynamics of beta-catenin-ligand interactions: the roles of the N- and C-terminal tails in modulating binding affinity. *The Journal of biological chemistry* **281**(2), 1027-38.
- Chong, C., Tan, L., Lim, L., and Manser, E. (2001). The mechanism of PAK activation. Autophosphorylation events in both regulatory and kinase domains control activity. *The Journal of biological chemistry* **276**(20), 17347-53.
- Critchley, D. R. (2004). Cytoskeletal proteins talin and vinculin in integrin-mediated adhesion. *Biochem Soc Trans* **32**(Pt 5), 831-6.
- Dephoure, N., Zhou, C., Villen, J., Beausoleil, S. A., Bakalarski, C. E., Elledge, S. J., and Gygi, S. P. (2008). A quantitative atlas of mitotic phosphorylation. *Proceedings of the National Academy of Sciences of the United States of America* **105**(31), 10762-7.
- Easley, C. A. t., Brown, C. M., Horwitz, A. F., and Tombes, R. M. (2008). CaMK-II promotes focal adhesion turnover and cell motility by inducing tyrosine dephosphorylation of FAK and paxillin. *Cell motility and the cytoskeleton* **65**(8), 662-74.
- El Sayegh, T. Y., Arora, P. D., Fan, L., Laschinger, C. A., Greer, P. A., McCulloch, C. A., and Kapus, A. (2005). Phosphorylation of N-cadherin-associated cortactin by Fer kinase regulates N-cadherin mobility and intercellular adhesion strength. *Molecular biology of the cell* **16**(12), 5514-27.
- El Sayegh, T. Y., Arora, P. D., Laschinger, C. A., Lee, W., Morrison, C., Overall, C. M., Kapus, A., and McCulloch, C. A. (2004). Cortactin associates with N-cadherin adhesions and mediates intercellular adhesion strengthening in fibroblasts. *Journal of cell science* **117**(Pt 21), 5117-31.
- Espeut, J., Gaussen, A., Bieling, P., Morin, V., Prieto, S., Fesquet, D., Surrey, T., and Abrieu, A. (2008). Phosphorylation relieves autoinhibition of the kinetochore motor Cenp-E. *Molecular cell* **29**(5), 637-43.
- Etienne-Manneville, S., and Hall, A. (2002). Rho GTPases in cell biology. *Nature* **420**(6916), 629-35.
- Friedman, D. S., and Vale, R. D. (1999). Single-molecule analysis of kinesin motility reveals regulation by the cargo-binding tail domain. *Nature cell biology* **1**(5), 293-7.
- Galbraith, C. G., Yamada, K. M., and Galbraith, J. A. (2007). Polymerizing actin fibers position integrins primed to probe for adhesion sites. *Science* **315**(5814), 992-5.

- Gatti, A., Huang, Z., Tuazon, P. T., and Traugh, J. A. (1999). Multisite autophosphorylation of p21-activated protein kinase gamma-PAK as a function of activation. *The Journal of biological chemistry* **274**(12), 8022-8.
- Geller, R. L., and Lilien, J. (1983). Repair of a calcium-dependent adhesive mechanism of embryonic neural retina cells: kinetic and molecular analysis. *Journal of cell science* **60**, 29-49.
- Geneste, O., Copeland, J. W., and Treisman, R. (2002). LIM kinase and Diaphanous cooperate to regulate serum response factor and actin dynamics. *The Journal of cell biology* **157**(5), 831-8.
- Guillaud, L., Wong, R., and Hirokawa, N. (2008). Disruption of KIF17-Mint1 interaction by CaMKII-dependent phosphorylation: a molecular model of kinesin-cargo release. *Nature cell biology* **10**(1), 19-29.
- Gumbiner, B. M. (1996). Cell adhesion: the molecular basis of tissue architecture and morphogenesis. *Cell* **84**(3), 345-57.
- Hackney, D. D., Levitt, J. D., and Suhan, J. (1992). Kinesin undergoes a 9 S to 6 S conformational transition. *The Journal of biological chemistry* **267**(12), 8696-701.
- Hall, A. (1998). Rho GTPases and the actin cytoskeleton. *Science* **279**(5350), 509-14.
- Hammond, J. W., Blasius, T. L., Soppina, V., Cai, D., and Verhey, K. J. (2010). Autoinhibition of the kinesin-2 motor KIF17 via dual intramolecular mechanisms. *The Journal of cell biology* **189**(6), 1013-25.
- Haraguchi, K., Hayashi, T., Jimbo, T., Yamamoto, T., and Akiyama, T. (2006). Role of the kinesin-2 family protein, KIF3, during mitosis. *The Journal of biological chemistry* **281**(7), 4094-9.
- Hart, M. J., de los Santos, R., Albert, I. N., Rubinfeld, B., and Polakis, P. (1998). Downregulation of beta-catenin by human Axin and its association with the APC tumor suppressor, beta-catenin and GSK3 beta. *Current biology* **8**(10), 573-81.
- Hart, M. J., Eva, A., Zangrilli, D., Aaronson, S. A., Evans, T., Cerione, R. A., and Zheng, Y. (1994). Cellular transformation and guanine nucleotide exchange activity are catalyzed by a common domain on the dbl oncogene product. *The Journal of biological chemistry* **269**(1), 62-5.
- Harvey, B. P., Banga, S. S., and Ozer, H. L. (2004). Regulation of the multifunctional Ca²⁺/calmodulin-dependent protein kinase II by the PP2C phosphatase PPM1F in fibroblasts. *The Journal of biological chemistry* **279**(23), 24889-98.
- Hatta, K., Takeichi, M., 1986. Expression of N-cadherin adhesion molecules associated with early morphogenetic events in chick development. *Nature* **320**, 447-449.
- Hoelz, A., Nairn, A. C., and Kuriyan, J. (2003). Crystal structure of a tetradecameric assembly of the association domain of Ca²⁺/calmodulin-dependent kinase II. *Molecular cell* **11**(5), 1241-51.

