Characterization of kindlin3 in Integrin functions

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**NANYANG
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UNIVERSITY**

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Integrin functions**

Feng Chen

School of Biological Sciences

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Abbreviations

ADMIDAS	adjacent to metal ion-dependent adhesion site
Amp	ampicillin
ATCC	American type culture collection
bp	base pair(s)
BSA	bovine serum albumin
C3G	cyanidin-3-glucoside;
CalDAG-GEFI	diacylglycerol-regulated guanine-nucleotide-exchange factor I
CRKL	Crk-like protein;
CSK	cytoskeleton stabilization
C-terminal	carboxy terminal
DAG	diacylglycerol
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulphoxide
dNTP	deoxynucleotide triphosphate
DTT	dithiothreitol
DNA	deoxyribonucleic acid
EB	Epidermolysis Bullosa
ECIS	Real-time electric cell-substrate impedance sensing
ECL	enhanced chemiluminescence
ECM	extracellular matrix
EDC	N-ethyl-N-(3-dimethylaminopropyl)- carbodiimide hydrochloride
EGTA	ethylene glycol tetraacetic acid
EI	expression index
EM	electron microscopy
Fab	fragment, antigen-binding
FACS	fluorescent activated cell sorter
FAK	focal adhesion kinase
FERM	four-point-one ezrin radixin moesin
FBS	fetal bovine serum

FITC	fluorescein isothiocyanate
FN	fibronectin
FPLC	fast performance liquid chromatography
FRET	fluorescence resonance energy transfer
GRAF	GTPase-activating protein
Grb	growth factor receptor-bound protein
GT	Glanzmann's Thrombasthenia
HA	hemagglutinin
HD	head domain
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
hnRNP K	heterogeneous nuclear ribonucleoprotein K
HRP	horseradish peroxidase
HUVEC	Human Umbilical Vein Endothelial Cells
JAM-1	junctional adhesion molecule-1
Kana	kanamycin
kDa	kilodaltons
I domain	inserted domain
ICAMs	intercellular adhesion molecules
I-EGF	integrin-epidermal growth factor-like fold
Ig	immunoglobulin
IGF-I	insulin-like growth factor-I
IgSF	immunoglobulin superfamily
IL2	interleukin 2
ILK	integrin-linked kinase
IMC	inner membrane association clasp
IP	immunoprecipitation
IP3	inositol-1, 4, 5-trisphosphate
IPTG	isopropyl- β -D-thiogalactopyranoside
ITAM	immunoreceptor tyrosine-based activation motif
LAD	leukocyte adhesion deficiency

LFA-1	lymphocyte function-associated antigen-1
mAb	monoclonal antibody
MCS	multiple cloning site
MIDAS	metal ion-dependent adhesion site
min	minute(s)
mRNA	messenger ribonucleic acid
Mst-1	macrophage-stimulating 1
MW	molecular weight
NHS	N-hydroxysuccinimide
NMR	nuclear magnetic resonance
N-terminal	amino terminal
O.D.	optical density
OMC	outer membrane association claps
p70S6K	70kDa ribosomal protein S6 kinases
PAGE	polyacrylamide gel electrophoresis
PAS	protein A-sepharose
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDE4D5	cAMP-specific 3',5'-cyclic phosphodiesterase 4D
PH	pleckstrin homology
PHA	phytohaemagglutinin
PIP5K1	1-phosphatidylinositol-4-phosphate kinase 1
PKC	protein kinase C
PLC γ	phospholipase C γ
PSI	plexins, semaphorins, and integrins
PTB domain	phosphor-tyrosine binding domain
RACK1	receptor for activated C kinase 1
Rap1	Ras-proximate-1 or Ras-related protein-1
RAPL	regulator of cell adhesion and polarization enriched in lymphoid tissues
RIAM	Rap1-GTP-interacting adaptor molecule

RNA	ribonucleic acid
RPMI	Roswell park memorial institute
rpm	revolutions per minute
RT	room temperature
s	second(s)
SD	standard deviation
SDF-1 α	stromal cell-derived factor 1 α
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SFKs	Src family kinases
SH3	src homology 3
SIC	spreading initiation centers
SyMBS	synergistic metal ion-binding site
T _m	melting point
T _M	transmembrane
Tris	tris (hydroxymethyl)-aminoethane
Tween 20	polyoxyethylene sorbitan monolaurate
UV	ultraviolet
v/v	volume per volume
w/v	weight per volume

The single letter and triplet codes for amino acid residues are used. Restriction enzymes are referred to by their three letter names derived from that of the source microorganism. Other abbreviations are defined in the text where first encountered.

Abstract

Integrins are heterodimeric cell adhesion molecules that are involved in many biological processes. Integrin ligand-binding requires its activation by the FERM (4.1 ezrin radixin moesin)-domain-containing cytoplasmic protein talin 1/2. Recently, another family of FERM-domain-containing cytoplasmic proteins known as kindlins has been shown to regulate talin-induced integrin conformation change and activation. The primary focus in this study is kindlin3, which is expressed in platelets, hematopoietic cells and endothelial cells. In the disease known as leukocyte adhesion deficiency (LAD) III, mutation(s) in *KINDLIN3* disrupts kindlin3 expression, leading to defective $\beta 2$ integrins-mediated leukocyte adhesion and integrin $\alpha \text{IIb}\beta 3$ -mediated platelet aggregation. LADIII patients therefore have a compromised immune system and show bleeding disorders. Although kindlin3 is well-established to be important in integrin ligand-binding, little is known of its role in integrin outside-in signaling, a process that is essential for integrin-mediated cell spreading and migration. In this study, we identified a novel interaction between kindlin3 and receptor for activated-C kinase 1 (RACK1). This interaction is dependent on the pleckstrin homology (PH) domain of kindlin3. We also provide evidence that integrin $\beta 2$ cytoplasmic tail, kindlin3 and RACK1 can potentially form a ternary complex. Kindlin3 and RACK1 localize to the lamellipodium of migrating T cells on integrin $\alpha \text{L}\beta 2$ ligand intercellular adhesion molecule-1 (ICAM-1). This was also observed in HUVECs spreading on integrin $\alpha 5\beta 1$ ligand fibronectin in which kindlin3 and RACK1 localized to the cell's leading edge. An interesting observation in HUVECs is that kindlin3 did not localize to mature focal adhesion sites. Rather kindlin3 appears to be important in nascent adhesion sites that contain cytoplasmic proteins necessary for direction sensing, for example RACK1, and possibly protein translational machineries.

Chapter 1 Introduction

1.1 Integrins: heterodimeric cell adhesion molecules

Integrins are a large family of type 1 transmembrane receptors that function as adhesion molecules. Apart from promoting cell-cell and cell-extracellular matrix (ECM) adhesions, they also transmit extracellular signals into cell. The name “integrin” was given to denote the functions of these receptors to maintain the integrity of the cytoskeletal-ECM linkage. Each integrin is a heterodimer having an α subunit and a β subunit. Each subunit has a large extracellular region and a transmembrane domain followed by a cytoplasmic tail. In humans, there are 24 integrins formed by specific non-covalent association of 18 α and 8 β subunits (**Figure 1.1**). They may be categorized into different subfamilies and their ligands are listed in **Table 1.1** (Barczyk et al., 2010; Humphries et al., 2006).

Despite having no intrinsic enzymatic properties, integrins play important roles in cell signaling that impinges on cell shape, motility, survival, and cell fate determination (Springer and Wang, 2004). Integrin serves as mechanical link between the cytoskeleton and the extracellular environment. Most integrins connect the ECM with the actin cytoskeleton (Larson et al., 1989) whereas integrin $\alpha 6 \beta 4$ connects the ECM to intermediate filaments (Nodari et al., 2008). Notably, it has been shown that the recruitment of intermediate filament protein vimentin to the cell surface is depended on integrin $\beta 3$ (Bhattacharya et al., 2009). Integrins are able to transduce bi-directional signals through the plasma membrane referred to as inside-out and outside-in signaling (Abram and Lowell, 2009b). Inside-out signaling involves conformational changes of

an integrin that are propagated from its cytoplasmic tails and along its transmembrane domains to the ligand-binding site in its extracellular regions (Margadant et al., 2011). Ligand-bound integrins and ligand-induced integrin clustering are known to trigger signaling events in cells that regulate various cellular processes, including cell migration, proliferation, cytokine secretion and degranulation, which is termed as outside-in signaling (Abram and Lowell, 2009b).

The importance of integrins is underscored by many debilitating diseases. The leukocyte adhesion deficiency (LAD) disorder type I is a life-threatening disease that is characterized by a compromised immune system (Anderson and Springer, 1987). Leukocytes from LAD I patient have poor expression levels of $\beta 2$ integrins due to mutations in the integrin $\beta 2$ subunit (Abram and Lowell, 2009a). As a consequence these leukocytes have defective adhesive and migratory properties, leading to persistent microbial infections in LAD I patients (Hanna and Etzioni, 2012). The bleeding disorder Glanzmann's Thrombasthenia (GT) is also caused by defective integrin expression. Under normal hemostasis, vascular injury triggers platelet aggregation to stop bleeding. GT individuals have a tendency to bleed because their platelets are defective in aggregation. The molecular basis of this defect for most of the cases is the lack of integrin $\alpha \text{IIb}\beta 3$ expression in platelets of GT individuals as a consequence of mutations in the integrin αIIb or $\beta 3$ subunit (van de Vijver et al., 2012). In 2008, six cases of GT patients suffered from bleeding tendency caused by acquired GT were diagnosed. The cause of acquired GT is due to the generation of the autoantibodies against integrin $\alpha \text{IIb}\beta 3$ (Porcelijn, L., 2008). More recently, another of LAD disorder termed LAD III has been reported. LAD III patients exhibit symptoms of LAD I and GT. The etiology of LAD III is the defective expression of the

cytoplasmic molecule kindlin3, which is a key regulator of $\beta 2$ and $\beta 3$ integrins (Svensson et al., 2009). Another example of a chronic disease due to defective integrin function and/or expression is the skin fragility and blistering disorder Epidermolysis Bullosa (EB) (Hogg and Bates, 2000). Mutations in integrin subunits $\beta 4$ and $\alpha 6$ have been identified in EB patients (Pulkkinen et al., 1998a; Pulkkinen et al., 1998b; Pulkkinen et al., 1997; Ruzzi et al., 1997; Vidal et al., 1995). These mutations disrupt the function of integrin $\alpha 6\beta 4$, a central component of the hemi-desmosome abundantly found in stratified epithelia. Poor formation of hemi-desmosome results in weak anchorage of epithelia cells to ECM and a propensity of these cells to detach under friction.

Integrin	α I	Ligands/ recognition sequences
$\alpha 5$ (CD49e, VLA5) $\beta 1$ (CD29)		Fibronectin (RGD) (Hemler, 1990; Hynes, 1992), L1CAM (Ruppert et al., 1995), endostatin (Rehn et al., 2001), maspin (Bass et al., 2009), ADAM15 (Nath et al., 1999)
αL (CD11a) $\beta 2$ (CD18)	✓	Intercellular adhesion molecule-1, -2, -3, -4, -5 (ICAM-1, -2, -3, -4, -5) (de Fougierolles and Springer, 1992; de Fougierolles et al., 1991; Ihanus et al., 2003; Marlin and Springer, 1987), junctional adhesion molecule-1 (JAM-1) (Ostermann et al., 2002)
αIIb (CD41, GpIIb) $\beta 3$ (CD61)		Fibrinogen (RGD or AGD) (Parise and Phillips, 1985; Phillips et al., 1988), fibronectin (RGD) (Plow and Ginsberg, 1981), vitronectin (Phillips et al., 1988), thrombospondin (Jurk et al., 2003), von Willebrand's factor (Kuijpers et al., 2004)

Table 1.1 Integrins and their ligands. α I: integrin α subunit that contains an I-domain.

Figure 1.1 Mammalian integrins. 8 β subunits combine with 18 α subunits to form 24 integrins. Based on evolutionary relationships and ligand specificities, these may be categorized into several subfamilies. The peach colored α subunit contains an I domain. Integrin heterodimers expressed on immune cells are shown with red lines.

1.2 General domain-organization and structure of integrins

In 2001, the crystal structure of the extracellular portion of integrin $\alpha V\beta 3$ (Xiong et al., 2001) was solved, which initiated many follow-on studies to relate integrin structure to function. Perhaps the most exciting feature observed in the integrin $\alpha V\beta 3$ structure is its bent conformation. Later it was reported by the same group that integrin $\alpha V\beta 3$ adopts the same overall conformation even in the presence of an RGD-containing ligand mimetic (Xiong et al., 2002). However, there are also other studies that suggest the requirement of an integrin to convert from a bent to an extended conformation in order to bind ligand. These will be discussed in the later section. In addition to the solved structures of integrin $\alpha V\beta 3$, the extracellular structures of integrins $\alpha I1b\beta 3$ and $\alpha X\beta 2$ have been reported (Xie et al., 2010; Zhu et al., 2008; Zhu et al., 2009) (**Figure 1.2**)

Figure 1.2 The linear domain-organization of human integrin with $\alpha X\beta 2$ as an example. Both subunits are type I membrane proteins with large extracellular regions, TM domains and cytoplasmic tails. The αI domain is inserted into the second and third blades of the β propeller domain of the αX subunit while the βI domain is inserted into the hybrid domain of the $\beta 2$ subunit. PSI: plexins, semaphorins, and integrins; I-EGF: integrin-epidermal growth factor-like fold; βT : β tail domain.

1.2.1 Extracellular domains of the integrin α subunit

The integrin α subunit has a large extracellular region and a transmembrane domain followed by a relatively short cytoplasmic tail. The extracellular region comprised a seven-bladed β propeller, a thigh domain and two calf domains termed calf-1 and calf-2. Nine of the human integrin α subunits contain an inserted (I) domain of about 200 amino acids (**Figure 1.3**) that is inserted between blade 2 and blade 3 of the seven-bladed β propeller (**Figure 1.2**). The I domain is the primary ligand-binding site in all I-domain-containing integrins. The I domain adopts the Rossmann nucleotide-binding fold characterized by a central β sheet surrounded by α helices as revealed by the solved I domain crystal structures of integrin αM , αL and $\alpha 2$ subunits (**Figure 1.3**) (Emsley et al., 2000; Lee et al., 1995; Legge et al., 2000). Found at the top of the I domain is the metal ion-dependent adhesion site (MIDAS) motif characterized by a conserved Asp-X-Ser-X-Ser (X: any amino acid) motif in all I domains (Bergelson and

Hemler, 1995; Tan, 2012). The side chains of the Asp and Ser residues in this motif together with two non-contiguous residues Thr and Asp in the I domain coordinate a metal divalent cation. The octahedral coordination sphere of the metal ion is completed when the acidic side chain of a residue, for example a Glu, from a ligand engages the MIDAS.

Figure 1.3 The integrin I domain. (A) Integrin $\alpha 2$ I domain engaging an acidic Glu from collagen with its MIDAS. (B) The MIDAS of integrin αL I domain engaging an acidic Glu from ICAM-1 is shown (Figure kindly provided by Dr. Tan Suet Mien (Tan, 2012))(Emsley et al., 2000;Shimaoka et al., 2003).

Although the blades (~60 amino acids each) of the α subunit's β propeller share weak sequence homology to each other, the β propeller structure has been verified by different groups based on structural studies (Xiao et al., 2004; Xiong et al., 2001; Zhu et al., 2008). The β propeller is required for ligand-binding in integrins that do not contain an I domain. From the crystal structure of integrin $\alpha V\beta 3$ with a bound RGD-containing ligand mimetic, it is evident that the αV β -propeller is involved in ligand-binding and it forms an interacting interface with $\beta 3$ subunit (Xiong et al., 2002). A cap subdomain is also seen in the crystal structures of integrin $\alpha IIb\beta 3$ (Xiao et al., 2004). It has been demonstrated by mutagenesis that this cap subdomain contributes to integrin ligand-binding (Kamata et al., 2001). Variation in lengths and sequences of different integrin cap subdomains suggests that it could play a role in determining ligand-binding specificity (Xiao et al., 2004). The β propeller also has four Ca^{2+} -binding sites that are located at the opposite face from the ligand-binding and β subunit-interacting interface, and may be important for the stability of the β propeller (Oxvig and Springer, 1998).

To the C-terminal of the β propeller are three immunoglobulin-like β -sandwich folds known as the thigh, calf-1 and calf-2 domains. The thigh domain and the calf-1 domain are connected by a Ca^{2+} binding loop known as the “genu” that is flexible and it serves as a pivot for the conversion of an integrin α subunit from a bent to an extended conformation (Xie et al., 2004).

1.2.2 Extracellular domains of the integrin β subunit

The integrin β subunit is a mosaic of different domains and folds. It comprises from

the N-terminus a plexins/semaphorins/integrin (PSI) domain, a hybrid domain, a β I domain, four integrin epidermal growth factor-like (I-EGF) folds and a β tail domain.

The integrin β I domain is a highly conserved domain of about 240 residues and is structurally similar to the α I domain. The β I domain is the ligand binding site in integrins that lack α I domain. In integrins that contain the α I domain, the β I domain regulates by allostery the ligand-binding property of the α I domain. There are two important segments in the β I domain: one is the specificity-determining loop which plays a role in ligand binding whereas another interacts with the α subunit's β propeller (Cheng et al., 2007). The β I domain also contains a MIDAS, and two other metal ion-binding sites known as the adjacent to MIDAS (ADMIDAS) and synergistic metal ion-binding site (SyMBS) that regulate its ligand-binding property (Raborn et al., 2011).

The PSI domain is formed by two non-contiguous primary sequences located at the N-terminal region and in between the hybrid domain and I-EGF-1. A long-range disulphide bond formed covalently links the PSI segments. Similar to the PSI domain, the hybrid domain is formed by two different segments flanking the β I domain (Shi et al., 2005; Xiong et al., 2001). The hybrid domain can undergo displacement referred to as the “swing-out” motion that regulates integrin ligand-binding (Mould et al., 2003). Many studies have shown that the hybrid domain is required for the propagation of activation signal in integrins (Xiao et al., 2004; Takagi et al., 2003; Tng et al., 2004).

The I-EGF folds of the integrin β subunit have a high content of Cys residues that are disulphide bonded. Perturbations of these disulphide linkages by site-directed mutations or by the addition of reducing agent dithiothreitol (DTT) have been shown

to induce integrin activation (Davis and Camarillo, 1993; Nolan et al., 2000). The segment between the I-EGF1 and I-EGF2 is flexible, which is also known as the knee of the integrin β subunit, and it allows the integrin to adopt either a bent or extended conformation (Nishida et al., 2006; Xiong et al., 2001).

The β tail domain contains an $\alpha + \beta$ fold (Xiong et al., 2001). The weak electron density of this domain observed in the α IIb β 3 crystal structure suggests a flexible connection to other domains of the β 3 subunit (Zhu et al., 2008).

1.2.3 The transmembrane domains of integrins

The transmembrane (TM) domains of an integrin not only serve as membrane anchors, they also regulate integrin function (Li et al., 2003). Integrins are maintained in their resting conformations by constraining associations of their TMs and cytoplasmic domains as determined by cryo-electron microscopy, fluorescence resonance energy transfer (FRET) analyses, biochemical assays and cell-based mutation analyses (Adair and Yeager, 2002; Dana et al., 1991; Kim et al., 2003; Luo et al., 2004a; Luo et al., 2005; Mehta et al., 1998; Partridge et al., 2005; Peterson et al., 1998). There are two key interfaces in the TM-TM packing referred to as the outer membrane association clasp (OMC) and the inner membrane association clasp (IMC) (Lau et al., 2009) (**Figure 1.4**). Based on disulphide crosslinking experiments and Rosetta computational modeling, a ridge-in-groove packing in the OMC of integrin α IIb β 3 TMs was reported (Zhu et al., 2009). In the IMC, two highly conserved juxtamembrane Phe residues from the integrin α IIb subunit insert into the inner leaflet of the plasma membrane and an electrostatic interaction between α IIb Arg 915 and β 3 Asp 723 was reported

(Hughes et al., 1996; Wang and Luo, 2010). The crossing angle between the integrin α IIb and β 3 TMs is $\sim 25^\circ$ (Lau et al., 2009). It was proven that disruption of the IMC by cytoplasmic activator, for example the cytoskeletal protein talin, can induce TM-TM unpacking leading to global conformational changes of the integrin extracellular regions (Anthis et al., 2009; Kim et al., 2003; Morin et al., 2008; Wegener et al., 2007). There are also lines of evidence that suggest integrin TMs having a role in integrin micro-clustering (Li et al., 2003; Vararattanavech et al., 2009).

Figure 1.4 Integrin TM domains. Sequence alignments of integrin TMs. The GxxxG-like motifs in β subunit TMs are shaded in pink. The GFFKR motifs in α subunit are shaded in green. The OMC (red) and IMC (blue) residues are highlighted.

1.2.4 Cytoplasmic tails of integrins

The cytoplasmic tails of integrins are much smaller than their extracellular domains (generally less than 50 amino acids). They have no enzymatic or actin-binding activities of their own, but they are essential in integrin signaling (O'Toole et al., 1994).

