Investigating the functions and signaling properties of kindlins

Tan, Hui Foon

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SINGAPORE

**INVESTIGATING THE FUNCTIONS AND
SIGNALING PROPERTIES OF KINDLINS**

TAN HUI FOON

SCHOOL OF BIOLOGICAL SCIENCES

2019

INVESTIGATING THE FUNCTIONS AND SIGNALING PROPERTIES OF KINDLINS

TAN HUI FOON

SCHOOL OF BIOLOGICAL SCIENCES

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

2019

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research done by me except where otherwise stated in this thesis. The thesis work has not been submitted for a degree or professional qualification to any other university or institution. I declare that this thesis is written by myself and is free of plagiarism and of sufficient grammatical clarity to be examined. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University and that the research data are presented honestly and without prejudice.

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Supervisor Declaration Statement

I have reviewed the content and presentation style of this thesis and declare it of sufficient grammatical clarity to be examined. To the best of my knowledge, the thesis is free of plagiarism and the research and writing are those of the candidate's except as acknowledged in the Author Attribution Statement. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University and that the research data are presented honestly and without prejudice.

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Tan Suet Mien

Authorship Attribution Statement

This thesis contains material from one paper published in Data in Brief journal in which I am listed as an author.

Chapter 6 is published as Han J. J., **Tan H. F.**, Feng C., Wee W.K., Tee S. Y., Tan S. M. Data on cell spread area and directional contraction in human umbilical vein endothelial cells on fibronectin and on collagen type I-coated micropost. Data In Brief, 2016. **6**: p. 803-810.

The contributions of the co-authors are as follows:

- Asst/ Prof Shan-You Tee and Assoc/ Prof Suet-Mien Tan provided the initial project direction
- Assoc/ Prof Suet-Mien Tan prepared the manuscript drafts. The manuscript was revised by Asst/ Prof Shan-You Tee, Dr. Jing-Jing Han, Dr. Feng Chen, Wei-Kiat Wee and I.
- Live cell imaging micropost work, immunofluorescence staining and confocal microscopy work were performed by me in the School of Biological Sciences.
- Dr. Jing-Jing Han performed all the statistical analyses.

19 August 2019

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Publications

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Abbreviations

Abbreviation	Full Name
ALIX	Apoptosis-linked gene 2-interacting protein X
APC/ C	Anaphase promoting complex/ cyclosome
ATAT1	α -tubulin acetyltransferase 1
BSA	Bovine serum albumin
BDNF	Brain-derived neurotrophic factor
CAMs	Cell adhesion molecules
cAMP/ PKA	Cyclic adenosine monophosphate/ protein kinase A
CD81	Cluster of differentiation 81
CDK1	Cyclin-dependent kinase 1
CDC20	Cell-division cycle protein 20
CSX	Cardiac specific homeobox protein
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGM	Endothelial cell growth media
eIF	Eukaryotic initiation factor
EMT	Epithelial to mesenchymal transition
ERK	Extracellular-signal regulated kinase
FAK	Focal adhesion kinase
FBS	Foetal bovine serum
FERM	4.1-ezrin-radixin-moesin
FRET	Fluorescence resonance energy transfer
GPCRs	G-protein-coupled receptors
GSK3 β	Glycogen synthase kinase 3 β
HDAC6	Histone deacetylase 6
HIF-1 α	Hypoxia-inducible factor-1 α
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase

HSP70	Heat shock protein 70
HUVEC	Human umbilical vein endothelial cell
ICAM	Intercellular adhesion molecule
IFN- γ	Interferon- γ
IgSF	Immunoglobulin superfamily
IGF-IR	Insulin-like growth factor receptor I
ILK	Integrin-linked kinase
IL-1	Interleukin-1
IRES	Internal ribosome entry sites
IRS-1/2	Insulin receptor substrate 1/2
JNK	Jun N-terminal kinase
LAD	Leukocyte adhesion deficiency
LFA-1	lymphocyte function-associated antigen 1
MAD2	Mitotic arrest deficient 2
MAPK	Mitogen-activated protein kinase
miR	MicroRNA
miRISC	miRNA-induced silencing complex
MMP1	Matrix metalloproteinase I
mRNA	messenger RNA
MVBs	Multivesicle bodies
NMDAR	N-methyl D-aspartate receptor
NMR	Nuclear magnetic resonance
NR2B	N-methyl D-aspartate receptor subtype 2B
OCT	Optimal cutting temperature
PBS	Phosphate-buffered saline
PDE	Phosphodiesterase
PEI	Polyethylenimine
PH domain	Pleckstrin homology domain
PI3K	Phosphoinositide 3-kinase
Plk-1	Polo-like kinase 1
PKC	Protein kinase C
PLC β	Phospholipase C β

PVDF	Polyvinylidene difluoride
PTB	Phosphotyrosine-binding domain
RACK1	Receptor for activated kinase 1
RPMI	Roswell park memorial institute medium
SAC	Spindle assembly checkpoint
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Sos-1	Son of sevenless-1
SPR	Surface plasmon resonance
STAT3	Signal transducer and activator of transcription 3
TAE	Tris-acetic-EDTA
TBST	Tris-buffered saline and tween-20
Tcf4	Transcription factor 4
TGF- β	Transforming growth factor- β
TNF- α	Tumour necrosis factor- α
TSG101	Tumour susceptibility gene 101 protein
uPA	urokinase type plasminogen activator
VEGF	Vascular endothelial growth factor
WRN	Werner syndrome ATP-dependent helicase
WT	Wild-type

Abstract

Kindlins are FERM-containing cytoplasmic proteins that have crucial roles in cell adhesion and migration. The three members kindlin-1, kindlin-2 and kindlin-3 have different tissue expression profiles and they do not show functional redundancy. The primary role of kindlins is to regulate the activity of integrins by binding directly to β integrin cytoplasmic tails. In this thesis, we examine the functional properties of kindlin-2 and kindlin-3. We show that kindlin-2 regulates mitotic spindle assembly in the neuroblastoma SH-SY5Y cell line. Mechanistically, it involves the Akt/ GSK3 β / Histone deacetylase 6 (HDAC6) or/and Paxillin/ HDAC6 signaling pathway that modulates the acetylation of α -tubulin, a post-translational modification that stabilizes microtubules. Depleting kindlin-2 in cells leads to mitotic spindle abnormalities and delays the progression of mitosis. Hypoxia, a hallmark of solid tumour microenvironment, up-regulates miR-138 that suppresses the expression of kindlin-2, leading to spindle abnormalities in cells and slow growth. We also provide preliminary evidence that kindlin-2 is required for the functions of small extracellular vesicles that have important roles in cancer cell dissemination. Extracellular vesicle derived from SH-SY5Y cells depleted of kindlin-2 exhibited functional defect in promoting early stage HUVECs tube formation. In a previous study, it has been shown that kindlin-3, but not other kindlin members, interacts with the scaffold protein RACK1. Here, we show that the sequence ³⁶¹IPRR³⁶⁴ in the PH domain of kindlin-3 long isoform is a specificity determinant for RACK1 interaction. We also provide preliminary data showing that over-expressed kindlin-3 in HUVECs reduces cell traction force and directional contractions on fibronectin. Taken together, these studies provide novel insights into the regulatory functions of kindlin-2 and kindlin-3.

Chapter 1 Introduction

1.1 Cell Adhesion Molecules (CAMs)

Cell adhesion is an important process in tissue morphogenesis, cell-cell communication, host defense and diseases. Cell adhesion involves Cell Adhesion Molecules (CAMs) located on the cell surface. There are four major families of CAMs, namely Immunoglobulin Superfamily (IgSF) molecules, cadherins, selectins and integrin [1]. IgSF CAMs contain Ig-like domains in their extracellular domains [2]. A number of IgSF CAMs bind integrins and others are involved in homophilic interactions [3, 4]. The expression of IgSF CAMs is tightly regulated. Endothelial cells, for example, expressed low level of Intercellular Adhesion Molecule (ICAM)-1 (CD54) under non-inflamed conditions [5-8]. ICAM-1 exists as an oligomer on the cell membrane [9-12] and it is a ligand for $\beta 2$ family of integrins that are expressed primarily in immune cells [13]. ICAM-1 expression is markedly up-regulated when endothelial cells are stimulated by inflammatory cytokines, including TNF- α , IL-1 and IFN- γ [14, 15]. In line with its crucial role in leukocyte adhesion, ICAM-1 deficient mice have been reported to have defective neutrophil emigration and poor T cell responses [16]. ICAM-3, another member of the IgSF CAMs, is expressed on T-lymphocytes [17] and it is the counter-receptor of Dendritic Cell-Specific ICAM-Grabbing Non-integrin (DC-SIGN) expressed on dendritic cells [18, 19]. Their interaction is crucial for antigen presentation and plays a role in Human Immunodeficiency Virus (HIV) infection [20].

Cadherins are a superfamily of calcium-dependent CAMs that are categorized into four types, namely classical, desmosomal, proto-cadherins and unconventional [21]. The most extensively studied type is the classical cadherins. Through membrane cis-interaction, they form homodimers on cell surface and interact with neighboring cell cadherin homodimers forming adherens junctions [22, 23]. Apart from their roles in normal cell physiology, changes in cadherin expression have been extensively studied in diseases. Loss of classical E-cadherin, for example, is considered a hallmark of epithelial-mesenchymal transition (EMT) in many cancers [24-27]. Another example of the importance of cadherins is the functional disruption of desmosomal cadherins (desmoglein and desmocollin) by autoimmune antibodies that leads to development of epidermal blistering disease known as pemphigus [28, 29].

Selectins are vascular CAMs that promote leukocyte rolling, which is an important initiation step in leukocyte transendothelial migration [30, 31]. The three members of the selectin family are E-selectin, L-selectin and P-selectin. They are mainly expressed in endothelial cells, leukocytes and platelets [32]. A key feature of selectins is the presence of an amino terminal C-type lectin domain which binds the minimal recognition sugar moiety sialyl-Lewisx (SLe^x) [33]. In Leukocyte Adhesion Deficiency (LAD) II, deficiency in SLe^x attenuates the binding of selectins to their ligands, leading to defective leukocyte tissue infiltration [34].

Perhaps the most complex in terms of function and regulation is the CAM superfamily integrins. These are cell surface transmembrane receptors that are obligate heterodimers [35]. There are many excellent detailed reviews on integrins, including that from our lab. Henceforth, I will only provide an overview of integrin structure, function and its regulation, which forms the basis for my project investigating the function of a small family of cytoplasmic proteins known as kindlins.

1.2 An overview of integrins

Integrins are divalent cation-dependent CAMs that mediate cell adhesion to extracellular matrix, cell-cell adhesion and cell-virus interaction [36]. Each integrin is composed of an α and a β subunit [37, 38]. In mammals, there are 24 different integrins (**Figure 1**). They can be sub-categorized based on their ligands and tissue expression. Arginine-glycine-aspartic acid (RGD) sequence is a major integrin binding site found in ligands. Examples of ligands containing the RGD sequence includes fibronectin, vitronectin, fibrinogen, von Willebrand factor, thrombospondin, laminin, entactin, tenascin, osteopontin and bone sialoprotein [39]. It should be noted that the RGD sequence is not always presented at the surface of protein and its presentation is dependent on the folding of the protein under different conditions [39]. For example, collagen type I contains RGD sequences that is only exposed under denatured condition [40].

Both integrin subunits consist of an extracellular domain, a transmembrane domain and a relatively short cytoplasmic tail, except that of $\beta 4$ subunit [41, 42]. The general domain organization of integrins is shown at **Figure 2**. It is noteworthy that nine of the α subunits contain an inserted domain referred to as the α I-domain. The α I-domain is the primary ligand-binding site in these integrins whereas integrins without the α I-domain bind ligands via the β -propeller of the α subunit and the I-like

domain of the β subunit (or β I-domain) [43, 44]. A list of extracellular ligands of integrins is summarized in **Table 1**.

Ligand	Integrin
A Disintegrin and Metalloprotease (ADAM) Family	$\alpha_4\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$, $\alpha_9\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_6$,
Adenovirus penton base protein	$\alpha_v\beta_3$, $\alpha_v\beta_5$
Bone sialoprotein	$\alpha_v\beta_3$, $\alpha_v\beta_5$
<i>Borrelia burgdorferi</i>	$\alpha_{IIb}\beta_3$
<i>Candida albicans</i>	$\alpha_M\beta_2$
Collagens	$\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_{10}\beta_1$, $\alpha_{11}\beta_1$, $\alpha_{Ib}\beta_3$
Connective Tissue Growth Factor	$\alpha_v\beta_3$, $\alpha_{IIb}\beta_3$
COMP	$\alpha_v\beta_3$, $\alpha_5\beta_1$
Cyr61	$\alpha_6\beta_1$, $\alpha_v\beta_3$, $\alpha_D\beta_2$, $\alpha_{IIb}\beta_3$
Cytotactin/ tenascin-C	$\alpha_8\beta_1$, $\alpha_9\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_6$
Denatured collagen	$\alpha_5\beta_1$, $\alpha_v\beta_3$, $\alpha_{IIb}\beta_3$
Decorsin	$\alpha_{IIb}\beta_3$
Disintegrins	$\alpha_v\beta_3$, $\alpha_{IIb}\beta_3$
E-cadherin	$\alpha_2\beta_1$, $\alpha_E\beta_7$
Echovirus 1	$\alpha_2\beta_1$
Epiligrin	$\alpha_3\beta_1$
ESM-1	$\alpha_L\beta_2$
Factor X	$\alpha_M\beta_2$
Fibronectin	$\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_4\beta_7$, $\alpha_5\beta_1$, $\alpha_8\beta_1$, $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_v\beta_6$, $\alpha_v\beta_8$, $\alpha_{IIb}\beta_3$, $\alpha_D\beta_2$
Fibrillin	$\alpha_5\beta_1$
Fibrinogen	$\alpha_5\beta_1$, $\alpha_M\beta_2$, $\alpha_v\beta_3$, $\alpha_X\beta_2$, $\alpha_{IIb}\beta_3$, $\alpha_D\beta_2$
Heparin & negative charges in denatured proteins	$\alpha_M\beta_2$, $\alpha_X\beta_2$
HIV Tat protein	$\alpha_v\beta_3$, $\alpha_v\beta_5$
iC3b	$\alpha_M\beta_2$, $\alpha_X\beta_2$
ICAM-1	$\alpha_M\beta_2$, $\alpha_L\beta_2$
ICAM-2,3,5	$\alpha_L\beta_2$
ICAM-4	$\alpha_L\beta_2$, $\alpha_4\beta_1$, $\alpha_M\beta_2$, $\alpha_X\beta_2$, $\alpha_v\beta_3$, $\alpha_{IIb}\beta_3$
Invasin	$\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$
Laminin	$\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_6\beta_1$, $\alpha_7\beta_1$, $\alpha_6\beta_4$, $\alpha_v\beta_3$, $\alpha_{10}\beta_1$, $\alpha_{11}\beta_1$,
LAP-TGF β	$\alpha_8\beta_1$, $\alpha_v\beta_5$
L1	$\alpha_5\beta_1$, $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_{IIb}\beta_3$
MAdCAM-1	$\alpha_4\beta_7$
Matrix metalloproteinase-2	$\alpha_v\beta_3$
Nephronectin	$\alpha_8\beta_1$
Neutrophil inhibitory factor	$\alpha_M\beta_2$
Osteopontin	$\alpha_v\beta_3$, $\alpha_4\beta_1$, $\alpha_4\beta_7$, $\alpha_9\beta_1$
Plasminogen	$\alpha_{IIb}\beta_3$, $\alpha_D\beta_2$
POEM	$\alpha_8\beta_1$
Prothrombin	$\alpha_v\beta_3$, $\alpha_{IIb}\beta_3$
Sperm fertilin	$\alpha_6\beta_1$
Tenascin	$\alpha_2\beta_1$
Thrombospondin	$\alpha_3\beta_1$, $\alpha_v\beta_3$, $\alpha_{IIb}\beta_3$, $\alpha_5\beta_1$, $\alpha_6\beta_1$
VCAM-1	$\alpha_4\beta_1$, $\alpha_4\beta_7$
Vitronectin	$\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_{IIb}\beta_3$, $\alpha_D\beta_2$
Von Willebrand Factor	$\alpha_v\beta_3$, $\alpha_{IIb}\beta_3$

Table 1. Extracellular ligands of integrins. The information was obtained from Humphries, J.D. et.al., 2006 [66] and Plow, E.F.et.al., 2000 [67].

Figure 1 Members of the integrins family. There are 24 heterodimer integrins in mammals. They can be divided into different subgroups based on their ligand binding types.

Figure 2 General domain organization of integrins. I-domain: Inserted domain; PSI: Plexins-semaphorin-containing integrin; I-EGF: cysteine-rich integrin epidermal growth factor-like domain; βTD: β-tail domain. Nine of the integrin α subunits (α1, α2, α10, α11, αE, αL, αM, αD, and αX) contain an I-domain, which is referred to as α I-domain. This should not be confused with the I-domain found in all β subunits, which is referred to as β I-domain. One major difference between the two I-domains is that α I-domain contains only one divalent cation-binding site whereas there are three divalent cation-binding sites in the β I-domain. Collective regions of the integrin are also given labels such as “head”, “legs”, “headpiece” and “tailpiece”, which are often used in literature to describe overall conformational changes during integrin activation.

The importance of integrins is underscored by wide ranging defects as a result of gene mutations that leads to the expression of dysfunctional or loss of expression of integrins. These include impaired leukocyte recruitment [45], skin blistering [46], haemorrhage [47], muscular dystrophy [48] and embryonic lethality [49, 50]. Given that integrins are essential CAMs in metazoans, it is not surprising that integrin function is tightly regulated. Studies using mouse knockout system have shown that many of these integrins are crucial in physiology as summarized in **Table 2**.

Integrin subunit	Phenotypes
α_1	Bone recovering problem and decreased in tumour angiogenesis [68]
α_2	Reduction of platelet adhesion and poor mammary gland branching [69]
α_3	Problems in kidney, lung and skin development [70]
α_4	Problems in placenta and cardiac development [71]
α_5	Embryonic lethal, defects in mesodermal development [72]
α_6	Prenatal death, skin blistering, problems in neurogenesis [73]
α_7	Muscular dystrophy [74]
α_8	Problems in kidney development [75]
α_9	Problems in lymphatic drainage [76]
α_{10}	Dwarfism, mild chondrodysplasia [77]
α_{11}	Dwarfism, high chances of death, defective teeth eruption [77]
α_v	Problems in placenta formation and in central nervous system and gastrointestinal blood cell formation, cleft palate [77, 78]
α_M	Impaired of phagocytosis, problems in neutrophil adhesion and degranulation and mast cells development [77, 79]
α_L	Defects in leukocyte recruitment and osteoclast development [77]
α_D	Higher chances for bacterial infection [77]
α_E	Reduction of lymphocyte numbers and skin inflammation [80]
α_X	Defects in T-cells, higher chances for bacterial infection [77]
α_{IIb}	Platelets abnormalities [77]
β_1	Reduction in inner cell mass [77]
β_2	Impaired leukocyte adhesion and migration, skin infections, osteoporosis [77, 81]
β_3	Defects in platelets [82]
β_4	Skin blistering [83]
β_6	Problems in TGF β activation, asthma and periodontal disease [77]
β_7	lymphocytes defects in gut [84]
β_8	Problems in placenta formation and in central nervous system and gastrointestinal blood cell formation, cleft palate [77]

Table 2. The phenotypes of integrin subunit knockout mice. Information in this table was obtained from review articles Bouvard D. et. al., 2013 [77] that cited others references.

Generally, integrin-mediated adhesion involves distinct stages. The term inside-out signaling, which is often used to describe integrin activation, involves recruiting intracellular proteins to integrin cytoplasmic tails during cellular activation [47, 51] (**Figure 3A**). Talin, which is one of the most studied positive regulators of integrins, is a large cytoskeletal protein that contains an N-terminal head region and a long helical C-terminal rod region [52, 53]. Two talin genes (*Tln1* and *Tln2*) are found in vertebrates. The head region is composed of a 4.1 protein, ezrin, radixin, moesin (FERM) domain within which resides the F1, F2 and F3 subdomains. The talin F3 subdomain has been shown to bind

to a highly conserved NPxY (x: other amino acid) motif located in the membrane proximal region of integrin β cytoplasmic tails [54]. This interaction disrupts a salt-bridge between the integrin α and β cytoplasmic tails, leading to the separation of the cytoplasmic tails, a pre-requisite of integrin activation [55]. This interaction is probably enhanced by the interaction between a basic patch in the talin F2 subdomain with the inner leaflet of the plasma membrane [56, 57]. The talin helical rod region is mainly involved in binding F-actin and vinculin [58, 59]. In this way, talin connects integrins to the cytoskeleton and up-regulates the ligand-binding property of integrins. The interaction between talin and integrins is regulated. Talin has been reported to form a dimer, which binds poorly to integrins [52]. Another mode of regulation involves steric exclusion of talin head domain from binding to the integrin β cytoplasmic tail [60]. This will be discussed in the following section.

Following inside-out activation, the extended integrin binds to its ligand that leads to the opening of the integrin headpiece and tailpiece. While binding to multivalent or multimeric ligands, clustering of integrins ensues. In turn, clustered integrins promotes activation of downstream signaling molecules, including src family kinases [61] and PI3K [62], which are important nodes in cell signaling. This process is referred to as integrin outside-in signaling (**Figure 3B**). Apart from inducing biochemical signals, integrins are mechanosensors of cells for detecting changes in their microenvironment, in particular the stiffness of the substrate [63]. It is well established that substrate stiffness plays an important role in regulating cell migration, proliferation and differentiation [64, 65].

Figure 3 Integrins activation and conformational changes. (A) Inside-out signaling refers to the process by which cytoplasmic proteins bind to and induce conformational changes in integrins, leading to their activation. For example, talins and kindlins are two families of cytoplasmic proteins that have crucial roles in activating integrins. (B) Outside-in signaling usually refers to cellular signaling induced by ligand-bound integrins. When the activated integrin binds to its ligand, this induces further conformational changes in the integrin. Multivalent or multimeric ligands also promote the clustering of integrins. Both events lead to the recruitment of and activation of effector proteins, for example src-family kinases, that initiate signaling cascades. O: Open headpiece extended (active form); B: Bent closed headpiece (inactive form); E: Extended closed headpiece.

1.3 Kindlins

As mentioned in the previous section, the cytoskeletal protein talin binds to and promotes integrin activation. A three-membered family of FERM-containing focal adhesion proteins known as kindlins have been shown to regulate the binding of talin to the integrin β cytoplasmic tails [85]. Kindlin-1 (FERMT-1), kindlin-2 (FERMT-2) and kindlin-3 (FERMT-3) are encoded by different genes *KIND1* (chromosome 20p12.3), *KIND2* (chromosome 14q22.1) and *KIND3* (chromosome 11q13.1) respectively [86]. They share highly identical sequences with one another. Based on the amino acid sequence alignment of human kindlins, 59 % identity is found between kindlin-1 and kindlin-3, and kindlin-2 is 65 % identical to kindlin-3 [87, 88].

1.3.1 Domain organization and structure of kindlins

The FERM domain of kindlins is divided into three subdomains which are F1, F2 and F3. An additional subdomain F0 is located N-terminus to the F1 subdomain. Unlike the FERM domain of talin, the F2 subdomain of kindlins is bisected by a pleckstrin homology (PH) domain [89, 90] (**Figure 4**). Although kindlins share highly similarity sequences with talins, this is only restricted to F2 and F3 subdomains [91]. The F1 subdomain of kindlins has a larger insert, compared to talin and this insert is not conserved [92]. Previous nuclear magnetic resonance studies reported partial structures of kindlins. These are the F0 subdomain of kindlin-1 [92], the long basic loop in the F1 subdomain of kindlin-3 [93], and the PH domain of kindlin-2 and kindlin-3 [94]. Recently, an almost complete structure of mouse kindlin-2 has been solved by X-ray crystallography [95]. Two salient features observed are its clover-leaf conformation and dimer formation (**Figure 5**). The latter is dependent on the F2 subdomain of kindlin-2.

Figure 4 General domain organization of kindlins. Kindlins are FERM-containing proteins. Within the FERM domain are the F1, F2 and F3 subdomains. A distinct feature of kindlins is the presence of a PH domain that inserts in the F2 subdomain, separating it into two halves. The two halves of the F2 subdomain fold into a single moiety based on the crystal structure of kindlins. In addition, a F0 subdomain is located at the N-terminal of the F1 subdomain. A variable region containing a long basic loop is found in the F1 subdomain.

Figure 5 Crystal structure of kindlin-2. (A) The crystal structure of mouse truncated kindlin-2 lacking the PH domain has been solved. The truncated kindlin-2 lacking the PH domain is indicated by the purple ribbon. The overall structure of kindlin-2 adopts a clover-leaf like conformation. (B) Mouse truncated kindlin-2 lacking the PH domain model forms homodimer to activate integrins via F3 subdomain binding to integrin β cytoplasmic tail. This figure is generated from the structural coordinate PDB 5XPZ, derived from Li, H., et al. [95].

1.3.2 The association of kindlins with integrin β cytoplasmic tails

The binding of kindlin and talin to the β cytoplasmic tails of integrins promotes integrin activation. There are two highly conserved NPxY/F motifs in the integrin β cytoplasmic tails. Whereas talin binds to the membrane proximal NPxY/F motif [96], kindlins bind to the membrane distal NPxY/F motif [97]. Kindlins do not interact with talin but they can simultaneously associate with an integrin β cytoplasmic tail forming a ternary complex [98]. A few models of kindlins regulating integrin activation have been proposed. One of these involves the displacement of integrin negative regulator. Filamin A, a large cytoskeletal protein, binds to and occludes the talin binding site in integrin β cytoplasmic tail thereby preventing talin activation of integrins [97]. The role of kindlins is to recruit the adaptor protein migfilin. Migfilin interacts with the same region in filamin A that is involved in binding integrin β cytoplasmic tail. Hence, the kindlin/ migfilin complex can dissociate filamin A from the integrin, in turn allowing talin to bind to the integrin β cytoplasmic tail [99]. In another model, kindlins promote integrin clustering. Studies have shown that kindlin-3 promotes clustering of α L β 2 and α IIb β 3 integrins [100, 101] but the detailed mechanism remains to be determined. Based on the recently solved crystal structure of mouse kindlin-2 dimer, it is suggested that kindlins promote integrin clustering by forming dimers and that this is dependent on the F3 subdomain [95]. However, more supporting data are needed because a truncated kindlin-2 lacking the PH domain was used in that study.

Apart from integrin mediated pathway, kindlins perform integrin independent functions. Study has shown that kindlin-2 forms a ternary complex with β -catenin and TCF4 to promote cancer progression [102]. Kindlin-2 has also been shown to localize in the nucleus of smooth muscle cells [103]. Both kindlin-1 and kindlin-2 are involved in cell-cell contacts in epithelial cells [104]. Finally, kindlin-3 has been shown in stabilizing erythrocytes' membrane cytoskeleton [105].

1.3.3 Kindlin-1

Kindlin-1 is mainly expressed in epithelial cells. Mutations occurred in kindlin-1 lead to kindler syndrome in human, characterized by skin blistering, skin atrophy, poikiloderma and high risk in intestinal complications [104, 106]. It is also involved in integrin-mediated adhesion [107, 108]. It interacts with focal adhesion proteins through outside-in signaling. Kindlin-1 connects integrins to actin cytoskeleton by binding to migfilin and integrin-linked kinase (ILK) [104, 106, 109, 110].

