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INTEGRIN-INTERACTING PROTEINS IN HUMAN CANCER PROGRESSION

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To my family

"Hope forever tells us that tomorrow will be better…"

ABSTRACT

Integrins are the major transmembrane receptors for the extracellular matrix (ECM) which regulate a diverse array of cellular functions crucial to tumor cell migration, invasion, proliferation and survival. Integrins contact the ECM via their N-terminal extracellular domains and connect to the intracellular environment via the C-terminal cytoplasmic domains. Therefore, studies on the integrins cytoplasmic domain binding proteins will help to better understand the mechanisms of tumor progression and make them appealing targets for cancer therapy. Kindlin and PAK (p21-activated kinase) family proteins have been identified as integrin-interacting proteins. The following studies in this thesis aimed to investigate the role of Kindlin-2 and PAK5 in human cancer progression.

In paper I, we investigated the expression of Kindlin-2 in a series of malignant mesothelioma (MM) and found it to be highly expressed and correlated to tumor cell proliferation. To evaluate the biological relevance of Kindlin-2 in MM, we also evaluated ILK (integrin-linked kinase) and Kindlin-1 expression levels. Notably, in *vitro* depletion of Kindlin-2 impaired tumor cell adhesion and migration. Our findings provide new evidence that Kindlin-2 contributes to MM progression and may therefore be a potential target for anti-cancer therapy in MM.

In paper II, we demonstrated a novel role of Kindlin-2 as a signaling molecule that controls a Wnt-paralleling signaling pathway. We showed that Kindlin-2 specifically activates small GTPase Cdc42, but not Rac1 and RhoA, and regulates β-catenin activation via a Cdc42 -PAR 6 -PKCζ -GSK-3β cascade. Overexpression of Kindlin-2 in zebrafish embryo xenograft promotes tumor growth, invasion and dissemination. Importantly, overexpression of Kindlin-2 correlates to a poor prognosis in malignant mesothelioma patients, suggesting an important role of Kindlin-2 in cancer progression. Our data indicates that Kindlin-2 controls a signaling pathway that regulates tumor cell invasive growth.

In paper III, we used the human prostate cancer cell line PC-3 as a working model and to analyze the role of Kindlin-2 in cell cycle regulation by a loss-of-function approach. We found that depletion of Kindlin-2 causes mitotic arrest during metaphase, with cyclin B1 accumulation, mitotic spindle disruption, γ tubulin mislocation and abnormal chromosome formation. In addition, we demonstrated that Kindlin-2 is involved in Cdc42 mediated functions at metaphase. Our results identify a novel role of Kindlin-2 in the regulation of cell cycle progression in mitosis.

In paper IV, we showed that PAK5 was overexpressed in colorectal carcinoma (CRC) and associated with CRCs progression from adenoma to carcinoma. Overexpression of PAK5 also correlated to CRC development from lower Duke's grades to higher grades and correlated to CRC cell differentiation. Depletion of PAK5 reduced CRC cell adhesion but promoted their migration. Our study demonstrated that PAK5 expression correlates to CRC progression and that PAK5 promotes CRC metastasis by regulating CRC cell adhesion and migration.

Taken together, our studies highlight the importance of Kindlin-2 and PAK5 association with human cancer. This work also strengthens the link between Kindlin-2 and PAK5 expression and tumor malignancy in general, and therefore, promotes Kindlin-2 and PAK5 as novel putative targets for anti-cancer therapies.

LIST OF PUBLICATIONS

I. **Zhengwen An**, Katalin Dobra, John G. Lock, Staffan Strömblad, Anders Hjerpe and Hongquan Zhang.
Kindlin-2 is expressed in malignant mesothelioma and is required for tumor cell adhesion and migration.
Int. J. Cancer. 2010 Feb 2. [Epub ahead of print]

II. **Zhengwen An***, Yunling Wang*, Katalin Dobra, Samantha Lin Chiou Lee, Pegah Rouhi , Anders Hjerpe, Weigang Fang, Yihai Cao, Staffan Strömblad, & Hongquan Zhang.
A Wnt-paralleling Kindlin-2/β-catenin signaling pathway controls tumor invasion and dissemination.
Manuscript Submitted.
*the authors contributed equally to this study

III. **Zhengwen An**, Minna Thullberg, Staffan Strömblad and Hongquan Zhang.
Kindlin-2 is required for cell cycle progression in mitosis.
Manuscript Submitted.

IV. Wei Gong, **Zhengwen An**, Yunling Wang, Xinyan Pan, Weigang Fang, Bo Jiang and Honquan Zhang.
P21-activated kinase 5 is overexpressed during colorectal cancer progression and regulates colorectal carcinoma cell adhesion and migration.
Int. J. Cancer. 2009 Aug 1;125(3):548-55.

CONTENTS

LIST OF ABBREVIATIONS

APC	Adenomatosis polyposis coli
Cdc42	Cell division control protein 42 homolog
CRC	Colorectal carcinoma
CRIB	Cdc42/Rac interactive binding
DKK1	Dickkopf-related protein 1
ECM	Extracellular matrix
FA	Focal adhesion
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FERM	Four-point-one-protein, Ezrin, Radixin, Moesin
GSK	Glycogen synthase kinase
GST	Glutathione *S*-transferase
IBD	Integrin-binding domain
IF	Immunofluorescence
IHC	Immunohistochemistry
ILK	Integrin-linked kinase
IP	Immunoprecipitation
IPTG	Isopropyl-1-thio-*β*-D-galactopyranoside
KD	Kinase domain
KS	Kindler syndromes
MM	Malignant mesothelioma
MMP	Matrix metalloproteinases
MTs	Microtubules
PAK	P21-activated kinase
PAR6	Partitioning-defective 6
PBD	P21 GTPases-binding-domain
PC	Prostate Cancer
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PKC	Protein kinase C
PVDF	Polyvinylidene fluoride
shRNA	Short hairpins RNA
siRNA	Small interference RNA
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TCF/LEF	T-cell factor/lymphoid enhancer-binding factor
TGF-β	Transforming growth factor-β
TNM	Tumor-nodes-metastases
URP	Unc-112-related protein
Wnt	Wingless and Int
WST-1	Water-soluble tetrazolium-1
wt	Wild type

1 INTRODUCTION

1.1 INTEGRINS

Integrin family and structure

Mammalian cells interact with the ECM via a family of cell surface heterodimeric receptors known as integrins [1]. At least 24 distinct integrin heterodimers are formed by the combination of 18 α-subunits and 8 β-subunits [2, 3] (Fig. 1A). Integrin subunits have large extracellular domains (approximately 800 amino acids) that connect with specific ligands, a single transmembrane domain (approximately 20 amino acids), and short cytoplasmic tails [2] (13-70 amino acids, except that of β4) that bind to intracellular proteins (Fig. 1B).

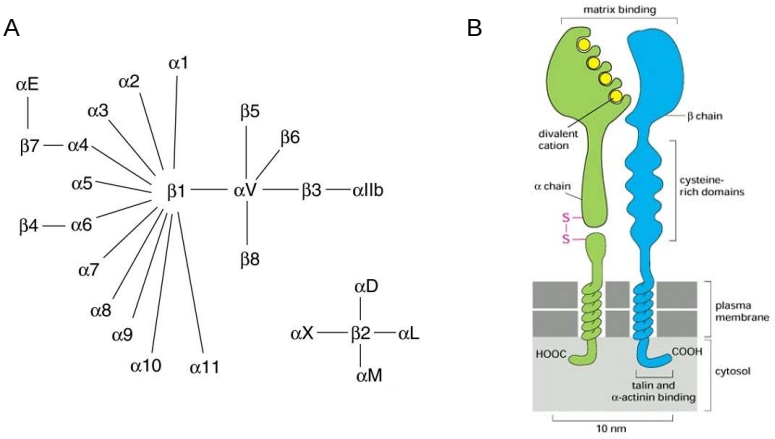

Figure 1. The members of the human integrin superfamily. (A) 18 α-subunits and 8 β-subunits generate 24 heterodimeric integrins. Each integrin has distinct ligand-binding specificity and tissue and cell distribution. (Takada et al., 2007 *Genome Biol*. Reprinted with permission from *BioMed Central*) (B) Schematic graph shown is heterodimeric integrin structure. By binding to a matrix protein outside the cell and to the actin cytoskeleton (via the attachment proteins talin and α-actinin) inside the cell, the protein serves as a transmembrane linker. (Alberts et al., 1994 *Molecular Biology of the Cell*. Reprinted with permission from *GARLAND SCIENCE-BOOKS*)

Although other transmembrane proteins also play a role in mediating ECM-cell communication (e.g., syndecan-4, selectins, immunoglobulins, cadherins, lymphocyte homing receptors), integrins provide the major functional link between the ECM and the intracellular compartments. Integrin signaling regulates diverse functions in tumor cells, including migration, invasion, proliferation and survival [4].

Integrin cytoplasmic tail binding proteins

The cytoplasmic tail of both α and β integrin subuits each make contributions to binding of integrin to intracellular kinases, adaptor proteins, and actin-binding proteins. The integrin α subunit binds to filamentous actin, caveolin, calreticulin, and paxillin, whilst more than 20 proteins are known to bind to one or more integrin β tails including

actin-binding proteins, signaling proteins and other proteins [5] (Table 1).

Table 1. Integrin cytoplasmic tail binding proteins (Liu et al., 2000 *J Cell Sci*. Reprinted with permission from *COMPANY OF BIOLOGISTS LTD*)

Binding partner	Integrin tail	Detection	Reference
Actin-binding protein			
Talin	β_{1A},β_{1D},β_2,β_3	COIP, PEP, EQ, INT, SLS	Horwitz et al., 1986; Knezevic et al., 1996; Pfaff et al., 1998; Goldmann, 2000
Filamin	β_{1A},β_2,β_3,β_7	COIP, PEP, 2HYB, SLS	Pavalko et al.,1989; Loo et al., 1998; Pfaff et al., 1998; Goldmann, 2000
α-actinin	β_{1A},β_2	PEP, INT, COIP, SLS	Otey et al., 1990; Pavalko et al., 1991; Cattelino et al., 1999
F-actin	α_2	PEP	Kieffer et al., 1995
Myosin	β_3	PEP, COIP	Jenkins et al., 1998; Sajid et al., 2000
Skelemin	β_1,β_3	2HYB, PEP	Reddy et al., 1998
Signaling protein			
ILK	β_1,β_3	2HYB, COIP	Hannigan et al., 1996
FAK	β_1,β_2,β_3	PEP, COIP	Schaller et al., 1995; Chen et al., 2000
Cytohesin-1	β_2	2HYB, COIP, PEP	Kolanus et al., 1996
Cytohesin-3	β_2	2HYB	Hmama et al., 1999
Other protein			
Paxillin	β_1,β_3,α_4	PEP, COIP	Schaller et al., 1995; Chen et al., 2000; Liu et al., 1999
Grb2	β_3	PEP	Law et al., 1996
Shc	β_3	PEP	Law et al., 1996
β3-endonexin	β_3	2HYB, INT, PEP	Shattil et al., 1995; Eigenthaler et al., 1997
TAP-20	β_5	PEP	Tang et al., 1999
CIB	α_{IIb}	2HYB, PEP, COIP	Naik et al., 1997; Shock et al., 1999; Valler et al., 199
Calreticulin	α	PEP, COIP	Rojiani et al., 1991; Leung-Hagesteijn et al., 1994; Coppolino et al., 1995
Caveolin-1	α	COIP	Wary et al., 1998
Rack1	β_1,β_2,β_5	2HYB, PEP, COIP	Liliental et al., 1998
WAIT-1	β_7	2HYB, PEP	Rietzler et al., 1998
JAB1	β_2	2HYB, PEP, COIP	Bianchi et al., 1998
Melusin	β_{1A},β_{1B},β_{1D}	2HYB, INT	Brancaccio et al., 1999
MIBP	β_{1A},β_{1D}	2HYB, PEP, COIP	Li et al., 1999
ICAP-1	β_{1A}	2HYB, PEP, INT	Chang et al., 1997; Zhang and Hemler, 1999
CD98	β_{1A},β_3	PEP	Zent et al., 2000
DRAL/FHL2	α_{5A},α_{3B},α_{7A},β	2HYB, PEP	Wixler et al., 2000

COIP--Coimmunoprecipitation; PEP--Synthetic/recombinant peptide studies; 2HYB--Yeast two-hybrid screen; INT--Binding to purified integrins; SLS--Static light scattering; EQ--Equilibrium gel filtration.