Data from FRET experiments have shown that in the resting state the integrin cytoplasmic tails are close to one another whereas the separation of the tails triggers integrin activation (Kim et al., 2003). Based on nuclear magnetic resonance (NMR) studies, the interactions of the integrin α and β cytoplasmic tails are weak (Bhunia et al., 2009; Chua et al., 2011; Vinogradova et al., 2002; Weljie et al., 2002) whereas another group has reported that the interaction was undetectable (Ulmer et al., 2001).

Despite the fact that integrin cytoplasmic tails are short, they serve as a cell signaling hub because they can interact with many cytoplasmic molecules, including signaling proteins (Anthis and Campbell, 2011; Gonzalez et al., 2010; Margadant et al., 2011). There are two highly conserved NXXY/F motifs (X: other amino acid) in integrin β cytoplasmic tails (**Figure 1.5**). The membrane proximal NXXY/F motif is a docking site for the well-established integrin activator talin, whereas the membrane distal NXXY/F motif has been reported to be the binding site for the integrin co-activators known as kindlins. These two proteins will be discussed in later section. Unlike the integrin β cytoplasmic tails that share significant sequence homology, the integrin α cytoplasmic tails are divergent in both sequence and length. In addition much less is known of cytoplasmic proteins interacting with integrin α cytoplasmic tails compared with that of β cytoplasmic tails.

Figure 1.5 Sequence alignment of integrin β subunit cytoplasmic domains. Two highly conserved NXXY/F motifs in the integrin β tails are highlighted with the exception of β 4 and β 8 subunits.

1.2.5 Conformation changes in integrin during its activation

Many lines of evidences suggest that integrin activation involves a three-state conformational transition that converts the integrin from a low to a high ligand-binding affinity (Hogg et al., 2011; Kinashi, 2005) (**Figure 1.6**).

The integrin with a bent conformation is well accepted to represent the low ligand-binding affinity state based on biochemical and electron microscopy (EM) studies (Luo and Springer, 2006; Nishida et al., 2006; Shimaoka and Springer, 2004; Takagi et al., 2002). In the bent conformation the headpiece of integrin is pointed towards the plasma membrane, hence it prevents the headpiece from binding to large ligands (Takagi et al., 2002). However the bent integrin may still engage small molecules as shown in the crystal structure of integrin α V β 3 that was bound to a cyclic RGD-containing peptide (Xiong et al., 2002). This was further evidenced by electron microscopy images of a bent integrin α V β 3 binding to fibronectin fragments (Adair et al., 2005).

Figure 1.6 Integrin conformational changes. The structure of integrin $\alpha X\beta 2$ in a bent conformation (left) and an extended conformation (right). The bent integrin $\alpha X\beta 2$ extracellular region was generated using the structure data PDB code 3K6S. The αX subunit cytoplasmic tail is shown in dotted line because the structure of it is unresolved. Green sphere, Ca^{2+} ; red sphere, Mg^{2+} . (The figure is kindly provided by Dr. Tan Suet Mien)

Upon inside-out activation of an integrin, the cytoplasmic tails and TMs are known to separate (Abram and Lowell, 2009b; Anthis and Campbell, 2011). This leads to integrin extension in a switch-blade motion that projects the integrin headpiece away from the plasma membrane, so that it is poised to bind ligands. Integrin extension can be reported by using reporter antibodies or based on EM analysis (Beglova et al., 2002; Ye et al., 2010). Based on EM studies of integrin $\alpha X\beta 2$, a population of extended but closed headpiece conformers was observed (Chen et al., 2010). The headpiece is considered a closed conformation when the β subunit's hybrid domain is juxtaposed to the α subunit's thigh domain. In this extended and closed headpiece conformation, the

integrin $\alpha\text{L}\beta 2$ adopts an intermediate ligand-binding affinity (Li et al., 2007).

The high ligand-binding affinity state is depicted by an extended integrin with an open headpiece in which the hybrid domain is oriented away from the thigh domain. The displacement of the hybrid domain is also known as the hybrid “swing-out” (Luo and Springer, 2006). Structural study on integrin $\alpha\text{IIb}\beta 3$ has shown that hybrid swing-out pulls downward on the $\alpha 7$ helix of the β I domain and changes the conformation of the metal ion binding sites from low to high affinity (Xiao et al., 2004). To convert from an extended close headpiece conformation to an extended open headpiece conformation, the integrin requires further structural changes that are induced by proteins interacting with its cytoplasmic tails. Based on molecular dynamic simulations, it was hypothesized that mechanical forces exerting on the integrin β cytoplasmic tail by acto-myosin contraction in the cell play an important role in the above conversion from a closed to an open headpiece (Tan, 2012). Recently it has been shown that the cytoplasmic protein kindlin3 is involved in the conversion of an extended integrin $\alpha\text{L}\beta 2$ from a closed to an open headpiece (Lefort et al., 2012). Taken together, there exists a dynamic equilibrium of integrin conformers on the cell that is highly dependent on the extracellular environment and intracellular signaling events.

1.3 The $\beta 2$ integrins

There are four members in the $\beta 2$ integrin family: $\alpha\text{L}\beta 2$, $\alpha\text{M}\beta 2$, $\alpha\text{X}\beta 2$, and $\alpha\text{D}\beta 2$. They are all I-domain-containing integrins and are expressed only on immune cells (**Figure 1.1**). As mentioned in previous section, the importance of $\beta 2$ integrins is underscored by the disorder LAD I. This project addresses mainly the function of

kindlins in outside-in signaling of integrins $\alpha\text{L}\beta 2$ and $\alpha 5\beta 1$. Thus, only these two integrins will be discussed in this session.

1.3.1 Integrin $\alpha\text{L}\beta 2$

Integrin $\alpha\text{L}\beta 2$ is also known as leukocyte function associated antigen-1 (LFA-1) or CD11a/CD18. It is expressed on all leukocytes and plays major functions in leukocyte adhesion, migration, and trafficking. It is also involved in leukocyte survival and proliferation (Hogg et al., 2011). The ligand-binding site of the integrin $\alpha\text{L}\beta 2$ lies in its I domain (**Figure 1.7**). Integrin $\alpha\text{L}\beta 2$ binds to six Ig-superfamily (IgSF) ligands which are ICAM-1 (CD54), ICAM-2 (CD102), ICAM-3 (CD50), ICAM-4 (CD242), ICAM-5 (telencephalin, TLN), and JAM-1 (Table 1). It has been shown that the MIDAS of αL I domain binds to the acidic side chain of a Glu in ICAM-1, ICAM-3, and ICAM-5 (Gahmberg, 1997; Shimaoka et al., 2003; Song et al., 2005; Stanley and Hogg, 1998; Zhang et al., 2008). It has also been shown that the three-state conformational transition plays a role in modulating the binding affinity of integrin $\alpha\text{L}\beta 2$ to different ligands (Tang et al., 2008). The extended integrin $\alpha\text{L}\beta 2$ with a closed headpiece is sufficient to bind ICAM-1 whereas an extended open headpiece conformation promotes ICAM-3-binding.

The OMC and IMC of integrin $\alpha\text{L}\beta 2$ TMs have been characterized (Vararattanavech et al., 2010). Unlike the prototype integrin $\alpha\text{IIb}\beta 3$ TMs, a polar interaction in the OMC of integrin $\alpha\text{L}\beta 2$ has been reported (Vararattanavech et al., 2010). Mutations that disrupt this polar interaction triggered constitutive activation of integrin $\alpha\text{L}\beta 2$. It has also been shown that disrupting the salt bridge in the integrin $\alpha\text{L}\beta 2$ IMC triggers its activation (Li et al., 2007). Hence, the packing of the integrin $\alpha\text{L}\beta 2$ TMs and

cytoplasmic tails constrain the integrin in a resting state.

Figure 1.7 General domain-organization of an I-domain-containing integrin. In the case of integrin $\alpha\text{L}\beta 2$, its I-domain binds to the ligand ICAM in the presence of Mg^{2+} . Red sphere represent Mg^{2+} . Cyto: cytoplasmic tail

1.3.2 Inside-out activation of integrin $\alpha\text{L}\beta 2$

Ras-proximate-1 or Ras-related protein-1 (Rap1) is one of the key players that mediate integrin $\alpha\text{L}\beta 2$ inside-out activation. The Rap1 signaling pathway is initiated by chemokines or TCR-CD3 ligation in immune cells. Chemokine or TCR engagement triggers early signaling events that activate phospholipase $\text{C}\gamma$ ($\text{PLC}\gamma$), leading to the formation of inositol-1, 4, 5-trisphosphate (IP3) and diacylglycerol (DAG). DAG and IP3 induce intracellular Ca^{2+} release that activates diacylglycerol-regulated guanine-nucleotide-exchange factor I (CalDAG-GEFI), leading to Rap1 activation and translocation to the plasma membrane. Integrins can also be translocated to the

membrane by effectors of Rap1 namely regulator of cell adhesion and polarization enriched in lymphoid tissues (RAPL) and macrophage-stimulating 1 (Mst-1) (Abram and Lowell, 2009b; Hogg et al., 2011). It has also been reported that the effector molecule Rap1-GTP-interacting adaptor molecule (RIAM) interacts with Rap1-GTP and talin1 to form an “integrin activation complex”, which leads to the recruitment of talin1 to the integrin (Hogg et al., 2011; Lee et al., 2009). Activated talin subsequently binds to the membrane proximal NPLF motif in the integrin $\beta 2$ cytoplasmic tail. This leads to the disruption of the integrin $\alpha L\beta 2$ IMC followed by its activation. Recently another family of cytoplasmic proteins known as kindlins is found to be important in this activation process and will be discussed later (**Figure 1.8**).

Figure 1.8 Illustration of integrin $\alpha L\beta 2$ inside-out activation. Inactive integrin $\alpha L\beta 2$ adopts a bent conformation in which the αL and $\beta 2$ extracellular, transmembrane and cytoplasmic domains are closely associated. Intracellular signals triggered by chemokine or TCR engagement lead to the separation of the integrin cytoplasmic tails by talin 1, leading to integrin extension and an increased in its ligand-binding affinity. CRKL: crk-like protein; C3G: cyanidin-3-glucoside; PIP5K1: 1-phosphatidylinositol-4-phosphate kinase 1

1.3.3 Outside-in signaling of integrin α L β 2

Following binding to multimeric or multivalent ligands that induces receptor clustering, a second wave of signal is elicited by the clustered integrins in a process termed outside-in signaling. Outside-in signaling drives cytoskeletal remodeling, which is essential for firm cell adhesion and spreading (Shattil, 2005). The Src family kinases are important for integrin outside-in signaling. Pull-down assay showed that Hck, Lyn, and c-Yes, but not c-Src, Fyn, and c-Fgr associate with the integrin β 2 cytoplasmic tail peptide, and these interactions are dependent on the Src homology 3 (SH3) domain of the Src family kinases (SFKs) (Arias-Salgado et al., 2003). SFK inhibitor reduces β 2 integrin-mediated neutrophils egress from circulation into tissues (Sarantos et al., 2008). Another group of kinases involved in integrin α L β 2 outside-in signaling is the Syk/ZAP-70 kinases. Syk and ZAP-70 each contains two N-terminal SH2 domains followed by a C-terminal kinase domain (Chu et al., 1998). It has been shown in monocytes that ligand bound β 2 integrin induces SFK phosphorylation of the immunoreceptor tyrosine-based activation (ITAM) motif found in the adaptor molecule DAP12. This leads to the recruitment of Syk to the vicinity of the SFKs, allowing its phosphorylation and activation (Mocsai et al., 2006). More recently, ZAP-70 has been shown to associate constitutively with integrin α L β 2 in T lymphocytes and it is involved in converting integrin α L β 2 from an intermediate to a high affinity conformation necessary for T cell spreading and migration (Evans et al., 2011).

Apart from the SFKs and Syk/ZAP-70, the non-receptor tyrosine kinase Pyk2 that belongs to the same family as focal adhesion kinase (FAK) is also involved in integrin α L β 2 outside-in signaling (Rodriguez-Fernandez et al., 2002). Pyk2 or FAK is known to amplify SFK mediated responses (Brown et al., 2005) because the phosphorylated

tyrosines on FAK, for example, are docking sites for growth factor receptor-bound protein (Grb), paxillin, and GTPase-activating protein (GRAF) (Brown et al., 2005; Chu et al., 2009; Clark et al., 1998). Collectively, the aforementioned kinases downstream of integrin α L β 2 play important roles in effecting cell cytoskeletal remodeling and spreading.

1.4 Talin

In vertebrates there are two talin isoforms referred to as talin1 and talin2 (Zemljic-Harpf et al., 2009). In terms of integrin activation, talin1 is better characterized as compared to talin2. Each talin consists of an N-terminal globular head domain that contains a FERM subdomain and a C-terminal rod domain (**Figure 1.9**). The FERM domain interacts with integrin β 1 (Bouaouina et al., 2008), β 2 (Lim et al., 2007), and β 3 (Calderwood et al., 1999) while the rod domain interacts with actin and serves as vinculin binding sites (Ye et al., 2011). Studies have established that talin induces the separation of the integrin α and β cytoplasmic tails, leading to integrin activation and up regulation of its ligand-binding affinity (Li et al., 2007). Activation of α L β 2 by TCR engagement was defective in human T cells treated with talin-targeting siRNA (Simonson et al., 2006). FRET analysis showed that talin 1 head domain induces the separation of integrin α L β 2 cytoplasmic tails (Kim et al., 2003). Platelets from mice lacking talin1 were unable to induce arterial thrombosis mediated by β 1 and β 3 integrins (Nieswandt et al., 2007; Petrich et al., 2007b).

A large number of studies have been performed to address the molecular basis of integrin activation by talin. The F3 subdomain in talin contains a phosphotyrosine-binding (PTB) fold that binds to the membrane proximal NXXY/F motif of integrin β tails (Anthis et al., 2009; Wegener et al., 2007). An ionic interaction between a basic

residue in talin F3 sub-domain (Lys327 in talin2) and an acidic residue in the integrin β tail (Asp759 in β 1D) is established, which disrupts the salt bridge formed by the same Asp residue in the integrin β tail and an Arg residue in the integrin α tail. This salt bridge maintains integrin in an inactive state. In addition to talin F3 subdomain, the F2 subdomain contains an acidic patch that can interact with the negatively charged head groups of the membrane phospholipids. Together, these interactions of talin with the integrin β tail lead to the separation of the integrin cytoplasmic tails and the disruption of TM-TM packing followed by integrin activation. The importance of talin interacting with integrins has been demonstrated in a study that made use of knock-in mice expressing integrin β 3 subunit that contains point mutations in its cytoplasmic tail (Petrich et al., 2007a). Platelets from these mice having integrin β 3 subunit mutation Y747A or L746A, which disrupts talin binding, failed to bind fibrinogen effectively even in the presence of platelet agonists such as ADP and epinephrine.

Figure 1.9 The structure of talin1. (A) The domain architecture of mouse talin1. (B) Ribbon diagram of the structure of the talin1 FERM domain. The illustration was generated by PyMOL byusing the structure data PDB code 3IVF.

1.5 Kindlins as co-activators of integrins

In addition to the well-studied talin 1 in integrin activation, another family of molecules known as kindlins has been reported to be key regulators of integrin function (Lai-Cheong et al., 2010; Moser et al., 2009b). Like talins, kindlins are FERM domain-containing cytoplasmic proteins that modulate integrin-mediated adhesion (**Figure 1.10**) (Malinin et al., 2009; Svensson et al., 2009). The three members of kindlins are the widely expressed kindlin1 (Unc112-related protein 1, URP1, and

FERMT1) and kindlin2 (Mitogen inducible gene 2, Mig2, and FERMT2), whereas kindlin3 (URP2, FERMT3) is expressed in the hematopoietic cells (Petrich, 2009; Svensson et al., 2009) and endothelial cells (Bialkowska et al., 2010; Moser et al., 2009b). Kindlin1, kindlin2, and kindlin3 are encoded by different genes, namely *KIND1* (chromosome 20p12.3), *KIND2* (chromosome 14q22.1), and *KIND3* (chromosome 11q13.1) (Rogalski et al., 2000; Siegel et al., 2003). They share considerable sequence and structure similarities. In humans, kindlin3 is approximately 59% identical to kindlin1 and 65% identical to kindlin2 based on amino-acid sequence. Kindlin3 from different species share very high sequence identity (**Fig. 1.11**) (Human kindlin3 share 93% sequence identity to mouse kindlin3 and 93% sequence identity to rat kindlin3).

Figure 1.10 The linear domain-organization of kindlin3 (mouse). The F0, F1, F2, and F3 domain of kindlin3 are shown in orange, dark blue, purple, and blue, respectively. The PH domain is indicated by a brown box. Unstructured regions are represented by line.

In keratinocytes, kindlin1 is predominantly present at focal adhesions. Loss of kindlin1 expression in patients with Kindler's syndrome causes skin blistering, photosensitivity, progressive poikiloderma, and extensive skin atrophy (Lai-Cheong et al., 2010). The poor or defective expression of kindlin1 in these patients is due to mutations in the gene *KIND1* (Has et al., 2011). Because kindlin1 is also expressed throughout the cytoplasm and basolateral sites of colonic epithelial cells, Kindler's syndrome individuals also have severe gastrointestinal symptoms (Kern et al., 2007; Ussar et al., 2008). Kindlin2 is expressed in fibroblasts and cells from the muscle, epithelium, and

endothelium (Mackinnon et al., 2002; Rogalski et al., 2000). Due to its wide tissue expression, kindlin2 knockout mice showed peri-implantation embryonic lethality (Montanez et al., 2008). Kindlin3 is expressed in hematopoietic cells and has a high expression level in megakaryocytes that produce platelets (Ussar et al., 2006). Patients with defective kindlin3 expression have symptoms that are similar to the disorders found in LAD I and GT. In these patients, their leukocytes and platelets are defective in integrin mediated adhesion, leading to a compromised immune system and a tendency to bleed. This disorder is now known as LAD III (Malinin et al., 2009; Manevich-Mendelson et al., 2009; McDowall et al., 2010; Svensson et al., 2009).

Kindlins are linearly organized into different subdomains known as F0, F1, F2, and F3 (Goult et al., 2009). A pleckstrin homology (PH) domain is inserted into the F2 subdomain. The N-terminal region mediates interactions with integrin-linked kinase (ILK) and migfilin while F3 subdomain contains a PTB fold resembling that of talin (Kloeker et al., 2004; Shi et al., 2007). The PTB fold allows kindlins to bind to the membrane distal NXXY/F motif of the integrin β cytoplasmic tail. This interaction also involves the amino acids Ser-Thr or Thr-Thr preceding the NXXY/F motif (Harburger et al., 2009; Ma et al., 2008; Moser et al., 2008). Unlike talin, kindlins are not direct activators of integrins. Instead, they serve to enhance talin-induced integrin activation. Studies have shown that kindlin1 and kindlin2 are poor activators of integrin α IIB β 3, but they could enhance talin-induced α IIB β 3 ligand-binding (Harburger et al., 2009; Ma et al., 2008). It has been observed that kindlin3 acts synergistically with talin 1 in integrin α L β 2 activation (unpublished data from our lab). Indeed talin induces an extended α L β 2 with a closed headpiece and the presence of kindlin3 is necessary for converting the headpiece into an open conformation (Lefort

et al., 2012).

