Regulation of Focal Adhesion Dynamics by a Kinesin Motor Kid/KIF22/Kinesin-10

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Submitted to the Graduate Faculty of the

Kenneth P. Dietrich School of Arts and Sciences in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

University of Pittsburgh

2016

UNIVERSITY OF PITTSBURGH

Kenneth P. Dietrich School of Arts and Sciences

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Cell adhesion is one of the fundamental mechanisms cells use to interact with their surrounding environment. It is mediated by multiprotein structures termed focal adhesions (FAs). The formation of FAs and the regulation of their assembly and disassembly dynamics are crucial to cell structure and viability. In my dissertation study, I explored the role of a kinesin motor Kid/kinesin-10/KIF22 in adhesion dynamics. I found that Kid localizes to the sites of FAs and functions as an adhesion disassembly factor by modulating the phosphorylation of two important components of FAs, focal adhesion kinase (FAK) and paxillin. Loss of Kid caused changes in the density, distribution, and size of FAs leading to defects in cell adhesion and migration. This study discovered a novel function of Kid during interphase and expanded our knowledge on how motor proteins play significant roles in adhesion dynamics.

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1.0 INTRODUCTION

Cell adhesions provide an interface network that connects the scaffolding and signaling machinery inside the cell to the physical and chemical microenvironment surrounding the cell (Geiger et al. 2001). Among diverse adhesion receptors cells express, the integrin family is the most widely studied. They form transmembrane heterodimers that initiate the building of multi-protein adhesion structures, which connect cells to the extracellular matrix (ECM) or to their neighboring cells (Geiger and Yamada 2011). Such interactions provide both physical support and signaling activity to maintain cell structure, regulate cell behavior, and determine cell fate. Recent bioinformatics analyses have revealed the association of genes involved in cell adhesions with a variety of diseases including cancers, cardiovascular diseases, immune disorders, and neurological conditions (Winograd-Katz et al. 2014). Expanding the adhesion network, identifying new interactions between adhesion components, and uncovering mechanisms controlling the temporal and spatial precision of adhesion dynamics, have significant impacts on our understanding of this highly dynamic and coordinated adhesion system.

1.1 INTEGRIN ADHESOME-MEDIATED CELL-ECM CROSSTALK

1.1.1 Properties of the ECM

The ECM is a collection of extracellular molecules secreted by cells which provides structural support and initiates signaling cues for cell homeostasis (Frantz et al. 2010). Cells and their ECM have intensive and dynamic crosstalk. As shown in Fig. 1, on one hand, cells secrete and remodel the ECM; on the other hand, the ECM provides a microenvironment for cells to live and is actively involved in regulating cell behavior and determining cell fate (Geiger et al. 2001; Geiger and Yamada 2011). Properties of the ECM contributing to the cell-matrix function include its molecular composition and mechanical properties.

Molecular composition is a key element of an ECM, which can vary a lot based on difference types of matrices. For example, tendons contain high levels of collagen I whereas cartilage contains high levels of collagen II and XI, and different collagens respond to various receptors including integrins, discoidin receptors, and glycoproteins (Ricard-Blum 2011). Another example is basement membranes, which are layered ECMs formed by a variety of matrices including laminin and collagen IV. These components interact with a number of cell surface receptors and other molecules including integrins, glycoproteins, and multiple growth factors such as transforming growth factor-β (TGF-β) and vascular endothelial growth factors (VEGF) (Yurchenco 2011).

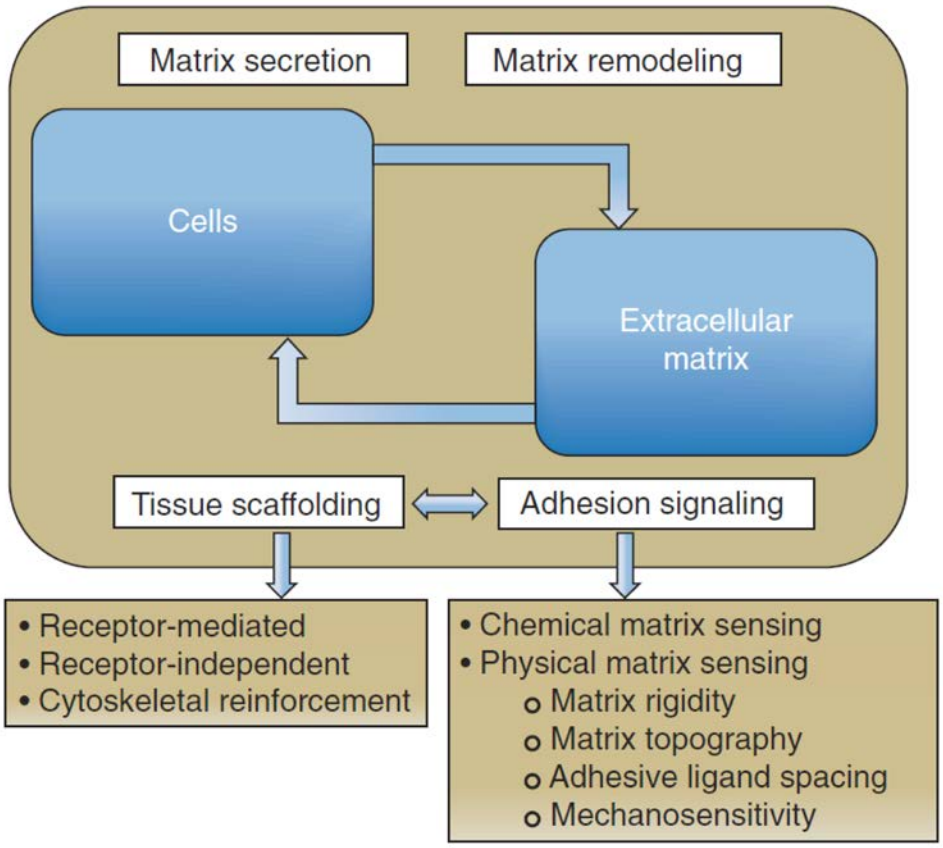


Figure 1. Schematic illustration highlighting the dynamic cross talk between cells and the ECM

Cells secrete and remodel the ECM, and the ECM contributes to the assembly of individual cells into tissues, affecting this process at both receptor and cytoskeletal levels. Adhesion-mediated signaling, based on the cells' capacity to sense the chemical and physical properties of the matrix, affects both global cell physiology and local molecular scaffolding of the adhesion sites. The molecular interactions within the adhesion site stimulate, in turn, the signaling process, by clustering together the structural and signaling components of the adhesome. This figure together with figure legend was taken from (Geiger and Yamada 2011).

Another feature of an ECM is its mechanical properties, which include elasticity or compliance, stiffness or rigidity, and heterogeneity of local porosity and cross-linking (Evans and Gentleman 2014). Elasticity is the property of an object or material to be able to restore itself after its original shape is distorted, whereas stiffness describes the resistance of an object or

material to deformation when a force is applied. During development, matrix elasticity and rigidity have a fundamental role in cell fate determination. For instance, mesenchymal stem cells will be directed into a neurogenic lineage in response to soft matrices; however, if they encounter stiffer matrices, they will be directed into myogenic and osteogenic lineages (Engler et al. 2006). In adults, the correlation between tissue specificity and matrix rigidity is also critical, which fine-tunes the specific cell type that resides in different tissues. For example, bone tissue is obviously much stiffer than brain tissue, therefore the survival, growth, and differentiation of osteoblasts favor stiffer matrices whereas neural cells favor highly compliant matrix (Butcher, Alliston, and Weaver 2009). Crosslinking is one of the post-translational modifications the ECM undergoes to modify its tension and viscoelasticity, which is the property of an object or material that have both viscous and elastic characteristics during deformation. Either mediated by enzymes or non-enzymatic mechanisms, crosslinking can change matrix deposition, tension, and stiffness, thereby affecting the mechanical properties of the ECM (Payne, Hendrix, and Kirschmann 2007). One example is that increased collagen crosslinking can lead to stiffened heart muscle which compromises normal cardiac function (Sivakumar et al. 2008). Glycation is the covalent bonding of a protein or lipid molecule with a sugar molecule without involving an enzyme. In diabetic patients, increased glycation due to high blood glucose level can affect appropriate glycation-mediated crosslinking, and therefore damage wound healing and cardiac function (Susic 2007). It is especially important to study these mechanical features in cancer research, as transformed cells have very different mechanical characteristics from normal cells. For example, in breast cancer, transformed cells often show abnormally high cell-generated force, increased compression force, and stiffened matrix (Butcher, Alliston, and Weaver 2009). These altered forces can affect focal adhesion maturation and disrupt the integrity of the cell-cell junction,

thereby enhancing the growth, survival, and invasion of transformed cells, resulting in rigid tumor tissue (F. Wang et al. 1998; Paszek et al. 2005).

In addition to its molecular composition and mechanical properties, the environment of the ECM, e.g., on a culture dish or inside a human body, can give rise to different functions. Most research so far has been using 2D substrates *in vitro* as the primary model system to study cell mechanosensing including adhesion and migration; however, 3D models either *in vitro* or *in vivo* provide unique properties of the ECM that cannot not be recapitulated in 2D (Doyle and Yamada 2016). Cells can respond differently to 2D surfaces, e.g., a tissue culture dish, from 3D environment, e.g., an organ. Most cells need specific cues from a 3D environment in order to recapitulate *in vitro* those physiological structures and chemical signals (Griffith and Swartz 2006). Molecular composition of 3D adhesion structures appear to be similar to 2D adhesion structures in general although variations can occur especially in the phosphorylation status of adhesion proteins (Cukierman et al. 2001; Pelham and Wang 1997).

1.1.2 The integrin family of adhesion receptors

Cells express various adhesion receptors including integrins, cadherins, and proteoglycans like syndecans, among which the integrin family is the best known (Parsons, Horwitz, and Schwartz 2010). Integrins are transmembrane heterodimers that bind to ECM molecules like fibronectin, vitronectin, collagen, and laminin, and bridge them to the intracellular cytoskeleton. Since the initial discovery of the first integrin (Tamkun et al. 1986), numerous studies have revealed the structure and activation of various integrin members, as well as their interaction with other proteins (Campbell and Humphries 2011).

As shown in Fig. 2, each integrin heterodimer is assembled from one α and one β subunit. Both subunits contain a single helix that spans the membrane and a cytoplasmic tail which is short and flexible. The α subunit determines the ligand specificity for the integrin, whereas the β subunit is connected to the actin cytoskeleton and affects multiple signaling pathways (Barczyk, Carracedo, and Gullberg 2010). There are 18 α and 8 β subunits in vertebrates which can form 24 different heterodimers with specificity to different ECM molecules, rendering distinct patterns of cell spreading and adhesion distribution.

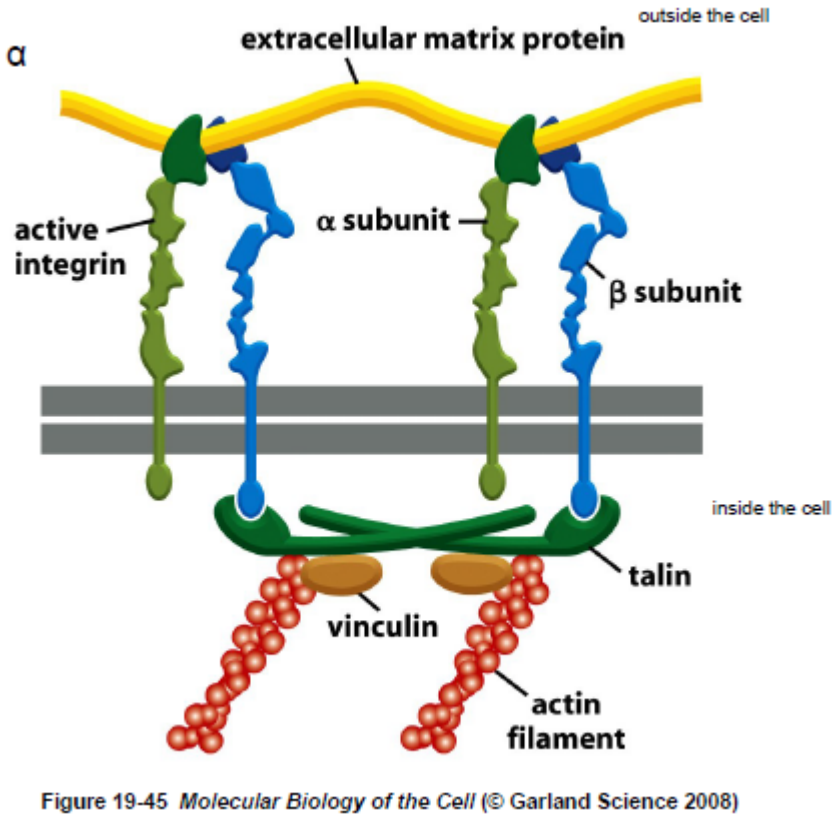


Figure 19-45 *Molecular Biology of the Cell* (© Garland Science 2008)

Figure 2. Schematic illustration highlighting the integrin heterodimers and their interaction with actin cytoskeleton

Each integrin heterodimer is assembled from one α and one β subunit. The α subunit determines the ligand specificity for the integrin, whereas the β subunit is connected to the actin cytoskeleton and affects multiple signaling pathways. This figure was taken from Molecular Biology of the Cell (© Garland Science 2008).

Integrins display three conformational states: a resting state which is inactive, an intermediate state which is partially activated, and a ligand-bound state which is fully activated (D. Cox, Brennan, and Moran 2010). Integrins themselves have no enzymatic activity; instead, they are involved in multiple signaling pathways through interactions with many signaling molecules including talin, FAK, paxillin, etc. Integrin activation involves the initial triggering events, the intermediate signaling events, and the so called “inside-out” signaling events in which the interaction of integrins with cytoplasmic regulators changes the integrin-ligand binding affinity (Shattil, Kim, and Ginsberg 2010; Carman and Springer 2003). Inside-out signaling controls cell adhesion, migration, and the assembly and remodeling of ECM. On the other hand, integrins can also act as traditional receptors transmitting information from surrounding environment into cells through “outside-in” signaling, which plays an important role in cell polarity, proliferation, and even gene expression (Shattil, Kim, and Ginsberg 2010). Defects in integrin family members have been implicated in various diseases including thrombosis, immune disorders, cancer, infection, etc., hence providing therapeutic opportunities (D. Cox, Brennan, and Moran 2010; Desgrosellier and Cheresh 2010).

1.1.3 The integrin adhesome

Once activated, integrins induce recruitment of scaffolding and signaling proteins to build multiprotein complexes known as adhesion sites, bridging cells to the ECM or to their neighboring cells. The whole network of these adhesion sites is termed “integrin adhesome”. As depicted in Fig. 3, more than 230 components and over 700 direct interactions between them have been identified so far, revealing a highly complex and connective network (Ronen Zaidel-Bar, Itzkovitz, et al. 2007; Horton et al. 2015). These components are either intrinsic residents of

the adhesion sites, or transient tenants that temporarily associate with adhesions and affect their structure and activity (Geiger, Spatz, and Bershadsky 2009). Functionally, adhesome molecules work as “scaffolding” contributors or “signaling” players. The former group includes integrin receptors, adaptor proteins, and actin-associated proteins, which together link integrins to the actin cytoskeleton, whereas the latter group contains various kinases, phosphatases, and GTPases regulating adhesion-mediated signaling pathways (Winograd-Katz et al. 2014).

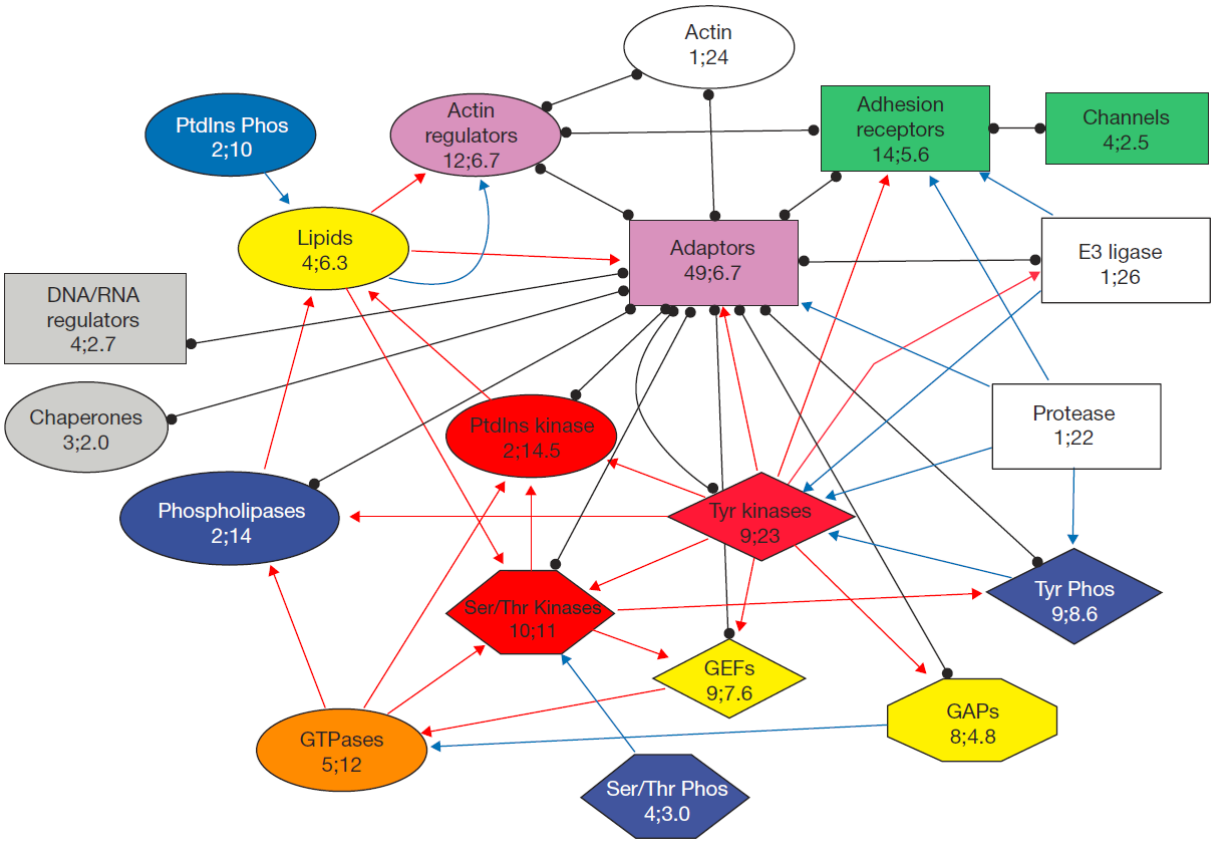


Figure 3. Interactions between functional families of adhesome components

Each protein in the adhesome was categorized into one of 20 groups according to its known biological activity. The families are shown in unique combinations of color and shape, indicating the number of family members followed by the average number of their interactions. In addition, the dominating interactions between families (red arrows, activating interactions; blue arrows, inhibiting interactions; black lines, binding interactions) are shown. This figure together with figure legend was taken from (Ronen Zaidel-Bar, Itzkovitz, et al. 2007).

Based on their size, structure, composition and function, integrin adhesions can be classified into different categories including nascent adhesions (NAs), focal complexes (FCs), focal adhesions (FAs), fibrillar adhesions, podosomes, and invadopodia (Albiges-Rizo et al. 2009). NAs are the earliest form of integrin-containing adhesion structures that can be visualized by the microscope (Choi et al. 2008; Nobes and Hall 1995). They either disassemble quickly or stabilize into FCs which can further develop into mature FAs by recruiting more components to adhesion sites (Geiger, Spatz, and Bershadsky 2009). Compared to FAs, fibrillar adhesions are morphologically more elongated and prominently located towards the central region of the cell (Geiger et al. 2001; R Zaidel-Bar et al. 2004). These ECM contacts are where integrin α5β1 is associated with fibronectin fibrils and involved in fibronectin fibrillogenesis, a cell-mediated matrix assembly process to form fibrillar network (R Zaidel-Bar et al. 2004; Wierzbicka-Patynowski and Schwarzbauer 2003; Mao and Schwarzbauer 2005). Podosome and invadopodia are two similar actin-rich structures controlling cell protrusion, and the term “podosome” is typically used in normal cells whereas “invadopodia” is commonly used in cancer cells (Murphy and Courtneidge 2011). Both protrusions represent sites where cells attach to the ECM or degrade the ECM and have been implicated in human diseases including genetic diseases and tumor progression and metastasis (Murphy and Courtneidge 2011). In my dissertation I focused on NAs, FCs, and FAs.

1.2 THE ASSEMBLY, MATURATION, AND DISASSEMBLY OF ADHESION STRUCTURES

1.2.1 The assembly and maturation of FAs

Early work by Ridley and Hall showed that microinjection of either recombinant RhoA or a constitutively activated RhoA stimulated formation of actin stress fibers and induced the assembly of FAs, which was later found to be driven by Rho-stimulated actomyosin contractility (Ridley and Hall 1992; Chrzanowska-Wodnicka 1996; Delanoë-Ayari et al. 2004). Specifically, it was believed that myosin II generates tension that helps actin bundling, which induces integrin clustering and promotes FA formation (Peterson et al. 2004; Chrzanowska-Wodnicka 1996; Webb, Parsons, and Horwitz 2002). The appearance of FAs was found to localize at the transition between the lamellipodium and lamellum of the cell where retrograde F-actin flow is near zero (Ponti 2004; Salmon, Adams, and Waterman-Storer 2002). As shown in Fig. 4, the lamellipodium is composed of a dense meshwork of F-actin located near the leading edge of a migrating cell, whereas the lamellum is the disc-like F-actin network between the lamellipodium and the cell body (Le Clainche and Carlier 2008). Retrograde F-actin flow runs fast in the lamellipodium but slowly in the lamellum (Salmon, Adams, and Waterman-Storer 2002).

One study published in 2008 further divided the formation of adhesions into assembly, stabilization, and maturation, and carefully examined the role of actin and myosin II in each step using two-color imaging and high resolution total internal reflection fluorescence microscopy (TIRFM, Choi et al. 2008). What they found was that NAs are constantly assembled and turned over with a half-life of less than 60s. Some NAs stabilize and grow into a transient entity of FCs,

which are further mediated by actin-crosslinking dependent on myosin II and α-actinin and mature into larger, elongated structures, FAs (Choi et al. 2008).

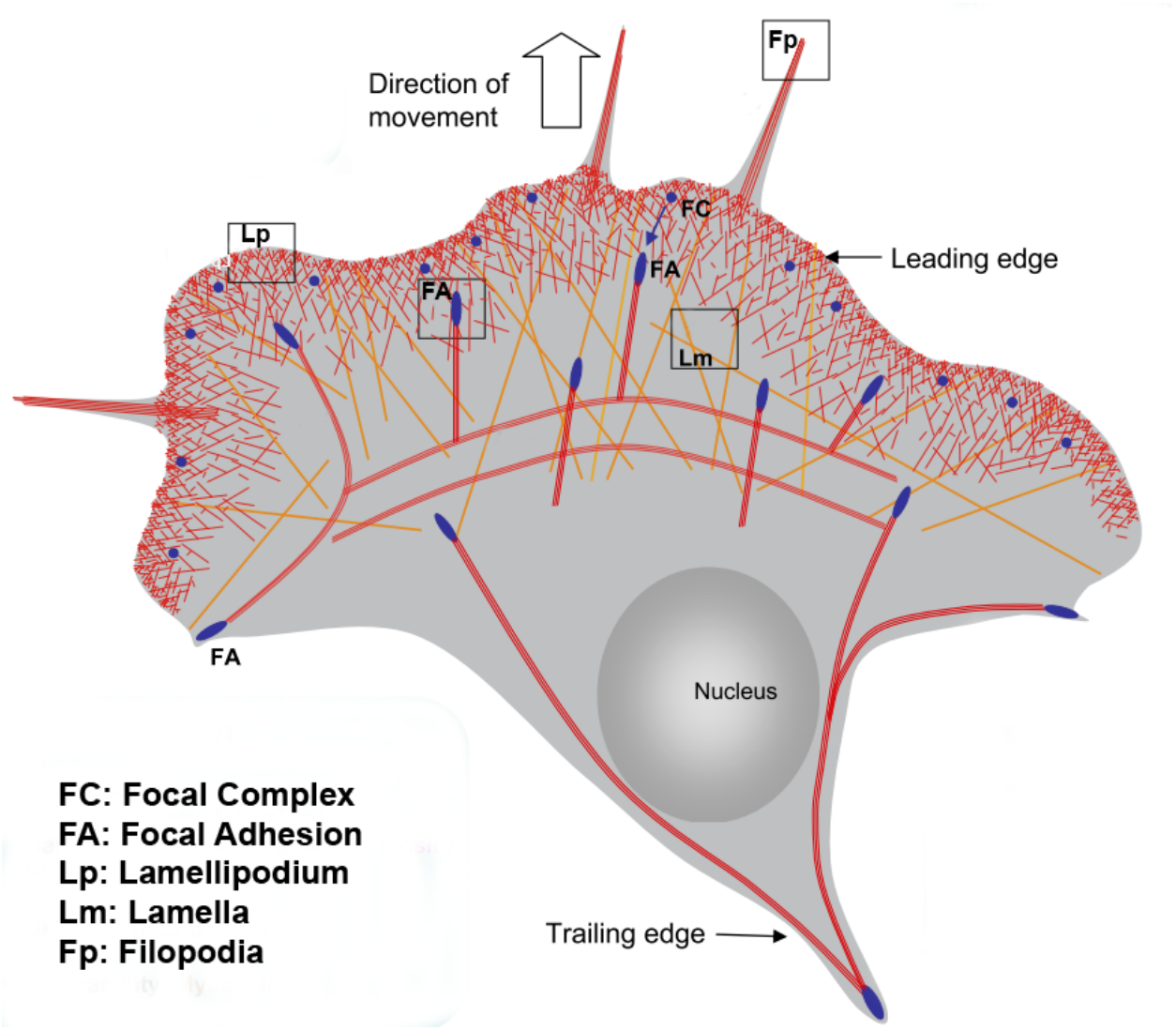


Figure 4. Schematic illustration of the actin cytoskeleton inclcluding the lamellipodium and the lamella in a migrating cell

In a migrating cell, the lamellipodium is composed of a dense meshwork of F-actin located near the leading edge of a migrating cell, whereas the lamellum is the disc-like F-actin network between the lamellipodium and the cell body. NAs that do not disassemble grow near the lamellipodium-lamella interface into FCs. Mature FAs are elongated and localized at the termini of actin stress fibers. This figure was modified from (Le Clainche and Carlier 2008).

NAs are located in the lamellipodium where F-actin retrograde flow runs fast, but NAs themselves are largely immobile (Alexandrova et al. 2008; Brown et al. 2006). The half-life of NAs is determined by the speed of actin flow, meaning that the faster the actin flow, the faster NAs turn over (Alexandrova et al. 2008; Choi et al. 2008). It was proposed that the lamellipodium with branched actin provides a scaffolding network for NAs to form, whereas actin depolymerization causes NAs to disassemble (M. Vicente-Manzanares and Horwitz 2011). The diameter of NAs is around 100nm or even smaller (Choi et al. 2008). NAs that do not disassemble grow near the lamellipodium-lamellum interface into FCs. FCs are larger than NAs but share similar molecular composition to NAs (Choi et al. 2008). They contain many well-known adhesion components including F-actin binding proteins (e.g., talin and vinculin), adaptor proteins (e.g., paxillin) and signaling proteins (e.g., FAK and p130Cas) (M. Vicente-Manzanares and Horwitz 2011). Some of these adhesion components, including FAK, Src, and paxillin, are heavily phosphorylated on serine, tyrosine, and threonine residues, which enable them to recruit additional molecules to adhesion sites to promote actin polymerization (Kirchner et al. 2003; Ronen Zaidel-Bar, Milo, et al. 2007; Kiyokawa et al. 1998; Nayal et al. 2006).

Adhesion maturation is a continuous process resulting in a heterogeneous population of FAs with a range of sizes from one to several square micrometers (Geiger et al. 2001; Geiger, Spatz, and Bershadsky 2009). In contrast to NAs and FCs, mature FAs are elongated and localized at the termini of stress fibers which are bundles of actin filaments containing associated proteins including α-actinin, filamin, and myosin II (Pellegrin and Mellor 2007). Growing adhesions remain in place while cells are moving forward and is dependent on actin-crosslinking mediated by α-actinin and nonmuscle myosin II (NMII) (Choi et al. 2008; Miguel Vicente-Manzanares, Xuefei Ma 2009). As listed in Table 1, mature FAs differ from NAs and FCs in

their location, morphology, enhanced life-time, protein composition (e.g., recruitment of zyxin to FAs), and protein phosphorylation (e.g., paxillin is less phosphorylated in FAs) (Zamir, Geiger, and Kam 2008; Ronen Zaidel-Bar, Milo, et al. 2007).