Table 2. Integrin β subunit binding proteins initially identified by our research group

Binding partner	Integrin tail	Detection	Reference
PAK4	β5	2HYB, COIP, PD	[6]
Myosin X	β1, β3, β5	2HYB, COIP, PD	[7]
Kindlins	β1, β2, β3	2HYB, COIP, PD	

2HYB: Yeast two-hybrid screen; COIP: Coimmunoprecipitation; PD: GST-pull down

Using aYeast two-hybrid screen and GST-pull down assays, we contributed to identify other novel integrin-interacting proteins in our research group a few years ago (Table 2). This thesis work is based on those previous findings, even though the identification of Kindlins as integrin-interacting and integrin activating proteins have been also shown by later studies during the past two years [8-15].

Isolated portions of the β integrin subunit cytoplasmic tail are sufficient to activate downstream signaling, including FAK activity, and can also regulate cell cycle progression and actin cytoskeleton assembly[16, 17] (Fig. 2). Therefore study on the integrins cytoplasmic domain binding proteins will help to better understand the biological function mediated by integrins.

Integrin activation

The affinity of integrin binding to extracellular lignads is tightly controlled by a spatial and temporal way, which is an important mechanism for the cells regulating integrin functions. This spatiotemporal control is brought about by rapid and reversible changes in the extracellular domains of the integrin, so-called integrin activation [18, 19].

Integrins are present and remain in a low-affinity binding state and that can be transformed to a high-affinity by the cellular stimulation, which is a key event that modifies cell adhesion [20, 21]. Binding of talin to integrin β cytoplasmic tails is a final step in integrin activation [22]. Talin is a critical integrin-activating protein, which is a FERM domain containing protein. FERM domains have three subdomains-F1,F2 and F3- and often mediate interactions with cytoplasmic tails of transmembrane proteins [23]. Kindlins are essential components of the integrin adhesion complex and recently have been identified as a co-activator of talin for integrin activation, thus Kindlins require talin, and talin is not sufficient to active integrins [14]. During the past, talin was considered as the only regulator of integrin activation, but it now shares the same importance with Kindlins [14].

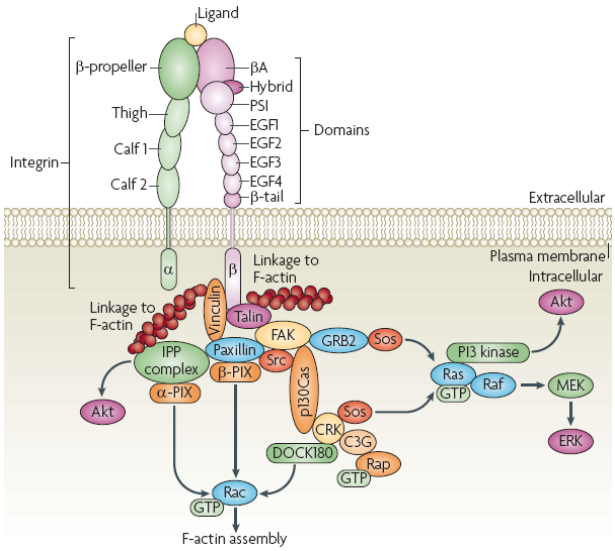

Figure 2. Integrin extracellular domains, transmembrane region and cytoplasmic domains. Integrin affinity for ligands is controlled by the binding of particular proteins (for example, talin) to the β-cytoplasmic domain, inducing conformational changes that activate ligand binding by the extracellular domains (inside-out signalling). Ligand binding triggers the propagation of extracellular conformational changes across the plasma membrane to the cytoplasmic domains, activating intracellular (outside-in) signaling. (Smith and Marshall, 2010 *Nat Rev Mol Cell Biol*. Reprinted with permission from *Nature Publishing Group*)

1.2 CELL ADHESION, MIGRATION AND INVASION

Cellular adhesion is the binding of a cell to a surface, ECM or adjacent cells by cell adhesion molecules such as selectins, integrins, and cadherins. The adhesive interaction between tumor cells, host cells or ECM plays a crucial role in cell migration, proliferation, differentiation, tissue organization and embryonic development [24]. Adhesion to the ECM is required for cell survival and growth, and influences both cell morphology and migration. Integrins are the principal cell surface adhesion receptors

mediating cell-matrix adhesion [25]. This attachment, mediated by integrins and adaptor proteins, provides both physical and regulatory links between the ECM and the cellular microfilament system [26]. Structural proteins provide a scaffold linking transmembrane ECM-binding integrins to the actin cytoskeleton, thereby anchoring cells to the substrate. The signaling proteins (kinases, phosphatases, exchange factors, etc.) respond to different environmental stimulus, and transmit signals to the intracellular environment. Numerous signaling pathways are activated by focal adhesion (FA) proteins, including those that control cell survival, division, differentiation, and migration [27, 28].

Cell migration is a crucial process in the development and maintenance of multicellular organisms. Tissue formation during embryonic development, wound healing and immune responses all require the well organized cell movement from one direction to the specific locations. Invasion is a hallmark of cancer characterized by cells breaking through the basement membrane from their origin in three-dimensional tissue, which causes a change in tissue structure and eventually leads to tissue destruction [29]. Cancer cells need to migrate and invade into surrounding tissues to metastasize and grow. Metastatic tumors spreading to different organs are the primary cause of death in cancer patients [30]. The ability to block the migratory and invasive capacity of tumor cells offers a new approach to treating patients with malignant diseases [30].

Cellular migration and invasion are controlled at both the extracellular and intracellular level by several factors. This process depends on the precise balanced dynamic interaction between cell and ECM. During cell migration, cells project lamellipodia that attach to the ECM, and simultaneously break the contacts of ECM and cells trailing edge. Cancer cell migration is typically regulated by integrins, matrix-degrading enzymes, cell-cell adhesion molecules and cell-cell communication [31]. Integrins are essential for cell migration and invasion, not only because they directly mediate adhesion to the ECM, but also because they regulate intracellular signaling pathways that control cytoskeletal organization, force generation and survival [30].

Reactivation of cell migration processes underlies invasion and metastasis of human cancers, making the study of morphogenic cell movements clinically relevant [32]. Understanding these processes will allow deeper understanding of the origin of form and how these mechanisms contribute to human disease. As disregulation of migration can cause diseases, an appreciation of the molecules involved in cell migration may also lead to novel therapeutic approaches aimed at the treatment and prevention of cancer invasion and metastasis.

1.3 KINDLINS

The Kindlin family

The Kindlin family consists of three members termed Kindlin-1,-2 and -3. They are structurally similar and evolutionarily conserved FERM-domain-containing proteins which represent a novel class of focal adhesion (FA) proteins. Recent studies highlighted their importance in integrin activation [8-10, 12, 13, 33-36]. The Kindlins are so named because of the association between Kindlin-1 mutations and Kindler

syndrome, an epidermal blistering/skin fragility syndrome [37, 38], which was used to describe a patient early in 1954 by Theresa Kindler [38].

Cellular and tissue localization of the Kindlins

The three Kindlins exhibit similar distributions within cells but distinct tissue distribution. Kindlin-1, also termed Unc-112-Related Protein 1(URP1), is localized predominantly to epithelial cells in skin and intestine [39], Kindlin-2/Mig-2[40, 41] is ubiquitously expressed, whilst Kindlin-3/URP2 expression is restricted to hematopoietic cells [42]. At the cellular level, Kindlin-1 and -2 are localized to FA [39, 41, 43], and Kindlin-3 is localized to podosomes [42] which are integrin-dependent adhesion sites found in hematopoietic cells. These patterns suggest that the Kindlins may have overlapping functions but exert their effects in a tissue and cell-dependent manner [9].

Kindlins structure

The Kindlin family consists of evolutionarily conserved and structurally related multi-domain proteins, which exhibit identical domain architecture and high sequence similarities. Kindlin-1 and -2 share 60% identity and 74% similarity, whilst Kindlin-3 shares 50% identity and 69% similarity to Kindlin-1 and 49% identity and 67% similarity to Kindlin-2 [42]. The amino acids of the Kindlins are organized into the same structure domains. A prominent structural of the Kindlinds is a FERM domain split by a PH domain (Fig. 3A). FERM domains are found in a number of proteins linking the membrane to the cytoskeleton [44]. Among these proteins, the FERM domain of talin is most homologous to the Kindlin FERM domain (Fig. 3B). Most of FERM-domain-containing proteins including talin have their FERM domains in the N-terminal region, but the Kindlins FERM domains are located at the C-terminus and split by a PH domain [45, 46]. The PH domain is known to mediate binding to phosphatidylinositol phosphates [47], the function and specificity of this domain have not been further identified in Kindlin proteins.

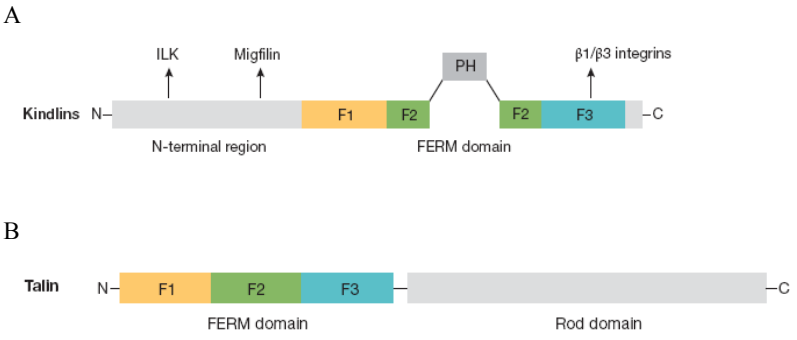

Figure 3. Schematic illustration is Kindlins and talin domain architecture. (A) Kindlins domain structure and binding partners. All members of the Kindlin protein family show identical domain architecture. Arrows indicate the regions of Kindlins that interact with β1-integrin and β3-integrin, ILK or migfilin. (B) Talin domain structure. (Larjave et al., 2008 *EMBO reports*. Reprinted with permission from *Nature Publishing Group*)

Kindlins do not contain catalytic domains and therefore their principle function is to mediate protein-protein interaction. So far, four Kindlin-binding proteins have been identified. They are ILK [43, 48, 49], migfilin [43, 50], and β1-integrin and β3-integrin [15, 45, 46, 49], and all of these proteins are components of cell-ECM adhesions.

Kindlins in focal adhesion (FA) and integrin activation

The first evidence that revealed Kindlins as a cell-ECM component was by Rogolski et al. [41], and studied later on by Tu et al. in 2003 [43]. Kindlin-1 association with the regulation of integrin function was first derived from studies on keratinocytes obtained from patients with Kindler syndromes (KS) [37, 39]. Deficiency of Kindlin-1 caused by Kindlin-1 mutation in keratinocytes impaired cell spreading, adhesion and migration. Futhermore, a decreased surface β1 integrin activation was detected in KS keratinocytes but total β1 integrin and talin expression levels remain unchaged [51]. In addition, Kindlin-1 deficient murine keratinocytes and intestinal epithelial cells display reduced β1 integrin activation [33]. Moreover, overexpression of mutant Kindlins with impared integrin binding ability in Chinese hamster ovary (CHO) cells also inhibits integrin activation. All these findings indicated the role of Kindlin-1 in integrin activation, but the exact mechanism remains to be further investigated.

Kindlin-2 needs integrin-linked kinase (ILK) [48] for its localization to FAs as well as recruitment of migfilin to FA sites [43]. Formation of this complex may also provide a link between the actin cytoskeleton and FA [43]. Kindlin-2 and actually all the Kindlins bind to the integrin β1 C-terminal NxxY motif [10]. Impairment of integrin binding show absence of Kindlin-2 localization at FA suggesting that integrin binding is required for Kindlin-2 localization [46]. Kindlin-2 is able to activate αIIβ3 integrin in CHO cells exogenously expressing this integrin [46]. Moreover, knock-down endogenous Kindlin-2 by siRNA in CHO cells makes integrins insensitive to talin overexpression [33]. These evidence suggest that both Kindlin and talin are required for integrin activation [14].