Mouse	1	MAGMKTASGDYIDSSWELRVFVGEEDPEAQSVTLRVGTGESHIGGVLLKIVEEINRKQDWS	60
Human	1	MAGMKTASGDYIDSSWELRVFVGEEDPEAESVTLRVGTGESHIGGVLLKIVEQINRKQDWS	60
Rat	1	MAGMKTASGDYIDSSWELRVFVGEEDPEA+SVTLRVGTGESHIGGVLLKIVE+INRKQDWS	60
Mouse	61	DHAIWWEQKRQWLLQTHWTLDKYGILADARLFFGQHRPVILRLPNRRVLRRLRASFSKPL	120
Human	61	DHAIWWEQKRQWLLQTHWTLDKYGILADARLFFGQHRPVILRLPNRRALRLRASFSQPL	120
Rat	61	DHAIWWEQK+QWLLQTHWTLDKYGILADARLFFGQHRPVILRLPNRR LRLRASFS+PL	120
Mouse	121	FQTVAACRLLSIRHPEELSLLRAPEKKEKKKKKEKEPEEEVHDLTKVVLAGGVAPTLFRG	180
Human	121	FQ VAAICRLLSIRHPEELSLLRAPEKKEKKKKKEKEPEEE++DL+KVVLAGGVAP LFRG	180
Rat	121	FQ VAAICRLLSIRHPEELSLLRAPEKKEKKKKKEKEPEEE++DL++VVLAGGVAP LFRG	180
Mouse	181	MPAHFSDSAQTEACYHMLSRPQPAPDPLLQLRLPRPSSLPDKTQLHSRWLDSSRCIMQQG	240
Human	181	MPAHFSDSAQTEACYHMLSRPQP PDPLLQLRLPRPSSL DKTQLHSRWLDSSRCIMQQG	240
Rat	181	MPAHFSDSAQTEACYHMLSRPQP PDPLLQLRLPRPSSL DKTQLHSRWLDSSRCIMQQG	240
Mouse	241	IKAGDVLWLRFKYYSFFDLDPKTDVRLTQLYEQARWDLLEEDCTEEEMMVFAALQYH	300
Human	241	IKAGDALWLRFKYYSFFDLDPKTDVRLTQLYEQARWDLLEEDCTEEEMMVFAALQYH	300
Rat	241	IKAGDVLWLRFKYYSFFDLDPKTDVRLTQLYEQARWDLLEEDCTEEEMMVFAALQYH	300
Mouse	301	INKLTLSGDVGELASGDLGLDDDLDAALNNLEVKLKGSAPSDMLDSLTTIPELKDHL----	356
Human	301	INKLSQSGEVGEFAGTDPGLDDDLVALSNLEVKLEGSAPTVDVDSLTTIPELKDHLRIFR	360
Rat	301	INK++ SG+VGE A DPGLDDDL AL+NLEVKL+GSAP+D+LDLSTTIPELKDHLRIF-	359
Mouse	357	RIFRPRKLTCLKGYRQYWVVKDTTLSYYKSQDEAPGDPQQNLNLKGCEVVPDNNVSGQKF	416
Human	361	IPRRPRKLTCLKGYRQHWVVKETTLSSYYKSQDEAPGDPQQNLNLKGCEVVPDNNVSGQKF	420
Rat	360	---RPRKLTCLKGYRQHWVIFKDTTLSSYYKSQDEAPGDPQQNLNLKGCEVVPDNNVSGQKF	416
Mouse	417	CIKLLVPSPEGMSEIYLRCQDEQQYAQWMAACRLASKGRTMADSSYASEVQAILAFSLQ	476
Human	421	CIKLLVPSPEGMSEIYLRCQDEQQYARWMAGCRLASKGRTMADSSYTSEVQAILAFSLQ	480
Rat	417	CIKLLVPSPEGMSEIYLRCQDEQQYAQWMAACRLASKGRTMADSSYASEVQAILAFSLQ	476
Mouse	477	RAGGSNGSGNKPQGPEAPAEGLNPYGLVAPRFQRKFKAQQLTPRILEAHQNVAQLSLTE	536
Human	481	R G +GG GN P GP+A AEGLNPYGLVAPRFQRKFKAQQLTPRILEAHQNVAQLSLAE	538
Rat	477	RAGGTNGSGNKPQGLEASAEGLNPNYGLVAPRFQRKFKAQQLTPRILEAHQNVAQLSLTE	536
Mouse	537	AQLRFIAWQSLPDFGISYVMVRFKGSRKDEILGIANNRLIRIDLAVGDVVKTWRFNSMR	596
Human	539	AQLRFIAWQSLPDFGISYVMVRFKGSRKDEILGIANNRLIRIDLAVGDVVKTWRFNSMR	598
Rat	537	AQLRFIAWQSLPDFGISYV+VRFKGSRKDEILGIANNRLIRIDLAVGDVVKTWRFNSMR	596
Mouse	597	QWNVNWDIRQVAIEFDEHINFAFSCVSASCRIVHEYIGGYIFLSTRERARGEELDEDLFL	656
Human	599	QWNVNWDIRQVAIEFDEHINFAFSCVSASCRIVHEYIGGYIFLSTRERARGEELDEDLFL	658
Rat	597	QWNVNWDIRQVAIEFDEHINFAFSCVSASCRIVHEYIGGYIFLSTRERARGEELDEDLFL	656
Mouse	657	QLTGGHEAF 665	
Human	659	QLTGGHEAF 667	
Rat	657	QLTGGHEAF 665	

Figure 1.11 Kindlin3 protein sequence alignment among species.

In addition to integrin activation, kindlins could be important in integrin outside-in signaling. Migfilin and ILK have been shown to interact with kindlin2, which are important to link the integrins to the cytoskeleton (Larjava et al., 2008; Moser et al., 2009b; Tu et al., 2003). In platelets, kindlin3 is essential for integrin $\alpha\text{IIb}\beta 3$ activation and subsequent integrin outside-in signaling. To date, there is still very little information on the cytoplasmic interactors of kindlin3. Identification of these proteins will allow us to better understand the function of kindlin3 in integrin outside-in signaling.

1.6 Receptor for activated C kinase 1 (RACK1)

RACK1 is a member of the WD40 repeat family of β -propeller proteins (McCahill et al., 2002). RACK1 is known to bind to the activated form of protein kinase C (PKC) and it translocates PKC to specific compartments in the cell (Grosso et al., 2008; Ron, 1994). RACK1 is ubiquitously expressed in many eukaryotic species ranging from *Chlamydomonas* to plants and humans and it is evolutionarily conserved among these species. RACK1 has been reported to regulate and promote cell migration in different cell types (Buensuceso et al., 2001; Cox et al., 2003; Kiely et al., 2006). The structure of human RACK1 has not been resolved. Nevertheless, the solved structure of the yeast orthologue ASC1 that shares 53% sequence identity to RACK1 is a seven-bladed β -propeller protein that is similar to the structure of the G protein β subunit (**Figure 1.12**) (Ullah et al., 2008).

Figure 1.12 Sequence alignment of RACK1 and ASC1, and the structure of ASC1. (A) Sequence alignment of yeast ASC1 and human RACK1 obtained from NCBI. The yeast ASC1 exhibits 53% sequence similarities to human RACK1. (B) Top view of yeast ASC1 showing a seven-bladed β -propeller structure. PDB code: 3FRX. (C) Side view of ASC1.

RACK1 is a scaffold protein that binds many cytoplasmic proteins. These include PKC, c-Src, the integrin β cytoplasmic tails, phospholipase C- γ , RasGAP, PTP μ , β -spectrin, dynamin, FAK, and Fyn (Buensuceso et al., 2001; Chang et al., 2001; Kiely et al., 2009; Liliental and Chang, 1998; Mourton et al., 2001; Rodriguez et al., 1999; Yaka et al., 2002). It has been shown that RACK1 may regulate the focal adhesion organization and cell migration through its interaction with c-Src and PKC (Chang et

al., 2001). RACK1 binds c-Src through Tyr246 (Cox et al., 2003) and negatively regulates c-Src activity (Liliental and Chang, 1998; Mamidipudi et al., 2004). RACK1 is also required for Src-mediated phosphorylation of Tyr31/118 on paxillin (Doan and Huttenlocher, 2007). In fact, RACK1 localizes to a subset of peripheral adhesions that contain paxillin (Mamidipudi et al., 2004). RACK1 is important for the proper function and localization of paxillin and talin at focal complexes. In addition, RACK1 binds to and stabilizes activated PKCs that are important for integrin outside-in signaling (Buensuceso et al., 2001). RACK1 has been shown to interact with FAK through its WD repeats 1-3 and this interaction is important for adhesion signaling (Kiely et al., 2009). The phosphorylation of RACK1 on Tyr 52 is essential for the regulation of FAK. Cells with Y52F mutation in RACK1 failed to adhere and grow. Based on the crystal structure of yeast RACK1, the Tyr52 phosphorylation state is important for RACK1 interacting with FAK. RACK1 is also required for insulin-like growth factor-I (IGF-I) mediated cell migration and proliferation by interacting with PP2A and $\beta 1$ integrin (Kiely et al., 2008).

1.7 Integrin $\alpha 5\beta 1$

Integrin $\alpha 5\beta 1$ is a fibronectin (FN) receptor. The binding of integrin $\alpha 5\beta 1$ to fibronectin plays a central role in endothelial cell development, migration, and survival (Hynes, 1992; Smyth et al., 1993). Lacking of integrin $\alpha 5$ or fibronectin results in early embryonic lethality (George et al., 1993; Goh et al., 1997). The binding of fibronectin to integrin $\alpha 5\beta 1$ requires two critical amino acid sequences, an RGD sequence and a Pro-His-Ser-Arg-Asn (PHSRN) sequence (Aota et al., 1994; Danen et al., 1995; Pierschbacher et al., 1983). The function of PHSRN sequence is for optimal binding of fibronectin to integrin $\alpha 5\beta 1$ and it is referred to as the synergy site (Akiyama, 1996).

In integrin $\alpha 5\beta 1$ mediated adhesion to fibronectin, the requirement of the PHSRN synergy site appears to depend on the activation state of integrin $\alpha 5\beta 1$. It has been shown that the PHSRN sequence stabilizes the binding of $\alpha 5\beta 1$ to RGD. When the integrin $\alpha 5\beta 1$ is in its high-affinity state, these contributions from PHSRN site are no longer necessary (Danen et al., 1995; Sechler et al., 1997).

The engagement of integrin $\alpha 5\beta 1$ with fibronectin can lead to integrin clustering, which results in FAK activation. FAK, in turn, can further enhance the pool of activated integrin (Michael et al., 2009). There are two steps involved in this process. Under low cell contractility, $\alpha 5\beta 1$ binds to the RGD motif in fibronectin. Secondly, under high cell contractility, integrin $\alpha 5\beta 1$ interact with the PHSRN synergy site on fibronectin. This allows adhesion strengthening, leading to the full activation of FAK (Friedland et al., 2009). Activated FAK is regulated for complex formation with Src-family kinases and phosphorylation of other substrate proteins within focal adhesion sites (Wu et al., 2008). At later stages of fibronectin-stimulated cell spreading, FAK forms a complex with Rho guanine nucleotide exchange factor (Rgnef) and paxillin and localized to focal adhesion site (Lim et al., 2008).

RACK1 has been shown to be present and colocalized with FAK at spreading initial centers in mouse embryonic fibroblasts when spreading on ECM (Serrels et al., 2007). The FAK FERM domain is involved in the interaction between FAK and RACK1 and controls RACK1 localization to spreading initial centers (Serrels et al., 2010). FAK, RACK1 together with PDE4D5 is a novel direction-sensing complex and recruits components of the cAMP second-messenger system to nascent integrin adhesion sites. Another study has shown that RACK1 colocalized with vinculin at cell spreading

initial complex structure but not at all in mature focal adhesions, indicating a role of RACK1 in cell spreading initiation centers (de Hoog et al., 2004).

1.8 Aims of this study

The mechanism by which talin regulates integrin activation has been well established. However, there are still gaps in knowledge concerning the functions of kindlins in integrin activation and outside-in signaling. This study comprises three parts that are described in three chapters. The first part of this study involves characterization of kindlin3 mAbs that are useful for subsequent analyses of kindlin3 functions. The second part of this study examines the role of kindlin3 in integrin outside-in signaling, focusing mainly on the leukocyte-restricted integrin α L β 2. The association of kindlin3 with RACK1 is also being investigated. The last part of this study investigates the role of kindlin3 and RACK1 in cell spreading initiation centers in HUVEC.

Chapter 2 Materials and Methods

2.1 General reagents

All general chemicals and reagents were of analytical grade, and were purchased from Sigma-Aldrich (St. Louis, MO, USA), BDH Chemicals (West Chester, PA, USA), BD (Franklin Lakes, NJ, USA), Pierce Biotechnology (Rockford, IL, USA), Calbiochem (San Diego, CA, USA), and Merck (Darmstadt, Germany) unless stated otherwise. Protein markers, restriction endonucleases, and other enzymes were obtained from New England Biolabs (Ipswich, MA, USA). T4 ligase was purchased from Promega (Madison, WI, USA). DNA markers and loading dye were bought from Fermentas (Burlington, CA, USA).

2.2 Vectors and cDNA

pcDNA3.0 and pcDNA3.1 (-) Zeo were purchased from Invitrogen (Carlsbad, CA, USA). pEGFP-C1, pECFP-C1 and pEYFP-N1 were generous gifts from Dr. D.X. Liu and Dr. H.Y. Li (School of Biological Sciences, Nanyang Technological University). pET-24a(+) and pET-31b(+) vectors were purchased from Novagen EMD Biosciences (Gibbstown, NJ, USA). pGEX-6P-1 was purchased from Amersham GE Healthcare (Piscataway, NJ, USA).

2.3 Antibodies

Name	Specificity	Species	Source
MHM24	anti-human α L	mouse	Hybridoma from Dr. A. J. McMichael (John Radcliffe Hospital, Oxford, UK) (Hildreth et al., 1983)
10E5	anti-human β 3	mouse	Kindly provided by B.S. Collier (the Rockefeller University, New York, NY, USA) (Collier et al., 1983; Luo et al., 2004b).
Anti-HA	anti-HA	rabbit	Purchased from Delta Biolabs (Gilroy, CA, USA)
Anti-kindlin3	anti-kindlin3	rabbit	Purchased from Abnova (Taipei, Taiwan, USA)
B-3	anti-human RACK1	mouse	Purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA)
Anti-MyH9	anti-human MyH9	rabbit	Purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA)
Clone C4	anti-human actin	mouse	Purchased from BD (Franklin Lakes, NJ, USA)
Clone 27	anti-human α L	mouse	Purchased from BD (Franklin Lakes, NJ, USA)
Anti-PKC β	anti-human PKC β	mouse	Purchased from BD (Franklin Lakes, NJ, USA)
3285	anti-human FAK	rabbit	Cell Signaling Technology (Danvers, MA, MA)
3283	anti-human P-FAK (Y397)	rabbit	Cell Signaling Technology (Danvers, MA, MA)
8d4	anti-human talin	mouse	Purchased from Sigma-Aldrich (St. Louis, MO, USA) (Ratnikov et al., 2005)
Anti-human Fc fragment	anti-human Fc fragment	goat	Purchased from Sigma-Aldrich (St. Louis, MO, USA)

Rat IgG	IgG from rat serum		Purchased from Sigma-Aldrich (St. Louis, MO, USA)
MOPC-31c	IgG from mouse serum		Purchased from Sigma-Aldrich (St. Louis, MO, USA)
Anti-kindlin2	anti-human kindlin2	rabbit	Purchased from ProteinTech Group (Chicago, IL, USA)
9202	anti-P-p70S6K (T389)	rabbit	Purchased from Cell Signaling Technology (Danvers, MA, MA)
9205	anti-p70S6K	rabbit	Purchased from Cell Signaling Technology (Danvers, MA, MA)
ab74030-100	anti-kindlin2	rabbit	Purchased from Abcam (Cambridge, MA, USA)
H-300	hnRNP K	rabbit	Purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA)
anti-CD49e	anti-human CD49e	mouse	Purchased from BD (Franklin Lakes, NJ, USA)
DB063	anti-His	rabbit	Purchased from Delta Biolabs (Gilroy, CA, USA)

HRP-conjugated donkey anti-rabbit IgG, HRP-conjugated sheep anti-mouse IgG and HRP-conjugated goat anti-rat IgG were purchased from GE Healthcare (Piscataway, NJ, USA). Alexa Fluor® 488-conjugated goat anti-mouse IgG (H+L), Alexa Fluor® 594-conjugated goat anti-mouse IgG (H+L), Alexa Fluor® 488-conjugated goat anti-rabbit IgG (H+L), Alexa Fluor® 594-conjugated goat anti-rabbit IgG (H+L), Alexa Fluor® 633-conjugated goat anti-rabbit IgG (H+L), Alexa Fluor® 488-conjugated donkey anti-rat IgG (H+L), and Alexa Fluor® 594-conjugated donkey anti-rat IgG (H+L) were all highly cross-adsorbed and purchased from Molecular Probes, Invitrogen Corporation (Carlsbad, CA, USA). FITC-conjugated sheep anti-mouse F(ab')₂ secondary antibodies were purchased from Sigma-Aldrich (St. Louis, MO,

USA). APC-conjugated goat anti-mouse secondary antibody was purchased from BD Pharmingen (Franklin Lakes, NJ, USA). Rat anti-kindlin3 mAbs clone 9, clone 181A, clone 157D, and clone 229A were generated using recombinant full length human kindlin3 as the immunogen (mAbs provided by supervisor Dr. Tan Suet Mien).

2.4 Expression plasmids of integrins and talin 1 head domain (HD)

Human integrin α L, β 2, β 2N329S and β 3N339S cDNAs in the expression plasmid pcDNA3.0 were described previously (Cheng et al., 2007; Tan et al., 2000; Tang et al., 2008). pEYFP- α L and pECFP- α L have been described before (Vararattanavech et al., 2009). The numbering of the integrin is based on the mature protein as described in Barclay et al (Barclay et al., 1997). Full-length human integrin α IIB and integrin β 3 pcDNA3.0 expression plasmid were kindly provided by Prof. P.J. Newman, Blood Centre of Wisconsin and medical college, Wisconsin, U.S.A. The expression plasmid pXJ40-HA containing the human talin 1 head domain (residues Met1-Gln435) has been reported by our group previously (Li et al., 2007).

2.5 Expression plasmids of kindlin2 and kindlin3

The numbering of the kindlin2 short form and long form are based on the protein information from NCBI (NM_006832.2). Kindlin2 pcDNA3.1 expression construct was generated by PCR cloning of kindlin2 from pXJ40-HA-kindlin2 (kindly provided by Dr. Li YF who was a staff in Dr. Tan SM's lab) using a forward primer containing an *Xba*I site and reverse primer containing a *Kpn*I site. The PCR product was digested with restriction enzymes *Xba*I and *Kpn*I and subcloned into the similarly digested mammalian expression plasmid pcDNA3.1(-) with an N-terminus HA tag to generate pcDNA3.1-HA-kindlin2. Forward (F) and reverse (R) primers used are shown:

F 5' CTAGTCTAGAACCATGTACCCATACGA 3'

R 5' CGGGGTACCTCACACCCAACCACTGG 3'

Kindlin2 was also later subcloned into pECFP-C1 (Clontech Laboratories) for FRET experiments. *XhoI* and *KpnI* were used to digest the PCR product and the vector. The forward primer contained two extra bases to allow fusion of kindlin2 to be in frame with the N-terminus ECFP in the expression plasmid pECFP-C1. Forward (F) and reverse (R) primers used are shown:

F 5' CCGCTCGAGCTATGGCTCTGGACGGGATAAG 3'

R 5' CGGGGTACC TCACACCCAACCACTGG 3'

For protein purification, kindlin2 was subcloned into pET24a plasmid (Novagen) with a 6His-tag at the C-terminus. pET24a-kindlin2-6His was generated by PCR cloning from pcDNA3.1-HA-kindlin2. The forward primer contained an *NdeI* site and reverse primer contained an *XhoI* site. Forward (F) and reverse (R) primers used are shown:

F 5' GGA ATTCCATATGGCTCTGGACGGGATA AGGATGCCA 3'

R 5' CCGCTCGAGCACCCA ACCACTGGTAAGTTTGTA 3'

The numbering of the kindlin3 amino acids is based on the protein information from NCBI (NM_178443). Kindlin3 construct was generated by PCR cloning of kindlin3 from pXJ40-HA-kindlin3 (kindly provided by Dr. Li YF who was a staff in Dr. Tan SM's lab) using forward primer containing an *EcoRI* site and reverse primer containing a *KpnI* site. The PCR product was cloned into pcDNA3.1 (-) that was digested with restriction enzymes *EcoRI* and *KpnI*, thereby generating an N-terminus HA-tagged kindlin3 (pcDNA3.1-HA-kindlin3). Forward (F) and reverse (R) primers used are shown:

F 5' CCGGAATTCACCATGTACCCATACGACGTG 3'

R 5' CGGGGTACCTCAGAAGGCCTCATGGCCC 3'

The kindlin3-PH Δ was generated by two consecutive procedures. The first set of primers (primer 1 and primer 2) was designed to generate by PCR the former part of kindlin3 (from Met1 to Pro350) and the HA tag from pXJ40. In addition, the 5'-end of the forward primer contained an *EcoRI* site. The 5'-end of the reverse primer also contained complementary sequence of the part of kindlin3 that is after the PH domain with three Gly in the middle of these two parts. The second set of primers (primer 3 and primer 4) was designed to amplify the part of kindlin3 that is after the PH domain (from Ala462 to Phe667). The 5'-end of the forward primer also contained complementary sequence to the former part of kindlin3 with three Gly in the middle of these two parts. The 5'-end of the reverse primer contained the *KpnI* site. PCR products from both sets of primers were used for overlapping extension PCR to generate a PH-deleted kindlin3. The product was digested with *EcoRI* and *KpnI* and subcloned into the similarly digested pcDNA3.1(-). Forward (F) and reverse (R) primers used are shown:

Primer 1 5' CCGGAATTCACCATGTACCCATACGACGTG 3'

Primer 2 5' CTGGTGTAGCTGCTGTCTGGCCCCCCCCCTGGGATGGTGGTGAGGCTGTCCA 3'

Primer 3 5' TGGACAGCCTCACCACCATCCCAGGGGGGGGGCCGACAGCAGCTACACCAG 3'

Primer 4 5' CGGGGTACCTCAGAAGGCCTCATGGCCCCC 3'

