Role of HECTD1 in regulating adhesion dynamics during cell movement

Inauguraldissertation

Zur
Erlangung der Würde eines Doktors der Philosophie
Vorgelegt der
Philosophisch-Naturwissenschaftlichen Fakultät
der Universität Basel

Von

Xiaoli Shen
aus Chongqing, China

Basel, 2015

Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät

auf Antrag von

Prof. Dr. Christoph Handschin

Prof. Dr. Christian De Geyter

Prof. Dr. Peter Scheiffele

Basel, 23. 06. 2015

Prof. Dr.Jörg Schibler

Dekan der Philosophisch-

Naturwissenschaftlichen Fakultät

Acknowledgements

First, I would like to thank my supervisor Prof. Christian De Geyter, for his continuous support during the last years. His trust and encouragement has motivated me to make progresses. Although being busy in clinic, he has always managed to organize our lab meeting and journal club meetings, during which I practiced to present scientifically and received many valuable suggestions. Moreover, I am also very respectful for his carefulness in correcting each report of me. Finally I would like to thank him for supporting me to join scientific retreats and conferences.

In particular, I would like to give my great thanks to my science advisor, Dr. Hong Zhang. I was fortunate to pursue my PhD under his guidance. His passion in work and strict attitude in science are positive examples for me. Whenever I was puzzled by the experiments, he could help me to think out of the box and find new ways. Also, he is always patient and gives me enough freedom to try out my own ideas. Under his support, I understand how important it is to speculate the different possibilities of one result, how much I should focus on my work and how many efforts have to do to finish a project. Outside work, I also thank him for his invitation to dinners, especially in Chinese New Year, it greatly relieved my homesickness.

As well, I want to thank my faculty members of my Committee, Prof. Peter Scheiffele and Prof. Christoph Handschin. Since the first meeting, Prof. Peter Scheiffele has been responsible for my research these years, his broad view in science and constructive suggestions have inspired me and will influence me in my future work. I am also respectful to his enthusiastic for discussing my project with me outside the annual committee meeting. I would like to sincerely thank Prof. Christoph Handschin for accepting as one of my committee members in my committee faculty, also thank for his comments and encouragement during my committee meeting and kind recommendation letter for me. I am also greatfrul for Prof. Anne Spang accepting as my Chiraman of my Committee.

I am very thankful to the MD-PhD committee of University of Basel, especially Prof. Christoph Hess, who gave me the precious opportunity to join the MD-PhD program. It is the trust of the committee that made it possible for me to pursue my PhD as a medical background student.

I would like to thank all the colleagues of Prof. De Geyter's group. The nice atmosphere here helped me to concentrate on my work. I would like to thank Brigitte Schneider, who not only helped me in experimental techniques, but also organized the whole lab work and all the nice parties which we enjoyed a lot. Many thanks give to Dr. Anne-Catherine Feutz, who generously offered her help whenever I had questions. My thanks also go to Dr. Sofia Forte and Flurina Pletscher, for their supports in both work and life. In particular, I would like to thank Xinggang Wang, for his countless help during our collaboration and for sharing all the difficulties and progresses in our projects. In addition, I would like to thank Dr. Zanhui Jia,

Shahidul Alam, Somayeh Khosravifarsani and Dr. Oliver Sterthaus, for helping me at the beginning of my PhD. Finally, I am thankful for our secretary Hanna Fluekiger, for her efforts and time offered to me during these years.

I am grateful for Michael Abanto, who instructing me on microscopes and the analysis software. I also thank Emmanuel Traunecker for teaching me FACS. As well, I would like to give my thanks to the whole Department of Biomedicine, for their generously sharing of reagents and antibodies when I needed for trials.

I am also thankful for Chinese Scholarship Council for financial support during my PhD and Chinese embassy in Switzerland for backing when I was far away from my country.

I am grateful for all my Chinese friends in Switzerland. Thank you all!

感谢我的家人，有你们的默默付出和支持，才有今天的这个我。无论走到哪里，你们都是我的根源和支柱。从大学开始，又是十年。终于，毕业也将来临，终于，将要学以所用。千言万语，汇成一句，谢谢你们！

It was fortune for me to complete my PhD study accompanied by my husband, Yuanfeng Zhang. Although the regional distance between us, I was deeply grateful that we could share this important period together. Thanks for your constant love, support and trust.

Catalog

Summary

E3 ubiqutin ligase for inhibin B receptor (HECTD1) has been demonstrated to play an indispensable role in embryonic development, including neural tube closure, placentation and eye formation, etc. The malformations caused by loss of HECTD1 are also found in humans, such as neural tube closure defects in HECTD1 knockout mouse model are comparable to anencephaly in humans. Therefore, the investigation of HECTD1 function in regulating neural tube closure and placental development may be helpful for understanding the underlying mechanisms of embryonic development and for the search of new approaches to prevent or treat the associated diseases. Normal embryonic development is the result of proper cell movement which is precisely regulated by complex cellular events. Although several mechanisms of HECTD1 function have been proposed recently, many aspects of the connection of HECTD1 with intracellular signaling pathways in cell movement are novel.

Using HECTD1 knockout mouse embryonic fibroblasts (MEFs) as the primary model, we first examined cell spreading and migration respectively. We found that loss of HECTD1 shortened the duration and reduced the area of cell spreading, while the velocity was enhanced and the directionality of cell migration impaired. Furthermore, as compared to wild-type cells, the cell adhesion proteins, paxillin and zyxin, were inhibited in maturing from focal complexes into focal adhesions in HECTD1 knockout cells. These defects in the formation of focal adhesions were associated with enhanced tyrosine phosphorylation of paxillin (paxillin-Y118), as well as higher activity of Rac1 and RhoA.

Screening with mass spectrometry has led to the identification of IQ motif containing GTPase activating protein 1 (IQGAP1) which is essential for the formation of cell polarity, stabilization of cell-cell adhesion and proper cell movement (Watanabe et al., 2004; Kuroda et al., 1998; Choi et al., 2013). Interestingly, we discovered that, in contrast to wild-type cells, IQGAP1 is overexpressed in HECTD1 knockout cells. Using co-immunoprecipitation and co-localization assay, we confirmed that IQGAP1 and HECTD1 physically interact with each other. Since HECTD1 is an E3 ubiquitin ligase, then we examined whether IQGAP1 acts as the substrate protein of HECTD1 by checking the ubiquitination and half life of IQGAP1. Our

results confirmed that in HECTD1 knockout cells ubiquitination was reduced thereby prolonging the half life of IQGAP1.

To answer the question whether the changes in adhesion proteins and cell movement observed in HECTD1 knockout cells were caused by overexpression of IQGAP1, we mimicked the observed effects by overexpressing GFP-IQGAP1 in wild-type cells and by siRNA knockdown of IQGAP1 in HECTD1 knockout cells. Intriguingly, overexpression of GFP-IQGAP1 in wild-type cells resulted in similar impaired expression of focal adhesions in HECTD1 knockout cells, while siRNA knockdown of IQGAP1 in HECTD1 knockout cells significantly rescued the maturation of focal adhesions, activity of RhoA, duration of cell spreading and velocity of cell migration.

Taken all data together, our findings indicate that HECTD1 plays a regulatory role in ubiquitination of IQGAP1, which in turn impacts on dynamics of focal adhesions and regulates cell spreading and migration.

1. Introduction

1. 1 Cell spreading and cell migration

In this chapter, I will introduce the principles of cell spreading and cell migration, including the basic steps for the complete cell movement behavior. Then, the different modes of cell migration will be mentioned.

1. 1. 1 Cell spreading

Cell spreading is the initial process of close contact between the cell and the substrate. In this process, when the cells take contact to a solid quasi-two-dimensional surface, it changes its shape from a spherical to a more flattened appearance. The process is characterized by the formation of filopodia and lamellipodia, representing the outward movement of the cellular membrane, including polymerizing actin and cytoskeletal complexes at the leading edge (Dubin-Thaler et al., 2004; Hall, 2005).

As cells start to spread on a substrate, a number of signals are emitted that are involved in various physiological functions such as cell migration (Lauffenburger and Horwitz, 1996; Woodhouse et al., 1997), morphogenesis (Gumbiner, 1996), differentiation (McBeath et al., 2004), growth (Folkman and Moscona, 1978) or metastasisof tumor cells (Woodhouse et al., 1997). Initial spreading is accompanied by formation of cellular adhesions and small actin bundles that are later remodeled into mature focal adhesions with reinforced stress fibers. In addition, spreading is also characterized by increasing area of the cell/substrate surface, which is regulated by the matrix or surface stiffness among other factors (Discher et al., 2005; Engler et al., 2004; Yeung et al., 2005).

Based on observations of fibroblasts, researchers (Dobereiner et al., 2004; Dobereiner et al., 2006; Dubin-Thaler et al., 2008) recently classified three distinct spreading phases with rapid inter-phase transitions: (a) early spreading, during which cells flatten until they reach a similar sectional area as in its beginning spherical shape, (b) intermediate spreading, during

which cells rapidly increase their interface and initiate contractile forces, and (c) late spreading, during which cells optimize their surface with increased cell adhesions and contractile cytoskeletal tension. While the later stages of cell spreading involve actin polymerization and myosin contraction, it is the earlier events that determine whether a cell will adhere to a surface (Cuvelier et al., 2007; McGrath, 2007). Various mechanisms are involved in the regulation of cell spreading, such as active CSK remodeling (Cai et al., 2006; Chamaraux et al., 2008), activation of focal adhesion kinase (FAK) and APR 2/3 (Serrels et al., 2007), actin polymerization and contractile forces (Loosli et al., 2010; Wakatsuki et al., 2003).

During cell spreading, focal complexes and focal adhesions are formed and function as cytoskeletal organizing centers (Borisy and Svitkina, 2000; Burridge and Chrzanowska-Wodnicka, 1996) and surface-sensing entities that locally and globally control adhesion-mediated signaling and coordinate the adhesive and migratory process (Cavalcanti-Adam et al., 2007; Geiger and Bershadsky, 2001; Zaidel-Bar et al., 2004).