Kindlin-3 is found in integrin-containing podosomes of hematopoietic cells [42] and is emerging as a key molecule in the control of hemostasis and thrombosis [15, 39, 42, 52]. In homozygous Kindlin-3 $^{-/-}$ mice, several glycoproteins such as β1 integrin, β3 integrin, CD9, GPVI were found reduced expression levels implicating that Kindlin-3 has an undefined role in the expression of these proteins [15]. Flow cytometry analysis showed reduced binding of Kindlin-3 null platelets to fibrinogen and restored binding in the presence of manganese, suggesting that Kindlin-3 is essential for integrin activation [15]. It is believed that this process results from the direct interaction of the F3 subdomain of Kindlin-3 and the cytoplasmic tail of β1 and β3 integrins on platelets, independent of talin expression [15]. Collectively, these studies provide the distinct evidence that Kindlin-3 play a role in integrin activation.

The interrelationship between Kindlins and integrins depends in part on their direct interaction with the integrin β subunits cytoplasmic tails and in part on their interaction with other components assembled into these adhesion complexes. The association of Kindlins and adhesion complex are probably change dynamically in composition when cells attach, spread and migrate [9].

Kindlins and human diseases

Kindlin-1 mutation causing Kindler syndrome (KS) was first reported in 2003 by Jobard and Siegel respectively [37, 39]. KS is an autosomal recessive genodermatosis characterized by skin blistering, skin atrophy and varying degrees of photosensitivity [38, 53-55]. In addition, there is also the involvement in gingivitis, periodontitis and non-melanoma skin cancer especially squamous cell carcinomas [39, 53-57]. Patients with KS may have severe gastrointestinal symptoms, resembling ulcerative colitis [33, 58, 59]. To further address the role of intestinal Kindlin-1, Kindlin-1-deficient mice were generated and it showed marked shin atrophy as well as shortened and swollen terminal ileum and colon with strictures in the distal colon [33].

The first observation of Kindlin-1 association with cancer was in 2003 showing an increased Kindlin-1 mRNA expression in up to 60% and 70% of lung and colon cancers respectively [60]. Interestingly, TGF-β which is known to contribute to tumor invasion and cancer progression by increasing the motility of tumor cells, induces overexpression of Kindlin-1[45]. Although KS is rare, it appears that there is an increased risk of cancer under this condition, particularly of squamous cell carcinomas (SCC) [56]. So far, SCC have been reported in 5 individuals with KS, four of whom have mutation in the Kindlin-1 gene [54, 56, 61-64].

Kindlin-2-deficient mice were generated to gain insight into this protein functions. The results showed that knockout mice died at or before embryonic 7.5 days due to severe detachment of the epiblast and endoderm resulting in peri-implantation lethality [49, 65]. Furthermore, Kindlin-2-deficient embryonic stem cells (ESCs) demonstrated reduced compactness of stem cell colonies with decreased adhesion to various ECM substrates such as laminin-111, laminin-332 and fibronectin [49]. Kindlin-2 deficiency also resulted in disruption of cardiac and skeletal muscle development [65, 66].

Kindlin-2 may also play a role in carcinogenesis [67]. Gene expression profiling showed reduced or almost absent levels of Kindlin-2 were in various colonic carcinoma cell lines as well as in the HT-1080 cell line, a highly metastatic fibrosarcoma cell line [67]. Kindlin-2 shows variable expressions in human cancers and may regulate mesenchymal cancer invasion. To date, Kindlin-2 is the only Kindlin protein that has not yet been implicated in disease pathophysiology [68].

In addition to its role in β1and β3 integrin activation in platelets, Kindlin-3 recently has been implicated in β2 integrin activation in leukocyte adhesion but not rolling [36]. Kindlin-3 is also expressed in red blood cells [52] and may play a role in the maintenance of the membrane skeleton of erythrocytes [69].

Kindlin-3 pathogenic mutations resulting in the rare autosomal recessive leukocyte adhesion deficiency syndrome-III (LAD-III) were reported in recent studies [12, 13, 70, 71]. It implies that Kindlin-3 might be a suitable target for anti-thrombotic protection in certain individuals with pro-thrombotic diseases or traits.

1.4 RHO GTPASES: CDC42

The family of Rho GTPase

Rho GTPase proteins were initially cloned on the basis of their similarity to the RAS oncogenes [72]. Mammalian Rho GTPases comprise a family of 20 intracellular signaling molecules, best documented for their important roles in regulating the actin cytoskeleton [73]. Most Rho GTPases switch between active GTP-bound form and inactive GDP-bound form, and the most studied Rho GTPases are RhoA, Rac1 and Cdc42. Apart from the role of Rho proteins in actin cytoskeleton regulation, it is clear that they also affect gene expression, cell proliferation and survival and these cellular functions are important in tumorigenesis.

To be able to invade the surrounding tissue, tumor cells need to alter their morphology to acquire this ability. It is clear that Rho GTPases are involved in the control of cell morphology and motility in untransformed cells [74]. Cdc42, Rac1 and RhoA are also involved in forming integrin-based cell-ECM contacts. Tumor cells response to mitogenic signals alteration and many studies have linked Rho family proteins to the deregulation of this process. Multiple pathways seem to link Rho family proteins to the control of cyclin D1 levels, indicating the role of Rho family proteins in cell cycle control [75-78]. Rho GTPases have also been implicated in both pro- and anti-apoptotic signaling, and in the apoptotic process itself [79].

Cdc42 and PAR6-PKCζ complex

Cdc42 has a conserved role in regulating cell polarity and the actin cytoskeleton in many eukaryotic organisms [73]. Cell polarization is the process by which a cell responds to an extracellular stimulus and produces a front and back of the cells. This is fundamental to many cellular processes, including migration, differentiation and morphogenesis. Cdc42 seems to function primarily through the polarity protein partitioning-defective-6 (PAR6) and thereby with atypical protein kinase C zeta (PKCζ) to induce polarity in several different animal models [80, 81]. For example, Cdc42 and the PAR complex (PAR6-PAR3-PKCζ) have been proposed to mediate the capture and stabilization of microtubules at the front of the cell and to orientate the Golgi and microtubules-organizing centre (MTOC) during the establishment of migratory polarity [82, 83]. Cdc42-dependent phosphorylation of GSK-3β occurs specifically at the leading edge of migrating cells, and induces the interaction of APC protein with the plus ends of microtubules [82]. Cdc42 and the PAR complex have been identified in a recent genome-wide screen for regulation of endocytic traffic, which indicates that this pathway could be important for targeting recycling endosomes to specific intracellular sites [84]. The same complex has also been implicated in numerous other polarity pathways, including asymmetric division, epithelial junction assembly and neuronal morphogenesis [85, 86].

1.5 P21-ACTIVATED KINASES (PAKS)

PAK family and structure

P21-activated kinases (PAKs) are a family of serine/threonine protein kinases, which are direct targets of the small GTPases Rac and Cdc42. The six members of PAK family are divided into two groups, Group I PAKs (PAK1-3), and Group II PAKs (PAK4-6), based on the structural and functional similarities. All PAKs consist of N-terminal GTPase-binding domain (PBD) and C-terminal kinase domain. Group I PAKs

additionally possess an autoinhibitory domain (PID) overlapping with the PBD [87] (Fig. 4A).

Group I PAKs are activated by extracellular signals through GTPase-dependent and GTPase-independent mechanisms, while the Group II PAKs are constitutively active [88, 89]. Group II PAKs are still able to bind GTP-Cdc42 and GTP-Rac, but this does not enhance their kinase activities [90, 91]. However, binding of Cdc42 and Rac may regulate localization of Group II PAKs and/or their interaction with other proteins [92]. PAKs have also been identified as integrin-interacting proteins with the identical amino acid region which is designated as the IBD (integrin-binding domain) present in all PAKs members [6] (Fig. 4B and C).

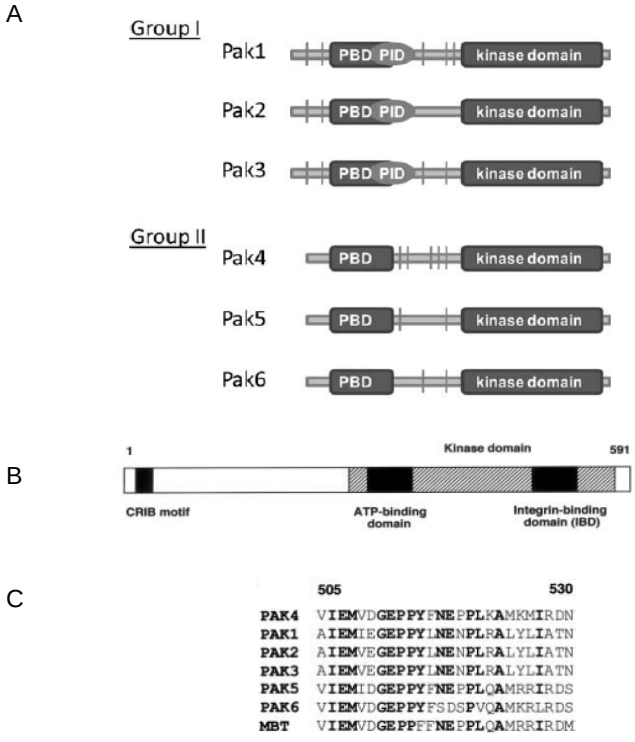

Figure 4. Schematic illustration of the PAKs domain structure. (A) All PAKs have p21 (Cdc42/Rac)-binding domain (PBD) and kinase domain. Group I PAKs additionally contain an autoinhibitory domain (PID) that is overlapping with the PBD (Dummler et al., 2009 *Cancer metastasis Rev*. Reprinted with permission from *Springer*). (B) PAKs have been also identified with integrin-binding domain (IBD) in the C-terminal region. (C) The corresponding sequences in other PAK family members are compared with PAK4 IBD with identical amino acids in bold (B). (© Zhang et al., 2002. *J. Cell Biol.* doi:10.1083/jcb.200207008. Reprinted with permission from *The Rockefeller University Press*)

PAKs signaling and human cancer

Dynamic changes in the cytoskeleton are necessary for cancer cell proliferation, survival and invasion to the surrounding tissue [92]. Small GTPases and their effectors, including PAKs are involved in the regulation of these processes (Fig. 5). PAKs are considered as key regulators of the actin cytoskeleton and motility. Besides the role of PAKs in cytoskeleton dynamics, PAKs recently have been shown to regulate various other cellular activities, including cell survival, mitosis and transcription. PAK1 appears to have a critical role during cell cycle progression, its kinase activity peaks at mitosis entry and maintains the activity levels during mitotic progression[93]. The ability of PAK to regulate the MAP kinase pathway may also contribute to cell proliferation [94-97]. PAK1 protects cell from intrinsic apoptotic signals via a PAK-Raf1-BAD pathway. PAK1 and PAK5 induce phosphorylation of Raf1 at Ser338 and stimulate translocation of a subpopulation of Raf1 to the mitochondria [98-100]. PAK1 also promotes cell survival by phosphorylating dynein light chain 1 (DLC1) and BimL [101]. PAK2 is the only protein kinase among the PAKs that has both pro- and anti-apoptotic functions [102-104].