The kindlin3 cDNA in pXJ40 was used as a template for the construction of the HA-kindlin3-F3 Δ in mammalian expression vector pcDNA3.1 (-). Because the HA-kindlin3-F3 Δ is a truncated protein, an extra stop codon (TGA) was introduced at the end of F3 domain of kindlin3 in the reverse primer. *EcoRI* and *KpnI* were used to

digest PCR product HA-kindlin3-F3Δ and the fragment was ligated into pcDNA3.1 (-) vector, which was digested with *EcoRI* and *KpnI*. Forward (F) and reverse (R) primers used are shown:

F 5' CCGGAATTCACCATGTACCCATACGACGTG 3'

R 5' CGGGGTACCCCGTCAGGGCAGGGACTGCCAGGCCTG 3'

The kindlin3, kindlin3-PHΔ and kindlin3-F3Δ were also later cloned into pECFP-C1 for FRET experiments. In all three cases, *HindIII* and *KpnI* were used to digest the PCR product and the vector. All of the forward primers contained two extra bases to allow fusion of kindlin3 to be in frame with the N-terminus ECFP in the expression plasmid pECFP-C1. Specific forward (F) and reverse (R) primers used are shown:

Primers for pECFP-C1-kindlin3:

F 5' CCCAAGCTTCGATGGCGGGGATGAAGAC 3'

R 5' CGGGGTACCTCAGAAGGCCTCATGGCC 3'

Primers for pECFP-C1-kindlin3-PHΔ

F 5' CCCAAGCTTCGATGGCGGGGATGAAGAC 3'

R 5' CGGGGTACCTCAGAAGGCCTCATGGCC 3'

Primers for pECFP-C1-kindlin3-F3Δ

F 5' CCCAAGCTTCGATGGCGGGGATGAAGAC 3'

R 5' CGGGGTACCCCGTCAGGGCAGGGACTGCCAGGCCTG 3'

For protein purification, kindlin3, kindlin3-PHΔ and kindlin3-F3Δ were subcloned into pET24a plasmid with a 6His-tag at the C-terminus. pET24a-kindlin3-6His, pET24a-kindlin3-PHΔ-6His and pET24a-kindlin3-F3Δ-6His were generated by PCR cloning from pXJ40-HA-kindlin3, pcDNA3.1-HA-kindlin3-PHΔ and pcDNA3.1-HA-kindlin3-

F3Δ. All of three forward primers contain an *NdeI* site and all three reverse primers contain an *XhoI* site. Forward (F) and reverse (R) primers used are shown:

pET24a-kindlin3-6His:

F 5' GGAATTCCATATGGCGGGGATGAAGACAGCC 3'

R 5' CCGCTCGAGGAAGGCCTCATGGCC 3'

pET24a-kindlin3-PHΔ-6His:

F 5' GGAATTCCATATGGCGGGGATGAAGACAGCC 3'

R 5' CCGCTCGAGGAAGGCCTCATGGCC 3'

pET24a-kindlin3-F3Δ-6His:

F 5' GGGAATTCCATATGATGGCGGGGATGAAGACAGCC 3'

R 5' CCGCTCGAGCAGGGACTGCCAGGCCTGGAT 3'

2.6 Expression plasmids of RACK1

The numbering of the RACK1 amino acids is based on the protein information from NCBI (NM_006098). Human RACK1 cDNA was generated by reverse transcription-PCR from a human leukocyte cDNA library using forward and reverse primers containing the *XbaI* and *KpnI* restriction sites, respectively. A Flag tag sequence in the forward primer was introduced to generate an N-terminus Flag tag in fusion with RACK1. The reverse transcription-PCR product was cloned into the mammalian expression plasmid pcDNA3.1 (-) digested with restriction enzymes *XbaI* and *KpnI*.

Forward (F) and reverse (R) primers used are shown:

F 5' GCTCTAGAGCATGGACTACAAGGACGATGATGACAAGATGACTGAGCAGATGACCCT 3'

R 5' GGGGTACCCCTAGCGTGTGCCAATGGTCACC 3'

RACK1 was later cloned into pEYFP-N1 (Clontech Laboratories) for FRET

experiments using forward primer containing a *Hind*III site and reverse primer containing a *Kpn*I site. The reverse primers contained two extra bases to allow fusion of RACK1 in frame with the C-terminus EYFP in the expression plasmid pEYFP-N1.

Forward (F) and reverse (R) primers used are shown:

F 5' CCCAAGCTTATGACTGAGCAGATGACCCT 3'

R 5' CGGGGTACCTTGCGTGTGCCAATGGTCACCTGC 3'

For protein purification, RACK1 was subcloned into pGEX-6P-1 plasmid (GE Healthcare) with a GST-tag at the N-terminus. pGEX-6P-1-GST-RACK1 was generated by PCR cloning of RACK1 from pcDNA3.1-FLAG-RACK1 using forward primer containing an *Sma*I site and reverse primer containing a *Not*I site. The forward primer contained one extra base to allow fusion of RACK1 to be in frame with the N-terminus GST tag in the expression plasmid pEGX-6P-1. Forward (F) and reverse (R) primers used are shown:

F 5' TCCCCCGGGGATGACTGAGCAGATGACCCT 3'

R 5' ATAAGAATGCGGCCGCCTAGCGTGTGCCAATGGTCACCTGC 3'

The truncated RACK1 (from blade 5 to blade 7) were generated by PCR cloning of RACK1 blade 5 to blade 7 using forward primer containing an *Sma*I site and reverse primer containing a *Not*I site. The boundaries of the blades of human RACK1 were based on the crystal structure of the yeast orthologue of RACK1, ASC1 PDB code 3FRX. The PCR product was digested and subcloned into pGEX-6P-1 plasmid for protein purification. The forward primers contained one extra base to allow fusion of RACK1 blade 5 to blade 7 to be in frame with the N-terminus GST tag in the expression plasmid pEGX-6P-1. Forward (F) and reverse (R) primers used are shown:

F 5' TCCCCCGGGTGGCCACACAGGCTATCTGAAC 3'

R 5' ATAAGAATGCGGCCGCCTAGCGTGTGCCAATGGTCACCTG 3'

All constructs were verified by DNA sequencing (1st base, Singapore)

2.7 Medium

All media were sterilized by autoclaving or filtered unless otherwise stated.

LB medium	1% (w/v) Bacto-tryptone (BD), 0.5% (w/v) yeast extract (BD), 1% (w/v) NaCl
LB agar	LB medium plus 1.5% (w/v) bacto-agar
Amp broth	LB medium containing 60 µg/mL ampicillin (Sigma-Aldrich)
Amp plate	LB agar plate containing 60 µg/mL ampicillin
Kana broth	LB medium containing 30 µg/mL kanamycin (Gibco, Grand Island, NY, USA)
Kana plate	LB agar plate containing 30 µg/mL kanamycin
Cell freezing medium	10% (v/v) DMSO in heat-inactivated FBS
RPMI wash buffer	RPMI containing 5% (v/v) heat-inactivated FBS and 10mM HEPES (pH7.4)

2.8 Solutions

10× PBS (phosphate buffered saline)	80 g NaCl, 2 g KCl, 14.4 g Na ₂ HPO ₄ , 2.4 g KH ₂ PO ₄ in 1 L ddH ₂ O, pH 7.4
TBS buffer	150mM NaCl, 10mM Tris, pH 8.0

Blocking buffer (western)	TBS-T (TBS with 0.1% (v/v) Tween-20 containing 5% (w/v) non-fat milk)
Blotting buffer (western)	0.19 M glycine, 25mM Tris
Lysis buffer	150 mM NaCl, 1% (v/v) Nonidet P (NP)-40, 10mM Tris, pH 7.5
10×SDS-PAGE running buffer	1.9 M glycine, 1% (w/v) SDS, 0.25 M Tris,
4×Resolution gel buffer	0.4% (w/v) SDS, 0.25 M Tris, pH 8.8
4×Stacking gel buffer	0.4% (w/v) SDS, 0.5 M Tris, pH 6.8
Sodium bicarbonate buffer	0.136% (w/v) sodium carbonate, 0.735% (w/v) sodium bicarbonate, pH 9.2
60 mg/mL Ampicillin	60 mg/mL in ddH ₂ O, filtered (0.22μm)
30 mg/mL Kanamycin	30 mg/mL in ddH ₂ O, filtered (0.22 μm)
TfBIC	30 mM KOAc, 100 mM KCl, 10 mM CaCl ₂ , 50 mM MnCl ₂ , 15% (v/v) glycerol
TfBII	10 mM MOPS, 75 mM CaCl ₂ , 10 mM KCl, 15% (v/v) glycerol, pH 7.0
Coomassie blue staining buffer	50% (v/v) methanol, 10% (v/v) glacial acetic acid, 40% (v/v) ddH ₂ O, 0.05% (w/v) bromophenol blue
Coomassie blue destaining buffer	30% (v/v) methanol, 10% (v/v) glacial acetic acid, 60% (v/v) ddH ₂ O
Wash buffer for kindlin2 and kindlin3 purification	150 mM NaCl, 10% (v/v) glycerol, 40 mM Tris, pH 8.0
Elution buffer for pET24a protein expression system	150 mM NaCl, 10% (v/v) glycerol, 300 mM imidazole, 40 mM Tris, pH 8.0
Wash buffer for RACK1 and RACK1-W5-7 purification	1 × PBS, pH 7.4

Elution buffer for pGEX-6P-1 protein expression system	1 × PBS, 30 mM reduced L-glutathione, pH 8.0
Pull-down buffer	150 mM NaCl, 40 mM Tris, pH 8.0
6×loading DNA dye	0.25% (w/v) Bromophenol blue, 0.25% (w/v) xylene cyanol FF, 30% (v/v) glycerol
HBSS buffer	400 mg/L KCl, 350 mg/L NaHCO ₃ , 60 mg/L KH ₂ PO ₄ , 8 g/L NaCl, 90 mg/L Na ₂ HPO ₄ • 7H ₂ O, 1 g/L Glucose or Dextrose, pH 7.4
Shear flow buffer	HBSS buffer , 0.5% BSA, 1 mM CaCl ₂ , 1 mM MgCl ₂ , 10 mM HEPES, pH 7.4
Strip buffer	673 mM 2-Mercaptoethano, 2% SDS, 62.5 mM Tris-HCl (pH6.8) in ddH ₂ O
CSK buffer	100 mM NaCl, 300 mM sucrose, 3 mM MgCl ₂ , 1 mM EGTA, 10 mM PIPES, pH 6.8
TAE buffer	20mM acetic acid, and 1mM EDTA, 40mM Tris, pH 8.0

2.9 Miniprep and midiprep of plasmid DNA

For small-scale purification of plasmid DNA, 5 mL LB with appropriate antibiotics was inoculated with a single colony of *Escherichia coli* that was transformed with recombinant plasmid from an agar plate and incubated at 37 °C with constant shaking overnight. Plasmid DNA was extracted using a miniprep kit (Axygen, CA, USA). For large scale preparation of plasmid DNA, a single colony of transformed *Escherichia coli* was inoculated into 100 mL LB with appropriate antibiotics and incubated at 37°C with constant shaking overnight. A plasmid midiprep kit (Axygen) was used to extract the plasmid.

2.10 Quantitation of DNA

The concentration of DNA was determined using a NanoDrop2000 Spectrophotometers (Thermo Fisher Scientific, Rockford, IL, USA) by its absorbance at wavelength 260 nm based on the calculation: 50 µg/mL double-stranded DNA gives and O.D.₂₆₀ of 1. The O.D.₂₈₀ was also read and the ratio of O.D.₂₆₀/O.D.₂₈₀ was calculated to estimate the purity of the DNA solutions. An O.D.₂₆₀/O.D.₂₈₀ ratio between 1.6 and 2.0 was considered satisfactory.

2.11 DNA electrophoresis

A 60 mL 1 % (w/v) agarose gel (agarose was melted in 1 × TAE) was regularly used for analysis of 0.1 – 8 kb DNA fragments. GelRed (Biotium, Hayward, CA, USA) was added at a dilution of 1:10000 followed by casting in a mini-gel apparatus.

Electrophoresis was carried out in a horizontal gel apparatus with the gel submerged in 1× TAE. DNA samples were mixed with loading dye and loaded into the wells. DNA fragments were visualized by fluorescence over a UV light (302nm, UV transilluminator TM-20, UVP, San Gabriel, CA, USA), under which DNA/GelRed complexes fluoresce and the image was recorded with a Gel Doc 1000 imaging system (Bio-RAD, Milano, Italy).

2.12 Purification of DNA fragments agarose gel

Agarose gel slice containing the DNA plasmid or fragment of interest was excised using a razor blade. DNA was extracted using a gel extraction kit (Axygen).

2.13 DNA ligation

DNA ligation was performed using appropriate vector DNA to insert DNA ratio using T4 DNA ligase (Promega) according to manufacturer's instructions.

2.14 Preparation of *Escherichia coli* competent cells

The *E. coli* strains DH5 α and BL21 were used to prepare the competent cells. A fresh plate of cells was prepared by streaking out cells from frozen stocks and grown at 37 °C overnight. An individual colony was inoculated in 10 mL LB medium without antibiotic and was cultured at 37 °C overnight. Thereafter, 5 mL of overnight culture was transferred into each of two flasks containing 250 mL LB medium followed by incubation at 37 °C in a shaking incubator until the culture reached an O.D.₆₀₀ of 0.6, which usually takes 2 h. the cells were transferred to centrifuge bottles and spun at 8000 rpm at 4 °C for 10 min. Pellets were gently resuspended in 100 mL ice cold TfbIC buffer. The cell suspension was centrifuged at 8000 rpm at 4 °C for 10 min. The pellet was then resuspended in 20 mL ice cold TfbII buffer. Competent cells were distributed into convenient aliquots (0.1 mL) in pre-chilled microcentrifuge tubes. Cells were stored at -80 °C. An aliquot of the cells was used to assay for viability and competence.

2.15 Transformation of plasmid DNA

Plasmid DNA (1 μ g) or ligation product (10 μ L) was added to the competent cells and incubated on ice for 30 min. The cell suspension was incubated in a 42 °C water bath for 1.5 min and on ice for 2 min. The cell suspension was cultured in 500 μ L LB medium at 37 °C with constant shaking at 150 – 200 rpm for 30 min to 1 h. Thereafter,

the cell suspension (50 – 500 μ L) was spread onto antibiotic-containing LB agar plates.

2.16 Standard PCR protocol

PCR was routinely performed in a 50 μ L reaction volume containing 0.1 μ g template DNA, 1 μ M of each oligonucleotide primer, 100 μ M of each dNTP, and 1 U DNA polymerase. KAPA HIFI DNA polymerase (KAPA biosystems, Woburn, MA, USA) was used for high fidelity DNA synthesis. PCR was performed on a DNA Thermal Cycler (MJ Research, Waltham, MA, USA).

2.17 mRNA extraction and reverse transcription PCR

mRNA kit (Roche, Switzerland) was used for mRNA extraction according to the manufacturer's instructions. Briefly, cells were washed twice with ice cold PBS and remove PBS. Cells were lysed with TRIZOL reagent (Invitrogen) followed by centrifuge at 12,000 g for 10 min at 2 to 8 $^{\circ}$ C to remove insoluble material. The cleared homogenate solution was transferred to a fresh tube, incubated at RT for 5 min and add 0.2 mL of chloroform per 1 mL of TRIZOL reagent. Cap sample tubes and shake vigorously for 15 min. Sample is incubated at RT for 3 min and centrifuged at 12,000 g for 15 min at 2 to 8 $^{\circ}$ C. The aqueous phase, which is the upper phase, is transferred to a fresh tube. 0.5 mL of isopropyl alcohol per 1 mL of TRIZOL reagent used for the initial homogenization was added into the sample. Samples were incubated at RT for 10 min followed by centrifuge at no more than 12,000 g for 10 min at 2 to 8 $^{\circ}$ C to precipitate the RNA. RNA was washed with 75% ethanol and pelleted at no more than 7,500 g for 5 min at 2 to 8 $^{\circ}$ C to pellet the RNA. RNA pellet was dissolved in RNase-free water. The reverse transcription of mRNA to cDNA and PCR

procedures was carried out by QIAGEN OneStep RT-PCR kit (QIAGEN, Valencia, CA, USA) at 50 °C for 30 min.

2.18 Expression and purification of recombinant kindlin2 and kindlin3 protein

Full-length kindlin2 or kindlin3 was expressed as a recombinant protein containing a non-cleavable C-terminus 6His-tag. *Escherichia coli* BL21 cells were transformed with pET24a-kindlin2-6His or pET24a-kindlin3-6His plasmid and grown at 37 °C in LB medium containing 30 µg/ml kanamycin until an O.D.₆₀₀ of 0.8 was reached. Isopropyl β-D-1-thiogalactopyranoside (IPTG) (0.2 mM) was added to the cell culture to induce fusion protein expression. After incubating for 6 h at 16 °C, cells were harvested, resuspended in wash buffer and lysed by sonication on ice. The clarified supernatant was applied onto a Ni-NTA resin column (Qiagen) for 6His-tag affinity purification. Bound kindlin2-6His or kindlin3-6His was eluted in elution buffer (150 mM NaCl, 10% (v/v) glycerol, 300 mM imidazole, 40 mM Tris, pH 8.0) The kindlin2-6His or kindlin3-6His was further purified by size exclusion chromatography using a superdex G200 column on a fast performance liquid chromatography (FPLC)-purification system (Amersham Biosciences).

2.19 Expression and purification of recombinant RACK1 and RACK1-W-5-7

pGEX-6P-1-RACK1 was transformed into BL21 and GST-RACK1 expression was induced by 0.4 mM IPTG at 25°C overnight. The same was performed using empty pGEX-6P-1 plasmid to induce the expression of GST. The cells were sonicated in PBS and pelleted by centrifugation at 20,000 g for 20 min at 4 °C. The clarified

supernatant was applied onto a glutathione sepharose column followed by extensive washing in PBS to generate GST-RACK1- or GST-immobilized sepharose beads. pGEX-6P-1-RACK1-W-5-7 was purified using the same procedure.

2.20 Mammalian cell culture

293T cells (human embryonic kidney cells with SV40 large T antigen), K562 (human leukemia cells), SKW3 (human T lymphoma cell line) and COS-7 cells (African green monkey kidney fibroblasts) were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA). HUVEC were purchased from Lonza Walkersville, Inc., and cultured in endothelial growth medium from the same company.

2.20.1 Culture of HEK293T cells

HEK293T cells (henceforth referred to as 293T cells) were cultured in DMEM medium containing 10% (v/v) heat-inactivated FBS, 100 IU/mL penicillin, and 100 µg/mL streptomycin at 37 °C in a humidified 5% CO₂ incubator. Cells were washed once in PBS, incubated in 0.25% (w/v) trypsin (Invitrogen) at 37 °C for 1 min, followed by tapping of each flask to dislodge the adherent cells. Trypsin was subsequently inactivated by adding complete medium. Cells were then seeded into culture flask or dish with fresh culture medium.

2.20.2 Culture of COS-7 cells

COS-7 cells were cultured in RPMI-1640 medium containing 10% (v/v) heat-inactivated FBS, 100 IU/mL penicillin, and 100 µg/mL streptomycin at 37 °C in a humidified 5% CO₂ incubator. Cells were washed once in PBS incubated in 0.25%

(w/v) trypsin (Invitrogen) at 37 °C for 3 min, followed by tapping of each flask to dislodge the adherent cells. Trypsin was subsequently inactivated by adding complete medium. Cells were then seeded into culture flask or dish with fresh culture medium.

2.20.3 Culture of K562 and SKW 3.0 cells

K562 and SKW 3.0 cells were cultured in complete RPMI-1640 medium as described in Section 2.20.2. Cells were passaged by diluting cells with fresh media after reaching the density of approximately 10^6 cells/mL.

2.20.4 Culture of HUVECs

HUVECs were cultured in EBM[®]-2 medium with EGM[™]-2 SingleQuots[®] supplement at 37 °C in a humidified 5% CO₂ incubator. Cells were washed once in PBS, incubated in 0.25% (w/v) trypsin (Invitrogen) at 37 °C for 1 min, followed by tapping of each flask to dislodge the adherent cells. Trypsin was subsequently inactivated by adding complete EBM[®]-2 medium with 10% (v/v) heat-inactivated FBS. Cells were then seeded into culture flask or dish with fresh culture medium.

2.21 Cell storage in liquid nitrogen

Cells were centrifuged at 400 g for 5 min and resuspended in cell freezing medium at a concentration of 5×10^6 cells/mL before dispensing into Cryo Vials (Greiner, Monroe, NC, USA). Cells were frozen in a NALGENE[™] Cryo 1 °C freezing container (Thermo Fisher Scientific) at -80 °C for 24 h. Thereafter the vials were transferred into liquid nitrogen for long-term storage.

2.22 Cell recovery from liquid nitrogen

Cells were removed from the liquid nitrogen storage and quickly thawed at 37 °C in a water bath. Cells were gently resuspended in 10 mL warmed medium and incubated at RT for 5 min. Cells were then centrifuged (400 g, 5 min) to remove DMSO. The cell pellet was resuspended in complete media and cultured at 37 °C in a humidified 5% CO₂ incubator.