The skin blistering phenotype observed in Kindler syndrome patients is phenocopied in kindlin-1 knockout mice in which the epidermal-dermal junction is defective. Interestingly, hyperproliferation of cutaneous stem cells was observed in these mice. The stem cell niche is regulated by the anti-proliferative effect of TGF β signaling and the pro-proliferative effect of Wnt/ β -catenin signaling. In kindlin-1 knock-out mice, loss of kindlin-1 caused dysfunctional α V β 6 integrin. In turn, a reduction of active TGF- β 1 was detected because α V β 6 integrin is needed for the release of TGF- β 1 from the latent form LAP-TGF- β 1. Concomitantly, there was an increase in Wnt/ β -catenin activity. Together, these lead to the hyperproliferation of cutaneous stem cells [111]. This may also lead to the development cutaneous squamous cell carcinoma [112-114].

Studies have shown that kindlin-1 plays a crucial role in cancer progression. The mRNA expression level of kindlin-1 was up-regulated in lung, breast and colon, hepatocellular carcinomas [115-117]. Kindlin-1 also promotes cell migration and invasion in several pancreatic cancer cell lines [118]. Mechanistically, these cancers involved dysregulated activation of integrin α V β 6 and mediated by kindlin-1, resulting in the release of active TGF- β 1 and promoting EMT [119, 120]. It is well established that β 1 integrins are important in promoting cell proliferation in cutaneous squamous cell carcinoma. β 1 integrin function-blocking antibodies inhibit tumorigenesis of human epithelial tissues [121]. In line with this study, a transgenic mouse model harboring an activating T1881A point mutation in the β 1 integrin has a propensity to develop cutaneous squamous cell carcinoma [122]. However, others have shown that the loss of kindlin-1 expression affects TGF- β 1 and Wnt/ β -catenin signaling pathways, leading to the aforementioned skin tumour development [123-125]. Hence, more studies are needed to explain this paradox.

Apart from integrins, kindlin-1 is involved in RhoGTPases signaling which regulates lamellipodia formation and cell migration [126]. RhoGTPases promote cell growth, cell spreading, migration and invasion in breast, lung, colorectal and pancreatic cancers [116, 118]. In addition, kindlin-1 has been shown to regulate mitotic spindle assembly. Polo-like kinase 1 (Plk-1), a cell cycle regulator serine/ threonine kinase, phosphorylates kindlin-1, which in turn regulates the acetylation of α -tubulin through histone deacetylase 6 (HDAC6) [127]. Studies have shown that the acetylation of microtubules promotes microtubule stability by enhancing microtubule interaction through microtubule associated proteins and motor proteins [128, 129]. Given that kindlin-1 is primarily expressed in

epithelial cells, whether kindlin-2 and kindlin-3 are involved in the regulation of mitotic spindle assembly in other cell types remains to be determined. An overview of cell cycle and mitotic spindle assembly is provided in section 1.4.

1.3.4 Kindlin-2

Kindlin-2 is expressed in most tissues except hematopoietic cells. Kindlin-2 gene knock-out in mice and zebra fish is embryonic lethal [130]. This may explain as to why there is, to date, no report of dysfunctional kindlin-2 gene mutations in humans. A number of non-integrin kindlin-2 binding partners have been identified. The association between kindlin-2 and integrins at nascent adhesion sites also leads to the recruitment of kindlin-2-binding proteins migfilin and ILK, the former is involved in displacing integrin negative regulator filamin A whereas the latter is involved in forming the IPP-complex together with PINCH and parvin [110, 131-133]. The kindlin-2 and migfilin complex has also been reported to bind to the transcription factor CSX in the nucleus, which contributes to cardiac development [134]. A recent study demonstrated that kindlin-2 directly binds to actin, hence establishing a role of kindlin-2 in connecting the integrins to F-actin [135]. A nuclear localization signal within its protein sequence may explain the detection of kindlin-2 in the nucleus [136], although more definitive studies are needed to demonstrate its translocation between the cytoplasm and the nucleus. Nonetheless, kindlin-2 has been shown to form a ternary complex with β -catenin and Tcf4 that activates myogenic promoter and increases myogenic expression in muscle [137]. In another study, kindlin-2 forms a ternary complex with β -catenin and Tcf4, enhancing the Wnt signaling pathway in cancer cells [136]. In wound healing, kindlin-2 becomes highly expressed in activated myofibroblasts. Kindlin-2 regulates focal adhesion formation and transforms the smooth muscle actin into stress fibers, promoting the process of wound closure and tissue repair [138]. Using a skin-equivalent model, it has been shown that kindlin-2 is required for maintaining the integrity of the stratified epidermal layers by regulating RhoA-mediated cytoskeleton re-organization [139]. In the setting of the chronic disease renal tubulointerstitial fibrosis in which tubular epithelial cells undergo EMT into myofibroblasts, kindlin-2 interacts with Ras and Sos-1, and regulates Erk1/2 and Akt signaling pathways [140]. Kindlin-2 is involved in TGF β signaling pathway by interacting with Smad3 at its N-terminus domain and the TGF β receptor I [141]. Kindlin-2 in endothelial cells interacts with clathrin to regulate endocytosis and indirectly regulates platelet aggregation and homeostasis [142].

Given the crucial role of kindlin-2 in an increasing number of signaling conduits, it is not unexpected that kindlin-2 is involved in different cancers [143-147]. Kindlin-2 has been reported to bind to and stabilize EGFR in breast cancer cells thereby promoting EGF-induced cell migration [148]. Interestingly, kindlin-2 down-regulates the expression of miR200b, which has a role in inhibiting EMT [102]. An inverse relationship between kindlin-2 and miR-200 has also been reported in esophageal squamous cell carcinoma undergoing metastasis in which a decrease in miR-200 leads to an increase in kindlin-2 expression [149]. Adding to the complexity of kindlin-2 signaling, kindlin-2 promotes prostate cancer cell survival via its interaction with Gli1 and anti-apoptotic Bcl2 proteins [150, 151]. Despite its importance in cancer progression, there are a few studies suggesting kindlin-2 having a tumour suppressor function. In mesenchymal cancer cells, kindlin-2 inhibits urokinase type plasminogen activator (uPA) secretion, suppressing cancer invasion [152]. In another study, kindlin-2 inhibits cancer progression by up-regulating the expression of estrogen receptor, which in turn promotes mesenchymal to epithelial transition [153].

It is well documented that the microenvironment plays a crucial role in cancer cell dissemination, survival and proliferation. In a seminal study by Hoshina et al., tumour-derived small extracellular vesicles known as exosomes have been shown to play important role in the metastasis of cancer cells to distant organs, and this is dependent on integrins found in exosomes [154]. An overview of exosome biogenesis and function is provided in section 1.6. Whether kindlins play a role in regulating integrin activity in exosomes remains to be determined.

1.3.5 Kindlin-3

Kindlin-3 is expressed in hematopoietic cells, endothelial cells and platelets [85]. Mutations in kindlin-3 disrupt integrin activation in platelets and immune cells, leading to leukocyte adhesion deficiency III syndrome (LAD III) characterized by bleeding disorder and defective immune responses and osteopetrosis [155]. Kindlin-3 homozygous knockout mice died within days from severe hemorrhages in multi-organs and osteopetrosis because of impaired integrin $\alpha\text{IIb}\beta 3$ activation [156]. Based on a study using kindlin-3 knockout mice to investigate the formation of mature lymphatic systems, kindlin-3 is required to activate platelet aggregation and separate the lymphatic vessel from existing vascular system [157]. In addition, kindlin-3 is crucial for integrins $\beta 1$ and $\beta 3$ activation on

platelets. Loss of kindlin-3 expression affects $\beta 2$ integrin-mediated leukocyte adhesion and arrests neutrophils on activated endothelial cells [158].

In B-cells, kindlin-3 is important for activation of lymphocyte function-associated antigen 1 (LFA-1), affecting cell adhesion under physiological shear flow condition [159]. Kindlin-3 also regulates B-cells trafficking and its immunoglobulin expression [160]. In addition, kindlin-3 plays crucial roles in T cell and natural killer cell function [161, 162]. It has been shown that kindlin-3 is important for $\alpha 4\beta 1$ and $\alpha L\beta 2$ integrin ligand binding [161]. Kindlin-3 regulates the stability of LFA-1/ICAM-1 binding in T-cells [163, 164]. Furthermore, the anti-tumour cytotoxicity activity of natural killer cells and their secretion of inflammatory cytokines are dependent on kindlin-3-integrin signaling pathway [165].

Higher expression of kindlin-3 in B-cell malignancies was detected in a proteomic study, but the role and function of kindlin-3 in blood cell malignancies are not well characterized [166]. In breast cancer tissues, an increase expression level of kindlin-3 mRNA has been reported. This apparently promotes Twist-mediated VEGF secretion that induces angiogenesis [167]. However, the presence of kindlin-3 in breast cancer tissues is intriguing given that kindlin-3 expression is mainly detected in hematopoietic cells. A possible explanation is that the kindlin-3 detected is derived from immune cells that have infiltrated the cancer tissue. Other group has also shown that kindlin-3 expression is reduced in melanoma, breast, lung and kidney tumours [87]. Hence, more studies are needed to clarify the role of kindlin-3 in cancer progression.

To date, few non-integrin binding partners of kindlin-3 have been identified. Kindlin-3 has been reported to interact with the hematopoietic-restricted adaptor protein ADAP and regulates integrin $\alpha IIb\beta 3$ activation in platelets [168]. Our group has shown that kindlin-3 interacts with the receptor for activated kinase C1 (RACK1) [101]. RACK1 is a ubiquitously expressed multifaceted scaffold protein with many interacting partners [169]. An overview of RACK1 structure and function is provided in section 1.5. In an early study, RACK1 has been shown to bind integrin β cytoplasmic tail [170]. As expected, integrin β cytoplasmic tail, RACK1 and kindlin-3 can form a ternary complex [101]. Interestingly, RACK1 is also a component of the 40S ribosome but its role in regulating protein translation remains to be fully characterized [171, 172]. Our group reported that kindlin-3 can associate with the 40S ribosome via RACK1 as the bridging molecule, and that this interaction may be

important for the proliferation of chronic myeloid leukemia cells [173]. However, the specificity determinant and binding interface between kindlin-3 and RACK1 require additional studies.

1.4 Cell Cycle

1.4.1 Cell cycle process

In this section, an overview of cell cycle progression is provided. Cell cycle is a process of cell growth, DNA replication and duplication, and separation of duplicated chromosomes into two daughter cells [174]. In cell cycle of eukaryotes, there are three phases: interphase, mitosis and cytokinesis. Interphase is divided into G₁, S, G₂ phase (**Figure 6**). At G₁ phase, cell grows without DNA replication [175]. DNA replication only occurred at S phase [176, 177]. At G₂ phase, cell continues to grow and prepares nutrients and proteins for mitosis [178]. An additional period in the cell cycle is referred to as the G₀ quiescent phase in which the cell is neither dividing nor preparing for cell division [179]. Cells remain at this phase until specific signal is received to induce their entry into the G₁ phase [180, 181]. However, cells such as mature cardiac muscle or nerve cells are permanently arrested at this phase and never enter the G₁ phase [182].

Figure 6 Cell cycle in eukaryotes. Cell cycle is divided into interphase, mitotic phase (M) and cytokinesis (C). Interphase includes G₁, S and G₂ phase. G₀ is the quiescent phase which cell arrest occurs. Mitotic phase consists of prophase, prometaphase, metaphase, anaphase and telophase. This figure was redrawn based on the reference Harashima, H., et al [183].

In mitosis, two genetically identical chromosomes are equally segregated into two daughter cells with proper mitotic spindles coupling [184]. Mitosis is divided into prophase, prometaphase, metaphase, anaphase and telophase (**Figure 7**). In prophase, chromosomes condensation occurs. Centrosomes are duplicated and move toward to the two opposite poles with spindles formation [185, 186]. In prometaphase, nuclear envelope breakdowns [187, 188]. Studies have reported that it is regulated by cyclin B1-CDK1 activity [189, 190]. Kinetochore, proteins locate at centromeres of each chromosome, attach to the mitotic spindles [174, 191, 192]. Sister kinetochores of chromosomes are attached to the opposite spindle poles and stably aligned at the middle of two centrosomes which is known as the metaphase plate [193-196]. Bi-orientated chromosomes formation in metaphase plate is required for the onset of anaphase [197-199]. When unattached kinetochores are detected by the spindle assembly checkpoint complex (SAC) [192, 197, 200], this inhibits the activity of anaphase-promoting complex/ cyclosome (APC/C) [197, 201, 202]. Sister chromatids of each chromosome are pulled apart toward the opposite poles of centrosomes in the anaphase [203-206]. During telophase, spindles are disassembled and nuclear envelope starts to reform around the two sets of decondensed chromosomes [187]. Lastly, two genetically identical chromosomes are separated by cleavage furrow into two daughter cells in cytokinesis [207-209].

Figure 7 Mitotic phase and cytokinesis process. Mitotic phase is divided into prophase, prometaphase, metaphase, anaphase and telophase. At prophase, chromosomes condense and become visible. Duplicated centrosomes sit at opposite poles and start to initiate spindles formation. At prometaphase, the nuclear envelope breaks down and chromosomes are attached to the spindles. At metaphase, chromosomes align at the middle of the metaphase plate and are attached to the opposite mitotic spindles. At anaphase, two genetically identical sister chromatids move toward the opposite poles. At telophase, the nuclear envelope reforms and mitotic spindles start to dissociate. In cytokinesis, the two identical sets of chromosomes are separated into two daughter cells. This figure was redrawn based on the reference Yanagida, M. [210].

1.4.2 Cell cycle checkpoints

Cell cycle is a tightly regulated process in order to maintain genome stability. Cell cycle progression is either delayed or terminated in the event of abnormal cellular processes detected by various checkpoint mechanisms [211]. Abnormal cell size, insufficient growth factors and nutrients, DNA damages are some examples of conditions that will lead to the arrest of cells at the G1/ S phase [212, 213]. During G2/ M phase, checkpoint mechanism ensures the proper and high fidelity of DNA replication before entry into mitosis [214]. As mentioned earlier, SAC in mitosis is activated in order to stop progression into anaphase when even one kinetochore is unattached or when chromosomes are not aligned at the metaphase plate [192, 197, 200, 215, 216]. Therefore, cell cycle checkpoints are crucial to ensure completion of one phase before the next [217-219].

1.4.3 Role of microtubules arrangement in cell cycle

Cancerous cells divide rapidly and bypass cell cycle checkpoints. In normal cell cycle, centrosomes play an important role in regulating equal number of chromosomes segregation through bipolar spindles coupling [220]. Duplicated centrosomes are precisely separated and serve as opposite spindle poles for mitotic spindle assembly in mitosis [220, 221]. On the other hand, abnormal centrosomes often lead to aberrant mitotic spindles formation, chromosomes mis-segregation, disruption of centrosome-associated cell cycle regulatory molecules and its cell polarity, which in turn promote cancer progression and genome instability [222-224]. Studies have shown that supernumerary centrosomes detected in solid tumours lead to aneuploidy and chromosome instability [225-228].

Centrosomes are also known as microtubule organizing centers. Centrosome consists of a pair of centrioles which is surrounded by pericentriolar materials [229]. At the onset of mitosis, duplicated centrosomes recruit proteins which are necessary for centrosome maturation in which microtubule nucleation increases [230]. Microtubule nucleation is facilitated by γ tubulin. It serves as the template for the initial event of microtubules formation and promotes polymerization of α and β tubulins heterodimers [231, 232]. Studies have shown that multipolar mitotic spindles (abnormal) are observed rarely in normal cells but commonly in cancerous cells (**Figure 8**) [233-239]. Multipolar cell division causes delay of chromosomes segregation to spindle poles during anaphase which cause lagging chromosomes [235, 236]. Even though chromosomes can align at supernumerary spindles

poles, multipolar cell division can lead to cell death [240]. Mis-segregation of chromosomes leads to unequal number of chromosomes in the daughter cells which is detrimental for cell survival. Therefore, cancerous cells overcome these problems of multipolar cell division and cell death by targeting several proteins such as microtubules-associated proteins, motor proteins, CLASP and sister chromatid cohesion proteins, which are involved in centrosomes clustering prior to anaphase onset [241-247]. Centrosomes clustering promote extra centrosomes to form two poles and perform bipolar division [223, 240, 248, 249]. This mechanism could reduce the consequences of chromosome instability [240].

Besides, microtubules dynamics are also regulated by post-translational modifications such as acetylation, tyrosination and glutamylation, through influencing the interaction of microtubules with microtubule-associated proteins and motor proteins [250, 251]. Acetylation of Lys40 in α tubulin is the only post-translational modification which occurs in the inner luminal tube of microtubules [252]. Acetylated tubulins are highly expressed in the microtubules of mitotic cells. A study has shown that kindlin-1 regulates mitotic spindle assembly through integrin activation or phosphorylation of Plk-1, which is one of the pericentriolar materials [127]. However, the role of kindlin-2 in mitosis remains to be characterized.

Figure 8 The arrangement of microtubules during metaphase. During normal metaphase, chromosomes align at the middle of the metaphase plate with (a) two centrosomes located at opposite poles and bipolar spindles formed between them. Abnormal spindle assembly includes (b) clustered supernumerary centrosomes and bipolar spindles formation; (c) multiple centrosomes and abnormal spindles formation. This figure was redrawn base on the reference Kamer, A., et al [253]

1.5 RACK1

1.5.1 Structure of RACK1

RACK1 is a scaffold protein [169] that is highly expressed in humans and higher mammals [254]. Human RACK1 is encoded by the gene *GNB2L1* (Guanine nucleotide binding protein (G-protein), beta polypeptide 2-like 1) [255]. It contains seven internal tryptophan-aspartate 40 repeat (WD-repeat) which form a seven bladed β propeller structure (**Figure 9**) [254].

1.5.2 RACK1 function

RACK1 regulates protein translocation, translation and cell migration [256, 257]. The WD repeats of RACK1 are docking sites for SH2 domains of Src and Fyn [258, 259], PH domains [260] and C2 domains of PKCs [261, 262]. RACK1 forms homodimer that promotes interactions with multiple binding partners [259, 263-267]. Homodimer RACK1 interacts with Fyn and NR2B subunit of methyl D-aspartate receptor (NMDAR) simultaneously [259, 266-268]. RACK1 also forms N-heterodimer with G β that is involved in cross-talk between signaling pathways mediated by GPCRs and ion channels especially cAMP/ PKA pathway and NMDAR [266].

Figure 9 Human RACK1 crystal structure. Top view (left) and side view (right) of RACK1. Numbering of WD repeats of bladed β propeller structure is from the N-terminus of RACK1. This figure is generated from the structural coordinate PDB 4AOW, derived from Ruiz Carrillo, D., et al., [269].

RACK1 binds to PKC β II and stabilizes its activated state [262, 270, 271]. In addition, RACK1 regulates site-specific substrate phosphorylation by PKC β II [271]. RACK1 also mediates cross-talks between PKC and MAP kinase (MAPK) pathways by serving as a scaffold protein that promotes PKC phosphorylation of JNK with MAPK [272]. It has also been shown that the depletion of nucleus Werner syndrome RecQ helicase like (WRN) protein caused RACK1 to translocate from the nucleus into the cytoplasm and to co-localize with PKC β II and PKC δ , suggesting that cytoplasmic PKC prevents RACK1 nuclear translocation [273].

RACK1 is involved in the cAMP/ PKA signaling pathway [266, 274, 275]. RACK1 competes with β -arrestin to bind to phosphodiesterase (PDE) isoform PDE4D5 [276]. Hydrolysis of cAMP is mediated through the interaction of RACK1 and PDEs [277]. In addition, cell polarity is regulated based on complex formation involving RACK1, FAK and PDE4D5 [278, 279]. Furthermore, activation of cAMP/ PKA pathway dissociates RACK1 from the complex, allowing its translocation into the nucleus, which promotes brain-derived neurotrophic factor (BDNF) transcription [268, 280]. Interaction of RACK1 and 14-3-3 ζ is also required for cAMP-dependent nuclear translocation of RACK1 that regulates BDNF transcription [281].

Interestingly, RACK1 serves as an inhibitor in Src and Fyn kinase interaction [259, 282]. In colorectal cells, cell growth was inhibited and the stabilization of E-cadherin and catenins at cell-cell contacts were observed as a result of Src inhibition through RACK1 binding [283, 284]. RACK1 interacts with NR2B subunit of NMDAR and Fyn concurrently, hence preventing NR2B phosphorylation by Fyn [259]. Upon activation of cAMP/ PKA pathway, RACK1 dissociates from this complex. This leads to the phosphorylation of NR2B by Fyn, which in turn up-regulates NMDAR channel activity [118, 259, 285].

RACK1 also associates with the C-terminus of Insulin-like Growth Factor Receptor I (IGF-IR) and recruits adaptor proteins, including Shc and IRS-1/2, phosphatase, Shp2 and transcription factor STAT3 [286-288]. An early study also showed that RACK1 interacts directly with the integrin β cytoplasmic tail and it is involved in integrin function [289].

Cell migration is an important process in embryonic development, wound healing and immune response. RACK1 co-localizes with paxillin at the leading edge of migrating cells [170] and it promotes FAK phosphorylation [290]. FAK phosphorylation is crucial for cell adhesion and spreading [286, 290,

291]. During cell migration, RACK1 interacts with PKC β [292] and PKC ϵ [293] that regulate RACK1 and Src interaction [294]. Association of RACK1, β integrins and Src regulate cell adhesion and spreading [170, 289]. Modulating paxillin during cell migration is dependent on RACK1-Src activity as well [295]. It has also been shown that RACK1 integrates signaling pathways of IGF-IR and integrins thereby promoting cancer cell migration [286, 287, 296]. RACK1 also regulates VEGF-mediated cell migration through PI3K/ Akt-Rac1 activation [297]. However, there is a study that showed that RACK1 binds to G β y, preventing PLC β and PI3K activation, leading to inhibition of T cell migration [263].

Notably, RACK1 is an eukaryote ribosomal protein, but it is not present in mammalian mitochondrial and protozoan ribosomes [172, 298]. RACK1 interacts directly with the 40S ribosome and it is located at the back of 40S subunit head region near the mRNA exit channel [172]. RACK1 is important for 80S ribosome formation. Mechanistically, RACK1 recruits PKC β II to the 40S ribosome, which allows PKC β II to phosphorylate Ser235 of eIF6 on the 60S ribosome. Phosphorylated eIF6 is released from the 60S ribosome, which promotes 60S and 40S association [299]. PKC β II also phosphorylates eIF4E to promote translation of growth and survival factors such as cyclin D1, c-Myc, survivin and Bcl-2 [300].

Specific mRNAs translation may be regulated by RACK1. RACK1 yeast homolog Asc1 is required for mRNA binding protein Scp160 to bind to the active form of translating ribosomes. Scp160 interacts with polyadenylated mRNAs translation [301]. Furthermore, recruitment of translation initiation ternary complexes eIF2-GTP-Met-tRNA is mediated by Asc1-eIF3c-40S interaction [302]. In addition, RACK1 plays an opposite role by recruiting miRNA-induced silencing complex (miRISC) in miRNA-mediated gene repression [303]. In yeast, RACK1 plays important role in nascent peptide-dependent translation arrest to modulate gene expression quality [304].

1.6 Exosomes

1.6.1 Overview of exosomes

Extracellular vesicles are a group of membrane-bound carriers which are released by cell. They include exosomes, microvesicles, microparticles and oncosomes [305]. Exosomes are small endocytic membrane derived-vesicles (approx. 40-100nm) that secreted by many cell types under physiological and pathological conditions [306, 307]. Exosomes contained a wide variety of

molecules, including mRNA, miRNA, DNA and proteins such as cytoskeletal proteins, major histocompatibility, signal transducers and heat shock proteins and those involved in membrane trafficking [308, 309]. CD63, CD81, CD9, TSG101, ALIX and HSP70 are frequently found in exosomes and they serve as markers in exosomes identification [310].

Exosomes are formed from the endocytic pathway. Multivesicle bodies (MVBs) are mature state of early endosomes. MVBs are either degraded by lysosomes or fused with plasma membrane to release exosomes into the extracellular space [310] (**Figure 10**). Information from producing cells is transferred to target cell through exosome uptake [311, 312]. Exosomes uptake via clathrin-mediated endocytosis have been identified in ovarian cancer [313], dendritic cells [314], and phagocytosis in monocytes and macrophage [315]. Exosomes can either fuse with the plasma membrane of target cell through ligand-receptor binding or they are internalized by receptor-mediated endocytosis [316]. Studies have also shown that exosomes are involved in antigen presentation, immune system stimulation, coagulation and cancer progression [317-319].

Figure 10 Exosome biogenesis. In brief, when early endosomes become MVBs, intraluminal vesicles are formed in the MVB by inward budding. Exosome-containing late MVBs are either degraded by lysosomes or released into the extracellular space as exosomes. This figure was redrawn based on the reference Raposo, G., et al [326].

1.6.2 Signaling properties of exosomes

Tumour-derived exosomes mediate cancer metastasis and angiogenesis [320, 321]. In addition, tumour-derived exosomes educate and recruit bone-marrow derived cells to form pre-metastatic niche [308]. Tumour-derived exosomes also transport immunosuppressive molecules to

inactivate T-lymphocytes or natural killer cells and promote Treg lymphocytes or myeloid cells [322]. Tumour-derived exosomes have been shown to stimulate matrix metalloproteinase 1 (MMP1) in gastrointestinal stromal cancer and promote cancer invasion [323]. In inflamed intestinal epithelial cell, exosomes are secreted as antigen presenting molecules which can regulate the response of distant immune cells [324]. In the respiratory system, isolated exosomes from bronchoalveolar fluid of sarcoidosis patients contain proinflammatory cytokines that promote progression of inflammation [325].

In the nervous system, exosomes are secreted from neurons and oligodendroglial cells [327, 328]. A recent study showed that synapsin I is packaged in glia-derived exosomes which mediate neurite outgrowth and neuronal survival in oxygen/ glucose deprivation conditions [329]. Exosomes are also secreted from cells associated with prions [330], α -synuclein [331], superoxide dismutase [332] and β -amyloid [333], thereby transmitting the pathogenesis to recipient cells.

Exosomes are present in healthy and pathological conditions. They are potential biomarkers in diagnosis of cancer, neurodegenerative, metabolic and infectious diseases [334]. Tetraspanin CD63 is a cancer protein marker which is highly expressed in exosomes derived from malignant cancer cells compared to healthy cells [335]. Another tetraspanin protein CD81 is a potential diagnostic marker on exosomes for hepatitis C [336]. Exosomes from brain tumour patients have been shown to contain epidermal growth factor receptor (EGFR), EGFRvIII and TGF- β [337]. In Alzheimer's disease patients, exosomes are found to contain β -amyloid peptides aggregate at brain plaques [333]. Together, exosomes are useful biomarkers in the diagnosis of different diseases. Exosomes could also have beneficial therapeutic effects in diseases such as myocardial ischemia reperfusion [338] and myocardial infarction [339]. They are also important in tissue regeneration [340, 341].

There is an increasing number of exosomes based therapeutic studies in recent years [342, 343]. Exosomes could be employed as a carrier for drug delivery. Exosomes could avoid phagocytosis and degradation and stay longer in the body [344]. Exosomes have been used to form complex with curcumin for treating inflammatory disease and cancer [345, 346]. 98% of potential drugs targeting the central nervous system are low in efficacy because they do not effectively pass through the blood-brain barrier [347]. Therefore, exosomes could be used to overcome this problem.

For example in Parkinson's disease, catalase was loaded into exosomes to form catalase-loaded exosomes (exoCAT) which efficiently delivered the antioxidant to targeted neurodegenerative region *in vivo* and *in vitro* [348]. In summary, exosomes are not only useful in diagnosis biomarkers, but also could be employed for drug delivery.

1.7 Objectives

This thesis contains four chapters of results.