- Horgan, C. P., Hanscom, S. R., Jolly, R. S., Futter, C. E., and McCaffrey, M. W. (2010). Rab11-FIP3 links the Rab11 GTPase and cytoplasmic dynein to mediate transport to the endosomal-recycling compartment. *Journal of cell science* **123**(Pt 2), 181-91.
- Howard, J., Hudspeth, A.J., Vale, R.D., 1989. Movement of microtubules by single kinesin molecules. *Nature* **342**, 154-158.
- Huber, A. H., Nelson, W. J., and Weis, W. I. (1997). Three-dimensional structure of the armadillo repeat region of beta-catenin. *Cell* **90**(5), 871-82.
- Huber, A. H., Stewart, D. B., Laurents, D. V., Nelson, W. J., and Weis, W. I. (2001). The cadherin cytoplasmic domain is unstructured in the absence of beta-catenin. A possible mechanism for regulating cadherin turnover. *The Journal of biological chemistry* **276**(15), 12301-9.
- Huttlin, E. L., Jedrychowski, M. P., Elias, J. E., Goswami, T., Rad, R., Beausoleil, S. A., Villen, J., Haas, W., Sowa, M. E., and Gygi, S. P. (2010). A tissue-specific atlas of mouse protein phosphorylation and expression. *Cell* **143**(7), 1174-89.
- Ichimura, T., Wakamiya-Tsuruta, A., Itagaki, C., Taoka, M., Hayano, T., Natsume, T., and Isobe, T. (2002). Phosphorylation-dependent interaction of kinesin light chain 2 and the 14-3-3 protein. *Biochemistry* **41**(17), 5566-72.
- Imamura, T., Huang, J., Usui, I., Satoh, H., Bever, J., and Olefsky, J. M. (2003). Insulin-induced GLUT4 translocation involves protein kinase C-lambda-mediated functional coupling between Rab4 and the motor protein kinesin. *Molecular and cellular biology* **23**(14), 4892-900.
- Ishida, A., Kameshita, I., and Fujisawa, H. (1998). A novel protein phosphatase that dephosphorylates and regulates Ca²⁺/calmodulin-dependent protein kinase II. *The Journal of biological chemistry* **273**(4), 1904-10.
- Iwai, S., Yonekawa, A., Harada, C., Hamada, M., Katagiri, W., Nakazawa, M., and Yura, Y. (2010). Involvement of the Wnt-beta-catenin pathway in invasion and migration of oral squamous carcinoma cells. *International journal of oncology* **37**(5), 1095-103.
- Jaffer, Z. M., and Chernoff, J. (2002). p21-activated kinases: three more join the Pak. *The international journal of biochemistry & cell biology* **34**(7), 713-7.
- Jang, Y. N., Jung, Y. S., Lee, S. H., Moon, C. H., Kim, C. H., and Baik, E. J. (2009). Calpain-mediated N-cadherin proteolytic processing in brain injury. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **29**(18), 5974-84.
- Jimbo, T., Kawasaki, Y., Koyama, R., Sato, R., Takada, S., Haraguchi, K., and Akiyama, T. (2002). Identification of a link between the tumour suppressor APC and the kinesin superfamily. *Nature cell biology* **4**(4), 323-7.