2.23 Isolation of human blood primary T cells

Peripheral blood was taken from donor by a nurse in a clinic and kept in an EDTA tube before use. T cells were isolated using RosetteSep[®] Human CD4 + T Cell Enrichment Cocktail kit (StemCell[™] Technologies, Vancouver, BC, Canada) according to the manufacturer's instructions. Briefly, RosetteSep[®] Human CD4 + T Cell Enrichment Cocktail reagent was added to whole blood at 50 µl/mL and incubate at RT for 20 min. Sample was diluted with an equal volume of PBS + 2% FBS and mixed gently. The mixture was layered on top of Ficoll[™] (GE Healthcare) and centrifuge at RT for 20 min at 1200 g, with the brake switched off. CD4+ T cells were retrieved from the Ficoll:plasma interface. The isolated T cells were expanded in RPMI containing 10% (v/v) heat inactivated FBS, 100 IU/mL penicillin, 100µg/mL streptomycin, 1nM interleukin 2 (IL2) and 2 µg/mL phytohaemagglutinin (PHA) for 3 days before use.

2.24 Transfection of 293T or COS-7 cells

293T or COS-7 cells were seeded into 6-cm culture dishes and cultured overnight to achieve 60-70% cell confluence at the day of transfection. Expression plasmid (4 µg each) was resuspended in cell culture medium without FBS and antibiotics in a total

volume of 150 μ l. Polyfect transfection reagent (QIAGEN) (40 μ l) was added to the plasmid solution and incubated at RT for 5-10 min to allow complex formation. Complete cell culture medium (1 mL) was added to the plasmid solution and transferred to cells in the culture dishes. Cells were harvested 24-48 hours after transfection for subsequent analyses.

2.25 Transfection of K562 cells

Expression plasmid (8 μ g each) were transfected into K562 (2×10^6 cells per reaction) by electroporation using Microporator MP-100 (a pipette type electroporator) and reagents according to the manufacturer's instructions (NanoEnTek, Korea). The cell pellet was resuspended in R buffer with desired plasmids. The samples were added into gold tips (100 μ l) which were connected to the provided pipette and air bubbles were avoided. The optimized microporation program used for K562 was 3 pulses of 1300 V pulse voltage with a duration of 10 ms for each pulse. Electroporated cells were added to the 6-well culture plates containing 2 mL of pre-warmed medium and cultured for 24-48 h before analysis.

2.26 Generation of stable K562 cells with reduced kindlin3 expression

A 3rd generation lentiviral-based siRNA transduction system was used to generate stable K562 cells with reduced kindlin3 expression according to manufacturer's instructions (Applied Biological Materials, BC, Canada). Briefly, four kindlin3-targeting piLenti-RNAi-GFP plasmids were generated. Each of these plasmids or the control si-RNA encoding plasmid was co-transfected with the packaging plasmids into 293T cells to allow production of pseudo-virions. The culture supernatant containing

virus particles was collected. 1×10^6 K562 cells were resuspended in infection medium which includes 1 mL complete RPMI medium, 1 mL of the pseudo-virions-containing supernatant and 6 $\mu\text{g/mL}$ polybrene (Sigma-Aldrich) and cultured for 1 day. The next day, the infection medium was replaced with fresh complete RPMI medium and cultured for at least two days. When GFP-expressing cells were observed under a fluorescence microscope, puromycin (Sigma-Aldrich) was added to the culture at a concentration of 1.5 $\mu\text{g/mL}$ to select for cells stably expressing kindlin3 si-RNA. One of the four kindlin3-targeting piLenti-RNAi-GFP plasmids produced K562 clones that showed significant reduction in kindlin3 expression. The sequence that encodes this kindlin3 si-RNA is TGGAGCAGATCAATCGCAA, which has been previously described (Malinin et al., 2009).

2.27 Coating of microtitre plates, glass bottom, Ibidi® μ -slide | 0.4 Luer chambers or 16-well E-plate dishes with ICAM-1, fibrinogen or fibronectin

Goat anti-human IgG (Fc specific) (100 μL , 5 $\mu\text{g/mL}$ in sodium bicarbonate buffer, pH 9.2) was dispensed into each well of a Polysorb microtitre plate (Nunc), 16-well E-plate device® (Acea Biosciences, Inc., San Diego, CA), or glass bottom dishes followed by incubation at 4 °C overnight. Thereafter, the solution was discarded and 150 μL of 0.5 % (w/v) BSA in PBS (blocking solution) was added to each well and incubated at 37 °C for 30 min. The blocking solution was discarded and 50 μL of ICAM-1/Fc (Minneapolis, MN, USA) (1 $\mu\text{g/mL}$ in PBS) was added to each well and incubate at RT for 2-3 h.

16-well E-plate device® with gold-electrodes at the bottom of each well for real-time electric cell-substrate impedance sensing (ECIS) measurements were coated with fibrinogen (ligand for integrin α IIb β 3) (Sigma-Aldrich). Fibrinogen (100 μ g/ml in sodium bicarbonate buffer, pH 9.2) was dispensed into wells and incubated at 37°C for 2 h.

Coverslip glass-bottom culture dishes or 96-well culture dish were precoated with 2.5 μ g/cm² fibronectin (ligand for integrin α 5 β 1) (Sigma-Aldrich) in bicarbonate buffer at RT for 1 h. The Ibidi® μ -slide | 0.4 Luer chambers were coated with 5 μ g/cm² fibronectin in PBS at RT for 1 h or 4°C overnight. 0.5 % BSA in PBS was used to block the chamber at RT for 1 h before use.

2.28 Preparation of protein A-sepharose with or without rabbit anti-rat IgG conjugation

Protein A-sepharose (PAS) (1 g) (Amersham, Buckinghamshire, UK) was swelled in PBS and rotated at 4 °C overnight. Thereafter, the PAS beads, which occupied a bed volume of approximately 4 mL, was sedimented by centrifugation (3000 g, 5 min, 4 °C), washed twice in PBS and resuspended in 12 mL of PBS to obtain a 25% (v/v) bead suspension.

Rabbit anti-rat IgG-coupled Protein A-Sepharose beads were prepared by adding 2 mg of rabbit anti-rat IgG (Sigma-Aldrich) to the Protein A-sepharose beads and incubated at 4 °C with rotation for at least 2 h. The beads were subsequently sedimented by centrifugation (300 g, 5 min, and 4 °C), washed twice in PBS and resuspended in 12 mL of PBS to obtain a 25% (v/v) beads suspension.

2.29 Western blot

Proteins separated by SDS-PAGE were transferred onto a PVDF membrane (Immobilon-P, Millipore, Bedford, MA) as standard protocols. The PVDF membrane was incubated in TBS-T containing 5 % (w/v) non-fat milk at RT for 1 h to block non-specific binding sites.

The PVDF membrane was incubated in blocking buffer containing the relevant primary antibody at RT for 2 h with rotation to detect integrin α L subunit, integrin β 1 subunit, kindlin3, kindlin2, actin, RACK1, talin, PKC β , FAK, P-FAK, p70S6K, P-p70S6K, His-tagged proteins or HA-tagged proteins. The primary antibody used and dilutions were as followed.

Name	Specificity	Species	Dilution
Clone 27	anti- α L	mouse	1:1000
Clone 9	anti-kindlin3	rat	1:2500
Clone 157D	anti-kindlin3	rat	1:2500
Clone 181A	anti-kindlin3	rat	1:2500
Clone 229A	anti-kindlin3	rat	1:2500
Clone C4	anti-actin	mouse	1:5000
B-3	anti-RACK1	mouse	1:2500
8d4	anti-human talin	mouse	1:2500

Anti-PKC β	anti-human PKC β	mouse	1:1000
Anti-HA	anti-HA	rabbit	1:2500
3285	anti-human FAK	rabbit	1:2500
3283	anti-human P-FAK (Y397)	rabbit	1:1000
Anti-kindlin2	anti-human kindlin2	rabbit	1:2500
9202	anti-P-p70S6K (T389)	rabbit	1:1000
9205	anti-p70S6K	rabbit	1:1000
H-300	hnRNP K	rabbit	1:2500
anti-CD49e	anti-human CD49e	mouse	1:2500
DB063	anti-His	rabbit	1:2500

The membrane was washed for three times in TBS-T and incubated in TBS-T containing HRP-conjugated sheep anti-mouse IgG antibody (1:5000 dilution) (GE Healthcare, UK), HRP-conjugated goat anti-rat IgG antibody (1:5000 dilution) (GE Healthcare, UK) or HRP-conjugated donkey anti-rabbit IgG antibody (1:5000 dilution) (GE Healthcare, UK) at RT for 1 h. The membrane was washed for three times in TBS-T and the Enhanced Chemiluminescence (ECL) substrate (ECL Plus Detection Kit, GE Healthcare, UK) was used to detect protein binds on the membrane.

To strip off the antibodies on PVDF membrane, membranes were incubated in strip buffer with gently shaking at 55°C for 30 min. Membranes were transferred to another

container and washed with TBS-T buffer for 10 times followed with blocking and antibodies incubation as described above.

2.30 Immunoprecipitation

293T transfectants were lysed in lysis buffer (10 mM Tris-HCl, 150 mM NaCl, 1% (v/v) NP-40, pH 7.5, and protease inhibitors cocktail). Cell lysates were precleared with 6 µg rabbit IgG Protein A-Sepharose beads (GE healthcare) for 1 h at 4°C. Protein A-Sepharose beads were spun down by centrifugation and the cell lysate collected. The cell lysate was incubated with 6 µg rabbit anti-HA antibody or 6 µg rabbit IgG and Protein A-Sepharose beads for 4 h at 4°C to precipitate the HA-tagged kindlin2, HA-tagged kindlin3 or mutants. Precipitated proteins were resolved on a 10% SDS-PAGE under reducing conditions, and co-immunoprecipitated RACK1 was detected by immunoblotting with mouse anti-RACK1 antibody as described above.

To assess the interaction between α L β 2, kindlin3 and RACK1 in SKW3.0 cells, 20 - 30 $\times 10^6$ cells in RPMI1640 medium containing stromal cell-derived factor 1 α (SDF-1 α) (100 ng/mL) (EMD4 Biosciences, Gibbstown, NJ) were seeded into ICAM-1-coated wells of Polysorb microtiter plates that were prepared as described in previous section. Cells were incubated for 15 min in a CO₂ incubator. Thereafter cells were collected and lysed in lysis buffer. For immunoprecipitation, irrelevant rat IgG or rat anti-kindlin3 (clone9) hybridoma supernatant was added to rabbit anti-rat IgG-coupled Protein A-Sepharose beads to precipitate kindlin3 and irrelevant rat IgG were included as control.

2.31 Real-time electric cell-substrate impedance sensing (ECIS) measurements

Dithiobis succinimidyl propionate (Pierce, Thermo Fisher Scientific) (4 mg/mL) in DMSO (40 μ L) was added to each microtitre well of a 16-well E-plate device® (Acea Biosciences, Inc., San Diego, CA) with gold-electrodes at the bottom of each well and incubated at RT for 30 min. Wells were washed twice in dd H₂O and coated with goat anti-human IgG (Fc specific) (100 μ L, 5 μ g/mL in sodium bicarbonate buffer, pH 9.2) followed by 150 μ L of 0.5 % (w/v) BSA in PBS (blocking solution) and 50 μ L of ICAM-1/Fc (1 μ g /mL in PBS). Wells were washed once in RPMI-1640 complete medium and refilled with 50 μ L medium in each well. Background scan of the wells were performed on a Real Time Cell Electronic System™ (Acea Biosciences, Inc.). K562 transfectants (8×10^4 cells per well) were then seeded into each well and AC impedance measurements taken with time with an interval of 1 min between scans. α L β 2N329S, α IIb β 3N339S and α 5 β 1 mediated cell adhesion and spreading were verified by including the function-blocking mAbs MHM24, 10E5 and anti-CD49e (10 μ g/mL each), respectively.

2.32 GST pull-down assays

After GST and GST-RACK1 were immobilized on the beads, the wash buffer for kindlin3 (in section 2.8) was used to equilibrate the column. The beads were blocked in wash buffer containing 5% (w/v) BSA at 4 °C overnight. Indicated amounts of purified kindlin2-6His, kindlin3-6His or its mutants were incubated with GST, GST-RACK1 or GST-RACK1-W5-7-immobilized sepharose beads at 4°C for 3 h. Beads were washed in wash buffer and proteins were resolved on a 10% SDS-PAGE under reducing conditions. Anti-kindlin3 and anti-kindlin2 antibodies were used to detect kindlin3 and kindlin2. GST and GST-RACK1 were detected by staining in Coomassie

brilliant blue solution.

The N-terminal biotin-conjugated full-length $\beta 2$ tail peptide (Lys702-Ser747) and its mutant (N741A) were synthesized, purified and ESI-MS-verified by ChinaPeptides, Shanghai, China. The peptide was incubated with streptavidin-agarose beads suspension (Sigma-Aldrich) (100 μ g peptides per 50 μ l beads) in pull-down buffer (in section 2.8) at 4 °C for 1 h. The beads were washed in pull-down buffer and incubated in the same buffer containing 0.5 % (w/v) BSA at 4 °C for 1 h to block non-specific binding sites. Thereafter, washed beads were incubated with indicated amount of purified recombinant kindlin3-6His or RACK1-6His.

2.33 Surface plasmon resonance (SPR) analyses

Sensor chip CM5, *N*-hydroxysuccinimide (NHS), *N*-ethyl-*N*-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC), ethanolamine and surfactant P20 were purchased from Biacore, GE Healthcare, UK. SPR assays were conducted at 30 μ l/min flow rate at 37°C on a Biacore 3000 system. Standard HBS-EP buffer was used for the analysis. GST-RACK1 were immobilized to 5000RU onto sensor chip CM5 using standard amine-coupling procedure as previously described (Myckatyn et al., 2002). GST was also immobilized to the same level onto another flow cell as control. Kindlin3-6His was verified not to interact with immobilized control GST. Two-fold serially diluted kindlin3-6His (1.56, 3.125, 6.25, 12.5, 25, 50 and 100 μ M) were separately injected across flow cells comprising an unmodified surface, the control surface and GST-RACK1 for 6 min allowed to dissociate for 5 min. Surfaces were regenerated by injected with a short pulse (30 s) of 15 mM HCl followed by HBS-EP buffer. All raw sensorgrams were double referenced (Jiang et al., 2002) by subtracting all resultant

sensorgrams with the control GST surface (to eliminate non-specific binding) and blank buffer injections (to eliminate equipment systematic error), and then globally fit to a simple bimolecular interaction model. Three replicates of the interaction were performed (with different batches of proteins) with similar results. This part of the work was performed with the help of Dr. Yau Yin Hoe, SPR core facility, School of Biological Sciences, NTU.

2.34 Flow cytometry

Flow cytometry analysis of integrin cell surface expression was performed as described previously (Tan et al., 2000). Briefly, cells were incubated with α L β 2 specific mAb MHM24, α IIb β 3 specific mAb 10E5 (20 μ g/ml) or α 5 β 1 specific mAb anti-CD49e (20 μ g/ml) in PBS at RT for 1 h. Thereafter, cells were washed. APC-conjugated goat anti-mouse secondary Ab (1:400 dilution; Sigma) were used for control siRNA K562 cells, kindlin3-targeting-siRNA K562 cells, and K562 cells transfected with α L β 2N329S and α IIb β 3N339S at RT for 45 min. Stained cells were washed once and analyzed on a FACSCalibur (BD Biosciences). Data were analyzed using the CellQuest software (BD Biosciences) or the Flowjo software (Tree Star Inc., Ashland, OR, USA)

2.35 Shear flow

The Ibidi® μ -slide | 0.4 Luer chambers was coated with fibronectin and blocked with BAS as described in **session 2.27**. Control siRNA K562 cells or kindlin3-targeting-siRNA K562 cells were collected and resuspended in cell culture medium to achieve a concentration of 6×10^5 cells/mL. 1 mL cell suspension was injected into The Ibidi®

μ -slide | 0.4 Luer chamber at a speed of 230 μ l/min (shear stress 0.3 dyn/cm²) using Harvard Apparatus PHD 2000 Syringe Pump Series instrument at 37°C. 3 more shear flow buffered were injected and passed through the chamber at a constant speed of 230 μ l/min (0.3 τ (dyn/cm²)) after the injection of cells. Cells attached to fibronectin in chambers were counted under microscope immediately after buffer injection.

2.36 FRET analyses

FRET analyses were performed using similar procedures as described (Vararattanavech et al., 2008). For FRET experiments, COS-7 transfectants expressing CFP and RACK1-YFP, CFP-kindlin3 and RACK1-YFP, CFP-kindlin3-PH Δ and RACK1-YFP, CFP-kindlin3-F3 Δ and RACK1-YFP or CFP-kindlin2 and RACK1-YFP were plated into a coverslip glass-bottom culture dish and cultured for 24 h. FRET was performed on a Zeiss LSM510 confocal microscope (Carl Zeiss, Inc., Thornwood, NY) to detect the interaction between RACK1 and kindlin3 or mutants. The settings for FRET used were: mCFP, excitation wavelength 458 nm, and the emission signal was detected with a BP 470-500 nm emission filter; mYFP, excitation wavelength 514 nm, and the emission signal was detected with a LP 530 nm emission filter. The mYFP of an entire cell was photobleached by scanning the entire cell 20 times using the 514 argon laser line that was set at maximum intensity. The mCFP signal of the entire cell was acquired before and after photobleaching the mYFP. The low density of kindlin3 in the nucleus of the cell was excluded from the analyses. EF (FRET efficiency) was calculated as a percentage using the equation $EF = (I_6 - I_5) \times 100 / I_6$, where I_n is the mCFP intensity at the nth time point. Bleaching was performed between the 5th and 6th time points. The mean noise computed as $NF = (I_5 - I_4) \times 100 / I_5$ in which the mCFP signals at the 4th and 5th time points before the bleaching process was close to

zero in all cases. For integrin α L β 2 clustering analyses in K562 cells, only the plasma membrane was selected as the region of interest for measurements of CPF signal pre- and post YFP photobleached.

2.37 Fluorescence microscopy

SKW3.0 cells or human primary T cells were washed in PBS once, resuspended in full medium with SDF-1 α (100 ng/mL) and then seeded into ICAM-1 precoated coverslip glass-bottom culture dishes. Cells were incubated in 37°C CO₂ incubator for 15 min, fixed in 3.7% (w/v) paraformaldehyde in PBS at RT for 10 min. Thereafter, cells were permeabilized with 0.3% (v/v) Triton X-100 in modified cytoskeleton stabilization (CSK) buffer (100 mM NaCl, 300 mM sucrose, 3 mM MgCl₂, 1 mM EGTA, 10 mM PIPES, pH 6.8) at RT for 1 min. Permeabilized cells were incubated with relevant primary antibodies (1 μ g each) at RT for 1 h. Cells were washed three times in PBS followed by incubation in PBS containing relevant secondary antibodies, Alexa Fluor® 488-phalloidin (0.27 ng/mL) or DAPI (0.1 μ g/mL) at RT for 1 h. Cells were washed three times in PBS and analyzed on a Zeiss LSM 510 or LSM 710 confocal laser scanning microscope equipped with a 63 $\times$ objective. Images were processed using software LSM 510, version 3.2.

HUVEC cells were collected, resuspended in EBM-2 medium and seeded into fibronectin precoated coverslip glass-bottom culture dishes. Cells were incubated at 37°C for 15 min or 45 min, fixed in 3.7% (w/v) paraformaldehyde in PBS at RT for 10 min. Thereafter, cells were permeabilized with 0.3% (v/v) Triton X-100 in modified CSK buffer at RT for 1 min. Permeabilized cells were incubated with relevant primary antibodies (1 μ g each) at RT for 1 h. Cells were washed three times in PBS followed

by incubation in PBS containing relevant secondary antibodies, Alexa Fluor® 594-phalloidin (0.27 ng/mL) or DAPI (0.1 µg/mL) at RT for 1 h. Cells were washed three times in PBS and analyzed on a Zeiss LSM 510 or LSM 710 confocal laser scanning microscope equipped with a 63×objective.

2.38 Statistical analysis

In all cases, student's t test (two tailed and unequal variance) was used in statistical determination of the difference observed between two groups. The difference was considered significant when $p < 0.05$.