1. Kindlin-1 has been demonstrated to regulate mitotic spindle assembly in breast cancer [127]. However, the roles of kindlin-2 and kindlin-3 in the regulation of mitotic spindle assembly remain to be determined. In Chapter 3, we investigate the role of kindlin-2 in mitosis of neuroblastoma cells.
2. Kindlin-3 has been shown to be important in leukemic cell proliferation. Our group has shown that kindlin-3, but not other kindlins, interacts with RACK1. However, the binding interface between kindlin-3 and RACK1 remains to be determined. In Chapter 4, we determine the specificity determinant in kindlin-3 that is crucial for its binding to RACK1.
3. Exosomes have been demonstrated to play important roles in cancer cell dissemination, and integrins in exosomes are essential for organotropic metastasis [154]. Whether exosomes contain kindlins and their functions remain to be determined. In Chapter 5, we examine the presence of kindlin-2 in exosomes derived from neuroblastoma cells and investigate its function.
4. Traction force is necessary for cell migration, cell shape and cellular homeostasis maintenance. Kindlin-3 has been reported to play a role in endothelial cell spreading and tube formation. In Chapter 6, we examine the effect on traction forces and directional contractions of HUVECs overexpressing kindlin-3.

Chapter 2 Methods and Materials

2.1 General materials and reagents

Most of the general chemicals and reagents were purchased from Sigma Aldrich (St. Louis, MO, USA), Merck (Darmstadt, Germany) and Thermo Fisher scientific (Waltham, Massachusetts, USA) unless stated otherwise.

Table 3 General Reagents

Name	Company
6x loading dye	Fermentas (Burlington, CA, USA)
DNA marker	
10 X PBS	Axil Scientific Pte Ltd (Singapore)
BSA	Sigma Aldrich (St. Louis, MO, USA)
β -mercaptoethanol	
DMSO	
Foetal Bovine Serum (FBS)	GE Healthcare (Little Chalfont, UK)
GelRed nuclei acid stain	Biotium Inc., USA
KAPA HIFI polymerase	KAPA Biosystems (Woburn, MA, USA)
Lipofectamine 2000 [®]	Thermo Fisher Scientific (Waltham, Massachusetts, USA)
Protein Marker	
Penicillin-Streptomycin	Hyclone Laboratories (South Logan, Utah, USA)
Polyfectamine transfection reagent	Qiagen (Venlo, Netherlands)
Sodium pyruvate	Gibco (Waltham, MA, USA)
Trypsin (0.25%, 1x, with 1mM EDTA.4Na)	
30 % Acrylamide/ Bis Solution, 37: 5: 1	Bio-rad (Hercules, CA, USA)
T4 ligase	Promega (Fitchburg, Wisconsin, USA)
TRIzol [®] reagent	Invitrogen (Carlsbad, CA, USA)

2.1.1 Media

Table 4 Media

Name	Reagent
Dulbecco's modified Eagle's medium (DMEM)	Hyclone Laboratories (South Logan, Utah, USA)
Endothelial cell Growth Medium-2 Bullet Kit (EGM) with supplements and growth factors	Lonza (Basel, Switzerland)
McCoy's 5A (modified) medium	Thermo Fisher Scientific (Waltham, Massachusetts, USA)
Roswell Park Memorial Institute medium (RPMI)-1640	Hyclone Laboratories (South Logan, Utah, USA)
Cell freezing medium	90 % (v/v) heat-inactivated FBS, 10 % (v/v) DMSO
LB broth	1 % (w/v) Bacto-tryptone (BD), 0.5 % (w/v) yeast extract (BD), 1 % (w/v) NaCl
LB agar	LB broth with 1.5 % (w/v) bacto-agar

2.1.2 Solution

Table 5 Solution

Name	Components
2 x Laemmli buffer	125 mM Tris (pH 6.8), 4 % (w/v) SDS, 20 % (v/v) Glycerol, 0.02 % Bromophenol Blue, 8 M Urea
Ampicillin	Stock: 60 mg/ mL ampicillin in miliQ ultrapure water
Cytoskeleton stabilization buffer	100 mM NaCl, 300 mM sucrose, 3 mM MgCl ₂ , 1 mM EGTA, 10 mM PIPES, pH 6.8
Kanamycin	Stock: 30 mg/ mL kanamycin in miliQ ultrapure water
Lysis buffer	10 mM Tris, 150 mM NaCl, 1 % (v/v) NP-40 pH 7.4
Puromycin	Stock: 10 mg/ mL puromycin in miliQ ultrapure water
Resolution gel buffer (4X)	1.5 M Tris (pH 8.8), 0.4 % (w/v) SDS
Stacking gel buffer (4X)	0.5 M Tris (pH 6.8), 0.4 % (w/v) SDS
SDS-PAGE running buffer (10x)	0.25 M Tris, 1.9 M glycine, 1 % (w/v) SDS
Stripping buffer	100 mM β -mercaptoethanol, 2 % (w/v) SDS, 62.5 mM Tris-HCl, pH 6.8
Tris-Acetic-EDTA (TAE) buffer (50x)	2 M Tris base, 5.71 % (v/v) acetic acid, 0.05 M EDTA, pH 8
Tris-Buffered Saline (TBST) buffer	25 mM Tris, 150 mM NaCl, 0.1 % (v/v) Tween-20
Western blot blotting buffer (10x)	25 mM Tris, 192 mM glycine, 20 % (v/v) Methanol

2.2 Experimental methods

2.2.1 Mammalian cell culture

All cells were cultured at 37 °C in a humidified 5 % CO₂ incubator.

2.2.1.1 Culture of HEK293T, MDA-MB-231 and SH-SY5Y cells

HEK293T cells (human embryonic kidney cells with SV40 large T antigen) and MDA-MB-231 (Human mammary gland epithelial cancerous cell) were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA) and SH-SY5Y human neuroblastoma cell line was kindly provided by Assistant Prof. Esther Wong (School of Biological Sciences, Nanyang Technological University). Cells were cultured in DMEM supplemented with 10 % (v/v) heat inactivated FBS, 100 IU/mL penicillin, 100 IU/mL Streptomycin and 1 mM sodium pyruvate.

2.2.1.2 Culture of HCT-116

HCT-116 cells (Human colorectal epithelial cancerous cell) were purchased from ATCC (Manassas, VA, USA) and cultured in McCoy's 5A (modified) medium containing 10 % (v/v) heat inactivated FBS, 100 IU/mL penicillin and 100 IU/mL Streptomycin.

2.2.1.3 Culture of HUVECs

HUVECs were purchased from LONZA (Basel, Switzerland) and cultured in EGM medium containing supplements and growth factors according to supplier's instructions.

2.2.1.4 Culture of COS-7 cells

COS-7 cells (African green monkey kidney fibroblasts) were purchased from ATCC (Manassas, VA, USA) and cultured in RPMI medium containing with 10 % (v/v) heat inactivated FBS, 100 IU/mL penicillin, 100 IU/mL Streptomycin and 1 mM sodium pyruvate.

2.2.2 Transfection and generation of stable mammalian cell lines

2.2.2.1 Neuroblastoma SH-SY5Y cell line

(a) SH-SY5Y 5HRE-GFP

SH-SY5Y cells (2×10^5) were seeded on 60 mm tissue culture dish the day before transfection. Transfection was performed using Lipofectamine[®] 2000. 3 µg 5HRE-GFP plasmid (#46926; Addgene, Watertown, Massachusetts, USA) and 3 µL of Lipofectamine[®] 2000 were mixed in individual tube which containing 250 µL serum free DMEM medium and incubated for 5 min at room temperature and followed by mixing and incubation for 20 min at room temperature. The cell culture medium of yesterday seeded SH-SY5Y cell was replaced with fresh DMEM full medium before adding plasmid-Lipofectamine mixture to the cells. Cells were maintained in medium with 900 µg/ mL G418-Sulphate (GE Healthcare, Piscataway, NJ, USA).

(b) SH-SY5Y pcDNA3.1 zeo(-) GFP-tagged kindlin-2

SH-SY5Y cells (2×10^5) were seeded on 60 mm tissue culture dish the day before transfection. Transfection was performed using Lipofectamine[®] 2000. 3 µg pcDNA3.1zeo(-) GFP-tagged kindlin-2 and 3 µL of Lipofectamine[®] 2000 were mixed in individual tube which containing 250 µL serum free DMEM medium and incubated for 5 min at room temperature and followed by mixing and incubation for 20 min at room temperature. The cell culture medium of yesterday seeded SH-SY5Y cell was replaced with fresh DMEM full medium before adding plasmid-Lipofectamine mixture to the cells. Cells were maintained in medium with 300 µg/ mL zeocin (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

(c) SH-SY5Y pGFP-C-shLenti-viral targeting kindlin-2

(i) Lentiviral supernatant preparation

HEK293T cells (2×10^6) were seeded on 100 mm tissue culture dish the day before recombinant lentivirus transfection. 15 μ g packaging lentivirus combo (1 μ g/ μ L) and 10 μ g pGFP-C-shLenti non-targeting shRNA control (# TR30021; Origene, Maryland, USA), pGFP-C-shLenti K2A (targeting sequence: GAAGAACTTATTGGAATTGCATACAACAG; #TL310359A; Origene, Maryland, USA) or pGFP-C-shLenti K2B (targeting sequence: ACAACAGTGACAAAGAAGTTGATGAAGTT ; #TL310359B; Origene, Maryland, USA) was prepared in individual tube which containing 1 mL of serum-free DMEM medium. 80 μ L PolyFectamine transfection reagent was diluted in 1 mL of serum free DMEM medium. Lentiviral plasmid combo and PolyFectamine solutions were mixed and incubated for 20 min at room temperature. Cell culture medium of yesterday seeded HEK293T was replaced with fresh DMEM medium before adding the corresponding lentiviral-PolyFect mixture. Medium of pGFP-C-shLenti-control shRNA, K2A shRNA and K2B shRNA transfected plates were removed and replaced with 10 mL fresh DMEM medium after 24 h of transfection. Viral supernatants of pGFP-C-shLenti-control shRNA, K2A shRNA and K2B shRNA were collected and centrifuged at 3000 r.p.m. for 15 min to remove cell debris after 48 h of transfection.

(ii) Lentivirus transduction

SH-SY5Y cells (2×10^5) were seeded on 6-well cell culture plate the day before the transduction. Cell culture medium was replaced with 1 mL fresh DMEM medium. 1 mL viral supernatants of pGFP-C-shLenti-control shRNA, K2A shRNA and K2B shRNA were added to corresponding well with 6 μ g/ mL final concentration Polybrene (Sigma-Aldrich, St. Louis, MO, USA). Cells were subjected to flow cytometry on a FACSAria flow cytometer (BD Biosciences, Mountain View, CA, USA) 24 h post-transfection. Fluorescent cells were sorted and maintained in medium with 1.5 μ g/ mL puromycin (Sigma-Aldrich, St. Louis, MO, USA).

(d) SH-SY5Y K2B shRNA targeting cells rescued with different K2B shRNA-resistant kindlin-2 constructs

K2B targeting shRNA SY5Y cells (1.5×10^5) were seeded on 6-well cell culture plate the day before transfection. Transfection was performed using TransIT-X2[®] reagent (Mirus Bio LLC, Madison, Wisconsin, USA). 2.5 μ g different K2B shRNA-resistant kindlin-2 plasmids (pcDNA3.1 zeo(-) siK2B targeting HA-tagged kindlin-2 QW/AA or Δ PH) and 7.5 μ L of TransIT-

X2[®] reagent were mixed in a tube containing 250 µL serum free DMEM medium and incubated for 30 min at room temperature. The cell culture medium of yesterday seeded cells was replaced with fresh DMEM full medium before adding plasmid-TransIT-X2[®] mixture to the cells. Cells were maintained in medium with 300 µg/ mL zeocin (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

2.2.2.2 Transfection of HEK293T cells

HEK293T cells (3×10^6) were seeded on 100 mm tissue culture dish the day before transfection. Transfection was performed using the Polyethylenimine (PEI) reagent (Sigma-Aldrich, St. Louis, MO, USA). In the single plasmid transfection, 9 µg plasmid (pcDNA3.11 zeo(-) HA-tagged kindlin-3 short form, long form and kindlin-3 long form truncated constructs) and 30 µg of PEI were mixed in 150 µL serum free DMEM medium. In the co-transfection procedure, 30 µg pcDNA3.1 zeo(-) HA-tagged kindlin-2, 10 µg mcherry-paxillin and 30 µg PEI were mixed in the serum free DMEM medium and followed by 15 min incubation at room temperature. The cell culture media was replaced with fresh DMEM full medium before adding the plasmid-PEI mixtures to the cells. Cells were harvested 24 h post-transfection for subsequent analyses.

2.2.2.3 Transfection of HUVECs

HUVECs (2×10^5) were seeded on each well of a 6-well cell culture plate the day before transfection. Transfection was performed using Lipofectamine[®] 2000. 1 µg of plasmid (EGFP-C1-vector or EGFP-C1-Kindlin-3) and 2 µL Lipofectamine[®] 2000 reagent were added into individual tube containing 250 µL of Opti-MEM[®] I reduced serum medium (Gibco, Waltham, MA, USA) and incubated for 5 min at room temperature. Thereafter, the reagents were mixed followed by incubation for 20 min at room temperature. The cell culture medium was replaced with fresh EGM-2 BULLET kit medium. The plasmid-Lipofectamine[®] 2000 solution was then added to the cells. Cells were analyzed 24 h post transfection.

2.2.2.4 Transfection of COS-7 cells

COS-7 cells (4×10^5) were plated onto glass-coverslip bottom 35 mm culture dish the day before transfection. Transfection was performed using the Polyfect transfection reagent (Qiagen, Venlo, Netherlands). 1 µg plasmid (YFP-N1-RACK1 with CFP-C1-vector, CFP-C1-kindlin-2 or CFP-C1-kindlin-3 long form) and 10 µL polyfect reagent were mixed in 100 µL serum free DMEM medium and

incubated for 10 min at room temperature. The cell culture medium was replaced with fresh DMEM full medium before adding the plasmid-Polyfect mixture to the cells. Cells were analyzed 24 h post-transfection.

2.2.3 DNA and RNA extractions

2.2.3.1 Miniprep and midiprep of plasmid DNA

A single colony of plasmid transformed *E.coli* DH5 α was inoculated in LB broth (6 mL for miniprep and 100 mL for midiprep) with appropriate antibiotics and cultured for 18 h at 37 °C on an orbital shaker. Bacteria were harvested by centrifugation and plasmids were extracted by using the Axygen miniprep (CA, USA) or Qiagen midiprep kit (Venlo, Netherlands).

2.2.3.2 RNA extraction

Total RNA was extracted from cells using the TRIzol[®] reagent according to manufacturer's instructions. RNA pellet was dissolved in RNase-free water (20-50 μ L depend on the cell pellet size).

2.2.3.3 DNA/ RNA concentration determination

Concentration of DNA/ RNA was measured by NanoDrop2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) based on the absorbance at wavelengths 230 nm, 260 nm and 280 nm.

2.2.4 Molecular cloning

2.2.4.1 Polymerase Chain reaction (PCR)

High fidelity PCR was performed using the KAPA HIFI DNA Polymerase with relevant primers. The PCR reaction was performed on a DNA Thermal Cycler (MJ Research, Waltham, MA, USA) or T100[™] Thermal Cycler (Bio-RAD, Hercules, CA). In KAPA HIFI DNA polymerase reaction, each reaction typically contained 100 ng DNA template, 0.3 mM dNTP mix, 0.3 μ M Forward and Reverse primers, 1x KAPA HiFi buffer and 1 unit of KAPA HIFI DNA polymerase.

2.2.4.2 Site directed mutagenesis

Nucleotide point mutations were generated using the KAPA HIFI DNA Polymerase. Appropriate overlapping primers containing the base substitution(s) were used in each reaction. Base substitution was verified by DNA sequencing (Bio Basic Asia Pacific Pte Ltd, Singapore).

2.2.4.3 Reverse transcription (RT) PCR

mRNA was reverse transcribed to complementary DNA (cDNA) using the Power SYBR[®] Green Cells-to-Ct[™] kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA) according to manufacturer's instructions. For the standard reverse transcription reaction for quantitative real-time PCR was performed at 37°C for 1 h and then 95 °C for 5 min.

2.2.4.4 Quantitative real-time PCR (qRT-PCR)

Quantitative real-time polymerase chain reaction (qRT-PCR) analysis was performed using either the Power SYBR[®] Green Cells-to-Ct[™] kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA) or KAPA SYBR Fast qPCR Master Mix Universal Kit (KAPA Biosystems, Woburn, MA, USA). Transcribed cDNA was mixed with qPCR Master Mix, forward and reverse primers and the reaction was performed on a CFX96[™] Real-time PCR detection system (Bio-RAD, Hercules, CA, USA) at 95 °C for 10 min, 95 °C for 30 sec, 60 °C for 30 sec and repeated from step 2 for 40 cycles. Primers for qRT-PCR were synthesized by Integrated DNA Technologies Singapore and they are listed below.

Table 6 Primers for qRT-PCR

Gene	Primers
Human VEGF-A	Forward: 5' GAAGTGGTGAAGTTCATGGATCTCTA 3' Reverse: 5' TGGAAGATGTCCACCAGGGT 3'
Human Kindlin2	Forward: 5' GAACAAGCAGATAACAGCGAGA 3' Reverse: 5' TGGAACCTTGCAATGAAGTG 3'
Hsa miR-138-5p	Forward: 5' GCGCAGCTGGTGTGTTGTGAATC 3' Reverse: 5' CTAAGTGTTGTGGTCGACGCG 3'
Human S18rRNA	Forward: 5' GTAACCCGTTGAACCCCAT 3' Reverse: 5' CCATCCAATCGGTAGTAGCG 3'
U6	Forward: 5' CTCGCTTCGGCAGCACATA 3' Reverse: 5' AACGCTTCACGAATTTGCG 3'
Human GAPDH	Forward: 5' GGTGAAGGTCGGAGTCAACG 3' Reverse: 5' CTCGCTCCTGGAAGATGGTG 3'

2.2.5 Expression plasmids generation

Expression plasmid pcDNA3.1 zeo(-) was purchased from Invitrogen (Carlsbad, CA, USA). The following plasmids were from Associate Prof. Suet-Mien Tan (Nanyang Technological University, Singapore): pcDNA3.1 zeo(-) HA-tagged kindlin-2, pcDNA3.1 zeo(-) GFP-tagged kindlin-2, pcDNA3.1 zeo(-) HA-tagged kindlin-3 long form, pcDNA3.1 zeo(-) HA-tagged kindlin-3 short form, CFP-C1

vector, CFP-C1 kindlin-2, CFP-C1 kindlin-3 long form, YFP-N1-RACK, mcherry-paxillin, EGFP- vector and EGFP-kindlin-3.

2.2.5.1 Human kindlin-3 constructs

(a) Kindlin-3 PH domain replaced with that of kindlin-2

Kindlin-3 PH domain (P350-Q480) replaced with kindlin-2 PH domain (P350-Q480) in pcDNA3.1zeo (-) HA-tagged kindlin-3 was generated by using the following primers indicated in Table 7, containing *EcoRI* and *KpnI* restriction sites. Set (a) primers were used to generate PCR product from *EcoRI* restriction site to region before PH domain (P350) of kindlin-3; set (b) primers were used to generate PCR product after PH domain (Q480) to the end of the kindlin-3 restriction site *KpnI*; set (c) primers were used to generate the PCR product of kindlin-2 PH domain (P350-Q480) with overhang sequences of kindlin-3. PCR products of using set (a) and (c) were used as templates for generating PCR product of the front portion of kindlin-3 with kindlin-2 PH domain by using primers (a-i) and (c-ii). Then this PCR product and PCR product of set (b) were used to generate the long form of kindlin-3 PH domain replaced with kindlin-2 PH domain (Kindlin-3 Δ PHK2) by primers (a-i) and (b-ii). The long form PCR product and pcDNA3.1zeo(-) vector were finally digested by restriction enzymes *EcoRI* and *KpnI* (New England Biolabs, Ipswich, Massachusetts, USA) and followed by T4 ligation at room temperature based on the manufacturer's instructions and transformed into *E.coli* DH5 α .

Table 7 Primers for Kindlin-3 PH domain (P350-Q480) replaced with kindlin-2 PH domain (P350-Q480)

	Primers	Sequences
(a)	(i) ECOR1-ACC-HA-F	5'CCGGAATTCACCATGTACCCATACGACGTGCCAGAC 3'
	(ii) K3F2-K2PH(FL)OH-R	5'GAAAACCTTTAATGTAGTCAGCAAGTTCAGGGATGGTGGTGAGGCTGTCCA 3'
(b)	(i) OHK2PH(FL)K3F2F	5'CAGAATATTCTTTCTTTCTGAAGATGCAGCGCACGGGCAGTGGGGGCCCG 3'
	(ii) KpnI- K3-R	5'CGGGGTACCTCAGAAGGCCTCATGGCCCCCGGT3'
(c)	(i) K3OH-K2PH (FL)-F	5'ACAGATGTGCTGGACAGCCTCACCACCATCCCTGAAC TTGCTGACTACATT 3'
	(ii) K2PH(FL)-K3OH-R	5'GTGGTTGCCCGGGCCCCCACTGCCCGTGCGCTGCATCTTCAGAAAGGAAAG 3'

Kindlin-3 partial PH domain (P350-V378, V379-K405, G406-Q480) substituted for corresponding kindlin-2 partial PH domain (P350-C378, T379-R405 G406-Q480) in pcDNA3.1zeo(-) HA-tagged kindlin-3 were also generated based on the aforementioned procedure. The following primers in Tables 8, 9 and 10 were used to generate the corresponding constructs.

Table 8 Primers for Kindlin-3 PH domain (P350-V378) replaced with to kindlin-2 PH domain (P350-C378)

	Primers	Sequences
(a)	(i) ECOR1-ACC-HA-F	5'CCGGAATTCACCATGTACCCATACGACGTGCCAGAC 3'
	(ii) K3F2-K2PH(FL)OH-R	5'GAAACTTTAATGTAGTCAGCAAGTTCAGGGATGGTGGTGAGGCTGTCCA 3'
(b)	(i) K2PHOH(350-378)K3PH(379)	5'ACTCTGAAAGGTTACAAACAATATTGGTGCGTGTTCAAGGAGAC CAACTG 3'
	(ii) KpnI- K3-R	5'CGGGGTACCTCAGAAGGCCTCATGGCCCCCGGT 3'
(c)	(i) K3OH-K2PH (FL)-F	5'ACAGATGTGCTGGACAGCCTCACCACCATCCCTGAACTTGCTG ACTACATT 3'
	(ii) PHK2(350-378)-OHK3-R	5'GTAGTAGGACAGTGTGGTCTCCTTGAACACGCACCAATATTGTT TGTAAACC 3'

Table 9 Primers for kindlin-3 PH domain (V379-K405) replaced with kindlin-2 PH domain (T379-R405)

	Primers	Sequences
(a)	(i) ECOR1-ACC-HA-F	5'CCGGAATTCACCATGTACCCATACGACGTGCCAGAC 3'
	(ii) K3(378)F2-K2PH(379)OH-R	5'ATAACAAGAAATGGATGTGTCTTTGAAGGTCACCCAGTGTGG CCGTAGCC 3'
(b)	(i) OHK2PH(405)-K3F2-F	5'GGCACACCAGCTCATCAGATGAACCTCAGGGGCTGTGAGGTG GTTCCCGAT 3'
	(ii) KpnI- K3-R	5'CGGGGTACCTCAGAAGGCCTCATGGCCCCCGGT 3'
(c)	(i) K3OH-K2PH (FL)-F	5'ACCCTGAAGGGCTACCGCCAACACTGGGTGACCTTCAAAGAC ACATCCATT 3'
	(ii) K2PH 405 – K3OH-R	5'GACGTTAACATCGGGAACCACCTCACAGCCCCTGAGGTTTCATC TGATGAGC 3'

Table 10 Primers for kindlin-3 PH domain (G406-Q480) replaced with kindlin-2 PH domain (G406-Q480)

	Primers	Sequences
(a)	(i) ECOR1-ACC-HA-F	5'CCGGAATTCACCATGTACCCATACGACGTGCCAGAC 3'
	(ii) PHK3 (405)-OHK2PH(406-480)-R	5'AAATGTTTACATCTGGGGTAACTTCACATCCCTTGAGGTTGAG CTGCTGAAT 3'
(b)	(i) OHK2PH(FL) K3F2-F	5'CAGAATATTCTTTCTTTCTGAAGATGCAGCGCACGGGCAGTG GGGGCCCG 3'
	(ii) KpnI- K3-R	5'CGGGGTACCTCAGAAGGCCTCATGGCCCCCGGT 3'
(c)	(i) K3PHOH(405)-K2PH(406-480)-F	5'TGGGGACCCCATTCAGCAGCTCAACCTCAAGGGATGTGAAGT TACCCAGAT 3'
	(ii) K2PH(FL)-K3OH-R	5'GTGGTTGCCCGGGCCCCCACTGCCCGTGCGCTGCATCTTCAG AAAGGAAAG 3'

(b) Human Kindlin-3 long form PH domain mutants

Kindlin-3 long form PH domain mutations were generated by site directed mutagenesis. Lys353 (K, underlined bold nucleotides) as indicated: ACC ACC ATC CCA GAG CTC **AAG** GAC CAT CTC CGA ATC TTT; His355 (H, underlined bold nucleotides) as indicated: ATC CCA GAG CTC AAG GAC **CAT** CTC CGA ATC TTT CGC ATC; His376 (H, underlined bold nucleotides) as indicated: CTG AAG GGC TAC CGC CAA **CAC** TGG GTG GTG TTC AAG GAG; Val378 (V, underlined bold nucleotides) as

indicated: GGC TAC CGC CAA CAC TGG **GTG** GTG TTC AAG GAG ACC ACA are used to design the relevant primer pairs for site directed mutagenesis. For generating all four mutations in one construct, site-directed mutagenesis was first performed using the following primers set (v-a) to create two mutations followed by mutations using primers set (v-b). The primers used are listed below.

(i) pcDNA3.1zeo(-) HA-tagged kindlin-3 long form K353A
K353A primers

Forward: 5' ACCACCATCCCAGAGCTCCGCGACCATCTCCGAATCTTT 3'

Reverse: 5' AAAGATTCCGAGATGGTCCCGCGAGCTCTGGGATGGTGGT 3'

(ii) pcDNA3.1zeo(-) HA-tagged kindlin-3 long form H355Y
H355Y primers

Forward: 5' ATCCCAGAGCTCAAGGACTATCTCCGAATCTTTCGCATC 3'

Reverse: 5' GATGCGAAAGATTCCGAGATAGTCCTTGAGCTCTGGGAT 3'

(iii) pcDNA3.1zeo(-) HA-tagged kindlin-3 long form H376Y
H376Y primers

Forward: 5' CTGAAGGGCTACCGCCAATACTGGGTGGTGTTCAGGAG 3'

Reverse: 5' CTCCTTGAACACCACCCAGTATTGGCGGTAGCCCTTCAG 3'

(iv) pcDNA3.1zeo(-) HA-tagged kindlin-3 long form V378C
V378C primers

Forward: 5' GGCTACCGCCAACAGTGGTGCGTGTTCAAGGAGACCACA 3'

Reverse: 5' TGTGGTCTCCTTGAACACGCACCACTGTTGGCGGTAGCC 3'

(v) pcDNA3.1zeo(-) HA-tagged kindlin-3 long form K353A H355Y H376Y V378C
(a) K353A H355Y primers

Forward: 5' ACCACCATCCCAGAGCTCCGCGACTATCTCCGAATCTTTCGCATC 3'

Reverse: 5' GATGCGAAAGATTCCGAGATAGTCCCGCGAGCTCTGGGATGGTGGT 3'

(b) H376Y V378C primers

Forward: 5' GGCTACCGCCAATACTGGTGCGTGTTCAAGGAGACCACA 3'

Reverse: 5' TGTGGTCTCCTTGAACACGCACCACTATTGGCGGTAGCC 3'

2.2.5.2 Human kindlin-2 constructs

(a) Kindlin-2 shRNA-resistant mutants

Silent mutations (underlined bold nucleotides) of pGFP-C-shLenti-K2B targeting site were generated in plasmid pcDNA3.1zeo(-) HA-tagged kindlin-2 and its mutant (kindlin-2 Q621W622/AA) and truncated (kindlin-2 ΔPH) construct as indicated: 5' AATAATAGCGAGAGGTGGACGAGGTC 3'.