- Kabsch, W., Mannherz, H. G., Suck, D., Pai, E. F., and Holmes, K. C. (1990). Atomic structure of the actin:DNase I complex. *Nature* **347**(6288), 37-44.
- Kanaseki, T., Ikeuchi, Y., Sugiura, H., and Yamauchi, T. (1991). Structural features of Ca²⁺/calmodulin-dependent protein kinase II revealed by electron microscopy. *The Journal of cell biology* **115**(4), 1049-60.
- Kawauchi, T., Sekine, K., Shikanai, M., Chihama, K., Tomita, K., Kubo, K., Nakajima, K., Nabeshima, Y., and Hoshino, M. (2010). Rab GTPases-dependent endocytic pathways regulate neuronal migration and maturation through N-cadherin trafficking. *Neuron* **67**(4), 588-602.
- Keil, R., Kiessling, C., and Hatzfeld, M. (2009). Targeting of p0071 to the midbody depends on KIF3. *Journal of cell science* **122**(Pt 8), 1174-83.
- Kitani, T., Ishida, A., Okuno, S., Takeuchi, M., Kameshita, I., and Fujisawa, H. (1999). Molecular cloning of Ca²⁺/calmodulin-dependent protein kinase phosphatase. *Journal of biochemistry* **125**(6), 1022-8.
- Knaus, U. G., Morris, S., Dong, H. J., Chernoff, J., and Bokoch, G. M. (1995). Regulation of human leukocyte p21-activated kinases through G protein--coupled receptors. *Science* **269**(5221), 221-3.
- Koh, C. G., Manser, E., Zhao, Z. S., Ng, C. P., and Lim, L. (2001). Beta1PIX, the PAK-interacting exchange factor, requires localization via a coiled-coil region to promote microvillus-like structures and membrane ruffles. *Journal of cell science* **114**(Pt 23), 4239-51.
- Koh, C. G., Tan, E. J., Manser, E., and Lim, L. (2002). The p21-activated kinase PAK is negatively regulated by POPX1 and POPX2, a pair of serine/threonine phosphatases of the PP2C family. *Current biology* **12**(4), 317-21.
- Kozma, R., Ahmed, S., Best, A., and Lim, L. (1995). The Ras-related protein Cdc42Hs and bradykinin promote formation of peripheral actin microspikes and filopodia in Swiss 3T3 fibroblasts. *Molecular and cellular biology* **15**(4), 1942-52.
- Kovacs, E.M., Goodwin, M., Ali, R.G., Paterson, A.D., Yap, A.S., 2002. Cadherin-directed actin assembly: E-cadherin physically associates with the Arp2/3 complex to direct actin assembly in nascent adhesive contacts. *Current Biology* **12**, 379-382.
- Kumar, R., Gururaj, A. E., and Barnes, C. J. (2006). p21-activated kinases in cancer. *Nature review cancer* **6**(6), 459-71.
- Lambert, M., Thoumine, O., Brevier, J., Choquet, D., Riveline, D., and Mege, R. M. (2007). Nucleation and growth of cadherin adhesions. *Experimental cell research* **313**(19), 4025-40.
- Lawrence, C. J., Dawe, R. K., Christie, K. R., Cleveland, D. W., Dawson, S. C., Endow, S. A., Goldstein, L. S., Goodson, H. V., Hirokawa, N., Howard, J., Malmberg, R. L., McIntosh, J. R., Miki, H., Mitchison, T. J., Okada, Y., Reddy, A. S., Saxton, W. M., Schliwa, M., Scholey, J. M., Vale, R. D., Walczak, C. E., and Wordeman, L.