Chapter 3 Characterization of kindlin3 monoclonal antibodies

3.1 Introduction

In general, kindlins consist of F0, F1, F2 and F3 subdomains with a PH domain inserted into the F2 subdomain. Different domains of kindlin have different functions. The F3 subdomain of kindlins has been well established to bind to the membrane-distal NXXY/F motif in the integrin β cytoplasmic tails (Harburger et al., 2009; Ma et al., 2008; Moser et al., 2008). The F0 subdomain has been shown to adopt an ubiquitin-like fold and it is involved in the translocation of kindlin1 to integrin α IIb β 3-containing focal adhesions (Goult et al., 2009). The PH subdomain of kindlin2 has been shown to bind membrane PI(3,4,5)P3 (Qu et al., 2011). To characterize the functions of kindlin3, it is essential to have antibodies that bind kindlin3. Rat mAbs against human kindlin3 were generated and provided by my supervisor Dr. Tan Suet Mien using full-length purified recombinant human kindlin3. However, many of these mAbs have not been characterized with respect to their epitopes. Whether these mAbs can be used in immunoprecipitation or immunofluorescence studies are also not determined. Hence, the first part of my study involved the characterization of these mAbs.

3.2 kindlin3 monoclonal antibodies

In this chapter, we characterized four mAbs to kindlin3: clone 9, clone 157D, clone 181A and clone 229A. All four mAbs reacted well with endogenous kindlin3 expressed in the chronic myelogenous leukemia cell line K562 as determined by western blotting (**Figure 3.1A**). All except clone 157D immunoprecipitated endogenous kindlin3 from K562 cells (**Figure 3.1B**). We also tested these mAbs in

immunofluorescence study. The human T lymphoma cell line SKW3 was treated with chemokine SDF-1 α and seeded onto glass-coverslip dishes coated with integrin α L β 2 ligand ICAM-1 to induce polarization and adhesion. The cells were fixed, permeabilized and stained with the respective mAbs followed by Alexa Fluor®488-conjugated anti-rat secondary antibody. The cells were also stained for actin and nuclei using Alexa Fluor®594-conjugated phalloidin and DAPI, respectively (**Figure 3.2**). Clone 9 and clone 157D, but not clone 181A and clone 229A, stained kindlin3 in these cells. Irrelevant rat IgG was included as a control.

3.3 The epitopes of the four mAbs in kindlin3

Next we sought to define the epitopes of these four mAbs in kindlin3 by immunoblotting. We generated five kindlin expression constructs that contained an N-terminal HA-tag in each of them (**Figure 3.3**). The two full-length constructs are HA-K2 and HA-K3. Kindlin2 and kindlin3 share ~50% sequence identity and ~70% sequence homology. We did not include kindlin1 because it is epithelial-specific whereas kindlin2 is widely expressed in many cell types. HA-K2 was included to assess the specificity of these four mAbs to kindlin3. The rest of the kindlin3 constructs are truncated or region-deleted variants. The expression levels of all these constructs were verified in transfected 293T cells by anti-HA western blotting (**Figure 3.4B**). 293T cells are human embryonic kidney cell line which doesn't express kindlin3. Kindlin3 transcript was not detected in total RNA extracted from 293T cells but was abundant in HUVEC as shown by RT-PCR (**Fig 3.4A**). All constructs were highly expressed albeit varying levels. The HA-K3F1 construct appears to have two protein bands, possibly due to degradation. Nevertheless, we made use of these cell lysates for western blotting using the four mAbs. Data obtained show that the epitope of clone

9 resides in the F3 subdomain of kindlin3. The epitope of clone 157D is found in the F0F1 region of kindlin3. The epitope of 181A is located in the PH domain and the epitope of clone 229A resides in the F3 subdomain. In all cases, none of the mAbs reacted with overexpressed HA-K2 (**Figure 3.4C-F**). All the data obtained are summarized in **Table 3.2**. This kindlin3 mAbs characterization study allows us to use these mAbs in subsequent studies.

Figure 3.1 Kindlin3 monoclonal antibodies can be used to detect endogenous kindlin3. (A) Cell lysates from K562 cells were subjected to immunoblotting using clone 9, clone 157D, clone 181A and clone 229A to detect endogenous kindlin3

expression. (B) Cell lysates from K562 cells were subjected to immunoprecipitation analyses with clone 9, clone 157D, clone 181A and clone 229A. Precipitated endogenous kindlin3 was detected by immunoblotting with clone 9.

Figure 3.2 Immunofluorescence staining of SKW3 cells with kindlin3 mAbs. SKW3 cells were seeded into ICAM-1-coated glass-bottom dishes in the presence of SDF-1 α at 37°C for 15 min. Cells were fixed, permeabilized and stained for kindlin3. Alexa Fluor® 488-conjugated phalloidin and DAPI were used to stain F-actin and

nucleus, respectively. SKW3 cells that were stained with clone 181A (O) and clone 229A(S) failed to show specific fluorescence within cells whereas cells stained with clone 9 (G) and clone 157D (K) showed green fluorescence. Rat IgG (C) were included as control. Scale bar represents 20 μ m.

Fig 3.3 The linear organizations of human kindlin2, human kindlin3 and its mutants used in this study.

Figure 3.4 Epitope screening of kindlin3 mAbs. (A) RNA extracted from 293T cells and HUVEC cells were used for RT-PCR experiments using primers for actin and kindlin3. (B-F) 293T cells were transfected with HA-tagged kindlin2 (HA-K2), HA-tagged kindlin3 (HA-K3), HA-tagged kindlin3 F0F1 domain (HA-K3F1), HA-tagged PH-domain-deleted kindlin3 (HA-K3PH Δ) and HA-tagged F3-domain-deleted kindlin3 (HA-K3F3 Δ). Cells were lysed and subjected to immunoblotting with indicated antibodies. (B) Anti-HA antibody. (C) Clone 9. (D) Clone 157D. (E) Clone

181A. (F) Clone 229A.

Name	Epitope localization	Usage		
		Immunoblotting	Co-ip	Immunofluorescence
Clone 9	F3 domain	√	√	√
Clone 157D	F0F1 domain	√	×	√
Clone 181A	PH domain	√	√	×
Clone 229A	F3 domain	√	√	×

Table 4.2 Summary of kindlin3 mAbs properties and epitopes.

3.4 Discussion

Kindlin3 has been found to be a co-activator of integrin. The function of kindlin3 has been described in section 1.5. Similar to talin1, kindlin3 contains a FERM domain, which consists of three subdomains called F1, F2 and F3. Kindlin3 also possess a C-terminal F0 domain and a PH domain, which is inserted into the F2 subdomain. The domain structure and organization is important for the function of kindlins. Kindlin1 F0 domain is required for the ability of kindlin1 to co-activate talin-induced integrin $\alpha\text{IIb}\beta 3$ activation and the localization of kindlin1 to focal adhesion sites (Goult et al., 2009). The interaction site of kindlin2 with migfilin and ILK lies in F1 domain (Mackinnon et al., 2002; Montanez et al., 2008; Tu et al., 2003). It has been well established that the PTB fold within kindlins F3 domain allows kindlins to bind to the integrin β tails (Kloeker et al., 2004; Shi et al., 2007). Therefore, generation of antibodies that bind to different domain of kindlin3 is crucial for studying its functions. In this study, we generate rat anti-kindlin3 monoclonal antibodies that specifically bind to F0F1, PH, and F3 domain of kindlin3. The application of the mAbs of kindlin3 have

also been tested and identified. This work will facilitate the function and structure studies of kindlin3 in our subsequent studies.

Chapter 4 Kindlin3 is important for integrin α L β 2 outside-in signaling and it interacts with the scaffold protein receptor for activated-C kinase 1 (RACK1)

4.1 Introduction

As described in earlier chapters, kindlin3 is a co-activator of integrins and it is important for β 2 integrin mediated leukocyte adhesion and spreading (Feigelson et al., 2011; McDowall et al., 2010; Moser et al., 2008; Moser et al., 2009b; Svensson et al., 2009). To better understand the role of kindlin3 in integrin functions, we first investigated its role in β 2 integrin outside-in signaling.

4.2 Kindlin3 plays an important role in integrin α L β 2 outside-in signaling

To this end, a kindlin3-targeting siRNA K562 cell line and its control siRNA K562 cell line were generated using a siRNA lentiviral system with GFP reporter. As shown in **Figure 4.1 A**, the expression level of kindlin3 in knock-down cell line was reduced compared with control cell line while the expression level of other cytoplasmic proteins RACK1, FAK, talin and PKC β were comparable in both cell lines. K562 cells express β 1 integrins but not β 2 integrins. Therefore we make use of the constitutively activated integrin mutant α L β 2N329S to bypass the need for kindlin3 integrin inside-out signaling. The mutant α L β 2N329S has been reported by our lab previously (Cheng et al., 2007). This allows us to study the role of kindlin3 in cell adhesion and spreading mediated by constitutively activated α L β 2 in K562 cells with reduced kindlin3 expression. The expression levels of α L β 2N329S in two K562 transfectants were analyzed by using mAb MHM24 followed by flow cytometry analyses and they were

comparable (**Figure 4.1 B**). Both transfectants were also GFP positive compared with wild-type K562 cells.

Real-time electrical cell-substrate impedance sensing (ECIS) measurements were performed to analyze K562 transfectants adhesion and spreading on immobilized ICAM-1. The extent by which cells adhere and spread on ICAM-1 is registered as cell index. Control si-RNA transduced cells expressing integrin $\alpha\text{L}\beta\text{2N329S}$ showed a marked increase in the cell index whereas the kindlin3-targeting si-RNA transduced cells expressing integrin $\alpha\text{L}\beta\text{2N329S}$ showed marginal increase in cell index (**Figure 4.1 C**). The function blocking mAb MHM24 (anti- αL) was used to demonstrate the specificity of integrin $\alpha\text{L}\beta\text{2N329S}$ -mediated cell adhesion and spreading. These data suggest that kindlin3 is not only important in integrin inside-out signaling, but also plays a role in integrin $\alpha\text{L}\beta\text{2}$ outside-in signaling. Similar experiments were performed with K562 cells transfected with constitutively activated integrin $\alpha\text{IIb}\beta\text{3N339S}$ (**Figure 4.1 D**). Consistent with that of $\alpha\text{L}\beta\text{2N329S}$, K562 cells with reduced kindlin3 expression showed inefficient cell adhesion and spreading despite expressing constitutively activated integrin $\alpha\text{IIb}\beta\text{3N339S}$. Again, ECIS measurements were used to analyze the cell adhesion and spreading to demonstrate the generality of kindlin3 in mediating integrin outside-in signaling (**Figure 4.1 E**).

Figure 4.1 Kindlin3 plays an important role in integrin α L β 2 outside-in signaling and micro-clustering. (A) K562 was transduced with control si-RNA or kindlin3-targeting si-RNA and selected for clones that stably express control si-RNA or kindlin3-targeting si-RNA. Cell lysates from both clones were subjected to SDS-PAGE followed with western blotting to assess the expression levels of kindlin3 and other

cytoplasmic proteins. Actin was used as loading control. (B) The control si-RNA K562 clone and kindlin3-targeting si-RNA K562 clone both expressed GFP as lentiviral siRNA reporter. Both clones were transfected with integrin mutant $\alpha\text{L}\beta\text{2N329S}$. Surface expression of $\alpha\text{L}\beta\text{2N329S}$ on transfectants was assessed by mAb MHM24 staining followed by APC-conjugated secondary antibody. Data were analyzed by two-color flow cytometry. FL1 is for GFP expression and FL4 is for $\alpha\text{L}\beta\text{2N329S}$ expression level. The expression level was presented as expression index (EI): % gated positive (GP) $\times$ geo-mean fluorescence intensity (GM). (C) The cell adhesion/spreading of K562 transfectants on immobilized ICAM-1 was monitored and recorded on a ACEA RE-CES® system (ECIS measurements) for 3 h. Measurements were taken and recorded at 1 min intervals and is plotted at 5 min intervals for clarity. The result is displayed as in the dimensionless parameter termed cell-index or impedance value, which is derived from electrode/solution interface. When cells migrate and adhere on the chips, the electrode/solution interface is affected which leads to an increase in cell index. Each time point measurement is the mean $\pm$ SD of triplicates. A representative experiment of three independent experiments is shown. Three experiments showed similar profiles and only margin variations were observed. (D) Kindlin3-targeting siRNA K562 cells and control siRNA K562 cells were transfected with integrin $\alpha\text{IIb}\beta\text{3N339S}$. Expression levels of integrin $\alpha\text{IIb}\beta\text{3N339S}$ in both cells were examined by staining cells with mAb 10E5 followed by APC-conjugated secondary antibody. Two color flow cytometry analyses were performed. In this experiment, FL1 is for GFP while FL4 is for integrin $\alpha\text{IIb}\beta\text{3N339S}$ expression. The expression level was presented by expression index EI. (E) ECIS measurements of K562 transfectants expressing $\alpha\text{IIb}\beta\text{3N339S}$ on immobilized fibrinogen. The specificity of $\alpha\text{IIb}\beta\text{3}$ mediated binding was demonstrated by including the function-blocking mAb 10E5. Each time point measurement is the mean $\pm$ SD of triplicates. A representative experiment of two independent experiments is shown. Two experiments showed similar profiles and only margin variations were observed.

4.3 Kindlin3 over-expression induced integrin $\alpha\text{L}\beta\text{2}$ micro-clustering in K562 cells.

Micro-clustering of integrin is the formation of laterally associated, micron-scale integrin aggregates. Micro-clustering is considered to play central roles in regulating integrin avidity and outside-in signaling. Whether kindlin3 plays a role in integrin micro-clustering process is not known. In this study, YFP-photobleach FRET-based assay was used to investigate if kindlin3 facilitates integrin $\alpha\text{L}\beta\text{2}$ micro-clustering in K562 cells. Monomeric CFP or monomeric YFP was fused to integrin αL subunit at its C-terminus, referred to as αLmCFP or αLmYFP respectively (Vararattanavech et al., 2009). If an αLmCFP is in the proximity (<10 nm) of an αLmYFP , which is the case

when micro-clustering of integrin occurs, FRET signal will be detected (**Figure 4.2 A**). On the other hand, a minimal FRET signal would be detected if α LmCFP and α LmYFP are more than 10 nm apart. K562 transfectants bearing α LmCFP, α LmYFP, β 2 and HA-kindlin3 showed significant increase in CFP signal after YFP photobleaching. By contrast, when YFP photobleaching was performed in K562 cells expressing α LmCFP, α LmYFP, β 2 and HA-kindlin3-PH Δ or α LmCFP, α LmYFP, β 2 and HA-kindlin3-F3 Δ , there was minimal increase in CFP signal. YFP photobleaching of K562 cells expressing α LmCFP, α LmYFP, β 2 and empty vector were also performed as controls (**Figure 4.2 B**).

These observations suggest that kindlin3 could facilitate integrin α L β 2 micro-clustering and both its PH and F3 domains are essential for this function. It has been showed that F3 domain of kindlin3 is the domain that mediates the interaction of kindlin3 with integrin (Harburger et al., 2009; Ma et al., 2008; Moser et al., 2008). This could explain why K562 cells expressing kindlin3- Δ F3 showed poor induction of integrin α L β 2 micro-clustering. The mechanisms by which the PH domain of kindlin3 takes part in integrin α L β 2 micro-clustering process will need further studies.

B

C

Figure 4.2 Kindlin3 over-expression induces the micro-clustering of integrin $\alpha\text{L}\beta 2$ in K562 cells. (A) Drawings that illustrate the detection of integrin $\alpha\text{L}\beta 2$ micro-clustering using FRET analysis. The integrin αL subunit was fused with either mCFP or mYFP at the C-terminus. (B) K562 cells were transfected with integrin subunits αLmCFP , αLmYFP , $\beta 2$ and HA-tagged kindlin3 or its mutants. The expression of these proteins was verified by immunoblots of lysates from K562 transfectants. (C) % FRET efficiency plot. Data are representative of three independent experiments. Each data point is the mean $\pm$ SD for 40 cells analyzed. * $p < 0.05$, Student's t test.

4.4 Kindlin3 interacts with the scaffold protein RACK-1

RACK-1 is a seven-blade propeller WD-repeat scaffold protein that is widely expressed in many cell types. RACK1 has been shown to interact with integrin β tails and is involved in integrin functions (Buensuceso et al., 2001; Liliental and Chang, 1998). Blades 5-7 of RACK1 bind the membrane proximal sequence Lys702-His706 of the integrin β 2 tail. Co-immunoprecipitation experiments has shown that RACK1 associated with integrin α L β 2 in PMA-treated JY cell lysate (Liliental and Chang, 1998). Latex bead coated with SDF-1 α induced the polarization of Jurkat T cells and RACK1 localized at the polarized front of these cells. RACK1 has also been observed to localize at the leading edge of migrating HL-60 cells (Chen et al., 2008). In another study, RACK1 is found to bind various PH domains from different proteins (Rodriguez et al., 1999). All these data therefore prompted us to examine the association of kindlin3 with RACK-1.

To assess the interaction between RACK1 and kindlin3, 293T cells were transfected with HA-kindlin3, HA-kindlin3-PH Δ , HA-kindlin3-F3 Δ or HA-kindlin2. Anti-HA polyclonal antibody was used to immunoprecipitate ectopically expressed kindlin3 or its mutants or kindlin2. Endogenous co-precipitated RACK1 was detected by immunoblotting with anti-RACK1 mAb. Kindlin2 was included in this experiment because it has been shown to be important in many integrin functions as well as cell adhesion and spreading (Larjava et al., 2008; Moser et al., 2009b; Tu et al., 2003). As shown in **Figure 4.3 A**, RACK1 co-precipitated with HA-kindlin3 and HA-kindlin3-F3 Δ , but not HA-kindlin2 and HA-kindlin3-PH Δ . It is interesting that RACK1 interacts with kindlin3 but not kindlin2. This result prompted us to further verify the interactions by pull-down assays using recombinant purified GST-RACK1, kindlin2-

6His and kindlin3-6His. The recombinantly purified kindlin2-6His (K2-6His) and kindlin3-6His (K3-6His) were verified by immunoblotting with their specific antibodies (**Figure 4.3 B**). Purified recombinant GST and GST-RACK1 were conjugated to sepharose beads followed by passing through kindlin2-6His or kindlin3-6His. Results from the pull-down assays are consistent with co-immunoprecipitation data, in which RACK1 associated with kindlin3 but not kindlin2 (**Figure 4.3 C**).

Next, FRET-based interaction studies in living cells were used to further verify the above data. Both kindlin2 and kindlin3 were fused with CFP at their N-terminus while RACK1 was fused with YFP at its C-terminus. COS-7 cells were co-transfected with RACK1-YFP with CFP-kindlin2 or CFP-kindlin3 followed by YFP-photobleach FRET analyses. COS-7 cells transfected with the CFP empty vector and RACK1-YFP were included as control (**Figure 4.3 D**). Cells transfected with RACK1-YFP and CFP-kindlin3 showed a significant increase in CFP signal when YFP was photobleached compared with others.

Figure 4.3 Interactions between kindlin3 and the scaffold protein RACK1. (A) 293T cells were transfected with HA-kindlin2 (HA-K2), HA-kindlin3 (HA-K3), HA-kindlin3-PH Δ (HA-K3PH Δ) and HA-kindlin3-F3 Δ (HA-K3F3 Δ). The 293T transfectants were lysed and subjected to immunoprecipitation (IP) analyses with anti-HA antibody. Immunoblotting (IB) was used to detect endogenous RACK1. (B) Anti-kindlin3 mAb clone 229A were used to immunoblot recombinant protein kindlin3-6His (K3-6His) while commercial anti-kindlin2 antibody were used to detect kindlin2-6His (K2-6His). Coomassie stained gel of purified kindlin2-6His and kindlin3-6His is

also shown. (C) GST-pull down assay using GST or GST-RACK1 conjugated beads and kindlin2-6His and kindlin3-6His. (D) FRET analyses of COS-7 cells transfected with CFP, CPF-K3, CFP-K2 and RACK1-YFP. Each data point represents mean $\pm$ SD of ≥ 50 cells analyzed. * $p < 0.05$, Student's t test.

Next, we examined which domain of kindlin3 is responsible for the interaction with RACK1. CFP was fused with kindlin3-PH Δ and kindlin3-F3 Δ at their N-terminus. COS-7 cells were co-transfected with two plasmids: RACK1-YFP and CFP-kindlin3 or RACK1-YFP and CFP-kindlin3-PH Δ or RACK1-YFP and CFP-kindlin3-F3 Δ . Again COS-7 cells transfected with the CFP empty vector and RACK1-YFP were used as control. We detected high FRET efficiency in cells transfected with RACK1-YFP and CFP-kindlin3 (**Figure 4.4 A**). This was specific to RACK1 and kindlin3 interaction because cells transfected with empty CFP vector and RACK1-YFP showed basal FRET signal. Cells transfected with RACK1-YFP and CFP-kindlin3-F3 Δ showed a moderate reduction in FRET signal whereas a significant reduction in FRET signal was detected in cells transfected with RACK1-YFP and CFP-kindlin3-PH Δ (**Figure 4.4 B**). These data suggest that the PH domain in kindlin3 plays an important role in RACK1-kindlin3 interaction and it corroborates well with our data in the previous sections.