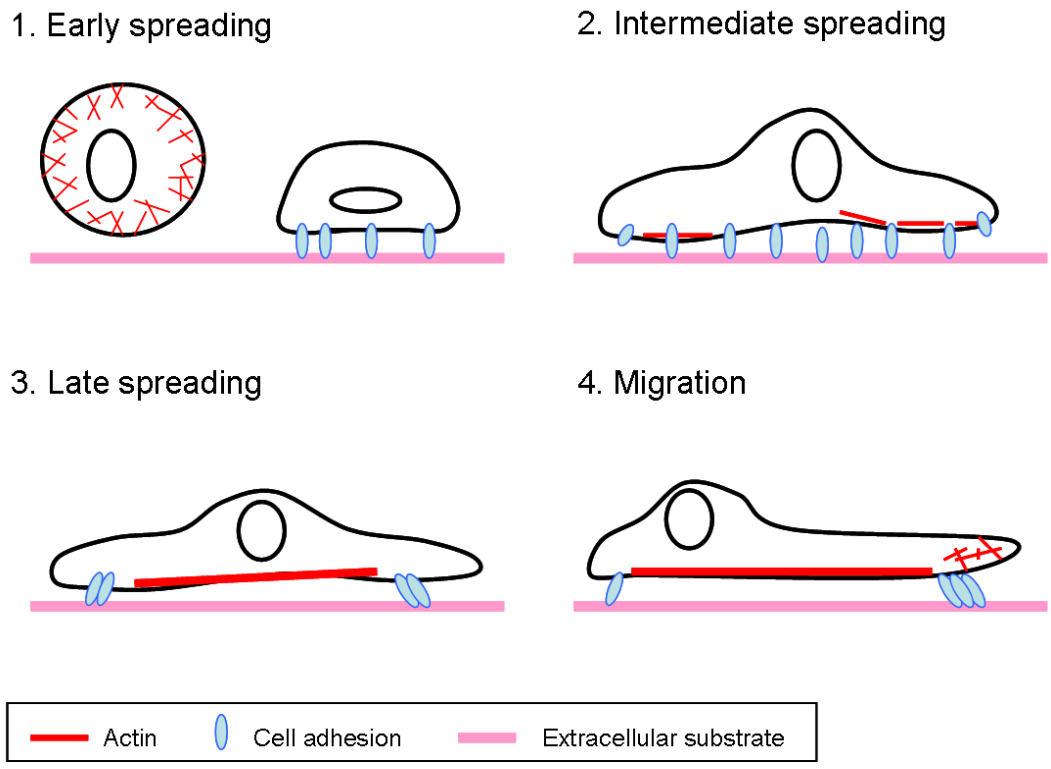


Figure 1. Schematic picture of cell spreading from suspension to migration. (1) Early spreading: contacts are initiated between cell and extracellular substrate and adhesions are generated. (2) Intermediate spreading: the cell continues to flatten, more adhesions are growing and actin

bundles begin to form. (3) Late spreading: cell reinforcement via reconstituting adhesions and actin bundles. (4) Migration: cell polarity is determined and starts to migration with maturing adhesions. (Adapted from Cuvelier et al., 2007)

1. 1. 2 Cell migration

Cell migration defined as a process indicating the translocation of cells from one site to another. Although cell migratory phenomena are apparent as early as in embryo implantation, cell migration orchestrates morphogenesis throughout embryonic development (S. F. Gilbert, Ed., Developmental Biology, 2003).

Our current understanding of cell migration is composite based on studies of various cell types and environments. Generally, cell migration can be conceptualized as a cyclic process (Lauffenburger and Horwitz, 1996). The initial step of a cell to a migration-stimulating compound is to polarize and forward protrusions in migrating direction. These protrusions can be either large and flat lamellipodia or spike-like filopodia, are normally driven by actin polymerization, and then are stabilized by adhering to the ECM or adjacent cells via transmembrane receptors linked to the actin cytoskeleton. The migration processes include cell polarization and protrusion, translocation of the cell body and retraction of the rear (Figure 2A). These steps are coordinated by extensive and transient signaling networks. These adhesions serve as traction sites at the front of migration and they are disassembled at the rear of cell, allowing to detach, in which way cell can move forward. The modes of cell migration are different depending on the cell type and the circumstance in which it is migrating (Figure 2B).

Pathologically, abnormal migratory signals may mislead the migration of cells, which may lead to catastrophic influences on tissue homeostasis and even overall health. Cell migration contributes to diverse important pathological processes, including vascular disease, osteoporosis, chronic inflammatory diseases such as rheumatoid arthritis and multiple sclerosis, cancer, and mental retardation. Thus, understanding the fundamental mechanisms underlying cell migration provides the value of effective therapeutic methods for treating diseases, preparation of artificial tissues and cellular transplantation (Ridley et al., 2003).

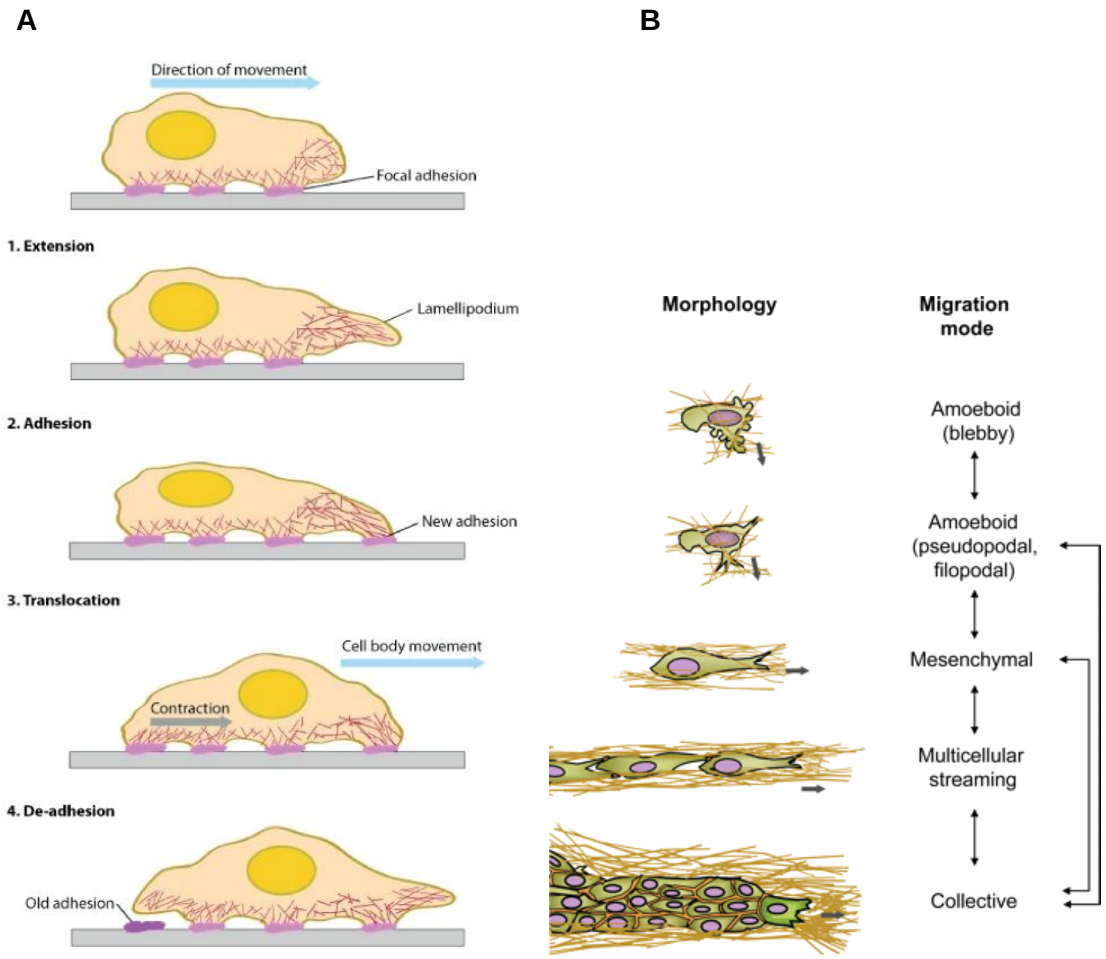


Figure 2. Conserved steps in directional cell migration and schematic mode for cell migration. Extension: actin polymerization is the driving force to form cell protrusion at the leading edge. Adhesion: new adhesion proteins are recruited at cell protrusion, linking actin filaments with transmembrane receptors to the ECM. Translocation: cell move forward by tension produced by activity of retrograde actin movement and contractile forces generated by stress fibers. De-adhesion: as the the continue to move forward, the complex of focal adhesion protein dissociates, allowing the cell to detach form the ECM and recruit the adhesion proteins to re-concentrate at another leading point (A) (Used by permission from MBInfo: www.mechanobio.info; Mechanobiology Institute, National University of Singapore). Cell migration modes is depended on cell morphology (rounded or spindle-shaped) and migrating pattern (individual, loosely connected or collective). Cell migration is transited between different patterns that regulated by specific molecular events. The thick arrows show the direction of migration (B) (Friedl and Wolf, 2010).

1. 1 .2 . 1 Polarization

Cell polarization represents the tendency of migrating cells to form a distinct, stable front and rear. This polarity usually arises from the cellular environment that provides a directional cue.

Whereas how this protrusive structure is formed at the molecular level in a cell is fragmentary known. It is believed that actin filaments and microtubules are essential for providing the polarity of a cell. Experimental evidence has shown that there is rapid actin polymerization at the cell's leading edge (Wang, 1985). This observation has raised the hypothesis that formation of actin filaments leads the leading edge forward and is the main motile power for pushing forward the cell's front edge (Mitchison and Cramer, 1996; Pollard and Borisy, 2003). Moreover, cytoskeletons could extensively and intimately interact with the plasma membrane of a cell (Doherty and McMahon, 2008).

Although microtubules have been reported to affect cell migration for many years, the precise mechanism by which they do so has still remained controversial. Studies have shown microtubules are not needed for the movement on a planar surface, but are required to establish and maintain directionality of cell movement and efficient protrusion of the leading edge (Ganguly et al., 2012; Meyer et al., 2012).

1. 1. 2. 2 Protrusion

Protrusion is the *de novo* formation of cell membrane extensions in the direction of migration. It includes three main components: the extension of the plasma membrane, the formation of cytoskeleton that supports membrane expansion, and the establishment of links with the substrate, which provides traction for the movement and signals that regulate actin polymerization (Horwitz and Parsons, 1999).

The two forms of protrusion are thought to play different roles: filopodia act as mechanosensory and exploratory devices, while lamellipodia offer wide surfaces that generate traction for advancing movement (Machesky, 2008).

1. 1. 2. 3 Cell body translocation and retraction of the rear

Rear retraction is the result of coordinated contraction of the actin filaments and disassembly of the cellular adhesions at the trailing edge. It is believed that several mechanisms contribute to accelerate adhesion disassembly: actomyosin contraction that brings power against the

adhesion promoting its mature, microtubule-induced adhesion turnover, integrin endocytosis, and proteolytic cleavage of focal adhesions that associate the integrins with actin (Ezratty et al., 2005; Jean et al., 2013; Webb et al., 2002).