Table 1. Differences between FCs and FAs

Adhesion structure	Nascent adhesions (NAs)	Focal complexes (FCs)	Focal adhesions (FAs)
Location	lamellipodium	Lamellipodium-lamella interface	termini of actin stress fibers
Morphology	dot-like	dot-like	elongated
Size	100nm	below 1μm	2-5μm
Life-time	$T_{1/2}$<60s	transition from NAs to FAs	minutes to an hour
Composition	paxillin, talin, vinculin, FAK, p130CAS	similar to NAs	many more components like α-actinin and zyxin
Protein phosphorylation	Some components including FAK and paxillin are heavily phosphorylated on serine, tyrosine, and threonine residues	similar to NAs	Proteins are less phosphorylation

1.2.2 The interplay between Rac and Rho GTPases in adhesion formation and maturation

Activated integrins upon ECM engagement will initiate a cascade of downstream signaling pathways leading to cell attachment and spreading on the ECM. During this process, Rho GTPases, specifically Rac1 and RhoA, have long been implicated in regulating the formation and maturation of adhesion structures associated with actin cytoskeleton (Nobes and Hall 1995). The formation of NAs is dependent on high activity of Rac1 associated with low activity of RhoA, whereas the maturation of FAs requires inhibition of Rac1 and a concomitant increased activation of RhoA (Lawson and Burridge 2014). Summarized in Fig. 5, various effectors have been identified to mediate the cross talk between Rac1 and RhoA by activating or inhibiting either of these two GTPases.

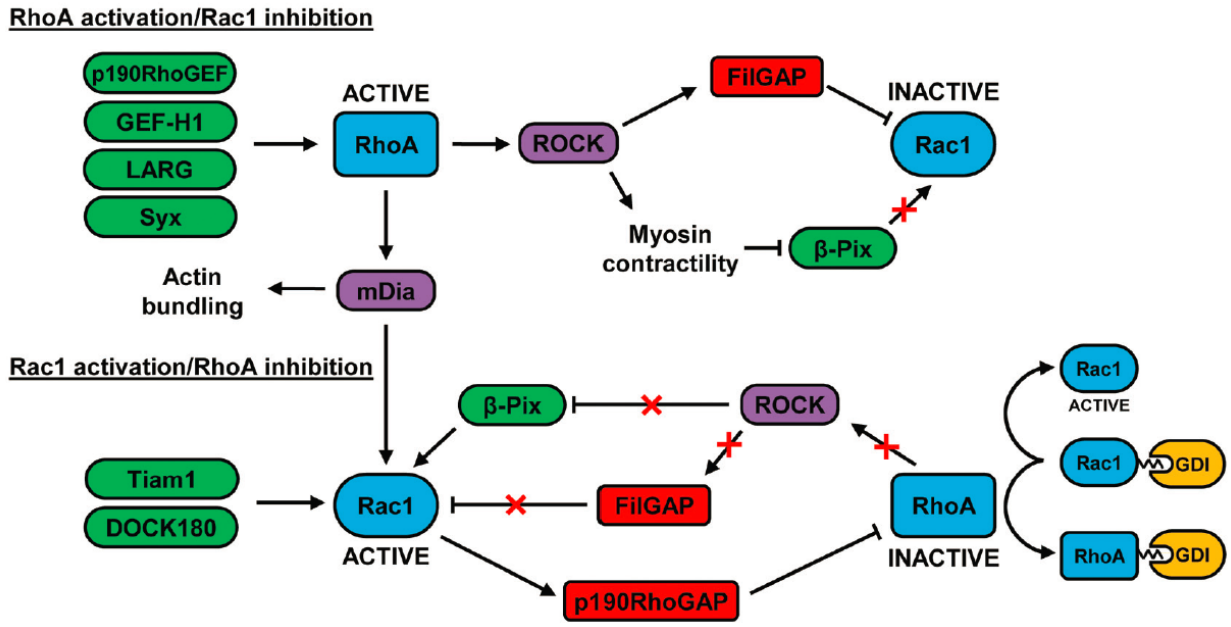


Figure 5. Schematic illustrating of cross talk between Rac1 and RhoA in migrating cells

Crosstalk between RhoA and Rac1 in migrating cells. At the leading edge of migrating cells, RhoA can be activated by GEFs such as p190Rho- GEF, GEF-H1, LARG, and Syx. At the cell rear, RhoA can restrict Rac1 activity via

FilGAP and by negatively regulating the localization of β-Pix. RhoA- stimulated mDia activity may contribute to the subsequent increase in Rac1 activity at the leading edge, possibly by activating Src-dependent GEFs such as Tiam1 and DOCK180. Rac1 can inhibit RhoA via p190RhoGAP and the decrease in RhoA activity may further activate Rac1 by preventing FilGAP activation and by relieving the inhibition of β-Pix. The association of inactive RhoA with RhoGDI could also increase Rac1 activity as a result of the competitive binding of these 2 GTPases to GDI. This figure together with the figure legend was taken from (Lawson and Burridge 2014).

The initiation of NAs occurs in the lamellipodium which requires Rac1 activation (Nobes and Hall 1995). Specifically, upon integrin activation and clustering, a non-receptor tyrosine kinase, FAK, binds to integrin and is autophosphorylated at tyrosine 397 residue (pY397FAK), which creates a binding site for another non-receptor tyrosine kinase, Src (Mitra and Schlaepfer 2006). The FAK-Src complex targets two downstream adaptor proteins, paxillin and p130Cas, for phosphorylation. Subsequently, paxillin and p130Cas bind to other adaptor proteins including GIT2 and Crk, which, respectively, recruit guanine nucleotide exchange factors (GEFs) β-Pix and DOCK180 to the sites of NAs, promoting the recruitment and activation of Rac1 at NAs. In the meanwhile, RhoA activity is suppressed by p190RhoGAP, a GTPase activating protein (GAP) specific for Rho. Activation of p190RhoGAP is triggered by integrin ligation dependent on Src (W T Arthur and Burridge 2001; William T Arthur, Petch, and Burridge 2000). In sum, the formation of NAs is associated with Rac1 activation and RhoA inhibition.

In contrast, the maturation of adhesions is associated with RhoA activation and Rac1 inhibition. A couple of GEFs have been shown to regulate the activation of RhoA. One study identified two GEFs, namely Lsc/p115 RhoGEF and LARG, involved in RhoA activation downstream of integrin engagement to fibronectin (Dubash et al. 2007). Another study found a connection between proline-rich kinase-2 (Pyk2) and FAK, and demonstrated that Pyk2

promoted the expression of p190RhoGEF which enhanced RhoA activation and FA formation in response to integrin engagement (Lim et al. 2008). A microtubule-associated GEF, GEF-H1, was identified as a mediator of RhoA activation via cell contractility dependent on microtubule disassembly and is activated downstream of FAK (Chang et al. 2008; Nalbant et al. 2009; Guilluy et al. 2011). Concomitant with the increased activity of RhoA, Rac1 activity at the stage of adhesion maturation is diminished, which is considered to result from crosstalk with the enhanced RhoA activity and actomyosin contractility.

1.2.3 The disassembly of adhesion structures

Cells migrate through four repetitive steps: protrusion, adhesion, contraction, and retraction (Miguel Vicente-Manzanares, Webb, and Horwitz 2005; Etienne-Manneville 2013). During this process, adhesion dynamics needs to be precisely regulated at the front, center, and rear of the cell (Broussard, Webb, and Kaverina 2008). At the leading edge of the cell, NAs either undergo rapid turnover or stabilize into FCs which further mature into FAs in response to contractile forces (Geiger, Spatz, and Bershadsky 2009). In order for cells to contract and relocate, adhesions need to be disassembled both underneath the cell body and at the rear of the cell (Rid et al. 2005). FAs that do not disassemble underneath the cell body turn into fibrillar adhesions involved in extracellular matrix modifications (Rid et al. 2005).

Compared to the mechanisms of adhesion assembly and maturation which have been extensively studied, how adhesions are disassembled is less well understood. Nonetheless, there are a couple of pathways regulating FA disassembly that have been identified so far. FAK has been recognized as a key regulator in adhesion turnover. It has been shown that fibroblasts from FAK-null mouse had an increased number and size of peripheral adhesions (Ilić et al. 1995).

Kinetics studies showed major changes in adhesion disassembly without FAK, although minor changes in adhesion assembly were also observed (Webb et al. 2004; Schober et al. 2007). Downstream pathways of FAK include modulation of actomyosin contractility via ERK and MLCK which promotes tension that destabilizes FAs (Webb et al. 2004). FAK is also found to be required for the phosphorylation and activation of p190RhoGAP at the sites of FAs, which suppresses actin stress fiber formation induced by Rho and inhibits FA stabilization (Schober et al. 2007). Paxillin, a downstream target of FAK-Src complex, is implicated in adhesion turnover dependent on its phosphorylation status (Ronen Zaidel-Bar, Milo, et al. 2007; Nayal et al. 2006). Interestingly, although fibroblasts from FAK-deficient mice showed a decreased migration rate and loss of directionality, knockdown of FAK or paxillin in HeLa cells caused increased cell motility by activating Rac1 (Yano et al. 2004). Microtubules were found to induce FA disassembly mediated by FAK and dynamin2 (Ezratty, Partridge, and Gundersen 2005). Later studies confirmed a direct interaction between FAK and dynamin2 which regulates FA disassembly in response to Src kinase (Y. Wang et al. 2011). The role of the microtubule cytoskeleton in adhesion disassembly will be discussed in detail in section 1.3.

Besides the phosphorylation events regulated by the FAK-Src complex, proteolysis of multiple FA components has also been shown to play a role in adhesion turnover. Calpain, an intracellular calcium-dependent protease induced by myosin II activity, has been shown to regulate FA turnover by mediating proteolysis of multiple FA components including talin and FAK (Franco et al. 2004; Chan, Bennin, and Huttenlocher 2010). Talin is a cytoskeletal protein that has been found to function in integrin activation (Tadokoro et al. 2003) and adhesion formation and reinforcement (Jiang et al. 2003; Giannone et al. 2003). Recruitment of talin and Type I Phosphatidylinositol Phosphate Kinase (PIP kinase) to FAs is dependent on FAK

signaling and the interaction between talin and PIP kinase is critical for adhesion assembly (Ling et al. 2002). At FAs, talin and PIP kinase stimulate local generation of phosphatidylinositol-4,5-bis-phosphate (PtdIns(4,5)P2), leading to recruitment of additional FA components (Ling et al. 2002). An elevated level of PtdIns(4,5)P2 is required for integrin endocytosis for adhesion disassembly mediated by clathrin (Ezratty et al. 2009; Chao and Kunz 2009; Chao et al. 2010). Proteolysis of talin by calpain also induces the dissociation of other components from FAs, including paxillin, vinculin, and zyxin (Franco et al. 2004). Interestingly, FAK is another substrate of calpain and has been shown to regulate dynamic turnover of talin at FAs (Chan, Bennin, and Huttenlocher 2010).

1.2.4 The role of tension in adhesion dynamics

The transition from adhesion assembly to adhesion maturation is triggered by increased tensile force through interactions with the actin cytoskeleton. A key player in this process is an actin-associated motor protein, myosin II, which provides contractile force required for adhesion maturation (Choi et al. 2008). Two major myosin II isoforms, myosin IIA and myosin IIB, function differently in cell protrusion. Whereas myosin IIB controls cell polarity through actin-crosslinking, myosin IIA regulates adhesion size and dynamics in the central region of the cell as well as adhesion disassembly at the rear of the cell (Miguel Vicente-Manzanares et al. 2007).

1.3 MICROTUBULE CYTOSKELETON IN ADHESION NETWORK

1.3.1 Microtubules in cell protrusion and migration

Microtubules are polymers derived from α- and β- tubulin dimers. Coordinated with accessory proteins including molecular motors and microtubule-associated proteins (MAPs), the microtubule cytoskeleton is well known for its role in cell division and vesicular transport (Green, Paluch, and Oegema 2012; Hendricks et al. 2010; Holzbaur and Goldman 2010; Etienne-Manneville 2010). The role of the microtubule cytoskeleton in cell migration was not recognized until 1970, however, numerous investigations have been carried on ever since (Vasiliev et al. 1970; Etienne-Manneville 2013). Microtubule-associated motors including kinesins and dynein transport cargos such as mRNA, protein, and vesicles to their destinations along the microtubule tracks (Gatlin and Bloom 2010; Y. Yu and Feng 2010; Vanneste, Ferreira, and Vernos 2011). There are two categories of MAPs: one interacts with the plus ends of microtubules and is therefore termed microtubule plus-end binding proteins (+TIPs); the other associates with the microtubule lattice where they modulate microtubule instability (Etienne-Manneville 2013). How +TIPs and kinesin motors function in the adhesion network will be discussed in more detail in 1.3.4 and 1.3.5, respectively.

Most cell types, including epithelial cells, endothelial cells, and fibroblasts, depend on both actin and microtubules to generate membrane protrusions. The regulation of membrane protrusion by microtubules relies on a number of their properties including mechanical properties, cellular trafficking capability, and signaling capacities (Etienne-Manneville 2010). First, microtubule polymerization can generate forces potentially used to push the plasma membrane (Etienne-Manneville 2013). In neurons, microtubule bundles can exert forces

sufficient for neurite outgrowth when actin polymerization is disrupted (Bradke and Dotti 1999). Secondly, multiple cargoes are delivered to and from the plasma membrane by microtubules to contribute to cell protrusion and migration. For example, microtubules are required for the trafficking of lipid rafts from the plasma membrane to recycling endosomes during cell detachment from the substratum and from recycling endosomes back to the plasma membrane when cells are replated onto the substratum (Balasubramanian et al. 2007). Rho family GTPases Rac and Cdc42 are delivered to the plasma membrane in a microtubule-dependent manner to ensure spatial restriction of signaling during cell migration (Palamidessi et al. 2008; Osmani et al. 2010). In addition, localization of mRNAs encoding proteins involved in actin polymerization, such as β-actin and subunits of Arp2/3 actin-nucleating complex, to the leading edge of the cell has been shown to rely on microtubules and microtubule-associated motor proteins (Shestakova, Singer, and Condeelis 2001; Mingle et al. 2005). The compartmentalization of β-actin mRNA enhances the stability of FAs and plays an important role in directional cell migration (Katz et al. 2012). Finally, microtubules can regulate FA dynamics and promote cell protrusion by indirectly affecting signaling molecules such as Rho family GTPases, which will be discussed in detail in 1.3.3.

1.3.2 Microtubules and FA dynamics

In migrating cells, microtubules often appear associated with FAs (Rinnerthaler, Geiger, and Small 1988). Kaverina *et al*. first demonstrated that microtubules repeatedly target FAs and these targeting events correlate with the areas of FA disassembly, indicating a link between microtubules and FA dynamics (I. Kaverina, Rottner, and Small 1998; I. Kaverina, Krylyshkina, and Small 1999). Later work by Ezratty *et al*. demonstrated that microtubules regrowth after

nocodazle treatment and release induces FA disassembly involving FAK and dynamin (Ezratty, Partridge, and Gundersen 2005). Several categories of important regulators controlling crosstalk between microtubules and FA dynamics will be discussed in 1.3.3, 1.3.4, and 1.3.5.

1.3.3 Microtubules and Rho GTPases

One way microtubules affect adhesion dynamics is by locally regulating signaling pathways of Rho family GTPases. Microtubule growth induced by nocodazole treatment and release in fibroblasts activates Rac1 which leads to actin polymerization in lamellipodial protrusion (Waterman-Storer et al. 1999). Later studies identified a number of guanine exchange factors (GEFs) of Rac delivered to or activated at protrusion sites by microtubules (Kawasaki et al. 2000; Rooney et al. 2010; Montenegro-Venegas et al. 2010). For example, the Rac GEF Sif and Tiam1- like exchange factor (STEF) mediates activation of Rac dependent on microtubules and regulates microtubule-mediated FA disassembly (Rooney et al. 2010). Depolymerization of microtubules has also been shown to cause an increase in GEF-H1 dependent RhoA activation, which influences cell contractility and adhesion assembly (Chang et al. 2008; Nalbant et al. 2009). In contrast, repolymerization of microtubules after nocodazole washout induced FA disassembly, which, however, is not dependent on Rho and Rac, suggesting a more complex modulation between Rho GTPases (Ezratty, Partridge, and Gundersen 2005).

1.3.4 Microtubules and microtubule plus-end binding proteins (+TIPs)

Great effort has been contributed to identify microtubule-associated proteins that regulate the crosstalk between microtu bules and FA dynamics. One group that has been categorized is the

microtubule plus-end binding proteins (+TIPs), including APC (Adenomatous polyposis coli), ACF7 (actin cross-linking family 7), and CLASPs (cytoplasmic linker–associated proteins). These proteins all localize close to the sites of FAs through interactions with EB1 (End-binding protein 1), and are involved in similar, however not redundant, processes controlling microtubules and FA dynamics during cell migration (Matsumoto et al. 2010; Jaulin and Kreitzer 2010; Wu, Kodama, and Fuchs 2008; Kumar et al. 2009; S. Stehbens and Wittmann 2012). EB1 is a protein concentrated at the distal tips of microtubules and regulate microtubule dynamics (Tirnauer and Bierer 2000). All three of these +TIPs bind to EB1 at the distal tips of microtubules and are required for directional cell migration (Akhmanova and Steinmetz 2008; Kumar and Wittmann 2012).

As shown in Fig. 6, a hierarchy of APC, the spectraplakin MACF1/ACF7, and CLASPs, was proposed. APC was initially identified as a tumor suppressor which is mutated in human colon cancer and has been found to regulate levels of β-catenin in the Wnt signaling pathway (Aoki and Taketo 2007). The interaction between APC and Dishevelled has been shown to increase the localization of paxillin at the leading edge of the cell and is involved in the Wnt5a-dependent FA dynamics during cell migration (Matsumoto et al. 2010). APC was also found to nucleate actin filaments and therefore was speculated to be involved in seeding new sites of FAs (Okada et al. 2010). ACF7 belongs to a family of F-actin and microtubule cross-linker proteins called spectraplakin (Suozzi, Wu, and Fuchs 2012). It localizes close to FAs which can be inhibited by glycogen synthase kinase 3 (GSK3β) phosphorylation (Wu et al. 2011). Cells deficient in ACF7 showed defects in targeting of microtubules to FAs and hence decreased FA turnover and cell migration (Wu, Kodama, and Fuchs 2008). CLASPs are microtubule-associated proteins which regulate the organization of microtubules, and similar to ACFs, the binding of

CLASPs to microtubules is also inhibited by GSK3β phosphorylation (Kumar et al. 2009). It has been shown that the clustering of CLASPs facilitates FA disassembly (S. J. Stehbens et al. 2014). Interestingly, unlike APC and ACF7, the localization of CLASPs is not dependent on microtubules, but on a peripheral membrane protein called LL5β. Depletion of LL5β resulted in a loss of FA-associated CLASPs and an increase in FA turnover (S. J. Stehbens et al. 2014).

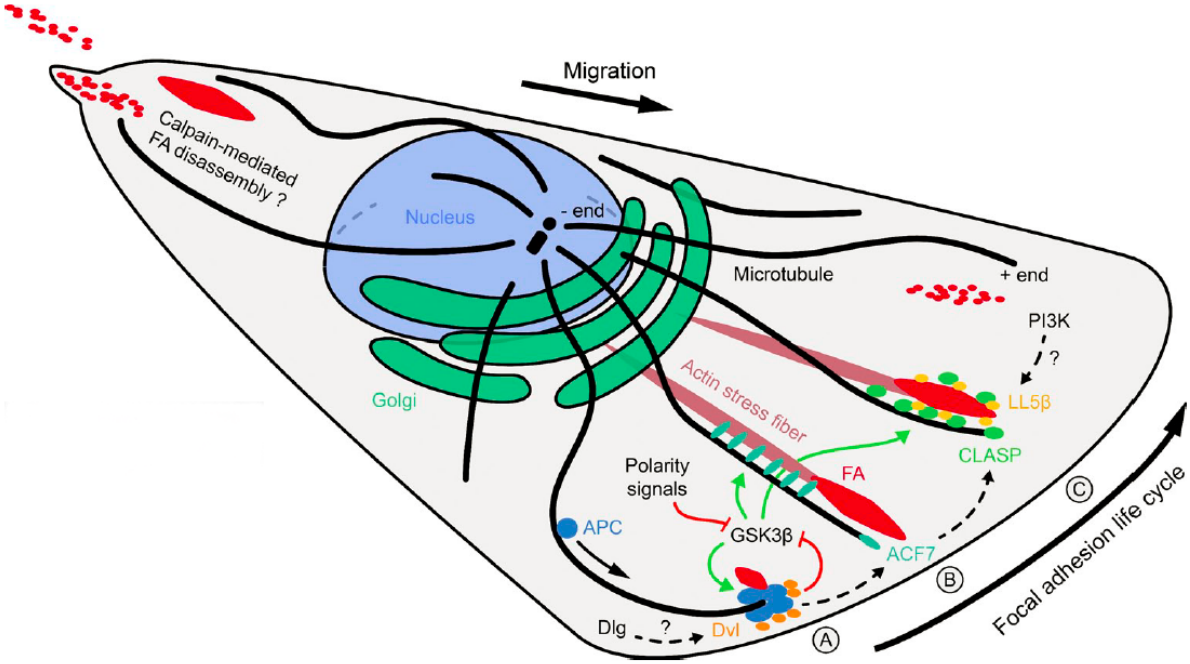


Figure 6. +TIPs regulate cross talk between the microtubule cytoskeleton and focal adhesion dynamics in the front of a migrating cell

Three types of +TIPs have been implicated in mediating microtubule–FA interactions. (A) APC is transported along microtubules to the cell edge and directly interacts with polarity signals such as the Wnt signaling pathway. APC may be involved in stabilizing nascent FAs. (B) MACF1/ACF7 mediates microtubule interactions with F-actin stress fibers, and is required to guide microtubule growth toward FAs. (C) CLASPs stabilize microtubules in a domain around mature FAs. CLASP accumulation near FAs depends on interactions with the PIP3-binding protein LL5β. FA disassembly in the retracting rear of the cells differs mechanistically from FA turnover in the front, and it is not known to what extent the same molecules are involved. Disassembling FAs are symbolized by red dots. This figure and figure legend were modified from (S. Stehbens and Wittmann 2012).

1.3.5 Microtubules and microtubule-associated motor proteins

Motor proteins are another group of microtubule-associated proteins that have been shown to be involved in regulating FA dynamics. They were traditionally known to move along microtubules and be powered by ATP hydrolysis for cargo transport (Hirokawa et al. 2009). However, many kinesins have additional functions beyond cargo transport and play important roles in cell division, adhesion and migration (Kashina et al. 1996; I. N. Kaverina et al. 1997).

Coordination between microtubules and kinesins has been implicated in adhesion dynamics for over a decade. Fig. 7 listed a number of kinesins that have been identified so far to function in adhesion dynamics. Inhibition of KIF5B/kinesin-1 in Xenopus fibroblasts induces an increase in the size and a decrease in the number of FAs, although not through perturbing microtubule dynamics (Krylyshkina et al. 2002). KIF1C/kinesin-3 transports α5β1-integrin and mediates the stabilization of trailing adhesions at the rear of the cell which is required for directional persistence of migrating cells (Theisen, Straube, and Straube 2012). KIF4A/kinesin-4 and KIF15/kinesin-12 are another two kinesins implicated in recycling α5β1-integrin and α2β1-integrin, respectively, which is thought to spatially regulate adhesions in different areas of the cell (Heintz et al. 2014; Eskova et al. 2014). Overexpression of KIF14/kinesin-3 causes defects in integrin activation and hence impaired cell adhesion and migration (Ahmed et al. 2012). KIF17/kinesin-2 is required for clustering of APC, one of the +TIPs, which is associated with FA assembly (Jaulin and Kreitzer 2010). Moreover, KIF1C/ kinesin-3 and KIF9/kinesin-9 are both involved in podosome dynamics, as cells after knockdown of either of these two kinesins showed impaired podosome formation and turnover (Cornfine et al. 2011; Efimova et al. 2014).

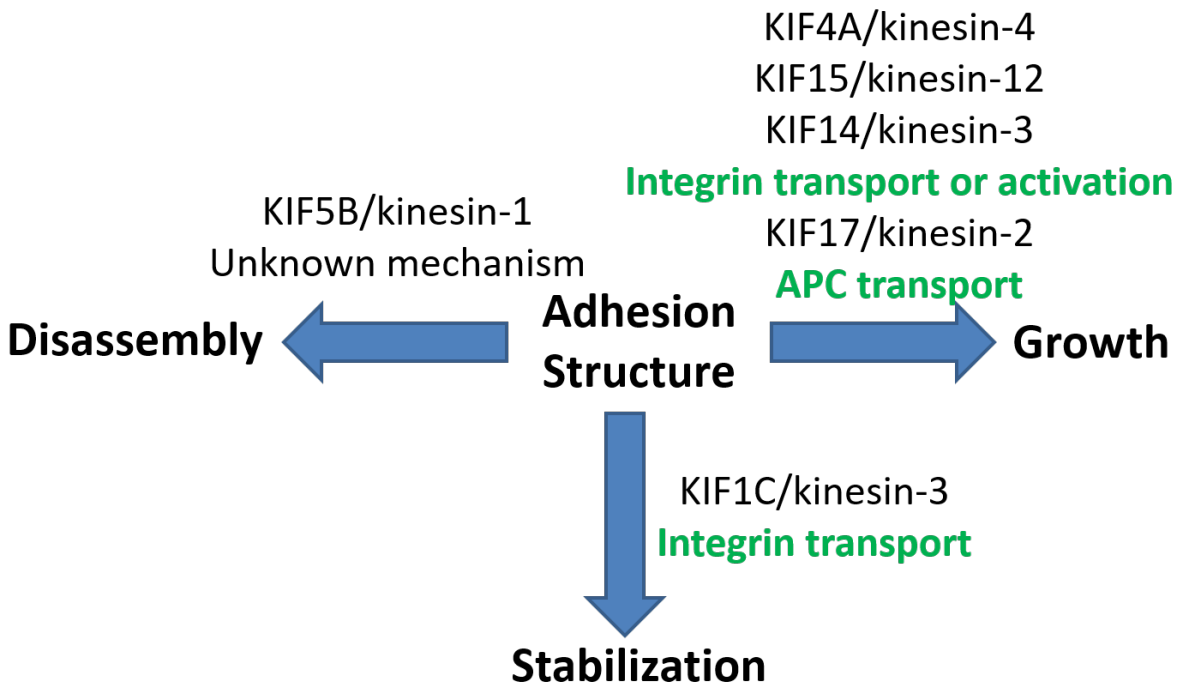


Figure 7. Known kinesins that function in adhesion dynamics

A number of kinesin motor proteins, which move towards the plus ends of MTs, have been implicated in FA dynamics. KIF4A/kinesin-4, KIF15/kinesin-12, and KIF14/kinesin-3 regulate adhesion formation by transporting or activating integrins or microtubule-associated proteins like +TIPs. KIF1C/kinesin-3 has been shown to mediate the dynamics of trailing adhesions at the tail of the cell, which is required for cells to migrate persistently towards one direction. Inhibition of KIF5B in Xenopus fibroblasts induced an increase in size and a decrease in number of FAs, although the mechanism controlling this is not clear.

1.4 KINESIN MOTOR KID/KIF22/KINESIN-10

Kid belongs to one particular class of kinesins termed chromokinesins, the canonical function of which is to assemble the mitotic spindle and power chromosome movement during mitosis (Vanneste, Ferreira, and Vernos 2011). As shown in Fig. 8, Kid contains an N-terminal catalytic motor domain which contains two microtubule-binding sites, a stalk domain which contains a

coiled-coil region for dimerization, and a C-terminal tail domain for cargo binding (Vanneste, Ferreira, and Vernos 2011; Shiroguchi et al. 2003). Although Kid has a predicted coiled-coil region, previous studies suggested that it functions as a monomer in human cells (Shiroguchi et al. 2003). In addition, a helix-hairpin-helix (HhH) motif located at the C-terminus of Kid was shown to be responsible for DNA binding (Tokai et al. 1996). In 2000, Funabiki and Murray found that in Xenopus, Xkid is necessary for chromosome alignment in metaphase and its degradation is required for chromosome movement in anaphase (Funabiki and Murray 2000). Studies using mouse embryos showed that Kid-mediated chromosome compaction is required for proper nuclear envelope formation (Ohsugi et al. 2008). In human cells, Kid was found to generate chromosome oscillations (Levesque and Compton 2001). A genetic link of Kid to skeletal disorders has been discovered in which mutations affecting two adjacent residues in the motor domain of Kid resulted in skeletal dysplasia and joint laxity (Boyden et al. 2011). Before my dissertation study, research has been mainly focused on the mitotic function of Kid. My study explored and discovered a novel function of Kid during interphase, which expanded our knowledge on how motor proteins play significant roles in adhesion dynamics.

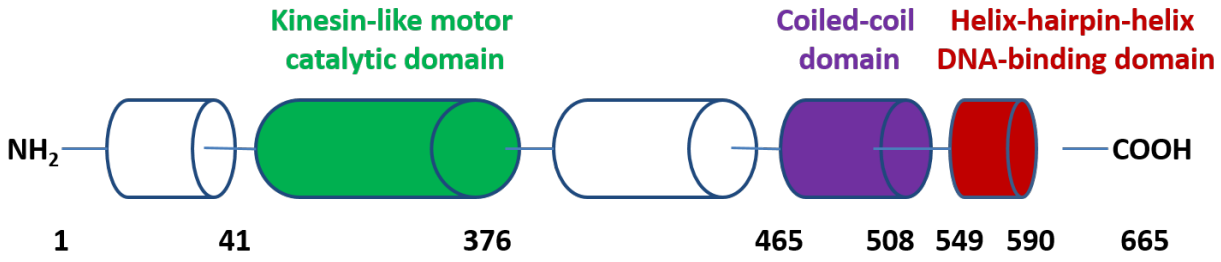


Figure 8. Schematic illustration of Kid protein domains

Kid contains a motor domain which binds to the microtubule, a stalk domain which contains a coiled-coil region for dimerization, and a tail domain for cargo binding. In addition, Kid has a DNA binding domain at its C-terminus, which is consistent with its role in chromosome alignment and segregation during mitosis.