- (2004). A standardized kinesin nomenclature. *The Journal of cell biology* **167**(1), 19-22.
- Lei, M., Lu, W., Meng, W., Parrini, M. C., Eck, M. J., Mayer, B. J., and Harrison, S. C. (2000). Structure of PAK1 in an autoinhibited conformation reveals a multistage activation switch. *Cell* **102**(3), 387-97.
- Lilien, J., and Balsamo, J. (2005). The regulation of cadherin-mediated adhesion by tyrosine phosphorylation/dephosphorylation of beta-catenin. *Current opinion in cell biology* **17**(5), 459-65.
- Lisman, J., Schulman, H., and Cline, H. (2002). The molecular basis of CaMKII function in synaptic and behavioural memory. *Nature reviews. Neuroscience* **3**(3), 175-90.
- Loo, T. H., Ng, Y. W., Lim, L., and Manser, E. (2004). GIT1 activates p21-activated kinase through a mechanism independent of p21 binding. *Molecular and cellular biology* **24**(9), 3849-59.
- Lukong, K. E., and Richard, S. (2008). Breast tumor kinase BRK requires kinesin-2 subunit KAP3A in modulation of cell migration. *Cellular signalling* **20**(2), 432-42.
- Lupas, A., Van Dyke, M., and Stock, J. (1991). Predicting coiled coils from protein sequences. *Science* **252**(5009), 1162-4.
- Manser, E. (2005). "Rho Family GTPases." Springer, Netherlands.
- Manser, E., Leung, T., Salihuddin, H., Zhao, Z. S., and Lim, L. (1994). A brain serine/threonine protein kinase activated by Cdc42 and Rac1. *Nature* **367**(6458), 40-6.
- Manser, E., Loo, T. H., Koh, C. G., Zhao, Z. S., Chen, X. Q., Tan, L., Tan, I., Leung, T., and Lim, L. (1998). PAK kinases are directly coupled to the PIX family of nucleotide exchange factors. *Molecular cell* **1**(2), 183-92.
- Mary, S., Charrasse, S., Meriane, M., Comunale, F., Travo, P., Blangy, A., and Gauthier-Rouviere, C. (2002). Biogenesis of N-cadherin-dependent cell-cell contacts in living fibroblasts is a microtubule-dependent kinesin-driven mechanism. *Molecular biology of the cell* **13**(1), 285-301.
- Mills, R. J., Frith, J. E., Hudson, J. E., and Cooper-White, J. J. (2011). Effect of Geometric Challenges on Cell Migration. *Tissue engineering. Part C, Methods*.
- Mitchison, T., and Kirschner, M. (1984). Dynamic instability of microtubule growth. *Nature* **312**(5991), 237-42.
- Morfini, G. A., You, Y. M., Pollema, S. L., Kaminska, A., Liu, K., Yoshioka, K., Bjorkblom, B., Coffey, E. T., Bagnato, C., Han, D., Huang, C. F., Banker, G., Pigino, G., and Brady, S. T. (2009). Pathogenic huntingtin inhibits fast axonal transport by activating JNK3 and phosphorylating kinesin. *Nature neuroscience* **12**(7), 864-71.
- Muresan, V., Abramson, T., Lyass, A., Winter, D., Porro, E., Hong, F., Chamberlin, N. L., and Schnapp, B. J. (1998). KIF3C and KIF3A form a novel neuronal heteromeric

- kinesin that associates with membrane vesicles. *Molecular biology of the cell* **9**(3), 637-52.
- Nishimura, T., Kato, K., Yamaguchi, T., Fukata, Y., Ohno, S., and Kaibuchi, K. (2004). Role of the PAR-3-KIF3 complex in the establishment of neuronal polarity. *Nature cell biology* **6**(4), 328-34.
- Nobes, C. D., and Hall, A. (1995). Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. *Cell* **81**(1), 53-62.
- Nogales, E., Whittaker, M., Milligan, R. A., and Downing, K. H. (1999). High-resolution model of the microtubule. *Cell* **96**(1), 79-88.
- Nollet, F., Kools, P., and van Roy, F. (2000). Phylogenetic analysis of the cadherin superfamily allows identification of six major subfamilies besides several solitary members. *Journal of molecular biology* **299**(3), 551-72.
- Olsen, J. V., Vermeulen, M., Santamaria, A., Kumar, C., Miller, M. L., Jensen, L. J., Gnäd, F., Cox, J., Jensen, T. S., Nigg, E. A., Brunak, S., and Mann, M. (2010). Quantitative phosphoproteomics reveals widespread full phosphorylation site occupancy during mitosis. *Science signaling* **3**(104), ra3.
- Ozawa, M., Ringwald, M., and Kemler, R. (1990). Uvomorulin-catenin complex formation is regulated by a specific domain in the cytoplasmic region of the cell adhesion molecule. *Proceedings of the National Academy of Sciences of the United States of America* **87**(11), 4246-50.
- Pan, C., Gnäd, F., Olsen, J. V., and Mann, M. (2008). Quantitative phosphoproteome analysis of a mouse liver cell line reveals specificity of phosphatase inhibitors. *Proteomics* **8**(21), 4534-46.
- Parrini, M. C., Lei, M., Harrison, S. C., and Mayer, B. J. (2002). Pak1 kinase homodimers are autoinhibited in trans and dissociated upon activation by Cdc42 and Rac1. *Molecular cell* **9**(1), 73-83.
- Parrini, M. C., Matsuda, M., and de Gunzburg, J. (2005). Spatiotemporal regulation of the Pak1 kinase. *Biochem Soc Trans* **33**(Pt 4), 646-8.
- Pearson, R. B., Woodgett, J. R., Cohen, P., and Kemp, B. E. (1985). Substrate specificity of a multifunctional calmodulin-dependent protein kinase. *The Journal of biological chemistry* **260**(27), 14471-6.
- Pirruccello, M., Sondermann, H., Pelton, J. G., Pellicena, P., Hoelz, A., Chernoff, J., Wemmer, D. E., and Kuriyan, J. (2006). A dimeric kinase assembly underlying autophosphorylation in the p21 activated kinases. *Journal of molecular biology* **361**(2), 312-26.
- Price, L. S., Leng, J., Schwartz, M. A., and Bokoch, G. M. (1998). Activation of Rac and Cdc42 by integrins mediates cell spreading. *Molecular biology of the cell* **9**(7), 1863-71.