1. 1. 3 Collective cell migration

As distinguished from single cell migration, collective cell migration is a process through which groups of cells are transported to new destinations and in which cells affect the movement of each other through physical coupling or signaling. It is well accepted that collective cell dynamics give rise to complex changes in multicellular tissue structures, including wound-healing (Martin, 1997), neurulation in embryogenesis (Theveneau and Mayor, 2012; Weijer, 2009), invasion of cell masses during cancer metastasis and sprouting of ducts in branching morphogenesis (Friedl and Gilmour, 2009). In these processes, cells migrate collectively in a set of modes, as sheet movement, sprouting and branching, streams or free groups. In accordance with individual cell migration, collective cell movement requires actomyosin polymerization and contractility together with cell polarity; however, some of the mechanisms involved in collective cell migration are different. The main difference is that the cells are coupled by cell to cell junctions in which two cells are in contact with each other (Friedl, 2004; Lecaudey and Gilmour, 2006; Rorth, 2007). The types of cell-cell junctions include adherens junctions, desmosomes, tight junctions and gap junctions. Adhesive cell-cell coupling collective cell migration is mediated by adherens junction proteins, including cadherins, α/β-catenin, integrins and immunoglobulin superfamily (Ilina and Friedl, 2009). The major roles of adherens junctions during collective cell migration are to maintain the integrity of the migrating cells and to promote the coordination of cells while allowing cellular rearrangements (Peglion et al., 2014). For example, neural crest cells which are collectively attracted toward a chemokine, N-cadherin. Protrusion formation is stimulated at the free edge of the cells while protrusion at cell-cell contacts is suppressed (Theveneau et al., 2010). In carcinoma cells, loss of E-cadherin with increased N-cadherins and neural cell adhesion molecules cause the onset of collective migration (Lee et al., 2006). Besides, wnt/β-catenin and Fgf signaling in leader cells regulates

coordinated migration via chemokine signaling (Aman and Piotrowski, 2008). Thus, studying the details of adherens junctions offers the opportunity to better understand collective cell movement.

1. 2 Factors influencing cell movement

In this chapter, I will introduce the factors that affect cell spreading and cell migration. Those mainly include the basic unit of cell movement, cell matrix adhesion, the cell cytoskeleton system, extracellular matrix and role of Rho GTPases in regulating cell movement, as well as the diseases associated with these factors.

1. 2. 1 Cell matrix adhesion

Cell adhesion, which acts as the physical interaction of a cell with its adjacent cell or with the ECM, is essential for both cell migration and tissue integrity. Cell-matrix adhesion is the best-studied form of adhesion that mediates cell migration. Different types of adhesions are defined by their subcellular location, size, composition and link to F-actin. Usually they are cataloged to four main structures: nascent adhesion, focal complexes, focal adhesions and fibrillar adhesions.

Nascent adhesions are the first emerging adhesive structures, showing up within the lamellipodium. Nascent adhesions are small and highly transient, normally vary in size between 0.5-1 μm with an average lifetime of 80 sec (Choi et al., 2008) (Figure 3), either maturing to focal complexes or disassembling, and are not easily detected in every type of cell (Alexandrova et al., 2008; Choi et al., 2008).

Focal complexes are cell adhesions in the early phases of maturation. They were original observed in cells expressing a active form of Rac (Zaidel-Bar et al., 2003). They are larger than nascent adhesions, rely on myosin II for the formation and maintenance, and locate at the border of the lamellum and lamellipodium (Giannone et al., 2007; Rottner et al., 1999). Rac1 activation and followed phosphoinositide production induce the recruitment of talin

homodimer in a F-actin and vinculin dependent manner (Banno et al., 2012; Campbell and Ginsberg, 2004; Martel et al., 2001). As scaffold proteins, the talin-mediated connection to actin cytoskeleton maintain the stabilization of the integrin-ECM linkage (Nishizaka et al., 2000) (Figure 3). Like nascent adhesions, focal complexes also tend to either disassemble or aggregate and elongate into focal adhesions (Choi et al., 2008). The presence of focal complexes and nascent adhesions is a marker of highly motile cells, their quick appearance and turnover correlate directly with the high velocity of protrusion and cell movement.

Focal adhesions: Focal adhesion is currently represented for mature adhesions that evolve continually over time (Zaidel-Bar et al., 2004), which maintain a mean size between 1-5 μm. Over 150 proteins have been discovered in the complexes (Zaidel-Bar et al., 2007). As focal adhesions continue to mature, more and more adhesion proteins are recruited to form bigger and bigger protein complexes, such as paxillin, FAK and zyxin. Among the focal adhesion components, the presence of zyxin distinguishes mature focal adhesions from their earlier forms. Increasing concentrations of zyxin are acquired via tyrosine phosphorylation processes (Beningo et al., 2001; Zaidel-Bar et al., 2004). Then, the redistribution of zyxin to stress fibers regulates the strength of the adhesion (Yoshigi et al., 2005). Focal adhesions locate at both the cell periphery and more centrally, bind with the ends of stress fibers in cells cultured on two-dimensional (2D) rigid surfaces (Wozniak et al., 2004). They are protein complexes that exert mechanical connections between intracellular actin bundles and the extracellular substrate in many cell types (Abercrombie and Dunn, 1975; Hotulainen and Lappalainen, 2006) (Figure 3). Disparate observations across cell types, such as fast-moving Dictyostelium discoileum and neutrophils vs. slowly moving fibroblasts, indicate an inverse relation between focal adhesion size and cell migration speed. For instance, in FAK-deficient fibroblasts, reduced focal adhesion number and size are associated with enhanced cell motility. In addition, using Dystrglycan deficient or overexpression fibroblasts as tool, it is suggested that decreased size of focal adhesions is related to higher velocity and impaired directionality of cells, and vice versa (Ilic et al., 1995; Nagasaki et al., 2009; Thompson et al., 2010). One study also suggests focal adhesion size maintains biphasic relationship with cell migration speed (Kim and Wirtz, 2013).

Fibrillar adhesions have been described in three-dimensional matrix systems or in cells

plated on 2D complex ECM and are thought to be derived from a subset of focal adhesion at more central positions (Cukierman et al., 2001, 2002). These are long, highly stable complexes that connect microfilament stress fibers with extracellular fibronectin (FN) fibers, as well as run parallel to bundles of FN *in vivo*. FBs are highly enriched in tensin, active α5β1 integrin and little or no phosphotyrosine, and indeed are sites of localised matrix deposition and FN fibrillogenesis beneath the cell (Pankov et al., 2000; Zamir et al., 2000).

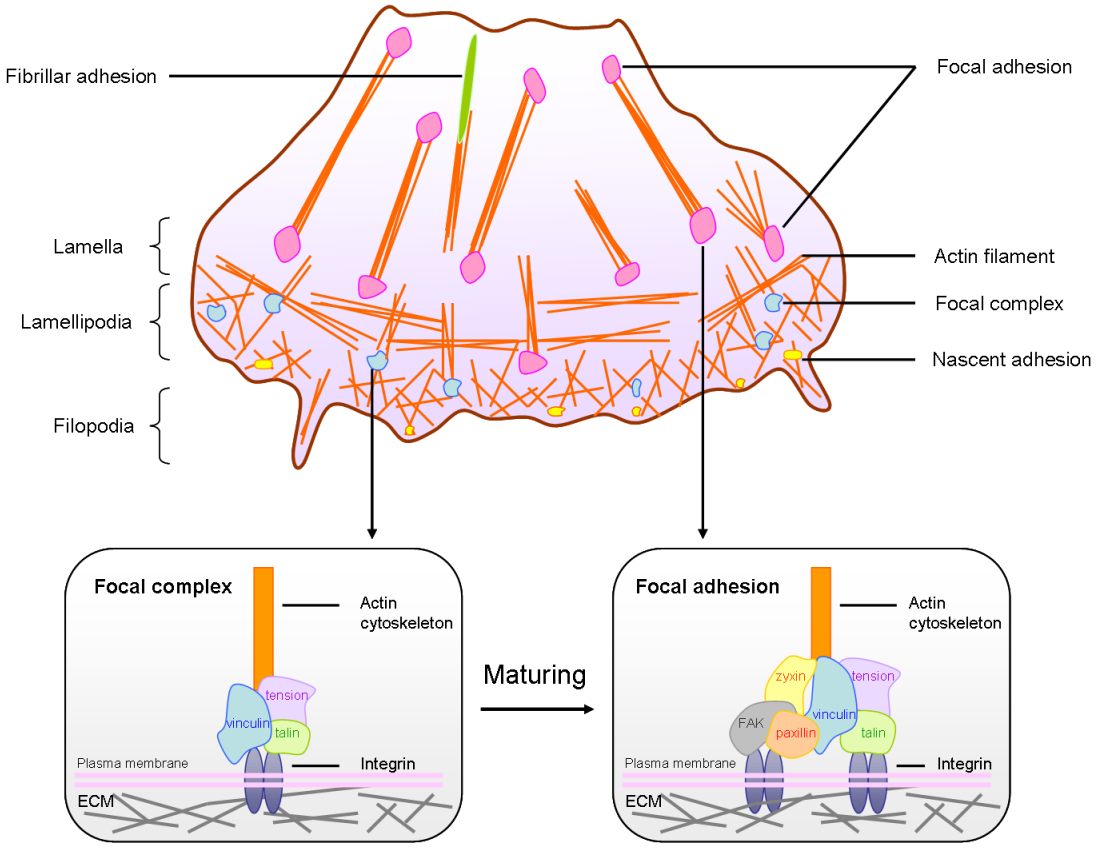


Figure 3. Schematic for type and composition of cell matrix adhesion. Cell matrix adhesions are formed as the cells enter in contact with extracellular substrates. Nascent adhesions (yellow patches in the upper panel) are the earliest form of cell matrix adhesion, locating at the front of lamellipodia, where the nascent adhesions turn into focal complexes (blue patches in the upper panel and left low panel), typically compose of talin and vinculin. As the focal complexes continue to mature, more adherens proteins (e. g. paxillin, zyxin and FAK, right low panel) are recruited, focal adhesions (pink patches in the upper panel) are generated and linked to actin bundles.

1. 2. 1. 1 Tyrosine phosphorylation

During activation of focal adhesions, one of the key signaling pathways is tyrosine phosphorylation. Using YFP-Src-SH2 domains as a live cell probe, Kirchner and coworkers indicated that recruitment of components such as FAK and paxillin to focal adhesions precedes important tyrosine phosphorylation. Tyrosine phosphorylation at the focal adhesion offers docking sites for the binding of SH2-containing proteins and modulates the activation of additional kinases and phosphatases subsequently (Kirchner et al., 2003). Two of the main kinases discovered in focal adhesions are FAK and Src, which bind to different substrates to regulate focal adhesion dynamics and cell behavior (Wozniak et al., 2004). Tyrosine phosphorylation allows paxillin to interact with a great number of signaling molecules: tyrosine residues (Y) 31 and 118 being especially predominant targets of phosphorylation by kinases (Bellis et al., 1997; Schaller and Parsons, 1995; Sudol, 1998), since several cytokines and growth factors also lead to tyrosine phosphorylation of paxillin (Turner, 1998) (Sattler et al., 2000). Therefore, it is indicated that the signals from both cytokine- and growth-factor receptors and also from integrins converge on paxillin (Iwasaki et al., 2002; Zaidel-Bar et al., 2007b).

1. 2. 1. 2 Protein phosphatase

Protein phosphatase 2A (PP2A) is believed to be a major serine/threonine phosphatase, while there are also some studies showing PP2A is also a regulator for protein tyrosine phosphatase activity (Jackson and Young, 2003). One study of Lewis lung cancer cells (LLC) showed that reduced PP2A activity results in serine hyper-phosphorylation and tyrosine dephosphorylation leading to unstable Src/FAK/paxillin complex formation and reduced adherence. Another paper concluded that inhibition of PP2A activity by okadaic acid resulted in paxillin serine hyperphosphorylation and tyrosine dephosphorylation (Jackson and Young, 2003). Together with these former studies, it is suggested that that PP2A may act as a regulator of the balance between phosphorylation and dephosphorylation of serine and tyrosine residues.