These mutations of pcDNA3.1zeo(-) siK2B HA-tagged kindlin-2 and its mutants do not change the amino acid coding sequence.

Kindlin-2 mutation of Gln621 and Trp622 (Q and W, underlined bold nucleotides) as indicated: TGG CGT TTC AGC AAC ATG AAA **CAG TGG** AAT GTC AAC TGG GAA ATC were generated by site directed mutagenesis. The primers used to mutate Gln621Trp622 in kindlin-2 into Ala are listed below:

pcDNA3.1zeo(-) siK2B HA-tagged kindlin-2 Q621W622/AA

Q621A W622A primers

Forward: 5' TGGCGTTTCAGCAACATGAAAGCGGCGAATGTCAACTGGGAAATC 3'

Reverse: 5' GATTTCCTCAGTTGACATTGCGCGCTTTTCATGTTGCTGAAACGCCA 3'

Kindlin-2 PH deleted (Δ PH) construct was generated by using the following primers which contained *XbaI* and *KpnI* restriction sites. Set (a) primers were used to generate the PCR product from the *XbaI* restriction site to the region before PH domain (P350); set (b) primers were used to generate PCR product from the region after PH domain (Q480) to the end of the restriction site *KpnI*. After the PCR product of set (a) and (b) were generated and used as templates for primers (a-i) and (b-ii) to synthesize the Kindlin-2 Δ PH construct and followed by *XbaI* and *KpnI* restriction enzymes (New England Biolabs, Ipswich, Massachusetts, USA) double digestion on the PCR product and pcDNA3.1 zeo(-) vector as well. The ligation of digested vector and PCR product were ligated by T4 ligase at room temperature based on the manufacturer's instructions and transformed into *E.coli* DH5 α

(a) (i) *XbaI*-HA-F1: 5' TGCTCTAGAACCATGTACCCATACGACGTGCCAGAC 3'

(ii) K2-F2 OH-R1: 5' GTCCGCCATGGTCTTGCCTTTAATGTAGTCAGCAAG 3'

(b) (i) OH-K2-F2-F2: 5' CTTGCTGACTACATTAAAGGCAAGACCATGGCGGAC 3'

(ii) *KpnI*-K2-R2: 5' CGGGGTACCTCACACCCAACCACTGGTAAGTTTGTA 3'

(b) Kindlin-2 PH domain (P350-Q480) replaced with kindlin-3 PH domain (P350-Q480)

Kindlin-2 PH domain (P350-Q480) replaced with kindlin-3 PH domain (P350-Q480) in pcDNA3.1zeo (-) HA-tagged kindlin-2 was generated by using primers listed in Table 11 containing *XbaI* and *KpnI* restriction sites. The procedure of generating this construct is the same as that used to generate kindlin-3 PH domain replaced with that of kindlin-2 (refer to section 2.2.5.1 (a)).

Table 11 Primers for kindlin-2 PH domain (P350-Q480) replaced with kindlin-3 PH domain (P350-Q480)

	Primers	Sequences
(a)	(i) XbaI-ACC-HA-F (K2)	5'TGCTCTAGAACCATGTACCCATACGACGTGCCAGAC 3'
	(ii) K2F2-K3PH(FL)OH-R	5'AAAGATTTCGGAGATGGTCCTTGAGCTCTGGAATGGAA GTAATGTCACCCAA 3'
(b)	(i) OHK3PH(FL)-K2F2-F	5'CAGGCCATCCTGGCCTTCCTCAGCCTGCAGCATTTAA ACCCAGATCCTCAG 3'
	(ii) KpnI-K2-R	5'CGGGGTACCTCACACCCAACCACTGGTAAGTTT 3'
(c)	(i) K2OH-K3PH(FL)-F	5'TCAACAATTTTGGGTGACATTACTTCCATTCCAGAGCT CAAGGACCATCTC 3'
	(ii) K3PH(FL)-K2OH-R	5'TGGTATTAACCTGAGGATCTGGGTTTAAATGCTGCAGG CTGAGGAAGGCCAG 3'

2.2.6 Protein detection

2.2.6.1 Immunoprecipitation

HEK293T transfectants were lysed in lysis buffer (refer to Table 5) containing protease inhibitor cocktail (Roche, Basel, Switzerland) on ice for 30 min. The lysate was pre-cleared with 3 µg of mouse serum IgG (Sigma-aldrich, St. Louis, MO, USA) and Protein A-Sepharose beads (GE Healthcare, Piscataway, NJ, USA) for 30 min at 4 °C on a roller. The pre-cleared lysate was separated into two equal portions. 3 µg mouse serum IgG was added to one portion and 3 µg Anti-HA mouse antibody (Merck, Darmstadt, Germany) was added into the other. Protein A-Sepharose beads were added to each portion. The samples were incubated for 3 h at 4 °C on a roller. Precipitated proteins on beads were washed three times in lysis buffer. Precipitated proteins were eluted by boiling the beads for 10 min in 2 x laemmli buffer (refer to Table 5) containing 40 mM DTT.

2.2.6.2 Immunoblotting

Cells were lysed in lysis buffer (refer to Table 5) with protease inhibitor (Roche, Basel, Switzerland) and phosphatase inhibitor (Nacalai Tesque, Kyoto, Japan). Total protein concentration was measured by using bicinchoninic acid assay kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA) based on the manufacturer's procedure. Proteins were separated by running SDS-PAGE under reducing conditions and electro-transferred onto polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories, Hercules, CA, USA). PVDF membrane was incubated in 5 % (w/v) non-fat milk in TBST blocking solution (refer to Table 5) at room temperature for 30 min. For phosphorylated protein immunoblotting, PVDF membrane was blocked in 5 % (w/v) BSA in TBST instead. The membrane was incubated with the relevant primary antibodies (Table 12) in blocking solution at 4 °C roller overnight. The membrane was washed three times with TBST and incubated with corresponding

HRP-conjugated secondary antibody (Table 13) at room temperature for 1 h. Membrane was washed three times in TBST before performing enhanced Chemiluminescence (ECL) detection of protein bands using the ECL-kit (Advansta Inc., Menlo Park, CA, USA). Protein bands were visualized on a Western Blot imaging system (Bio-RAD, Hercules, CA, USA) or by conventional X-ray film exposure method.

Table 12 Primary antibodies

Antibody	Source	Specificity	Species	Dilution	Concentration
Clone 9	Generated and purified by TSM lab	Anti-kindlin-3 (F3 subdomain)	Rat	1: 1000	1.4 µg/ mL
Clone 181A	Generated and purified by TSM lab	Anti-kindlin-3 (PH domain)	Rat	1: 1000	0.51 µg/ mL
RACK1 (B-3)	Santa Cruz Biotechnology (Santa Cruz, CA, USA)	Anti-RACK1	Mouse	1: 2500	0.08 µg/ mL
Kindlin-2	Sigma-aldrich (St. Louis, MO, USA)	Anti-Kindlin-2	Rabbit	1: 1000	1 µg/ mL
Anti-acetylated tubulin		Anti-acetylated tubulin	Mouse	1: 1000	1 µg/ mL
Anti-β tubulin		Anti-β tubulin	Mouse	1: 2500	0.4 µg/ mL
GAPDH	Cell Signalling Technology (Danvers, MA, USA)	Anti-GAPDH	Rabbit	1: 2500	0.0168 µg/ mL
Histone		Anti-Histone	Mouse	1: 1000	0.116 µg/ mL
Alix		Anti-Alix	Mouse	1: 1000	0.1 µg/ mL
Cyclin B1 (D5C10)		Anti-Cyclin B1	Rabbit	1: 1000	0.92 µg/ mL
Anti-AKT		Anti-AKT	Rabbit	1: 1000	0.034 µg/ mL
Anti-phosphorylated AKT (Ser473)		Anti-phosphorylated Ser473 AKT	Rabbit	1: 1000	0.01 µg/ mL
Anti-GSK3β (27C10)		Anti-GSK3β	Rabbit	1: 1000	0.013 µg/ mL
Anti-phosphorylated GSK3β(Ser9) (5B3)		Anti-phosphorylated Ser 9 GSK3β	Rabbit	1: 1000	0.057 µg/ mL
Anti-HDAC6 (D2E5)		Anti-HDAC6	Rabbit	1: 1000	0.046 µg/ mL
anti-HIF-1α (D1S7W)		anti-HIF-1α	Rabbit	1: 1000	0.1 µg/ mL
Anti-Actin Ab-5		Anti-actin	Mouse	1: 1000	0.25 µg/ mL
CDK1/Cdc2 (pY15)		Anti-CDK1/Cdc2	Mouse	1: 1000	0.25 µg/ mL
Anti-HA tag	(Merck, Darmstadt, Germany)	Anti-HA	Mouse	1: 1000	1 µg/ mL
Paxillin (5H11)		Anti-Paxillin	Mouse	1: 1000	1 µg/ mL
Anti-phosphorylated CDK1/Cdc2 (pY15)	Abcam (Cambridge, UK)	Anti-pY15 CDK1/Cdc2	Mouse	1: 1000	0.25 µg/ mL
Annexin V		Anti-Annexin V	Rabbit	1: 1000	0.5 µg/ mL
TSG101		Anti-TSG101	Mouse	1: 1000	1 µg/ mL
HSP70		Anti-HSP70	Mouse	1: 1000	1 µg/ mL
Anti-phosphorylated HDAC6 (Ser22)	Thermo Fisher Scientific (Waltham, Massachusetts, USA)	anti-phosphorylated Ser22 HDAC6	Rabbit	1: 1000	1 µg/ mL
Anti-α tubulin		Anti-α tubulin	Rabbit	1: 1000	1 µg/ mL

Table 13. Secondary antibodies

Antibody	Source	Dilution
Amersham™ ECL™ Anti-Rat IgG, HRP-Linked Whole Antibody (From goat)	GE Healthcare (Piscataway, NJ, USA)	1: 5000
Goat-anti-mouse IgG (H+L) HRP conjugate	Advansta (Menlo Park, CA, USA)	1: 10000
Goat-anti-Rabbit IgG (H+L) HRP conjugate	Advansta (Menlo Park, CA, USA)	1: 10000

2.2.6.3 Membrane stripping for reblotting

In some experiments, after the first round of protein detection, the membrane was stripped of antibodies in stripping buffer (refer to Table 5) for 30 min at 55 °C. The membrane was washed and incubated in TBST buffer (refer to Table 5) every 10 min for six times. The membrane was then subjected to a second round of immunoblotting with a different antibody.

2.2.7 Fluorescence Resonance Energy Transfer (FRET) analysis

FRET analyses were performed according to modified published protocol [349, 350]. COS-7 cells were co-transfected YFP-N1-RACK1 with CFP-C1 empty vector, CFP-C1-kindlin-2 or CFP-C1-kindlin-3 long form. FRET was performed on a Zeiss LSM 510 confocal microscope (Carl Zeiss, Inc., Thornwood, NY, USA). The excitation wavelength and emission filter of eCFP are 458 nm and BP 470-500 nm; and eYFP are 514 nm and LP 539 nm. eYFP of whole cell was photobleached at 5th time point to 6th time point by 20 times scanning of the whole cell using 514 argon laser line that was set at maximum intensity. eCFP signal of whole cell excluded nucleus was acquired before and after photobleaching. FRET Efficiency = $(I_6 - I_5) / I_6 \times 100 \%$, where I is the eCFP intensity before and after eYFP bleaching at n^{th} time point. Mean noise = $(I_6 - I_5) / I_6 \times 100\%$, where I is the eCFP intensity without eYFP bleaching on the cell of at n^{th} time point. Mean noise was close to zero in all cases.

2.2.8 Micro-posts experiments

Micro-posts were generated and kindly provided by Prof. Jianping Fu (University of Michigan, USA). The parameters of the micro-posts are: Height 8.3 μm , diameter 1.83 μm , spacing between posts 6 μm , spring constant 7.22 nN/ μm and effective modulus 5.65 kPa. Micro-posts were fixed on cover-glass culture dishes.

2.2.8.1 Live cell imaging

Micro-posts were either coated with 20 $\mu\text{g}/\text{mL}$ fibronectin (Sigma Aldrich, St Louis, MO, USA) in PBS or 20 $\mu\text{g}/\text{mL}$ rat tail collagen type I (BD Bioscience, Mountain View, CA, USA) in 0.02 N acetic acid at

37 °C for 2 h. Micro-posts were rinsed with PBS before HUVECs seeded. HUVECs (4×10^5 cells) were seeded on ECM-coated micro-posts and incubated for 10 min at 37 °C in a humidified 5 % CO₂ incubator before placing in an adaptor which was connected with 5 % CO₂, on the stage of inverted fluorescence microscope (Olympus, IX70, 40X|0.60 Ph2 objective, equipped with CCD camera) (Olympus, Tokyo, Japan). It was customized with a built-in plastic box which was connected to a 37 °C temperature incubator. Individual cell was selected randomly within 3 min after micro-posts were placed in the adaptor. Cell in contact with another cell was not selected as this would affect the analyses of cell spreading and defection of posts. Phase contrast live cell images were captured every minute for 2 h using MetaMorph software.

Micro-posts were cleaned by ultrasound in a water bath sonicator (Elma, Schmidbauer GmbH, Germany). After that, micro-post was either re-coated with appropriate ECM proteins or left un-coated in water for repeating experiments.

The calculations based on the data obtained were performed by our collaborator Dr. Jing-Jing Han from the School of Physical and Mathematical Sciences, Nanyang Technological University, Singapore.

2.2.8.2 Immunofluorescence staining and imaging

HUVECs (4×10^5 cells) were seeded on ECM-coated micro-posts and incubated for 30 min at 37 °C, humidified 5 % CO₂ incubator. Cells were fixed with 3.7 % (w/v) paraformaldehyde in PBS for 10 min at room temperature and permeabilized in cytoskeleton stabilization buffer (refer to Table 5) with 0.25 % (v/v) Triton X-100 for 1 min at room temperature. Cells were washed by three times in PBS and non-specific binding sites were blocked by incubating cells in PBS containing 1 % (w/v) BSA (Sigma-aldrich, St. Louis, MO, USA) for 30 min at room temperature. Cells were stained with 5 µg/ mL anti-paxillin (clone 5H11) (Merck, Darmstadt, Germany) in 1 % BSA at 4 °C overnight. Cells were washed five times before staining with Alexa Fluor® 488-conjugated goat anti-mouse IgG secondary antibody (1:600 dilution) (Invitrogen, Carlsbad, CA, USA). Images of cells were captured under confocal laser microscope (Zeiss LSM510, Carl Zeiss, Germany) with 63X oil immersion objective lens. Data were analyzed using the Zen 2012 software.

2.2.9 Neuroblastoma SH-SY5Y mitosis and hypoxia experiments

2.2.9.1 Electric cell-substrate impedance sensing (ECIS) measurement

16-well ACEA chip (ACEA Biosciences, San Diego, CA) was incubated with 4 mg/ mL Dithiobis succinimidyl propionate (Pierce; Thermo Fisher Scientific, Rockford, IL) in DMSO (Thermo Fisher Scientific, Waltham, Massachusetts, USA) at room temperature for 30 min. Wells were rinsed with autoclaved water twice before coating with 2.5 $\mu\text{g}/\text{cm}^2$ fibronectin per well at room temperature for 3 h. Wells were rinsed with autoclaved water three times and 100 μL DMEM fresh medium was added to each well. Background signal was measured by real-time cell electronic system (ACEA Biosciences, San Diego, USA). pGFP-C-shLenti control shRNA and K2B shRNA transfected SH-SY5Y cells (8×10^4 per well) were seeded. Data was measured 5 min per interval for 1120 min. Cell index represents cell adhesion or spreading. Each datum point represents standard deviation of technical triplicates points.

2.2.9.2 3D Transwell Assay

1×10^6 Cells (SH-SY5Y WT, pGFP-C-shLenti control shRNA and K2B shRNA transfected SH-SY5Y) were seeded on 100 mm culture dish the day before the experiment. On the day of experiment, cells were starved in serum-free DMEM medium for 6 h at 37 °C, humidified 5 % CO_2 incubator and followed by seeding (6×10^4) cells with serum-free medium on 2.5 $\mu\text{g}/\text{cm}^2$ fibronectin coated 6.5 mm Transwell® with 8 μm pore polycarbonate membrane insert (Corning Incorporated, New York, USA) which was inserted into 24-wells culture plate containing DMEM full medium for 18 h in 37 °C, humidified 5 % CO_2 incubator. After that, inserted transwell was fixed with 3.7 % (w/v) paraformaldehyde in water for 10 min at room temperature and permeabilized in cytoskeleton stabilization buffer (refer to Table 5) with 0.25 % (v/v) Triton X-100 for 2 min at room temperature. Inserted transwell was washed with 1 X PBS before staining with 0.5 % (w/v) crystal violet in 1 X PBS for 20 min at room temperature. Inserted transwell was rinsed in 1 X PBS several times before adding cytoskeleton stabilization buffer (refer to Table 5) with 0.25 % (v/v) Triton X-100 for crystal violet dissociation. The crystal violet readings were measured at absorbance 595 nm by Benchmark Plus microplate spectrophotometer (Bio-RAD, Hercules, CA, USA)

2.2.9.3 Wound healing analysis

Commercial culture inserts (Ibidi GmbH, Martinsried, Germany) was used to perform the 2D wound healing assay. Culture insert was placed on uncoated 35 mm cell-culture dish. SH-SY5Y cells (4×10^4) were resuspended in 70 μ L culture media and seeded into each chamber of the culture insert. Cells were incubated at 37 °C in a 5 % CO₂ incubator for 30 min. The culture insert was removed and 2 mL of culture media was added to fill up the culture dish. The culture dish was captured using 4X objective (UPLFLN-PH, Olympus) on a motorized Olympus IX83 inverted microscope system equipped with 37 °C and 5 % CO₂ incubator stage and a cooled monochrome digital camera. The Phase contrast live cell images were captured every 30 min for 22.5 h by using CellSens Dimension software.

2.2.9.4 Soft agar spheroid tumour formation

SH-SY5Y WT, pGFP-C-shLenti control shRNA and K2B shRNA transfected SH-SY5Y cells (8×10^3 in 100 μ L DMEM medium per well) were seeded on 1 % autoclaved agarose gel (Vivantis Inc., USA) coated 96-well plate. Seeded plates were centrifuged at 500 r.p.m. for 5 min before incubating at 37 °C, humidified 5 % CO₂ incubator for 7 days. The phase contrast spheroids images were captured and measured using 4X objective (UPLFLN-PH, Olympus) by using CellSens Dimension software.

2.2.9.5 Mitotic cells synchronization

Cells (SH-SY5Y WT, pGFP-C-shLenti control shRNA and K2B shRNA transfected SH-SY5Y or kindlin-2 WT and its mutants re-expressed in K2B targeting shRNA SH-SY5Y cells) were seeded on 2 μ g/ cm² laminin- (Roche, Basel, Switzerland) or 2.5 μ g/ cm² fibronectin- (Sigma Aldrich, St. Louis, MO, USA) coated culture dish in the first day morning. Medium was discarded and changed with fresh media containing thymidine (Sigma Aldrich, St. Louis, MO, USA) at a final concentration of 2 mM in the evening and incubated for 14 h at 37 °C in a humidified 5 % CO₂ incubator. Medium was discarded and cells were washed in 1 x PBS twice before adding fresh media containing deoxycytidine (Sigma Aldrich, St. Louis, MO, USA) at a final concentration of 24 μ M. Cells were incubated in this supplemented medium for 9 h at 37 °C in a humidified 5 % CO₂ incubator and then adding thymidine (final concentration 2 mM) incubated for another 14 h at 37 °C, humidified 5 % CO₂ incubator. Cells were washed in 1 x PBS twice and replaced with fresh media containing final concentration of 24 μ M deoxycytidine for 2 h at 37 °C in a humidified 5 % CO₂ incubator. Cells were

washed with 1 x PBS twice before adding fresh media containing final concentration of 10 μ M RO3306 (Sigma Aldrich, St. Louis, MO, USA) for 19 h at 37 °C in a humidified 5 % CO₂ incubator. After 19 h RO3306 release, cells were washed in 1 x PBS twice and added with fresh medium either immediately captured live-cell imaging or incubated for 90 min at 37 °C in a humidified 5 % CO₂ incubator and then harvested or fixed for mitotic cells for immunoblotting or immunofluorescence staining. For tubacin experiment, cells were subjected to 2 μ M tubacin (Sigma-aldrich, St. Louis, MO, USA) for 16 h before RO3306 release. For manganese (II) chloride treatment, cells were subjected to a final concentration of 0.2 mM manganese (II) chloride (Sigma-aldrich, St. Louis, MO, USA) supplemented DMEM fresh media for 90 min after RO3306 release.

2.2.9.6 Live-cell imaging of mitotic cells and calculation of mitotic rate

SH-SY5Y WT, pGFP-C-shLenti control shRNA, K2B shRNA transfected SH-SY5Y and the re-expression kindlin-2 WT and its mutants on K2B shRNA targeting SY5Y cells (4×10^4) were seeded on 2 μ g/ cm² laminin- (Roche, Basel, Switzerland) or 2.5 μ g/ cm² fibronectin- (Sigma Aldrich, St. Louis, MO, USA) coated 12-well cell culture plate in the first day morning and then followed the cells synchronization steps which have mentioned earlier (refer to section 2.2.9.5). After 19 h RO3306 treated, cells were washed in 1 x PBS twice and fresh medium added. Plate was immediately placed on a motorized Olympus IX83 inverted microscope system equipped with 37 °C and 5 % CO₂ incubator stage and a cooled monochrome digital camera. The capturing field was selected randomly within 5 min after the plate was placed in the adaptor. Phase contrast live cell images were captured using 10X objective (UPLFLN-PH, Olympus) every 2 min for 6 h by using the CellSens Dimension software. Mitotic rate was calculated based on the time-point when a mitotic cell divides into two daughter cells.

2.2.9.7 HDAC6 activity assay on mitotic cells

SH-SY5Y WT, pGFP-C-shLenti control shRNA and K2B shRNA transfected SH-SY5Y (4×10^5) were seeded on 2.5 μ g/ cm² fibronectin- (Sigma Aldrich, St. Louis, MO, USA) coated 100 mm cell culture plate in the first day morning and then followed the cells synchronization steps mentioned in previous section (refer to section 2.2.9.5). After 90 min RO-3306 release, mitotic cells were harvested for HDAC6 activity assay based on manufacturer's procedure (BioVision incorporation Inc., San Francisco, CA, USA). First portion of mitotic cells were lysed in HDAC6 lysis buffer from the assay kit

whilst second portion was lysed in lysis buffer (refer to Table 5) containing protease inhibitor (Roche, Basel, Switzerland). The second portion of lysis buffer was used to measure the total protein concentration by using bicinchoninic acid assay kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA) based on the manufacturer's procedure. Same amount of total protein concentration of each sample was added to 96 wells clear flat bottom white polystyrene plate (Sigma Aldrich, St. Louis, MO, USA) and each well was topped up with HDAC6 assay buffer from the assay kit to a final volume of 50 μ L. 50 μ L of HDAC6 substrate mix was added into each sample's well and incubated at 37 °C in the dark for 30 min. Developer reagent was added to each well for 10 min at 37 °C in dark to terminate the reaction. The fluorescence signal was measured at Ex/ Em: 380/ 490 nm. Each group of data was normalized based on its SH-SY5Y WT mitotic cells in the same group.

2.2.9.8 Hypoxia treatment

MDA-MB-231, HCT-116 and SH-SY5Y cells (2×10^6 for 24 h and 48 h hypoxia treatment; 4×10^5 for 96 h hypoxia treatment) were seeded on 100 mm tissue culture dishes the day before treatment. Cells were seeded on two dishes for each time point (normal and hypoxia conditions). Fresh medium was replaced before placing them in the hypoxia incubator chamber. A flow meter (Dwyer Instruments Inc., MICH, USA) was connected to the outlet tube of the hypoxic mixture gas (95 % N_2 , 5 % CO_2) and inlet tube of the hypoxia incubator chamber. The hypoxic chamber was flushed with the hypoxic gas mixture at the 10 L/ min for 3 min. After which, both inlet and outlet switches of the chamber were closed. The chamber was flushed twice with the hypoxic gas mixture on the same day.

2.2.9.9 Cell Proliferation and viability assay

Cell number was measured using a ADAM-MC™ Automatic Cell Counter (NanoEnTek, Inc., Korea). Cell proliferation and viability were analyzed and compared between time points of cells subjected to normoxia or hypoxia conditions.

2.2.9.10 3D spheroid formation and flow cytometry analyses of cells

SH-SY5Y 5HRE-GFP cells (8×10^3 in 100 μ L DMEM medium per well) were seeded on 50 μ L 1 % autoclaved agarose gel (Vivantis Inc., USA) coated 96-well plate. Seeded plates were centrifuged at 500 r.p.m. for 5 min before incubating at 37 °C in a humidified 5 % CO_2 incubator for 7 days. Spheroids were trypsinized and resuspended in 1 x PBS containing 20 % FBS. Cells were filtered (to eliminate cell clumps) and subjected to cell sorting on a FACSAria flow cytometer (BD Biosciences,

Mountain View, CA, USA). Cells were sorted into high fluorescent and low fluorescent groups and data were analyzed using the FlowJo software.

2.2.9.11 Immunofluorescence staining and imaging

SH-SY5Y WT, pGFP-C-shlenti control shRNA, K2B shRNA transfected SY5Y cells and re-expression kindlin-2 WT and its mutants on K2B shRNA targeting SY5Y cells (3×10^3) were seeded on $2.5 \mu\text{g}/\text{cm}^2$ fibronectin-(Sigma Aldrich, St. Louis, MO, USA) coated glass bottom 14 mm microwell dish (MatTek Corporation, Ashland, MA, USA) in the first day morning and then followed the cells synchronization steps which have mentioned earlier (refer to section 2.2.9.5). Cells were incubated 90 min before immunofluorescence staining procedure. Cells or tumour slides were fixed with 3.7 % (w/v) paraformaldehyde in PBS for 10 min at room temperature and permeabilized in cytoskeleton stabilization buffer (refer to Table 5) with 0.25 % (v/v) Triton X-100 for 1 min at room temperature. Cells or tumour slides were washed three times with 1 x PBS and non-specific binding sites were blocked by incubating cells with 1 x PBS containing 5 % (w/v) BSA (Sigma Aldrich, St. Louis, MO, USA) for 30 min at room temperature. Cells or tumour slides were then stained with relevant primary antibody (Table 14) in 5 % BSA at room temperature for 1 h. Cells or tumour slides were washed three times before staining with corresponding secondary antibody (Table 15) in 1 x PBS with DAPI (0.1 mg/ mL) or together with Alexa Fluor-594-phalloidin (0.27 ng/ mL, for cyro-sectioned tumour slide). Images of cells or tissue were captured under confocal laser microscope (Zeiss LSM710, Carl Zeiss, Germany) with 10X or 63X oil immersion objective lens. Data were analyzed using Zen 2012 software. Normal and abnormal spindles were determined by analyzing the alignment of the chromosomes and spindle distribution while at metaphase. Fluorescence intensity ratio of acetylated tubulin and α -tubulin was processed and calculated by using the image-J software.