- Qi, J., Wang, J., Romanyuk, O., and Siu, C. H. (2006). Involvement of Src family kinases in N-cadherin phosphorylation and beta-catenin dissociation during transendothelial migration of melanoma cells. *Molecular biology of the cell* **17**(3), 1261-72.
- Rosenberger, G., and Kutsche, K. (2006). AlphaPIX and betaPIX and their role in focal adhesion formation. *European journal of cell biology* **85**(3-4), 265-74.
- Scholey, J. M., Porter, M. E., Grissom, P. M., and McIntosh, J. R. (1985). Identification of kinesin in sea urchin eggs, and evidence for its localization in the mitotic spindle. *Nature* **318**(6045), 483-6.
- Schonteich, E., Wilson, G. M., Burden, J., Hopkins, C. R., Anderson, K., Goldenring, J. R., and Prekeris, R. (2008). The Rip11/Rab11-FIP5 and kinesin II complex regulates endocytic protein recycling. *Journal of cell science* **121**(Pt 22), 3824-33.
- Sells, M. A., Knaus, U. G., Bagrodia, S., Ambrose, D. M., Bokoch, G. M., and Chernoff, J. (1997). Human p21-activated kinase (Pak1) regulates actin organization in mammalian cells. *Current biology* **7**(3), 202-10.
- Setou, M., Nakagawa, T., Seog, D. H., and Hirokawa, N. (2000). Kinesin superfamily motor protein KIF17 and mLin-10 in NMDA receptor-containing vesicle transport. *Science* **288**(5472), 1796-802.
- Sharma, M., and Henderson, B. R. (2007). IQ-domain GTPase-activating protein 1 regulates beta-catenin at membrane ruffles and its role in macropinocytosis of N-cadherin and adenomatous polyposis coli. *The Journal of biological chemistry* **282**(11), 8545-56.
- Shimizu, K., Shirataki, H., Honda, T., Minami, S., and Takai, Y. (1998). Complex formation of SMAP/KAP3, a KIF3A/B ATPase motor-associated protein, with a human chromosome-associated polypeptide. *The Journal of biological chemistry* **273**(12), 6591-4.
- Singh, P., Gan, C. S., Guo, T., Phang, H. Q., Sze, S. K., and Koh, C. G. (2011). Investigation of POPX2 phosphatase functions by comparative phosphoproteomic analysis. *Proteomics* **11**(14), 2891-900.
- Sotiropoulos, A., Gineitis, D., Copeland, J., and Treisman, R. (1999). Signal-regulated activation of serum response factor is mediated by changes in actin dynamics. *Cell* **98**(2), 159-69.
- Stein, U., Arlt, F., Walther, W., Smith, J., Waldman, T., Harris, E. D., Mertins, S. D., Heizmann, C. W., Allard, D., Birchmeier, W., Schlag, P. M., and Shoemaker, R. H. (2006). The metastasis-associated gene S100A4 is a novel target of beta-catenin/T-cell factor signaling in colon cancer. *Gastroenterology* **131**(5), 1486-500.
- Stock, M. F., Guerrero, J., Cobb, B., Eggers, C. T., Huang, T. G., Li, X., and Hackney, D. D. (1999). Formation of the compact conformation of kinesin requires a COOH-terminal heavy chain domain and inhibits microtubule-stimulated ATPase activity. *The Journal of biological chemistry* **274**(21), 14617-23.