1.5 IMAGING TECHNOLOGIES

1.5.1 Total Internal Reflection Fluorescence Microscopy (TIRFM)

The idea of TIRFM was first described by E. J. Embrose and extended by D. Axelrod (AMBROSE 1956; Axelrod 1981). As shown in Fig. 9, this technology applies the unique properties of an evanescent wave which selectively excites fluorophores in a limited region of a specimen immediately adjacent to the interface between the specimen and a glass coverslip. The evanescent wave is generated when the incident angle is totally internally reflected at the specimen-glass interface and decays exponentially in intensity from the interface. Therefore, the area in which fluorophores are excited is restricted to a depth of less than 100nm, which makes this imaging technology a powerful tool in visualizing the surface area of a cell, e.g., the plasma membrane and sites of FAs.

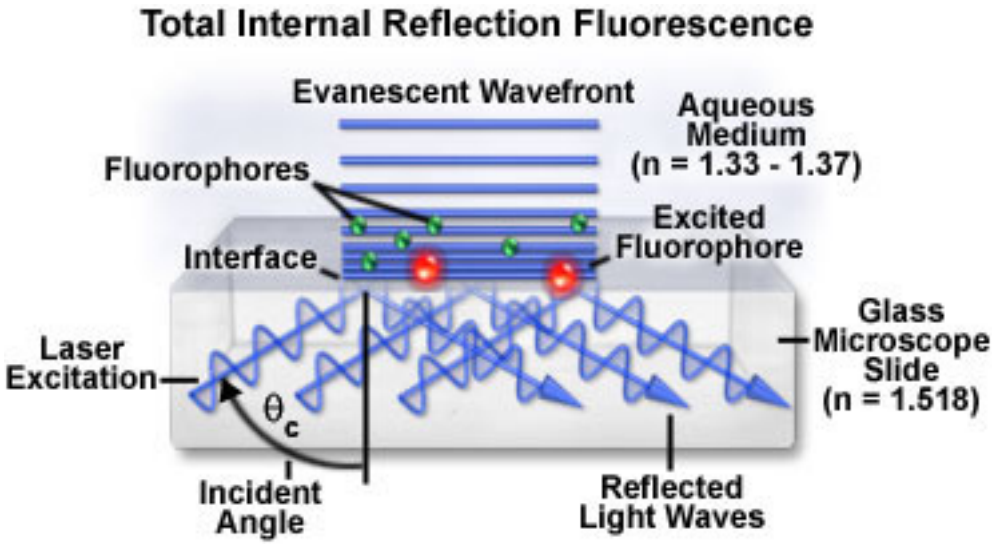


Figure 9. Total Internal Reflection Fluorescence Microscopy (TIRFM)

An evanescent wave is generated when incident angle is totally internally reflected which only excites fluorophores at the specimen-glass interface and enables a selective visualization of a restricted region less than 100nm in depth. This figure was taken from http://www.microscopyu.com/.

1.5.2 Scanning Electron Microscopy (SEM)

Instead of using light, SEM uses electrons to form an image, which has many advantages over traditional microscopy. It was invented by K. McMullan to study the targets of television camera tubes and the first commercial SEM was for sale in 1965 (McMullan 1995). Compared to traditional microscopy, SEM has a deeper field and higher resolution, thus allowing more of a specimen to be at focus and magnified at higher levels.

As illustrated in Fig. 10, at the top of the microscope, an electron gun produces a beam of electrons, which passes through the microscope vertically. The electron beam is focused by one or two condenser lenses and then passes through scanning coils which deflect the electron beam to the x and y axes. Once the beam hits the sample, X-rays, backscattered electrons, and secondary electrons are ejected and detected by their corresponding detectors, followed by conversion into signals which are sent to a TV scanner to produce the final image.

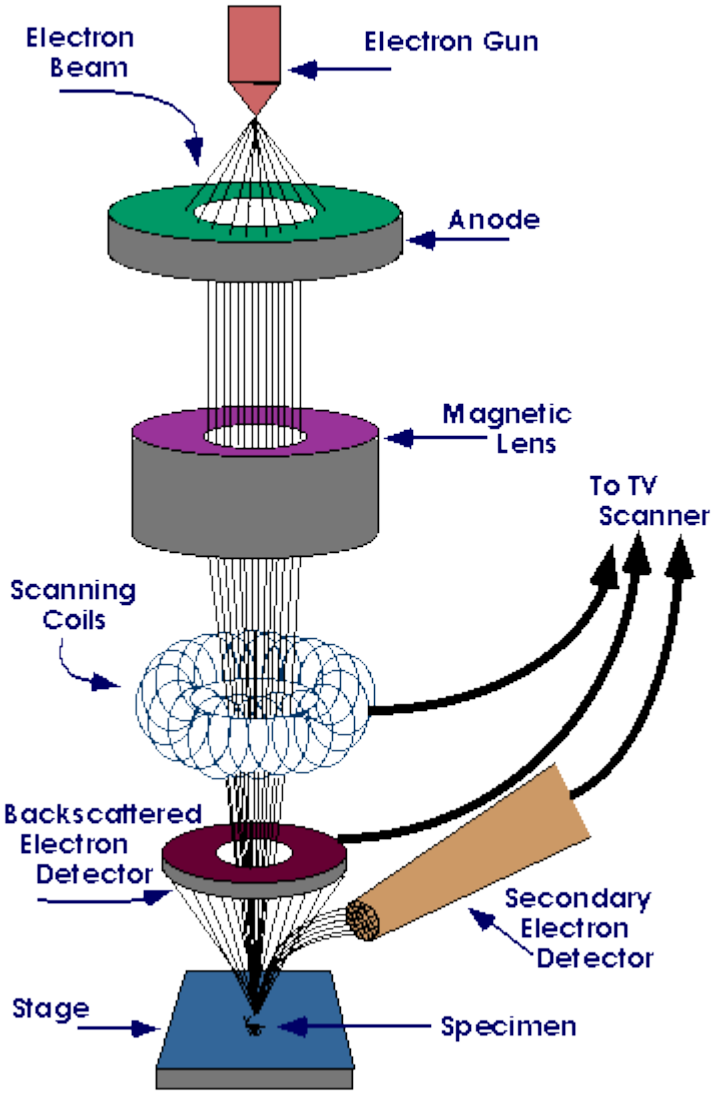


Figure 10. Scanning Electron Microscopy (SEM)

A beam of electrons produced by an electron gun passes through the microscope and is focused by one or two condenser lenses. The electron beam then passes through scanning coils which deflect the electron beam to the x and y axes. Once the beam hits the sample, X-rays and electrons are ejected and detected by their corresponding detectors, followed by conversion into signals which are sent to a TV scanner to produce the final image. The image was taken from https://www.purdue.edu/ehps/rem/rs/sem.htm.

1.5.3 Stimulated Emission Depletion Microscopy (STEDM)

Conventional light microscopy is limited by its resolution which is defined as the largest distance between which two objects can distinguish from each other. STEDM is one of the super-resolution microscopy techniques that bypass the diffraction limit to enhance imaging resolution (Schermelleh, Heintzmann, and Leonhardt 2010). Developed by S. Hell and J. Wichman, STEDM uses a non-linear saturation response to deactivate previously excited fluorophores that are slightly off the center of the excitation point spread function, a term that describes how a point-like object spreads in an image (Hell and Wichmann 1994). Therefore, STEDM overcomes diffraction limit by taking images below diffraction limit, which is different from photoactivated localization microscopy (PALM) and stochastic optical reconstruction microscopy (STORM) that take diffraction-limited images and uses mathematical models to reconstruct from those images and improve resolution.

2.0 MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Cell Culture

All cell lines were maintained in a 37°C incubator with 5% CO_2. HeLa cells, HEK293 cells were cultured in DMEM (Dulbecco's Modified Eagle's Medium; Sigma Cat# D5796) supplemented with 10% FBS (Fetal Bovine Serum; Atlanta Biologicals Cat# S11150). OSCC cells were cultured in MEM (Minimum Essential Medium Eagle; Sigma Cat# M4655) supplemented with 10% FBS and 1% non-essential amino acids (Gibco Cat# 11140050). RPE1-hTERT cells were cultured in DMEM F-12 (HyClone Cat# SH30023.01) supplemented with 10% FBS. NIH-3T3 cells were cultured in DMEM supplemented with 10% BCS (Bovine Calf Serum; HyClone Cat# SH30072.03).

2.1.2 Antibodies

Primary antibodies: rabbit α-kif22 (Novus Biologicals Cat# NBP1-82876); rabbit α-kif22 (Cytoskeleton Cat# AKIN12-A); mouse α-vinculin (Sigma Cat# V9131); rat α-tubulin (Novus Biologicals Cat# NB600-506); mouse α-alpha tubulin (Sigma Cat# T9026); mouse α-gamma tubulin (Sigma Cat# T6557); mouse α-zyxin (Life Technologies Cat# 396000); mouse α-paxillin

(abcam Cat# ab3127); rabbit α-paxillin (Santa Cruz Cat# sc-5574); rabbit α-pY118paxillin (Life Technologies Cat# 44722G); mouse α-FAK (Thermo Scientific Cat# MA5-15588); rabbit α-FAK (abcam Cat# ab40794); rabbit α-pY397FAK (Life Technologies Cat# 700255); mouse α-N-cadherin (Invitrogen Cat# 41390). Secondary antibodies for Western blot: ECL™ Mouse IgG, HRP-linked whole antibody (from sheep) (GE Healthcare Cat# NA931-1ML); ECL™ Rabbit IgG, HRP-linked whole antibody (from donkey) (GE Healthcare Cat# NA934-1ML). Secondary antibodies for immunofluorescence: Goat anti-Mouse IgG AF488 (Life Technologies Cat# A11001); Goat anti-Rabbit IgG AF568 (Life Technologies Cat# A-11011); Goat anti-Rabbit IgG AF488 (Life Technologies Cat# A-11034); Goat anti-Mouse IgG Cy5 (Jackson ImmunoResearch Cat# 115-175-146); Goat anti-Rat IgG Cy3 (Jackson ImmunoResearchf Cat# 112-165-143).

2.1.3 Plasmids and siRNAs

Plasmids and siRNAs: tGFP-Kif22 (OriGene Cat# RG200397); mCherry-paxillin (a gift from Dr. Simon Watkins at Center for Biologic Imaging, University of Pittsburgh); siC (Qiagen Cat# 1027280); siK#1 (Qiagen Cat# SI03019856); siK#2 (Life Technologies Cat# s7913).

2.1.4 Reagents

Nocodazole (Sigma Cat# M1404); Rho inhibitor (Cytoskeleton Cat# CT04-A); Rhodamine-conjugated Phalloidin (Cytoskeleton Cat# PHDR1); FAK inhibitor (Santa Cruz Cat# sc-203950); 16% Paraformaldehyde (VWR Cat# AA43368-9M); Triton X-100 (Sigma Cat# T9284); Bovine Serum Albumin (Sigma Cat# A2153); ProLong Gold Antifade Mountant with DAPI (Life Technologies Cat# P36931); Opti-MEM Reduced Serum Medium (Life Technologies Cat#

31985-062); Lipofectamine RNAiMAX (Life Technologies Cat# 13778150); Lipofectamine 2000 (Life Technologies Cat# 11668019); MitoTracker® Green FM (Invitrogen Cat# M7514); MitoSOX™ Red Mitochondrial Superoxide Indicator (Invitrogen Cat# M36008).

2.2 METHODS

2.2.1 Immunofluorescence

To examine the localization of Kid during interphase, cells were passaged the day before immunofluorescence to reach 70-80% confluency. Cells were fixed in 2% paraformaldehyde at room temperature for 15min, permeabilized in 0.1% Triton X-100 for 15min, and blocked in 5% normal donkey serum for 45min. Cells were then incubated with primary antibodies diluted in 0.5% BSA probing for kid (rabbit α-kif22 1:250), vinculin (mouse α-vinculin 1:250), and tubulin (rat α-tubulin 1:500) together either at room temperature for 1h or at 4°C overnight, followed by secondary antibodies (Goat anti-Rabbit IgG AF488 1:500; Goat anti-Mouse IgG Cy5 1:1000; Goat anti-Rat IgG Cy3 1:1000). Cells were mounted and imaged after 24h using either Olympus Fluoview 1000 Confocal Microscope with a 60X objective or Olympus BX60 Epifluorescence Microscope with a 100X objective. To examine if the localization of Kid to FAs is microtubule-dependent, 70-80% confluent HeLa cells were either left untreated or treated with 10μM nocodazole for 4h before immunofluorescence as described above.

2.2.2 Transfection

siRNA transfection: $1.2x10^5$ HeLa cells were seeded onto each 35mm dish 4-5h before transfection. For each transfection reaction, 50µl of Opti-MEM and 3µl of Lipofectamine RNAiMAX were mixed in a 1.5ml microcentrifuge tube; 50µl of Opti-MEM and 1.5µl of one of the siRNAs (siC or siK#1 or siK#2, 5µM) were mixed in another microcentrifuge tube. Mixtures from the two tubes were combined and incubated at room temperature for 15-20min. 2.4ml of fresh medium (DMEM+10%FBS) and 100µl of transfection reaction mix was added to each dish. The final concentration of siRNA was 3nM. Cells were incubated for 48h before further experiments and medium was changed 24h post-transfection. The RNAi efficiency was determined by Western blotting Kid.

Plasmid transfection: $4x10^5$ HeLa cells were seeded onto each 35mm dish the day before transfection to reach ~90% confluency upon transfection. For each transfection reaction, 150µl of Opti-MEM and 4µl of Lipofectamine 2000 were mixed in a 1.5ml microcentrifuge tube; 150µl of Opti-MEM and 1µg of tGFP-Kif22 plasmid were mixed in another microcentrifuge tube. Mixtures from the two tubes were combined and incubated at room temperature for 5min. 1.7ml of fresh medium (DMEM+10%FBS) and 300µl of transfection reaction mix was added to each dish. Cells were incubated for 24h before further experiments.

2.2.3 Cell Spreading Assay

HeLa cells were transfected with either control siRNA (siC) or siRNA targeting Kid (siK#1 or siK#2) for 48h and replated at the same density onto fibronectin-coated coverslips in serum-reduced medium (DMEM+1%FBS). Cells were fixed at various time points (10min, 20min,

30min, 60min, and 24h) and stained for F-actin using Rhodamine-conjugated Phalloidin. Images were taken using Olympus Fluoview 1000 Confocal Microscope with a 60X objective and the areas of cells spreading at each time point were measured and quantified by NIS-Elements software.

2.2.4 Wound Healing Assay

HeLa cells were transfected with either siC or siK#1 for 48h on 35mm glass-bottom culture dishes (MatTek Cat# P35G-1.5-14-C). When the plate reached 100% confluency, a clearing was scratched using a pipet tip and DIC images were taken every half an hour for 24h (or until the wound closed). Rates of cell migration were determined by how fast cells migrated to close the wound. 12 different regions of the plate were analyzed to diminish random variation. Live cell imaging videos were analyzed using NIS-Elements software.

2.2.5 Live Cell Imaging with MitoTracker or MitoSox

HeLa cells were seeded onto 35mm glass-bottom dishes and transfected with either control siRNA (siC) or siRNA targeting Kid (siK#1) for 48h. Old medium was removed and replaced with prewarmed (37°C) staining solution containing 20nM MitoTracker® Green FM or 5μM MitoSOX™ Red mitochondrial superoxide indicator. Cells were incubated at 37°C for 15min and washed three times with prewarmed (37°C) buffer. Cells were imaged using Leica SP5 Microscope for 5min.

2.2.6 Preparation for TIRFM

90% confluent HeLa cells were transfected with mCherry-paxillin plasmid DNA for 24h, followed by transfection with either control siRNA (siC) or siRNA targeting Kid (siK#1 or siK#2) for 48h on 35mm glass-bottom culture dishes. Cells were subjected to Nikon Eclipse Ti-E microscopy using 568nm laser to monitor the intensity of mCherry-paxillin signal over time. Images were taken by Dr. Simon Watkins every 10min for 1h and analyzed by myself using Imaris software.

2.2.7 Rho Inhibitor Assay

HeLa cells were transfected with either control siRNA (siC) or siRNA targeting Kid (siK#1 or siK#2). After 44h, cells were incubated in serum-free medium (DMEM) either with or without 0.5 μg/ml Rho inhibitor for 4h, followed by immunostaining against vinculin and F-actin. Images were taken using Olympus Fluoview 1000 Confocal Microscope with a 60X objective.

2.2.8 Microtubule-Induced FA Disassembly Assay

HeLa cells were transfected with either control siRNA (siC) or siRNA targeting Kid (siK#1 or siK#2). After 24h, cells were washed several times with serum-free medium (DMEM+10mM HEPES) and left in serum-free medium (DMEM+10mM HEPES) for 18h. Cells were then treated with 10μM nocodazole for 1h and released for various time points (0min, 15min, 30min, 60min, and 120min), followed by immunostaining against paxillin. Images were taken using Olympus BX60 Epifluorescence Microscope with a 100X objective.

2.2.9 FAK Inhibitor Assay

HeLa cells were transfected with either control siRNA (siC) or siRNA targeting Kid (siK#1 or siK#2). Medium was changed after 24h with fresh DMEM+10%FBS and cells were incubated either with or without 5μM FAK inhibitor for another 24h, followed by immunostaining against vinculin. Images were taken using Olympus Fluoview 1000 Confocal Microscope with a 60X objective.

2.2.10 Preparation for SEM

HeLa cells were seeded onto 12mm coverslips and transfected with 3nM siRNA targeting Kid (siK#1) for 48 hours. Untreated HeLa cells (Un), and cells treated with control siRNA (siC) were used as controls. After 48h, samples were prepared for SEM under room temperature as follows. First, cells were fixed with 2.5% glytaraldehyde for 1h followed by three times of wash with PBS. Secondly, cells were post-fixed with OsO4 for 1h followed by three times of wash with PBS. Then, cells were dehydrated by washing orderly with 30% Ethanol once, 50% Ethanol once, 70% Ethanol once, 90% Ethanol once, and 100% Ethanol once. Finally, cells were treated with hexamethyldisilazane (HMDS) for 15min and air dried overnight. Samples were sputter coated with palladium (4.5nm thick) and imaged using JSM 6330F Scanning Electron Microscope.

2.2.11 Preparation for STEDM

HeLa cells were passaged the day before immunofluorescence to reach 70-80% confluency. Cells were rinsed three time with PBS and fixed in 2% PFA for 15min. Cells were then rinsed three times with PBS followed by three times of washing with PBS with 5min for each time. After that, cells were permeabilized with 0.1% Triton for 10min followed by three times of rinsing with PBS. Cells were blocked with 2% BSA for 1h and incubated with primary antibodies for 1h followed by three times of washing with PBS with 5min for each time. Cells were incubated with secondary antibodies for 1h followed by three times of washing with PBS with 5min for each time. Cells were mounted and imaged using Leica TCS SP8 STED 3X microscope.

3.0 RESULTS AND DISCUSSION

3.1 LOCALIZATION OF KID TO THE SITES OF FAS

3.1.1 Kid localizes to the sites of FAs

Our immunofluorescence results from several cell lines showed that Kid colocalized with vinculin, which is a marker for FAs (Fig. 11). Note that a major population of Kid localized to the nucleus, which is consistent with a previous discovery that Kid contains a nucleic acid binding domain and binds to chromosomes during mitosis (Tokai et al. 1996). In order to test the specificity of this antibody, HeLa cells were treated with either of two different siRNAs (siK#1 and siK#2) targeting Kid. The signal of Kid greatly diminished both in the nucleus and at the FAs (Fig. 12, Kid and vinculin). Western blot analysis showed more than 90% depletion of Kid after knockdown using either of the two siRNAs (Fig. 13). In addition, a second antibody which recognizes a different epitope of Kid showed staining of Kid at FAs in NIH-3T3 and RPE1-hTERT cells (Fig. 14). These controls confirmed Kid's localization at the sites of FAs.

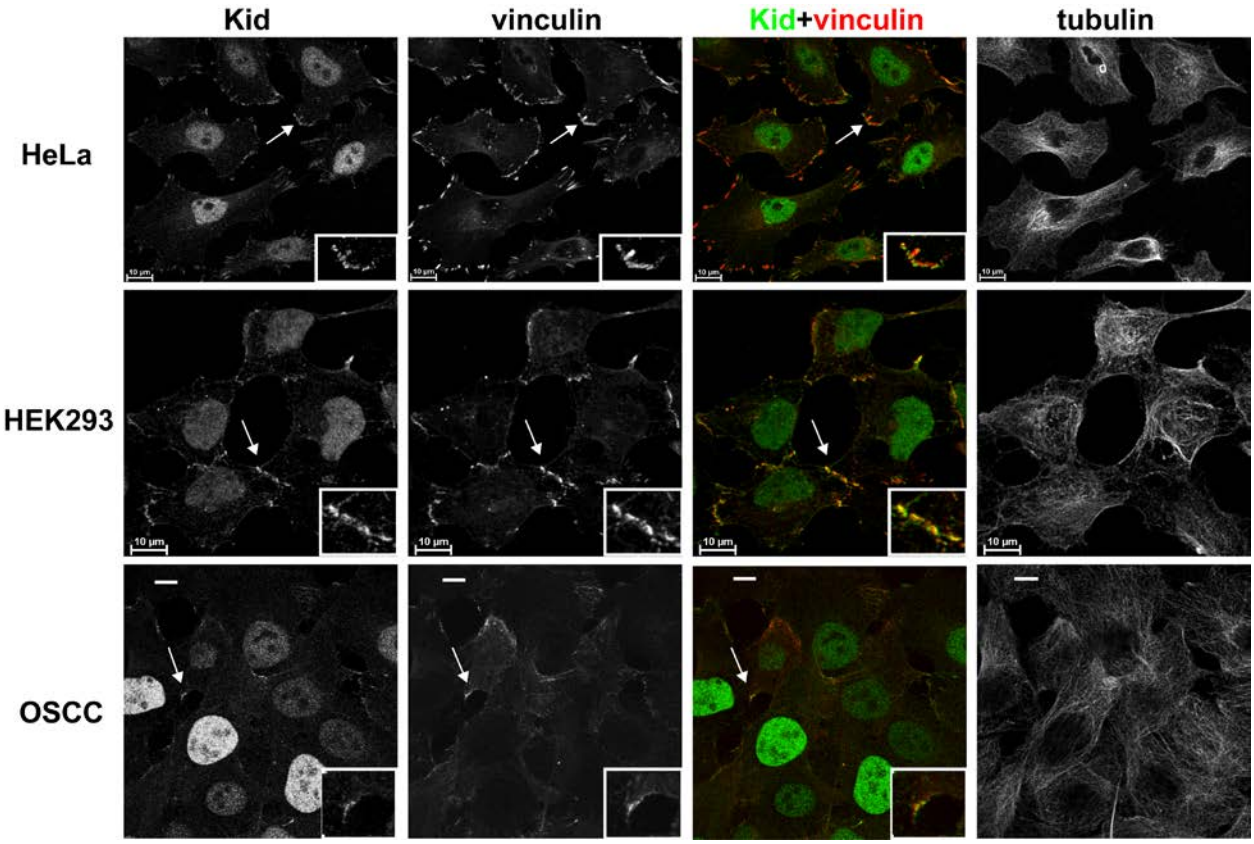


Figure 11. Kid localizes to the sites of FAs

HeLa, HEK293, and OSCC cells were immunostained against Kid, vinculin, and tubulin. Images were taken using an Olympus Fluoview 1000 Confocal Microscope with a 60X objective. Arrows point to sites of FAs which were zoomed in and shown in the inset boxes. Scale bar, 10µm.

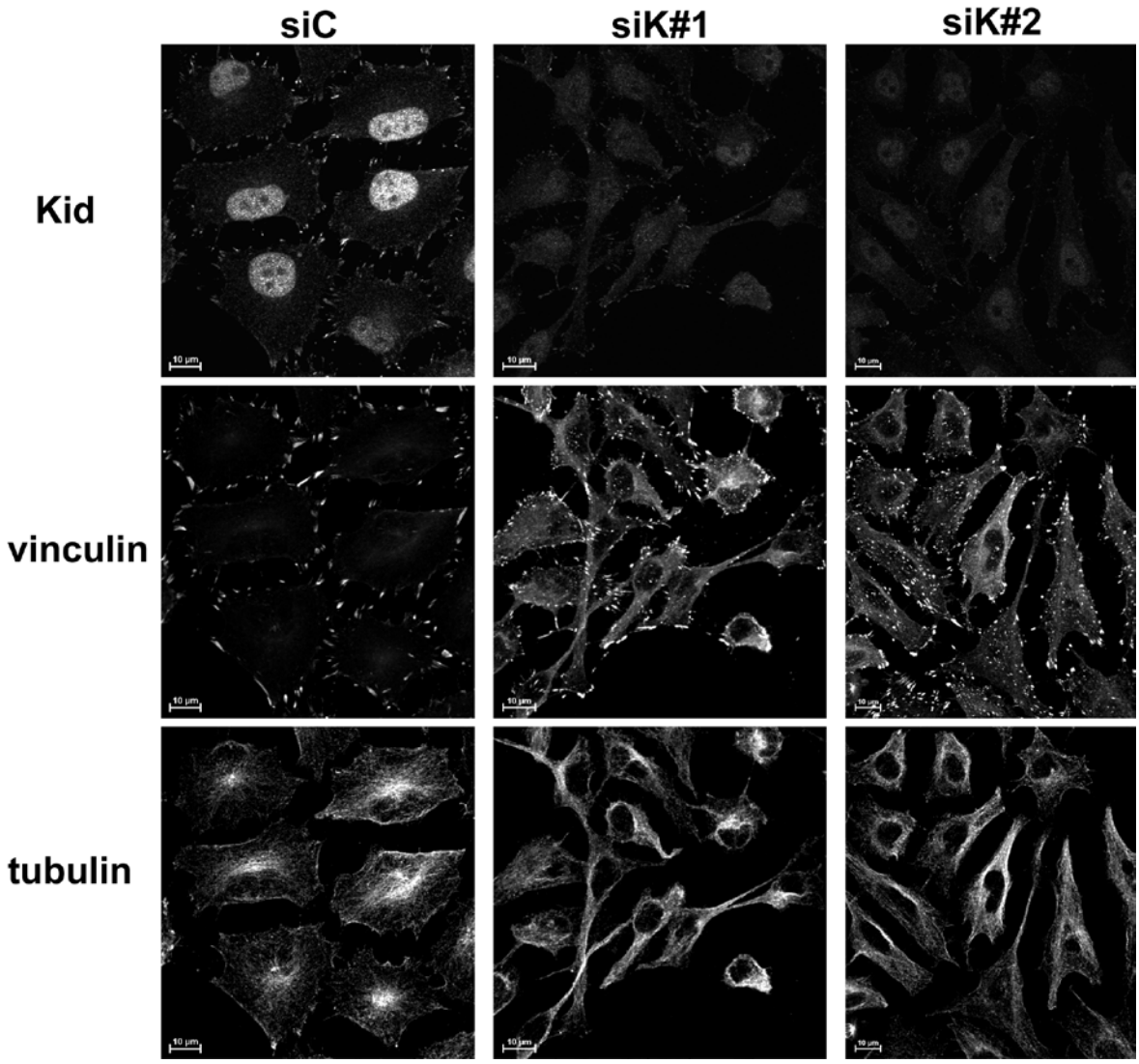


Figure 12. The signal of Kid greatly diminished both in the nucleus and at the FAs after knockdown of Kid

HeLa cells were transfected with either control siRNA or siRNA targeting Kid for 48h and immunostained against Kid, vinculin, and tubulin. Images were taken using an Olympus Fluoview 1000 Confocal Microscope with a 60X objective. Scale bar, 10μm.

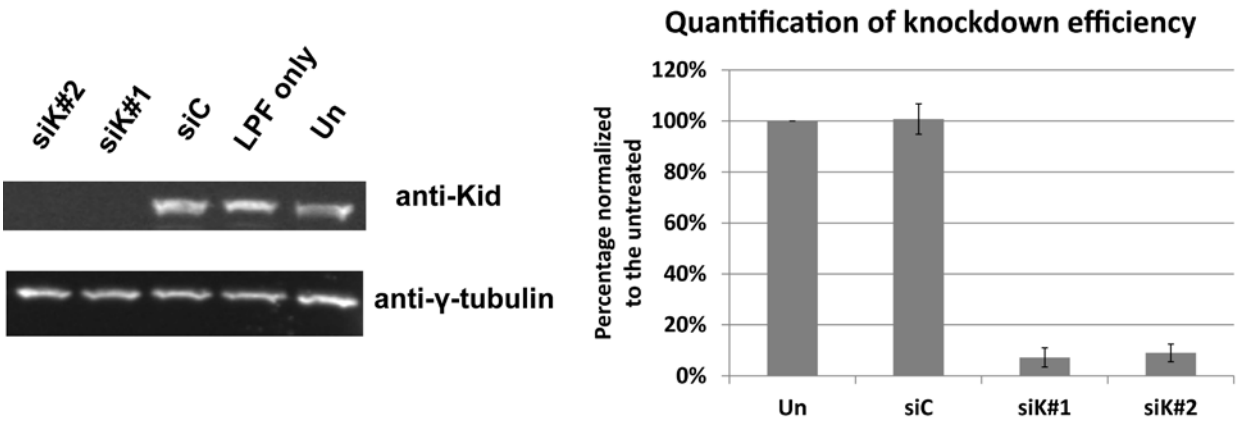


Figure 13. Knockdown efficiency of siRNAs targeting Kid

(A) Western blot was performed to examine knockdown efficiency of two different siRNAs targeting Kid in HeLa cells. (B) Quantification of knockdown efficiency from three independent experiments.