1. 2. 2 Adherens junctions

The sites, where migrating cells encounter each other to form a protein complexes at cell-cell junctions are known as adherens junctions, which cytoplasmic face is connected to the actin cytoskeleton (Guo et al., 2007). Traditionally, adherens junctions serve as a bridge linking the actin filaments of adjacent cells. They can show as both bands encircling the cell or as spots of attachment to the substrates. The composition of adherens junctions include: cadherins, which serve as calcium dependent transmembrane proteins; p120, which binds the juxtamembrane area of the cadherin; α-catenin, which indirectly binds the cadherin via β-catenin and links the actin cytoskeleton with cadherin; and γ-catenin which binds the catenin-binding region of the cadherin (McNeill et al., 1993). It is widely believed that E-cadherin binds β-catenin directly upon export of the proteins from endoplamic reticulum, while α-catenin adding the complex after being recruited to the plasma membrane (Hinck et al., 1994) (Figure 4).

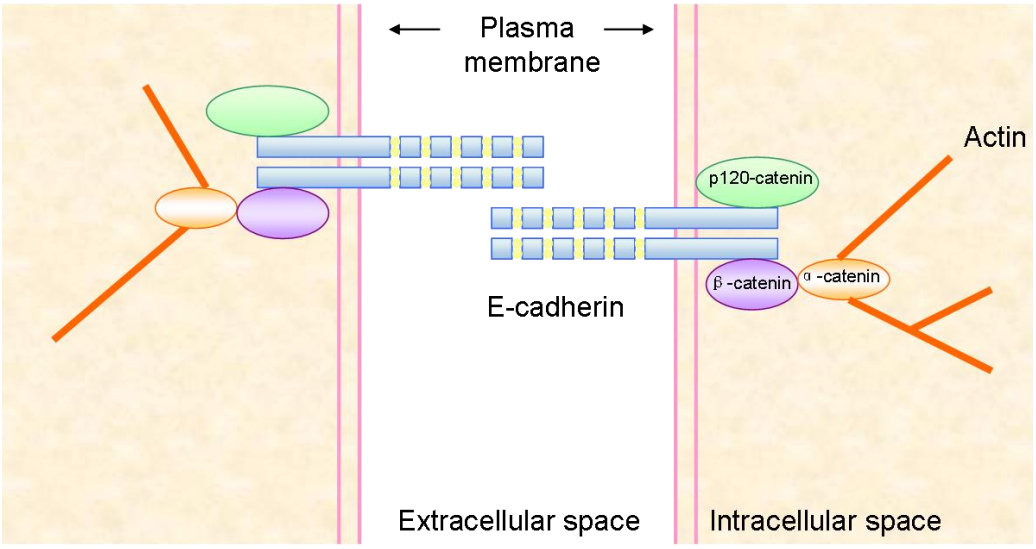


Figure 4. Schematic for adherens junctions in cell-cell contact. Cadherins are localized at the lateral cell membrane of neighboring cells, and their glycosylated residues forward to the extracellular space, overlapping and generating homodimers in a calcium-dependent manner. Inside the cell, the cytoplasmic domains of the cadherins are supported by binding with p120-catenin and β-catenin. To form the complexes of adherens junctions, β-catenin links α-catenin which in turn connects with actin filaments.

1. 2. 3 The Cytoskeleton

The eukaryotic cytoskeleton is a network formed by three long filament systems, which constitute with dynamic proteins that repetitive assembly and disassembly. The cytoskeleton system creates the cell shape by providing an internal architecture via elaborate linkage(s) to itself, the plasma membrane and internal organelles. The structure of cytoskeleton is regulated by adhesion to adjacent cells or to the extracellular substrates. The force and the type of these adhesions are critical for modulating the assembly and disassembly of the cytoskeleton components. Governed by strengths from both internal and external, the cytoskeleton dynamic property promotes cell movement (Le Clainche and Carlier, 2008; Small et al., 1999).

There are three major components of the cytoskeleton. These are unique networks with distinct compositions that exert slightly different yet interdependent functions. They are actin filaments, microtubules and intermediate filaments (Figure 5).

Actin filaments, also called F-actin, are shown as microfilaments in cytoskeleton system assembled by globular actin (G-actin) into linear actin polymers. They locate below the plasma membrane and are normally assembled at the cell periphery from focal adhesion or membrane extension sites (Egelman, 1985). Actin filaments provide the structure and shape of cells links the inside of cells with the outside environment, acting as key regulators to form the dynamic cytoskeleton, and signal transducer form external surroundings to the interior of the cell (Pollard and Cooper, 2009). Besides, they act to promote cell motility, such as through the formation and function of filopodia or lamellipodia (Insall and Machesky, 2009). Therefore, actin filaments play an important role in embryogenesis, the healing of wounds and the migration of cancer cells (Kellogg et al., 1988; Yamaguchi and Condeelis, 2007).

Microtubules are the largest component of the cytoskeleton and are substantially longer than actin filaments. They are stiff and hollow filament structures formed through the lateral association of tubulin protofilaments. They are highly dynamic, undergoing rapid cycles of polymerization and depolymerization. Besides cytokinesis, microtubules also play important roles in directional cell migration. Microtubules emerge from a microtubule organizing center (MTOC) where their minus end is embedded and the plus end grows towards the cell

periphery. The MTOC is a main location of microtubule nucleation, which reorientation toward cell leading edge determines cell polarity and finally contributes to directional cell migration (Gundersen, 2002; Ridley et al., 2003; Watanabe et al., 2005).

Intermediate filaments are the third type of cytoskeletal filament and share similar structures and functions with the actin filaments and microtubules. Intermediate filaments form an elaborate network both in the cytoplasm and in the nucleus in the form of lamina. Although highly flexible, they are much more stable than both actin filaments and microtubules. Intermediate filaments have no known clue in cell motility however they do offer mechanical stability to cells at sites of cell-cell contacts (Albert et al, 2002).

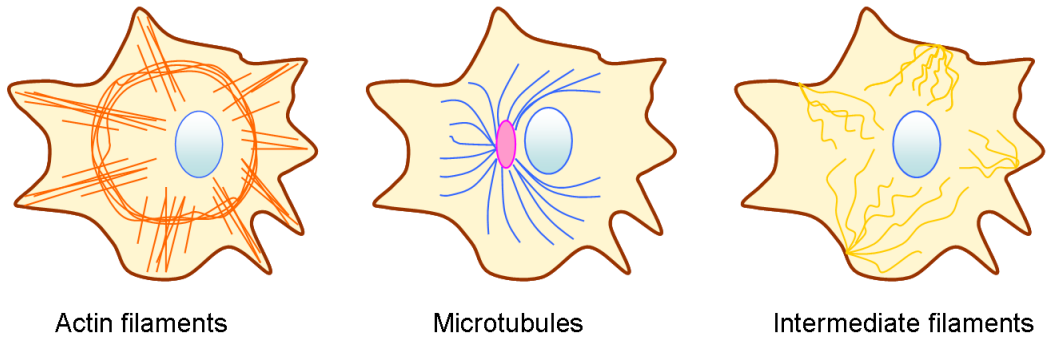


Figure 5. Overview of cytoskeleton. There are three major components of the cytoskeleton: including actin filaments, microtubules and intermediate filaments. They distinguish each other in structure while exerting interdependent functions.

1. 2. 3. 1 Lamellipodia

The lamellipodium is a cytoskeletal protein projection on cell leading edge, which contains quasi-two-dimensional actin mesh, and the whole structure promotes the cell across a extracellular substrate (Albert et al, 2002). Within the lamellipodia, when the ribs of actin (also known as microspikes) spread beyond the lamellipodium frontier, are called filopodia (Small et al., 2002). The lamellipodium is formed out off actin nucleation in the plasma membrane of the cell (Albert et al, 2002) and is the initial area of actin formation. Both lamellipodia and filopodia are common actin-dependent structures which are responsible to detect the cellular surroundings before of migrating cells. Actin filaments assembly in lamellipodia and retrograde movement of the filaments produces protrusive strengths that

guide cell motility in a certain orientation. These protrusive and retractive processes are intensively controlled by the small GTPases Rac1 and Rho, which respectively trigger actin polymerization and actomyosin contractility (Ballestrem et al., 2001; Cavalcanti-Adam et al., 2007; de Rooij et al., 2005; Wittmann et al., 2003).

The region immediately behind the lamellipodium has been defined as lamella, which comprises contractile bundles of actin filaments (Vallotton and Small, 2009) and frequently associate with sites of adhesions (Choi et al., 2008). Lamellipodia and lamella are different from each other both in structure and molecular composition. The lamellipodium consists of dendritic F-actin network (Koestler et al., 2008), whereas lamella are comprised of bundled actin filaments (Burnette et al., 2011). In contrast to lamellipodia which contain abundant Arp2/3 and ADF/cofilin, lamella are enriched in myosin II and tropomyosin (Ponti et al., 2004; Rottner and Stradal, 2011).

1. 2. 3. 2 Stress fibers

Stress fibers are higher **o**rder form of cytoskeletal structures contained cross-lined actin filament bundles (Cramer et al., 1997). Commonly, they connect to focal adhesions, and therefore are critical in mechanotransduction. In mammalian cells, stress fibers undergo cyclic assembly and disassembly, that enable them to maintain cellular tension and adjust changes in response to different forces (Hirata et al., 2007; Kaunas et al., 2005). Thus, stress fibers have since been shown to indispensibly be involved in cell motility and contractility, affording force various cellular events and morphogenesis (Tojkander et al., 2012).

1. 2. 4 Extracellular matrix

Extracellular matrix in tissue is involved in regulating tissue stability, integrity and functions. During tissue development the produced ECM gradually turns into tissue-specific based on specific composition and topology (Frantz et al., 2010). Basically, it is constituted from a complex mesh work of insoluble molecules to anchorage cells. The ECM is composed of two major types of macromolecules: proteoglycans (PGs) and fibrous proteins (Jarvelainen et al.,

2009; Schaefer and Schaefer, 2010). During the cell-ECM interactions, the tissue-specific nature of ECM compositions indicates specific functions of the ECM proteins such as FN, collagen and laminin that bind to integrin receptors which in turn are activated by the cytoskeletal components (Anselme, 2000; Koyama et al., 1996; Shekaran and Garcia, 2011; Stegemann et al., 2005) (Albert et al, 2002) (Rozario and DeSimone, 2010) (Figure 6).

FN is a high-molecular weight glycoprotein of the extracellular matrix that links to integrins. FN exists as a protein dimer, including two closely identical monomers connected by a pair of disulfide bonds (Pankov and Yamada, 2002). It also contains additional cell-binding domains and cryptic sites that are exposed in response to force and are involved in matrix assembly (Klotzsch et al., 2009; Smith et al., 2007). FN is important for controlling cell migration during embryonic development (Darribere and Schwarzbauer, 2000). Collagen is the main component of connective tissue and most abundant protein in mammals (Di Lullo et al., 2002), making up from 25% to 35% of the whole-body protein content. The fibroblast is the most common cell creating collagen. Collagen I is the most abundant collagen of the human body. Gelatin is a mixture of peptides and constitutes an irreversibly hydrolyzed form of collagen. During hydrolysis, the natural molecular bonds between individual collagen strands are broken down into a form that rearranges more easily. Its chemical composition is, in many respects, closely similar to that of its parent collagen.