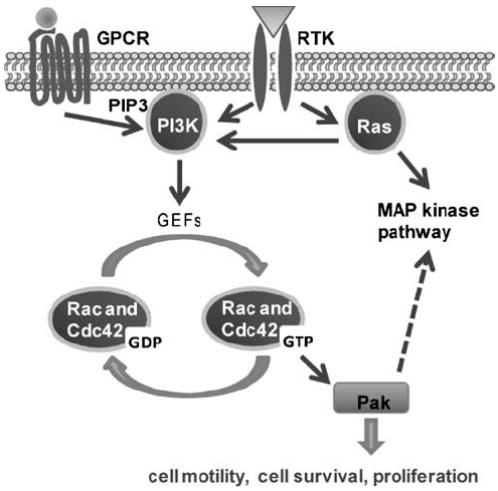

Figure 5. Schematic diagram of PAKs activation by the small GTPases Cdc42 and Rac. Signals from receptor tyrosine kinases, (e.g. insulin, EGF, PDGF and VEGF receptors) and G protein-coupled receptors lead to activation of PAKs via GTP-bound Cdc42 and Rac. Activated PAKs in turn initiates signaling cascades that culminate in the cellular response. In addition, activated PAKs potentiate activation of the MAP kinase pathway (Dummler et al., 2009 *Cancer metastasis Rev*. Reprinted with permission from *Springer*).

PAKs are evolutionally conserved and widely expressed in a variety of tissues and are either up-regulated or hyper-activated in multiple cancer types, such as breast, ovary, colorectal, thyroid and pancreatic cancers [105, 106]. Besides their role in cytoskeletal dynamics, PAKs have recently been found to be key regulators of cancer-cell signaling

networks [106] including motility, survival, mitosis, transcription and translation. Several distinct molecular mechanisms have been identified that cause aberrant PAKs signaling in cancers, including gene amplification and alteration of upstream regulators. Both PAK1 and PAK4 are localized to genomic regions, which are frequently amplified in cancers. PAK1 has been reported association with bladder, ovary and breast cancer progression [107-109]. PAK1 is overexpressed in human breast cancer and its expression levels increased in correlation with the progression stages in a series of MCF10A mammary epithelial cells suggesting a role for PAK1 in the early stages of cell transformation [110]. PAK4 gene amplification has been found in colorectal and pancreatic cancers [111-113]. PAK6 expression and hyperactivation have been reported in both primary and metastatic prostate cancer and tamoxifen resistant breast cancer cell lines and play a role in hormone signaling as well as being involved in hormone-dependent and -independent types of cancers [106, 114, 115].

PAK5 is structurally related to PAK4 and PAK6 in the Group II PAKs. PAK5 has different roles depending on its localization, such as activating the JNK kinase pathway in the cytosol and promoting survival signals in mitochondria [91, 116]. PAK5 protects cells from apoptosis by phosphorylation of Bad on Ser-112 and preventing its localization to mitochondria [100], but the function of PAK5 in cancer was still unknown. Recent study revealed that point-mutated PAK5 contributed to human neuroblastoma [116]. The importance of PAKs in cell and animal models of tumorigenesis and metastasis provides a principle for the development of PAKs inhibitors as anti-cancer therapeutics.

1.6 HUMAN CANCER

Cancer is characterized by a group of cells displaying *uncontrolled growth*, *invasion*, and sometimes *metastasis*. These three malignant properties of cancers differentiate them from benign tumors which are self-limited, and do not invade or metastasize. Tumorigenesis at the cellular level can either occur through a loss of function of tumor suppressor proteins that trigger cell apoptosis, or through a dominant gain-of-function of cell survival and/or cell proliferation signals, e.g. through overexpresion of oncogenes [117].

A decade ago, Robert A. Weinberg and Douglas Hanahan pointed out the six hallmarks of cancer which consist of novel capabilities acquired during tumor development: self-sufficiency in growth signals, insensitivity to growth inhibitory signals, evasion of programmed cell death or apoptosis, limitless replicative potential, sustained angiogenesis, and lastly, tissue invasion by metastasis [118].

Cancer is a complex disease with low incidence rate but high mortality. The improved understanding of molecular biology and cellular biology due to cancer research has led to a number of new, effective treatments for cancer since President Nixon declared "War on Cancer" in 1971. But the five/ten year survival rates in most cancers are still low and remain a challenge to cancer researchers. Discovery of novel tumor suppressor genes, mutations, molecular pathways and tumor markers will continuously contribute to overcome these diseases.

1.6.1 Malignant Mesothelioma (MM)

Malignant mesothelioma (MM) is an aggressive and invasive primary tumor of the pleura associated with asbestos exposure, the median survival ranging from 4-12 months [119-121]. Despite intense therapeutic efforts, average survival is only marginally improved. Several mechanisms have been associated with development of MMs such as chromosomal damage, interference with the mitotic spindle[122], free radical action[123], genetic susceptibility to asbestos-induced carcinogenesis[124] and activation of Wnt signaling[125] . Due to the poor response to therapy, new therapeutic methods based on an improved molecular understanding of MM are sorely needed.

1.6.2 Prostate Cancer (PC)

Prostate cancer (PC) represents a major health issue and its incidence is rising globally. PC is the most commonly diagnosed cancer and the second commonest cause of cancer related death in men in the Western countries [126]. Treatment options for patients with hormone-resistant PC are very limited and, even with toxic therapy, the life expectancy is only improved by a median of 2 months [127, 128]. Studies in molecular oncology have identified key signaling pathways that are considered to be impulsive events in prostate carcinogenesis. Many signaling pathways have been found to be important in prostate carcinogenesis and, in recent years, targeted therapy has emerged as a key focus for PC research [129]. Better understanding the signaling pathways involved in prostate carcinogenesis should lead to the development of a number of potential new drugs for anti-cancer therapies.

1.6.3 Colorectal Cancer (CRC)

Colorectal cancer (CRC) is the third leading cause of cancer-related death in Western countries. Its prognosis is closely related to the disease stage at the time of diagnosis [130]. CRC arises from a benign adenomatous polyp, which develops into an advanced adenoma with high grade dysplasia and then progresses to an invasive cancer [131]. Invasive cancers that are confined within the wall (TNM stages I and II) are curable, but if untreated they spread to regional lymph nodes (stages III) and then metastasize to distant sites (stages IV). Stages I, II and 73% of cases of stage III are curable by surgery combined with adjuvant chemotherapy, but stages IV is usually incurable [132]. Molecular studies have recently widened the opportunity for testing new possible markers. The challenges are to understand the molecular basis of an individual's susceptibility to CRC and to determine factors that initiate the development of the tumor and determine its reactivity to anti-tumor agents [133]. In future, a complete panel of clinical biomarkers are expected to use in every setting of CRC disease, and determine the prognostic significance by their expression [130].

1.7 ZEBRAFISH MODEL IN CANCER RESEARCH

The zebrafish has developed into an important model organism for biomedical research over the last decades. Originally the main focus was on developmental biology because of the advantages of zebrafish model in the laboratory such as large clutch size, transparent embryos and *ex utero* development of the embryo. Nowdays, zebrafish has been found to spontaneously develop almost any tumor type known from human, with similar morphology and comparable signaling pathway [134-136], which created its

own niche in cancer research. Different groups have been experimenting with transplantation of mammalian cancer cells into zebrafish embryos. This creates an *in vivo* system in which the advantages of cultured human cancer cells are combined with those of the transparent zebrafish embryos in which development can be followed [137]. Transplanted fluorescently labeled human metastatic melanoma cells into zebrafish blastula-stage embryos showed that these cells survive, migrate and divide [138, 139].

However the tumor incidences in zebrafish are generally lower and the onset is later as compared with the orthologous mouse models. Moreover, the zebrafish tumors developed from mammalian cancers do not compromise the organism as an animal model. Even though, it can nevertheless help to unravel mechanisms in carcinogenesis, complementary to the other models [140]. Although the area of cancer research in zebrafish is relatively young, it can still be expected to contribute to novel insights in tumor biology and cancer drug development.

1.8 WNT PATHWAY IN CANCER

Wnt pathway and cancer

Wnt (Wingless and int) proteins are a large family of secreted glycoproteins that activate signal transduction pathways which control a wide variety of cellular processes such as determination of cell fate, proliferation, migration, and polarity. The canonical Wnt pathway strictly controls the levels of a cytoplasmic protein known as β-catenin (Fig. 6), which has crucial roles in both cell adhesion and activation of Wnt target genes in the nucleus.

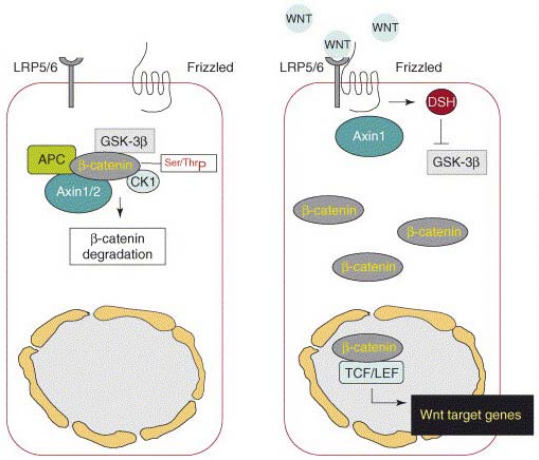

Figure 6. Schematic illustration of canonical Wnt/β-catenin signal transduction pathway. Left: in the absence of Wnt ligands, the destruction complex earmarks β-catenin for ubiquitination and proteolytic degradation by Ser-Thr phosphorylation. Right: in the presence of Wnt ligands, formation of the destruction complex is inhibited, resulting in the intracellular accumulation and nuclear translocation of β-catenin. (Fodde and Brabletz, 2007 *Curr Opin Cell Biol*. Reprinted with permission from *Elsevier*)

Wnt/β-catenin signaling was initially linked to cancer development when the adenomatous polyposis coli (APC) tumor suppressor was found to be mutated in inherited familial adenomatous polyposis (FAP) [141-143] and sporadic colorectal tumors [142, 144, 145]. Moreover, dysfunctional Wnt/β-catenin signaling has been implicated in a wide range of human cancers [146-148], including malignant melanomas [149], hepatocellular carcinomas [150, 151], ovarian carcinomas [152], Wilms' tumors [153], breast cancer [154] and prostate cancer[155]. Numerous studies suggest that activation of the Wnt/β-catenin signaling pathway plays an important role in human tumorigenesis [156-158]. The Wnt/β-catenin pathway is considered to be crucial not only for cancer initiation, but also for cancer progression [159]. The identification of many important regulatory genes and the mechanism of their function offer an opportunity to develop new anti-cancer therapies targeting this pathway.

Target genes in Wnt/β-catenin pathway

The key role of Wnt/β-catenin signaling pathway in all the cancers is the activation of target genes. More than 20 Wnt/β-catenin target genes have been identified, and many of them are regulators of cell proliferation, development control and genes involved in tumorigenesis, such as Axin-2, TCF, c-myc, cyclin D, MMPs, APC [160, 161] and many others.

β-catenin. The central task of Wnt signaling pathway is to stabilize the cytoplasmic β-catenin protein. The oncogenic role of β-catenin was highlighted by the discovery in which activating β-catenin mutations were detected in approximately 50% of the colorectal cancers that contained wild type APC [145]. In fact, the critical role of β-catenin in tumorigenesis has been demonstrated in a variety of animals models [162, 163], whereas mutations in the β-catenin gene have been frequently demonstrated in tumors induced by either carcinogens or activated oncogenes [157].

APC. The APC (adenomatosis polyposis coli) gene was first found as the genetic cause for familial adeomatous polyposis (FAP). It is the most well known dysfunctional gene in the Wnt/β-catenin pathway, and is mutated in 70% to 80% of sporadic colon tumors. The well established functions of APC tumor suppressor gene are considered as a gatekeeper in colorectal tumorigenesis [164].

GSK-3β. GSK-3β (Glycogen synthase kinase-3β) is a multifunctional serine/threonine kinase that participates in numerous signaling pathways involved in diverse physiological processes [165]. GSK-3β phosphorylates β-catenin and together with APC and axin consists of a complex that tightly regulates cytosolic levels of β-catenin. Wnt signaling inhibits the kinase activity of GSK-3β in this complex, leading to stabilization of β-catenin that can then move to the nucleus, where it is association with transcription factors of the TCF/LEF family induces Wnt target gene transcription [166].

Tcf/LEF. TCF/LEF (T-cell factor/lymphoid enhancer-binding factor) is a family of DNA-binding transcriptional modulators with DNA-binding activity in respective promoter regions of target genes. Target genes include c-myc and cyclin D1 [148, 167]. Initially, TCF/LEF proteins were identified as downstream effectors of Wnt/β-catenin signaling [168]. Dominant-negative LEF/TCF forms, able to bind to DNA but defective in interaction with β-catenin, are able to interfere with signaling mediated by Wnt or β-catenin [169].