- Straub, F. B., and Feuer, G. (1950). [Adenosine triphosphate, the functional group of actin]. *Kiserletes orvostudomány* **2**(2), 141-51.
- Sueyoshi, N., Nimura, T., Ishida, A., Taniguchi, T., Yoshimura, Y., Ito, M., Shigeri, Y., and Kameshita, I. (2009). Ca(2+)/calmodulin-dependent protein kinase phosphatase (CaMKP) is indispensable for normal embryogenesis in zebrafish, *Danio rerio*. *Archives of biochemistry and biophysics* **488**(1), 48-59.
- Susila, A., Chan, H., Loh, A. X., Phang, H. Q., Wong, E. T., Tergaonkar, V., and Koh, C. G. (2010). The POPX2 phosphatase regulates cancer cell motility and invasiveness. *Cell cycle* **9**(1), 179-87.
- Takeda, S., Yamazaki, H., Seog, D. H., Kanai, Y., Terada, S., and Hirokawa, N. (2000). Kinesin superfamily protein 3 (KIF3) motor transports fodrin-associating vesicles important for neurite building. *Journal of cell biology* **148**(6), 1255-65.
- Takeda, S., Yonekawa, Y., Tanaka, Y., Okada, Y., Nonaka, S., and Hirokawa, N. (1999). Left-right asymmetry and kinesin superfamily protein KIF3A: new insights in determination of laterality and mesoderm induction by *kif3A*^{-/-} mice analysis. *The Journal of cell biology* **145**(4), 825-36.
- Takeichi, M. (1991). Cadherin cell adhesion receptors as a morphogenetic regulator. *Science* **251**(5000), 1451-5.
- Tamura, K., Shan, W. S., Hendrickson, W. A., Colman, D. R., and Shapiro, L. (1998). Structure-function analysis of cell adhesion by neural (N-) cadherin. *Neuron* **20**(6), 1153-63.
- Tan, K. M., Chan, S. L., Tan, K. O., and Yu, V. C. (2001). The *Caenorhabditis elegans* sex-determining protein FEM-2 and its human homologue, hFEM-2, are Ca²⁺/calmodulin-dependent protein kinase phosphatases that promote apoptosis. *The Journal of biological chemistry* **276**(47), 44193-202.
- Tanuma, N., Nomura, M., Ikeda, M., Kasugai, I., Tsubaki, Y., Takagaki, K., Kawamura, T., Yamashita, Y., Sato, I., Sato, M., Katakura, R., Kikuchi, K., and Shima, H. (2009). Protein phosphatase Dusp26 associates with KIF3 motor and promotes N-cadherin-mediated cell-cell adhesion. *Oncogene* **28**(5), 752-61.
- Teng, J., Rai, T., Tanaka, Y., Takei, Y., Nakata, T., Hirasawa, M., Kulkarni, A. B., and Hirokawa, N. (2005). The KIF3 motor transports N-cadherin and organizes the developing neuroepithelium. *Nature cell biology* **7**(5), 474-82.
- Terasawa, M., Tokumitsu, H., Kobayashi, R., and Hidaka, H. (1991). Ca²⁺/calmodulin-dependent protein phosphorylation associated with the cytoskeleton of quiescent rat fibroblast (3Y1) cells. *Journal of biochemistry* **110**(3), 417-22.
- Tombes, R. M., Faison, M. O., and Turbeville, J. M. (2003). Organization and evolution of multifunctional Ca(2+)/CaM-dependent protein kinase genes. *Gene* **322**, 17-31.

- Tominaga, T., Sahai, E., Chardin, P., McCormick, F., Courtneidge, S. A., and Alberts, A. S. (2000). Diaphanous-related formins bridge Rho GTPase and Src tyrosine kinase signaling. *Molecular cell* **5**(1), 13-25.
- Tweedie-Cullen, R. Y., Reck, J. M., and Mansuy, I. M. (2009). Comprehensive mapping of post-translational modifications on synaptic, nuclear, and histone proteins in the adult mouse brain. *Journal of proteome research* **8**(11), 4966-82.
- Vagnoni, A., Rodriguez, L., Manser, C., De Vos, K. J., and Miller, C. C. (2011). Phosphorylation of kinesin light chain 1 at serine 460 modulates binding and trafficking of calyculin-1. *Journal of cell science* **124**(Pt 7), 1032-42.
- Van Aelst, L., and D'Souza-Schorey, C. (1997). Rho GTPases and signaling networks. *Genes development* **11**(18), 2295-322.
- Verhey, K. J., and Rapoport, T. A. (2001). Kinesin carries the signal. *Trends in biochemical sciences* **26**(9), 545-50.
- Walker, R. A., O'Brien, E. T., Pryer, N. K., Soboeiro, M. F., Voter, W. A., Erickson, H. P., and Salmon, E. D. (1988). Dynamic instability of individual microtubules analyzed by video light microscopy: rate constants and transition frequencies. *The Journal of cell biology* **107**(4), 1437-48.
- Wang, Z. W. (2008). Regulation of synaptic transmission by presynaptic CaMKII and BK channels. *Molecular neurobiology* **38**(2), 153-66.
- Watanabe, N., Madaule, P., Reid, T., Ishizaki, T., Watanabe, G., Kakizuka, A., Saito, Y., Nakao, K., Jockusch, B. M., and Narumiya, S. (1997). p140mDia, a mammalian homolog of Drosophila diaphanous, is a target protein for Rho small GTPase and is a ligand for profilin. *The EMBO journal* **16**(11), 3044-56.
- Wedaman, K. P., Meyer, D. W., Rashid, D. J., Cole, D. G., and Scholey, J. M. (1996). Sequence and submolecular localization of the 115-kD accessory subunit of the heterotrimeric kinesin-II (KRP85/95) complex. *The Journal of cell biology* **132**(3), 371-80.
- Wisniewski, J. R., Nagaraj, N., Zougman, A., Gnäd, F., and Mann, M. (2010). Brain phosphoproteome obtained by a FASP-based method reveals plasma membrane protein topology. *Journal of proteome research* **9**(6), 3280-9.
- Wozniak, M. J., and Allan, V. J. (2006). Cargo selection by specific kinesin light chain 1 isoforms. *The EMBO journal* **25**(23), 5457-68.
- Xie, Y., Tan, E. J., Wee, S., Manser, E., Lim, L., and Koh, C. G. (2008). Functional interactions between phosphatase POPX2 and mDia modulate RhoA pathways. *Journal of cell science* **121**(Pt 4), 514-21.
- Yamazaki, H., Nakata, T., Okada, Y., and Hirokawa, N. (1995). KIF3A/B: a heterodimeric kinesin superfamily protein that works as a microtubule plus end-directed motor for membrane organelle transport. *The Journal of cell biology* **130**(6), 1387-99.