SPR analysis was performed using the purified kindlin3-6His and GST-RACK1 to obtain binding constants for their interaction. GST-RACK1 was captured and immobilized using anti-GST antibody on a CM5 sensor chip followed by injections of kindlin3-6His at different concentrations at 10 μ l/min flow rate. The interaction between kindlin3 and RACK1 (colored lines) follows a simple bimolecular model (orange lines) with $k_a = 0.2 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$, $k_d = 4.1 \times 10^4 \text{ s}^{-1}$, $K_D = 1.8 \text{ }\mu\text{M}$. Three replicates using different batches of purified proteins gave an average affinity constant of $1.60 \pm 0.08 \text{ }\mu\text{M}$ (**Figure 4.4 C**). This part of the work was performed with the help

of Dr. Yau Yin Hoe, SPR core facility, School of Biological Sciences.

Figure 4.4 Kindlin3 PH domain is involved in the interaction with RACK1. (A) FRET analyses of COS-7 cells transfected with CFP, CFP-K3, CFP-K3PH Δ , CFP-K3F3 Δ and RACK1-YFP. Each data point represents mean $\pm$ SD of ≥ 50 cells analyzed. $*p < 0.05$, Student's *t* test. Data are representative of three independent experiments. (B) Drawing that

illustrates the result of FRET-based detection of interactions between RACK1 and kindlin3 or its mutant. (C) SPR analyses of K3 and RACK1 interaction. Purified K3-6His was injected at different concentrations across the CM5 sensor chips that were coated with GST or GST-RACK1. The results were double-referenced as described in (Nishida et al., 2006). Briefly, the sensorgrams from GST-RACK1 flow cell were subtracting all resultant sensorgrams with GST surface (to eliminate non-specific binding) and blank buffer injections (to eliminate equipment systematic error). The data obtained (colored lines) fit well to a simple bimolecular model (orange lines) with $k_a = 0.2 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$, $k_d = 4.1 \times 10^4 \text{ s}^{-1}$, $K_D = 1.8 \text{ }\mu\text{M}$. Three replicates of experiments were performed with different batches of purified proteins and the average affinity constant is $1.60 \pm 0.08 \text{ }\mu\text{M}$.

To further determine the interaction sites of kindlin3 and RACK1, GST pull-down assays using recombinant purified proteins were performed. Recombinant GST or GST-RACK1 were immobilized onto sepharose beads and the beads were incubated with different amount (0, 2, 20, 200 μg) of purified kindlin3-6His or kindlin3-PH Δ -6His or kindlin3-F3 Δ -6His. Proteins were resolved on a 10% SDS-PAGE under reducing conditions. Consistent with the previous data, kindlin3-6His and kindlin3-F3 Δ -6His, but not kindlin3-PH Δ -6His were detected (**Figure 4.5 A**). Both of kindlin3-6His and kindlin3-F3 Δ -6His detected in GST-RACK1 samples were concentration-dependent, whereas no kindlin3 was detected in the control GST samples. RACK1 folds into a seven-blade propeller structure with docking sites for different cytoplasmic proteins (McCahill et al., 2002). We attempted to examine which of these blades of RACK1 actually interacts with kindlin3 by performing pull-down assay of kindlin3 with different RACK1 blades. However, only GST-RACK1 blades 5-7 Gly 190-Arg317 out of all the construct of RACK1 blades can be purified in a soluble form. Therefore, GST-RACK1-blade-5-7 was used to pull down kindlin3. Again, GST-, GST-RACK1- or GST-RACK1-blade-5-7-immobilized sepharose beads were generated and incubated with kindlin3-6His. As shown in **Figure 4.5 B**, GST-RACK1 and GST-RACK1-blade-5-7 but not control GST pulled-down kindlin3. These data suggest that kindlin3 interacts directly with RACK1 and the interaction sites reside in kindlin3 PH domain and RACK1 blade 5-7.

In order to further investigate the interaction between the PH domain of kindlin3 and RACK1 blade 5-7, we attempted to express and purify kindlin3 PH domain in *E.coli* expression system. However the yield of kindlin3 PH domain was low possibly due to degradation. To circumvent this problem, kindlin3 PH domain with it flanking F2 sub-domains (6His-kindlin3 PH-F2) were constructed and expressed in an *E.coli* system. As shown in **Figure 4.5 C**, 6His-kindlin3 PH-F2 was pulled down by GST-RACK1 or GST-RACK1-blade-5-7, but not control GST. Taken together, all the data support the direct interaction between RACK1 and kindlin3 and RACK1 blade 5-7 and the PH-F2 domain of kindlin3 are involved in this interaction.

It has been reported that RACK1 binds to the integrin $\beta 2$ tail in a yeast two-hybrid system (Buensuceso et al., 2001). We therefore asked if integrin $\beta 2$ tail, RACK1 and kindlin3 could form a ternary complex. To test this hypothesis, N-terminal biotin-labeled full-length wild-type $\beta 2$ or mutant $\beta 2$ N741A integrin tails were conjugated to streptavidin-agarose beads, which are used for pull-down assay with different amounts of purified recombinant kindlin3-6His or RACK1-6His (**Figure 4.5 D** top panel). It has been well characterized that kindlin3 bind to integrin β tails at the membrane distal NXXF/Y motif (Harburger et al., 2009; Manevich-Mendelson et al., 2009), hence $\beta 2$ N741A mutant was generated by replacing Asn of the membrane distal N741PKF motif in the $\beta 2$ tail with Ala. As shown in **Figure 4.5 D** middle panel, kindlin3-6His can associate with wild-type $\beta 2$ tail and the association was concentration dependent. On the other hand, the level of kindlin3-6His that associated with N741A $\beta 2$ tail was diminished. The amounts of RACK1 pulled down by wild-type $\beta 2$ tail or N741A $\beta 2$ tail were comparable, which indicates that the interaction of RACK1 with integrin $\beta 2$ tail is not dependent on the membrane distal N741PKF motif. This is consistent with

the results that RACK1 interacts with the membrane proximal sequence of the integrin β 2 tail (Liliental and Chang, 1998).

Next, we tested if integrin β 2 tail, kindlin3 and RACK1 can form a ternary complex. The integrin β 2 tail peptide-conjugated beads were incubated with RACK1-6His followed by different amount of kindlin3-6His (**Figure 4.5 D** bottom panel). The rationale is that if indeed a ternary complex were formed by β 2 tail, kindlin3 and RACK1, then the poor interaction between kindlin3 and β 2N741A will be rescued by RACK1 (**Figure 4.5** illustration). As shown in **Figure 4.5 D** middle panel, both wild-type integrin β 2 peptide conjugated beads and β 2N741 mutant conjugated beads can pull-down comparable amount of kindlin3 through 6His-RACK1, which indicate a ternary complex was formed and RACK1 served as the bridging molecule for kindlin3 and integrin β 2N741. Taken together, integrin β 2 tail, kindlin3 and RACK1 can form a ternary complex.

Figure 4.5 PH domain of kindlin3 (K3) and blade 5-7 of RACK1 play important roles in kindlin3-RACK1 interactions. (A) Recombinant GST and GST-RACK1 were immobilized on Glutathione S-sepharose beads and pull down assay performed with recombinant K3-6His and its truncated proteins. (B and C) K3-6His or 6His-K3 PH-F2 were immobilized on Ni-NTA beads and pulled down with purified GST, GST-RACK1 or GST-RACK1 W5-7. In all A, B and C, GST-tagged proteins were analyzed by coomassie staining while the kindlin3 and its truncated proteins were detected by immunoblotting with relevant antibodies. (D) N-terminal biotin labeled wild-type integrin β2 tail or β2 N741A tail-peptide that were bound to streptavidin-agarose beads were used to perform pull-down assays with recombinant K3-6His

and 6His-RACK1. The wild-type $\beta 2$ or $\beta 2$ N741A tail-peptide bound to streptavidin-agarose beads were detected with commassie staining. Top panel, K3-6His or 6His-RACK1 that was associated with wild-type $\beta 2$ or $\beta 2$ N741A tail-peptide. Middle panel, an illustration of the ternary complex formation of integrin $\beta 2$ tail, kindlin3 and RACK1. A ternary complex of integrin $\beta 2$ N741A, kindlin3 and RACK1 can still form due to the interactions between kindlin3 and RACK1. Dotted line denotes interaction between proteins. Bottom panel, K3-6His that was associated with wild-type $\beta 2$ or $\beta 2$ N741A integrin tail-peptide in the presence of 6His-RACK1.

4.5 Interaction study of kindlin3 and RACK1 in T cells

The interaction between kindlin3 and RACK1 was further examined in SKW3 cells by with co-immunoprecipitation assays. SKW3 is a human T lymphoma cell line and it expressed kindlin3 and RACK1 endogenously (Shima et al., 1986). SKW3 cells were collected and resuspended in cell culture medium and seeded to empty or ICAM-1-coated on microtitre wells. After incubation for 30 min at 37°C, cells were collected, washed with PBS and lysed with lysis buffer on ice. Clone 9 was used to precipitate endogenous kindlin3 from the SKW3 cell lysate. Immunoblottings of precipitated kindlin3 and co-precipitated RACK1 were performed using clone 229A and anti-RACK1 mAb. As shown in **Figure 4.6**, high level of RACK1 co-precipitated with kindlin3 from lysate of cells treated with SDF-1 α and adhering to immobilized ICAM-1, whereas lesser amount of RACK1 was detected in kindlin3 immunoprecipitates of cell treated with SDF-1 α alone. In both samples, integrin $\alpha L\beta 2$ could be detected in the kindlin3 immunoprecipitates.

Figure 4.6 Co-immunoprecipitation of endogenous kindlin3 and RACK1 in a T cell line. SKW3 human T cell line expressing endogenous kindlin3 and RACK1 were treated with SDF-1 α and seeded into empty or ICAM-1-coated microtitre wells. After incubation for 15 min, cells were lysed and subjected to co-immunoprecipitation analyses with kindlin3 mAb clone 9. The precipitated kindlin3 was detected by immunoblotting using anti-kindlin3 mAb clone 229A while RACK1 and integrin α L β 2 were detected with anti-RACK1 mAb (clone B-3) and anti- α L mAb (clone 27), respectively. Rat IgG was used as a control.

4.6 Localization of kindlin3 and RACK1 in T cells

Immunofluorescence microscopy was performed to study the distributions of kindlin3 and RACK1 in polarized T cells on ICAM-1. SKW3 cells were collected and resuspended in medium with SDF-1 α and seeded onto ICAM-1-coated dishes to adhere and become polarized. Actin and nuclei were stained with Alexa Fluor® 488-conjugated phalloidin and DAPI, respectively. MyH9 is a cytoplasmic protein that has been reported to localize at the rear of migrating T cells and therefore was included as control (Morin et al., 2008). As shown in **Figure 4.7**, kindlin3 and RACK1 both localized at the migrating front whereas MyH9 localized at the rear of the SKW3 cells. Representative intensity plots indicating the distributions of these proteins in migrating T cells are also shown (**Figure 4.7** insets). In immunofluorescence microscopy experiments, kindlin3 and RACK1 were observed to co-localize at the front of the migrating cells (**Figure 4.7** and **Figure 4.8**).

Figure 4.7 Localization of endogenous kindlin3, RACK1 and MyH9 in SKW3 cells. SKW3 cells were seeded into ICAM-1-coated glass-bottom dishes in the presence of SDF-1 α and incubated at 37°C for 15 min. Cells were fixed, permeabilized and incubated with kindlin3 mAb clone 9 (E), anti-RACK1 (A) or anti-MyH9 (I) followed by relevant secondary antibodies. Nucleus and F-actin were stained with DAPI (C, G and K) and Alexa Fluor® 488-conjugated phalloidin (B, F and J), respectively. Scale bar represents 20 μ m. Top panels, intensity plots of selected cells.

Figure 4.8 Co-localization of endogenous kindlin3 and RACK1 in SKW3 cells. SKW3 cells were resuspended in cell culture medium with SDF-1 α and seeded into ICAM-1-coated glass-bottom dishes. After incubation at 37°C for 15 min, cells were fixed, permeabilized and stained for kindlin3 and MyH9 (A) or kindlin3 and RACK1 (D). (B and E) Selected cells (*) are shown. (C and F) intensity plots of B and E. Scale bar represents 20 μm .

Next, human T lymphoblasts were used to perform the same experiment. T lymphoblasts from three different donors were used to perform the experiments to rule out inter-donor variations (**Figure 4.9-4.11**) and the results are in line with that observed in the SKW3 cell line with kindlin3 and RACK1 showing colocalization at the migratory front of the cells.

Figure 4.9 Co-localization of kindlin3 and RACK1 in human primary T lymphoblasts. Human T lymphoblasts were isolated and expanded as described in

material and methods. Cells were seeded into ICAM-1-coated glass-bottom dishes in the presence of SDF-1 α at 37°C for 15 min. Cells were fixed, permeabilized and stained for kindlin3 and MyH9 (A) or kindlin3 and RACK1 (D). (B and E) Magnified images of representative cells are shown (*). (C and F) Intensity plots of B and E. Scale bar represents 10 μ m.

Figure 4.10 Co-localization of kindlin3 and RACK1 in human primary T lymphoblasts. Human T lymphoblasts were seeded into ICAM-1-coated glass-bottom dishes in the presence of SDF-1 α at 37°C for 15 min. Cells were fixed, permeabilized and stained for kindlin3 and MyH9 (A) or kindlin3 and RACK1 (D). (B and E) Magnified images of representative cells are shown (*). (C and F) Intensity plots of B and E. Scale bar represents 10 μ m.

Figure 4.11 Co-localization of kindlin3 and RACK1 in human primary T lymphoblasts. Human T lymphoblasts were seeded into ICAM-1-coated glass-bottom dishes in the presence of SDF-1 α at 37°C for 15 min. Cells were fixed, permeabilized and stained for kindlin3 and MyH9 (A) or kindlin3 and RACK1 (D). (B and E) Magnified images of representative cells were shown (*). (C and F) Intensity plots of B and E. Scale bar represents 20 μ m.

4.7 Discussion

In this study, we have shown by various methods a novel interaction between kindlin3 and RACK1, and the PH domain of kindlin3 is important for this interaction. We have also provided preliminary data showing kindlin3, RACK1, and α L β 2 forming a ternary complex upon ligand-binding. However, more studies are needed to fully-characterize these interactions. It has already been shown that kindlin3 plays an important role as a co-activating factor alongside talin 1 in integrin activation (Moser et al., 2008) and helps to convert the closed headpiece of the integrin α L β 2 into an open one (Lefort et al., 2012). In this study, we further demonstrated that kindlin3 could also be important for the recruitment of cytoplasmic molecule(s) in integrin outside-in signaling that promotes adhesion strengthening. This is critical for leukocytes outside-in signaling. Based on our preliminary findings, we hypothesize that the binding of kindlin3 to α L β 2 not only stabilizes the conformation of activated α L β 2, but also induces intracellular signaling involving RACK1, which serve as a nexus for subsequent signaling cascades that impinge on cytoskeletal remodeling.

We also planned to conduct a number of future experiments. First, it is not known which WD40 repeat of RACK1 interacts with kindlin3. Thus, subsequent mapping studies will be performed to address this question. Data generated will be important because it would allow us to determine whether kindlin3 and other RACK1-associating proteins can concomitantly interact with RACK1 or the interaction is

sequential. Second, several lines of evidence have shown that the PTB fold in F3 domain of kindlins binds to the membrane distal NXXY/F motif in the β integrin tails (Harburger et al., 2009; Ma et al., 2008; Moser et al., 2008). It would be interesting and important to show the effect of disrupting this interaction during cell spreading and migration process. To this end, we will design small peptides that are cell permeable that disrupt the interaction between kindlin3 and the integrin β tails. Finally, we will also examine the role of kindlin3 in integrin α M β 2 mediated cell adhesion, phagocytosis and signaling. To this end, we will use either siRNA or cell permeable peptides aforementioned to examine the function of kindlin3 in monocytes that highly expresses integrin α M β 2.

Chapter 5 Investigating the role of kindlin3 in cell spreading initiation centers

5.1 Introduction

Recently, it has been shown that kindlin3 and kindlin2 are both expressed in Human Umbilical Vein Endothelial Cells (HUVEC) (Bialkowska et al., 2010). Although from the same protein family, kindlin2 and kindlin3 have different subcellular localizations. In fully spread HUVEC on fibronectin or vitronectin, kindlin3 was observed to localize to regions of extending membranes. Kindlin3 was noticeably absent from $\beta 3$ and $\beta 1$ integrins-containing mature focal adhesions. By contrast, kindlin2 was observed to associate dynamically with $\beta 3$ integrin during spreading and it was also observed in mature focal adhesion sites. Kindlin3 plays a role in integrin-mediated adhesion of endothelial cells that is distinct from that of kindlin2. The detailed mechanisms warrant further investigations.

5.2 Kindlin3 plays an important role in integrin $\alpha 5\beta 1$ outside-in signaling

K562 cells are human myelogenous leukemia cells that expressed integrin $\alpha 5\beta 1$. Therefore they were used in part of the study to investigate the role of kindlin3 in regulating integrin $\alpha 5\beta 1$ functions. The expression levels of integrin $\alpha 5\beta 1$ on control siRNA K562 cells and kindlin3-targeting-siRNA K562 cells were comparable as determined by flow cytometry (**Figure 5.1 A**). Binding assay was used to determine the adhesion profiles of the two K562 siRNA cells to fibronectin, which is the ligand for integrin $\alpha 5\beta 1$. As shown in **Figure 5.1 B**, although both control siRNA K562 and kindlin3-targeting siRNA K562 showed basal adhesion to fibronectin even without

Mn²⁺ (activating agent for $\alpha 5\beta 1$), kindlin3-targeting siRNA K562 cells showed a significant reduction in adhesion to fibronectin regardless the present of Mn²⁺. Adhesion to fibronectin is specifically mediated by integrin $\alpha 5\beta 1$ because the integrin $\alpha 5\beta 1$ function-blocking antibody abolished binding in both control siRNA K562 cells and kindlin3-targeting-siRNA K562 cells (**Figure 5.1 B**). Next, shear flow assay was used to assess the adhesion properties of these cells. The Ibidi® μ -slide | 0.4 Luer chambers were coated with fibronectin as described in Materials and Methods. Control siRNA K562 cells or kindlin3-targeting-siRNA K562 cells were perfused through the chamber at a constant shear stress of 0.3 dyn/cm². After a 5-min period of accumulation, the number of Control siRNA K562 cells firmly attached to fibronectin was determined to be 69.6 ± 0.5 cells with the present of Mn²⁺ or 42 ± 1.7 cells without Mn²⁺ (**Figure 5.1 C**). Kindlin3 knockdown in K562 cells reduced significantly the number of cells firmly arrested. Next, ECIS measurements were performed to analyze control siRNA K562 cells or kindlin3-targeting-siRNA K562 cells adhesion and spreading on fibronectin. Control siRNA K562 cells showed a marked increase in the cell index in the presence of Mn²⁺ whereas kindlin3-targeting-siRNA K562 cells showed almost basal level of cell index (**Figure 5.1 D**). Control siRNA K562 cells showed marginal increase in cell index without Mn²⁺ while kindlin3-targeting-siRNA K562 cells showed minimal increase in cell index, comparable to those cells treated with integrin $\alpha 5$ functional blocking mAb (anti-CD49e). Taken together, these data suggest that kindlin3 plays an important role in integrin $\alpha 5\beta 1$ -mediated adhesion and spreading on fibronectin.

Figure 5.1 Kindlin3 is involved in integrin $\alpha 5 \beta 1$ outside-in signaling. (A) The surface expression of integrin $\alpha 5 \beta 1$ on K562 cells was detected by staining with integrin $\alpha 5$ functional blocking mAb anti-CD49e, followed by flow cytometry. The expression level is presented as expression index EI. (B) Adhesion of control si-RNA K562 cells or kindlin3-targeting-si-RNA K562 cells on fibronectin. Adhesion specificity was demonstrated using the $\alpha 5$ function-blocking mAb CD49e. (C) The Ibidi® μ -slide | 0.4 Luer chambers were coated with fibronectin. Control si-RNA K562 cells or kindlin3-targeting-si-RNA K562 cells were collected and resuspended in shear flow buffer with or without Mn²⁺. Cells were perfused and passed through the chamber at 37°C at 0.3 dyn/cm². (D) ECIS measurements of control si-RNA K562 cells or kindlin3-targeting-si-RNA K562 cells on fibronectin. Adhesion and spreading specificity was demonstrated using the $\alpha 5$ function-blocking mAb anti-CD49e.