Axin. The tumor suppressor Axin is an intracellular protein that binds to the APC/GSK-3β/CK1α complex and play a central role in regulating β-catenin degradation [170]. Hence, the loss of function of Axin results in elevated nuclear β-

catenin and consequently increases expression of the target genes such as cyclin D1 and c-myc [164].

Cyclin D1. Cyclin D1 is crucial for cell proliferation and tumorigenesis [75]. Evidence show that cyclin D1 is overexpressed in many colon carcinomas and inhibition of cyclin D1 expression causes growth arrest in colon carcinoma cell lines [171]. Cyclin D1 has been identified as a target gene for transcriptional activation by the β-catenin/TCF complex [160]. β-catenin activates the transcription of cyclin D1 through TCF-binding sites within the promoter. Expression of cyclin D1 is strongly dependent on β-catenin/TCF and has a direct effect on cell proliferation[160].

MMPs. MMPs (matrix metalloproteinases) consist of a family of 25 neutral Zn^{2+}-binding proteinases and play a role in multiple steps of tumor cell intravasation and extravasation, and the formation of distant metastasis [172, 173]. Because the Wnt signaling promotes the transcriptional activity of β-catenin, together with the evidence showed that several MMPs are transcriptional up-regulated by the β-catenin/LEF-TCF transcriptional complex, these data suggest that MMPs may be downstream targets of Wnt signaling and play a role in Wnt1-induced tumorigenesis [174].

The identification of many important regulatory genes and the mechanism of their functions offer an opportunity to develop new therapies targeting the Wnt pathway.

1.9 CELL CYCLE: MITOSIS

The cell cycle is a tightly controlled process divided into four distinct phases: G1 phase, S phase, G2 phase and mitosis (M phase) [175] (Fig. 7A). During the S and M phases, the cell replicates its genome and separates the duplicated genome over the two daughter cells respectively. Both phases are followed by a gap phase, designated G1 and G2 [176].

Mitosis is a process of cell division which results in the production of two daughter cells from a single parent cell. Mitosis exhibits the most apparent visual changes of cell morphology according to the chromatin condenses and the dynamic microtubule based spindle structure [177]. These processes can be followed directly by high-resolution microscopy. In a typical animal cell, mitosis can be divided into four principal stages that are defined largely by the organization and behavior of the chromosomes: prophase, metaphase, anaphase and telophase (Fig. 7B), and starts when cyclin B1 translocates into the nucleus and the chromatin condenses [178].

During *prophase* the chromosomes become progressively condensed inside the nucleus and microtubules become more dynamic [179]. Nuclear envelope breakdown marks the transition between prophase and prometaphase, during which the attachment of the microtubules to the chromosomes begins [180]. At *metaphase*, the bipolar microtubule-based spindle is attached to chromosomes [181]. All chromosomes are positioned at the spindle equator and aligned in the middle of the cell. Any abnormal organization of spindle microtubules might cause a cell not to be able to enter the next step of the cell cycle. At *anaphase*, new daughter chromosomes move poleward and the poles separate from each other. During the next stage, *telophase*, the chromosomes decondense as the nuclear envelopes reform around the two daughter nuclei. The cell is divided in two by cytokinesis, but the sister cells remain connected by a thin bridge termed the midbody.

Finally, abscission of midbody results in the complete separation of the two daughter cells [180].

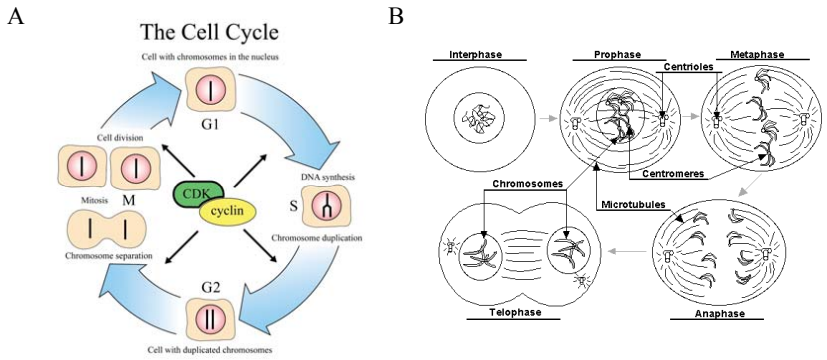

Figure 7. The different phases of the cell cycle (A). In the first phase (G1) the cell grows. When it has reached a certain size it enters the phase of DNA-synthesis (S) where the chromosomes are duplicated. At the next phase (G2) the cell prepares itself for division. During mitosis (M) the chromosomes are separated and segregated to the daughter cells, which thereby get exactly the same chromosome set up. The cells are then back in G1 and the cell cycle is completed (From *Key regulators of the cell cycle*. Reprinted with permission from *Nobel Web Team*). Schematic illustration of the stages of mitosis (B). Interphase: DNA is replicated. Prophase: duplicated chromosomes condense, the nuclear envelope dissolves, and centrioles divide and move to opposite ends of the cell. Metaphase: chromosomes line up at the equator of the cell. Anaphase: chromosomes begin to separate. Telophase: chromosomes migrate to opposite ends of the cell, two new nuclear envelopes form, and the chromosomes uncoil. (From *Answers Animal Cell Mitosis*. Reprinted with permission from *Enchanted Learning*)

The *spindle* is a microtubule-based structure that facilitates accurate chromosome segregation during mitosis and meiosis [182]. As a result, microtubules between the spindle poles are organized into an anti-parallel array, and microtubules outside of the spindle body form two radial asters that merge on the spindle poles [180] (Fig. 8).

During mitosis, the *centrosomes* are located at the spindle poles and are involved in formation of the bipolar spindle [183] (Fig.8). Centrosomes consist of a pair of cylindrical centrioles surrounded by pericentriolar material (PCM). PCM is formed by pericentrin and other structural proteins and the capability is to provide binding site for γ-tubulin [184-186]. γ-tubulin plays a key role in the nucleation of microtubules and assembly of the spindle [187].
Centromeres generally appear as constricted regions of mitotic chromosomes and serve as the foundations for the *kinetochores*, which are the transient structure assembled on the top of the centromeres just before and during the very early stages of mitosis [188]. The kinetochores are the sites where the spindle fibers attach. Kinetochores and the spindle apparatus are responsible for the movement of the two sister chromatids to opposite poles of dividing cell nucleus during anaphase (Fig. 8).

The pulling and pushing forces exerted by the mitotic spindle are fundamental for the chromosomes proper alignment at the metaphase plate and their subsequent segregation towards the spindle poles [183]. The forces creating tensions are required for silencing the spindle checkpoint and subsequent transition from metaphase to anaphase [189].

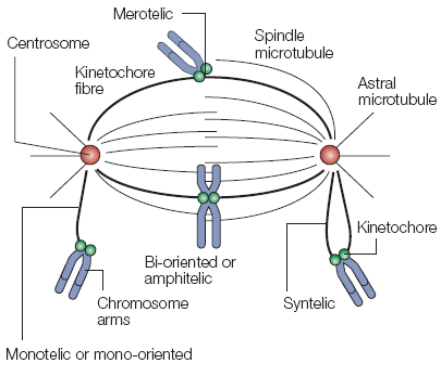

Figure 8. Schematic representation of a metaphase spindle with the centrosomes/spindle poles (red), the chromosomes (blue) and kinetochores (green). Spindle and astral microtubules are represented by thin black lines, whereas kinetochore fibres, which contain about 10 microtubules, are shown as thicker black lines. One correctly bi-oriented chromosome (lower centre), with the sister kinetochores attached to opposite poles, is shown, along with three mal-oriented chromosomes (upper, lower left, lower right). Monotelic/mono-oriented means that only one kinetochore is attached to one pole; merotelic means that one kinetochore is attached to both poles; amphitelic/bi-oriented means that kinetochores are attached to opposite poles; and syntelic means that both kinetochores are attached to the same pole. (Keen et al., 2004 *Nat Rev Cancer*. Reprinted with permission from *Nature Publishing Group*)

2 AIM OF THE STUDIES

The aim of this thesis is to investigate the role of novel integrin-interacting proteins in human cancer progression, therefore to better understand the functions and molecular mechanisms of these proteins in human cancers and to identify possible novel targets for anti-cancer therapies.

Specific aims:

Paper I: To investigate Kindlin-2 expression levels in MM and its cellular functions in MM cells and to explore possible association of Kindlin-2 with human cancers.

Paper II: To elucidate the potential mechanism of Kindlin-2 in controlling tumor cell migration and invasion. To further understand the phenotype changes in tumor cells caused by Kindlin-2 alteration.

Paper III: To determine the role of Kindlin-2 in tumor cell cycle progression and to uncover the underlying mechanism of Kindlin-2 in the regulation of tumor cell growth.

Paper IV: To evaluate the role of PAK5 expression in colorectal cancer.

3 COMMENTS ON METHODOLOGY

3.1 KINDLIN-2 CDNA CLONING, MUTANT GENERATION

Human Kindlin-2 full-length (amino acids 1-680) cDNA was cloned by polymerase chain reaction (PCR) from a prostate cancer cDNA library (Clontech) in the current investigation. Point and deletion mutants were generated by site-directed mutagenesis or gene deletion strategy using the QuickChange kit (Stratagene, CA, USA) and confirmed by DNA sequencing.

3.2 TRANSFECTION AND THE ESTABLISHMENT OF STABLE CLONES

In this work, we have used several transfection reagents for transient and stable transfection in different cells.
For *transient transfection with siRNA*, we used Oligofectamine Reagent according to the manufacturer's instruction. Cells were harvested at 48-72 h after transfection.
For *transient transfection with DNA*, we used Lipofectamine 2000 in COS-7, MCF-7 and PC-3 cells. FuGENE 6 transfection reagent was used for Hela, 293T, SW480 and HCT116 cells according to the manufactures instruction. Cells were harvested at 24-48 h post transfection.
For *stable clone*, PC-3 cells with stable expression of Kindlin-2 shRNA/control shRNA were generated by co-transfection of pcDNA3 and pSuper-Kindlin-2 shRNA/pSuper-control shRNA at a ratio of 1:20 using Lipofectamine 2000. Clones were selected by G418 (800 μg/ml) for 2 weeks. Mixed clones were maintained in RPMI medium with addition of G-418 (200 μg/ml), 10% FBS and 0.5% Gentamycin. The stable clones were grown with the presence of 200 μg/ml G418 for maintain drug selection.

3.3 RNAI

RNA interference (RNAi), an effective technique for regulating or silencing specific genes, can be applied to knock down particular genes of interest. Two types of small RNA molecules-microRNA (miRNA) and small interfering RNA (siRNA)-are central to RNA interference [190]. Since its discovery in 1998 [191], RNAi has emerged as a powerful tool for therapeutic gene silencing because of its unique specificity, broad applicability, and high efficiency [192]. Small RNAs (siRNA and shRNA) regulate gene expression by transcriptional and posttranscriptional gene-silencing mechanisms [193]. The effector RNA molecules of RNAi consist of about 20-30 nucleotides, which are complexed with the RNA-induced silencing complex to generate a cascade effect, causing sequence-specific messenger RNA cleavage or translation repression [194].

A *small hairpin RNA* or *short hairpin RNA (shRNA*) is a sequence of RNA that makes a tight hairpin turn that can be used to silence gene expression via RNA interference. The shRNA hairpin structure is cleaved by the cellular machinery into siRNA, which is then bound to the RNA-induced silencing complex (RISC). This complex binds to and cleaves mRNAs which match the siRNA that is bound to it.

Kindlin-2 siRNA was designed according to the human Kindlin-2 cDNA sequence, targeting to the region of nucleotides 325-343 counted from the start codon ATG, with the sense targeting sequence: AAGCUGGUGGAGAAACUCG synthesized by Qiagen.

An irrelevant chemically synthesized dsRNA with the sense sequence: CGAGUGGUCUAGUUGAGAA was used as control.