- Yamazaki, H., Nakata, T., Okada, Y., and Hirokawa, N. (1996). Cloning and characterization of KAP3: a novel kinesin superfamily-associated protein of KIF3A/3B. *Proceedings of the National Academy of Sciences of the United States of America* **93**(16), 8443-8.
- Yu, J. S., Chen, W. J., Ni, M. H., Chan, W. H., and Yang, S. D. (1998). Identification of the regulatory autophosphorylation site of autophosphorylation-dependent protein kinase (auto-kinase). Evidence that auto-kinase belongs to a member of the p21-activated kinase family. *The Biochemical journal* **334** (Pt 1), 121-31.
- Zenke, F. T., King, C. C., Bohl, B. P., and Bokoch, G. M. (1999). Identification of a central phosphorylation site in p21-activated kinase regulating autoinhibition and kinase activity. *The Journal of biological chemistry* **274**(46), 32565-73.
- Zhang, Y., Hancock, W.O., 2004. The two motor domains of KIF3A/B coordinate for processive motility and move at different speeds. *Biophysical journal* **87**, 1795-1804.
- Zhao, Z. S., Manser, E., Loo, T. H., and Lim, L. (2000). Coupling of PAK-interacting exchange factor PIX to GIT1 promotes focal complex disassembly. *Molecular and cellular biology* **20**(17), 6354-63.
- Zheng, Y., Glaven, J. A., Wu, W. J., and Cerione, R. A. (1996). Phosphatidylinositol 4,5-bisphosphate provides an alternative to guanine nucleotide exchange factors by stimulating the dissociation of GDP from Cdc42Hs. *The Journal of biological chemistry* **271**(39), 23815-9.

Selected publications

Susila, A., Chan, H., Loh, A. X., **Phang, H. Q.**, Wong, E. T., Tergaonkar, V., and Koh, C. G. (2010). The POPX2 phosphatase regulates cancer cell motility and invasiveness. *Cell cycle* 9(1), 179-87.

Singh, P., Gan, C. S., Guo, T., **Phang, H. Q.**, Sze, S. K., and Koh, C. G. (2011). Investigation of POPX2 phosphatase functions by comparative phosphoproteomic analysis. *Proteomics* 11(14), 2891-900.

Phang, H.Q. and Koh, C. G. (2012). Regulation of KIF3 motor complex by the POPX2 phosphatase. (Manuscript in preparation).

Appendix

Genotyping of putative *Popx2*^{+/-} heterozygous mice

To gain insights into the functions of POPX2 *in vivo*, we attempted to generate *Popx2* knockout transgenic mouse. The targeting vector for generating POPX2 knockout has been constructed by Swee and Koh (unpublished data) and was transfected into mouse embryonic stem cells. The first exon of *Popx2* was replaced by a cassette encoding neomycin resistance (*Neo*) after homologous recombination in ES cells (Figure S1). Selected ES cells were injected into C57BL/6 blastocysts before implantation into pseudo-pregnant mice. The chimeric mice were mated to check for germline transmission of the targeted allele in the F1^{+/-} heterozygous mice.

Figure S1. Targeted disruption of the *Popx2* gene. (A) Schematic representation of the wild type allele and targeting vector.