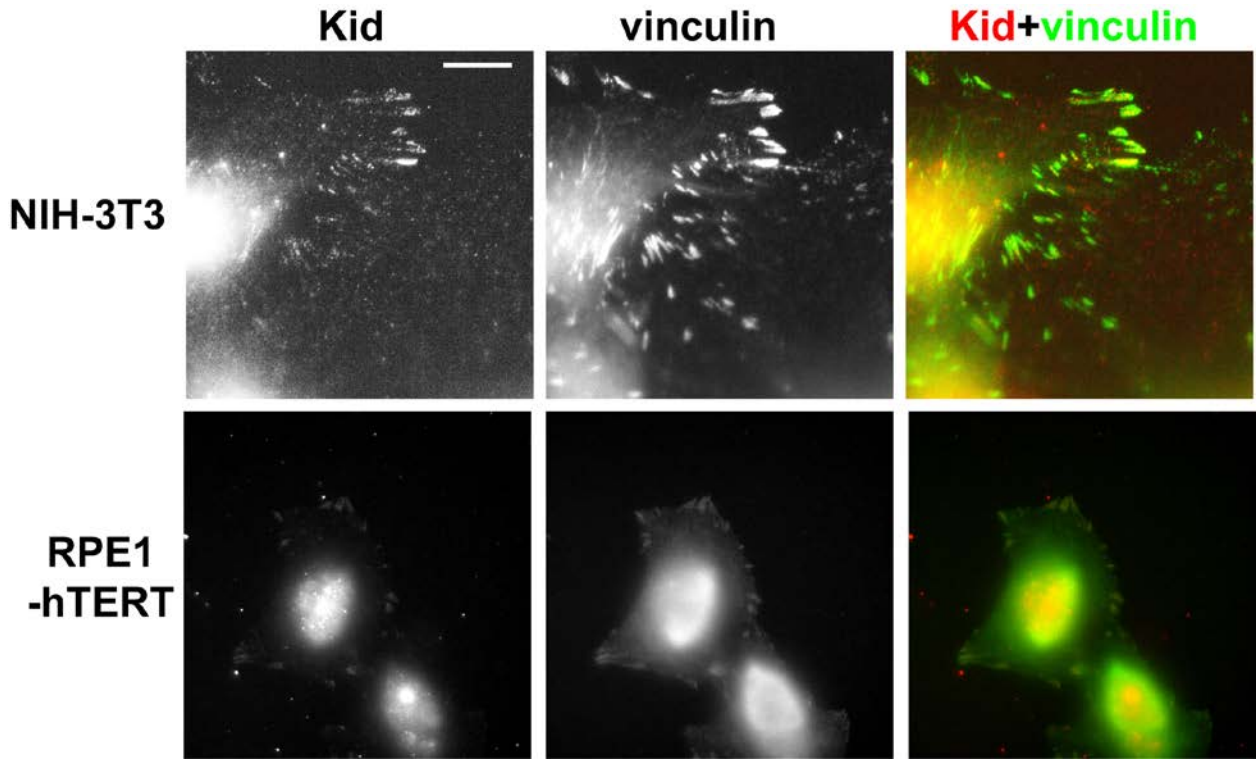


Figure 14. Detection of Kid at FAs in NIH-3T3 and RPE1-hTERT cells using a second antibody

NIH-3T3 or RPE1-hTERT cells were immunostained against Kid and vinculin. Images were taken using an Olympus BX60 Epifluorescence Microscope with a 100X objective. Scale bar, 10μm. Images of RPE1-hTERT cells were contributed by Dr. Kristen Bartoli, a former graduate student in the lab.

3.1.2 Localization of Kid to the sites of FAs is microtubule-independent

Since Kid is a microtubule motor and microtubules are involved in adhesion disassembly, we tested if the localization of Kid to the sites of FAs is dependent on intact microtubules. We treated HeLa cells with 10μM nocodazole for 4h which was sufficient to eliminate microtubule staining (Fig. 15, tubulin); however, Kid still localized to FAs when the structure of microtubules was not intact (Fig. 15, Kid+vinculin). Although we cannot rule out the possibility that microtubules are still required for the initial delivery of Kid to FAs, this result suggested that at least the maintenance of Kid's localization at FAs does not depend on intact microtubules.

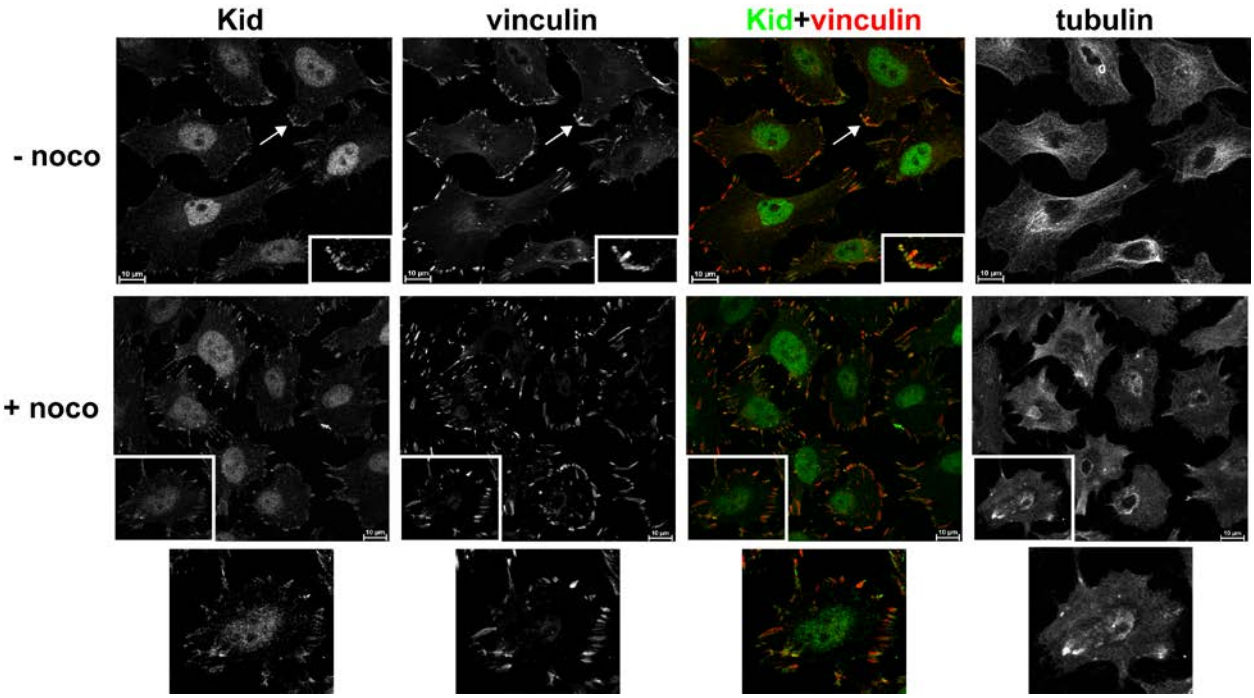


Figure 15. Kid localizes to the sites of FAs in a microtubule-independent manner

HeLa cells were treated with 10μM nocodazole for 4h followed by immunostaining against Kid, vinculin, and tubulin. Lower images were zoomed in of cells in the boxes of upper images. Images were taken using an Olympus Fluoview 1000 Confocal Microscope with a 60X objective. Scale bar, 10μm.

3.1.3 Exploring localization of Kid at different strata of FAs

Using super-resolution photoactivated localization microscopy (iPALM), Kanchanawong *et al.* discovered three layers at the core region of FAs between integrins and actin cytoskeleton, which include an integrin-signaling layer with marker proteins integrin, FAK and paxillin, a middle force-transduction layer with marker proteins vinculin and talin, and an actin-regulatory layer with marker proteins α-actinin and zyxin (Kanchanawong et al. 2010). We had the opportunity to use another super-resolution microscopy technique called STED (STimulated Emission Depletion) to explore to which layer of this FA core region Kid localizes. HeLa cells were immunostained with Kid and paxillin, or Kid and vinculin, or Kid and zyxin. However, Kid was

found to colocalize with all of these proteins using STED (Fig. 16). It was probably due to the different resolutions between these two types of microscopy technologies. The core region of an FA is about 40nm and iPALM allows localization accuracy of 20nm in the lateral dimension which was able to differentiate between those multiple layers (Kanchanawong et al. 2010). In comparison, the lateral resolution of STED is 30-80nm and might not be sufficient to separate the strata.

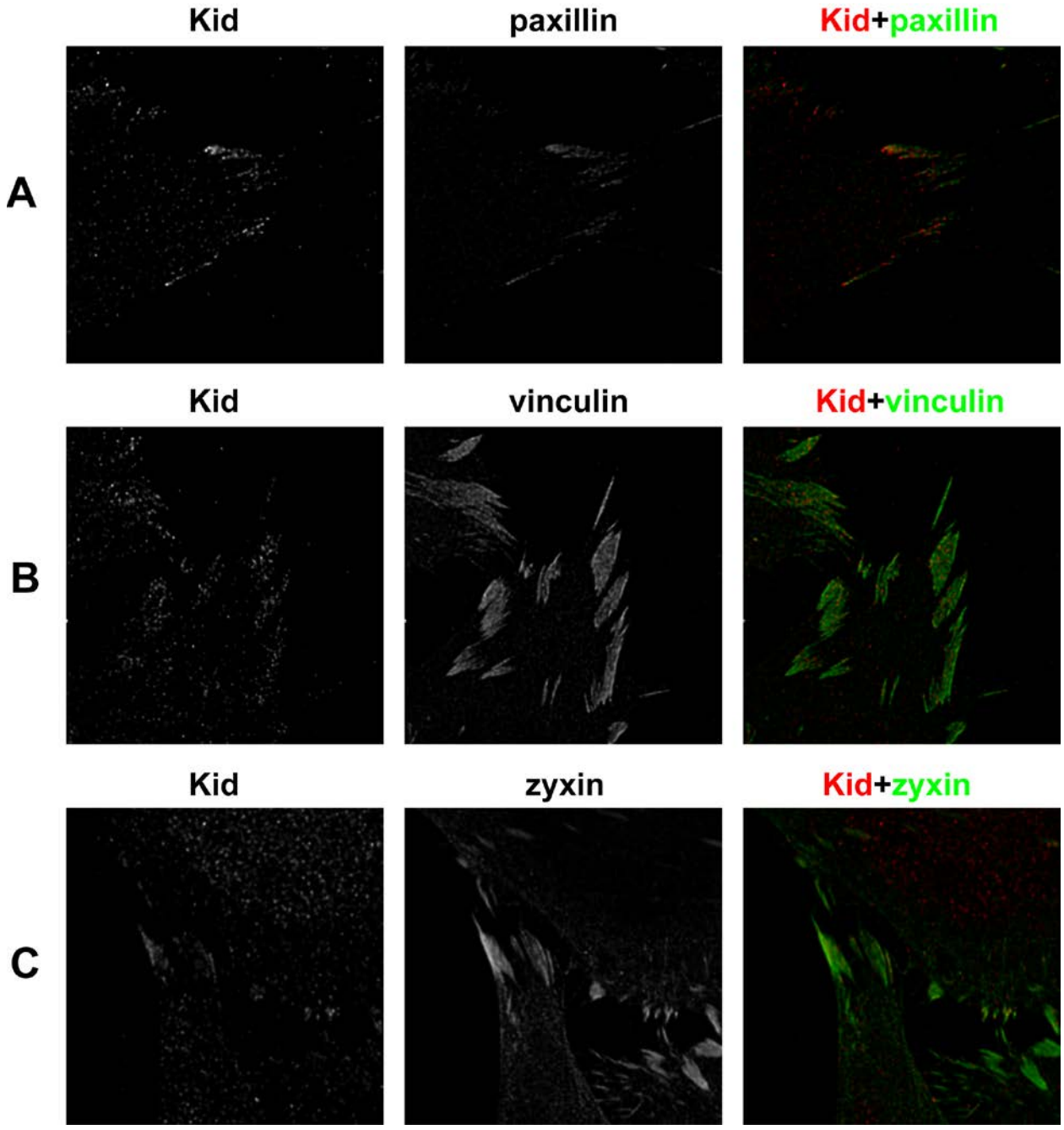


Figure 16. Colocalization of Kid with FA components using STED microscopy

HeLa cells were immunostained against (A) Kid and paxillin, or (B) Kid and vinculin, or (C) Kid and zyxin. Images were taken by Kristofer Fertig using a Leica TCS SP8 STED 3X microscope.

3.1.4 Discussion and future direction

Previous studies have shown that Kid mainly localizes to the nucleus, which is consistent with its role as a chromokinesin. In agreement with that, our immunofluorescence experiments showed prominent staining of Kid in the nucleus. Interestingly, we also observed a distinct localization of Kid at FAs, which was confirmed by independent antibodies and the reduction in immunofluorescence signal following siRNA targeting Kid. Unfortunately, a tGFP-tagged plasmid encoding Kid failed to localize to FAs in most of the cells albeit it still localizes to the nucleus. This could be due to the suppression of pY397FAK in those cells overexpressing Kid and it would be interesting to see if the localization of endogenous Kid is also abolished in the presence of tGFP-Kid.

We attempted to use super-resolution microscopy STED to dissect which layer of FA Kid belongs to. If localized to the membrane-exposed integrin signaling layer (marker proteins: FAK and paxillin), it could suggest a role for Kid in signaling delivery, activation, or transduction. If localized to a force-transduction layer (marker proteins: vinculin and talin), it might suggest a role for Kid in force generation required in adhesion maturation. If localized to the actin-regulatory layer (marker proteins: α-actinin and zyxin), it could suggest involvement of Kid in the actin cytoskeleton. Due to resolution limitation, STED was not able to fully separate those three strata of FAs under our experimental setting and Kid showed colocalization with all three marker proteins for each layer (paxillin, vinculin, and zyxin). However, Kid appeared to show slightly stronger signal in the colocalization with paxillin, which is consistent with its role in regulating phosphorylation levels of FAK and paxillin in adhesion disassembly, which will be shown in Section 3.4.

What surprised us initially was that localization of Kid to FAs did not seem to depend on microtubules, as pharmacological depolymerization of microtubules using nocodazole did not abolish the colocalization, yet Kid is still involved in the process of microtubule-induced FA disassembly. Although this result cannot rule out the possibility that microtubules are still required for the initial recruitment of Kid to FAs, it suggested that at least maintenance of Kid at FAs is microtubule-independent. It is, however, not uncommon that localization of some FA components does not rely on microtubules. One group of the microtubule +TIPs proteins, CLASPs, has been shown to localize around FAs in a microtubule-independent manner. It binds to a peripheral membrane protein LL5β which recruits CLASPs to FAs to facilitate FA disassembly, although how LL5β clusters form around FAs is not well understood (S. J. Stehbens et al. 2014). Clathrin and dynamin, which are proteins regulating endocytosis known to contribute to FA turnover, also accumulate at FAs independent of microtubules (Ezratty et al. 2009) (Chao and Kunz 2009). Kid could be recruited to FAs in a non-canonical way like these examples.

3.2 LOSS OF KID LEADS TO CHANGES IN THE DENSITY, DISTRIBUTION, AND SIZE OF ADHESIONS

3.2.1 Knockdown of Kid results in an increase in adhesion density and a change in adhesion distribution

As Kid localizes to the sites of FAs, we wondered whether loss of Kid may cause any change, i.e., number and/or distribution, to FAs. Since HeLa cells showed the most robust Kid signal at FAs among all cell lines we checked, they were chosen for further studies. Briefly, HeLa cells were transfected with siRNAs targeting Kid for 48h, followed by immunostaining against vinculin. In HeLa cells either left untreated (Un) or treated with a scrambled control siRNA (siC), most FAs localized to the peripheral edges of the cell (Fig. 17). After knockdown of Kid using either of the two different siRNAs (siK#1 or siK#2), there were more visible adhesion structures, and they were dispersed throughout the surface of the cell (Fig. 17). We performed quantitative analysis which showed significant differences in the adhesion density and distribution between control cells and cells after knockdown of Kid (Fig. 18). Loss of Kid caused an increase in the density of adhesions (defined as the number of adhesions in each cell divided by the area of the cell, $1/\mu m^2$) (Fig. 18A). This resulted from increased densities both at the cell periphery and in the central cell surface (Fig. 18, C-D). Consistent with the increased adhesion density, the distribution of adhesions changed from mostly at the cell periphery to the whole ventral cell surface (Fig. 18B).

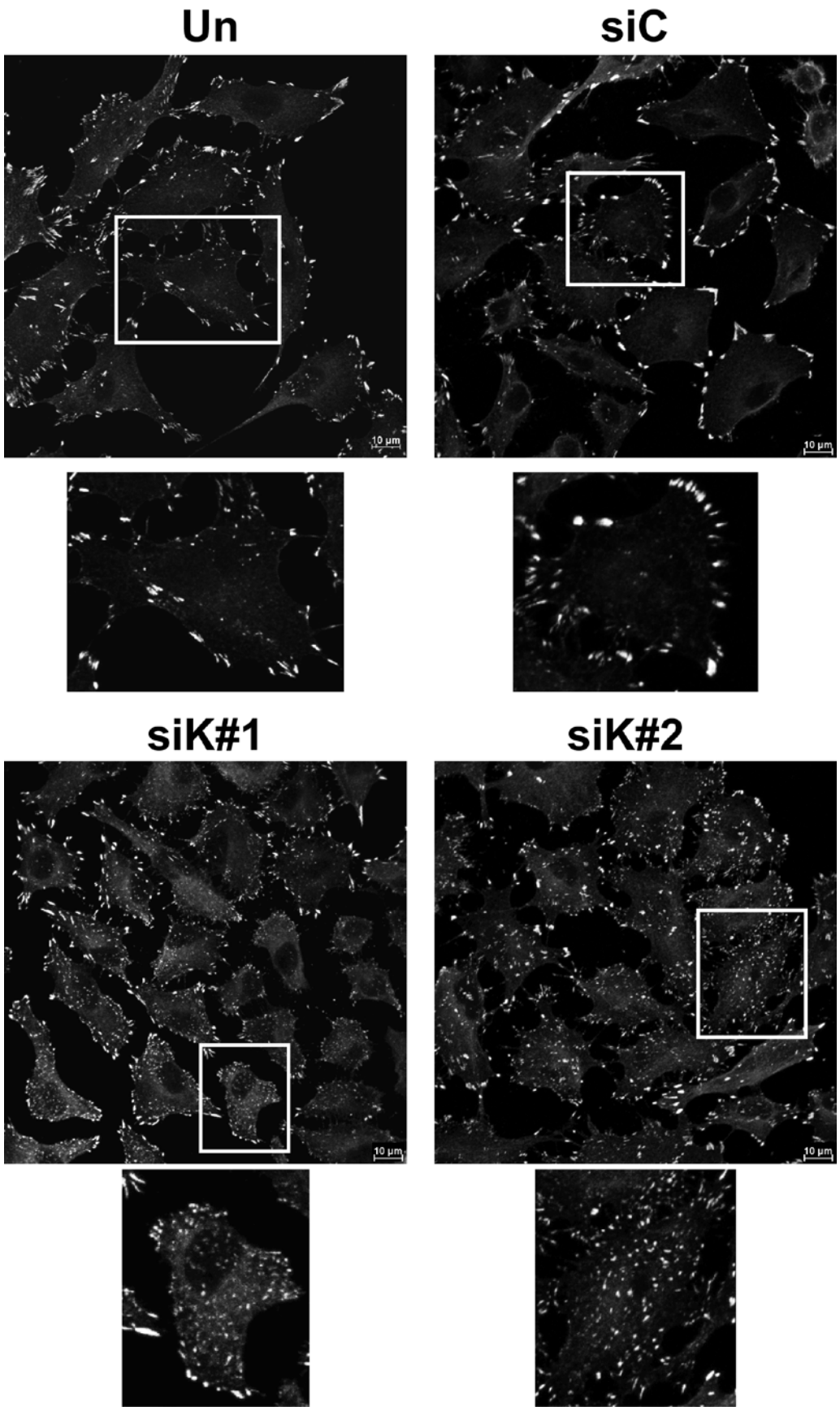

Un
siC
siK#1
siK#2
10 µm
10 µm
10 µm
10 µm

Figure 17. Knockdown of Kid caused changes in the density and distribution of adhesions

HeLa cells were either left untreated (Un) or transfected with control siRNA (siC) or siRNA targeting Kid (siK#1 or siK#2) for 48h and immunostained against vinculin. Images were taken using an Olympus Fluoview 1000 Confocal Microscope with a 60X objective. Representative cells under each condition were boxed and zoom-in images were shown below their corresponding original images. Scale bar, 10μm.

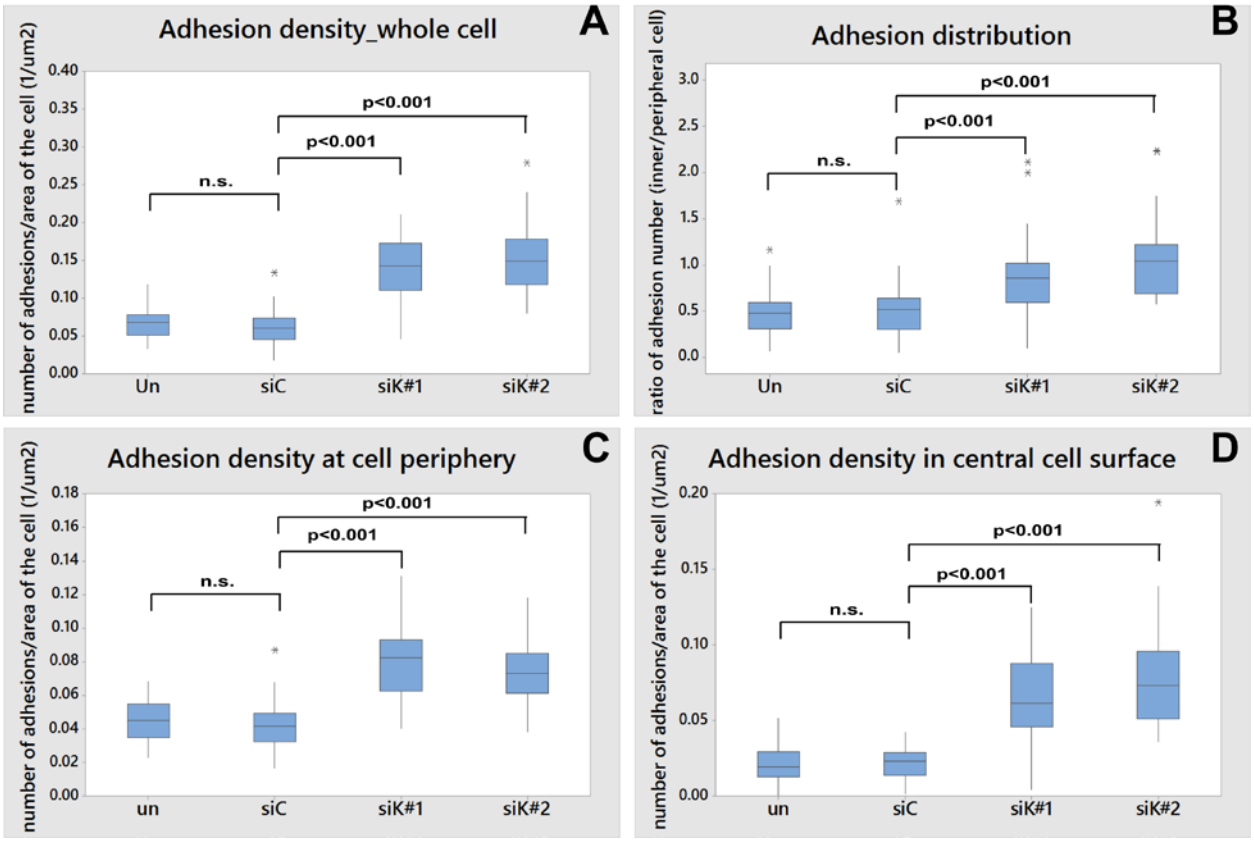


Figure 18. Quantification of adhesion density and distribution after knockdown of Kid

50 cells in each group (Un, siC, siK#1, and siK#2) from three independent experiments were quantitatively analyzed using Minitab software. Asterisks (*) in the boxplot represent data points that are outliers within the analyzed group. P values were calculated by 2-sample t-test in this figure and all subsequent figures of this type. Results showed that knockdown of Kid resulted in (A) an increase in adhesion density, including both at peripheral cell (C) and in the central cell surface (D), and (B) a change in adhesion distribution from peripheral edges of a cell to the whole cell surface.

3.2.2 Knockdown of Kid results in an increased population of nascent adhesions or focal complexes

As shown in Fig. 17, the newly appearing puncta positive for vinculin staining throughout the cell surface after knockdown of Kid seemed to be much smaller than the peripheral adhesions in control cells. Therefore, we analyzed and compared the size of adhesions before and after knockdown of Kid. We categorized the percentage of nascent adhesions or focal complexes (defined as 0-1μm, FCs for short throughout the rest of this dissertation) and mature FAs (defined as 1-5μm). Our data showed an increase in the proportion of FCs in cells after knockdown of Kid compared to control cells (Fig. 19).

Furthermore, we investigated the composition of those newly appearing puncta throughout the cell surface. Two markers were selected: paxillin phosphorylated at Tyrosine 118 (pY118pax), which is prominent in FCs, and zyxin, which is a scaffolding protein that incorporates into adhesions at a later maturation stage (Ronen Zaidel-Bar et al. 2003; Ronen Zaidel-Bar, Milo, et al. 2007; Zamir, Geiger, and Kam 2008). Most of those puncta were positively stained for pY118pax but did not stain for zyxin, therefore likely to be FCs instead of mature FAs, suggesting defects in adhesion maturation and/or disassembly (Fig. 20).

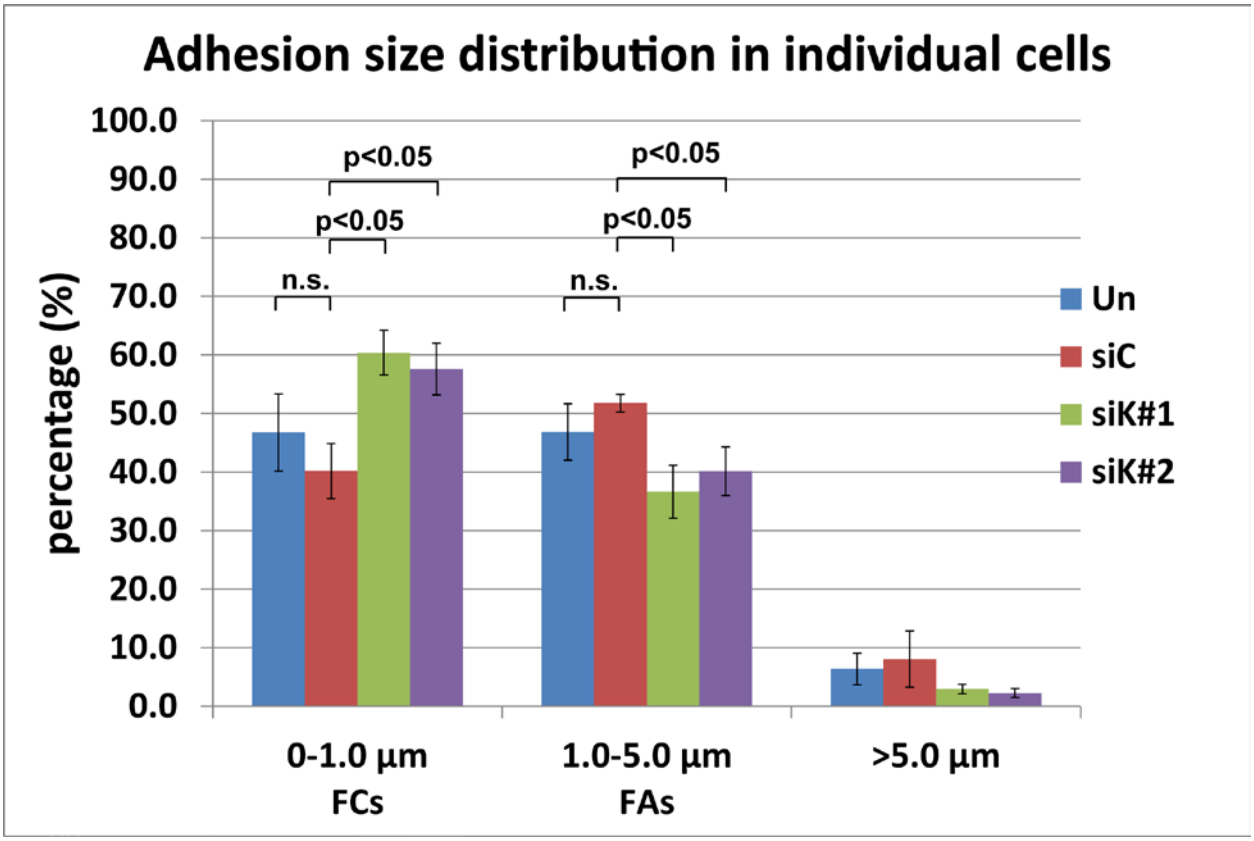


Figure 19. Knockdown of Kid caused an increase in the proportion of nascent adhesions or focal complexes versus mature focal adhesions

50 cells in each group (Un, siC, siK#1, and siK#2) from three independent experiments were quantitatively analyzed using Minitab software. P values were calculated by 2-sample t-test in this figure and all subsequent figures of this type. Results showed that knockdown of Kid resulted in an increase in the proportion of nascent adhesions or focal complexes (FCs) versus mature focal adhesions (FAs).