Kindlin-2 shRNA was designed according to the human Kindlin-2 cDNA sequence, targeting to the region of nucleotides 261-281 counted from the start codon ATG. A pair of 64-nucleotide complementary oligonucleotides was synthesized respectively with additions of a *Hind*III site at the 5′ end and a *Bgl*II site at the 3′ end, which allows to be cloned into pSuper vector[195]. The forward primer sequence was 5′-GATCCCC **AAGCTGGTGGAGAAACTCG** TTCAAGAGA**CGAGTTTCTCCACCAGCTT** TTTTTGGAAA-3′; the bold letters represent the Kindlin-2 shRNA sequence. The annealed 64-bp cDNA fragment with Kindlin-2 shRNA was cloned into the *Hin*dIII–*Bgl*II sites of the pSuper vector for producing shRNA in transfected cells. An irrelevant scrambled shRNA was described in the reference[7]

3.4 ADHESION AND MIGRATION ASSAY

Adhesion and migration are basic responses of living cells to environmental stimuli. It also contributes to pathological circumstances, including vascular and inflammatory diseases, as well as tumor growth and metastasis. These cellular responses depend on engagement of adhesion receptors by components of the extracellular matrix or molecules present on the surface of other cells. Hence, cell adhesion and migration assays are crucial methods in cell biology.

Cell adhesion assay using untreated 48-well plates coated with 10 μg/ml Collagen type I overnight at 4˚C. 1 % heat-denatured BSA was applied to block non-specific adhesion. Cells were seeded into triplicate wells at 2×10^4cells/well in cell adhesion buffer (RPMI 1640, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 0.2 mM $MnCl_2$ and 0.5 %BSA) and allowed to attach for 30 and 60 min at 37°C. After careful washing with adhesion buffer to remove unbound cells, cells were fixed with 2 % formaldehyde, followed by crystal violet staining to quantify the number of attached cells. Typically 18 microscopic fields were randomly chosen for analyses.

Cell migration assays were performed using Transwell chambers with 8.0 μm pore size. The lower surface of Transwell membranes were coated with Collagen type I (10μg/ml) overnight at 4°C. Cells were seeded on the upper surface of the Transwell membranes at 5×10^4 cells/well in migration buffer (RPMI 1640, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 0.2 mM $MnCl_2$ and 0.5 %BSA) at 37°C for 5h incubation. The Transwell membrane was then fixed with 2 % formaldehyde for 30 min and stained by crystal violet. 18 microscopic fields were randomly chosen for analyses.

3.5 CELL WOUND HEALING AND INVASION ASSAY

The wound-healing assay is simple, inexpensive, and one of the earliest developed methods to study directional cell migration *in vitro*. This method mimics cell migration during wound healing *in vivo*. The basic steps involve creating a "wound" in a cell monolayer, capturing the images at the beginning and at regular intervals during cell migration to close the wound, and comparing the images to quantify the migration rate of the cells. It is particularly suitable for studies on the effects of cell-matrix and cell-cell interactions on cell migration [196].

The ability of tumor cells to invade is one of the hallmarks of the metastatic phenotype. To elucidate the mechanisms by which tumor cells acquire an invasive phenotype, *in vitro* assays have been developed that mimic the *in vivo* process. The most commonly used *in vitro* invasion assay is a modified Boyden chamber assay using a basement membrane matrix preparation, Matrigel, as the matrix barrier and the conditioned media as the chemoattractant. The results obtained using this assay show a strong correlation between the ability of tumor cells to invade *in vitro* and their invasive behavior *in vivo*, which validates this assay as a measure of invasive potential [197].

Wound healing assays were performed 48 h after transfection, when the cells formed a confluent monolayer. A standard 100μl pipette tip was used to produce a wound approximately 400μm wide. The monolayers were then washed twice to remove non-adherent cells and the wound area was observed with a 20 × objective (Zeiss Axiovert s100, MRC, UK) after 12 h. Six randomly selected microscopic fields for each dish were observed and analyzed using Image J software.

Cell invasion were performed on Matrigel. After transfection with siRNA 72h, cells were serum-starved over night and plate onto the upper wells of the Modified Boydean Chamber coated with Matrigel. Cells pass through the membrane were counted. Cell invasion was plotted as number of cells/well that invaded through the membrane.

3.6 CELL PROLIFERATION ASSAY

There are several methods for cell proliferation assay. In this thesis, we used the water-soluble tetrazolium-1 (WST-1) cell proliferation assay. The WST-1 assay is based on the cleavage of the tetrazolium salt WST-1 by metabolically active cells. In our study, to determine the role of Kindlin-2 in cell growth, STAV-AB or MCF-7 cells were trypsinized and seeded into 96-well plates overnight at a density of 5000 cells /well after 8h transfection. The cell culture medium was then replaced with serum-free RPMI 1640 for 18h. Cells transfected with or without Kindlin-2 siRNA and overexpression of Kindlin-2 or control vector were measured for cell proliferation using a tetrazolium salt WST-1-based colorimetric assay (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1, 3-benzene disulfonate) at 24 h interval for 72 h. The resulting product was measured at wavelength of 440 nm with background subtraction at 650 nm using ELISA reader.

3.7 WESTERN BLOT

Cells were trypsinized and washed with PBS and lysed in PBSTDS lysis buffer containing 1X cocktail inhibitor. Total cell lysates were obtained by centrifugation at 13,000 rpm for 15 min at 4ºC. Equal amount of cell lysate was added to equal amount of SDS loading buffer, resolved by SDS-PAGE, blotted onto PVDF membranes (pore size 0.45 μm) and probed with the primary antibodies, followed by addition of the secondary antibodies. Finally, it visualized by enhanced chemiluminescence (Pierce).

3.8 CO-IMMUNOPRECIPITATION

Immunoprecipitation (IP) is the technique for precipitating a protein antigen out of solution using an antibody that specifically binds to that particular protein

Immunoprecipitation of intact protein complexes is known as co-immunoprecipitation (Co-IP). Co-IP works by selecting an antibody that targets a known protein that is believed to be a member of a larger complex of proteins. By targeting this known member with an antibody it may become possible to pull the entire protein complex out of solution and thereby identify unknown members of the complex. Co-IP is a powerful technique that is used regularly by molecular biologists to analyze protein-protein interactions.
In our studies, IP is described as before [6, 7]. Briefly, COS-7 cells were co-transfected with Flag-Kindlin-2 and c-myc-PAR6, and 500 μg pre-cleared cell lysates were used for co-IP using an anti-Flag tag mab or an anti-c-myc tag mab respectively. Precipitated c-myc-PAK6 or Flag-Kindlin-2 was probed by an anti-c-myc or an anti-Flag tag mabs.

3.9 GST-PULL DOWN ASSAY

The glutathione S-transferase (GST) pull-down assay is a relatively easy, straightforward method to identify potential protein-protein interaction *in vitro*. The pull-down method relies on the immobilization of a GST fusion protein on glutathione sepharose beads that serve as a solid phase. The first step requires the expression of GST-fusion protein. After binding of the GST fusion protein to the glutathione sepharose matrix, the mixture is incubated with a purified protein. Unbound material is washed off the column, and subsequently the binding complex is eluted. Upon elution, the mixture is resolved by SDS-PAGE and analyzed by Western blot.

To prepared GST-fusion protein, PAK-CRIB-domain was cloned into pGEX2TK (Pharmacia) at BamH1 and EcoR1 site and expressed in 0.1 mM isopropyl-1-thio-*β*-D-galactopyranoside (IPTG)-induced *E. coli* strain $BL_{21(DE3)}$. Harvested the bacteria and pellet was resuspended in bacterial lysis buffer After sonication and centrifugation, the supernatant was saved and incubated with 50 % glutathione Sepharose 4B beads slurry (Amersham, Pharmacia) for 30 min at 4°C and washed by lysis buffer. 150 μg total cell lysate (COS-7 cells with Kindlin-2 overexpression) or 800 μg total cell lysate (STAV-AB cells with knockdown endogenous Kindlin-2) were used for GST pull-down assay. Five μg purified GST-Cdc42 was used to pull down COS-7 expressed Flag-Kindlin-2 (upper panel) or c-myc-PAR6 (lower panel) (100 μg cell lysate for each pull-down), with GST as control. Flag-Kindlin-2 and c-myc-PAR6 were probed by an anti-Flag or an anti-c-myc tag mabs separately.

3.10 DUAL LUCIFERASE ASSAY

Dual luciferase assays were performed using Dual-luciferase Reporter Assay System.
The term “dual reporter” refers to the simultaneous expression and measurement of two individual reporter enzymes with a single system. In this assay, the activities of firefly and Renilla luciferases are measured sequentially from a single sample. The firefly luciferase reporter is measured first by adding Luciferase Assay Reagent II to generate a stabilized luminescent signal. After quantifying the firefly luminescence, the reaction is quenched, and the Renilla luciferase reaction is initiated by simultaneously adding Stop & Glo Reagent to the same tube and produces a stabilized signal from the Renilla luciferase. This method provides rapid quantification of both reporters in transfected cells. In our study, cells were lysed in 100 μl 1 × PLB (passive lysis buffer), incubated for 15 min at room temperature and 10 μl of cell lysate was transferred into

luminometer tubes containing 50 µl LAR using FB12 luminometer and its software (Berthold DETECTION SYSTEMS, USA). Firefly luciferase activity was measured first and then Renilla luciferase activity was measured after the addition of 50 µl of Stop & Glo Reagent.

3.11 IMMUNOHISTOCHEMISTRY

Immunohistochemistry (IHC) assays of tumor tissue were formalin-fixed and paraffin-embedded. After deparaffinization and hydration, tissue sections were treated in a microwave for antigen retrieval in 10 mM sodium citrate buffer, (PH 6.0) for 15 min. The staining was performed following the MSIP protocol using a DakoCytomation TechMate^{TM} Instruments. Non-specific binding was blocked by washing with 0.5 % BSA-TBST buffer, and endogenous peroxidase activity was abolished by ChemMate^{TM} Peroxidase-Blocking Solution. We used affinity-purified polyclonal anti-Kindlin-1, anti-Kindlin-2 and anti-PAK5 antibodies at 2 µg/ml, anti-ILK at 2 µg/ml (Santa Cruz), and Ki-67 (DAKO) at a 1:2000 dilution employed in this work. Reaction products were visualized via the streptavidin-biotin-peroxidase method using diaminobenzidine as the substrate-chromogen and with haematoxylin as counterstain.

3.12 IMMUNOFLUORESCENCE

Immunofluorescence (IF) is labeling of antibodies or antigens with fluorescent dyes. This technique is often used to visualize the subcellular distribution of biomolecules of interest. Immunofluorescent-labeled tissue sections or cultures are studied using a fluorescence microscope or by confocal microscopy.
Immunofluorescence was processed with 4 % PFA fixation for 10 min and 0.2 % Triton X-100 permeablization for 10 min, followed by 5 % Goat serum blocking at 4°C overnight. Primary antibodies were incubated at RT for 30 min followed by secondary antibodies for 45 min. Hoechst or DRAQ5 stained for 2-5 min for DNA detection, then coverslipped the slides for microscopy.

3.13 MICROSCOPY AND IMAGING

In this thesis, we used several microscopes for the images acquisition. All IHC images acquisition was carried out by ***Nikon microscope***, (ECLIPSE E1000, 10X, 20X and 40X dry objectives) with Nikon ACT-1 software.

The fluorescent images were captured by ***Olympus fluorescent microscope*** (Olympus 1X71 Inverted Microscope, plan Apo 60X/1.40 Oil/0.17 objective) and ***Confocal microscope*** (Zeiss LSM510, plan-Apochromat 63X/1.4 Oil DIC objective).The excitation for Alexa Flour 488 nm and Alexa Flour 568 nm used 488 and 543 argon lasers. DraQ5 used 633 He-Ne lasers.

The random cell motility and cell division was monitored using ***Time-lapse video microscope***, (Leica DMIRE2 Inverted Laboratory Microscope, USA).
For the random motility assay, STAV-AB cells were seeded in 35 mm glass bottom dishes (MatTek, USA) after 48 h post-transfection, and incubated at 37°C for 6 h before transferred to a Leica DMIRE2 Inverted Laboratory Microscope for imaging.