Initial genotyping of the putative *Popx2*^{+/-} heterozygous mice was performed by PCR analysis. Genomic DNA was extracted from both wild type C57BL/6 mouse and putative *Popx2*^{+/-} heterozygous mice (2 males, 2 females). Quality of the genomic DNA and efficiency of PCR were verified by amplification of the β -actin gene (Figure S2). The POPX2 exon 1 was amplified by the indicated primers to produce a 403-bp amplicon. Primers which annealed to the internal sequences of the *neo* resistant gene produced an amplicon of 410-bp in all the putative *Popx2*^{+/-} DNA samples but not in the DNA sample from the wild type mouse. Wild type and knockout alleles were further identified using primers that flanked the insertion site of the *neo* resistance cassette. Accordingly, the wild type allele consisting of exon 1 would generate a ~1000-bp product while the knockout allele containing the *neo* resistance cassette would generate a ~2500-bp product. However, amplification of the putative *Popx2*^{+/-} DNA with the flanking primers, failed to produce the two different bands. Only the ~1000-bp amplicon was observed. This led us to hypothesize that the *neo* resistant cassette had integrated elsewhere in the mouse genome. To end this, we performed long range PCR with primers (X2INF) that anneal to sequences outside the right arm of homology. As shown in Figure S2, only a single band of ~3500-bp was produced in the putative *Popx2*^{+/-} DNA samples. In addition, no bands were produced when the primer X2INF was used in combination with the *Neo-R* primer. From these results, we conclude that the *neo* resistant cassette had integrated into a different locus and rendered the POPX2 knockout unsuccessful.

Figure S2. PCR genotyping of the putative *Popx2*^{+/-} heterozygous mice. Genomic DNA was extracted from mice of different *Popx2* genotypes and subjected to amplification by PCR with the indicated primers. Wild Type (WT); F1 putative *Popx2*^{+/-} (01, 02, 06, 07); Targeting vector (Vec).

Primer name	Sequence
Exon1F	5' ATGGCCTCTGGAGCCGCACAG 3'
Exon1R	5' TCAGGAAGAGACGGCACTGGATG 3'
NeoF	5' CAAGATGGATTGCACGCAGGT 3'
NeoR	5' CGATGTTTTGCTTGGTGGTCG 3'
FlankF	5' ATAGCTCTACACTAATGGCCCCTG 3'
FlankR	5' CCTGCCCAAATGAGTCTTCTATC 3'
X2INF	5' AGCTCTGCCTGTGTACAACTCTC 3'

Table S1. DNA sequences of primers used in the PCR genotyping.

Genotyping by PCR has two major technical limitations: 1) the detection limit of the amplification reaction and 2) the resolution limit to distinguish the different alleles which vary in size and sequence. To overcome these technical limitations, we have performed Southern blot to further analyze the genotypes of the mice. Genomic DNA from the wild type and putative *Popx2*^{+/-} mice were digested with the restriction enzyme EcoRI to generate the DNA fragments shown in Figure S3A. The digested DNA were separated on agarose gel and subjected to Southern analysis with the ³²P-dCTP-labelled DNA probe that targets to the *neo* resistant cassette (P1). Three of the putative *Popx2*^{+/-} DNA samples but not the wild type sample, were found to contain the *neo* resistant cassette (Figure S3A). The genomic DNAs were then double-digested with EcoRI/EcoRV or XhoI/EcoRV and were subjected to Southern analysis using the indicated ³²P-dCTP-labelled DNA probe (P2). Wild type

allele digested with EcoRI/EcoRV should result in a band corresponding to 7.7 kb, while the knockout allele should yield a band of 5.7 kb (Figure S3B). However, only the 7.7 kb band was detected in both the wild type and putative *Popx2*^{+/-} DNA. Similarly, digestion of the wild type and putative *Popx2*^{+/-} DNA with XhoI/EcoRV should give rise to a band of 5.4 kb and 7.0 kb, respectively. Nevertheless, only the 5.4 kb band was detected in all samples (Figure S3C). Collectively, these results indicate that the *neo* resistant cassette was targeted to a different locus. During homologous recombination, correct targeting events occur at a very low frequency of ~1%. By including a negative selection marker such as thymidine kinase (lost after correct double homologous recombination), the correct targeting events can be enriched by 10 folds. Therefore, it is not unexpected that the *neo* resistant cassettes in the putative *Popx2*^{+/-} mice are integrated into a wrong locus since the targeting vector did not contain any negative selection markers.

Figure S3. Southern analysis of the putative *Popx2*^{+/-} heterozygous mice. Genomic DNA was extracted from the tails and was digested with the indicated restriction enzymes. DNA fragments were separated on agarose gel, transferred to nylon membrane and hybridized with the ³²P-dCTP-labelled DNA probes. Left panels show images of the blots analyzed by the Typhoon scanner and right panels show the schematics of the digested alleles. P1 represents a 410 bp ³²P-dCTP-labelled DNA probe that targets the *neo* resistant cassette (A) while P2 represents a 427 bp ³²P-dCTP-labelled DNA probe that binds the intron region downstream of the right arm of homology (B, C). Two DNA fragments which are 5.7 kb (B) and 5.4 kb (C) in length respectively were generated by PCR to act as controls. Wild type (WT), F0 Chimera (Chi); F1 putative *Popx2*^{+/-} (01, 02, 06, 07).