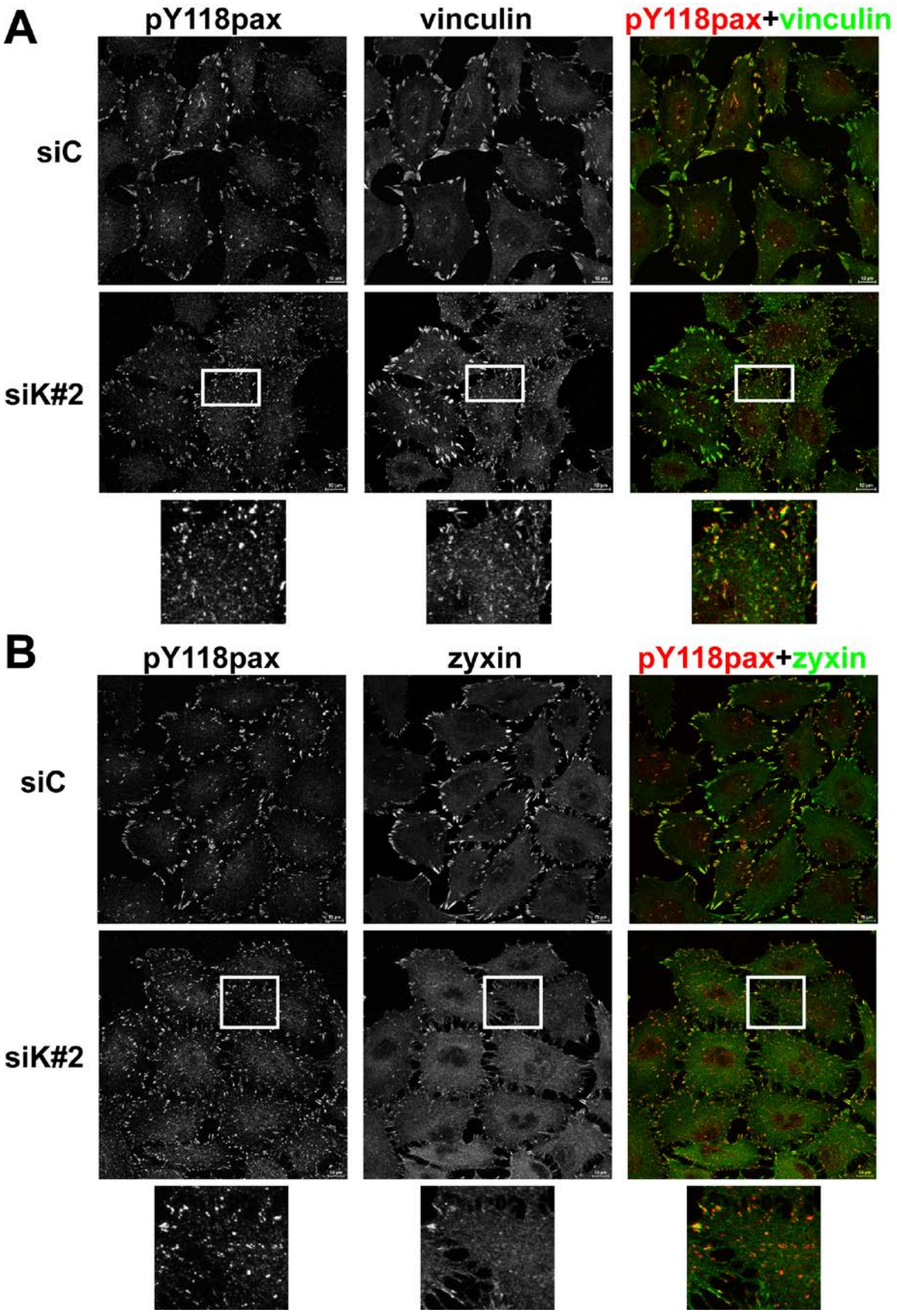

A
pY118pax
vinculin
pY118pax+vinculin
siC
siK#2
B
pY118pax
zyxin
pY118pax+zyxin
siC
siK#2

Figure 20. Newly appearing adhesion structures distributed throughout the central cell surface after knockdown of Kid are likely nascent adhesions or focal complexes

HeLa cells transfected with siRNA for 48h were immunostained against either (A) vinculin and pY118paxillin, or (B) zyxin and pY118paxillin. Images were taken using an Olympus Fluoview 1000 Confocal Microscope with a 60X objective. Representative areas were boxed and zoom-in images were shown below their corresponding original images. Scale bar, 10μm.

3.2.3 The phenotypic changes of adhesions due to loss of Kid requires actin polymerization

Initial formation of NAs requires actin polymerization. Therefore, we treated cells with Cytochalasin D, an inhibitor for actin polymerization by capping the barbed end of actin filaments, to see if it can suppress the phenotypic changes of adhesions caused by loss of Kid. Indeed, both control cells and cells after knockdown of Kid lost almost all adhesion structures when treated with Cytochalasin D (Fig. 21), indicating that the increased adhesion density due to loss of Kid requires actin polymerization.

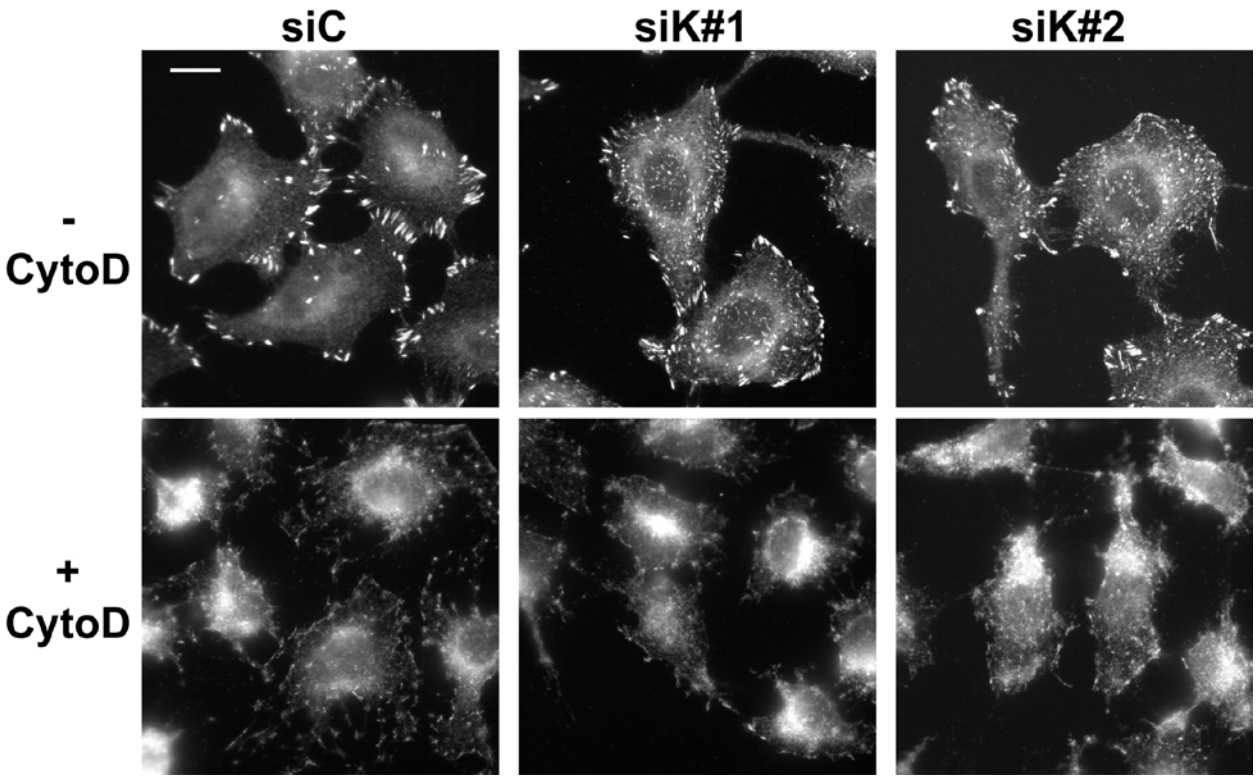


Figure 21. The phenotypic changes of adhesions after knockdown of Kid requires actin polymerization

HeLa cells were transfected with control siRNA or siRNA targeting Kid for 48h and then incubated with fresh medium either with or without 5μM Cytochalasin D for 45min. Cells were immunostained for vinculin. Images were taken using an Olympus BX60 Epifluorescence Microscope with a 100X objective. Scale bar, 10μm.

3.2.4 The phenotypic changes we observed after knockdown of Kid are not due to altered mitotic frequency or cell proliferation

As Kid is known to play important roles during mitosis, it is necessary to rule out the possibility that the phenotypes we observed due to loss of Kid were caused by indirect effects from changes in the cell cycle or cell proliferation. In order to examine this, HeLa cells were stained for chromatin to check the mitotic index (defined as the percentage of cells undergoing mitosis in a population at a random time point) as well as Ki67 which is a marker for cell proliferation. As

shown in Fig. 22, knockdown of Kid for 48h neither changed the percentage of cells in a population that undergo mitosis nor affected cell proliferation, supporting that the phenotypic changes we observed after knockdown of Kid were not due to altered mitotic frequency or cell proliferation.

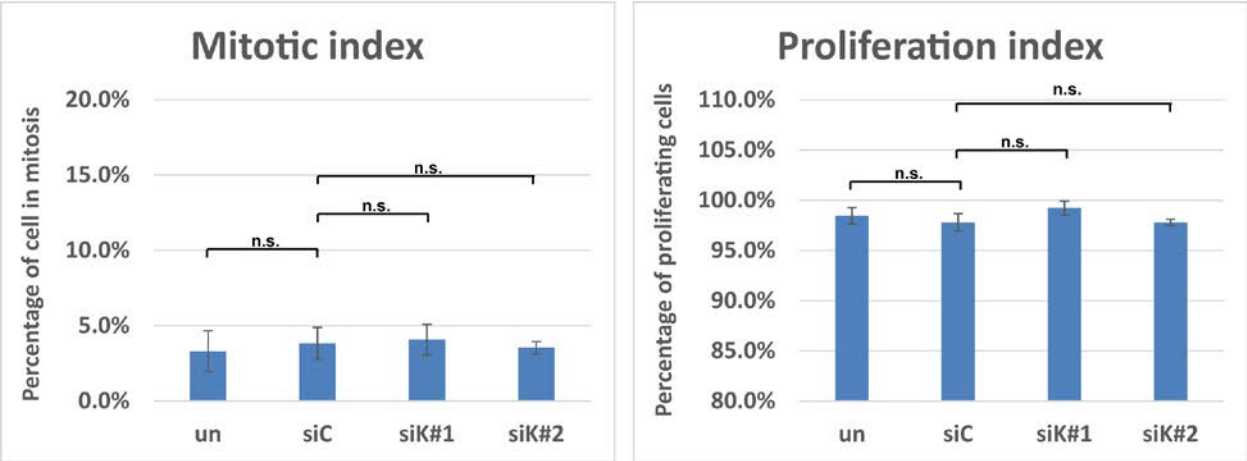


Figure 22. Knockdown of Kid for 48h did not affect mitotic index or cell proliferation

HeLa cells transfected with either control siRNA or siRNA targeting Kid for 48h were stained for either chromatin to check mitotic index or Ki67 to check proliferation index. Error bars represent standard deviations calculated from the results of three independent experiments.

3.2.5 Discussion and future direction

Studies exploring correlation between adhesion strength and FA organization demonstrated that only when cells at intermediate adhesion strength do they reach optimal FA number, size, and distribution across the lamellum leading to fastest migration (Gupton and Waterman-Storer 2006). At low adhesion strength, cells only showed peripheral FAs associated with peripheral F-actin bundles (E. A. Cox, Sastry, and Huttenlocher 2001; Gupton and Waterman-Storer 2006). At high adhesion strength, cells developed small FAs distributed throughout the ventral cell surface, correlated with increased rates in FA assembly and disassembly as well as decreased

rates in cell protrusion and retraction (Gupton and Waterman-Storer 2006). Knockdown of Kid caused phenotypic changes in adhesion size and distribution resembling that of cells at high adhesion strength. Our TIRF data showing an increased lifetime of FAs and wound healing assay showing a decreased migration rate also agreed with that of cells at high adhesion.

Adhesion strength affects distribution and activity of myosin II in the contractile module, which is located between the lamellipodium and near the nucleus and consists of the lamellum, which could in turn affect cell migration rate (Salmon, Adams, and Waterman-Storer 2002). Therefore, the decreased cell motility we observed after knockdown of Kid could be due to changes in myosin II distribution and/or activity. In addition, myosin II-dependent actin-crosslinking has been shown required for FA maturation (Choi et al. 2008). In our study, the lack of a maturation marker in most of the adhesions distributed at the central cell region after knockdown of Kid indicated potential defects in adhesion maturation, suggesting Kid might play a role in adhesion maturation. It would be interesting to investigate if myosin II functions downstream of Kid regulating adhesion strength and promoting adhesion maturation.

Different adhesion components incorporate into adhesion structures at different time. The lack of zyxin in a great number of adhesions at the ventral cell surface suggested that there might be a pool of focal complexes that are defective in maturation. Alternatively, this population could be due to impaired adhesion disassembly. One future direction is to use TIRF microscopy to explore the order of different adhesion components, including paxillin, FAK, vinculin, zyxin, to incorporate into adhesion structures and turn over after knockdown of Kid. In the meanwhile, we should be able to separate the different stages of individual adhesions (formation, maturation, turnover) and see which one(s) are affected by loss of Kid.

3.3 LOSS OF KID AFFECTS CELL SPREADING AND MIGRATION

3.3.1 Loss of Kid impaires the ability of cells to spread on fibronectin

As we observed those phenotypic changes of adhesions after knockdown of Kid, we wondered whether these changes have any consequence to cell behavior. As a preliminary test, HeLa cells treated with either control siRNA or siRNA targeting Kid for 48h were replated onto fibronectin-coated coverslips for either 15min or 60min and immunostained against vinculin and paxillin. For each time point, cells were categorized into two groups, rounded cells and spread cells. As shown by representative cells in Fig. 23 and Fig. 24, rounded cells were the ones that had settled down on the fibronectin-coated coverslips however not yet spread; whereas spread cells were the ones that had spread on the fibronectin and started protruding with the distinct structure of filopodia at cell periphery. At 15min, although both control cells and cells after knockdown of Kid had attached and presented filopodia, control cells appeared to spread much faster evident from their larger size and longer filopodia they exhibited (Fig. 23). This observation also held true for the 60min time point (Fig. 24). Quantification analysis showed that in control cells, 64% of cells already attached at 15min and the number went up to 97% at 60min. In contrast, only 37% of cells attached at 15min and 73% at 60min after knockdown of Kid, suggesting loss of Kid slowed cells spreading on fibronectin (Fig. 25).

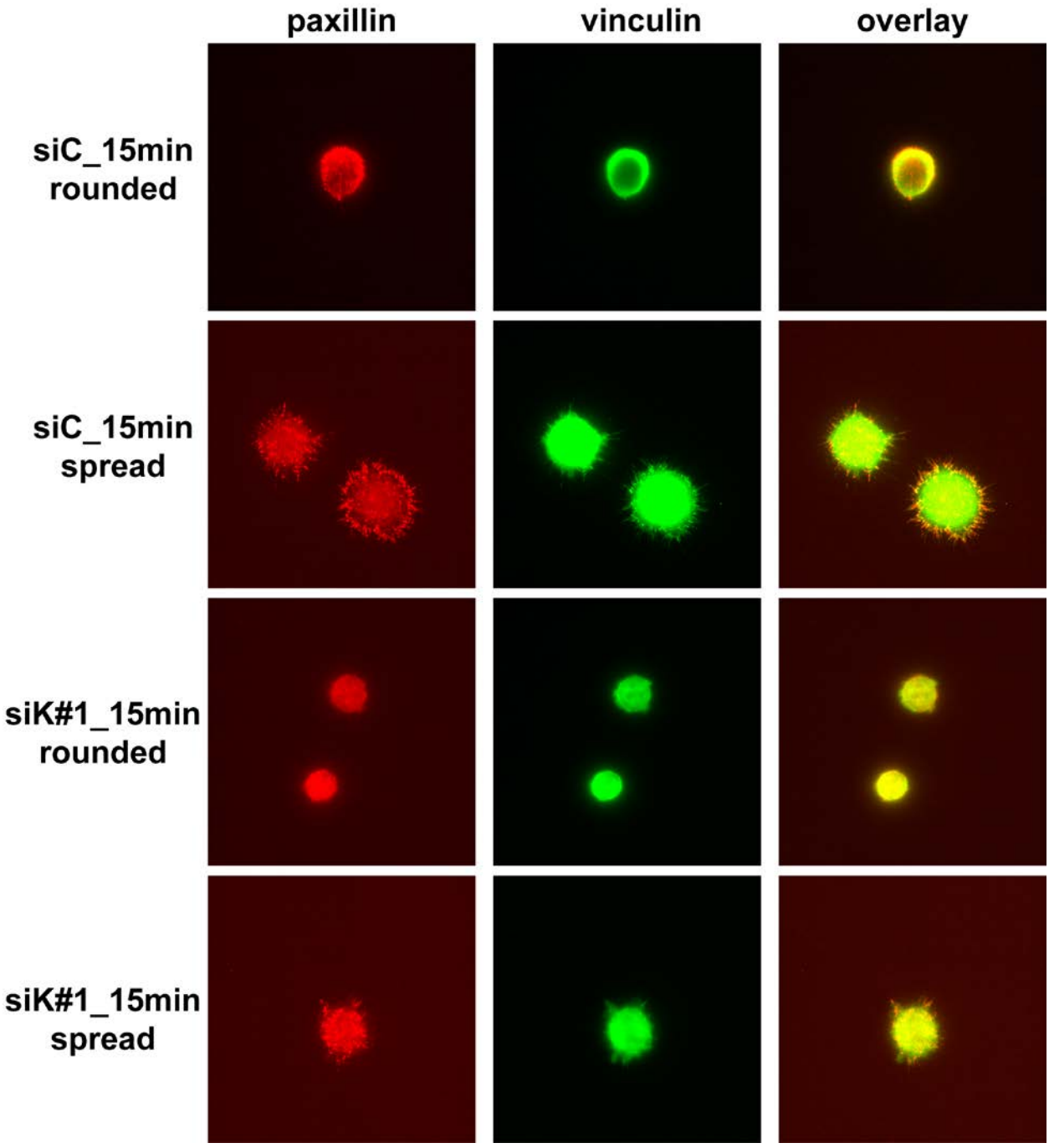


Figure 23. Knockdown of Kid impaired cell spreading on fibronectin

HeLa cells transfected with either control siRNA or siRNA targeting Kid for 48h were replated onto fibronectin-coated coverslips for 15min and immunostained against vinculin and paxillin. Images were taken using an Olympus BX60 Epifluorescence Microscope with a 100X objective.

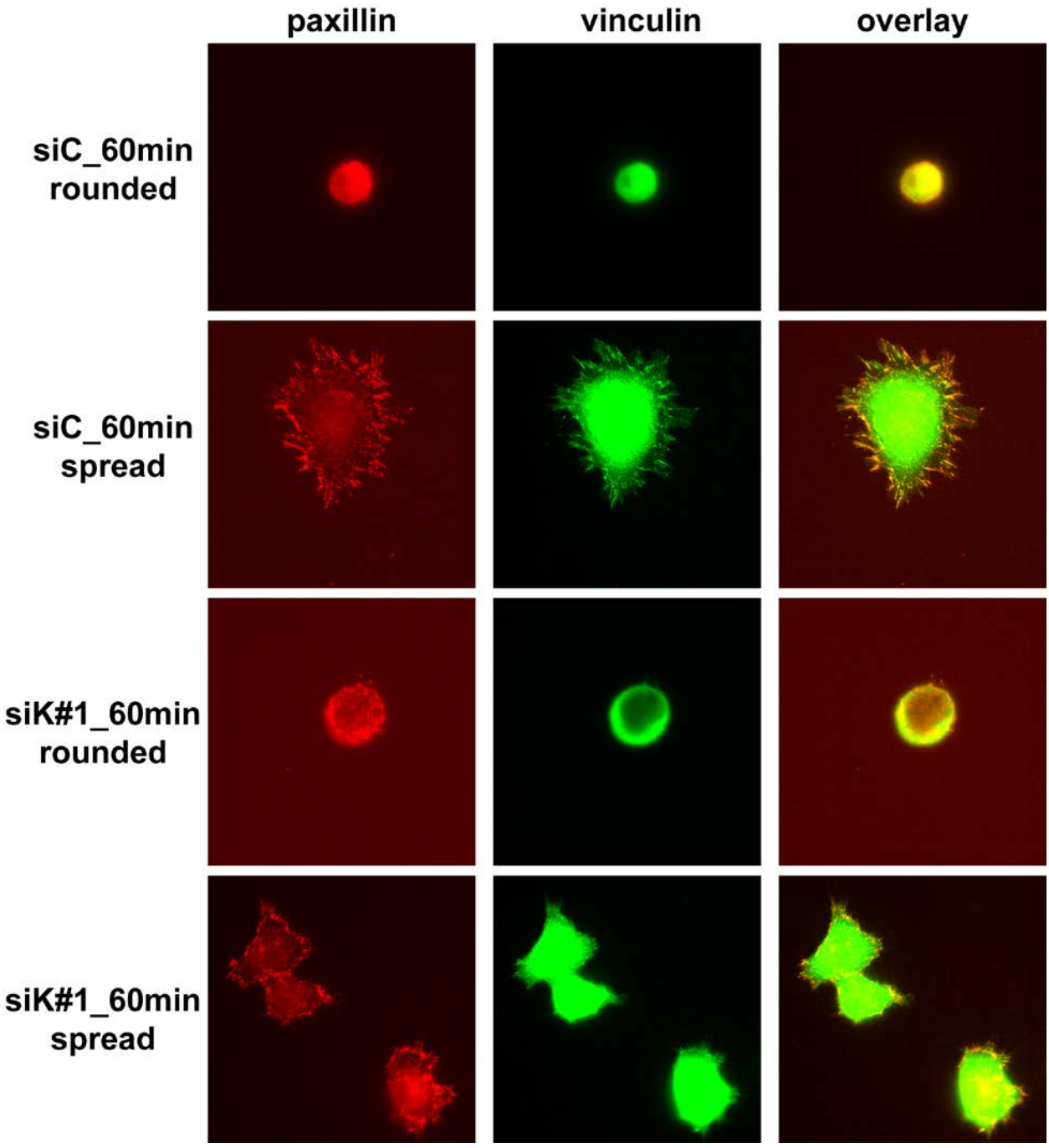


Figure 24. (Continued from Fig. 13) Knockdown of Kid impaired cell spreading on fibronectin

HeLa cells transfected with either control siRNA or siRNA targeting Kid for 48h were replated onto fibronectin-coated coverslips for 60min and immunostained against vinculin and paxillin. Images were taken using an Olympus BX60 Epifluorescence Microscope with a 100X objective.

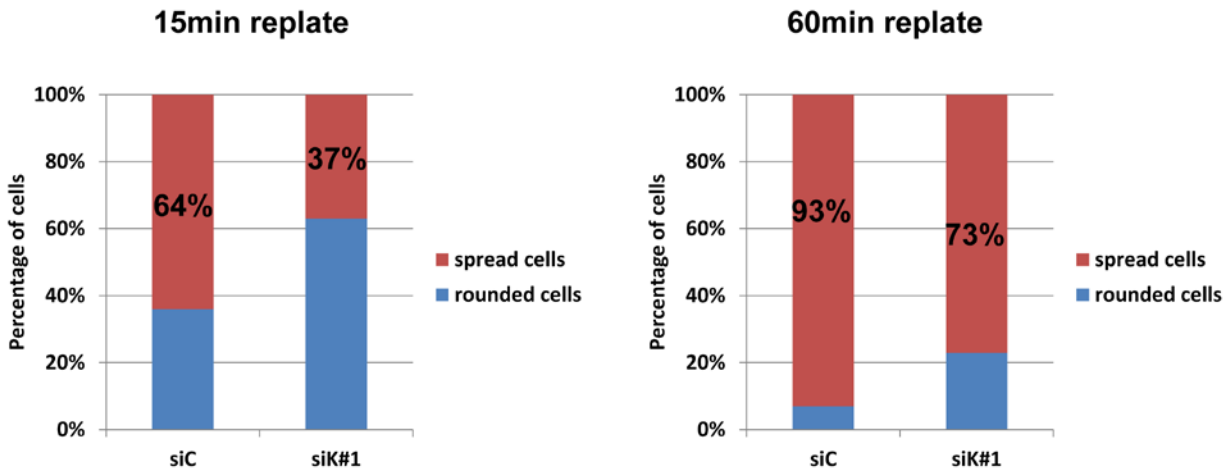


Figure 25. (Continued from Fig. 23 and Fig. 24) Knockdown of Kid impaired cell spreading on fibronectin

HeLa cells transfected with either control siRNA or siRNA targeting Kid for 48h were replated onto fibronectin-coated coverslips for 15min or 60min and immunostained against vinculin and paxillin. 200 cells from each time point of each group (siC, siK#1) was analyzed and quantified.

Next, we performed a time-course cell spreading assay in which both control cells and cells after knockdown of Kid were replated onto fibronectin for various time points and stained for F-actin and chromatin. As shown in Fig. 26A, at 10min, the earliest time point with sufficient attached cells to examine, both control and knockdown cells had just settled down and not yet spread. At 20min, control cells presented filapodia, indicating that cells were undergoing spreading. In comparison, cells treated with either of the two siRNAs targeting Kid (siK#1 or siK#2) spread more slowly; very few cells showed distinct filapodia, and even in those that did, their filapodia were much fewer and shorter. At 60min, while cells after knockdown of Kid were still in the process of spreading, most control cells had completely spread on the fibronectin-coated coverslips. Quantification analysis confirmed a decrease in the rate of cell spreading after knockdown of Kid (Fig. 26B).

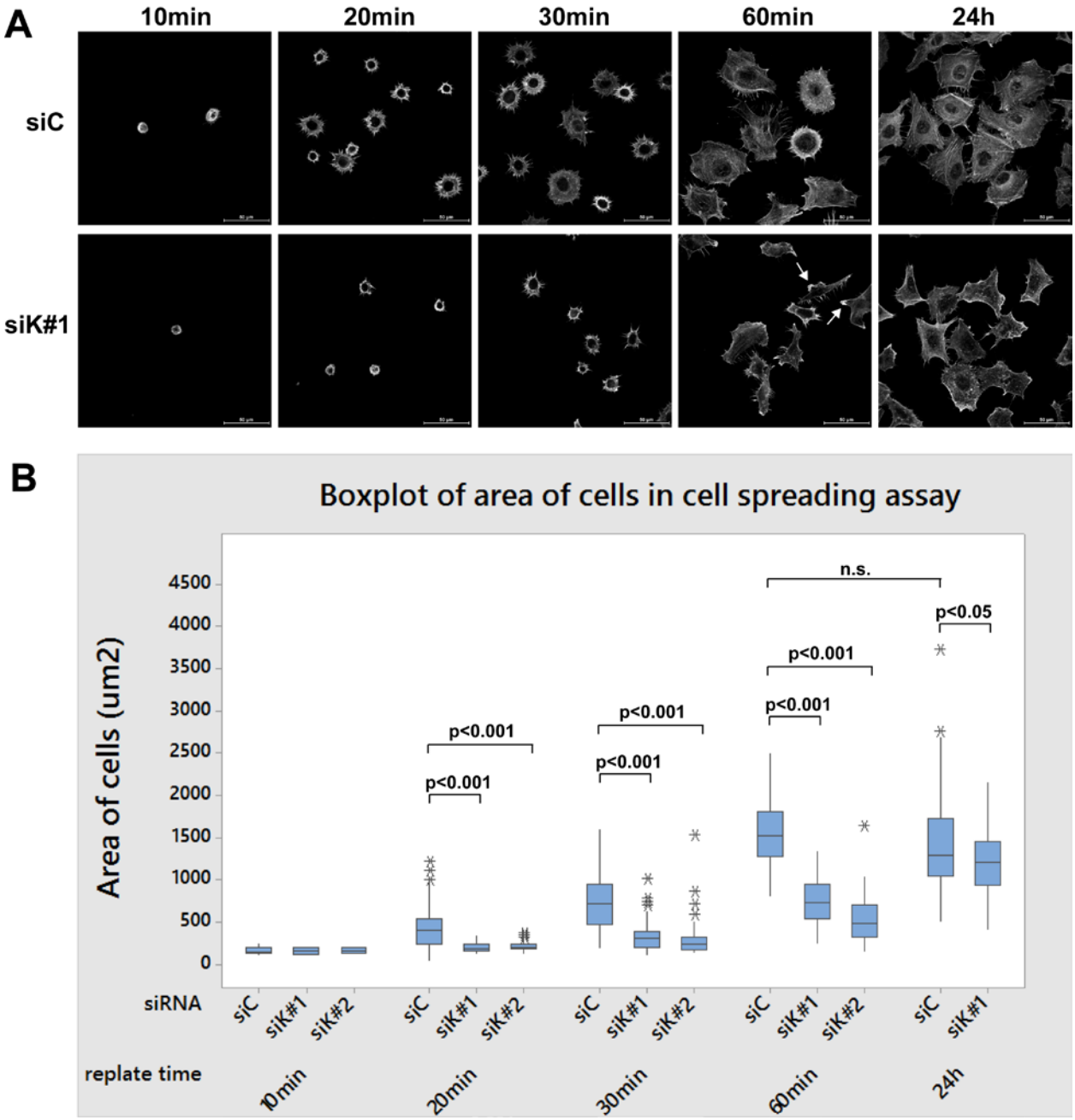


Figure 26. Loss of Kid caused a reduced rate in cell spreading

(A) HeLa cells transfected with control siRNA or siRNA targeting Kid for 48h and replated onto fibronectin-coated coverslips were fixed at different time points as indicated and stained for F-actin and chromatin. Images were taken using an Olympus Fluoview 1000 Confocal Microscope with a 60X objective. Arrows point to examples of localized condense F-actin. Scale bar, 50μm. (B) Quantification analysis by Minitab on 20-30 cells from each group at each time point demonstrated a reduced rate of cell spreading after knockdown of Kid.