5.3 RACK1 interacts with kindlin3 but not kindlin2

Kindlin2 and kindlin3 are from the same protein family and they share 65% sequence identity to each other. On the other hand, it has been shown in the previous section that RACK1 interacts with kindlin3 but not kindlin2 in T cells. Here we further investigate the interaction between RACK1 and different isoforms of kindlin2 and kindlin3. 293T cells were transfected with HA-kindlin2S (HA-K2S), HA-kindlin2L (HA-K2L), HA-kindlin3S (HA-K3S) or HA-kindlin3L (HA-K3L). Ectopically expressed kindlin3 or kindlin2 were precipitated with anti-HA polyclonal antibody. Endogenous co-precipitated RACK1 was detected by immunoblotting with anti-RACK1 mAb. As shown in **Figure 5.2 A**, RACK1 co-precipitated with both HA-kindlin3S and HA-kindlin3L, but not HA-kindlin2S or HA-kindlin2L. This result prompted us to further evaluate the nature and specificity of the interaction between RACK1 and kindlin3. HUVECs are primary endothelial cells isolated from the vein of the umbilical cord. It has been shown that HUVEC express both kindlin2 and kindlin3 (Bialkowska et al., 2010) therefore it is an ideal cell system for this study. As shown in **Figure 5.2 B**, HUVECs expressed RACK1, kindlin2, integrin β 1 and kindlin3, albeit at a low level. The expression levels of FAK, 70kDa ribosomal protein S6 kinases (p70S6K), heterogeneous nuclear ribonucleoprotein K (hnRNP K) and actin were also determined.

Figure 5.2 RACK1 associates with kindlin3 but not kindlin2. (A) 293T cells were transfected with HA-kindlin2S (HA-K2S), HA-kindlin2L (HA-K2L), HA-kindlin3S (HA-K3S) and HA-kindlin3L (HA-K3L). The 293T transfectants were lysed and subjected to immunoprecipitation (IP) analyses with anti-HA antibody. Immunoblotting (IB) was used to detect endogenous RACK1. IgG: irrelevant rabbit IgG. (B) HUVEC were lysed with lysis buffer and subjected to SDS-PAGE followed with western blotting to assess the expression levels of FAK, p70S6K, RACK1, β 1, kindlin2, actin, kindlin3 and hnRNP K.

5.4 Localization of kindlin3 and RACK1 in spreading HUVEC

HUVEC were collected and resuspended in EBM-2 full medium and seeded onto fibronectin-coated dishes at 37°C for 15 min. Actin and nuclei were stained with Alexa Fluor® 594-conjugated phalloidin and DAPI, respectively. Kindlin3 and RACK1 were stained with clone 157D and B-3, respectively. As shown in **Figure 5.3 A**, kindlin3 localized to regions of membranes protrusions, which is consistent with published data from other group (Bialkowska et al., 2010). Importantly, it is interesting to observe that RACK1 also localized at the membrane protrusions (**Figure 5.3 B**). These data are consistent with our earlier findings that kindlin3 and RACK1 co-localized to lamellipodia of migrating T cells.

Figure 5.3 Localization of endogenous kindlin3 and RACK1 in spreading HUVEC. HUVEC were collected and seeded onto fibronectin-coated glass-bottom dishes at 37°C for 15 min. Cells were fixed, permeabilized and incubated with kindlin3 mAb clone 157D (A) or anti-RACK1 (B) followed by relevant secondary antibodies. Alexa Fluor® 594-conjugated phalloidin and DAPI were included to stain F-actin and nuclei, respectively. Scale bar represents 20 μ m.

5.5 Localization of FAK, kindlin2, kindlin3, RACK1, and hnRNP K in HUVEC on fibronectin.

FAK has been shown to be a leading edge organizer in the regulation of cell motility (Tomar and Schlaepfer, 2009). FAK co-localized with RACK1 at spreading initiation centers and nascent protrusive structures of mouse embryonic fibroblasts on ECM (Serrels et al., 2007). A complex of FAK, RACK1, and cAMP-specific 3',5'-cyclic phosphodiesterase 4D (PDE4D5) has been found to be a novel 'direction-sensing' complex that recruits second-messenger system to the leading edge of spreading cells (Serrels et al., 2010). These data prompted us to examine the localization of FAK in HUVEC on ECM. HUVEC were seeded onto fibronectin-coated glass bottom dish and incubate in a 37°C CO₂ incubator for 15 min. Cells were then stained with B-3, 157D and anti-kindlin2 followed with relevant secondary antibodies and DAPI. As shown in **Figure 5.4 A**, kindlin2, kindlin3 and RACK1 localized at the nascent adhesion sites of HUVEC. An enlarged view of a selected field of **Figure 5.4 A** was also shown in **Figure 5.4 B**. Next, HUVEC were stained for FAK, kindlin3 and RACK1 under the same condition. As shown in **Figure 5.5 A** and **Figure 5.5 B**, FAK, kindlin3 and RACK1 localized to nascent cell adhesion sites of HUVEC.

Ribosomes and translation initiation factors have been observed in integrin-enriched adhesion complexes in spreading MRC5 (fetal lung tissue cells line) on fibronectin and rat vitronectin (Willett et al., 2010). RNA binding proteins such as hnRNP K and hnRNP E1 have also been found to localized to these nascent adhesion complexes known as cell spreading initiation centers (de Hoog et al., 2004). Furthermore, RACK1 was found to be a constituent of the eukaryotic ribosome. Data from cryo-electron microscopy study showed that it docks to the 40S subunit in the vicinity of the

messenger RNA (mRNA) exit channel (Sengupta et al., 2004). Therefore it is interesting to examine if RNA binding proteins are localized with kindlin3 and RACK1 in HUVEC. As shown in **Figure 5.6 A** and **Figure 5.6 B**, hnRNP K co-localized with RACK1 and kindlin3 to the leading edge of spreading HUVEC on fibronectin.

We further examine the localization of kindlin2, kindlin3, RACK1, FAK and hnRNP K in fully spread HUVEC. HUVEC were collected, resuspended in cell culture medium and seeded onto fibronectin-coated dishes at 37°C for 45 min instead of 15 min. Cells were fixed, permeabilized and stained for kindlin3, RACK1 together with kindlin2, FAK and hnRNP K respectively. As shown in **Figure 5.4 C-D** and **Figure 5.5 C-D**, kindlin2 and FAK translocated to the mature focal adhesion site in fully spread HUVEC. In fully spread HUVEC, kindlin3 and RACK1 were less abundant at the edge of cells and they were enriched in cytoplasm. hnRNP K was also less prominent at the edge of cells and we observed hnRNP K localized to micro-tube-like structures in the cytoplasm (**Figure 5.6 C, D**). Taken together, FAK, RACK1, kindlin3, kindlin2 and hnRNP K were recruited to the leading edge of HUVEC spreading on fibronectin. However, once spread, FAK and kindlin2 localized to mature focal adhesion sites, while kindlin3, RACK1, and hnRNP K were enriched in cytoplasm.

Figure 5.4 Localization of kindlin2, kindlin3 and RACK1 in spreading HUVEC and fully spread HUVEC. HUVEC were resuspended in EBM-2 full medium and seeded into fibronectin-coated glass-bottom dishes at 37°C for 15 (A, B) min or 45 min (C, D). Cells were fixed, permeabilized and stained for kindlin3 and RACK1 together with kindlin2 (A), and (C). (B) and (D), Selected field of cells of (A) and (C), respectively were shown. Scale bar represents 20 μm.

Figure 5.5 Localization of FAK, kindlin3 and RACK1 in spreading HUVEC and fully spread HUVEC. HUVEC were resuspended in EBM-2 full medium and seeded into fibronectin-coated glass-bottom dishes at 37°C for 15 (A, B) min or 45 min (C, D). Cells were fixed, permeabilized and stained for kindlin3 and RACK1 together with FAK(A), and (C). (B) and (D), Selected field of cells of (A) and (C), respectively were shown. Scale bar represents 20 μ m.

Figure 5.6 Localization of hnRNP K, kindlin3 and RACK1 in spreading HUVEC and fully spread HUVEC. HUVEC were resuspended in EBM-2 full medium and seeded into fibronectin-coated glass-bottom dishes at 37°C for 15 (A, B) min or 45 min (C, D). Cells were fixed, permeabilized and stained for kindlin3 and RACK1 together with hnRNP K (A), and (C). (B) and (D), Selected field of cells of (A) and (C), respectively were shown. Scale bar represents 20 μ m.

5.6 Integrin $\alpha 5\beta 1$ mediated cell spreading leads to the phosphorylation of FAK and p70S6K

P70S6K is responsible for the phosphorylation of 40S ribosomal protein S6 and S17. It is involved in many cellular processes. These include protein synthesis, mRNA processing, cell growth and survival (Jefferies et al., 1997; Patel et al., 1996). We have shown that RNA binding protein hnRNP K localizes to nascent adhesion sites of HUVEC spreading on fibronectin which implies the involvement of mRNA processing and protein synthesis in this process. Here we examine if p70S6K and FAK are phosphorylated during HUVEC spreading. Equal number of HUVEC was collected, resuspended in cell culture medium, seeded onto fibronectin-coated dishes and incubated in CO₂ incubator for 15 min or 45 min. Cells were also seeded onto uncoated dishes as control. Cells were collected and lysed followed by western blotting with anti-p70S6K and anti-pFAK(Y397). Actin was used as loading control. As shown in **Figure 5.7 A and B**, both FAK and p70S6K were phosphorylated after HUVEC were seeded onto fibronectin for 15 or 45 min. Membranes were subsequently stripped and re-blotted for total FAK and p70S6K. These data suggest that HUVEC spreading on fibronectin leads to the phosphorylation of not only FAK, but also p70S6K.

Figure 5.7 The ligand engagement of integrin $\alpha 5 \beta 1$ leads to the phosphorylation of FAK and p70S6K. HUVEC were collected, resuspended in full EBM-2 medium. Equal number of HUVEC was seeded into un-coated or fibronectin-coated cell culture dishes for 15 or 45 min. Cells were collected and lysed in lysis buffer followed by western blotting using relevant antibodies. Actin serves as loading control.

5.7 Discussion

The adhesion of cells to ECM mediated by integrin is essential for many cellular or tissue responses including cell spreading, proliferation, differentiation, tissue remodeling, development, wound healing and tumor cell metastasis (Sastry and Burridge, 2000; Turner et al., 2001). In early stages of cell spreading, nascent adhesion sites termed as spreading initiation centers play important roles in establishing mature focal adhesions. A series of proteins including focal adhesion markers and RNA binding proteins have been shown to localize to spreading initiation centers (de Hoog et al., 2004; Serrels et al., 2010). In this study we confirmed the observation from other group that integrin co-activator kindlin3 is localized to the spreading initiation center during HUVEC spreading on fibronectin. We also showed that FAK, RACK1, kindlin2 and hnRNP K were involved and localized to spreading initiation center at the early stage of HUVEC spreading. When HUVEC were fully spread, FAK and kindlin2 translocated to the mature focal adhesion sites in HUVEC whereas kindlin3 and RACK1 were found mainly in the cytoplasm. We also showed that HUVEC spreading

on fibronectin leads to the phosphorylation of FAK and P70S6K.

RACK1 has shown to localize to enter cell spreading initiation center and interacts with FAK and PDE4D5 (Serrels et al., 2011; Serrels et al., 2010). We showed that RACK1 interacts specifically with kindlin3 long or short form but not kindlin2 long or short form in transfected 293T cells. In HUVECs, RACK1 showed similar distribution profile to kindlin3 but not kindlin2. We are still attempting to perform Co-ip in HUVECs that express much lower level of kindlin3 compared with kindlin2.

It has been reported that RACK1 localized to the head region of the 40S subunit (Sengupta et al., 2004) and may involve in mRNA processing and protein synthesis. The interaction of kindlin3 and RACK1 may provide a molecular bridge for RNA binding protein, ribosome and protein synthesis machinery components to translocate to the cell spreading initiation center. Next, we will examine the involvement of ribosome and mRNAs of adhesion related proteins in spreading initiation centers and their relations with RACK1 and kindlin3.

Chapter 6 Discussion

Since the discovery of integrins in 1980s (Plow et al., 1986; Pytela et al., 1986; Tamkun et al., 1986), there have been numerous studies that addressed integrin structure and function. Despite the burgeoning literature on integrins, recent studies have thrown up a few surprises concerning integrin activation and its regulation. Kindlins are a family of three paralogs (kindlin1, kindlin2 and kindlin3) that are found to be important co-activators with talin of integrins (Ye and Petrich, 2011). Like talins, kindlins are FERM domain-containing cytoplasmic proteins. The F3 subdomain in kindlins binds to the membrane distal NXXY/F motif that is highly conserved in integrin β cytoplasmic tails. Another NXXY/F motif that is membrane proximal is the binding site of talins. The importance of kindlins in integrin-mediated cell adhesion is underscored by the skin fragility disease Kindler syndrome and the immune compromised and bleeding disorder LAD III (Mory et al., 2008). Etiologies of these diseases are mutations that ablate the expressions of kindlin1 and kindlin3, respectively. Mouse studies have also shown that *KINDLIN2* gene ablation is embryonic lethal (Dowling et al., 2008).

Kindlins are not direct activators of integrins, rather they enhance talin-induced integrin-mediated adhesion (Kahner et al., 2012). It has been shown in neutrophils that talin induces integrin α L β 2 extension and an intermediate ligand-binding affinity state while kindlin3 promotes head-piece opening and a high ligand-binding affinity state (Lefort et al., 2012). Moser et al., proposed three working models for the synergistic effect of kindlins on talin-induced integrin activation (Moser et al., 2009a). The sequential model suggests that talin and kindlin binding to the integrin β cytoplasmic

tail are temporally separated. The *trans* co-operation model suggests that talin binds to one integrin β cytoplasmic tail whereas kindlin binds to another. The *cis* co-operation model places both talin and kindlin on the same integrin β cytoplasmic tail. A recent study has shown that talin head domain and kindlin2, which do not interact with each other, can bind to the same integrin $\beta 3$ cytoplasmic tail (Bledzka et al., 2012), supporting the *cis* co-operation model.

In addition to integrin activation, kindlins participate in integrin outside-in signaling. Kindlin1 has been reported to control the formation of integrin $\beta 1$ -mediated lamellipodia while kindlin2 knockdown abolished integrin $\beta 1$ -mediated cell adhesion and proliferation via Rac1, Akt and AP-1 (Has et al., 2009; Jung et al., 2011). Notably, kindlin2 has been reported to associate with β -catenin and enhances Wnt signaling (Yu et al., 2012). In this project, we examined the role of kindlin3 in integrin outside-in signaling. We generated mAbs to human kindlin3 using full-length recombinant kindlin3 as the immunogen. We identified and characterized four mAbs against kindlin3 with regards to their epitopes and their applications in western blotting, immunoprecipitation and immunofluorescence staining experiments. Some of these mAbs were used subsequently for functional studies of kindlin3.

We provide direct evidence that kindlin3 is required for integrin $\alpha L\beta 2$ and integrin $\alpha IIb\beta 3$ outside-in signaling by using constitutively activated $\alpha L\beta 2$ or $\alpha IIb\beta 3$ integrins. Despite expressing these activated integrins, transfected K562 cells failed to spread effectively on appropriate ligands when kindlin3 expression level was reduced. We also demonstrated that kindlin3 can induce integrin $\alpha L\beta 2$ micro-clustering, an important process that contributes significantly to the avidity of cell adhesion. The

mechanistic property of kindlin3 for inducing integrin α L β 2 micro-clustering is not known at present, but we hypothesize that the PH domain of kindlin3 could play an essential role based on the following observations. The PH domain of kindlin2 has been shown to bind to phosphatidylinositol 4,5-bisphosphate (PIP2) and phosphatidylinositol 3,4,5-trisphosphate (PIP3) (Liu et al., 2011; Qu et al., 2011)(Liu et al., 2011; Qu et al., 2011). Studies performed by another student from our lab show that kindlin3 binds PIP2 (data not shown). In addition, a poly-lysine-containing inserted loop in the F1 subdomain of kindlin1 could support its recruitment to plasma membrane by binding to the negatively-charged lipid headgroups (Bouaouina et al., 2012). Collectively, these observations suggest that kindlins could be localized to lipid microdomains thereby promoting integrin micro-clustering. A recent study reveals that kindlin3 is required for TCR-induced clustering of activated α L β 2 on ICAM-1 (Feigelson et al., 2011).

There is very little information on the proteins that kindlin3 interacts with other than integrins. In this project, we demonstrated for the first time that kindlin3 interacts with RACK1 using biochemical and cell-based systems. Our data provide the groundwork to understand how integrin-derived cell signaling can be transduced via kindlin3, which itself is not an enzyme. RACK1 is not an obscure molecule as it has been widely reported to function in various biological processes (Gibson, 2012) and a search for its interacting partners using the free online software STRING 9.0 retrieved at least 50 candidates, including integrins. The panoply of interacting partners with RACK1 stems from the fact that it has a seven-bladed propeller fold that potentially permits each blade to bind a distinct protein. Like kindlin3, RACK1 is not an enzyme, but its capacity as a scaffold protein allows recruitment of various signaling proteins,

including activated PKCs and SFKs. Perhaps the most important observation made is the localization of kindlin3 and RACK1 to lamellipodia of migrating T cells on ICAM-1. These data resonate with previous observations from others that RACK1 regulates cell protrusion and localize to leading edge of polarized immune cells (Chen et al., 2008; Cox et al., 2003). In fact, it has also been shown that RACK1 together with FAK and PDE4D5 play important role in directional sensing in cancer cells (Serrels et al., 2010). It is also interesting to note that RACK1/FAK/PDE4D5 signals to the small GTPase Rap1 (Serrels et al., 2011), which is well reported to regulate integrin activation (Carmona et al., 2009). Our data show that the binding sites of kindlin3 and RACK1 are the PH domain and its flanking regions of kindlin3 and blade 5-7 of RACK1. We were unable to narrow down the blade(s) of RACK1 that associates with kindlin3 due to poor expression and solubility of individual RACK1 blade. Our data suggest that association of kindlin3 with RACK1 permits additional molecules to dock onto RACK1, for example PKC β II that binds to blade 3 of RACK1 (McCahill et al., 2002). We have shown by immunoprecipitation and pull-down assays that kindlin3, RACK1 and integrin α L β 2 can form a ternary complex. It is therefore tempting to speculate that this association may provide a mechanism by which FAK and PDE4D5 are recruited to the leading edge of migrating cells. Consequently, this promotes further Rap1-induced integrin activation that reinforces nascent adhesion sites. At present, we do not exclude the possibility that kindlin3 associating with RACK1 in the absence of integrins as we do not have data addressing the sequence of events.

Another noteworthy observation made in this project is the lack of interaction between kindlin2 and RACK1. Kindlin3 and kindlin2 share 65% sequence identity while kindlin3 is 59% identical to kindlin1. Our observation may not be surprising given that

there is gaining evidence supporting kindlins having unique functions despite their similarities. In keratinocytes, both kindlin1 and kindlin2 associate with $\beta 1$ integrins, but only kindlin1 associates with integrin $\alpha V\beta 6$, and it was shown that defective kindlin2 impaired keratinocyte spreading despite an intact kindlin1 expression (Bandyopadhyay et al., 2012). In HUVECs spreading on integrin ligands, kindlin2 but not kindlin3 localizes to mature focal adhesion sites (Bialkowska et al., 2010). Further, nuclear localizations of kindlin1 and kindlin2, but not kindlin3, have been reported (Bialkowska et al., 2010; Lai-Cheong et al., 2010). In the last part of this project, we verified that the localizations of kindlin3 and kindlin2 are markedly different in HUVECs spreading on fibronectin for 45 min. Kindlin2 and FAK showed punctate localizations to mature focal adhesion sites, whereas kindlin3 and RACK1 were absent from these structures. Kindlin3 and RACK1 were observed to localize primarily to the leading edge of spreading HUVEC, especially at the early time point of 15 min. This is in line with our previous observations that both molecules localize to lamellipodia of migrating T cells.

A similar observation was made on RACK1 in spreading MRC5 human lung fibroblasts and the authors proposed that RACK1 functions mainly in nascent adhesive contacts known as spreading initiation centers (SICs) (de Hoog et al., 2004). Perhaps what is most interesting about this study is that the authors observed localization of RNA and RNA-binding proteins, for example hnRNPK, to these SICs where RACK1 is present, suggesting that SICs could be active compartments for protein translation. Adding confidence to this hypothesis is the fact that RACK1 has been shown to interact with the 40S ribosomal subunit (Rabl et al., 2011). Although the mechanism by which RNA-binding protein(s) are recruited to SICs is not known, it is possible that

RACK1 is involved. In our study, we show that kindlin3, RACK1 and hnRNPK localize to membrane protrusions in spreading HUVECs on fibronectin. The future direction of our study will be to investigate the role of kindlin3 in RNA-binding protein(s) recruitment to these nascent adhesion sites that are typically enriched in integrins. We will also clarify if RACK1 serves as the bridging molecule or RACK1 recruits RNA-binding proteins to these sites without contribution from kindlin3 since RACK1 has been reported to bind integrin β cytoplasmic tails (Buensuceso et al., 2001; Buensuceso et al., 2005; Liliental and Chang, 1998).

In conclusion, we have shown that kindlin3 plays an important role in outside-in signaling of integrins α L β 2, α IIb β 3 and α 5 β 1. Kindlin3 can associate with the scaffold protein RACK1, and formation of a ternary complex consisting of kindlin3, RACK1 and integrin β 2 cytoplasmic tail is demonstrated. We also provide preliminary observations suggesting that the association of kindlin3 and RACK1 may be important in nascent focal adhesion sites in HUVECs.

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