Table 14 Primary antibodies for immunofluorescence staining

Antibody	Source	Specificity	Species	Dilution	Concentration
Kindlin-2	Sigma-aldrich (St. Louis, MO, USA)	Anti-Kindlin-2	Rabbit	1: 100	10 $\mu\text{g}/\text{mL}$
Anti-acetylated tubulin		Anti-acetylated tubulin	Mouse	1: 100	10 $\mu\text{g}/\text{mL}$
Anti- α tubulin	Thermo Fisher Scientific (Waltham, Massachusetts, USA)	Anti- α tubulin	Rabbit	1: 300	3.33 $\mu\text{g}/\text{mL}$
Anti-MAD2		Anti-MAD2	Rabbit	1: 300	3.33 $\mu\text{g}/\text{mL}$
Anti-centromere	Antibodies incorporated (Davis, CA, USA)	Anti-centromere	Human	1: 300	3.33 $\mu\text{g}/\text{mL}$

Table 15 Secondary antibodies for immunofluorescence staining

Antibody	Source	Dilution
Alexa Fluor-674 conjugated goat anti-mouse IgG	Invitrogen (Carlsbad, CA, USA)	1: 600
Alexa Fluor-594 conjugated goat anti-rabbit IgG		1: 600
Alexa Fluor-635 conjugated goat anti-rabbit IgG		1: 600
Alexa Fluor-488 conjugated goat anti-rabbit IgG		1: 600
Rhodamine Red-X-conjugated donkey anti-human IgG	Jackson Immunoresearch Laboratories Inc. (West Grove, PA, USA)	1: 10000

2.2.9.12 Xenograft tumour formation and cyrosectioning

SH-SY5Y 5HRE-GFP cells (5×10^6) resuspended in 1 x PBS containing Matrigel[®] Growth Factor Reduced (GFR) Basement Membrane Matrix, Phenol Red-free (Corning[®], New York, USA) in a total volume of 100 μ L were subcutaneously injected into the right flank of immune-compromised Rag -/- / IL2R -/- null mice and monitored for 24 days. The mice were sacrificed, and tumours were excised. Each tumour was rinsed in 1 x PBS before immersing into 3.7 % (w/v) paraformaldehyde in 1 x PBS (50 x volume of the tumour size) and incubated at 4 °C overnight. Tumour was immersed into 15 % sucrose in 1 x PBS (50 x volume of the tumour size) and incubated at 4 °C until the tumour sank to the bottom followed by immersion in 30 % sucrose in 1 x PBS (50 x volume of the tumour size). After the tumour sank to the bottom of 30 % sucrose solution, the tumour was embedded on a plastic embedding mold with OCT compound (Baxter, Deerfield, Illinois, USA). Tumour block was frozen immediately in liquid nitrogen. Tumour was sectioned at 8 μ m per slide on a Leica Cyrostat 1950 machine (Wetzlar, Germany). Tumour slide was used for immunofluorescence staining (refer to section 2.2.9.11).

2.2.10 Neuroblastoma SH-SY5Y extracellular vesicles (EVs) experiments

2.2.10.1 EVs isolation

SH-SY5Y WT, pGFP-C-shLenti control shRNA and K2B shRNA transfected SH-SY5Y cells were cultured in 20 dishes of 100 mm tissue culture dish. When the cells were confluent, the media was replaced with that containing 7 % (v/v) exosome-depleted FBS (Gibco, Waltham, MA, USA). Cells were then cultured for 24 h. The culture supernatant was collected and centrifuged at 480 g for 5 min followed by 2000 g for 10 min to remove cells and cell debris. The culture supernatant was then filtered using a Supor Syringe Filters, 25 mm, 0.2 μ m (Pall Corporation, New York, USA) and concentrated using the vivaspin 20 concentrator (100,000 MWCO) PES (Sartorius, Göttingen, Germany). The concentrated culture supernatant was subjected to ultra-centrifugation using a TLA

120.1 rotor (Beckman Coulter, Brea, California, USA) at 55000 rpm (corresponding to ~ 100 000 g) for 2 h at 4 °C. The culture supernatant was discarded and the EVs pellet was re-suspended in PBS and centrifuged at 55000 rpm (corresponding to ~ 100 000 g) for 1 h at 4 °C by using TLA 120.1 rotor. The supernatant was removed and pellet was dissolved in PBS for Nanosight examination, angiogenesis assay, flow cytometry and electron microscopy. EVs pellet was re-suspended in lysis buffer for immunoblotting.

2.2.10.2 Angiogenesis assay (EVs)

Matrigel[®] basement membrane matrix (50 µL) (BD Bioscience, Mountain View, CA, USA) was added into each well of pre-chilled 96-well sterile plate followed by incubation for 1 h at 37 °C to allow the solidification of the Matrigel. 10 µg of SH-SY5Y WT, pGFP-C-shLenti control shRNA and K2B shRNA transfected SH-SY5Y cells-derived EVs or PBS alone was mixed with HUVECs (1.5×10^4) in 120 µL growth medium. The cell suspension was then layered on top of the solidified matrigel. Endothelial tube formation was imaged on a motorized Olympus IX83 inverted microscope system (4X objective) (UPLFLN-PH, Olympus) that was equipped with a 37 °C and 5 % CO₂ incubator stage, a cooled monochrome digital camera and the CellSens Dimension software. Phase contrast live cell images were captured every 30 min. The total length, total branching length, number of junctions or number of nodes were analyzed using the ImageJ angiogenesis analyzer plugin.

2.2.10.3 Flow cytometry analysis (EVs)

Control group SureBeads[™] Protein G magnetic Beads (5 µL) (Bio-RAD, Hercules, CA, USA) were coated with 5 µg mouse serum IgG (Sigma-aldrich, St. Louis, MO, USA) in 400 µL PBS and sample group SureBeads[™] Protein G magnetic Beads (15 µL) were coated with 2 µg mouse monoclonal CD81 antibody (5A6) (Santa Cruz Biotechnology, CA, USA) in 400 µL PBS and incubated at 4 °C overnight on a roller. Sample group beads were incubated in 400 µL PBS containing 5 µg mouse serum IgG at 4 °C for 5 h on a roller to block non-specific binding sites. Both groups of beads were then mixed with 20 µg EVs in 400 µL PBS at 4 °C overnight on a roller. Thereafter, beads were washed once in PBS. Control beads were re-suspended in 10 µL PBS containing FITC anti-human CD81 (TAPA-1) (5A6) (Biolegend[®], San Diego, USA). Sample beads were re-suspended in 30 µL PBS and separated into three groups (each containing 10 µL of beads). Each of these groups was then incubated with 2.5 µg of relevant antibody for 30 min on ice. The following antibodies were used

FITC anti-human CD81 (TAPA-1) (5A6) antibody, FITC anti-human CD63 (clone H5C6) antibody (MOPC-21) (Biolegend®, San Diego, USA) and FITC mouse IgG1, isotype Ctrl (Fc) (MOPC-21) (Biolegend®, San Diego, USA). Samples were subjected to flow cytometry on a FACSCalibur flow cytometer (BD Biosciences, Mountain View, CA, USA) and data were analyzed using the FlowJo software.

2.2.11 Statistic Analysis

Statistical analyses were performed either using Excel software or GraphPad Prism 6 software. In most analyses, Student's *t* - test (two tailed and unequal variance) was used unless otherwise stated in the figure legend. Only $p < 0.05$ is considered significant.

2.2.12 Animal Experimentation

Animal studies were approved by the Nanyang Technological University, Institutional Animal Care and Use Committee (IACUC). IACUC number A18028.

Chapter 3 Kindlin-2 regulates mitotic spindle assembly by modulating α -tubulin acetylation in neuroblastoma cells

3.1 Overview

It has been shown that kindlin-1 regulates mitotic spindle assembly through integrin activation or phosphorylation of Plk-1, leading to a delay in cell cycle in breast cancer cells [127]. Given that kindlin-1 is expressed primarily in epithelial cells, kindlin-2 and kindlin-3 may have similar functions in other cell types. A genome-wide RNAi screen reveals a role of kindlin-2 homolog Fit-1 in suppressing multipolar cell division in *drosophila* S2 cells [241]. Whether mammalian kindlin-2 shares similar function remains unknown. Acetylated tubulins are abundant in the microtubules of mitotic cells. Acetylated tubulins facilitate microtubules to interact with microtubule associated proteins and motor proteins, promoting microtubule stability [128, 129]. This is critical for the assembly bipolar spindle, the alignment of chromosomes during metaphase, and the precise segregation of sister chromatids. A hallmark of cancer is rapidly dividing cells that bypass cell cycle checkpoints. Multipolar mitotic spindles are rarely observed in normal cells but are relatively common in many cancer cells [233-239]. Multipolar cell division delays chromosomes segregation to spindle poles during anaphase which causes lagging chromosomes [235, 236].

Neuroblastoma is one of the most common cancers in children and the survival of children with advanced stage neuroblastoma is poor [351, 352]. Studies on brain tumour have shown that protein involved in cell cycle including Aurora kinase A and CDK can serve as potential pharmacological targets to stop the growth of the tumour [353, 354]. Therefore in this study, we examine the role of kindlin-2 in mitosis using the widely reported human neuroblastoma SH-SY5Y cell line.

3.2 Silencing kindlin-2 expression in SH-SY5Y cells

In order to exclude functional compensation by kindlin-1 or kindlin-3 as a result of loss of kindlin-2 expression, we first determine the expression of kindlins in SH-SY5Y cells by immunoblotting using relevant antibodies. Only kindlin-2 was detected in SH-SY5Y cells (**Figure 11A**). We then silenced kindlin-2 expression in these cells by using a commercial GFP-shRNA 3rd generation lentiviral expression system. Four kindlin-2 targeting shRNA sequences and a control sequence in lentiviral expression vector were provided in this system. Cells were transduced with lentivirus and

subjected to cell sorting based on GFP signal on a FACS sorter. Stable cell lines were generated by puromycin (1.5 µg/ mL) selection. Cells expressing one of the kindlin-2 targeting shRNA sequence (henceforth referred to as K2B shRNA) showed marked reduction in kindlin-2 expression at both protein and mRNA levels as compared to wild-type (WT) and control shRNA-expressing cells (**Figure 11B**). We also did not detect any up-regulation of kindlin-1 or kindlin-3 expression (**Figure 11C**). The colorectal cancer DLD-1 and erythroleukemia K562 cell lines were included as positive controls.

3.3 Morphology, cell adhesion and migratory properties of SH-SY5Y cells expressing kindlin-2 targeting shRNA

SH-SY5Y WT, control shRNA and K2B shRNA cells were cultured in tissue culture dishes pre-coated with ECM protein fibronectin (2.5 µg/ cm²) or laminin (2 µg/ cm²) for 24 h and viewed under a phase contrast microscope. Whereas WT and control shRNA cells formed areas of lamellipodia, K2B shRNA cells exhibited filopodia (red arrows) with weak adhesion (**Figure 12A**). These observations are in line with the established role of kindlin-2 in the maturation of focal adhesion that is required for lamellipodia formation [355]. To provide quantitative data on the cell adhesion and spreading properties of K2B shRNA cells, electric cell-substrate impedance sensing assay was performed. In brief, this assay measures the impedance of current flow (given the arbitrary term Cell Index) when cells adhere onto gold surface-electrodes. K2B shRNA cells exhibited diminished adhesion and spreading on both ECM proteins compared with WT and control shRNA cells although the extent of difference varies between fibronectin and laminin (**Figure 12B**). We then performed *in vitro* wound healing assay to examine the migratory properties of K2B shRNA cells (**Figure 13A**). Wound closure was slower in K2B shRNA cells compared with control shRNA cells. In line with this observation, K2B shRNA cells exhibited slow migration compared with WT and control shRNA cells in 3D transwell migration assay (**Figure 13B**). Taken together, these data demonstrate that kindlin-2 is essential for cell adhesion and migration in SH-SY5Y cells. This is expected given that kindlin-2 is a positive regulator of integrin-mediated cell adhesion and migration.

3.4 Kindlin-2 is required for the proliferation of SH-SY5Y cells

Kindlin-2 is involved in the growth and proliferation of different cancers [102, 130, 356, 357]. We noted that K2B shRNA SH-SY5Y cells proliferate slower under conventional 2D cell culture conditions compared with WT and control shRNA cells based on the frequency of cell passage (data not shown). To verify this observation, we performed *in vitro* 3D spheroid culture of these cells in soft

A

B

C

Figure 11 Silencing of kindlin-2 expression in SH-SY5Y cells. (A) SH-SY5Y cell lysate was subjected to immunoblotting to detect kindlin-1, -2 or -3 using relevant antibodies. GAPDH was included as total protein loading control. (B) Immunoblotting (left panel) and qRT-PCR (right panel) were performed to determine the expression level of kindlin-2 in wild-type (WT), control shRNA (control), and K2B shRNA (K2B) cells. GAPDH was used as loading control for immunoblotting. Numbers below immunoblot represent fold-change relative to that of WT cells (normalized to 1.0) based on densitometry measurements obtained from three independent experiments. Both GAPDH and S18rRNA were used as housekeeping genes for qRT-PCR. Each Datum point represents mean $\pm$ standard deviation (S.D.) from three independent experiments. Statistics: only $p < 0.05$ is considered significant. (C) Kindlin-1 immunoblot (left panel). The human colorectal cancer DLD-1 cell line was included as a positive control. Kindlin-3 immunoblot (right panel). The human erythroleukemia K562 cell line was included as a positive control. GAPDH was used as loading control.

Figure 12 Morphology of SH-SY5Y cells depleted of kindlin-2. (A) Cells were seeded on $2.5 \mu\text{g}/\text{cm}^2$ fibronectin or $2 \mu\text{g}/\text{cm}^2$ laminin pre-coated 6-well culture plate and images were captured on a phase contrast microscope the following day. Red arrow points to filopodia or actin microspikes in K2B shRNA cells. Scale bar: $25 \mu\text{m}$. (B) Cells were seeded on $2.5 \mu\text{g}/\text{cm}^2$ fibronectin or $2 \mu\text{g}/\text{cm}^2$ laminin pre-coated 16-well ACEA chip. Cell index (an arbitrary unit of measurement that correlates with cell area) was measured over time. Each datum point represents mean $\pm$ S.D. of technical triplicates from three independent experiments.

A

• pGFP-C-shlenti-control shRNA

▲ pGFP-C-shlenti-K2B shRNA

pGFP-C-shlenti

B

Figure 13 Migratory properties of SH-SY5Y cells depleted of kindlin-2. (A) *In vitro* wound healing assay was performed over a period of 22.5 h and the wound area (μm^2) of each hour was measured and the data was plotted by wound area at time t (X_t) divided by wound area at time $= 0$ (X_0). Data represent mean $\pm$ S.D. from four independent experiments. Wound closure was monitored and imaged by live-cell imaging. Representative images at 0, 9, 17 h time points are presented. Scale bar: 200 μm . (B) Cells were seeded on 2.5 $\mu\text{g}/\text{cm}^2$ fibronectin-coated 3D-transwells and migration of cells to the bottom chamber was determined by staining cells with crystal violet followed by solubilization and readings at absorbance 595 nm were taken. Each datum point represents mean $\pm$ S.D. from five independent experiments. Statistics: only $p < 0.05$ is considered significant.

agar. As we noticed that 3D culture mimics better the microenvironment of solid tumours such as cells and extracellular matrix composition, cell-cell interactions, oxygen and nutrients supply, adhesive and mechanical forces environment [358-370]. K2B shRNA cells formed significantly smaller spheroids compared with those from WT and control shRNA cells (**Figure 14A**). These data suggest that K2B shRNA cells exhibit slow cell division.

To obtain mechanistic insights into the role of kindlin-2 in cell division in SH-SY5Y cells, we examined the mitotic rate of K2B shRNA cells. Cells were allowed to adhere on fibronectin or laminin followed by cell G2/ M phase synchronization and subsequently released into mitosis (describe in Materials and Methods section 2.2.9.5). G2/ M phase synchronization of cells is necessary because the frequency of cells undergoing mitosis at the same time is low in a population of cells under normal culture conditions. Live-cell imaging was captured for 6 h after RO-3306 was removed and mitotic rate (the time point of rounded mitotic cells to separate completely into daughter cells) was measured (**Figure 14B**). The mitotic rate of K2B shRNA cells was significantly slower than that of WT or control shRNA cells when cells were cultured on fibronectin but not laminin, suggesting that kindlin-2 is required for signaling from integrins for fibronectin but not laminin that impinges on cell cycle.

Activated form of CyclinB1-CDK1 complex triggers mitosis [371]. The expression level of active CyclinB1-CDK1 is up-regulated during centrosomes separation through prometaphase. Spindle assembly checkpoint ensures proper chromosome attachment and maintains high level of active form CyclinB1-CDK1 until proper bi-oriented chromosomes formation [372, 373]. WT, control shRNA and K2B shRNA cells were seeded on 2.5 $\mu\text{g}/\text{cm}^2$ fibronectin-coated cell culture dish followed by G2/ M synchronization. RO-3306 was removed and mitotic cells were then maintained under normal culture conditions for 90 min before being harvested. Consistent with the slower mitotic rate observed in K2B shRNA cells, low CyclinB1 and high phosphor-Y15 CDK1 (inactive form) were detected in K2B shRNA cells compared with WT and control shRNA cells by immunoblotting (**Figure 14C**).

3.5 High propensity of abnormal mitotic spindles detected in kindlin-2 silenced SH-SY5Y cells

In normal mitosis, the arrangement of mitotic spindles in a bipolar orientation is essential for proper chromosome segregation. Abnormal mitotic spindles often lead to lagging and uneven chromosomes separation, which may cause cell death [240]. We therefore examined the mitotic

A

B

C

Figure 14 SH-SY5Y K2B cells exhibited slower proliferation. (A) WT, control shRNA and K2B shRNA cells were seeded on soft agar to form 3D spheroids. Each datum point in the plot represents the 2D area (μm^2) of the spheroid based on image captured. Statistics: only $p < 0.05$ is considered significant. Representative images are shown (right panel). Scale bar: 200 μm . (B) G2/ M synchronization was performed on WT, control shRNA and K2B shRNA cells seeded on either 2.5 $\mu\text{g}/\text{cm}^2$ fibronectin- or 2 $\mu\text{g}/\text{cm}^2$ laminin-coated 12-wells plate. RO-3306 block was released to allow cells to enter into mitosis. Live-cell imaging was performed on an Olympus ix83 microscope fitted with a stage incubator. Images were captured at an interval of 2 min for 6 h. The time taken for each mitotic cell to divide into two daughter cells was recorded and plotted. Each datum point represents one cell. Data represent mean $\pm$ S.D. from three independent experiments. Statistics: only $p < 0.05$ is considered significant. (C) G2/ M synchronization assay of SH-SY5Y WT, control shRNA (control) and K2B shRNA (K2B) cells were performed on 2.5 $\mu\text{g}/\text{cm}^2$ fibronectin coated 10 cm^2 plate. Mitotic cells were harvested and lysed after 90 min. Cyclin-B1, phosphor CDK1 (pCDK1) and total CDK1 were verified by immunoblotting. Actin was used as loading control and housekeeping gene. Numbers below immunoblot represent fold-change relative to that of WT cells (normalized to 1.0) based on densitometry measurements obtained from three independent experiments of intensity ratio of phosphor proteins/ total proteins.

spindle assembly in K2B shRNA SH-SY5Y cells. Immunofluorescence staining of mitotic cells was performed using anti- α -tubulin antibody and DAPI (nucleus staining), and images were captured on a confocal laser scanning microscope (**Figure 15A**, upper panel). A higher percentage of abnormal mitotic spindles (a collection of multipolar spindles, supernumerary centrosomes bipolar spindles and bipolar spindles with lagging chromosomes) was observed in K2B shRNA cells compared with WT or control shRNA cells (**Figure 15A**, lower panel).

Spindle assembly checkpoint (SAC) regulates the onset of anaphase to ensure that kinetochores of sister chromatids are stably attached to the mitotic spindles. If unattached kinetochores are detected, it will inhibit the activity of APC/ C activity [191, 196, 199-201]. Mitotic arrest deficient 2 (MAD2) is one of the key components of SAC proteins, and it is recruited to the unattached kinetochores. It binds to the Cdc20, which is APC/ C coactivator [374, 375], and inhibits the activity of APC/ C that is needed for anaphase onset [376-378]. MAD2 was readily detected in the abnormal mitotic spindles of K2B shRNA cells (**Figure 15B**). Centromeres of the cells were stained and used as a reference (**Figure 15B**, upper panel). These data suggest that a large population of mitotic K2B shRNA cells contained unattached kinetochores.

3.6 A role of kindlin-2 in the regulation of α -tubulin acetylation

High level of acetylation of lysine 40 in α -tubulin is detected in mitotic cells, and tubulin acetylation stabilizes microtubules [128, 129]. Based on immunofluorescence staining, low level of acetylated α -tubulin was detected in the mitotic spindles of K2B shRNA cells compared with WT and control shRNA cells (**Figure 16**, upper panel). This difference was quantitated by taking the ratio of the fluorescence intensity of acetylated α -tubulin staining over that of total α -tubulin staining (**Figure 16**, lower panel). This suggest that acetylation of α -tubulin in K2B shRNA cells was defective.

All the experiments described above were based on using SH-SY5Y cells expressing a kindlin-2 targeting shRNA (K2B shRNA). In order to exclude potential off-target effects, we generated another stable SH-SY5Y cell line expressing kindlin-2 shRNA that targets another region of kindlin-2 mRNA (henceforth referred to as K2A shRNA). The reduced expression of kindlin-2 in these cells was verified by immunoblotting (**Figure 17A**). Similar to K2B shRNA cells, K2A shRNA cells exhibited slow mitotic rate compared with WT and control shRNA cells on fibronectin (**Figure 17B**). A significant reduction in α -tubulin acetylation in mitotic spindles was detected in K2A shRNA cells compared with

WT and control shRNA cells (**Figure 17C**). Taken together, these data lend support to the findings that kindlin-2 is required in SH-SY5Y cells during mitosis and that it has a role in the mitotic spindle assembly by regulating α -tubulin acetylation.

A

Fibronectin

Figure 15 SH-SY5Y K2B shRNA cells exhibited higher level of abnormal mitotic spindles and unattached kinetochores. (A) Mitotic cells of G2/ M synchronized mitotic SH-SY5Y WT, control shRNA and K2B shRNA cells were fixed and stained with anti- α -tubulin and DAPI (nucleus staining). Representative images are shown (upper panel). Scale bar: 2 μ m. The percentage of normal vs. abnormal mitotic spindles was plotted and shown (lower panel). Data represent mean $\pm$ S.D. from three independent experiments. Statistics: only $p < 0.05$ is considered significant. (B) Mitotic cells of SH-SY5Y WT, control shRNA and K2B shRNA were fixed and immunofluorescence staining was performed using anti-MAD2 antibody, anti-centromere antibody (ACA) and DAPI. Representative images are shown (upper panel). Scale bar: 2 μ m. The percentage of MAD2 detected in mitotic cells was plotted and shown (lower panel). Data represent mean $\pm$ S.D. from three independent experiments. Statistics: only $p < 0.05$ is considered significant.

Figure 16 Kindlin-2 depleted SH-SY5Y cells affected acetylated α-tubulin level. Mitotic cells of G2/ M synchronized mitotic SH-SY5Y WT, control shRNA and K2B shRNA cells were fixed and immunofluorescence staining was performed using anti-acetylated α-tubulin antibody, anti-α-tubulin antibody and DAPI. Representative images are shown (upper panel). Scale bar: 2 μm. The fluorescence intensity ratio of acetylated α-tubulin vs. α-tubulin was plotted and shown (lower panel). Each datum point represents one cell. Data represent mean ± S.D. from three independent experiments. Statistics: only $p < 0.05$ is considered significant.

A **B**

C

Figure 17 Silencing kindlin-2 in SH-SY5Y cells using another shRNA (K2A shRNA) also reduced α-tubulin acetylation. (A) Immunoblotting was performed to determine the expression level of kindlin-2 in WT, control shRNA (control), and K2A shRNA (K2A) cells. GAPDH was used as loading control for immunoblotting. (B) G2/ M synchronization was performed on WT, control shRNA and K2A shRNA cells seeded on 2.5 μg/ cm² fibronectin-coated 12-wells plate. Live-cell imaging was performed on an Olympus ix83 microscope fitted with a stage incubator. Images were captured at an interval of 2 min for 6 h. The time taken for each mitotic cell to divide into two daughter cells was recorded and plotted. Each datum point represents one cell. Data represent mean ± S.D. from three independent experiments. Statistics: only $p < 0.05$ is considered significant. (C) Mitotic cells of G2/ M synchronized mitotic SH-SY5Y WT, control shRNA and K2A shRNA cells were fixed and immunofluorescence staining performed using anti-acetylated α-tubulin antibody, anti-α-tubulin antibody and DAPI. Representative images are shown (upper panel). Scale bar: 2 μm. The fluorescence intensity ratio of acetylated tubulin vs. α-tubulin was plotted and shown (lower panel). Each datum point represents one cell. Data represent mean ± S.D. from three independent experiments. Statistics: only $p < 0.05$ is considered significant.

3.7 Mechanistic insights into kindlin-2 regulation of α -tubulin acetylation

Histone deacetylase-6 (HDAC6) removes the acetyl moiety in acetylated α -tubulin, leading to microtubule instability [379-381]. Tubacin is a selective HDAC6 inhibitor that increases the level of acetylated α -tubulin in cells [305]. Synchronized WT, control shRNA and K2B shRNA cells were treated with 2 μ M tubacin for 16 h followed by immunofluorescence staining (**Figure 18A**, upper panel). In contrast to the low level of acetylated α -tubulin/ total α -tubulin detected in the mitotic spindles of K2B shRNA cells (**Figure 16**), there was no significant difference in the level of acetylated α -tubulin/ total α -tubulin between K2B shRNA, control shRNA and WT cells under tubacin treatment (**Figure 18A**, lower panel). In line with this observation, there was no significant difference in the mitotic rate of tubacin-treated cells (**Figure 18B**), suggesting that tubacin treatment rescued the acetylation defect in K2B shRNA cells. These data also suggest that HDAC6 functions downstream of kindlin-2 in regulating α -tubulin acetylation.