3.3.2 Knockdown of Kid results in a decreased rate in cell migration

We also examined if cell migration was affected by loss of Kid. A classic wound healing assay was performed and the migration rates between control cells and cells after knockdown of Kid were compared. We found that loss of Kid resulted in a ~3-fold decrease in edge migration rate, strongly suggesting a defect in cell motility (Fig. 27).

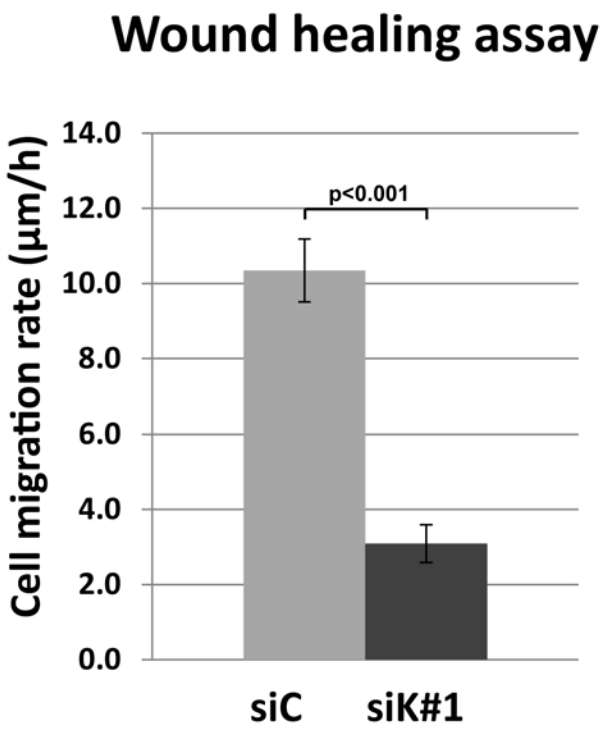


Figure 27. Loss of Kid caused a reduced rate in cell migration

Quantification of the wound healing assay showed a ~3-fold reduction of cell migration rate after loss of Kid. Error bars represent standard deviations.

3.3.3 Loss of Kid does not affect mitochondria morphology or dynamics

While we checked cell motility using live cell imaging, we observed in some cells fast-moving mitochondria. Therefore, we used MitoTracker® Green FM, a dye that labels mitochondria in live cells, to check mitochondria dynamics and morphology after knockdown of Kid. Mitochondria are very dynamic structures constantly undergoing fission and fusion which is required for maintaining their function when cells are challenged with environmental stress (Youle et al. 2012). Fast-moving mitochondria could be an indication of cellular stress. However, we did not observe a noticeable difference between control cells and cells after knockdown of Kid in either mitochondria dynamics or morphology using HeLa cells (Fig. 28). This is consistent with the observation that knockdown of Kid did not cause an increase in superoxide ROS production, indicated by staining cells with MitoSOX™ Red Mitochondrial Superoxide Indicator, suggesting that mitochondria did not undergo oxidative stress and likely function properly after knockdown of Kid (Fig. 29). Interestingly, in RPE1-hTERT cells, we noticed a change in cell morphology. After knockdown of Kid, RPE1-hTERT cells became much more elongated than control cells, indicating there might be some defects in cell polarity. In many of these cells, mitochondria staining appeared to be denser and exhibited aggregation. It would be interesting to explore if Kid plays a role in cell polarity in RPE1-hTERT cells.

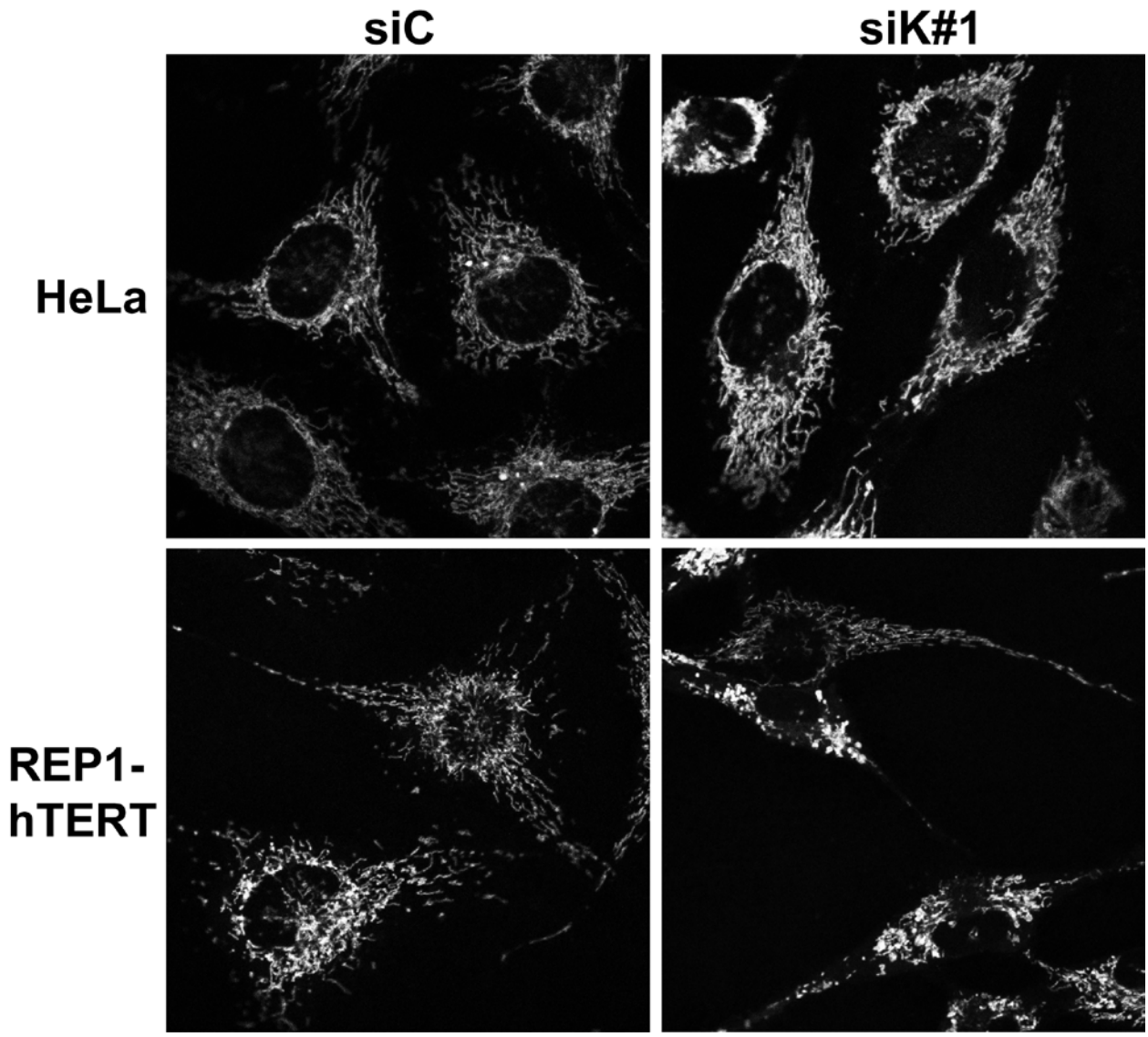


Figure 28. Knockdown of Kid did not affect mitochondria morphology

HeLa cells and RPE1-hTERT cells were seeded onto 35mm glass-bottom dishes and transfected with either control siRNA or siRNA targeting Kid for 48h. Cells were stained with MitoTracker® Green FM and imaged using a Leica SP5 Microscope for 5min.

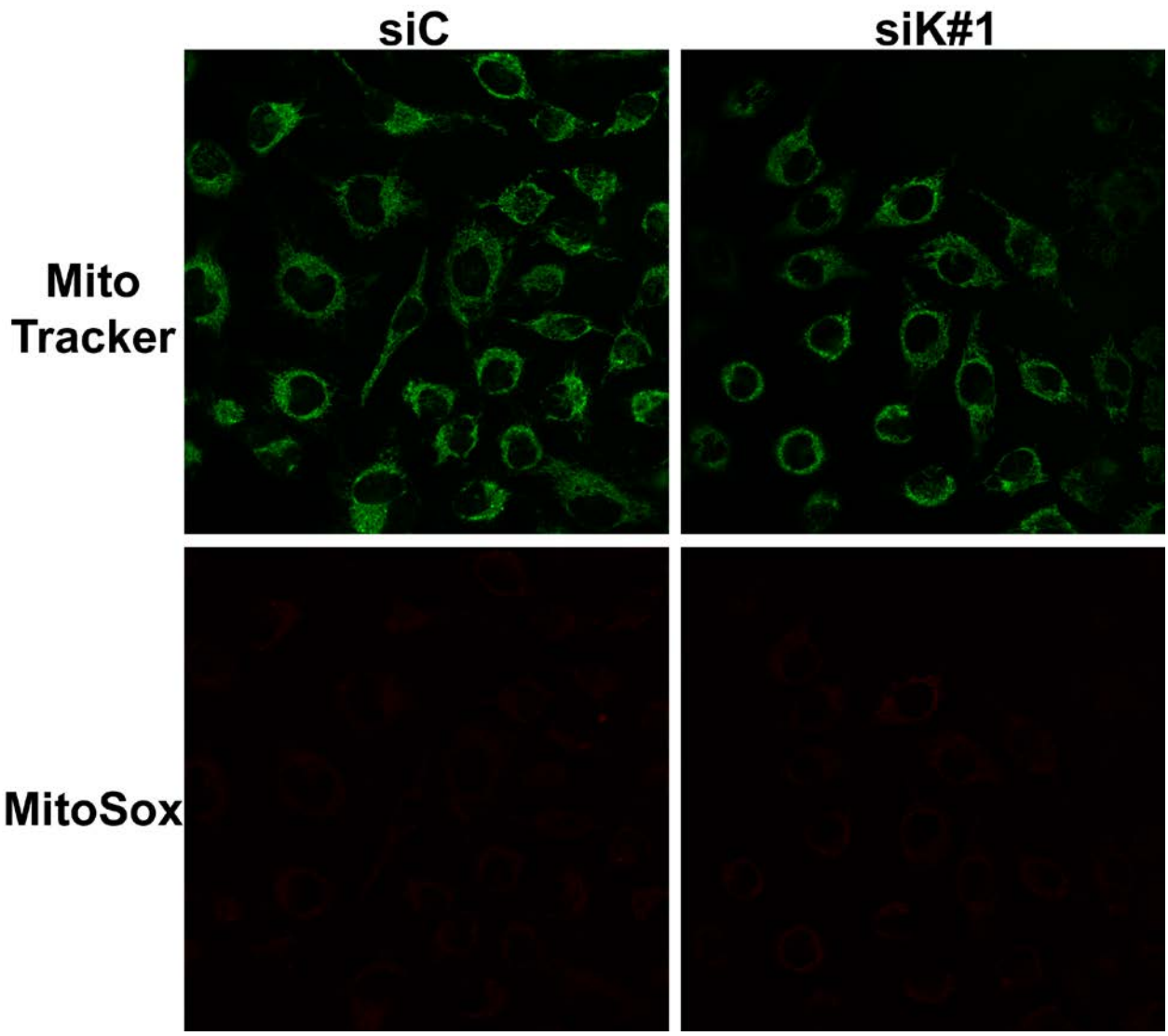


Figure 29. Knockdown of Kid did not increase the superoxide ROS production in HeLa cells

HeLa cells were seeded onto 35mm glass-bottom dishes and transfected with either control siRNA or siRNA targeting Kid for 48h. Cells were stained with MitoTracker® Green FM and MitoSOX™ Red mitochondrial superoxide indicator and then imaged using a Leica SP5 Microscope for 5min.

3.3.4 Discussion and future direction

Previous studies have suggested a relationship between the number of adhesions in cells with cell adhesion and motility. Cells from FAK-deficient mice showed increased number of peripheral

FAs which resulted in decreased cell motility (Ilić et al. 1995), which is consistent with our observation that knockdown of Kid resulted in an increased density of peripheral FAs and decreased rates in cell spreading and migration, supporting a correlation between FAK and Kid. The connection between FAK and Kid was explored and shown in Section 3.4.3-3.4.5. However, knockdown of Kid had additional phenotypic changes besides increased peripheral FAs, including alteration in distribution as well as an increase in the proportion of nascent adhesions or focal complexes with smaller sizes than mature FAs, suggesting that regulation of Kid at FAs via FAK is only part of the story. It has been shown that cells from vinculin-null mouse embryonic fibroblasts (MEFs) had smaller FAs; however, the number of FAs were fewer and cells were less spread and more motile (Rodríguez Fernández et al. 1992; Saunders et al. 2006), suggesting that the phenotypic changes due to loss of Kid does not involve vinculin. In future, it would be interesting to see if reversing the phenotypic changes of adhesions due to loss of Kid, e.g., by treating cells with FAK inhibitor (Section 3.4.5), is able to reverse the decreased spreading and migration rates after knockdown of Kid.

There are two isoforms of myosin II, myosin IIA and myosin IIB. Whereas myosin IIA regulates adhesion size and dynamics, myosin IIB controls cell polarity through actin-crosslinking (Miguel Vicente-Manzanares et al. 2007). This might explain the defects in cell polarity evident from elongated cell morphology after knockdown of Kid in RPE1-hTERT cells. Future investigation can focus on whether there is any change in the level or distribution of myosin IIB after knockdown of Kid in RPE1-hTERT cells.

3.4 REGULATION OF KID IN CELL ADHESION DISASSEMBLY

3.4.1 Knockdown of Kid increases the lifetime of FAs

The increased density in adhesion structures and the change in their distribution indicated that Kid is involved in adhesion dynamics. In order to examine this directly, we tracked FA lifetime by TIRF microscopy. HeLa cells were transfected with either control siRNA or siRNA targeting Kid (siK#1 or siK#2), as well as a plasmid DNA encoding mCherry-paxillin to track adhesion signals over time. Images were taken every 10min for 1h. We defined the track duration time as the time for an individual adhesion to go through assembly and disassembly. Initially we only analyzed FAs that went through the complete process of assembly and disassembly in that one-hour period of imaging time, e.g., an FA that appeared at 10min, remained at 20min, but disappeared at 30min will be categorized into “10-20min”. However, knockdown of Kid led to a large population of FAs that lasted greater than 30min but either started assembly before the imaging or had not completed disassembly by the end of the imaging. Examples include FAs that were already present at 0min and disappeared at 50min, or FAs that started to form at 20min and were still present at 60min. This category of FAs was much fewer in control cells. In order to better include those longer lasting FAs, we categorized them into groups “>30min” and “>1h”. Results showed significant decreases in the percentages of FAs whose track duration time was shorter after knockdown of Kid (Fig. 30B, 10-20min and 20-30min). In contrast, we observed a ~20% increase in the FAs with duration time longer than 30min and a ~10% increase in those longer than 1h in cells after knockdown of Kid (Fig. 30B, >=30min and >=1h). These data directly demonstrate that loss of Kid prolongs the lifetime of FAs, supporting our hypothesis that Kid functions to regulate adhesion dynamics.

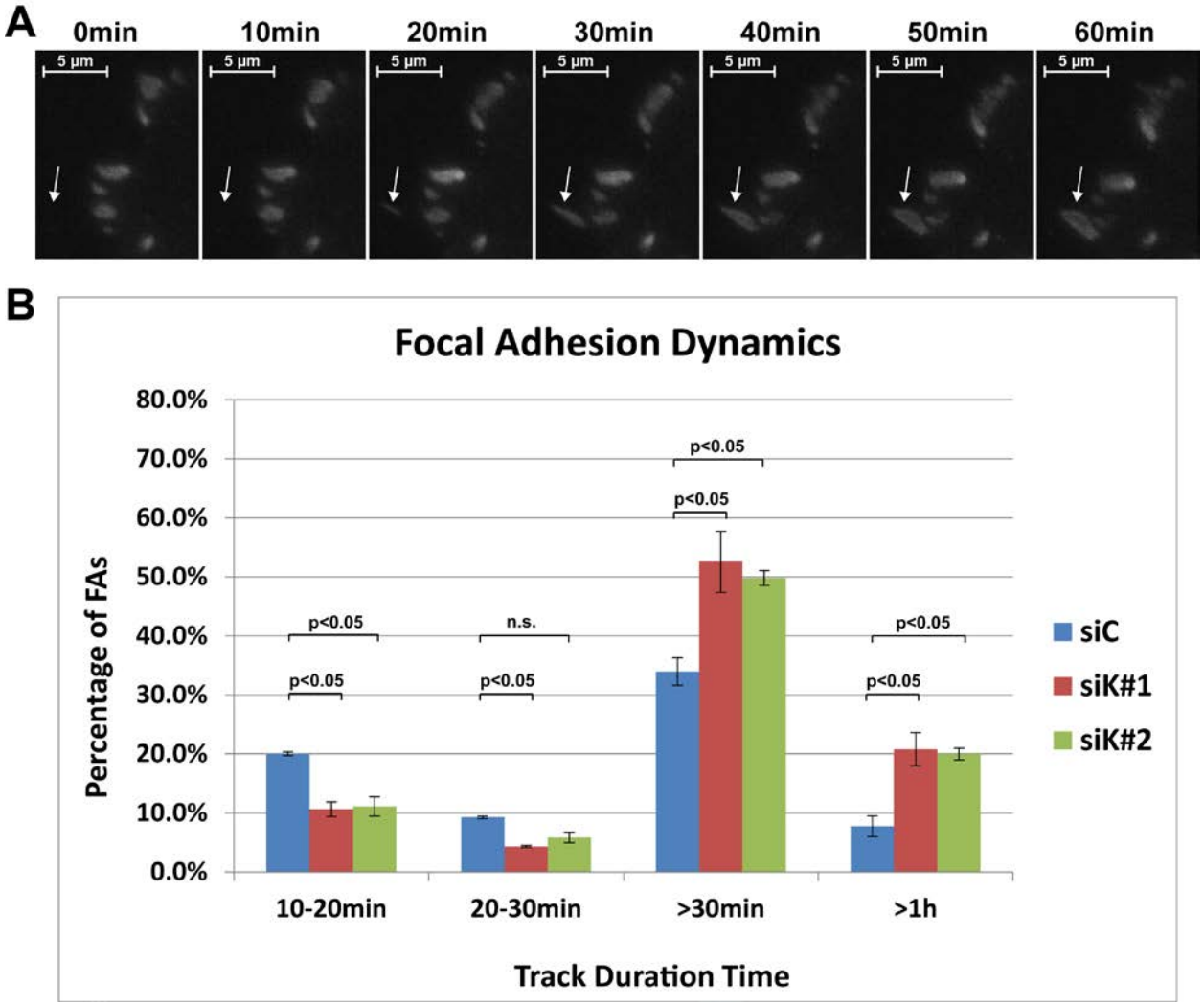


Figure 30. Loss of Kid resulted in prolonged lifetime of FAs

HeLa cells transfected with siRNA and a plasmid DNA encoding for mCherry-paxillin were imaged with a Nikon Eclipse Ti-E microscope to track FA intensity over time. Images were taken every 10min for 1h and the final movie was analyzed using Imaris software. (A) An area of FAs in siK#2 was shown as an example. Arrow points to one FA structure that appeared at 20min of imaging which grew and persisted until the end of the imaging. (B) In total about 20 cells from duplicated experiments containing more than a thousand focal adhesions for each condition were analyzed. Quantification results showed an increased percentage of FAs whose lifetime was longer than 30min or 1h. Error bars represent standard deviations.

3.4.2 Loss of Kid disrupts microtubule-induced adhesion disassembly

While tracking FA lifetime using TIRF microscopy, we noticed that once an adhesion was formed, it appeared to take a longer time to disassemble in cells after knockdown of Kid compared to that in control cells. Therefore, we examined if Kid plays a role in adhesion disassembly.

Since Kid is a microtubule-associated motor protein and microtubules have been implicated in adhesion disassembly, a reasonable hypothesis is that Kid regulates adhesion dynamics through microtubules. In order to test this hypothesis, we performed an assay that directly examines the disassembly of FAs triggered by microtubules and checked if knockdown of Kid is able to slow down this process. The logic behind this assay is that serum-starved cells have few FAs; after nocodazole treatment, FA formation is stimulated and there is an increase in both the size and number of FAs (Bershadsky et al. 1996). After nocodazole washout, microtubules repolymerize and grow to target FAs for disassembly (Ezratty, Partridge, and Gundersen 2005). In NIH-3T3 cells, FAs were completely disassembled at 60min after nocodazole washout and reformed at 120min when cells reestablished their homeostasis (Ezratty, Partridge, and Gundersen 2005). We adapted this assay to HeLa cells and hypothesized that if Kid affects FA disassembly, then knockdown of Kid should be able to delay this process. After treating cells with nocodazole, microtubules depolymerized and FAs formed and stabilized since there were no microtubules targeting them for disassembly (Fig. 31, 10μM nocodazole). Interestingly, after nocodazole washout, when FAs in control cells were completely disassembled, there were still a lot of adhesions present in the cells after knockdown of Kid (Fig. 31, 30min release), indicating that Kid is required for microtubule-based adhesion disassembly after nocodazole washout. Even at later time points, those adhesion structures in cells after

knockdown of Kid were still not disassembled and always appeared bigger compared to those in control cells (Fig. 31, 60min release and 120min release). Knockdown of Kid did not visibly affect the integrity of the microtubule network (Fig. 12, tubulin). All these results showed that loss of Kid disrupted microtubule-induced FA disassembly, indicating that Kid plays a role in adhesion disassembly.

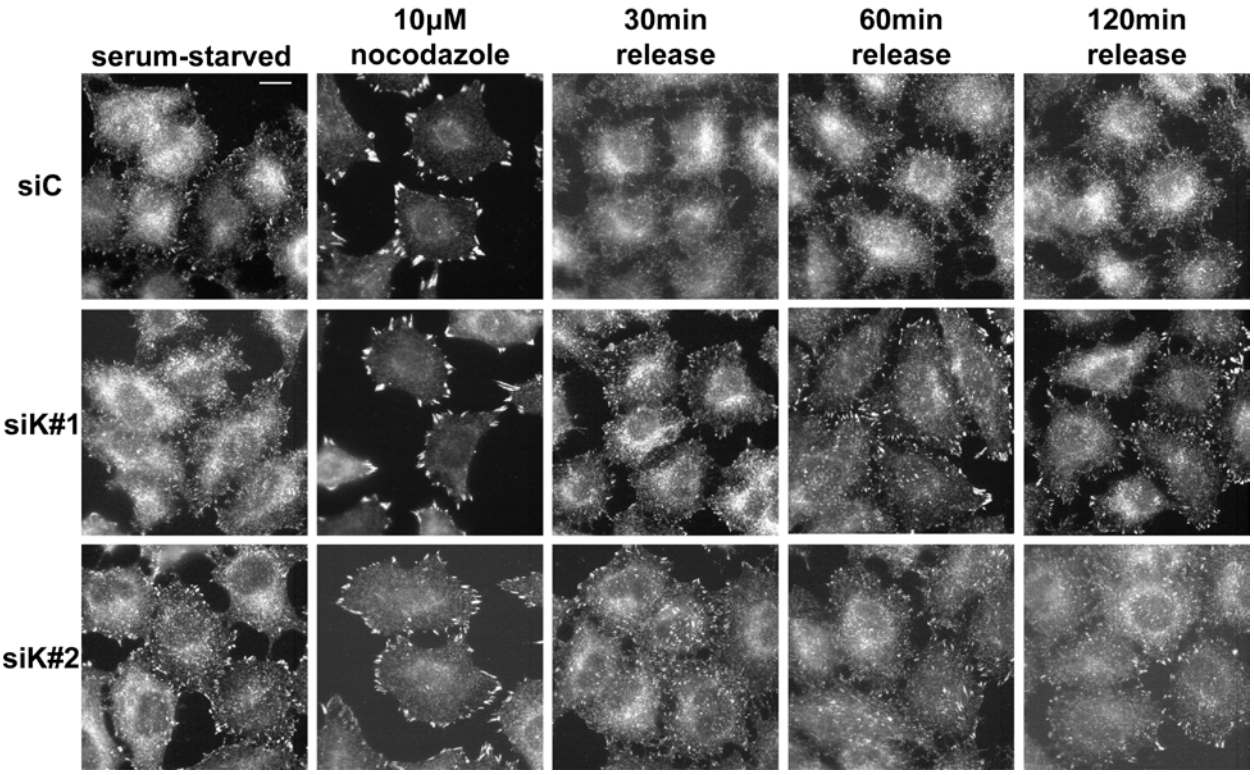


Figure 31. Loss of Kid disrupts microtubule-induced FA disassembly

HeLa cells were transfected with either control siRNA or siRNA targeting Kid for 24h and serum-starved for 18h. Cells were then treated with 10μM nocodazole for 1h followed by release for different time points as indicated and immunostained against paxillin. Images were taken using an Olympus BX60 Epifluorescence Microscope with a 100X objective. Scale bar, 10μm.

3.4.3 Kid modulates levels of pY397FAK and pY118pax in microtubule-induced adhesion disassembly

Dephosphorylation of FAK at Tyrosine 397 (pY397FAK) is induced after extension of microtubules to FAs, which serves as a prerequisite step for the successive disassembly process (Ezratty, Partridge, and Gundersen 2005). We therefore hypothesized that, during the process of microtubule-induced FA disassembly, pY397FAK levels would be elevated due to loss of Kid. As shown in Fig. 32, cells treated with control siRNA had continuously decreased levels of pY397FAK at 30min, 1h, and 2h after being released from nocodazole. At 3h, the level of pY397FAK went back up, although it had not fully recovered when compared to cells without nocodazole treatment (Fig. 32, A-B). In comparison, the loss of pY397FAK after microtubule regrowth was greatly diminished in cells treated with siRNA targeting Kid. There was more than 80% of the original pY397FAK left at all time points examined after nocodazole release. A similar result was observed using a second siRNA targeting Kid (Fig. 33, A-B). As loss of pY397FAK is correlated with active adhesion disassembly mediated by microtubules, an elevated level of pY397FAK suggested that cells cannot properly disassemble FAs after knockdown of Kid. In order to confirm the increased FAK activity in the process of adhesion disassembly due to loss of Kid, we looked at the levels of phosphorylated paxillin at tyrosine 118 (pY118pax), which is a downstream target of FAK and has also been documented previously to be involved in adhesion disassembly (Bellis, Miller, and Turner 1995; Ronen Zaidel-Bar, Milo, et al. 2007). As expected, we observed a similar effect on the levels of pY118pax in the microtubule-induced FA disassembly assay (Fig. 32, C-D and Fig. 33, C-D), supporting our conclusion that Kid is required for the dephosphorylation of pY397FAK which promotes microtubule-induced adhesion disassembly.