The molecular signals responsible for the selective guidance of ECM are complicated because of the different ligand-binding possibilities with integrins (Barczyk et al., 2010; Levental et al., 2009). Cell fate-like survival, proliferation or differentiation are controlled by a bidirectional signaling divisible into inside-out for the formation of focal contacts and outside-in cascades to guide the cell behavior through integrins. Different ECM proteins have been reported to bind to various integrin subtypes. FN has been described to bind $\alpha_1\beta_1$, $\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_5\beta_1$, $\alpha_8\beta_1$, $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_6$, and $\alpha_4\beta_7$ integrins (Adams, 2001; Altankov et al., 1997; Letourneau et al., 1992; Tzu and Marinkovich, 2008), and for collagen I, $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_{10}\beta_1$, $\alpha_{11}\beta_1$, and $\alpha_v\beta_8$ integrins are the binding motif (Hidalgo-Bastida and Cartmell, 2010; Letourneau et al., 1992; Tzu and Marinkovich, 2008) (Adams, 2001; Mineur et al., 2005). Moreover, laminin can bind integrin $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$, $\alpha_6\beta_4$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, and $\alpha_7\beta_1$ (Letourneau et al., 1992; Loeser et al., 2000; Tzu and Marinkovich, 2008). Besides, the

integrin motifs expressing in different cell types vary a lot, while in fibroblasts, $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$ and $\alpha_5\beta_1$ integrins were identified (Mineur et al., 2005). Therefore, there are some integrin subtypes mutual active in different ECM and fibroblasts (Schlie-Wolter et al., 2013).

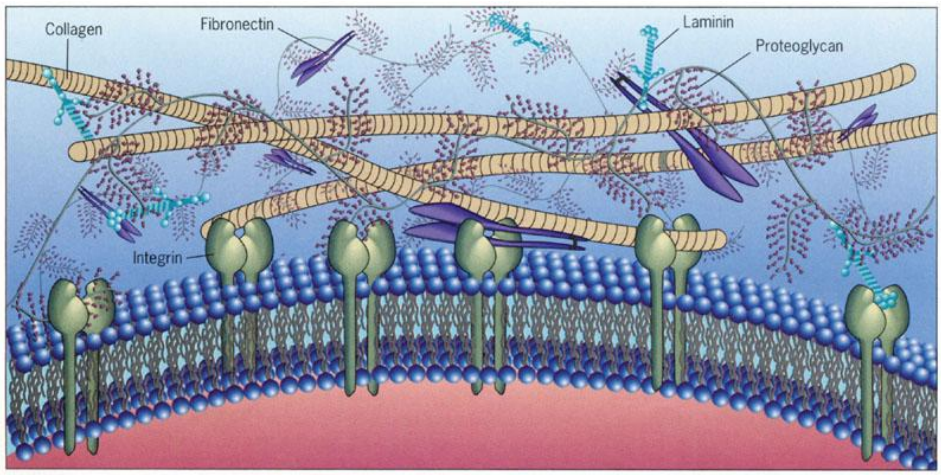


Figure 6. Illustration of the molecular structure of an extracellular matrix. The ECM proteins collagen, fibronetin, laminin, as well as proteoglycans, crosslink each other and interact with intracellular signals via cross-membrane proteins, i.e. integrins. (Adapted from Cell and Molecular Biology Concepts and Experiments by Karp, 2010)

1. 2. 5 Rho GTPases

The Rho family of GTPases is a family of small signaling G proteins and belongs to the subfamily of the Ras superfamily. Rho proteins act as “molecular switches” to play a role in organelle development, cytoskeletal dynamics, cell movement and other common cellular functions (Boureux et al., 2007; Bustelo et al., 2007) (Figure 7).

Three members of this family have been studied intensively: Rac1, RhoA and Cdc42, whose activation is highly related to the formation of stress fibers, lamellipodia and filopodia through various effector proteins, including kinases and focal adhesion proteins (Jaffe and Hall, 2005; Pleines et al., 2013). To be more specific, activation of Rac enables the formation of focal complexes, whereas Rho activity induces the induction and growth of focal adhesions (Nobes and Hall, 1995) and enhances the formation of fibrillar adhesions (Chrzanowska-Wodnicka and Burridge, 1996; Hotchin and Hall, 1995; Ridley and Hall, 1992; Zaidel-Bar et al., 2007b). Focal adhesion proteins, such as paxillin and FAK, serve as

scaffolds of phosphorylation-dependent signaling, through which proteins are recruited which modulate the activities of Rac and Rho, and finally regulate adhesion dynamics and protrusion extension (Choi et al., 2011; Schaller and Parsons, 1995).

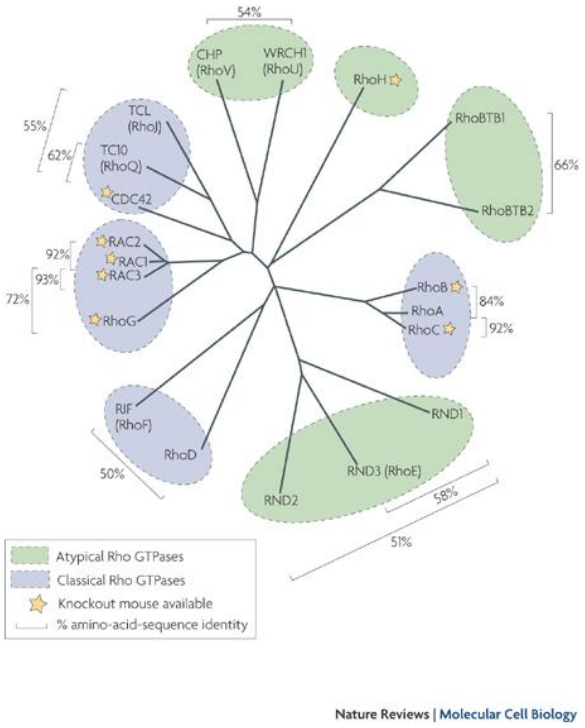


Figure 7. The Rho GTPase family. The unrooted phylogenic tree depicts the relationship between the different family members of GTPases proteins. The classical members of GTPases include Rho, Rac and cdc42 (Heasman and Ridley, 2008).

The activity of mall GTPases Rac1 is regulated by integrin-mediated adhesion (Del Pozo and Schwartz, 2007) and thereby dynamically regulates cell migration by promoting cytoskeletal re-organization and membrane protrusion (Ridley, 2006; Ridley et al., 2003). Disruption of Rac1 impaired of cell directionality on fibrillar matrices (Bass et al., 2007; Pankov et al., 2005) and thereby inhibited wound healing (Tscharntke et al., 2007). Consistent with these findings, overexpression of active Rac1(V12) impairs cell-cell adhesion and enhances directed cell motility and migration, while dominant negative Rac1(N17) induces the opposite effects (Hage et al., 2009).

Active RhoA stimulates the formation of both stress fibers and focal adhesions (Ridley and Hall, 1992). Accurate regulation of RhoA is important for efficient cell migration. Since Rho proteins are involved in dynamic cellular events, such as migration, their activity is closely controlled by positive and negative regulators in the mean time (Van Aelst and D'Souza-Schorey, 1997). RhoA activity is required for migration possibly due to the maintain

sufficient adhesions to the substrate (Nobes and Hall, 1999; Takaishi et al., 1994). Previous studies proved that adhesion to FN is sufficient for promoting RhoA-dependent stress fiber formation (Barry et al., 1997). Ren *et al.* (Ren et al., 1999) directly measured activity of RhoA, suggesting that RhoA activity is controlled by adhesion to FN in a triphasic way, where RhoA is transiently inhibited after early binding to FN at 10-30 minutes, followed by activation between 60 and 90 minutes and then slightly decreasing after 2-3 hours. However, RhoA activity has also been reported to maintain an inverse relationship with cell motility and cell migration. One study demonstrated that localized RhoA inactivation by p190RhoGAP promote to efficient cell movement by enhancing membrane protrusion and cell polarity as well (Arthur and Burridge, 2001).

Overall, recent studies show that both RhoA and Rac1 are active at the leading edge of migrating cells with spatial and temporal distinctions (Hodgson et al., 2010). Rottner and coworkers have shown that in response to Rac-upregulation focal complexes are differentiated into focal contacts, while down-regulation of Rac promotes the enlargement of focal contacts (Rottner et al., 1999). However, RhoA and Rac1 have been shown in migrating cells to act as mutual antagonism. For instance, the oxidative cascade including Rac1, reactive oxygen species (ROS) and a p190RhoGAP phosphatase has been shown to associate with the antagonistic crosstalk between Rac1 and RhoA (Nimnual et al., 2003). Moreover, during study of epithelial morphogenesis, through quantification of RhoA- and Rac1-dependent signaling pathway markers over the apical basal axis of lens pit cells, Chauhan and coworkers have found that in RhoA deficient cells there was a Rac1 signaling pathway gain of function and vice versa, suggesting the balanced activity of both Rac1 and RhoA regulates cell shape and drives invagination in epithelia (Chauhan et al., 2011).

As the “molecular switches” in cellular events, Rho GTPases are regulated in a cyclic way. On one side, the GTPases are activated by guanine nucleotide exchange factors (GEFs), which catalyze GDP release allowing GTP to bind and subsequently activate the proteins. On the other side, the return to an inactive state of Rho proteins upon hydrolysis of GTP to GDP, is dominated by GTPase-activating proteins (GAPs). Besides, Rho GTPases are regulated by guanine nucleotide disassociation inhibitors as well, which enable Rho proteins to extract from the plasma membrane (Olofsson, 1999). To understand the precise regulation of Rho

family GTPases, it is critical to depict the activity of GAPs, GEFs and guanine nucleotide disassociation inhibitors, altered by changing extracellular signals (Katoh and Negishi, 2003; Nishiya et al., 2005) (Figure 8).