Cells were maintained during imaging in an incubator with humidified air (95 %) and 5 % CO_2 at 37°C. 10-12 observation fields were randomly selected and time-lapse imaging was performed every 15 min over 16 h using a 40 X dry objective. The obtained images were converted to video files using Leica DMIRE2 software. Cell tracking was followed by Image J software and a graph was created using Microsoft Excel. Cell spreading was measured by Image J software.
For monitoring cell division, PC-3 cells were grown in 6-well glass bottom dishes (MatTek, USA) and synchronized for 72 h by serum-free media starvation. After the cell cycle was released by serum for 20 h, the cells were transferred for imaging. Cells were maintained in an incubator apparatus with humidified air (95 %) and 5 % CO2 at 37°C during imaging. The images were taken every 15 min over 16 h using a 40 X dry objective. The obtained images were converted to video files using Leica DMIRE2 software.

3.14 FLOW CYTOMETRY

Flow cytometry is a technique for counting and examining microscopic particles, such as cells and chromosomes, by suspending them in a stream of fluid and passing them by an electronic detection apparatus. It allows simultaneous multiparametric analysis of the physical and/or chemical characteristics of up to thousands of particles per second.

In our study, cells were trypsinized and resuspended in PBS /0.5 % BSA, fixed in 70 % cold ethanol, and stained with Propidium Iodide. The cell cycle distribution was then analysed with a FACScalibur Flow Cytometer (BD, Biosciences) and CellQuest software (BD, Biosciences).

3.15 ZEBRAFISH MODEL

Fertilized Zebrafish eggs were incubated at 28°C in fish tank water. After two days post-fertilization, zebrafish embryos of the FTA pigment defective strain (fms-tra-albino strain) kindly provided by Tomasiewic (US). After 48 h post fertilization, embryos were dechorionated with help of a sharp tip forceps and anesthetized with 0.04 mg/ml of tricaine (Sigma). Anesthetized embryos were transferred on a modified agarose gel for microinjection[198]. Approximately 100 MCF-7 wild type or Kindlin-2-transfected human breast cancer cells resuspended in serum-free medium (DMEM, Sigma) of 10 nl were injected into each embryo using an Eppendorf microinjector (FemtoJet 5247 and Inject Man NI2 microinjection apparatus, Eppendorf, Hamburg Germany) equipped with Borosilicate Glass Capillaries (1 mm in diameter, World Presicion Instruments, Inc. USA). Prior to injection, tumor cells were labeled *in vitro* with 2 µg/ml 1, 1´-Dioctadecyl-3, 3, 3´, 3´-tetramethylindocarbocyanine perchlorate (DiI, Fluka, Germany). After injection, the fish embryos were immediately transferred into housing-keeping water containing a final concentration of 10 nM 17β estradiol. The injected embryos were kept in a 32^{o} C and examined daily for tumor growth and invasion using a fluorescent microscope (Nikon, Japan).

4 RESULTS AND DISCUSSION

4.1 PAPER I

The Kindlins recently have been recognized as the new members to the club of focal adhesion proteins [35] and function to regulate cell adhesion to the ECM via integrin signaling. Although Kindlin-1 and -3 have been reported to associate with human diseases [9, 34], no human diseases has yet been associated with Kindlin-2 gene pathology[68].

In this study, we are the first to demonstrate an association of Kindlin-2 expression with human cancer, malignant mesothelioma. Our study revealed high Kindlin-2 expression in MM cells in Western blot analyses and in tumor tissue by IHC. We estimated cell proliferation by applying Ki-67 with IHC in consecutive tumor section, and found a correlation between Kindlin-2 expression and cell proliferation in a tumor type-dependent manner.

Focal adhesion proteins play critical roles in cell motility, adhesion-dependent signaling and tumor progression and invasion [199-201]. Integrin-linked Kinase (ILK) is an integrin-interacting protein that binds to Kindlin-2 [43, 48, 49] regulates tumor growth, invasion and angiogenesis [202, 203]. Both Kindlin-2 and ILK are components of focal adhesion complexes. To evaluate the biological relevance of Kindlin-2 and ILK in MM, we did IHC on parallel sections of MM tumors and found that they have an identical staining pattern with stronger expression at the invasive front and weaker expression toward the center of the tumors. Notably, we also detected a similar subcellular distribution of Kindlin-2 and ILK in the nucleus indicating a role for Kindlin-2/ILK-complex in malignant transformation and tumor progression.

Kindlin-1 and -2 share an identical domain architecture and high sequence similarities. Both of them are widely expressed, however, the expression patterns are not identical [34]. Pleural adenocarcinoma metastases are always difficult to differentiate from MM. Although comparison with Kindlin-1 and -2 in 10 MMs and 10 pleural adenocarcioma metastases failed to demonstrate difference between the two tumor types because of the strong reactivity to Kindlin-2 and the weak to Kindlin-1 showed in both, the distinct expression patterns of Kindlin-1 and -2 indicate the different roles for these two Kindlin members.

To further investigate the role of Kindlin-2 in the cellular function, we knocked down endogenous Kindlin-2 with RNAi in MM cells. This changed the cell morphology and diminished cell spreading and adhesion. Cell migration assay were performed using Transwell chambers, together with the cell wound healing and random cell motility assay monitored by time-lapse microscope, our results provide a functional evidence for a role of Kindlin-2 in the promotion of cell motility and correlates well with our detection of Kindlin-2 overexpression at the invasion front of MM. Knocking down of Kindlin-2 does not change the ILK expression level, but transfection with active (S343D) ILK leads to a partial reversal of the decreased effects of cell adhesion and migration caused by Kindlin-2 depletion, indicating a cross-link between Kindlin-2 and ILK in the regulation of MM progression.

Overall, our findings provide new evidence that Kindlin-2 contributes to tumor progression and may therefore be a potential target for anti-cancer therapy for MM.

4.2 PAPER II

In the first paper, we demonstrated an association of Kindlin-2 expression with human cancer [204]. However, the mechanisms of Kindlin-2 in the regulation of human cancer progression are still perplexing.

In this work, we showed that high Kindlin-2 expression was correlated to poor prognosis in MM patients, which strengthened the role of Kindlin-2 in cancer progression. Importantly, we identified that Kindlin-2 as a novel signaling molecule that regulates β-catenin activation as examined by Western blot and TCF transcriptional assays.

Glycogen synthase kinase-3β (GSK-3β) is a key component of the canonical Wnt pathway, controlling β-catenin turnover [147, 205, 206]. Interestingly, knockdown of endogenous Kindlin-2 decreased GSK-3β-Ser9 phosphorylation, while using GSK-3β inhibitor LiCl could rescue the lowered β-catenin level caused by Kindlin-2 depletion. This suggests that Kindlin-2 signals to β-catenin via modulation of GSK-3β activation.
To test if Kindlin-2 controls β-catenin activation through integrin binding, we generated an integrin β1-binding deficient Kindlin-2 mutant (Kindlin-2-QW). We found that overexpression of Kindlin-2-QW inhibits GSK-3β activation and increased the β-catenin level to a similar level as that of wt Kindlin-2. Our results suggest that Kindlin-2 controls β-catenin activation via a signaling pathway independent of its integrin binding.

To identify additional signaling components acting between Kindlin-2 and β-catenin, we performed a GST-pull down assay and found that overexpression of Kindlin-2 activated Cdc42, but not Rac1 and RhoA. We then employed the dominant negative Cdc42-N17 mutant and found it could markedly block Kindlin-2-induced β-catenin activation. The results indicate a requirement of Cdc42 in Kindlin-2 regulation of β-catenin. We therefore looked for molecules downstream of Cdc42 that were involved in this process. It has been shown that Cdc42 interacts with PAR6-PKCξ complex and that regulates GSK-3β together with adenomatous polyposis coli (APC) [82, 207, 208], and controls β-catenin turnover [209]. Using purified GST-Cdc42 in GST-pull down assay, we identified an association of Cdc42 with both Kindlin-2 and PAR6. Furthermore, we demonstrated an association of Kindlin-2 and PAR6 by co-immunoprecipitation (Co-IP) experiments. We also detected activated PKCξ upon Kindlin-2 expression, while blocking PKCξ activation by Gö6983 or PKCξ pseudosubstrate inhibited β-catenin activation induced by Kindlin-2 expression. Taken together, we hypothesize that Kindlin-2 signals to β-catenin activation via Cdc42-PAR6-PKCξ-GSK-3β cascades.

To investigate the relationship between the Kindlin-2/ β-catenin pathway and the canonical Wnt pathway, we used Wnt3a and found it could rescue the β-catenin reduction effect caused by Kindlin-2 depletion. However, whilst blocking the Wnt

pathway by dickkopf 1 (DKK1), the β-catenin levels could be rescued by ectopically expressed Kindlin-2. The reciprocal rescue indicates that the Kindlin-2/ β-catenin pathway is an independent pathway that run parallel with the canonical Wnt pathway in tumor cells (Fig. 9).

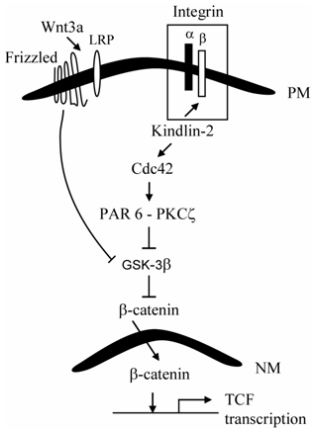

Figure 9. A working model of the Kindlin-2/β-catenin signaling pathway. Kindlin-2 activates Cdc42, and then Cdc42 binds to PAR6 followed by activation of PKCξ, which inhibits GSK-3β phosphorylation of β-catenin. Stabilized cytoplasmic β-catenin then translocates to the nucleus and initializes TCF transcription. This pathway parallels with the canonical Wnt/β-catenin pathway and they are cross-linked at GSK-3β. The black box shows that Kindlin-2 activates integrin as identified by recent findings.

In the Wnt/β-catenin pathway APC acts as a tumor suppressor and determines β-catenin turnover [210-212]. To address APC function in the Kindlin-2/β-catenin pathway, we employed APC non-functional colon cancer cells (CRC) SW480 and APC functional CRC HCT116 to examine the Axin-2 levels and TCF transcriptional activities. Similarly, ectopic expression of Kindlin-2 in SW480 and HCT116 cells both led to upregulated β-catenin and Axin-2 and also functionally activated TCF transcription. These finding suggest that the Kindlin-2/β-catenin pathway can override the APC function.

To further investigate the functional role of Kindlin-2 in tumor cells, we used loss-of-function and gain-of-function approaches. We found that Kindlin-2 regulates cyclin D1 and MMP13 levels examined by Western blot. Together with the data of cell proliferation assay and cell migration assay on Matrigel, our data indicates a role for Kindlin-2 in tumor cell proliferation and invasion. Moreover, overexpression of Cdc42-L61 rescued cell migration upon Kindlin-2 depletion and inhibition of PKCξ decreased the cell invasion upon ectopic expression of Kindlin-2. Our data suggest that Kindlin-2 controls tumor cell invasion through Cdc42 and PKCξ.

We used a zebrafish embryo model to examine the effects of Kindlin-2 overexpression *in vivo*. Consistently, we found that Kindlin-2 stably transfected tumor cells grew, invaded and metastasized greatly more than the vector-transfected tumor cells.

Collectively, our studies demonstrate a novel role of Kindlin-2 as a signaling molecule that regulates aWnt-parallel pathway independent of integrin binding and controls tumor progression.

4.3 PAPER III

In paper II, we discovered a novel role of the Kindlin-2/β-catenin pathway that controls tumor progression. We are also interested in the finding that loss of Kindlin-2 slowed down cell growth in a WST-1 cell proliferation assay and this was also obviously visible during normal cell culture. We therefore aimed to investigate the possible role of Kindlin-2 in cell cycle regulation.

We detected a high level of Kindlin-2 expression in human prostate cancer bone-metastasis cell line, PC-3, but not the soft tissue-metastasis cell line, LNCaP and DU145. To this end, we established cell clones in PC-3 cells with stable expression of shRNA against Kindlin-2 and performed the experiments by a loss-of-function approach. We analyzed cell cycle distribution by FACS and found a significant accumulation of cells in the G2 and/or M phases.