Inhibition of HDAC6 activity increases the level of acetylated tubulin in cells [380, 382]. It has been shown that activated GSK3 β phosphorylates Ser22 in HDAC6, which increases HDAC6 activity, leading to de-acetylation of α -tubulin [383]. Upstream of GSK3 β , AKT has been shown to inhibit the activity of GSK3 β by phosphorylating Ser9 in GSK3 β [384, 385]. To determine whether kindlin-2 regulates α -tubulin acetylation via the AKT/ GSK3 β / HDAC6 signaling axis, we performed immunoblotting experiments of lysates of mitotic WT, control shRNA and K2B shRNA cells on fibronectin using a panel of antibodies (**Figure 19A**). Densitometry measurements from at least three independent experiments were performed to provide quantitative data on the levels of phosphorylated proteins detected (in this case the intensity ratio of phosphor proteins/ total proteins was calculated). A significant reduction in phosphor Ser473 AKT (activated form) and phosphor Ser9 GSK3 β (inactivated form) was detected in K2B shRNA cells compared with WT and control shRNA cells. Phosphor Ser22 HDAC6 (activated form) level was also higher in K2B shRNA cells as compared to others. This was also verified by measuring the activity of HDAC6 using a commercial kit (**Figure 19B**). In line with this observation, acetylated α -tubulin level was lower in K2B shRNA cells. These data also suggest that despite the presence of integrin ligand, fibronectin, a lack of kindlin-2 leads to a decrease in activated AKT, an increase in GSK3 β activity, which in turn activates HDAC6, leading to de-acetylation of α -tubulin. Kindlins are required for integrin inside-out activation and they are important in integrin outside-in signalling. We repeated the study but included manganese in the

A

B

Figure 18 Tubacin reverted the acetylated α -tubulin level in kindlin-2 depleted SH-SY5Y cells

(A) G2/ M synchronized mitotic SH-SY5Y WT, control shRNA and K2B shRNA cells were treated with 2 μ M tubacin for 16 h. Mitotic cells were fixed followed by immunofluorescence staining using anti-acetylated α -tubulin antibody, anti- α -tubulin antibody and DAPI. Representative images are shown (upper panel). Scale bar: 2 μ m. The fluorescence intensity ratio of acetylated α -tubulin vs. α -tubulin was plotted and shown (lower panel). Each datum point represents one cell. Data represent mean $\pm$ S.D. from three independent experiments. Statistics: only p < 0.05 is considered significant. (B) G2/ M synchronization was performed on control shRNA and K2B shRNA cells seeded on 2.5 μ g/ cm² fibronectin-coated 12-wells plate. One group of K2B shRNA cells were treated with 2 μ M tubacin for 16 h before RO-3306 block was released. Live-cell imaging was performed on an Olympus ix83 microscope fitted with a stage incubator. Images were captured at an interval of 2 min for 6 h. The time taken for each mitotic cell to divide into two daughter cells was recorded and plotted. Each datum point represents one cell. Data represent mean $\pm$ S.D. from three independent experiments. Statistics: only p < 0.05 is considered significant.

culture media (**Figure 19C**). Manganese is an exogenous activating agent that directly activates integrins. The inclusion of manganese, which bypasses the need for kindlin-2 in integrin activation, did not alter the activation profiles of AKT, GSK3 β , and HDAC6 in K2B shRNA cells compared with WT and control shRNA cells. This suggests that kindlin-2 plays an important role in integrin outside-in signalling that regulates the AKT/ GSK3 β / HDAC6 signalling axis.

Paxillin is a scaffold protein which integrates several signalling pathways [386, 387]. Interestingly, paxillin has been shown to promote microtubule acetylation through either enhancing the activation of acetyltransferases or inhibition of HDAC6 activity [388, 389]. Kindlin-2 has been demonstrated to recruit paxillin to nascent focal adhesion, which in turn regulates membrane protrusion [355]. Therefore, we performed immunoprecipitation assay on HEK293T cells that were co-transfected with mcherry-paxillin and HA-tagged kindlin-2 plasmids. Both mcherry-paxillin and endogenous HDAC6 were detected in the co-precipitate of HA-tagged kindlin-2 (**Figure 20**), suggesting another possible kindlin-2 mediated signalling pathway that could regulate the acetylation α -tubulin through HDAC6 pathway.

3.8 Integrin binding and paxillin engagement are required for kindlin-2 to regulate α -tubulin acetylation

Our data suggest that kindlin-2 regulates α -tubulin acetylation via HDAC6. The activity of HDAC6 can be regulated through kindlin-2/ AKT/ GSK3 β or kindlin-2/ paxillin signalling, and they need not be mutually exclusive. We extended the study by performing rescue experiments using K2B shRNA cells. These cells were transfected with kindlin-2 shRNA-resistant expression plasmid containing HA-tagged kindlin-2 (WT), kindlin-2 Q621W622/AA (integrin-binding defective) mutant (Q621W622 in kindlin-2 long form correspond to Q614W615 in kindlin-2 short form) [390] or kindlin-2 Δ PH (paxillin binding defective) mutant [391] followed by selection in 300 μ g/ mL zeocin to generate stable lines. Their expression levels were comparable as determined by immunoblotting (**Figure 21A**). G2/ M cell synchronization was performed and the mitotic rate of these cells was determined. Re-expression of kindlin-2 (WT) in K2B shRNA cells increased the mitotic rate to a level that is comparable to control shRNA cells (**Figure 21B**). However, this was not observed in cells re-expressing kindlin-2 Δ PH mutant or kindlin-2 Q621W622/AA, suggesting that paxillin and integrin binding are both important in the function of kindlin-2 in mitosis.

A **B**

C

Figure 19 Regulation of acetylated α -tubulin level via kindlin-2/ AKT/ GSK3 β / HDAC6. G2/ M synchronization assay of SH-SY5Y WT, control shRNA (control) and K2B shRNA (K2B) cells were performed on 2.5 μ g/ cm² fibronectin coated 10 cm² plate. Mitotic cells were harvested and lysed after 90 min. (A) Phosphor AKT (pAKT), total AKT, phosphor GSK3 β (pGSK3 β), total GSK3 β , phosphor HDAC6 (pHDAC6), total HDAC6, acetylated tubulin and α -tubulin were verified by immunoblotting. Numbers below immunoblot represent fold-change relative to that of WT cells (normalized to 1.0) based on densitometry measurements obtained from at least three independent experiments of intensity ratio of phosphor proteins/ total proteins. (B) The fluorescence intensity of HDAC6 activities of SH-SY5Y WT, control shRNA and K2B shRNA mitotic cells were measured at Ex/ Em: 380/ 490 nm. Data represent mean $\pm$ S.D. from eight independent experiments. Statistics: only $p < 0.05$ is considered significant. Student's paired t - test was used. (C) G2/ M synchronization assay of SH-SY5Y WT, control shRNA (control) and K2B shRNA (K2B) cells were performed on 2.5 μ g/ cm² fibronectin coated 10 cm² plate with 0.2 mM manganese (II) chloride for 90 min before mitotic cells harvesting. Phosphor AKT (pAKT), total AKT, phosphor GSK3 β (pGSK3 β), total GSK3 β , phosphor HDAC6 (pHDAC6), total HDAC6, acetylated tubulin and α -tubulin were verified by immunoblotting. Numbers below immunoblot represent fold-change relative to that of WT cells (normalized to 1.0) based on densitometry measurements obtained from at least three independent experiments of intensity ratio of phosphor proteins/ total proteins.

Figure 20 Regulation of acetylated α -tubulin level via kindlin-2/ paxillin/ HDAC6 signaling pathway. HEK293T cells were co-transfected with HA-tagged kindlin-2 and mcherry-paxillin and immunoprecipitation was performed by using mouse anti-HA Ab. HA-tag, co-precipitated paxillin and endogenous HDAC6 were detected by immunoblotting.

A **B**

Figure 21 Loss of interaction of kindlin-2 with integrin or paxillin delayed mitosis in SH-SY5Y.

(A) Immunoblotting was performed to determine the re-expression of K2B shRNA resistant HA-tagged kindlin-2 WT (K2 WT), kindlin-2 Q621W622/AA (K2 QW/AA) or kindlin-2 ΔPH (K2 ΔPH) in SH-SY5Y K2B shRNA cells. GAPDH was included as total protein loading control. (B) G2/ M synchronization was performed on control shRNA, K2B shRNA and K2B shRNA HA-tagged re-expressed K2 WT, K2 QW/AA and K2 ΔPH cells seeded on 2.5 μg/ cm² fibronectin-coated 12-wells plate. Live-cell imaging was performed on an Olympus ix83 microscope fitted with a stage incubator. Images were captured at an interval of 2 min for 6 h. The time taken for each mitotic cell to divide into two daughter cells was recorded and plotted. Each datum point represents one cell. Data represent mean ± S.D. from three independent experiments. Statistics: only p < 0.05 is considered significant.

In addition, we observed that K2B shRNA cells re-expressing kindlin-2 (WT) but not kindlin-2 Δ PH mutant or kindlin-2 Q621W622/AA exhibited recovery of acetylated α -tubulin level comparable to control shRNA cells (**Figure 22A**). Finally, the frequency of cells showing abnormal mitotic spindles was comparable between K2B shRNA cells re-expressing kindlin-2 (WT) and control shRNA cells (**Figure 22B**). By contrast, a high frequency of cells showing abnormal mitotic spindles was still observed in K2B shRNA cells re-expressing kindlin-2 Δ PH mutant or kindlin-2 Q621W622/AA. Taken together, these data point to kindlin-2 having an important role in the regulation of mitotic spindle assembly, and this involves two levels of control. One involves kindlin-2/ AKT/ GSK3 β / HDAC6 whereas the other involves kindlin-2/ paxillin/ HDAC6, but they need not be mutually exclusive.

3.9 Hypoxia reduces kindlin-2 expression in SH-SY5Y cells

Our data suggest that kindlin-2 plays an important role in mitosis by regulating mitotic spindle assembly via α -tubulin acetylation. We asked whether kindlin-2 expression may be down-regulated under certain conditions leading to abnormal mitotic spindle assembly. A hallmark of solid tumours is the formation of hypoxic regions within the tumour mass due to poor blood vascularisation [392, 393]. Hypoxia is well established to induce changes in gene expression and it alters cell signalling pathways [394].

To test whether hypoxia can lead to a decrease in kindlin-2 expression, SH-SY5Y cells were subjected to hypoxia culture conditions (< 1 % O₂) for 24, 48 or 96 h followed by immunoblotting and qRT-PCR assays to detect the expression level of kindlin-2 protein and mRNA, respectively (**Figure 23A & B**). Interestingly, kindlin-2 expression decreased gradually over time when cells were cultured under hypoxic conditions. The effect was specific because both expression levels of HIF-1 α and VEGFA, biomarkers of many cell types subjected to hypoxia, were elevated [395].

MicroRNA (miR) regulates gene expression by directly targeting to the specific sequences in the 3' UTR of mRNA thereby inhibiting protein translation [396, 397]. It has been shown that miR-138 represses kindlin-2 expression and β 1 integrin signalling, increasing the sensitivity of prostate cancer cells to chemotherapy drug docetaxal [398]. Our data showed that the miR-138 expression level was up-regulated in SH-SY5Y cells under hypoxia as compared to those under normoxia (**Figure 23C**). This suggest that the down-regulation of kindlin-2 expression in SH-SY5Y cells is most likely attributed to concomitant up-regulation of miR-138 induced by hypoxia.

A

B

Figure 22 Loss of interaction of kindlin-2 with integrin or paxillin reduced α-tubulin acetylation.

(A) Mitotic cells of control shRNA, K2B shRNA and K2B shRNA HA-tagged re-expressed Kindlin-2 WT (K2 WT), kindlin-2 Q621W622/AA (K2 QW/AA) and kindlin-2 ΔPH (K2 ΔPH) cells were fixed and immunofluorescence stained of anti-acetylated α-tubulin and anti-α-tubulin. The fluorescence intensity ratio of acetylated tubulin vs. α-tubulin was plotted and shown. Each datum point represents one cell. Data represent mean ± S.D. from three independent experiments. Statistics: only $p < 0.05$ is considered significant. (B) The percentage of normal vs. abnormal mitotic spindles was plotted and shown. Data represent mean ± S.D. from three independent experiments. Statistics: only $p < 0.05$ is considered significant; $p > 0.05$ is considered not significant (n.s.).

A **B**

C

Figure 23 Hypoxic stress reduced kindlin-2 expression level in SH-SY5Y cells (A) Immunoblotting was performed to determine the kindlin-2 protein expression level of SH-SY5Y cells under normoxia and hypoxia conditions for 24, 48 and 96 h. β -tubulin was used as loading control for immunoblotting. Numbers below immunoblot represent fold-change relative to cells under normoxia condition at that time point (normalized to 1.0) based on densitometry measurements obtained from three independent experiments. (B, C) qRT-PCR were performed to determine the expression level of kindlin-2, VEGFA and miR138 in SH-SY5Y cells under normoxia and hypoxia conditions for 24, 48 and 96 h. S18rRNA were used as housekeeping genes for qRT-PCR. Each datum point represents mean $\pm$ S.D. from three independent experiments. Statistics: only $p < 0.05$ is considered significant.

We then determined cell proliferation under hypoxia of SH-SY5Y cells. Perhaps not unexpected, there was a significant reduction in cell proliferation under hypoxia (**Figure 24A**, upper panel). The reduced cell proliferation observed was not attributed to a high level of cell death because cells were still viable (**Figure 24A**, lower panel), suggesting a slowdown in cell cycle. Given that kindlin-2 is important for mitotic spindle assembly which affects cell cycle, we compared the mitotic spindles of cells subjected to hypoxia and normoxia culture conditions by performing immunofluorescence staining of α -tubulin in these cells (**Figure 24B**, upper panel). Because it is not possible to perform hypoxia culture conditions and G2/ M synchronization of cells at the same time, we performed wide field scanning of cells to identify mitotic cells for further analysis. Interestingly, the frequency of abnormal spindles was higher in mitotic cells under hypoxia as compared to that under normoxia (**Figure 24B**, lower panel).

We went further to examine the effect of hypoxia on kindlin-2 expression and mitotic spindle assembly in human colorectal cancer HCT-116 and breast cancer MDA-MB-231 cell lines (**Figure 25**). Consistent with data from SH-SY5Y cells, kindlin-2 expression was reduced and a high frequency of abnormal mitotic spindles was detected in both cell lines under hypoxia compared with normoxia condition. These data also suggest that down-regulation of kindlin-2 induced by hypoxia is not specific only to SH-SY5Y cells.

To further validate these data, we generate stable SH-SY5Y cells over-expressing GFP-kindlin-2 by transfecting cells with GFP-kindlin-2 expression plasmid followed by selection in 300 μ g/mL zeocin. We reasoned that the expression level of GFP-kindlin-2 would be minimally affected by hypoxia because the kindlin-2 cDNA does not contain the 3'-UTR region targeted by miR-138. This allowed us to determine if over-expressed GFP-kindlin-2 prevents abnormal mitotic spindle assembly in cells under hypoxia. Cells were cultured under normal and hypoxia conditions for 96 h, followed by immunoblotting or immunofluorescence staining of α -tubulin. Although there was a slight reduction in GFP-kindlin-2 expression level in cells under hypoxia compared with normoxia, the frequency of abnormal mitotic spindle assembly was minimal in the presence of over-expressed GFP-kindlin-2 similar to the cells under normoxia (**Figure 26**).

As mentioned in previous section, 2D cell culture is not representative of the complexity of 3D culture, especially that of the tumour microenvironment [358-370]. To examine hypoxic stress-induced

A

Figure 24 Hypoxic stress reduced cell proliferation and caused abnormal mitotic spindle assembly in SH-SY5Y cells. (A) Cell proliferation and viability assay were performed on SH-SY5Y cells subjected to normoxia or hypoxia culture conditions over a period of 24, 48 or 96 h. Each datum point represents mean $\pm$ S.D. from three independent experiments. Statistics: only $p < 0.05$ is considered significant; $p > 0.05$ is considered not significant (n.s.). (B) 96 h normoxia and hypoxia treated SH-SY5Y cells were fixed and immunofluorescence staining performed using anti- α -tubulin antibody and DAPI to examine the mitotic spindle assembly. Representative images of SH-SY5Y under normoxia or hypoxia condition are shown (upper panel). Scale bar: 2 μ m. The percentage of normal vs. abnormal mitotic spindles was plotted and shown (lower panel). Data represent mean $\pm$ S.D. from three independent experiments. Statistics: only $p < 0.05$ is considered significant.

MDA-MB-231

HCT-116

Figure 25 Hypoxic stress reduced kindlin-2 expression and caused abnormal mitotic spindle assembly in MDA-MB-231 and HCT-116 cells. Immunoblotting and immunofluorescence staining were performed on MDA-MB-231 (upper panel) and HCT-116 (lower panel) cells subjected to 96h of normoxia or hypoxia condition. β-tubulin was used as loading control for immunoblotting. Numbers below immunoblot represent fold-change relative to cells under normoxia condition (normalized to 1.0) based on densitometry measurements obtained from three independent experiments (left panel). α-tubulin immunofluorescence staining was performed to examine the mitotic spindle arrangement. The percentage of normal vs. abnormal mitotic spindles was plotted and shown (right panel). Data represent mean ± S.D. from three independent experiments. Statistics: only $p < 0.05$ is considered significant.

SH-SY5Y- pcDNA3.1zeo(-)GFP-K2

Figure 26 SH-SY5Y over-expressing GFP-kindlin-2 reverted the mitotic spindle assembly. Immunoblotting and immunofluorescence staining were performed on SH-SY5Y over-expressing GFP-kindlin-2 cells under normoxia and hypoxia condition for 96 h. β-tubulin was used as loading control for immunoblotting. HIF-1α was included as positive control for hypoxia condition. Numbers below immunoblot represent fold-change relative to cells under normoxia condition (normalized to 1.0) based on densitometry measurements obtained from three independent experiments (upper panel). Immunofluorescence staining of anti-α-tubulin was performed to examine the mitotic spindle arrangement. The percentage of normal vs. abnormal mitotic spindles was plotted and shown (lower panel). Data represent mean ± S.D. from three independent experiments. Statistics: p > 0.05 is considered not significant (n.s.).

kindlin-2 down-regulation in 3D culture *in vitro*, we first generated SH-SY5Y stable cell line expressing GFP under the control of a 5HRE (five copies of human VEGF hypoxia-responsive element) [399]. Under hypoxia, cells will be induced to express GFP, which allows isolation of these cells for further analysis. Cells were seeded on soft agar for 7 days to form 3D spheroids, which will form hypoxic regions. Spheroids were harvested and cells were dissociated followed cell sorting based on GFP low (GFP^{lo}) or GFP high (GFP^{hi}) expression on a FACS sorter (**Figure 27A**). High GFP signal was detected in a population of cells from the dissociated spheroids, suggesting that hypoxic region(s) was present in the spheroids. Based on immunoblotting and qRT-PCR assays, low level of kindlin-2 expression was detected in GFP^{hi} cells compared with GFP^{lo} cells (**Figure 27B**). In addition, high VEGFA and miR138 were detected in GFP^{hi} cells (**Figure 27C**). These data are consistent with that obtained from 2D culture under hypoxia (**Figure 23**).

Finally, we performed *in vivo* study to verify these observations. To this end, stable SH-SY5Y cells expressing 5HRE/ GFP were xeno-transplanted into the right flank of immune-compromised Rag-/- / IL2R-/- immune-compromised mice (**Figure 28**). Tumours were excised and cyro-sectioned followed by immunofluorescence staining to detect kindlin-2 and actin. We were able to detect GFP signal in certain regions of the tumour sections, suggesting that these regions are hypoxic. Based on the intensity plot of the images, regions high in GFP signal tend to have low kindlin-2 expression level GFP signal (**Figure 28**, lower panel). Taken together, both the *in vitro* and *in vivo* data suggest that hypoxia reduces kindlin-2 expression in SH-SY5Y cells and tumour.

A

Day 7 : SH-SY5Y 5HRE/ GFP Spheroid

B

SH-SY5Y 5HRE/ GFP
Spheroids

C

SH-SY5Y 5HRE/ GFP
Spheroid

Figure 27 Hypoxia reduced kindlin-2 expression level in SH-SY5Y 5HRE/ GFP cells. (A) SH-SY5Y 5HRE/ GFP cells were seeded on soft agar to form 3D spheroids for 7 days. Representative spheroid image was shown (left panel). Scale bar: 200 μ m. Cells were sorted by flow cytometry cell sorting based on GFP low (GFP^{lo}) and GFP high (GFP^{hi}) group (right panel). (B) Dissociated GFP low (GFP^{lo}) and GFP high (GFP^{hi}) cells from 3D spheroids were harvested and lysed to perform immunoblotting for kindlin-2 expression. β -tubulin was used as loading control for immunoblotting. Numbers below immunoblot represent fold-change relative to that of GFP^{lo} cells (normalized to 1.0) based on densitometry measurements obtained from three independent experiments. (C) qRT-PCR was performed on dissociated GFP low (GFP^{lo}) and GFP high (GFP^{hi}) cells from 3D spheroids. S18rRNA was used as housekeeping gene for kindlin-2 and VEGFA. U6 was used as housekeeping gene for miR138. Data represent mean $\pm$ S.D. from three independent experiments. Statistics: only $p < 0.05$ is considered significant.

Figure 28 Hypoxia reduced kindlin-2 expression level in SH-SY5Y 5HRE/ GFP tumours. SH-SY5Y 5HRE/ GFP cells were xeno-transplanted into the right flank of the immune-compromised Rag-/- / IR2R-/- mice for 2 weeks. Tumours were excised and cyro-sectioned at 8 μm per slide followed by immunofluorescence staining to detect kindlin-2 and F-actin. Hypoxic region is indicated by GFP signal. The intensity plot was presented on kindlin-2 and GFP region. Representative images are presented. Scale bar: 100 μm (upper right), 20 μm (lower).

3.10 Discussion

The primary function of kindlins is to regulate integrin-mediated cell adhesion. However, there are many lines of evidence suggesting that kindlins have non-integrin dependent functions. In this study, we showed that kindlin-2 plays an important role in regulating mitosis in human neuroblastoma cell line SH-SY5Y. A summary of our findings is shown in **Figure 29**. Mechanistically, kindlin-2 regulates the acetylation of α -tubulin that affects microtubule stability and mitotic spindle assembly. Kindlin-2 does not directly regulate α -tubulin acetylation. Rather two distinct signaling pathways, which need not be mutually exclusive, could be involved. The first involves the AKT/ GSK3 β / HDAC6 signaling axis. In this case, kindlin-2 plays an important role in regulating the phosphorylation and activation of AKT via integrin outside-in signaling. This is perhaps not unexpected because integrin outside-in signaling has been shown to activate AKT. Activated AKT phosphorylates GSK3 β resulting in its inhibition. Because active GSK3 β is required for the activation of HDAC6, which de-acetylates α -tubulin, AKT is a positive regulator of α -tubulin acetylation.

The second regulatory pathway involves kindlin-2/ paxillin/ HDAC6. In this case, the primary inhibitory effect comes from paxillin, which has been shown to inhibit HDAC6 activity. Our data suggest that kindlin-2 may play a role in recruiting paxillin to integrin-adhesion sites, which then inhibits HDAC6.

Acetylation of Lys40 in α -tubulin is abundant in stabilized microtubules [400-402]. Recent studies have also shown that it maintains the structural integrity of microtubules under mechanical stress [403, 404]. In mitotic cells, a high level of acetylated-tubulin can be found at interpolar and kinetochore microtubules but not astral microtubules [402, 405-408]. Acetylation of α -tubulin is regulated by α -tubulin acetyltransferase 1 (ATAT1) and HDAC6 [401]. Our data therefore suggest that kindlin-2 inhibits HDAC6 activity because silencing kindlin-2 expression led to an increase in HDAC6 activity and a concomitant decrease in the acetylation of α -tubulin in mitotic spindles. This effect was reversed when cells were treated with tubacin, a HDAC6 inhibitor. It has been reported that kindlin-1 localizes at centrioles in mitotic cells and regulates acetylation of α -tubulin at mitotic spindles by directly interacting with HDAC6 [127, 409]. Despite several attempts to detect kindlin-2 at centrioles of mitotic SH-SY5Y cells by immunofluorescence staining, we were unsuccessful. Given that SH-SY5Y cells only expressed kindlin-2, it suggests an alternative mechanism by which kindlin-2

regulates acetylation of α -tubulin at mitotic spindles. This involves the Akt/ GSK3 β / HDAC6 or paxillin/ HDAC6 or both signaling pathways. A key point is that both signaling pathways most likely than not emanate from integrin outside-in signaling involving kindlin-2.

Figure 29 An illustration of kindlin-2 in the regulation of mitotic spindle assembly. Kindlin-2 mediates integrin outside-in signaling. In this case, (1) kindlin-2 promotes AKT activation. Active AKT inhibits GSK3 β activity. Because active GSK3 β is required to activate HDAC6 that deacetylates α -tubulin, kindlin-2 is crucial to prevent this process. (2) Kindlin-2 may also recruit paxillin that has been shown to inhibit directly the activity of HDAC6. (3) In SH-SY5Y cells, hypoxia induces an up-regulation of miR-138, which could potentially inhibit the protein expression of kindlin-2. This could lead to abnormal mitotic spindle assembly and negatively affects cell proliferation.

A hallmark of adherent cells undergoing mitosis is cell rounding. It seems paradoxical that integrins are required for both signaling pathways in mitotic cells when the cell detaches from the ECM. However, the contact between cells and the ECM before entry into mitosis has been shown to be crucial for proper spindle assembly [410, 411]. Integrins have also been shown to regulate the

growth of microtubules from centrosome and the formation of normal bipolar spindles [412]. Interestingly, punctate active $\beta 1$ integrin contacts with ECM are maintained between mitotic cells and the ECM, suggesting that integrin-mediated adhesion is still maintained in mitotic cells but scaled down [413]. Hence, integrin outside-in signaling mediated by kindlin-2 may play an important role in modulating the activity of HDAC6 prior to or during mitosis or both. In fact kindlin-1 has also been shown to regulate mitotic spindle assembly that is dependent on integrins [127].

During mitosis, entry from metaphase into anaphase requires all kinetochores to be attached to microtubule spindles, which is tightly regulated by the SAC network of signaling molecules. Based on our data, depletion of kindlin-2 expression caused abnormal mitotic spindle assembly. In many of these cells, the SAC protein MAD2 was readily detected at the kinetochore of mis-segregated chromosomes, suggesting that these sites are not attached to microtubule spindles. This also accounts for the delay in mitosis of cells with kindlin-2 depletion. Our data also lend support to a previous genome-wide RNAi study in *Drosophila* S2 cells that Fit1, the *Drosophila* homolog of kindlin-2, suppresses abnormal spindle assembly [414].

Another key observation from our study is that hypoxia reduces kindlin-2 expression in SH-SY5Y cells. Mechanistically, hypoxia induces the up-regulation of miR138, which has been reported to inhibit kindlin-2 expression [415]. Hypoxia has a negative effect on cell cycle in many cell types [416]. Indeed, slow proliferation of SH-SY5Y cells was observed when under hypoxia culture conditions. Importantly, hypoxia could induce chromosomal instability [417]. Our data suggest that the decrease in kindlin-2 expression in cells under hypoxia could be one of the many factors inducing chromosomal instability. Given the crucial role of kindlin-2 in integrin-mediated cell motility [418] and hypoxia is strongly linked to tumour metastasis [419], it seems intriguing that hypoxia inhibits kindlin-2 expression in cancer cells. However, cancer progression is a complex process. During metastasis, cancer cells detach from the tumour and migrate into lymphatic or blood vessels. This process can be mediated by mesenchymal and amoeboid movements, which are interchangeable depending on the tumour microenvironment [420, 421]. In mesenchymal-type movement, cells adopt an elongated morphology and they are highly dependent on integrins. By contrast, amoeboid-like movement of cancer cells involves weak ECM adhesion and cells are highly deformable. Interestingly, a recent study provided evidence that prolonged and severe hypoxia stimulated head and neck squamous

carcinoma cells in 3D spheroid culture to adopt amoeboid migration [422]. Taken together, it is possible that in regions of severe hypoxic stress in tumours, kindlin-2 expression is down-regulated, which promotes cells to adopt amoeboid migration. Indeed, our *in vivo* data provide evidence that kindlin-2 expression was down-regulated in hypoxic regions of SH-SY5Y tumours although the demonstration of cells undergoing amoeboid movement requires further studies.

In conclusion, we provide first evidence that kindlin-2 regulates mitotic spindle assembly in cells. This involves the Akt/ GSK3 β / HDAC6 or the paxillin/ HDAC6 or both signaling pathways. We also showed for the first time that hypoxia down-regulates kindlin-2 expression, which may be important during cancer cell amoeboid movement-mediated metastasis.