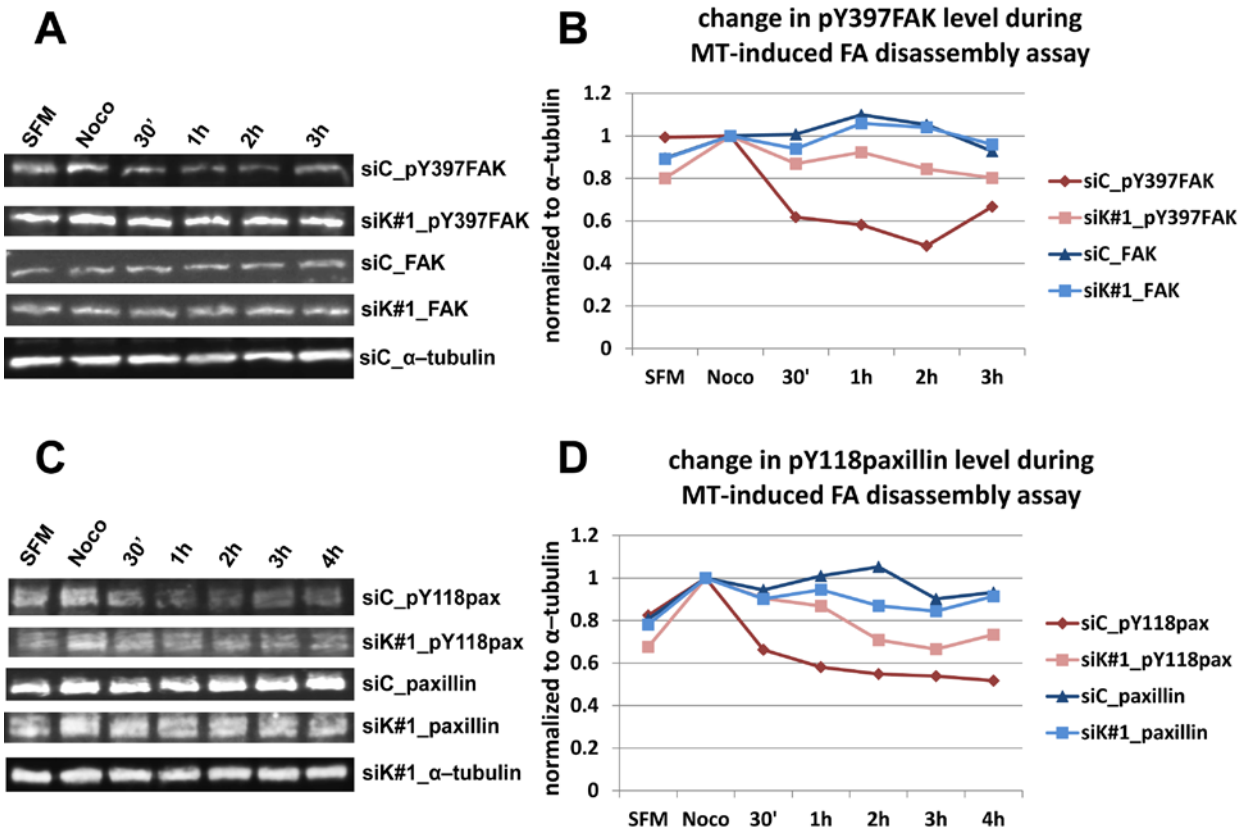


Figure 32. Loss of Kid caused increased FAK activity during the process of microtubule-induced FA disassembly

HeLa cells transfected with either control siRNA or siRNA targeting Kid for 48h were treated with 10μM nocodazole for 1h and lysed after being released for various time points as indicated. Cell lysates were subjected to immunoblotting for (A) pY397FAK, total FAK, and α–tubulin; or (C) pY118paxillin, total paxillin, and α–tubulin. Levels of protein of interest at each time point were first normalized to corresponding levels of α–tubulin to get ratios. Then ratios at different time points were normalized to the sample with nocodazole treatment only (Noco) to make the line charts shown in (B) and (D).

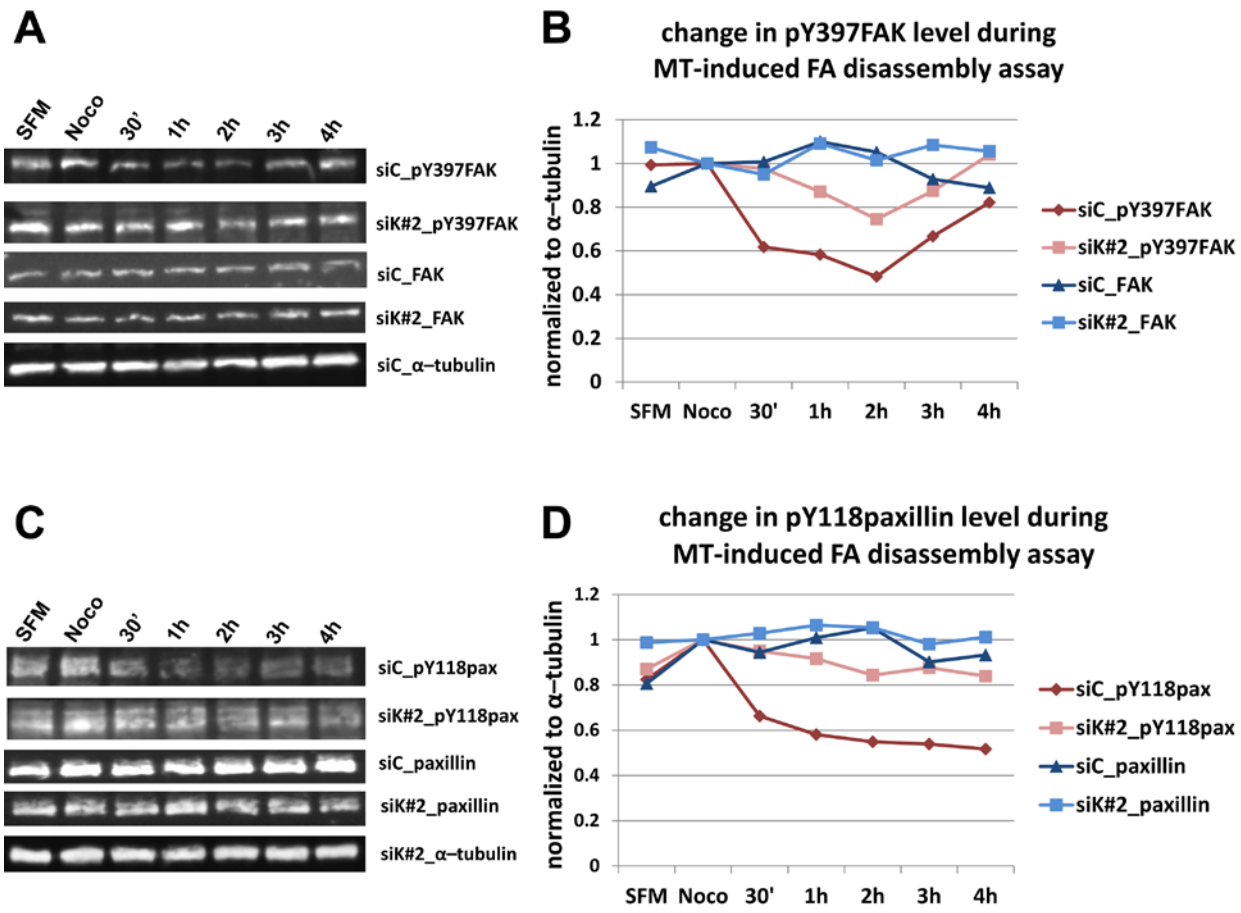


Figure 33. Knockdown of Kid using a different siRNA from that shown in Fig. 32 also caused increased FAK activity during the process of microtubule-induced FA disassembly

HeLa cells transfected with either control siRNA or siRNA targeting Kid for 48h were treated with 10μM nocodazole for 1h and lysed after being released for various time points as indicated. Cell lysates were subjected to immunoblotting for (A) pY397FAK, total FAK, and α–tubulin; or (C) pY118paxillin, total paxillin, and α–tubulin. Levels of protein of interest at each time point were first normalized to corresponding levels of α–tubulin to get ratios. Then ratios at different time points were normalized to the sample with nocodazole treatment only (Noco) to make the line charts shown in (B) and (D).

3.4.4 Overexpression of Kid leads to decreased pY397FAK and pY118pax

Based on the conclusion above, we then hypothesized that overexpression of Kid would lead to decreased phosphorylation of pY397FAK. In order to test this, we took advantage of a plasmid encoding an improved variant of green fluorescence protein (GFP), TurboGFP (tGFP)-tagged Kid. We transfected HeLa cells with tGFP-Kid plasmid and immunostained against tGFP and pY397FAK. HeLa cells tolerated the expression of exogenous Kid 24h post-transfection but not 48h post-transfection. Unexpectedly, very few cells showed localization of Kid at FAs. It could be due to the change of Kid's localization by adding the tGFP tag, however, we did observe colocalization of Kid and pY118pax in some cells (Fig. 34D). When we compared cells expressing tGFP-Kid to their neighboring cells that did not show tGFP staining, we found that cells with high expression levels of the exogenous Kid showed dramatic decreases in the levels of pY397FAK and pY118pax immunostaining (Fig. 34, A-B, arrows). Cells expressing moderate levels of tGFP-Kid showed minor decreases in levels of pY118pax (Fig. 34B, arrowhead). This phenomenon was specific to pY397FAK and pY118pax, as immunostaining cells against tGFP and paxillin did not show such a correlation (Fig. 34C). These observations confirmed our hypothesis that Kid influences phosphorylation of FAK and paxillin.

In addition, we examined the level of pY397FAK after overexpression of Kid using western blot. However, we did not observe a noticeable decrease. This was probably due to the fact that only ~10% of the total transfected cells strongly expressed tGFP-Kid, and only those cells showed significant decreased levels of pY397FAK and pY118paxillin by immunofluorescence.

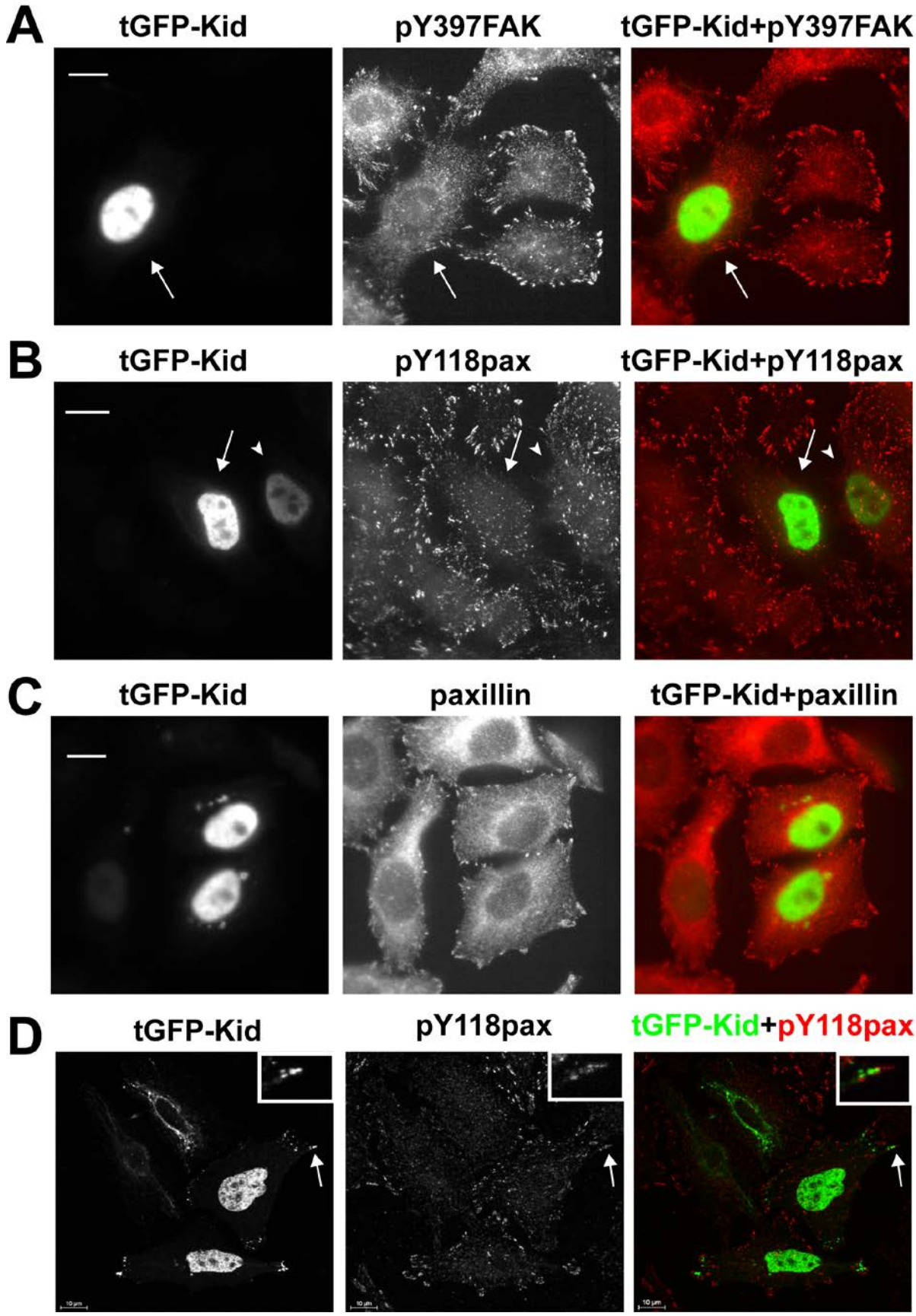

A
tGFP-Kid
pY397FAK
tGFP-Kid+pY397FAK
B
tGFP-Kid
pY118pax
tGFP-Kid+pY118pax
C
tGFP-Kid
paxillin
tGFP-Kid+paxillin
D
tGFP-Kid
pY118pax
tGFP-Kid+pY118pax

Figure 34. Overexpression of Kid was correlated with decreased levels of pY397FAK and pY118pax, but not paxillin

HeLa cells were transfected with a tGFP-Kid plasmid for 24h and immunostained against (A) tGFP and pY397FAK; or (B) tGFP and pY118pax; or (C) tGFP and paxillin. In (B), arrows point to a cell with high expression of tGFP-Kid whereas arrowheads point to a cell with moderate expression of tGFP-Kid. Examples of cells with localization of tGFP-Kid to FAs were shown in (D). Arrows point to sites of FAs which were zoomed in and shown in the inset boxes. Scale bar, 10μm.

3.4.5 FAK inhibitor reversed the phenotypic changes of adhesions due to loss of Kid

In order to rule out the possibility that the phenotypic defects after knockdown of Kid were due to some indirect effect rather than changes in FAK activity, we used a FAK inhibitor which selectively targets the autophosphorylation site of FAK (pY397FAK) and tested if it can suppress the phenotypic changes of adhesions due to loss of Kid. As shown in Fig. 35, knockdown of Kid caused changes in adhesion density and distribution (Fig. 35, -FAK inhibitor). As predicted by our model, treating cells with FAK inhibitor completely abolished these phenotypic changes (Fig. 35, +FAK inhibitor). This result confirmed that the phenotypic changes after knockdown of Kid were caused directly by perturbation in FAK activity. Together, these observations substantiated Kid's function in the cell as an adhesion disassembly factor by affecting levels of pY397FAK.

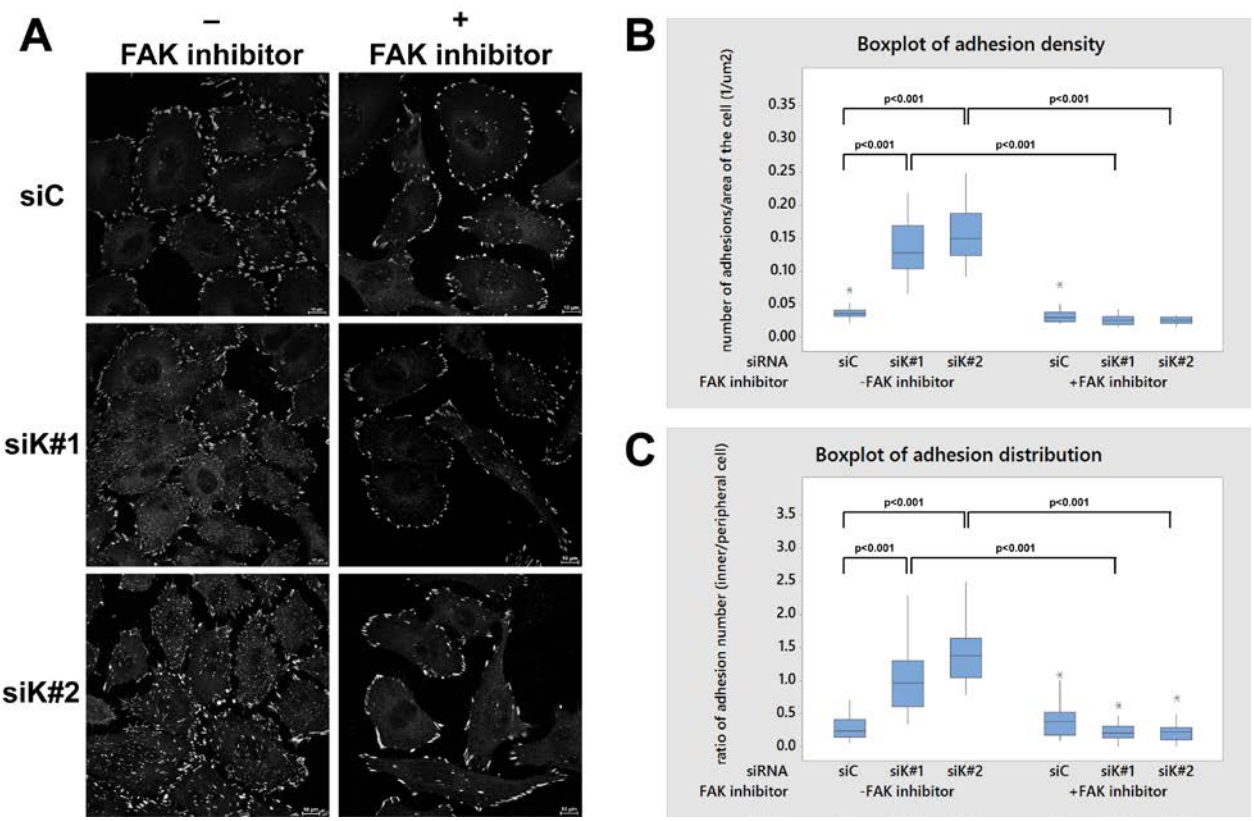


Figure 35. FAK inhibitor reversed the phenotypic changes in adhesion density and distribution caused by knockdown of Kid

(A) HeLa cells were transfected with siRNA for 24h and then incubated with fresh medium either with or without 5μM FAK inhibitor for 24h. Cells were immunostained against vinculin and images were taken using Olympus Fluoview 1000 Confocal Microscope with a 60X objective. Scale bar, 10μm. (B) 30 cells in each group (siC, siK#1, siK#2, siC+FAK inhibitor, siK#1+FAK inhibitor, siK#2+FAK inhibitor) from two independent experiments were quantitatively analyzed using Minitab software.

3.4.6 Discussion and future direction

In our study, we found that knockdown of Kid disrupted the process of adhesion disassembly induced by microtubules through inhibiting dephosphorylation of FAK at Tyrosine 397 (pY397FAK). As tyrosine 397 is an autophosphorylation site triggered upon integrin-activation,

it is less likely that Kid physically interacts with FAK and somehow triggers its activation. Our immunoprecipitation experiment supported this reasoning. We tried immunoprecipitating Kid to see if some of the FA components can be pulled down together with Kid, but did not see a direct interaction between Kid and FAK or vinculin. Introducing a cross-linker before immunoprecipitation did not render the interaction either, hence not supporting a transient interaction between Kid and FAK. These results suggested an indirect link between Kid and FAK via some other effector(s).

More likely, Kid acts upstream of FAK activation. Another human chromokinesin, KIF4A, has been found to recycle integrin β1 which could spatially regulate adhesions in a cell (Heintz et al. 2014). If Kid regulates FAK activity through integrin activation, it would not be surprising that adhesion assembly will be affected when Kid's function is impaired. Another possibility is that Kid interacts with some phosphatase which in turn acts on FAK. It has been shown before that lack of phosphatases like protein tyrosine phosphatase (PTP)-PEST or SHP-2 caused an increase in adhesion number and defects in cell migration (D.-H. Yu et al. 1998; Angers-Loustau et al. 1999). These are good candidates to explore if Kid regulates phosphorylation of FAK via phosphatase.

In our study, we explored phosphorylation of paxillin, a downstream target of FAK. The significance of tyrosine phosphorylation of paxillin in cells resides on both adhesion assembly and disassembly. One study showed that expression of phosphomimetic paxillin enhanced FCs, whereas expression of non-phosphorylatable paxillin caused more stable adhesions (FAs) (Zaidel-Bar et al. 2007). Overexpression of tGFP-Kid is correlated with dephosphorylation of pY118pax whereas loss of Kid diminished dephosphorylation of pY118pax in the process of microtubule-based FA disassembly. This could in turn enhance adhesion formation which fits

our observation that cells after knockdown of Kid showed increased proportion of FCs. It might be worth exploring if other downstream phosphorylation events of pY397FAK, both on multiple phosphorylation sites of FAK and Src, are altered as a result of the change in pY397FAK levels during adhesion disassembly due to loss of Kid.

It remains to be investigated if other downstream effectors of FAK are also affected. One study confirmed a direct interaction between FAK and dynamin2 which regulates FA disassembly in response to Src kinase (Wang et al. 2011). FAK and Src are two tyrosine kinases considered to be central regulators of FA turnover. Upon integrin-activation, FAK autophosphorylates at Tyrosine 397 which is required for binding and activating Src by disrupting the autoinhibitory confirmation of Src (Yeatman 2004). Subsequently, the FAK-Src complex promotes the phosphorylation of two scaffolding proteins, paxillin and p130 Crk-associated substrate (p130CAS) (Bellis, Miller, and Turner 1995; Cary et al. 1998). Future effort can be made to examine if the interaction between FAK-Src is affected by knockdown of Kid, which could lead to defects in paxillin phosphorylation that we observed. Other phosphorylation events including phosphorylation on other sites of FAK are also worth exploring. Phosphorylation at Tyrosine 861 (pY861FAK) is required for the interaction of FAK with paxillin and talin (Nagano et al. 2012). Phosphorylation at Tyrosine 925 (pY925FAK) mediates the interaction between FAK and Grb2 which recruits dynamin to FAs and function in adhesion turnover (Ezratty, Partridge, and Gundersen 2005). Exploring these downstream events could help better understand the mechanism contributing to the defects in adhesion disassembly due to loss of Kid.

3.5 LOSS OF KID AFFECTS ACTIN CYTOSKELETON

Formation, maturation, and disassembly of adhesion structures are tightly correlated with actin cytoskeleton mediated by Rho GTPases (Nobes and Hall 1995; Lawson and Burridge 2014). One interesting observation we had in cell spreading assay was that some of the cells with loss of Kid showed abnormal condensed F-actin (Fig. 26A, siK#1, 60min, arrows), which has been previously shown to correlate with localized activation of RhoA (Nalbant et al. 2009). Therefore, we performed a couple of experiments to examine the actin cytoskeleton.

3.5.1 Knockdown of Kid causes a change in actin organization

First, we simply knocked down Kid in HeLa cells and stained for F-actin. In control cells, actin stress fibers are mostly presented as dorsal stress fibers with a few ventral stress fibers (Fig. 36, Un and siC). In comparison, cells after knockdown of Kid had a lot of linear ventral stress fibers (Fig. 36, siK#1). This observation is consistent with the changed distribution of adhesion structures from mainly at the edges of the cell to throughout the ventral cell surface, indicating a potential link between actin cytoskeleton and Kid.

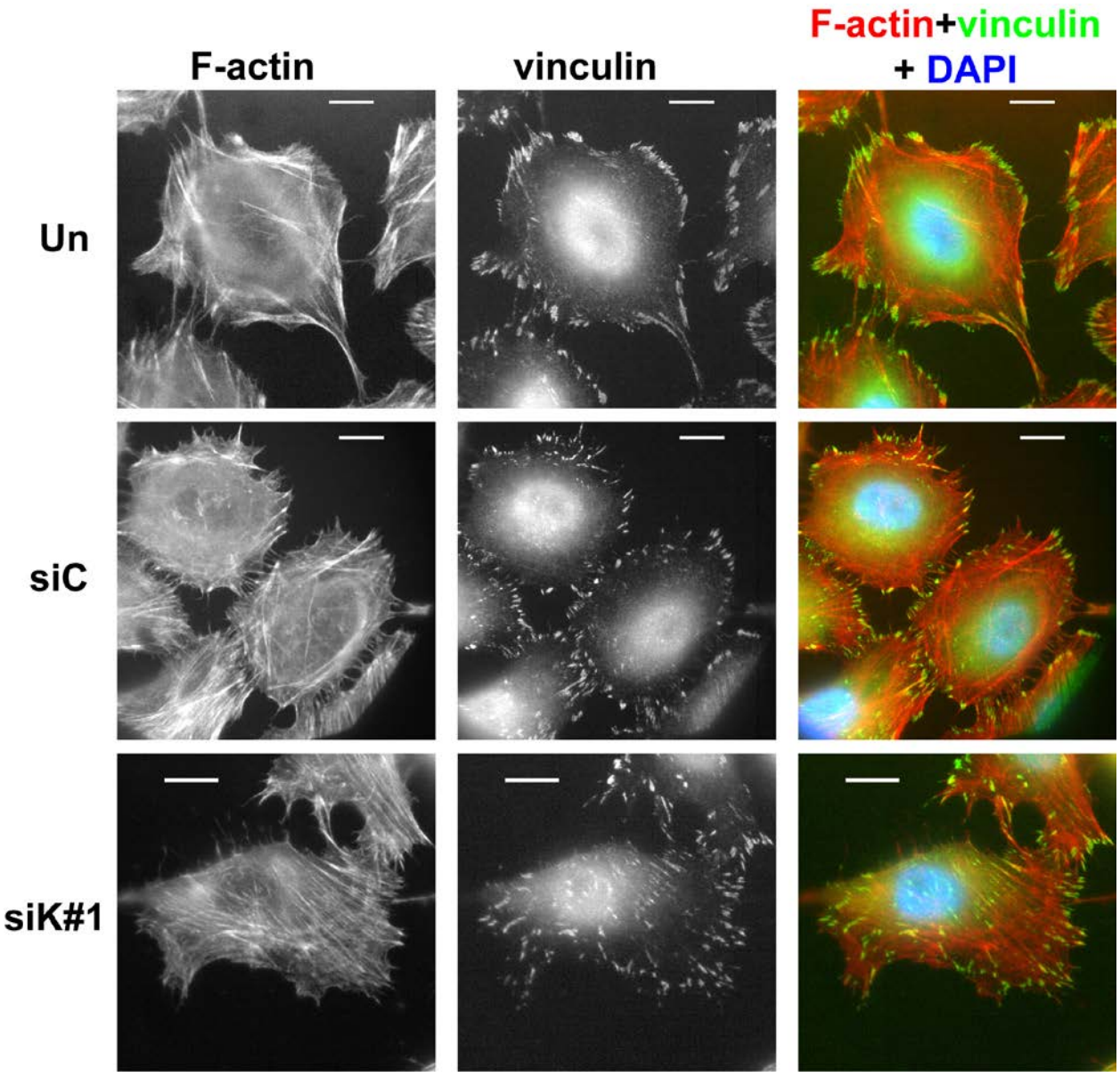


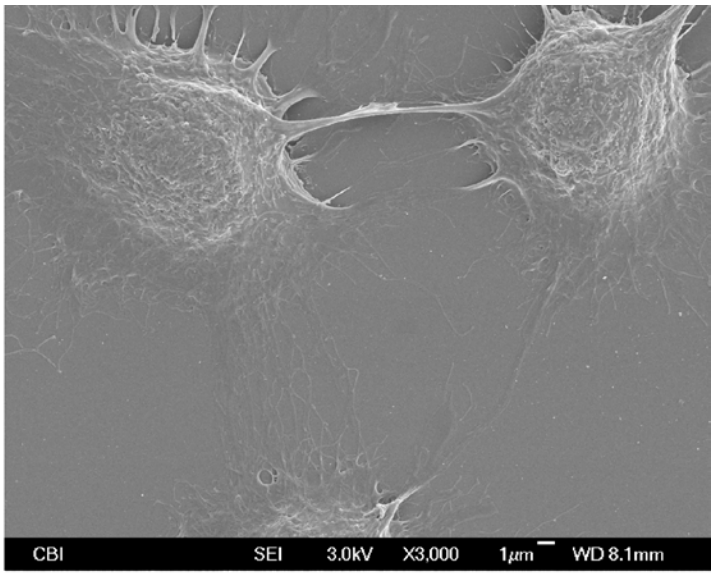
Figure 36. Knockdown of Kid caused a change in actin organization

HeLa cells were transfected with control siRNA or siRNA targeting Kid for 48h and immunostained against vinculin and Rhodamine-conjugated Phalloidin. Images were taken using an Olympus BX60 Epifluorescence Microscope with a 100X objective. Scale bar, 10μm.

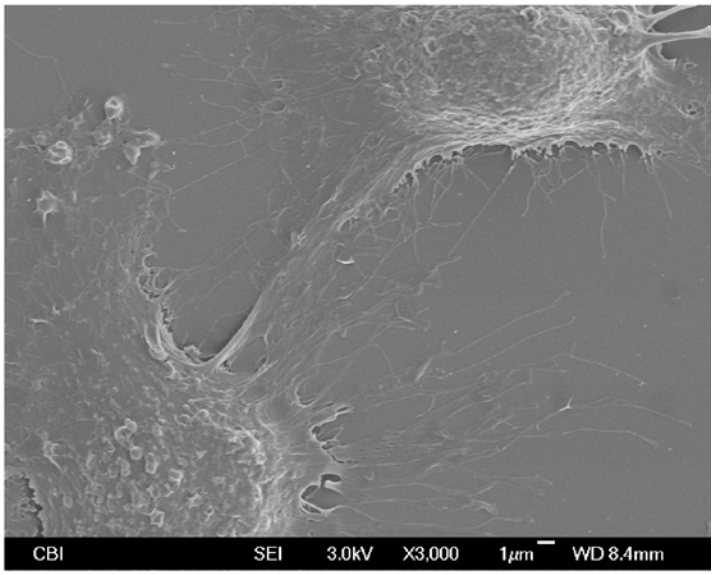
3.5.2 Knockdown of Kid results in abnormal structures of filopodia

Filopodia are “finger-like” bundled actin filaments that contain plasma membrane protrusions at the leading edge of migrating cells. These structures play essential roles in various cellular processes like cell adhesion, migration, and cell-cell contacts. To better examine if there is any structural change of filopodia at cell protrusion due to loss of Kid, we used SEM which gives a closer and clearer visualization compared to immunofluorescence. As shown in Fig. 37, in untreated HeLa cells, filopodia extended from the surface of one cell, either to a long distance away from cell body, or to make connections with their counterparts in an adjacent cell. HeLa cells treated with control siRNA presented similar structures (Fig. 37, siC). Interestingly, when Kid was knocked down, filopodia in HeLa cells appeared abnormal (Fig. 37, siK#1). These long protruded structures became shorter compared to those in control cells and showed disorganized appearance and a lot of the filopodia were broken. In addition, in some cells, the structures of plate-like extensions, called lamellipodia, also seem to be disrupted. Lamellipodia are composed of actin filaments and involved in cell motility and mechanosensing. These results are consistent with other data showing that Kid affects actin polymerization and cell migration.