To further investigate at which stage that Kindlin-2 affects the cell cycle, we used cyclin A and cyclin B1 as indicators to distinguish the G2 or M phases. The results showed the accumulation of cyclin B1 in Kindlin-2 depletion cells while cyclin A level was unaffected, indicating that the cells were arrested in early mitosis. Using α-tubulin and DNA staining to analyze the microtubules (MTs) structure by IF, we detected that the cells were arrested in metaphase in particular. Time-lapse video microscopy also revealed that the cells need a long time to pass through the metaphase with the depletion of Kindlin-2.

Microtubules (MTs) organization is vital in bipolar spindle formation as in properly chromosome alignment [181]. Any abnormal organization of spindle MTs might cause metaphase arrest. To analyze the MTs structure, we stained cells with an antibody against α-tubulin and found a high frequency of multipolar spindles and several abnormal chromosome localizations in Kindlin-2 depleted cells as compared to control cells. Confocal microscope images demonstrated that Kindlin-2 was not co-localized with MTs structure by co-staining with antibodies against Kindlin-2 and α-tubulin respectively. Our data indicates that Kindlin-2 regulates MTs organization not through direct interaction with α-tubulin, but instead Kindlin-2 may act as an indirect signal.

The centrosomes are the primary microtubule-organization centers [213]. Two essential proteins, γ-tubulin and pericentrin are localized to the centrosomes [184]. Disruption of γ-tubulin expression *in vitro* activates spindle assembly checkpoint (SAC), and subsequently promotes spindle MTs defects and chromosome segregation errors [186, 214]. To evaluate the role of Kindlin-2 in centrosome organization, we analyzed γ-tubulin and pericentrin localization in control cells and Kindlin-2 depleted cells. Interestingly, we detected that depletion of Kindlin-2 impaired γ-tubulin localization to the centrosomes; whereas, pericentrin showed normal localization. We therefore examined the γ-tubulin protein level by Western blot and found that it was not affected upon Kindlin-2 expression. Our results show that Kindlin-2 interrupts γ-tubulin accumulation in the centrosomes but not protein expression. Together with the data showing the abnormalities of MTs spindle and chromosome localization caused by Kindlin-2 depletion, our data suggest that Kindlin-2 maybe involved in coordinating centrosome maturation and SAC activation in mitosis.

We then raised the question of how Kindlin-2 works at metaphase and regulates metaphase progression. It is known that the small GTPase Cdc42 works in metaphase by regulating bi-orient attachment of spindle MTs to chromosomes [215, 216]. Inhibition of Cdc42 activation leads to spindle MTs failure to bind to chromosomes in the correct bipolar manner, and chromosomes fail to congress at the metaphase plate [215]. Recent findings showed Kindlin-1 regulates Cdc42 activation in Keratinocytes [205]. We also demonstrated an association between Kindlin-2 and Cdc42 in a GST-pull down assay. Inhibition of Cdc42 activation by overexpression of dominant negative form mutant Cdc42-N17 showed a similar effect of increased cyclin B1 levels with Kindlin-2 depletion. By contrast, overexpression of constitutive active mutant Cdc42-L61 and a Kindlin-2 mutant resistant to Kindlin-2 shRNA could rescue the cyclin B1 accumulation and abnormality of spindle MTs caused by Kindlin-2 depletion. All together, our results suggest that Cdc42 is involved in the Kindlin-2 regulation of metaphase progression.

We conclude that depletion of Kinldin-2 induces metaphase arrest, thus providing a novel role of Kindlin-2 as a cell cycle regulator.

4.4 PAPER IV

This work is based on the previous finding in our group that p21-activated kinase 4 (PAK4) is a novel integrin-interacting protein identified by yeast two-hybrid assay, and it shares an identical sequence of the integrin-binding domain with the other PAK family members [6]. PAKs are serine/threonine kinase family proteins consisting of Group I PAKs (PAK1-3) and Group II PAKs (PAK4-6) functionally as small GTPase effectors that play important roles in cell motility, proliferation and survival [217-219].

In this work, we analyzed the expression of PAK5 in human colorectal carcinoma (CRC) and its cellular function in CRC cells.
We cloned the PAK5 N-terminal domain from the human PAK5 cDNA library and purified PAK5 protein after expression in *E.coli*. $BL_{21(DE3)}$. Using purified PAK5 protein, we generated a polyclonal antibody against PAK5. We detected the high expression of PAK5 in most of CRC cell lines but not primary normal colon epithelium cells. Interestingly, PAK5 was found to be particularly highly expressed in LoVo cells which derived from Duke's D than SW116 and SW480 cells which derived from Duke's A, suggesting an association with PAK5 expression and CRC progression.

Based on these findings, we set out to examine the expression of PAK5 in human CRC tumor tissues by IHC. Our results revealed that PAK5 expression was negative in normal colon mucosa and hyperplastic polyps, and weakly expressed in Peutz-Jeghers Syndrome (PJS) polyps and adenomas, but increased in expression from primary tumors and lymph node to liver-metastasized tumors. An increased expression of PAK5 was also observed with CRC progression from Dukes A to D and correlated with tumor differentiation as well. These data suggest that PAK5 is involved in CRC progression.

Overexpression of PAK5 triggered both filopodium formation and neurite development [91]. In addition, PAK5 was found to be shuttled from mitochondria to the nucleus

[100, 116]. In our studies, we found that PAK5 was localized not only in the cytoplasm and nucleus, but also at focal adhesion of HCT116 and SW480 cells. We then examined the cellular function of PAK5 in CRC cells. Using gain-of-function and loss-of-function approaches, we demonstrated that overexpression of PAK5 increased cell adhesion and decreased cell migration, indicating that PAK5 regulates integrin-mediated cell adhesion and migration in CRC cells.

Altogether, our data for the first time demonstrate a correlation between PAK5 expression and human CRC progression and the regulation of cell adhesion and migration by PAK5 in CRC cells.

5 CONCLUSIONS AND FUTURE PERSPECTIVES

In this thesis, we demonstrate the role of novel integrin-interacting proteins Kindlin-2 and PAK5 in human cancer progression.

In **paper I**:

1. Kindlin-2 is overexpressed in MM.
2. Kindlin-2 expression level correlates to cell proliferation.
3. Localization of Kindlin-2 and ILK at the invasive front and nucleus indicates the role of Kindlin-2 and its association proteins in MM progression.
4. Kindlin-2, but not Kindlin-1 highly expressed in pleural MM and pleural metastasized lung adenocarcinomas suggests the potential role of Kindlin-2 in tumor metastases.
5. Kindlin-2 regulates MM cell adhesion and migration.

In this study, we are the first to demonstrate an association of Kindlin-2 expression with human cancer progression. Our results indicate that heightened expression of Kindlin-2 contributes to tumor progression in MM, therefore suggesting that Kindlin-2 is a potential target for anti-cancer therapy in MM. This finding also strengthens a link between Kindlin-2 expression and tumor malignancy in general, creating new opportunities for future investigation.

In **paper II**:

1. Kindlin-2 expression correlates to a poor prognosis in MM patients.
2. Kindlin-2 as a novel signaling molecule activates Cdc42 and controls β-catenin stability via Cdc42-PAR6-PKCξ-GSK-3β cascade.
3. Kindlin-2 regulates β-catenin activation paralleling with the canonical Wnt pathway.
4. Kindlin-2/β-catenin pathway controls tumor cell proliferation, migration and invasion independent of its binding to integrin β1 tail and overrides APC function.
5. Kindlin-2 promotes tumor cell growth and dissemination in a zebrafish embryo model *in vivo*.

In this work, we reveal a novel role of Kindlin-2 as a signaling molecule that controls tumor invasion and dissemination through the Kindlin-2/β-catenin pathway that functionally and mechanistically parallels with the canonical Wnt pathway. From this work, we can also see several highlighted perspectives. Firstly, we identify Kindlin-2 not only as a focal adhesion molecule involved in integrin function, but also as a signaling molecule regulating the signaling pathway that controls tumor cell behavior. The investigation of the components of the signaling complex from Kindlin-2 to β-catenin will help us to better understand and then control the tumor malignancy. Secondly, because it functions parallel to the Wnt pathway, targeting the Kindlin-2/β-catenin pathway could be important for complete blockading tumor invasive growth and metastasis. Thirdly, Kindlin-2 expression correlates to MM patients' short survival time predicting the prognostic value of Kindlin-2 in MM. Fourthly, our studies on the zebrafish model provides a powerful system for rapid *in vivo* screening of anti-cancer

metastasis drugs. Overall, we present a novel integrin-binding independent function of Kindlin-2 that plays an important role in cancer progression, which may shed light on a therapeutic intervention for cancer.

In **paper III**:

1. Kindlin-2 is expressed in human prostate cancer cell line, PC-3 and its reduction causes delayed cell cycle progression.
2. Depletion of Kindlin-2 causes cell cycle arrest at metaphase.
3. Loss of Kindlin-2 induces MTs structural abnormality and chromosomes misalignment.
4. Kindlin-2 is required for γ-tubulin recruitment to the centrosome.
5. Cdc42 is involved in Kindlin-2 regulated metaphase progression.

We report here another novel role of Kindlin-2 as a cell cycle regulator working at metaphase to regulate cell cycle progression mediated by Cdc42. In our study, depletion of Kindlin-2 interrupts γ-tubulin recruitment to the centrosome suggesting a role of Kindlin-2 in coordinating centrosome maturation. Our data indicates γ-tubulin mislocation probably contributes to the abnormalities of MTs organization, chromosome misalignment and SAC activation, and consequently leads to metaphase arrest. Furthermore, loss of Kindlin-2 might activate SAC through inactivation of Cdc42. Thus it may provide an explanation for why the bipolar spindles in the absence of Kindlin-2 can neither align chromosomes efficiently nor generate sufficient tension to shut off the SAC. Nevertheless, how Kindlin-2 is involved in γ-tubulin localization raises interesting questions for future investigation. Uncovering the role of Kindlin-2 is likely to expand and sharpen our view on the versatility of Kindlin family proteins function, thereby, producing a great understanding the role of Kindlins proteins in cell biology and human diseases.

In **paper IV**:

1. PAK5 is highly expressed in human CRC cell lines.
2. Increased PAK5 expression correlates to CRC progression from normal colon mucosa to metastatic carcinoma with marked high levels in the invasive and metastatic CRCs.
3. PAK5 expression associates with CRC cell differentiation.
4. PAK5 regulates CRC cell adhesion and migration.

Our studies for the first time demonstrate that the increased PAK5 expression is associated with CRC progression and that PAK5 might promote CRC metastasis by regulating CRC cell adhesion and migration. Interestingly, we found that PAK5 localized not only in the cytoplasm and nucleus, but also in focal adhesions which implies a potential role of PAK5 in the regulation of integrin-mediated cellular function. However, the role of cytoplasmic and nuclear localized PAK5 in CRC cells will remain of interest for further elucidation.

The data presented in this thesis gained novel insights into the role of integrin-interacting proteins Kindlin-2 and PAK5 in human cancers. We establish a link between Kindlin-2 expression and human cancer progression. We then elucidate the molecular mechanisms of Kindlin-2 in controlling tumor cells behavior. We identify

that Kindlin-2 as a signaling molecule that regulates β-catenin activation and controls tumor invasion and dissemination through a pathway that runs parallel with the canonical Wnt pathway. The finding that Kindlin-2 regulated cell proliferation motivated us to further investigate the role of Kindlin-2 in cell cycle regulation. We conclude that Kindlin-2 is required for cell cycle progression in mitosis. As a following work of this thesis investigation, we have another interesting study demonstrating that downregulation of Kindlin-2 sensitizes prostate cancer cells to cisplatin-induced cell death (unpublished data). Finally, we evaluate PAK5 expression in CRC and find a correlation between PAK5 expression and CRC progression. Our studies highlight the role of novel integrin-interacting proteins Kindlin-2 and PAK5 in human cancer progression, which may lead to identification of potential targets for anti-cancer therapies.

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