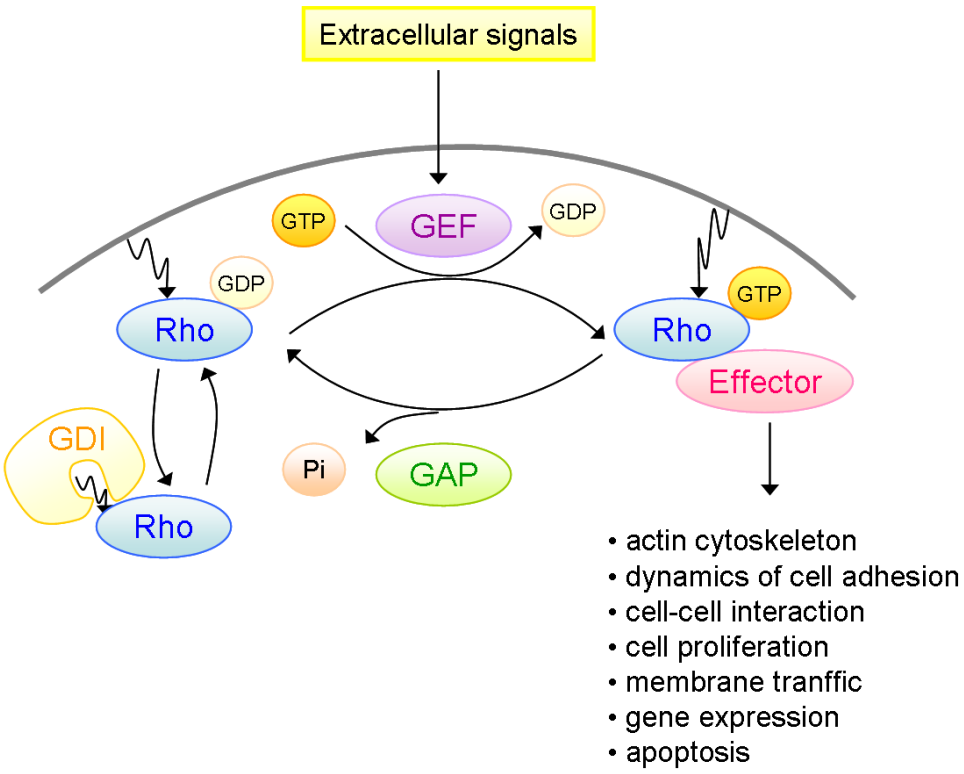


Figure 8. The Rho GTPases switch between active (GTP-bound) forms and inactive (GDP-bound) forms. The switch is mainly regulated by three classes of proteins: nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs) and Guanine-nucleotide dissociation inhibitors (GDIs). GEFs promote the formation of GTP-bound Rho GTPases, which interact with their substrate effectors and further regulate the downstream cellular events. In contrast to GEFs, GAPs exert the opposite influence to balance the cycle. While GDIs inhibit nucleotide dissociation and regulate cycling of Rho GTPases between membrane and cytosol.

1. 2. 6 Embryonic Development and Organogenesis

Embryonic layers (endoderm, mesoderm and ectoderm) are formed through the migration of large groups of cells within the blastocyst embryo. During these migrations, these cells involve in differentiation programs as precursors and migrate to their final targets where they undergo terminal differentiation developing the various organs. For instance, in the developing brain, neuronal precursors migrate from the neural tube to the distinct layers of tue

future brain. The cells migrate through various embryonic layers to their final destinations and then send projections as axons and dendrites through the layers to form subsequent specific interactions. Generally, this cell migration is the best understood model among all embryonic migrations. In this model, the cells derive from the upper part of the neural tube and move to a plenty of locations including bone, cartilage, peripheral nervous system and skin (Kuo and Erickson, 2010).

1. 3 Other proteins involved in cell movement

IQGAP1 and Hax1 are two potential binding partners to HECTD1 that have been shown to be highly involved in regulating cell movement. Here, I will give a brief review about the functions of the two proteins in cell movement.

1. 3. 1 IQGAP1 in cell movement

IQGAP1 belongs to the IQGAPs family which serves as scaffolding proteins with multiple domains, shorts for the IQ motifs containing GAP related domains. Despite the homology of amino-acid sequence with GAP, IQGAP1 do not have GTP hydrolysis activity (Brill et al., 1996; Hart et al., 1996; Kuroda et al., 1996). In eukaryotic cells, IQGAP1 localizes to actin-containing structures such as lamellipodia, membrane ruffles, cell-cell adhesions and the actomyosin ring formed during cytokinesis. IQGAPs perform their multiple functions through association with various proteins including filamentous actin, GTPases, calcium-binding proteins, microtubule binding proteins, kinases and receptors (Figure 9).

To date, our understanding of IQGAP1 in regulating cellular motility and morphogenesis is becoming more comprehensive and diverse (Mateer et al., 2003). IQGAP1 acts as a positive regulator in cell motility and cell migration through different cellular mechanisms in a range of cell lines. For instance, accumulating evidence has shown IQGAP1 is up-regulated in various types of tumor cells, including colorectal carcinoma (Hayashi et al., 2010; Nabeshima et al., 2002), gastric cancer (Takemoto et al., 2001), hepatocellular carcinoma

(Chen et al., 2010), pancreatic cancer (Wang et al., 2013) and ovarian carcinoma (Dong et al., 2008). Silencing the overexpression of IQGAP1 results in inhibiting the invasion of tumor cells (Dong et al., 2008; Wang et al., 2014). Mechanistically, as an effector of Rac1, IQGAP1 binds to activated Rac1(V12) to reduce the association of IQGAP1 and β-catenin, indicating the involvement of Rac1 in the inhibition of cellular adhesion thereby promoting metastasis (Hage et al., 2009). Moreover, the interaction of IQGAP1 with Platelet-derived growth factor receptor-β (PDGFR) and focal adhesion proteins (paxillin, vinculin and FAK) in response to stimulation of Platelet-derived growth factor (PDGF), progresses PDGF receptor (PDGFR) activation and focal adhesions formation that lead to cell migration (Kohno et al., 2013). Under normal physiological conditions, as coordinating with small GTPase, Rac1, RhoA and Cdc42, IQGAP1 supports cell movement via regulating adherens junctions, actin filaments and microtubules. Initially, IQGAP1 has been identified as a target of Rac1 and Cdc42. Overexpression of IQGAP1 reduces the activity of E-cadherin-mediated cell-cell adhesive activity (Kuroda et al., 1998). In addition, activation of Rac and Cdc42 in response to stimulation signals leads to the recruitment of IQGAP1, APC and CLIP-170, forming a complex which connects to the actin cytoskeleton and microtubules promoting cell polarization and directional cell migration (Fukata et al., 2002; Watanabe et al., 2004). Another mechanism also proposed that IQGAP1 requires PIPKIγ for targeting to the leading edge of cells and be activated specifically by PIP2 to promote actin polymerization and cell migration (Choi et al., 2013).

However, IQGAP1 may also negatively impact on cell migration. One study demonstrated in the microenvironment of tumors that IQGAP1 suppresses TβRII- and TGF-β-dependent myofibroblastic differentiation thereby inhibiting tumor growth (Liu et al., 2013). Besides, anti-GTPase activity of IQGAP1 sustains the amount of GTP-bound Rac1 at sites of cell-cell contact, resulting in stable adhesion (Noritake et al., 2005).

Taken all findings together, the involvement of IQGAP1 in cell migration deserves more interest in studies as an important potential target for tumor cell invasion and directional cell migration.

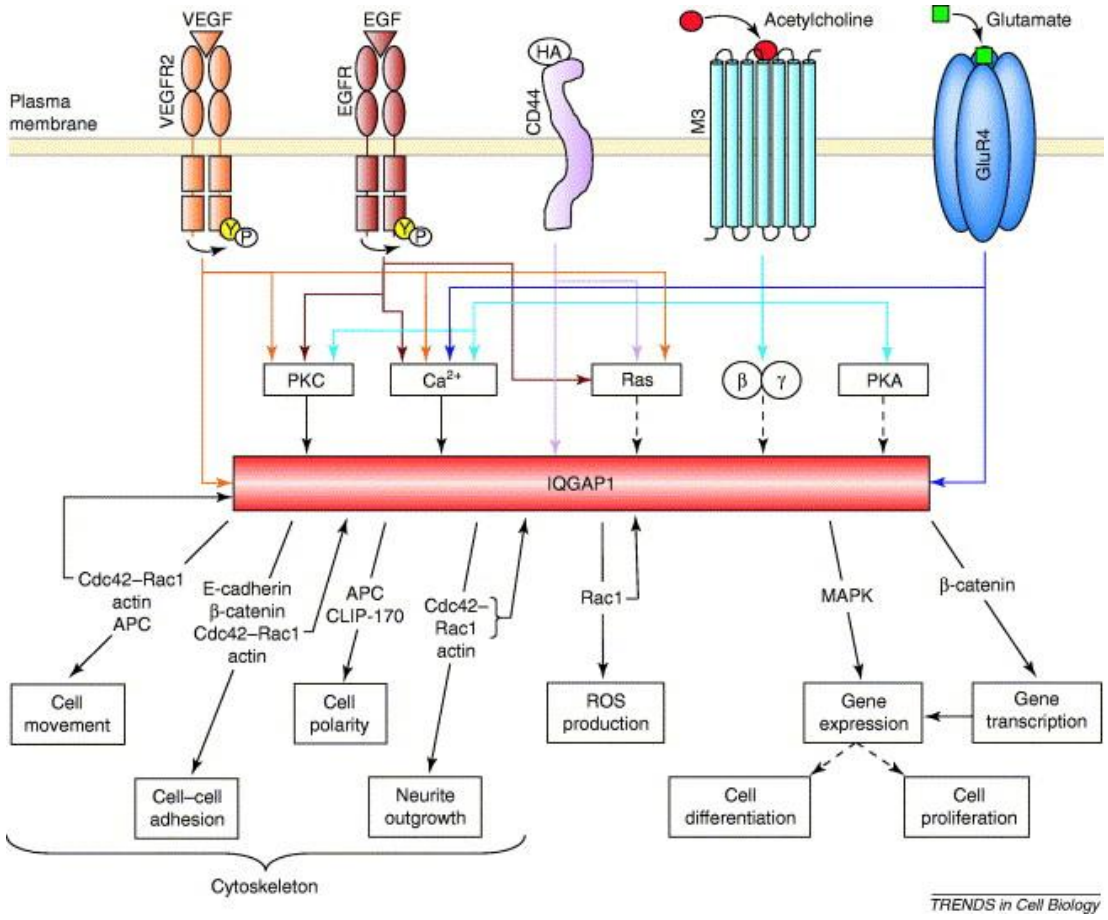


Figure 9. Schematic representation of IQGAP1-mediated multiple receptor signaling pathways. Activation signaling transferred from membrane receptors to downstream messengers or IQGAP1. Subsequently, IQGAP1 regulates a set of targets controlling cell behavior, such as cell movement, cell-cell adhesion, cell differentiation and proliferation. Rac1 and Cdc42 can act as both upstream and downstream regulators of IQGAP1, indicating a possible feedback loop between IQGAP1 and Rac1/Cdc42 (Brown and Sacks, 2006).

1. 3. 2 Hax1 in cell movement

Hax1, known as HS1-associated protein X1, is a 35 kDa, ubiquitously expressed protein, which subcellular localization depends on the cell type, such as mitochondria (Cilenti et al., 2004), lamellipodia, endoplamic reticulum and nuclear enveloppe (Gallagher et al., 2000)). It directly associates with HS1, a substrate of Src family tyrosine kinases. One study demonstrates that Hax1 is a short-lived protein and the fast degradation depends on its PEST sequence (Li et al., 2012).

Initially, Hax1 has been demonstrated to be consistently implicated in diseases with

compromised apoptosis, such as cancer and neutropenia. Different studies have revealed that Hax1 is up-regulated in a broad variety of cancers, including hepatoma, melanoma, breast and lung malignancies (Rhodes et al., 2004; Trebinska et al., 2010). One mechanism proposes that Hax1 exerts its anti-apoptotic role through inhibition of caspase-9 (Han et al., 2006). Another finding suggests that Hax1 interacts with the sarco(endo)plasmic reticulum (SR) Ca^2 transport ATPase (SERCA2) and modulates the protein level to promote cell survival (Vafiadaki et al., 2009).