Un


CBI SEI 3.0kV X3,000 1μm WD 8.1mm

siC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siK#1

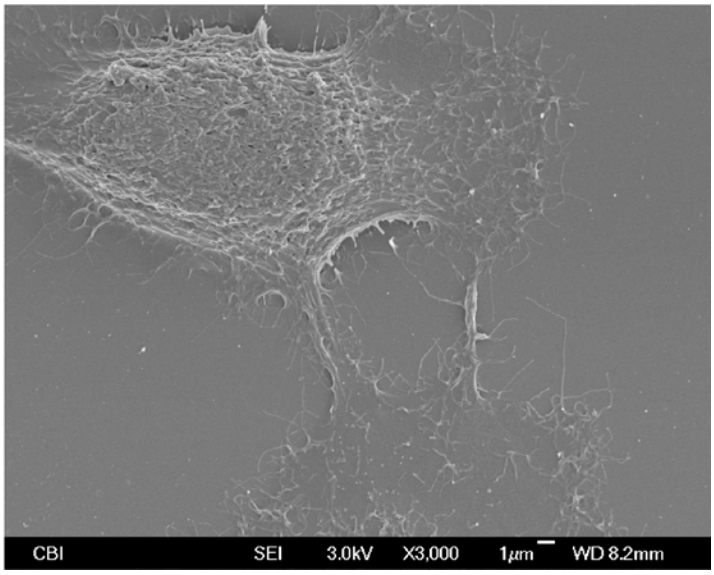
CBI SEI 3.0kV X3,000 1μm WD 8.2mm

Figure 37. Knockdown of Kid resulted in abnormal structures of filopodia

HeLa cells were seeded onto 12mm coverslips and transfected with 3nM siRNA targeting Kid for 48 hours followed by preparation for SEM. Images were taken using a JSM 6330F Scanning Electron Microscope.

3.5.3 Knockdown of Kid partially reverses the phenotypic changes of adhesions caused by Rho inhibitor

As Rho activity is required for the formation of actin stress fibers and maturation of FAs, the change in actin organization after knockdown of Kid led to the question whether Rho is involved in the pathway through which Kid regulates adhesion dynamics. In order to test this, HeLa cells were treated with a Rho inhibitor after knockdown of Kid and we observed the phenotypic changes due to Rho inhibitor were suppressed by loss of Kid. As shown in Fig. 38, FAs were mostly eliminated in cells treated with siC and Rho inhibitor (Fig. 38, vinculin). In comparison, cells treated with siRNA targeting Kid had a lot more FAs after Rho inhibitor treatment (Fig. 38, vinculin), suggesting that knockdown of Kid was able to partially suppress the loss of FAs due to Rho inhibition. F-actin staining in cells treated with the Rho inhibitor displayed phenotypes including loss of stress fibers and protrusion of dendritic extensions, further supporting that the inhibitor was working properly (Fig. 38, F-actin). These results suggested that Kid could be triggering the activation of Rho in the presence of Rho inhibitor and promoting maturation of FAs. However, this interpretation appeared to be controversial to our earlier observation that knockdown of Kid led to an increased proportion of focal complexes, which suggested maturation defects which could be due to inhibition of RhoA. In order to resolve this controversy, it would be helpful to examine whether and how RhoA activation is affected by knockdown of Kid using a RhoA activation assay directly measuring levels of active RhoA.

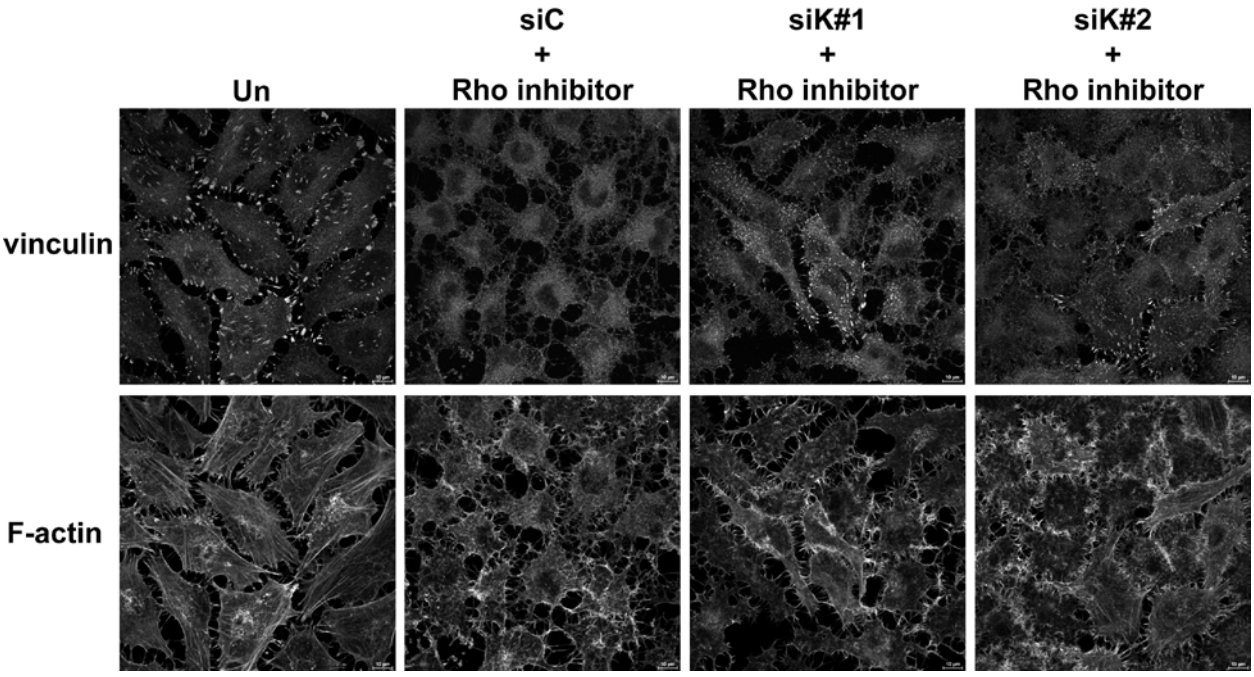

Figure 38. Loss of Kid diminished defects in FA maturation caused by Rho inhibitor

HeLa cells transfected with either control siRNA or siRNA targeting Kid for 44h were treated with 0.5μg/ml Rho inhibitor for 4h followed by immunostaining against vinculin and F-actin. Images were taken using an Olympus Fluoview 1000 Confocal Microscope with a 60X objective. Scale bar, 10μm.

3.5.4 Knockdown of Kid resulted in patches of FAs left by cells

While we examined FAs after knockdown of Kid, we observed an interesting phenotype. There were a number of cell bodies after knockdown of Kid without nucleus staining (Fig. 40). As we found that Kid plays a role in adhesion disassembly, these patches may be from the cells failing to overcome the stabilized adhesions while moving forward after knockdown of Kid, causing detachment of cell fragments.

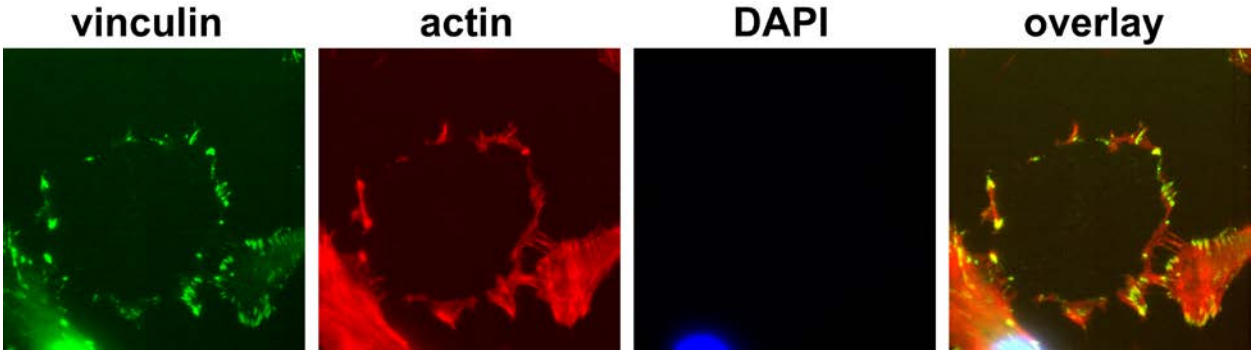

Figure 39. Cells after knockdown of Kid left patches of FAs

HeLa cells were transfected with control siRNA or siRNA targeting Kid for 48h and immunostained against vinculin and Rhodamine-conjugated Phalloidin. Images were taken using an Olympus BX60 Epifluorescence Microscope with a 100X objective.

3.5.5 Discussion and future direction

In the time-course cell spreading assay we noticed that some of the cells with loss of Kid showed abnormally condensed F-actin, which has been previously reported to correlate with localized activation of RhoA (Nalbant et al. 2009). If loss of Kid somehow activates RhoA, which counteracts the effect caused by a Rho inhibitor, we would expect to observe more FAs in cells after knockdown of Kid. As our results showed, inhibition of RhoA alleviated, although did not reverse, the phenotypic changes of adhesions caused by loss of Kid, indicating that these changes due to loss of Kid could be partially dependent on RhoA and Kid could play an additional role in adhesion maturation besides adhesion disassembly. Rho activation induces cell contractility leading to actin bundling, integrin clustering, and FAK activation. The increased levels of pY397FAK after knockdown of Kid in microtubule-induced adhesion disassembly may be attributed to increased RhoA activation. Additionally, Rho family GTPases, including Rac, Rho,

and Cdc42, play critical roles in actin organization (Aspenstrom, Fransson, and Saras 2004). In HeLa cells, we observed a change in actin organization after knockdown of Kid from mostly dense dorsal stress fibers to dramatically increased ventral stress fibers. This could be due to aberrant activation of Rho GTPases and contribute to the change in adhesion distribution after knockdown of Kid as adhesions grow along actin filaments. Therefore, it would be interesting to examine the activity of Rho family GTPases, especially Rac1 and RhoA, after knockdown of Kid and see if Kid also functions in adhesion assembly/maturation via these signaling molecules.

4.0 CONCLUSION

In this study, we specifically explored the unconventional role of a microtubule motor protein Kid in cell adhesion and migration during interphase of the cell cycle. We used multiple imaging techniques including confocal microscopy, TIRF microscopy, and live cell imaging to investigate the interphase cellular localization and function of Kid. We applied quantitative analysis to more precisely represent the phenotypes caused by knockdown of Kid. We have found that 1) Kid localizes to the sites of FAs; 2) Knockdown of Kid results in changes in the density, distribution, and size of FAs; 3) Such changes have effects on the ability of cells to spread onto the ECM and to migrate; 4) Kid functions in adhesion disassembly through regulating phosphorylation events of adhesion components including FAK and paxillin.

A long-term question for Kid's function in adhesion dynamics is whether it still acts as a traditional microtubule-associated motor protein or if it possesses other properties required for controlling adhesion dynamics beyond its motor function. If the former, then what is the cargo it transports to FAs to function? If the latter, how is it localized to FAs and what are the partners it cooperates at FAs? Although in our study microtubules were not required for Kid's localization at FAs, initial delivery of Kid to FA sites could still be microtubule-dependent. Creating mutants defective in motor domain of Kid would help reveal whether its motor property is required for its function in adhesion dynamics. A systematic study like Mass Spectrometry identifying binding

partners for Kid, especially those at the cell surface, would point to more specific proteins and pathways for further investigation.

Known as a microtubule motor that plays essential roles during mitosis, the function of Kid during interphase has long been overlooked. In this study we identified a novel role for Kid in adhesion disassembly during interphase of the cell cycle. By modulating the phosphorylation of FAK and FAK's downstream effector paxillin, Kid promotes adhesion disassembly, hence maintaining the appropriate density, distribution, and size of adhesions needed for functional cell adhesion and migration. Our discovery enhanced our knowledge at this motor protein as well as the dynamic regulatory network of cell adhesion.

5.0 EXPLORING A ROLE FOR KID IN PROTEIN SYNTHESIS

5.1 INTRODUCTION

Previous studies have discovered the existence of ribosomes, translational initiation factors, and RNA-binding proteins at nascent adhesion structures in the lamellipodium of migrating cells (Willett et al. 2010). We found that Kid also participates in nascent polypeptide synthesis. A previous graduate student from our lab showed that Kid localized to the nucleolus ~80% of the time in non-cancer cell lines versus ~35% of the time in cancer cell lines that have been tested. It is known that ribosomal biogenesis occurs in the nucleoli and ribosomal maturation occurs in the cytoplasm (Granneman and Baserga 2004). In the nucleolus, rDNA is transcribed into rRNA which is then incorporated into 90S pre-ribosomal subunits (Schäfer et al. 2003). Early pre-40S and pre-60S ribosomal subunits are then generated from cleavage and post-transcriptional modification of the 90S in the nucleoplasm (Schäfer et al. 2003). Once transported from the nucleus to the cytoplasm, pre-40S and pre-60S ribosomal subunits become mature 40S and 60S ribosomal subunits which can bind to mRNAs and form the 80S ribosomes to initiate translation (Henras et al. 2008). Here we used RPE1-hTERT cells and HeLa cells and explored the role of Kid in protein synthesis.

5.2 MATERIALS AND METHODS

5.2.1 Immunofluorescence

RPE1-hTERT cells were passaged the day before to reach 70-80% confluency for immunofluorescence experiment. After fixation and permeabilization in 4% paraformaldehyde supplemented with 0.2% Triton X-100, cells were incubated with primary antibodies probing for rabbit α-Kid and mouse α-vinculin at 4^0C overnight, followed by incubation in secondary antibodies under room temperature for 1h.

5.2.2 Polysome Profiling Assay

In order to determine whether Kid is associated with pre-ribosomal subunits, cellular fractionation was performed to separate nucleoli from cytoplasm. Then, nucleoli were disrupted and the DNA pellet was removed by centrifugation. The supernatant was loaded onto a 10-25% continuous sucrose gradient and centrifuged for 4 hours at 27,000rpm. The different fractions were separated according to their densities and the positions of the ribosomal subunits were determined by absorbance at 254nm. Gradient fractions were purified and concentrated using StrataClean® Resin and immunoblotted to reveal the position of the motors relative to the ribosomal subunits.

5.2.3 ^{35}S incorporation assay

Radiolabeled amino acids were added into HeLa cells after knockdown of Kid for 30min, followed by cell lysis, TCA precipitation, and scintillation counting.

5.3 RESULTS

5.3.1 Kid localizes to the nucleoli

As shown in Fig. 31, immunofluorescence results showed that Kid colocalizes with fibrilarin, which is a marker protein for the nucleolus. This observation is consistent with the fact that Kid contains a DNA-binding domain and suggested that Kid may play a role in ribosome biogenesis or some other aspect of protein translation.

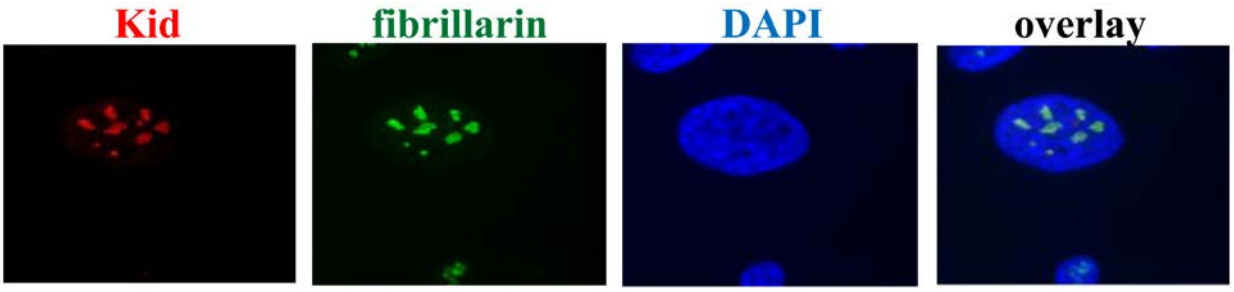

Figure 40. Localization of Kid to nucleoli during interphase

RPE1-hTERT were immunostained against Kid, fibrillarin, and DAPI. Images were contributed by Dr. Kristen Bartoli, a former graduate student in the lab.

5.3.2 Kid is associated with pre-ribosomal subunits in the nucleus

Next we examined if Kid is associated with ribosomal subunits in the nucleus. From Fig. 32, we can see that Kid was present in the nuclear fractions of both pre-40S and pre-60S, some in free fraction, but not in the gap between pre-40S and pre-60S. Also we noted that when density of markers for ribosomal subunits increased, level of Kid was increased as well. The close co-sedimentation of Kid and ribosomal markers suggested that Kid is associated with pre-ribosomal subunits in nucleoli.

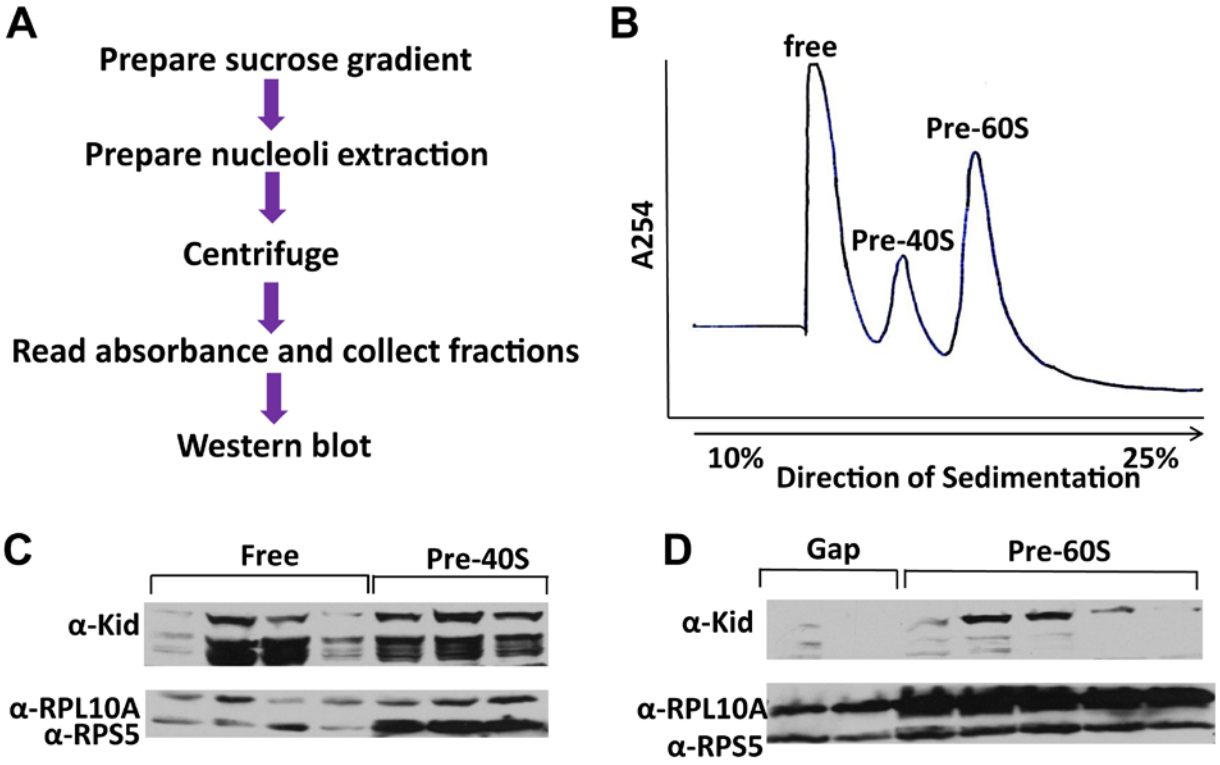

Figure 41. Kid is associated with pre-ribosomal subunits

A work flow chart is shown in (A). RPE1 cells were fractionated and nucleoli fraction was loaded onto sucrose gradient and centrifuged. The sample was read at the absorbance of 254nm and then collected as shown in (B). Proteins were concentrated and subjected to western blotting. Antibodies probing for Kid, RPL10A (60S), and RPS5 (40S) were used and results were shown in (C) and (D).

5.3.3 Knockdown of Kid caused an increase in nascent protein synthesis

Since ribosomes are units for protein translation, we checked if Kid knockdown has any effect on protein synthesis. ^{35}S incorporation assay was performed in which radiolabeled amino acids were added into HeLa cells after knockdown of Kid for 30min, followed by cell lysis, TCA precipitation, and scintillation counting. As shown in Fig. 33, knockdown of Kid resulted in a ~40% increase in nascent protein synthesis compared to untreated cells whereas cells treated with control siRNA showed a ~10% increase in nascent protein synthesis.

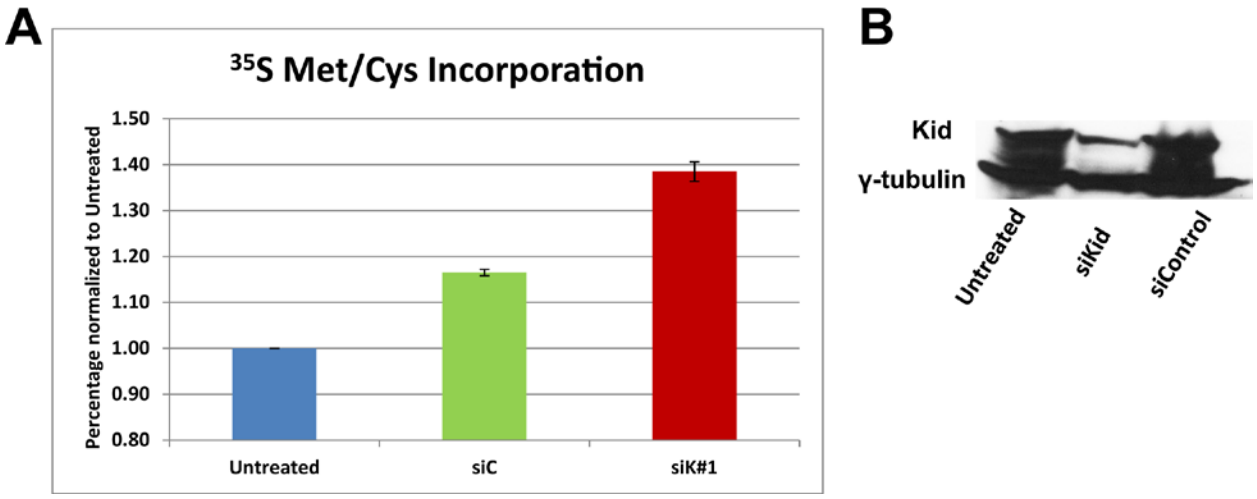

Figure 42. Knockdown of Kid resulted in an increase in nascent protein synthesis

HeLa cells were transfected with control siRNA or siRNA targeting Kid for 48h, followed by an ^{35}S Met/Cys incorporation assay. (A) Quantification of nascent protein synthesis from three independent experiments. (B) Knockdown efficiency of siRNA targeting Kid examined by western blotting.

5.4 DISCUSSION AND FUTURE DIRECTION

So far, we have found that 1) Kid also localized in nucleoli; 2) Kid was suggested to be associated with ribosomes; and 3) Knockdown of Kid resulted in an increase in nascent protein synthesis. Since knockdown of Kid resulted in a ~40% increase in protein synthesis, it might function as a translational repressor. And considering that Kid contains a nucleic acid-binding domain, it can regulate protein synthesis either through affecting ribosomes or through mRNAs.

If Kid directly binds to ribosomal DNA (rDNA) in nucleoli, which further results in a change in protein synthesis, we can perform chromatin immunoprecipitation (ChIP) to examine this. If Kid directly binds to mRNAs, cross-linking RNA-immunoprecipitation can be performed to identify mRNAs interacting with Kid. In this scenario, one possible outcome is that Kid binds to mRNAs in a global manner and therefore affects protein synthesis in general; another possibility is that Kid binds to certain mRNAs, e.g., mRNAs for FA components, and regulates their translation specifically. In spreading and migrating mammalian cells, ribosomes and translation initiation factors have been shown to localize to integrin-based adhesion complexes (Willett et al. 2010). One mechanism that has been found in regulating gene expression of some FA components is localization-coupled mRNA translation. For example, localized expression of the integrin α3 protein, which is a component of FA, has been shown regulated at the level of RNA localization (Adereth et al. 2005). Another example is that compartmentation and translation of β-actin is regulated by ZBP1, which enhances FA stability and directs cell migration (Katz et al. 2012).

This mechanism could potentially explain the observation of newly appearing FAs throughout the cell surface after Kid knockdown. It would be interesting to test the model in which Kid binds to mRNAs of certain FA proteins and inhibits their expression until they reach

the peripheral edges of cells where FA proteins function. After Kid knockdown, these mRNAs are translated into proteins on their way to be transported to destination, and this could explain why those FAs start to appear all over the cells.

APPENDIX A

KNOCKDOWN OF KID DID NOT AFFECT CELL-CELL CONTACTS

Previous study has revealed roles of integrin signaling molecules, including FAK, paxillin, Cas, and Pyk2, in cadherin-based cell-cell adhesion (Yano et al. 2004). As Kid regulates phosphorylation of FAK and paxillin in microtubule-induced adhesion disassembly, we examined if Kid also functions in cell-cell contacts. In order to check this, HeLa cells and RPE1-hTERT cells were seeded onto fibronectin-coated coverslips and transfected with either control siRNA or siRNA targeting Kid for 48h followed by immunostaining against N-cadherin. As shown in Fig. 34, there did not appear to be a difference in cell-cell contacts represented by N-cadherin between control cells and cells after knockdown of Kid, suggesting Kid is not involved in regulating cadherin-based cell-cell adhesion.

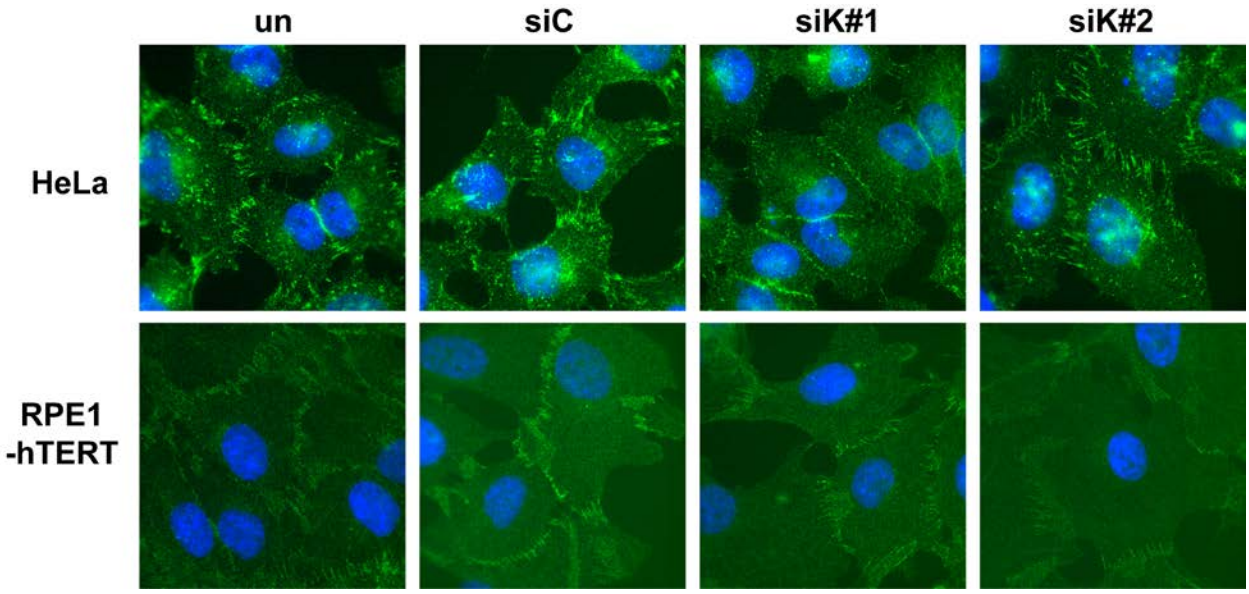

Figure 43. Knockdown of Kid does not affect cadherin-based cell-cell contacts

HeLa cells and RPE1-hTERT cells were seeded onto fibronectin-coated coverslips and transfected with either control siRNA or siRNA targeting Kid for 48h followed by immunostaining against N-cadherin. Images were taken using an Olympus BX60 Epifluorescence Microscope with a 100X objective.

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