Chapter 4 Identifying the molecular determinant in kindlin-3 for its interaction with RACK1

4.1 Overview

Alternative splicing of messenger RNA (mRNA) creates variable gene products with different functions which enhances biodiversity and functional complexity in the human proteome. Alternative splicing occurs naturally but abnormal splicing has also been reported during disease progression [423, 424]. There are at least three spliced isoforms of kindlin-3. The longest spliced variant contains an additional four amino acids ³⁶¹IPRR³⁶⁴ in the PH domain. It has been reported that this additional sequence enhances the phosphoinositide-binding property of the PH domain because of the two positively-charged Lys residues, but B cells from LAD III patient reconstituted with this isoform exhibited defective migratory property [425]. This suggests that the kindlin-3 long isoform may have functions that are unrelated to integrin-mediated cell adhesion and migration.

Kindlin-1, kindlin-2 and kindlin-3 share high sequence homology but they have many distinct functions apart from regulating integrin activity [87, 88, 426]. Our group has previously demonstrated that the PH domain of kindlin-3 (long isoform) interacts with blades WD 5-7 of RACK1 [101, 173]. However, fine mapping of the interacting regions has not been performed. In this study, we identify a short sequence (³⁶¹IPRR³⁶⁴) within the PH domain of kindlin-3 (long isoform; henceforth referred to as LF) that is crucial for the interaction between kindlin-3 and RACK-1.

4.2 Mapping of RACK1 interaction site in the PH domain of kindlin-3 LF

Kindlin-3 consists of F0, F1, F2 and F3 subdomains. Unlike other FERM proteins, a PH domain bisects the F2 subdomain in kindlin-3 [104]. Our group has previously shown that kindlin-3 PH domain is involved in RACK1 interaction [101, 173]. However, amino acid residue(s) within the PH domain involved in RACK1 binding has not been determined. Our lab has previously generated two mAbs named clone 9 and clone 181A. Data from epitope mapping study performed by a previous PhD student (Ms. Chen Feng) from our lab suggest that the epitope of mAb clone 9 resides in the F3 subdomain whereas that of mAb clone 181A resides in the PH domain. Both mAbs can be used for immunoblotting and immunoprecipitating kindlin-3 but it was noted that in co-immunoprecipitation assays using cell lysates, endogenous RACK1 was detected in the co-precipitate of mAb clone 9 but not clone 181A despite the presence of kindlin-3 in both samples (data not shown). One explanation

for this observation is that when kindlin-3 LF interacts with RACK1, it prevents the binding of mAb clone 181A to its epitope in the PH domain or mAb clone 181A dissociates RACK1 from kindlin-3 LF.

To uncover their interaction sites, another member (Ms. Belinda Wong Shu-Ee) of our lab generated five truncated constructs of N-terminus HA-tagged kindlin-3 LF with successive deletions of the PH domain (**Figure 30**). These were used herein to identify the region within the PH domain that contains the epitope of mAb clone 181A, which will also provide information on the RACK1 interaction site.

HEK293T cells were transfected with these expression plasmids followed by immunoblotting using anti-HA, mAb clone 181A and mAb clone 9 (**Figure 31**). HA-kindlin-2 transfected cells were included as a negative control to demonstrate the specificity of the two anti-kindlin-3 mAbs. mAb clone 9 failed to detect any of the mutant kindlin-3, which is consistent with its epitope found in the F3 subdomain. mAb clone 181A detected most of the kindlin-3 mutants except the shortest that contains the N-terminal 27 amino acids of the PH domain. It was also observed that relatively weak signal was detected for the second shortest mutant kindlin-3 PH2. In all cases, anti-HA immunoblotting revealed protein bands of HA-kindlin-2, HA-kindlin-3 LF and its mutants. Taken together, these data suggest that the epitope of mAb clone 181A lies within the first Glu351-Lys405. Based on the crystal structure of human kindlin-3 LF (unpublished data of the lab in collaboration with another group), this region involves one surface of the PH domain that is dominated by β strands (**Figure 32**).

4.3 ³⁶¹IPRR³⁶⁴ of kindlin-3 PH domain is crucial for RACK1 binding

Although truncated mutants of kindlin-3 LF were useful in mapping the epitope of mAb clone 181A by immunoblotting, they are inherently unsuitable for testing the interaction site between kindlin-3 LF and RACK1 because large deletions, as in the case of the five mutants, can cause severe protein mis-folding and degradation. Hence, an alternative strategy was employed in subsequent experiments. Instead of deleting regions within the kindlin-3 LF, chimeras of kindlin-3 and another kindlin family member can be generated. The basis of this strategy lies in the fact that kindlin-3 interacts with RACK1 whereas kindlin-2 interact minimally, if any. Indeed, YFP-photobleach FRET assay using COS-7 cell transfected with YFP-N1-RACK1 and CFP-C1-Kindlin-2, CFP-C1-Kindlin-3 LF or CFP-C1 empty vector showed significant interaction between kindlin-3 LF and RACK1 as compared to that of kindlin-2 (**Figure 33A**).

A

1	MAGMKTASGD	YIDSSWELRV	FVGEEDPEAE	SVTLRVTGES	HIGGVLLKIV	50
51	EQINRKQDWS	DHAIWWEQKR	QWLLQTHWTL	DKYGILADAR	LFFGPQHRPV	100
101	ILRLPNRRAL	RLRASFSQPL	FQAVAAICRL	LSIRHPEELS	LLRAPEKKEK	150
151	KKKEKEPEEE	LYDLSKVVL	GGVAPALFRG	MPAHFSDSAQ	TEACYHMLSR	200
201	PQPPPDPLLL	QRLPRPSSL	DKTQLHSRWL	DSSRCLMQQG	IKAGDALWLR	250
251	FKYYSFFDL	PKTDPVRLTQ	LYEQARWDL	LEEIDCTEEE	MMVFAALQYH	300
301	INKLSQSSEV	GEPAGTDPGL	DDL DVALSNL	EVKLEGSAPT	DVLDLTTIP	350
351	ELKDHLRIFR	IPRRPRKLT	KGYRQHWVF	KETTLSSYYKS	QDEAPGDPIQ	400
401	QLNLKGCEVV	PDVNVSGQKF	CIKLLVPSPE	GMSEIYLRCQ	DEQQYARWMA	450
451	GCRLASKGRT	MADSSYTSEV	QAILAFLSLQ	RTGSGGPGNH	PHGPDASAEG	500
501	LNPYGLVAPR	FQRKFKAKQL	TPRILEAHQN	VAQLSLAEAQ	LRFIQAWQSL	550
551	PDFGISYVMV	RFKGSRKDEI	LGIANNRLIR	IDLAVGDVVK	TWRFSNMRQW	600
601	NVNWDIRQVA	IEFDEHINVA	FSCVSASCRI	VHEYIGGYIF	LSTRERARGE	650
651	ELDEDLFLQL	TGGHEAF	667			

B

Figure 30 Kindlin-3 expression constructs. (A) Human kindlin-3 (LF) amino acid sequence. PH domain is highlighted in yellow. Stop codon was introduced in each of these mutant kindlin-3 constructs denoted as PH1, PH2, PH3, PH4, PH5. (B) The five kindlin-3 truncated mutants (PH1, PH2, PH3, PH4, PH5) generated were shown.

Figure 31 Determining the epitope of anti-kindlin-3 mAb clone 181A by immunoblotting. Immunoblotting was performed on HEK293T cells transfected with HA-tagged Kindlin-2 (K2), HA-tagged kindlin-3 LF (K3 LF), HA-tagged K3 PH1, HA-tagged K3 PH2, HA-tagged K3 PH3, HA-tagged K3 PH4 or HA-tagged K3 PH5 truncated constructs. These antibodies were used for immunoblotting: anti-HA, anti-kindlin-3 clone 181A and clone 9. Immunoblotting of GAPDH was performed to determine total protein loading.

Figure 32 Crystal structure of kindlin-3 long form PH domain. The β strands ribbon (orange) represents the kindlin-3 PH region 350 to 405. The additional short sequence ($^{361}\text{IPRR}^{364}$) within the PH domain of kindlin-3 long form is indicated by purple.

Next, we generated two expression constructs. One was HA-kindlin3 LF with its PH domain substituted with that of kindlin-2 (HA-kindlin-3 Δ PHK2), and the other was HA-kindlin-2 with its PH domain substituted with that of kindlin-3 LF (HA-kindlin-2 Δ PHK3L). HEK293T cells were transfected HA-kindlin-3 LF, HA-kindlin-2, HA-kindlin-3 Δ PHK2 or HA-kindlin-2 Δ PHK3L followed by co-immunoprecipitation using anti-HA antibody. As expected, endogenous RACK1 was detected in co-precipitates of HA-kindlin-3 LF and very weakly in that of HA-kindlin-2. Endogenous RACK1 could also be detected in co-precipitates of HA-kindlin-2 Δ PHK3L and very weakly in that of HA-kindlin-3 Δ PHK2 (**Figure 33B**). These data lend support to the importance of kindlin-3 PH domain in its interaction with RACK1, and there are specificity determinants within kindlin-3 LF PH domain.

We then generated additional expression constructs of HA-kindlin-3 LF that has shorter segments of its PH domain substituted for that of kindlin-2. These are HA-kindlin-3 Δ PHK2 (350-378), HA-kindlin-3 Δ PHK2 (379-405) and HA-kindlin-3 Δ PHK2 (406-480) (**Figure 33C**). Co-immunoprecipitation assay was performed as described above. Interestingly, RACK1 was minimally detected in co-precipitate of HA-kindlin-3 Δ PHK2 (350-378) but not others, suggesting that this region is important for RACK1 binding.

Based on amino acid sequence alignment of kindlin-3 LF and kindlin-2, most residues within the PH domain region 350-378 are highly conserved except Lys353, His355, His375, Val378 and Ile361-Arg364 of kindlin-3 LF (**Figure 34A**). We therefore generated four HA-tagged kindlin-3 LF mutants in which Lys353, His355, His375 or Val378 was substituted for the corresponding residue of kindlin-2 (**Figure 34B**). In addition, a composite mutant in which all four amino acids were substituted with that of kindlin-2 was generated. From the co-immunoprecipitation assay, we did not detect complete ablation of RACK1 interaction with any of these mutants, except the composite mutant in which the RACK1 signal was slightly diminished. These data suggest that these amino acids play a role but are not essential for kindlin-3 LF interaction with RACK1.

We then examined the region Ile361-Arg364 in kindlin-3 LF that is absent in kindlin-2 and kindlin-1. Based on co-immunoprecipitation assay, RACK1 was readily detected in the co-precipitate of HA-kindlin-3 LF but very weakly in that of the other spliced variant HA-kindlin-3 short form (SF), which lacks these four amino acids (**Figure 34C**). These data suggest that region Ile361-Arg364 in kindlin-3 LF is a specificity determinant of kindlin-3 interaction with RACK1. Given that both kindlin-3

LF and kindlin-3 SF are detected in immune cells, it also suggests that different spliced isoforms have different roles, which warrants further studies.

Figure 33 Region 350-378 in the PH domain of kindlin-3 is crucial for RACK1 binding (A) FRET analysis was performed by YFP-photobleaching COS-7 cells co-transfected with YFP-N1-RACK1 and CFP-C1 vector, CFP-C1-Kindlin-2 or CFP-C1-Kindlin-3 LF. Each datum point represents one cell, 60 cells per group from three independent experiments were analyzed. Data represents mean $\pm$ S.D. from three independent experiments. Statistic: $p < 0.05$ considered significant. (B, C) Immunoprecipitation was performed by using mouse anti-HA Ab on HEK293T transfected HA-kindlin-2, HA-kindlin-3 LF, HA-kindlin-3 Δ PHK2 or HA-kindlin-2 Δ PHK3L, HA-kindlin-3 Δ PHK2 350-378, HA-kindlin-3 Δ PHK 379-405 or HA-kindlin-3 Δ PHK2 406-480 plasmid. Mouse IgG was used as control antibody (IgG). Co-precipitated endogenous RACK1 was detected by western blotting. Red box indicates the absence of endogenous RACK1 in HA-Kindlin-2, HA-kindlin-3 Δ PHK2 and HA-kindlin-3 Δ PHK2 350-378 immunoprecipitates.

A

Kindlin-3 long form	HUMAN	350	PEL	K [*] D [*] H	L	R	I	F	R	I	P	R	R	P	R	K	L	T	L	K	G	Y	R	H	W	V	378	
Kindlin-2	HUMAN	372	PE	L	A	D	Y	I	K	V	F	K	---	P	K	K	L	T	L	K	G	Y	K	Q	Y	W	C	396

B

C

Figure 34 ³⁶¹IPRR³⁶⁴ of kindlin-3 PH domain is important for RACK1 binding. (A) Amino acid sequence alignment of kindlin-3 LF region 350-378 with corresponding regions of kindlin-2. Residues in kindlin-3 LF that are not identical to that of kindlin-2 are highlighted in yellow and marked with asterisk. (B) HEK293T cells were transfected with HA-Kindlin-3 LF or HA-kindlin-3 mutants and immunoprecipitation was performed by using mouse anti-HA Ab. Mouse IgG was used as control antibody (IgG). Co-precipitated endogenous RACK1 was detected by immunoblotting. (C) HEK293T cells were transfected with HA-Kindlin-3 LF or HA-Kindlin-3 SF and immunoprecipitation was performed by using mouse anti-HA Ab. Mouse IgG was used as control antibody (IgG). Co-precipitated endogenous RACK1 was detected by western blotting. Red box indicates weak endogenous RACK1 detected in HA-Kindlin-3 SF immunoprecipitate.

4.4 Discussion

Apart from integrins, there is an expanding list of proteins that have been shown to interact with kindlins [427]. A number of these interacting partners of kindlins are not directly involved in integrin-mediated cell adhesion and migration, suggesting additional functions of kindlins. Our group has previously reported that the PH domain of kindlin-3 long form (LF) interacts with blades WD 5-7 of RACK1 [101, 173]. However, there is little information on which specific amino acid residue(s) within this PH region is important for RACK1 interaction.

Here, we showed that a stretch of four amino acids, namely ³⁶¹IPRR³⁶⁴, in the PH domain of kindlin-3 LF is the specificity determinant for the interaction between kindlin-3 LF and RACK1. These four amino acids are absent from kindlin-2, which interact weakly, if any, with RACK1. An interesting observation based on the crystal structure of kindlin-3 LF is that these four amino acids reside in a flexible loop in the PH domain that is important for the binding of phosphoinositides. In a previous study, it has been shown that PH domain phosphoinositide-binding property of kindlin-3 LF is enhanced because of the two additional basic Lys residues but kindlin-3 LF failed to rescue the defective adhesion and migration properties of LAD III B cells as compared to that kindlin-3 SF (that is, without the additional four amino acids) [425]. These data suggest that kindlin-3 LF may have additional functions that are not directly linked to integrins.

Due to time constraint, we are unable to perform further experiments to determine the functional differences between kindlin-3 LF and kindlin-3 SF. Nonetheless, in future studies, we plan to generate cell lines expressing either kindlin-3 LF or SF. Our lab has generated CRISPR-Cas9n knock-out of kindlin-3 in the human erythroleukemia K562 cell line. We plan to reconstitute the expression of either kindlin-3 LF or SF in this cell line and examine the functional properties of these cells. Previously, our lab has shown that silencing of kindlin-3 expression in K562 cells diminished c-Myc protein but not mRNA expression. Given that kindlin-3 was detected in ribosomes containing RACK1, it is possible that kindlin-3 LF regulates protein translation although we do not have the mechanistic details. We also plan to generate another CRISPR-Cas9n kindlin-3 KO cell line for our experiments. The human monocytic-like THP-1 cell line may be a potential cell line to be used because these cells can be easily differentiated into macrophage-like cells in the presence of phorbol ester PMA stimulation. This would allow us to interrogate the function of kindlin-3 LF versus SF in a

macrophage-like cell. We also plan to express and purify recombinant proteins of kindlin-3 LF PH domain and RACK1 with N15 and C13 isotopes labelling in order to perform solution NMR studies to provide molecular insights into the interface between kindlin-3 LF PH domain and RACK1. This will be performed in collaboration with NMR structural biology lab in our school. This approach is required because conventional methods of deletion mutations in cell-based assays are not amenable for the fine-mapping of kindlin-3 binding site on RACK1. Although blade 5-7 of RACK1 interacts with kindlin-3 LF, further deletion or expression of individual blade is not possible because of mis-folding and degradation. Finally, we will examine whether there are specific conditions that specifically induce the splice insertion that generates the kindlin-3 LF.

Chapter 5 The role of kindlin-2 in the function of neuroblastoma cell-derived extracellular vesicles

5.1 Overview

Exosome is a subset of extracellular vesicles (EVs). It carries a wide variety of molecules, including mRNA, miRNA, DNA and proteins such as cytoskeletal proteins, major histocompatibility, signal transducers and heat shock proteins and those involved in membrane trafficking [308, 309]. Information from producing cells (or sometimes referred to as donor cells) is transferred to target cells through exosome uptake [311, 312].

There are increasing numbers of exosome-based therapeutic studies in recent years [342, 343]. Studies have shown that tumour-derived exosomes mediate cancer metastasis and angiogenesis [320, 321]. In addition, tumour-derived exosomes educate and recruit bone-marrow derived cells to form pre-metastatic niche [308]. It has been shown that integrins expression profiles on tumour-derived exosomes specify the metastasis sites [154]. Kindlins are known as one of the regulating partners of integrins. Kindlin-2 is involved in clathrin-mediated endocytosis [142] and exosome uptake depends on clathrin-mediated endocytosis [313-315], suggesting that kindlin-2 may be important in regulating exosome uptake of target cells. Kindlin-2 may also play an important role in the function of exosome given the presence of integrins on exosomes. In this chapter, we determine whether kindlin-2 in EVs is required for their uptake by target cells.

5.2 Isolation and validation of exosomes from kindlin-2 shRNA targeting SH-SY5Y cells

Studies have shown that tumour-derived exosomes are involved in cancer progression [320, 428-430]. We first performed exosome isolation in our lab using the human neuroblastoma SH-SY5Y cells. Based on published protocols, we cultured SH-SY5Y cells in full culture medium to > 90% confluence followed by replacement of the full medium with one that was serum free. This step was necessary because FBS is well known to contain large amount of exosomes, which will have adverse effect on the purity of exosomes derived from SH-SY5Y cells. After 24 h in serum free culture medium, the cell culture supernatant was collected and exosomes were isolated as described in Methods and Materials section 2.2.10.1. To verify the exosomes preparation, we performed immunoblotting using antibodies against exosome markers, namely Alix, HSP70, TSG101 and CD81 [307] (**Figure 35A**). Most of these markers were detected in the isolated exosomes. It was noted that

CD81 was readily detected in exosome preparation but minimally in the cell lysate. This could be attributed to the fact that CD81 is known to be highly enriched in exosomes [307]. We also detected kindlin-2 in the exosomes albeit at a relatively low level. To verify that kindlin-2 was derived from exosomes rather than apoptotic bodies, histone and annexin V immunoblottings were performed [431]. Both histone and annexin V were detected in the exosomes preparation, suggesting that there could be apoptotic bodies in the exosomes preparation, and that kindlin-2 detected may be from apoptotic bodies (**Figure 35A**). In this method, cells were serum starved (i.e. absence of growth factors) for 24 h before the isolation of exosomes. This could lead to cell stress and apoptosis. Indeed SH-SY5Y cells showed long-spindle-like morphology under serum-starvation (data not shown).

To circumvent this problem, the protocol was modified by culturing the cells in medium containing 4 % (v/v) exosome-depleted FBS for 24 h before the isolation of exosomes. Minimal if any histone or annexin V was detected in the exosomes preparation but we were unable to detect convincing kindlin-2 signal (**Figure 35B** & data not shown). We reasoned that the use of 4 % exosome-depleted FBS effectively reduced cell apoptosis but it is still sub-optimal and this could, in a yet-unknown mechanism, affect the incorporation of kindlin-2 into the exosomes.

We therefore repeated the experiments with 7 % (v/v) exosome-depleted FBS (**Figure 35C**). Kindlin-2 was readily detected in the exosomes and there were minimal apoptotic bodies as verified by the lack of annexin V signal. These preliminary results suggested that kindlin-2 is found in exosomes derived from the neuroblastoma SH-SY5 cells. To further verify our exosomes preparation, we performed flow cytometry bead-based exosomes analysis. Exosomes were confirmed by CD63 and CD81 staining (**Figure 35D**). Relevant controls mouse IgG coated beads (green histogram) and FITC mouse IgG (orange histogram) were included. We also performed size determination of the exosomes using Nanosight instrument (**Figure 36**). The mean of the size distribution was 148.2 nm, mode was 134.1 nm and standard deviation was 41.0 nm. Despite the different analysis to determine the surface markers and size of purified exosomes, we cannot exclude that the possibility of the preparation still contains others extracellular vesicles. Therefore in the subsequent discussion, we will use the extracellular vesicles (EVs) term to describe the exosomes preparation.

To determine whether kindlin-2 is important for the function of EVs, we isolated EVs from pGFP-C-shLenti kindlin-2 shRNA (K2B shRNA) and control shRNA targeting SH-SY5Y cells (these

stable cell lines were mentioned in Chapter 3). EVs were isolated from WT, control shRNA and K2B shRNA cells that were cultured in media containing 7 % (v/v) exosome-depleted FBS. Immunoblotting was performed to confirm the EVs isolation (**Figure 37A**). The

D

Figure 35 Validation of exosomes isolation. SH-SY5Y cells were treated with (A) serum free medium, (B) 4 % (v/v) exosome-depleted FBS or (C) 7 % (v/v) exosome-depleted FBS for 24 h. Cell lysates and isolated exosomes were lysed followed by immunoblotting using antibodies against- Alix, HSP70, TSG101 and CD81. The presence of apoptotic bodies were examined by annexin V and histone immunoblotting. (D) Exosomes derived from cells cultured in medium containing 7 % (v/v) exosome-depleted FBS were confirmed by flow cytometry analysis using FITC-anti-CD63 (red) and FITC-anti-CD81 (blue). The relevant controls green and orange histograms were included.

Figure 36 Size distributions of exosomes by Nanosight. Size determination was performed on exosomes-derived from SH-SY5Y cells by Nanosight instrument. Size (nm) of SH-SY5Y-derived exosomes was plotted against concentration (particles/ ml). Merged data (solid black line) was calculated based on three replicate measurements. We thank DKSH Technology Pte Ltd, Singapore, for kindly allowing us to test our samples on their Nanosight demo unit.

level of kindlin-2 in K2B shRNA cells-derived EVs was lower than that in WT and control shRNA cells. EVs markers (Alix, HSP70, TSG101 and CD81) were also detected. There were no apoptotic bodies observed in EVs preparation from all (**Figure 37A**).

In addition, EVs were subjected to negative staining electron microscopy (performed by our collaborator Associate Prof. Yong-Gui Gao's lab member Ms. Wen-Ting Bu). The images were captured to examine the size of the isolated EVs (**Figure 37B**). The size of the isolated EVs of SH-SY5Y WT, control shRNA and K2B shRNA in the electron microscopy images were around 50 - 80 nm. This seems to be smaller than that reported using Nanosight instrument but this could be due to the dehydration steps involved in the sample preparation step for electron microscopy.

5.3 SH-SY5Y derived EVs promote tube formation *in vitro*

Tumour-derived exosomes are taken up by endothelial cells and they promote angiogenesis [428]. To test whether EVs isolated from SH-SY5Y cells are functional. HUVECs were plated onto matrigel-coated well of 96-well culture plate and co-cultured with 10 µg EVs derived from SH-SY5Y WT cells. The clustering and branching of the HUVECs were determined by analyzing the images using the ImageJ angiogenesis analyzer software (**Figure 38A**). Number of nodes formation and total branching length were found to gradually increase in cells treated with 10 µg EVs compared to that in control group from 1.5 h to 2.5 h. However, the number of nodes and total branching length started to reach the saturation point and decrease at 3.5 h (**Figure 38B and C**).

We then examined the effects of EVs derived from K2B shRNA and control shRNA cells on HUVEC angiogenesis. HUVECs were plated onto matrigel-coated well of 96-well culture plate and co-cultured with or without 10 µg WT, control shRNA- or K2B shRNA-derived EVs. Images were captured and analyzed (**Figure 39A**). At the 1.5 h time point, fewer number of junctions and shorter total tube-length were observed in cells treated with EVs derived from K2B shRNA cells compared with that from other groups (**Figure 39B**). However, beyond 2 h, there was no significant difference between them, suggesting that the effect of EVs was during the early phase of HUVEC tube formation.

A **B**

Figure 37 Validation of EVs derived from SH-SY5Y WT, control shRNA and K2B shRNA cells. (A) SH-SY5Y WT, control shRNA and K2B shRNA cells were treated with 7 % (v/v) exosome-depleted FBS for 24 h. Cell lysates and isolated EVs were lysed to perform immunoblotting using antibodies against- Alix, HSP70, TSG101 and CD81. The presence of apoptotic bodies were examined by annexin V immunoblotting. (B) Electron microscopy images of EVs derived from SH-SY5Y WT, control shRNA and K2B shRNA cells (from left to right panel) were captured by our collaborator, Associate Prof. Yong-Gui Gao's lab member Ms. Wen-Ting Bu. Each red arrow points to an EV. Scale bar: 100 nm.

Figure 38 Induction of vascular tube formation by SH-SY5Y-derived EVs. SH-SY5Y-derived EVs in PBS were added to HUVECs on matrigel. (A) HUVECs tube formation on matrigel in the presence of PBS only (control) and 10 μ g of EVs. Tube formation was monitored on a live-cell imaging system for 3.5 h. Nodes (red dots) and branching length measurements (yellow, blue and green lines) were analyzed by ImageJ angiogenesis analyzer. Scale bar: 100 μ m. (B) Number of nodes and (C) total branching length were plotted against 1.5, 2.5 and 3.5 h time points. Data was plotted from one experiment.

Figure 39 Reduction of vascular tube formation in SH-SY5Y K2B shRNA cells-derived EVs. SH-SY5Y WT, control shRNA or K2B shRNA cells-derived EVs in PBS were added to HUVECs on matrigel. (A) HUVECs tube formation on matrigel in the presence of PBS only (control) and 10 μ g of different SH-SY5Y WT, control shRNA or K2B shRNA cells-derived EVs. Tube formation was monitored on a live-cell imaging system for 2.5 h. Junctions and total length measurements (yellow, blue and green lines) were analyzed using the ImageJ angiogenesis analyzer. Representative images are shown. Scale bar: 200 μ m. (B) Number of junctions and total length (μ m) were plotted at 1.5, 2 and 2.5 h time points. Data represent mean $\pm$ S.D. from three independent experiments. Statistics: * $p \leq 0.05$; ** $p \leq 0.01$; only $p < 0.05$ is considered significant; (n.s.) $p > 0.05$ is considered not significant.

5.4 Discussion

Studies have shown that tumour-derived exosomes promote cancer initiation, progression, metastasis and drug resistance [320, 428-430]. Kindlin-2 has been implicated in clathrin-mediated endocytosis [142] and integrins on exosomes are important for exosomes uptake by recipient cells [154]. We were able to isolate intact extracellular vesicles (EVs) that exhibited properties of exosomes after several rounds of optimization of protocol. A key point to take note of during the isolation process is to prevent harvesting of apoptotic bodies, which will complicate downstream analyses. We provide evidence that EVs isolated from SH-SY5Y cells contained kindlin-2. Importantly, we showed that a lack of kindlin-2 in EVs derived from K2B shRNA cells affects their function. Based on tube formation using HUVECs, which is a conventional *in vitro* angiogenesis assay, we showed that HUVECs tube formation was enhanced when treated with EVs from SH-SY5Y WT cells as compared to those without treatment. Our data also suggest that kindlin-2 is important for the EVs to exert their effect on HUVECs. However, we noted that this effect was most significant during the initial phase of HUVECs tube formation. Whether this is due to a defect in the binding of EVs onto the cell surface of HUVECs as a result of kindlin-2 depletion remains to be determined. We hypothesize that a lack of kindlin-2 in EVs may affect the activity of integrins in EVs. Due to limited time, this project is being put on hold.