Besides its role in anti-apoptosis, Hax1 also facilitates cell migration through coordinating with GTPases, interacting with actin cytoskeleton and other interacting proteins. Peter and coworkers have shown that loss of Hax1 results in increased integrin-mediated adhesion and reduced RhoA activity, while depletion of RhoA displays enhanced neutrophil adhesion and impaired migration, suggesting that Hax1 regulates neutrophil adhesion and chemotaxis via RhoA (Cavnar et al., 2011). Furthermore, Hax1 has been identified to form a complex with cortactin and the α-subunit of the heterotrimeric G protein G13 ($G\alpha_{13}$), that enhances $G\alpha_{13}$-mediated Rac activity while inhibits Rho activity, by which cell movement is promoted (Radhika et al., 2004). Hax1 is localized in the actin cytoskeleton (Burnicka-Turek et al., 2010), where Hax1 interacts with polycystic kidney disease protein (PKD2) and F-actin-binding protein cortactin, suggests a role of Hax1 in the formation of cell-matrix contacts in a PKD dependent manner (Gallagher et al., 2000). Another Hax1-binding partner, integrin β_6 cytoplasmic tail and Hax1 are required for the clathrin-mediated endocytosis of $\alpha_v\beta_6$ integrins, which further regulate cell motility and invasion (Ramsay et al., 2007). In addition, the urokinase-type plasminogen activator receptor (uPAR) has also been reported to interact with Hax1, which overexpression augments formation of cell adhesion, cell proliferation and migration in uPAR-stimulated cells (Mekkawy et al., 2012).

Although different mechanisms have been proposed in Hax1-mediated cell movement, our understanding about the precise role of Hax1 is still incomplete, that requires more efforts in research.

1.4 Ubiquitination

In this chapter, general principles of the ubiquitin cascade and chain formation, protein degradation by proteasome are discussed. Furthermore, I introduce the role of HECTD1, as a E3 ubiquitin ligase, as involved in protein turn over.

1. 4. 1 Ubiquitin-proteasome system

E3 ubiquitin ligase is involved in physiological degradation of proteins. The attachment of ubiquitin to a target protein requires the sequential function of three enzymes in a cascade, called E1 (ubiquitin-activating enzymes), E2 (ubiquitin-conjugating enzymes) and E3 (ubiquitin ligases). E3 ligases recruit E2 enzymes that have been loaded with ubiquitin, to identify a target protein and to catalyze the delivery of ubiquitin from the E2 to the target. Generally, E3 ligases polyubiquitinate their substrate proteins and target the substrates for disassembly by the proteasome. Ubiquitination by E3 ligases regulates various cellular events such as cell trafficking, cell cycle control, signal transduction, tranciptional regulation stress response, DNA repair and apoptosis (Teixeira and Reed, 2013). The human genome is capable of coding more than 600 putative E3s, resulting in enormous variety in substrates (Li et al., 2008) (Figure 10).

1. 4. 1. 1 Ubiquitin activation

Ubiquitin-activating enzyme (E1) is responsible for the initial process in ubiquitin-protein isopeptide bond formation and plays a crucial role in the initiation of *in vitro* conjugation reactions. By adenylating with ATP, E1 first activates ubiquitin, and to be completely active, links the residue to the sulphydryl side chain moiety of a cysteine residue in E1 with formation of high energy thiol ester bond and with the release of AMP. The activated ubiquitin is delivered to the lysine of substrates afterwards via the E2/E3 conjugation cascade. This covalent bond of targeted proteins with ubiquitin or ubiquitin-like proteins is the main mechanism for regulating protein function in eukaryotic organisms (Schulman and Harper, 2009).

1. 4. 1. 2 Ubiquitin conjugation

During the second step of ubiquitination reaction, ubiquitin is linked to the E2 enzymes, also known as ubiquitin-conjugating enzymes. The family of E2 enzymes is characterized by the existence of a conserved ubiquitin-conjugating (UBC) domain, which contain the ATP-activated ubiquitin or ubiquitin-like (UBL) protein through a covalently bonded thioester onto the active site residue. E2s act through specific protein-protein interactions with the E1s and E3s and link activation to covalent modification. So far, 35 active E2 enzymes have been recognized in humans (van Wijk and Timmers, 2010).

1. 4. 1. 3 Ubiquitin ligation

With the help of E3 ligase, ubiquitin is transferred from the E2 enzyme to a lysine residue on a substrate, leading to an isopeptide bond linking the substrate lysine and the C-terminus of ubiquitin, in which process E3 ligases control the substrate specificity. The E3s are a large group of proteins, characterized by defining motifs and classifies to four main types, including a Homologous to E6-associated protein C-terminus (HECT), Really Interesting New Gene (RING) or a modified RING motif without the full complement of Zn^{2+}-binding ligands (U-box) domain and The Plant Homeodomain (PHD) finger (Nakayama and Nakayama, 2006). In mammals, there are ~30 HECT domain E3 ligases that are directly participated in catalysis during ubiquitination. Among their many functions, HECT E3s have remarkable roles in immune response, protein trafficking, cellular growth and proliferation (Rotin and Kumar, 2009). The conserved HECT domain locates at the C-terminus of these enzymes, whereas their N-terminal domains are various and mediate substrate protein targeting. Whereas RING and U-box E3s function as adaptor-like molecules facilitate protein ubiquitination. Taken together, the many sided properties and interactions of E3s provide a critical and unique mechanism for protein removal within all cell types of eukaryotic organisms. The importance of E3s is reflected not only by the number of cellular events they regulate, but also the impressive amount of diseases related with their damaging of function or inappropriate targeting (Ardley and Robinson, 2005).

1. 4. 1. 4 Ubiquitin elongation

A poly-ubiquitin chain is formed by linking additional ubiquitin molecules to the first one, which occurs through a special type of E3 ligase referred to as a ubiquitin-elongation enzyme. Seven different lysine residues that could be used to bind ubiquitin molecules together result in various structures. Moreover, the length of the ubiquitin chain is also important, such as with Lys (48) poly-ubiquitin chains that its length influences its affinity for proteasomes (Chau et al., 1989). Therefore, E3 ligases exhibit the exquisite specificity in terms of which proteins should be targeted with ubiquitin, how many ubiquitin molecules are added to the substrate and at what positions the poly-ubiquitin molecules are linked, thereby leading to the future of the protein and the precise role it will play (Ikeda and Dikic, 2008).

1. 4. 1. 5 Proteasome

The targeted protein linked with a chain of multiple copies of ubiquitin for degradation by the intracellular protease, 26S proteasome, a large (> 60 subunits) complex with a 20S barrel-shaped proteolytic core consisting of alternating α and β subunits and two 19S regulatory "caps" at either end. The 19S caps recognize de-ubiquitinylate and unfold the substrate protein before being dragged through the hollow core of the 20S catalytic center, where it is dissembled into reusable amino acid components (Arrigo et al., 1988; Burger and Seth, 2004; Gerards et al., 1998). Proteasome-mediated protein degradation is commonly recognized as an integral part of cellular protein turnover and homeostasis. Proteasomes localize both in the nucleus and in the cytosol and build up to 1% of the cellular protein content in eukaryotes (Gerards et al., 1998). The proteins targeted by ubiquitin system are short-lived proteins and mainly regulatory proteins, which are regulated by rapid synthesis and degradation. As such, the ubiquitin system plays a vital role in controlling the concentration of key signaling proteins.

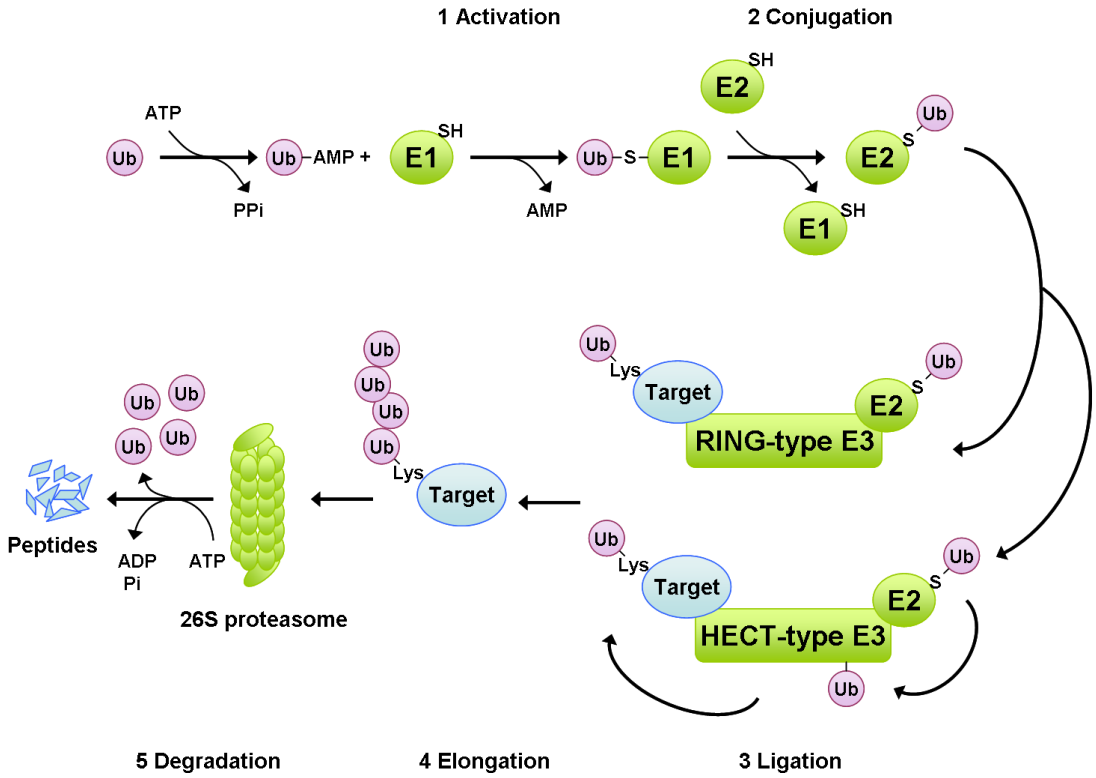


Figure 10. The ubiquitin-proteasome system. Ubiquitination of degradation of substrate proteins is completed by cascade reactions mediated by enzymes of ubiquitin-proteasome system (UPS). Initially, ubiquitin is activated by ubiquitin-activating enzyme (E1) in an ATP-dependent manner, connecting E1 to C-terminal glycine residue of ubiquitin by a high energy thioester bound. The activated ubiquitin is subsequently transferred to a ubiquitin-conjugating enzyme (E2), followed by sending it to an ubiquitin ligase (E3). The E3-conjugated ubiquitin binds to a lysine residue of the substrate protein and this binding mediates substrate specificity. The two classical E3s mediate different processes of reaction. Ring finger-type E3 carries a ubiquitin moiety directly from E2 to the target protein. While HECT-type E3 ligases ligate ubiquitin by first transferring ubiquitin from E2 to HECT domain, and then to a lysine residue of the target protein. The process of ubiquitin ligation could be repeated to form a polyubiquitin chain on the target protein. Finally, polyubiquitination linked substrate is targeted for 26S proteasome-dependent protein degradation (Adapted from Vucic et al., 2011).