In the future experiments, we plan to do *in vivo* angiogenesis assay by introducing EVs-containing matrigel plug into flanks of mice to verify angiogenic sprouting. To study the contribution of kindlin-2 in EVs biogenesis in SH-SY5Y cells, we plan to perform comparative analyses of EVs yield from control and kindlin-2 silenced SH-SY5Y cells. Hypothetically, silencing of kindlin-2 should reduce the yield of EVs production in these cells since kindlin-2 is involved in endocytosis. To interrogate the activity of the integrins on kindlin-2 silenced EVs, we plan to perform flow cytometry bead-based EVs analyses using active integrin reporter antibodies. This will also be complemented by electron microscopy studies using nano-gold conjugated integrin reporter antibodies.

Chapter 6 Investigating the traction force, cell spread area and contraction directionality of Human Umbilical Vein Endothelial Cells (HUVECs) on fibronectin and collagen pre-coated micro-posts

6.1 Overview

Fibronectin and collagen type I are abundant extracellular matrix proteins that modulate cell mechanics and they regulate angiogenic sprouting [432]. Fibronectin and collagen are known to be ligands for specific integrins [66]. Fibronectin is a homodimer covalently linked by disulfide bond at their C-termini [433]. Collagen is formed by three polypeptide II-type helical conformation coil triple helix [434]. The binding affinity between fibronectin and integrin is approximately $0.8 \mu\text{M}$ [435]; Collagen type I binds to $\alpha 1\beta 1$ and $\alpha 2\beta 1$ integrins with a K_D of $0.09 \pm 0.06 \mu\text{M}$ and $0.21 \pm 0.02 \mu\text{M}$, respectively [436]. Study has been shown that the proliferation and migration rates are different between Human Umbilical Vein Endothelial Cell (HUVEC) on fibronectin and collagen [437]. Furthermore during wound healing, temporal difference in the synthesis and deposition of fibronectin and collagen has been shown to modulate the angiogenic response [432].

The differences in mechano-sensing as a result of different ECM substrates and stiffness can lead to distinct cellular responses. HUVEC expressed fibronectin-binding integrins $\alpha 5\beta 1$ and $\alpha v\beta 3$ and collagen-binding integrins $\alpha 1\beta 1$ and $\alpha 2\beta 1$ [438-440]. Ligand-bound integrins play an important role in mechano-signal transduction by providing physical linkages between the ECM and the cytoskeleton, and they serve as nucleating factors for the maturation of focal adhesions [441]. Study has shown that HUVEC expressed kindlin-2 and kindlin-3. Although the expression level of kindlin-3 is lower than kindlin-2, silencing of kindlin-3 expression by siRNA significantly reduced HUVEC spreading and tube formation [85]. Given that HUVECs respond differently to fibronectin and collagen substrates, and that traction force has an important role in cellular responses, we therefore performed comparative analyses of traction forces and directional contractions of HUVECs on fibronectin- and collagen-coated microposts. Because kindlin-3 plays a role in endothelial cell spreading and tube formation, we extend the study to examine the effect on traction forces and directional contractions of HUVECs overexpressing kindlin-3.

6.2 Different morphologies of HUVECs on fibronectin and collagen type I

HUVECs were seeded on glass-bottom culture dish that was pre-coated with either fibronectin or collagen type I (henceforth referred to as collagen). Cell shape differences were

observed between fibronectin and collagen coated glass bottom dish at 60 min (**Figure 40A & B**). HUVECs on fibronectin exhibited larger cell spread area compared to those on collagen. HUVECs were subjected to immunofluorescence staining to detect actin filaments and focal adhesion protein paxillin and analyzed by confocal laser scanning microscopy (**Figure 40C**). Although punctuate focal adhesions (paxillin staining) and actin filaments were detected in both groups of cells, the distribution of focal adhesions were distinct. Whereas focal adhesions could be readily observed within the inner regions of cells spreading on fibronectin, focal adhesions were localized mainly at the lamellipodia and filopodia of on collagen. Taken together, these data suggest that there are differences in the formation of focal adhesions that would lead to distinct traction forces within these cells.

Figure 40 Cell shape differences of HUVECs on fibronectin and collagen at 60 min time point. Phase contracts images of HUVECs spreading on (A) fibronectin or (B) collagen type I coated glass bottom dish. Scale bar: 50 µm (C) Fibronectin (upper) and collagen (lower) pre-coated glass bottom dish on confocal microscopy images. Actin (Red), Paxillin (Green), DAPI (Blue). Scale bar: 20 µm.

6.3 HUVECs on fibronectin exert greater total traction force compared to those on collagen

Cells sense and respond to mechanical stimuli. Force sensing is required for cells to establish stable adhesion and spreading on ECMs. Integrins are involved in cell mechano-signal transduction. Integrins $\alpha 5\beta 1$ and $\alpha V\beta 3$ bind to fibronectin and integrins $\alpha 1\beta 1$ and $\alpha 2\beta 1$ bind to collagen [438-440]. HUVECs express all these integrins [436, 438, 442]. We therefore hypothesize that HUVECs on different ECMs exhibit different traction forces because different integrins are involved. This is physiologically relevant in wound healing, fibronectin and collagen synthesis and deposition are temporally distinct [432]. Micro-posts (micro-pillar) arrays have been used to measure traction forces of single cell or cell monolayer [443, 444]. In collaboration with Assistant Professor Shang-You Tee and Dr. Jing-Jing Han (School of Physical and Mathematical Sciences, NTU), we examined HUVECs traction forces on different ECM pre-coated micro-posts arrays. HUVECs were seeded on uncoated, fibronectin or collagen pre-coated micro-posts followed by live-cell imaging for 2 h at 37 °C under 5 % CO₂ on a microscope stage equipped with a CCD camera. Total absolute traction force and number of pillars exerted by each cell were calculated and plotted against time (**Figure 41**). In the experimental groups, the total traction force per cell increased with time and plateau after 40 min (**Figure 41A, E, I**). The total absolute traction force per cell was greater on fibronectin than on collagen pre-coated micro-posts from 0-20 min. At the plateau (i.e. > 20 min), the average traction force of cells on fibronectin pre-coated group was ~ 200 nN whereas that of cells on collagen pre-coated group was ~ 150 nN. The total absolute traction force per cell on uncoated micro-posts (i.e. control group) was the lowest at an average of ~ 100 nN. Next, we determined the number of pillars under each cell, which corresponds to the cell spread area. The number of pillars contacts per cell in the order to form highest to lowest was fibronectin, collagen and uncoated (**Figure 41B, F and J**).

Average force per pillar under each cell was then calculated and plotted against time (**Figure 41C, G and K**). Although the average force exerted on each pillar increased with time, there was a difference in time taken to reach the saturation plateau for the experimental groups. HUVECs on fibronectin and on collagen reached saturation plateau around 10 and 25 min, respectively. HUVECs on uncoated micro-posts showed the lowest average force per pillar for each cell compared with others. In an earlier study that measured cell contractile response using micro-posts array, it was observed that the number pillars in contact with a cell was inversely proportional to average force exerted per pillar [445]. In our study, although each cell was in contact with a higher number of

fibronectin-coated than collagen-coated pillars, there was apparently no significant difference in the average force exerted per pillar at the plateau phase. Normalized force histograms over 45000 data points of all pillars from all cells in each time frame were presented (**Figure 41D, H and L**). There was no significant difference in force distributions (Force ranges: 0 to 2 nN, 2 to 4 nN, 4 to 6 nN, 6 to 7 nN and above 7 nN) between cells on fibronectin and cells on collagen. Approximately 60 % pillars exerted traction force less than 2 nN in both groups. Throughout the 2 h live-cell imaging, only HUVECs on micro-posts that retained their capacities to spread and contract, suggesting that cells were viable, were analyzed. For subsequent experiments, HUVECs on uncoated pillars were not included because of the low traction force detected.

6.4 Pronounced directional contraction and higher correlation value were observed in HUVECs on fibronectin than on collagen

Both the magnitude and direction of force transmission are important in regulating cell spreading and migration. Our data showed that force vectors were directed towards the center of each cell that adhered to fibronectin (**Figure 42A**). By contrast, a random distribution of force vectors was observed in cells that adhered to collagen (**Figure 42D**). To facilitate the data interpretation, we calculated the directionality of contraction on these ECMs. Directionality of contraction is defined as the magnitude of the sum of force vectors divided by the sum of their magnitudes [446]. A value of 1 defines parallel forces and a value of 0 defines balanced forces. The average value of directionality of contraction was higher in cells on fibronectin than on collagen (**Figure 42B and E**). We also determined the correlation value of force vectors of neighboring pillars by calculating the cosine of the angle between the two neighboring force vectors. When the two neighboring pillars are in parallel, the angle is 0 and the correlation value is 1. A higher correlation value was determined in cells on fibronectin compared to those on collagen (**Figure 42C and F**). Taken together, these data suggest that the marked differences in directional contraction and correlation values between cells on fibronectin versus cells on collagen could lead to distinct focal adhesion assembly and the alignments of actin filaments. To further investigate the extent of focal adhesion formation, HUVECs on either fibronectin or collagen pre-coated micro-posts were fixed at different time point (2, 17 and 37 min) by immunofluorescence staining of focal adhesion protein paxillin [447] (**Figure 43A and C**). Horse-shoe shape focal adhesion formation on ECM micro-patterns was observed [448, 449]. In line with these studies, we observed similar structure around each pillar. The sum of focal adhesion areas of both

groups increased with time and it also correlated with an increase in cell spread area (**Figure 43B and D**).

Figure 41 Force measurements of HUVECs on uncoated (A-D), fibronectin (E-H) and collagen type-I (I-L) pre-coated micro-posts. (A, E and I) Total absolute force (nN) against time (min) of HUVECs on micro-posts. (B, F and J) Number of pillars exerted by each cell against time (min) on micro-posts. (C, G and K) Average force per pillar on each cell against time (min) on micro-posts. (D, H and L) Normalized force distribution of all pillars from all cells in each time frame throughout 2 h. N=7 for each plot. Data represent as mean (dark blue line) $\pm$ S.D. (pale blue region).

Figure 42 Measurement of directionality contraction and correlation on HUVECs fibronectin (A, B, C) and collagen (D, E, F) pre-coated micro-posts. (A and D) Representative HUVEC force map on pre-coated micro-posts at 60 min time point. The yellow arrow indicated the force vector on each pillar. Scale bars for force magnitude (yellow) and cell image (white) were 5 nN and 10 μ m, respectively. (B and E) Directionality of HUVECs on pre-coated micro-posts against time (min) (Duration: 2 h). (C and F) Average correlation of force on all neighboring pillar pairs for HUVECs on pre-coated micro-posts against time (min). N= 7 for each plot. Data represent as mean (dark blue line) $\pm$ S.D. (pale blue region).

Figure 43 Focal adhesion formation of HUVECs on fibronectin (A and B) and collagen (C and D) pre-coated micro-posts. (A and C) Representative focal adhesion image of HUVECs cultured on pre-coated micro-posts coated at 17 min time point. Scale bar: 10 μm (B and D) Total focal adhesion area against cell area for HUVECs on pre-coated micro-posts at 2 min, 17 min and 37 min. Each datum point represents one cell.

6.5 HUVECs overexpressing EGFP-kindlin-3 exhibited a reduction in total traction force and directional contraction on fibronectin pre-coated micro-posts

Kindlin-3 plays an important role in HUVEC spreading and tube formation [85, 108]. Studies have also shown that kindlin-3 is involved in cancer progression, but whether overexpression of kindlin-3 promotes cancer growth and metastasis remains to be clarified because of conflicting observations from different studies [87, 167]. Therefore, we examined the traction force and directional contraction in HUVECs overexpressing EGFP-kindlin-3. Expression levels of EGFP-kindlin-3 and EGFP-vector in transfected cells were determined by immunoblotting and flow cytometry analysis (**Figure 44A**). Reduction of traction force and fewer pillars underneath cell were observed in HUVECs overexpressing EGFP-kindlin-3 compared to cells overexpressing the EGFP-vector (**Figure 44B, C, F and G**). These data suggest that HUVECs overexpressing EGFP-kindlin-3 spread less effectively than cells overexpressing the EGFP-vector. Furthermore, HUVECs overexpressing EGFP-kindlin-3 showed moderately higher average force per pillar at the early phase of cell attachment and spreading (beginning 50 min) (**Figure 44D and H**). There was no major difference between EGFP-vector and EGFP-kindlin-3 cells with regard to force distribution although there appeared to be an increase in % force in the range of 2-4 nN in EGFP-kindlin-3 cells (**Figure 44E and I**).

We then determined the directional contraction of EGFP-kindlin-3 cells. Interestingly, EGFP-kindlin-3 cells showed attenuated directional contraction compared with EGFP-vector cells (**Figure 45A, B, D and E**). A similar trend was observed in the correlation value calculated (**Figure 45C and F**).

— HUVECs (non-transfected)
— HUVECs (EGFP-vector)
— HUVECs (EGFP-kindlin-3)

Figure 44 Force measurements of EGFP-vector control HUVECs (B-E) and EGFP-kindlin-3 overexpressing HUVECs (F-I) on fibronectin pre-coated micro-posts. (A) Immunoblotting and flow cytometry analyses of transfectants to detect EGFP-vector and EGFP-kindlin-3. Actin was used as loading control. (B and F) Total absolute force (nN) plotted against time (min) of EGFP-vector and EGFP-kindlin-3 overexpressing HUVECs on fibronectin pre-coated micro-posts. (C and G) Number of pillars exerted by each cell against time (min) on micro-posts. (D and H) Average force per pillar on each cell against time (min) on micro-posts. (E and I) Normalized force distribution of all pillars from EGFP-vector and EGFP-kindlin-3 overexpressing HUVECs in each time frame. N= 5 for each plot. Data represent as mean (dark blue line) $\pm$ S.D. (pale blue region).

Figure 45 Directionality contraction and measurements and correlation values of HUVECs transfected with EGFP-vector (A, B, C) and EGFP-kindlin-3 (D, E, F) on fibronectin pre-coated micro-posts. (A and D) Representative force map images at the 70 min time point. Each yellow arrow depicted the force vector on a pillar. Scale bars for force magnitude (yellow) and cell image (white) were 5 nN and 10 μ m, respectively. (B and E) Directionality of contraction plotted against time (min) (Total duration: 2 h). (C and F) Correlation values plotted against time (min). N= 5 for each plot. Data represent as mean (dark blue line) $\pm$ S.D. (pale blue region).

6.6 Discussion

Cell traction force on ECM is required for many physiological processes such as wound healing, inflammation, embryogenesis and angiogenesis [432, 450]. Cell traction forces ranges from pN – nN and the dimension (length) ranges from nm – μm [451]. Study has shown that the physical properties of stromal environment are shown to play an important role in cancer metastasis. Cancer cells experience different cellular traction forces in the heterogeneous nature of the stromal environment [452]. Studies have shown that ECM mediated cell adhesion can promote cell survival [453, 454]. FAK plays an important role in mechanosignal transduction in cancer cells [455, 456]. FAK is expressed in many cancers and is crucial for cell adhesion and migration [457, 458]. FAK knock-out cells exhibited slower migration and less contraction as compared to wild type cells. Therefore, FAK can be a useful target in the development of cancer therapy [459].

Fibronectin and collagen type I are abundant ECM proteins. In this chapter, traction force of HUVECs on fibronectin and collagen type I were examined. From our observation, cell spread area and total traction force of HUVECs on fibronectin are larger compared to those on collagen. Because HUVECs expressed fibronectin-binding integrins $\alpha 5\beta 1$ and $\alpha v\beta 3$ and collagen-binding integrins $\alpha 1\beta 1$ and $\alpha 2\beta 1$, the differences observed are not due to a lack of relevant integrins binding to fibronectin and collagen [436, 438-440, 442]. However, there were no significant differences between both groups in average force exerted per pillar and force range distribution. These data suggested that force transmission signaling differences observed in both groups of cells were unlikely a result of different force magnitudes. In terms of directional contraction, cells on fibronectin exhibited higher directional contraction compared with cells on collagen. This was evident from the almost parallel force vectors in cells on fibronectin compared to those on collagen. Force transmission in cells requires the interaction of actin cytoskeleton and focal adhesion sites coupled with myosin activity. In another micro-posts array study, there was positive correlation observed between cell spread area and total force adhesion area per HUVEC [460]. From our study, we also observed that cell spread area and total force adhesion increased with time in both groups. However, cells on fibronectin showed larger cell spread area and total force adhesion especially at later time point, 37 min. Different integrins bind to different ECM proteins and they induce specific signaling pathway which could lead to differences in cell spread area and total traction force. Indeed, a study has shown that JNK activation kinetics was faster in endothelial cells on fibronectin compared to collagen under flow

conditions [461]. In addition, Rho family of GTPases such as RhoA, Rac1 and Cdc42 are necessary for downstream signaling pathway of ligands-bound integrins and cytoskeleton remodeling [462]. RhoA generates contractile force. However, Rac1 and Cdc42 regulate lamellipodium protrusion and filopodium extension respectively. HUVECs on fibronectin and on collagen could trigger different levels of Rho protein activation, leading to differences in directional contractility. These differences could be physiologically important because in wound healing the synthesis and deposition of fibronectin and collagen at wounding site are different and these could influence the angiogenic responses of the endothelial cells [432].

Next, kindlins are important in integrin activation, clustering and outside-in signaling [100, 101, 126, 158, 463-466]. Kindlin-3 is expressed predominantly in hematopoietic cells, endothelial cells and platelets [85]. Aberrant kindlin-3 expression is involved in cancer progression but whether it is a positive or negative regulator of cancer metastasis remains controversial [87, 167]. HUVEC expressed kindlin-3 and kindlin-2, and silencing kindlin-3 expression in HUVECs has been shown to impede tube formation [85]. Therefore, we investigated the role of overexpressed kindlin-3 on traction force and directional contractility in HUVECs. For this study, it is not possible to examine traction force of kindlin-3 silenced HUVECs because the cells would not be able to attach to the micro-posts. From our observation, the total absolute force was reduced in HUVECs overexpressing kindlin-3 on fibronectin pre-coated micro-posts. We chose to use fibronectin instead of collagen because our previous analyses showed that cells on fibronectin but not collagen exhibits larger traction force and pronounced directional contraction. Unexpectedly, HUVECs overexpressing kindlin-3 exhibited smaller cell spread area compared to control cells. Force range was shifted from 0-2 nN to 2-4 nN in HUVECs overexpressing kindlin-3 compared to control cells. And there was a reduction in directional contractility in HUVECs overexpressing kindlin-3. These data may seem intriguing since kindlin-3 is well established to be important in integrin activation, which is necessary for cell adhesion to many ECM proteins. However, we conjectured that kindlin-3 plays a different role compared to other kindlins in that it promotes transient focal adhesion (also referred to as nascent adhesion sites) rather than facilitating mature focal adhesion formation. Indeed, kindlin-3 can be detected at the leading edges of migratory HUVECs but not at mature focal adhesions unlike kindlin-2 [85]. Overexpression of kindlin-3 in HUVECs could also lead to overt activation of RhoAGTPases. Interestingly, enhanced RhoA activity has been shown to inhibit migration of lung adenocarcinoma cells and fibroblasts on

fibronectin [426, 467]. Another possibility is that the overexpressed kindlin-3 disrupts the functions of the endogenous kindlin-2 that is necessary the maturation of focal adhesions. Although we would like to continue with this study along the lines of kindlin-3 in mechano-signal transduction, our collaborators from School of Physical and Mathematical Sciences have left NTU. Hence, we have to temporarily place this project on hold.

Chapter 7 Discussion and conclusion

It has long been assumed that talins are master regulators of integrin activation given that it directly binds and changes the conformation of the integrins [52]. Now it is clear that kindlins are also crucial regulators of integrin activation although kindlins have not been reported to induce conformational changes in integrins. Kindlins directly bind integrin β cytoplasmic tails in the region containing the membrane distal NxxY/F motif [97]. This is distinct from the membrane proximal NxxY/F motif that is the binding site of talin [96]. Many studies have shown that kindlins are crucial for integrin activation. One model of integrin activation by kindlins is that of kindlin-2 recruiting migfilin that displaces filamin A, a negative regulator of integrins, from its binding site in the integrin β cytoplasmic tail [99]. However, more studies are needed to establish the generality of this model because direct evidence of migfilin binding to kindlin-1 and kindlin-3 is still lacking. Another model of kindlins serving as positive regulators of integrin function is the induction of integrin clustering by kindlins. This has been demonstrated by our group and others based on kindlin-3 with integrins $\alpha\text{L}\beta 2$ and $\alpha\text{IIb}\beta 3$, respectively [101]. Mechanistically, it is improbable that one kindlin molecule cluster a minimum of two integrins because there is only one integrin β cytoplasmic tail binding site in each kindlin molecule, which is the PTB domain in the F3 subdomain. This is evident from the recent crystal structure of murine kindlin-2 in complex with integrin β cytoplasmic tail peptide. From the same study, it was proposed that kindlin-2 forms homodimers based on two kindlin-2 molecules juxtaposed in an asymmetric crystal unit. This leads to the model of a kindlin homodimer inducing integrin clustering. However, the crystal structure of kindlin-2 in that study was generated using a kindlin-2 mutant that lacks the PH domain and the F1 loop [95]. Based on our lab's collaboration with structural biologists, the formation of a homodimer from full-length kindlins seems unlikely (unpublished observation). Hence, more studies are needed to determine the detailed mechanism by which kindlins positively regulate integrin functions.

Apart from integrins, it is becoming clear that kindlins have non-integrin dependent functions. Some of these functions have been described in the Introduction section. Kindlin-1 has been demonstrated to regulate mitotic spindle assembly in breast cancer [127]. In this study, we showed that kindlin-2 also regulates mitotic spindle assembly but the detailed mechanism appears different from that of kindlin-1. Kindlin-1 has been detected at the centrioles of mitotic cells [127] but we were unable to detect significant localization of kindlin-2 at the centrioles. Our data suggest that kindlin-2

regulates mitotic spindle assembly by modulating acetylation of α -tubulin. We identified two possible signaling pathways that may be involved, the AKT/ GSK3 β / HDAC6 and the paxillin/ HDAC6 pathways. Our data also suggest that both pathways need not be mutually exclusive and they are dependent on kindlin-2-mediated integrin outside-in signaling. Depleting kindlin-2 in SH-SY5Y cells lead to abnormal mitotic spindle assembly. Our data suggest that abnormal spindle assembly in these cells is a consequence of defective α -tubulin acetylation, which can be rescued by treating cells with tubacin, a specific inhibitor of HDAC6. Another interesting observation made in this study is that hypoxia induces down-regulation of kindlin-2 expression. Given that hypoxia can induce abnormal mitotic spindle assembly, the loss of kindlin-2 expression may account, in part, for this phenomenon. This is not cell-type specific because SH-SY5Y, MDA-MB-231 and HCT-116 cells showed abnormal spindle assembly and loss of kindlin-2 expression under 96 h hypoxia condition. It will be interesting in future studies to determine whether kindlin-3 also regulates mitotic spindle assembly in immune cells. In the study by Patel et. al., it has been shown that Plk-1 phosphorylates Thr30 in kindlin-1 in mitotic cells [127]. Whether phosphorylation of kindlin-2 in mitotic cells plays a crucial role in its function remains to be determined.

Apart from demonstrating that kindlin-2 plays an important role in mitotic cell spindle assembly, we obtained preliminary evidence that kindlin-2 is detected in EVs. Tumour-derived exosomes have been shown to promote cancer initiation, progression, metastasis and drug resistance [320, 428-430]. We showed that EVs derived from SH-SY5Y cells depleted of kindlin-2 were unable to induce HUVECs tube formation as effectively as those obtained from wild-type cells. Although we do not have detailed mechanistic insights, we reasoned that the lack of kindlin-2 could lead to defective integrins in EVs, which prevents the efficient docking of EVs onto recipient cells. The integrin-mediated docking of exosomes onto recipient cells is a key step in exosome uptake. Although it has been shown that integrins on exosomes play an important role in organotropic cancer cell metastasis [154], it remains to be determined the ligand on cells that these integrins on exosomes bind. Nonetheless, the activity of integrins on EVs can be examined in future studies using the method of SPR apart from the bead-based flow cytometry method discussed in previous chapter. In this case, integrin ligand such as fibronectin can be coated on the SPR chip followed by infusion of EVs derived from either kindlin-2 depleted or wild-type SH-SY5Y cells. This will allow detection of integrin binding affinity differences between the two groups of EVs. Given that the effect on HUVECs was also

significant during the early time point, additional assays could be performed to determine any difference in EVs uptake. EVs can be labeled with lipophilic fluorescent dye. These labeled EVs can be provided to cells and the fluorescence signal in cells to be determined by either flow cytometry or immunofluorescence microscopy.

Within the scope of this study, we also determined the specificity determinant of kindlin-3 and RACK1 interaction. Our group provided evidence that kindlin-3 interacts with the scaffold protein RACK1 [101]. Based on mapping experiments, we identified ³⁶¹IPRR³⁶⁴ in the PH domain of kindlin-3 to be important for its interaction with RACK1. Interestingly, this sequence is only present in one of the splice variants of kindlin-3 designated the long-form (LF). This sequence is also absent from kindlin-1 and kindlin-2 (**Figure 46**), which may account for the lack of interaction between these kindlins and RACK1. Both kindlin-3 LF and kindlin-3 SF are expressed in immune cells but there are many unknowns, including whether kindlin-3 LF is constitutively expressed or is up-regulated under specific conditions. It will also be interesting to determine whether the changes in the splice isoform populations occur during blood cancer progression. A previous study has shown that kindlin-3 LF binds with high affinity to phosphoinositides compared with that of kindlin-3 SF, and B cells derived from LADIII patients reconstituted with the former exhibited defective adhesion and migratory properties [425]. The authors suggested that kindlin-3 LF may be a dysfunctional isoform. However, we hypothesize that kindlin-3 LF may have functions that are distinct from kindlin-3 SF based on its binding to RACK1. One of this may be to recruit activated PKC(s) to integrin-containing nascent adhesion sites during cell migration. Thus, further studies are warranted to examine the physiological functions of kindlin-3 LF.

In the last result chapter of this thesis, we examined HUVECs traction force on different ECM proteins, namely fibronectin and collagen type I, both are abundant proteins differentially expressed during wound healing. Perhaps the most interesting observation is that there was a reduction in directional contractility in HUVECs overexpressing EGFP-kindlin-3 compared with cells overexpressing EGFP-vector. These data seem contradictory to the role of kindlins in general as positive regulators of cell adhesion and spreading. However, we conjectured that kindlin-3 plays a different role from other kindlins in that it promotes transient nascent focal adhesion as opposed to mature focal adhesion. Indeed, kindlin-3 was shown to localize to the leading edges of migratory

HUVECs but not at mature focal adhesions unlike kindlin-2 [85]. Our group also showed that overexpression of kindlin-3 in melanoma cells, which do not normally express kindlin-3, impeded cell migration and metastasis in vivo [468]. Due to unforeseen circumstances, we are unable to continue with this part of the project.

In conclusion, kindlins have emerged as crucial regulators of integrin functions, and their roles in non-integrin dependent functions are beginning to unfold. Our study provides first evidence that kindlin-2 regulates spindle assembly in mitotic cells and that kindlin-2 may have an important role in EVs function. We also determine the specificity determinant in kindlin-3 that facilitates its association with RACK1. Our preliminary data on cell traction force suggest that kindlin-3 may have functions that diverge from other members of the same family.

Figure 46 Amino acid sequence alignment of PH domain of kindlins. The amino acid sequence ³⁶¹IPRR³⁶⁴ is absent in kindlin-1 and kindlin-2. : represents two of the conserved amino acid. * represents identical amino acid.

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