1. 4. 1. 6 Disease association

Inappropriate ubiquitin-mediated protein degradation has been recognized in a range of pathological conditions, especially neurodegenerative disorders, such as Alzheimer's disease (Upadhya and Hegde, 2007), Parkinson's disease (Stieren et al., 2011) and amyotrophic lateral sclerosis (ALS) (Leigh et al., 1991), in which protein aggregation and inclusion body formation play a role. This degradation pathway is also implicated in certain forms of cancer

subtypes as well.

E3 ubiquitin ligases regulate homeostasis, cell cycle and DNA repair. Since the HECT E3s have a direct and broad role in catalysis during ubiquitination, it is becoming increasingly clear that HECT E3s play an essential role in sporadic and hereditary human diseases including cancer (stomach cancer, renal cell carcinoma and liver cancer) (de Martino et al., 2015; Hou and Deng, 2015; Tang et al., 2014), cardiovascular disease (Liddle's syndrome) (Botero-Velez et al., 1994) and neurological (Angelman syndrome) disorders (Tomaic and Banks, 2015), and/or in other disease-relevant processes including bone homeostasis, immune response and retroviral budding (Chen and Matesic, 2007; Lu et al., 2008; Melino et al., 2008). Furthermore, as an E3 ubiquitin ligase, HECTD1 (HECT domain containing E3 ubiquitin protein ligase 1) has been shown to play a critical role in the formation of the neural tube and placenta during embryogenesis (Sarkar et al., 2014; Sarkar and Zohn, 2012; Zohn et al., 2007).

Thus, molecular approaches that target the activity of HECT E3s, regulators, and/or HECTD1 E3s substrates may become valuable for the future development of novel approaches for the treatment of relevant diseases.

1. 4. 2 HECTD1

HECT domain containing E3 ubiquitin protein ligase 1, short for HECTD1, belongs to the HECT E3s family and was first identified as a E3 ubiquitin ligase for inhibin B receptor in 2003 (Zhang et al., 2003, unpublished). It has been reported that HECTD1 plays an essential role in the formation of the neural tube and the placenta. Neural tube defects rank among the most common human congenital malformations with an incidence of approximately 0.1% of live births (Copp et al., 2003). Neural tube closure is a complicated morphogenic process where the neural plate rolls into a tube-like structure which forms the central nervous system (Copp, 2005; Copp et al., 2003). Since the etiology of human neural tube defects complexes has been attributed to various genetic and environmental factors, the study of the mechanic factors involved constitutes a big challenge.

By positional cloning Zohn's group identified the opm mutation in HECTD1 that is ubiquitously expressed throughout early mouse embryonic development, they first proposed

that HECTD1 may be essential for neural tube development, as up to 20% of mutant heterozygotes showing neural tube defects (Zohn et al., 2007). The substrate-specificity of the ubiquitin pathway arises from the interaction of the E3 ligases with their substrates and the HECT domain ligases typically interact directly with their substrates. During searching for the substrates of HECTD1, they found that Hsp90 is a binding partner of HECTD1 as a ubiquitin ligase,. Then they demonstrated that HECTD1-dependent ubiquitination of Hsp90 affects its intracellular localization and negatively regulates its secretion. When HECTD1 ubiquitination ligase activity is lost, the levels of extracellular Hsp90 are elevated (Sarkar and Zohn, 2012). Wnt signaling pathway is widely involved in the embryonic development of a variety of organisms (Gilbert et al, 2010). Several proteins in the core of wnt signaling pathway are known to be key regulators in neural tube development, including Frizzled (Fz), Van Gogh (Vang) and Disheveled (Dsh). In the present of deletions of each of these genes mutant mouse embryos demonstrated different aspects of neural tube defects (Hamblet et al., 2002; Kibar et al., 2001; Wang et al., 2006). Zohn's group also suggested that HECTD1 promotes the adenomatous polyposis coli (APC) protein-Axin interaction to negatively regulate Wnt signaling, by modifies APC with Lys-63 polyubiquitin, whereas loss of HECTD1 reduces APC ubiquitylation, disrupts the APC-Axin interaction and augments Wnt3a-induced β-catenin stabilization and signaling (Tran et al., 2013). After that, a new regulatory mechanism of HECTD1 was been proposed, ubiquitination of PIPKIγ90 by HECTD1 and consequent degradation modulates the on-site production of PIP2, finally regulating focal adhesion dynamics and cell migration (Li et al., 2013).

Ubiquitin-conjugated targets are abroad distributed in the endometrium and in the decidua of both rodents and primates (Bebington et al., 2000). Studies of mouse mutants prove that ubiquitin-dependent modification plays an important role in placental development (Verstrepen and Beyaert, 2012). Recently, HECTD1 was reported to be transcribed and translated in several trophoblast-derived cell types throughout placental development, while loss of HECTD1 leads to altered placenta structure, apoptosis and proliferation (Sarkar et al., 2014). However, as multiple molecular pathways are implicated in development of neural tube and placenta, the role of HECTD1 is likely very complicated.

1. 6 Aim of the research

Although the function of HECTD1 remains largely unknown, as an E3 ubiquitin ligase, it is possibly involved in the turnover of the inhibin-binding protein (InhBP), the putative receptor of inhibin A. We have established HECTD1 knockout mice model to elucidate the effects of HECTD1-deficient in mouse development. The HECTD1 knockout mice proved to be not viable at the late foetal stage and the phenotype was characterized by severe neural tube defects (exencephaly), growth retardation and impaired placental development. To better understand the function of HECTD1 in organogenesis during embryonic development, the main goals of our research are:

a. Study the role of HECTD1 in cell spreading and migration
b. Identification of HECTD1 interaction partners
c. Investigation of HECTD1 function in intracellular signaling

2. Materials and Methods

2. 1 Materials

2. 1. 1 Antibodies

Antibodies	Supplier
Mouse 6x-His Epitope Tag Antibody (HIS.H8)	Thermo Scientific, Waltham, USA
Rabbit α/β-Tubulin Antibody	Cell Signalling, Danvers, USA
Mouse Acetylated alpha Tubulin [6-11B-1]	Abcam, Cambridge, UK
Mouse α-Actinin, clone BM-75.2	Sigam-Aldrich, St. Louis, USA
Mouse Golgin-97, CDF4	Invitrogen, Carlsbad, USA
Mouse PP2A C subunit, Clone 1D6	Millipore, Darmstadt, Germany
Rabbit β-Catenin (D10A8)	Cell Signalling, Danvers, USA
Mouse Cortactin (p80/85), clone 4F11	Millipore, Darmstadt, Germany
Rabbit Phospho-Cortactin (Tyr421)	Cell Signalling, Danvers, USA
Mouse Src (active), clone28	MBL international corporation
Mouse GFP (GF28R)	Thermo cientific, Waltham, USA
Mouse H2A.X (Ser139), clone JBW301	Millipore, Darmstadt, Germany
Rabbit HAX-1 (FL-279)	Santa Cruz Biotech, Dallas, USA
Rabbit HECTD1(M03), clone 1E10	Abnova, Taipei, Taiwan
Rat Anti-Mouse CD29 (Integrin beta	BD Biosciences, San Jose, USA
Rabbit IQGAP1 (H-109)	Santa Cruz Biotech, Dallas, USA
Mouse Integrin beta6	R&D, Minutesneapolis, USA
Rabbit Paxillin (N-term)	Epitomics, Burlingame, USA
Rabbit Paxillin (phospho Y118**)**	Abcam, Cambridge, UK
Mouse Talin (Rod domain)	ECM biosciences, Versailles, USA
Rabbit Talin (Ser-425), phospho-specific	ECM biosciences, Versailles, USA
Mouse Ubiquitin (P4D1)	Santa Cruz Biotech, Dallas, USA
Rabbit Integrin alpha 5	Cell Signalling, Danvers, USA
Rabbit Integrin beta1 (D2E5)	Cell Signalling, Danvers, USA

Rabbit PIP5K1A	Cell Signalling, Danvers, USA
Rabbit PIP5K1B	Abcam, Cambridge, UK
Rabbit PIP5K1C	Cell Signalling, Danvers, USA
Mouse E3 ubiquitin-protein ligase HECTD1	CUSABIO, Wuhan, China
Mouse Vinculin [SPM227]	Abcam, Cambridge, UK
Rabbit Zyxin	Epitomics, Burlingame, USA
Rabbit GAPDH (14C10)	Cell Signalling, Danvers, USA
Rabbit UVRAG	Cell Signalling, Danvers, USA
Rabbit PPP2R5D	Novus, Littleton, USA

2. 1. 2 Reagents

Reagent	**Supplier**
1,4-dithiointhreitol	Roth, Karlsruhe, Germany
2-Propanol	Sigma, St. Louis, USA
A/G plus-Agarose beads	Santa Cruz Biotech, Dallas, USA
Acrylamide	Roth, Karlsruhe, Germany
Agarose	Sigma, St. Louis, USA
Ammonium persulfate	Sigma, St. Louis, USA
Ampicillin	Roth, Karlsruhe, Germany
Albumin Fraktion V	Roth, Karlsruhe, Germany
BCA protein assay Kit	Thermo Scientific, Waltham, USA
Bovine serum albumine	Roth, Karlsruhe, Germany
Bromphenol blue	Sigma, St. Louis, USA
Calcium chloride	Roth, Karlsruhe, Germany
DAPI	Roth, Karlsruhe, Germany
Dimethyl sulfoxide	Sigma, St. Louis, USA
Dulbecco’s modified Eagle’s medium	Sigma, St. Louis, USA
ECL plus Solution A and B	Thermo Scientific, Waltham, USA
Ethanol	Merck, Darmstadt, Germany
Fetal bovine serum (FBS)	Sigma, St. Louis, USA
Formaldehyde	Thermo Scientific, Waltham, USA

FuGENE6	Qiagen, Venlo, Nederland
Fuji Film medical X-ray	Fuji, Tokyo, Japan
Glycerin	Roth, Karlsruhe, Germany
Glycine	Roth, Karlsruhe, Germany
L-glutamine	Sigma, St. Louis, USA
LB-Medium	Roth, Karlsruhe, Germany
Magnesium chloride	Sigma, St. Louis, USA
Magnesium sulfate	Roth, Karlsruhe, Germany
Methanol	Roth, Karlsruhe, Germany
MG132	Peptide Institute, Osaka, Japan
Pro-long mounting medium	Invitrogen, Carlsbad, USA
NucleoSpin® Extract II Gel Extraction kit	Macherey-Nagel, Düren, Germany
PBS	Sigma, St. Louis, USA
Potassium chloride	Sigma, St. Louis, USA
PVDF	Bio-Rad laboratories, Hercules, USA
QYAprep® spin Mini Prep Kit	Qiagen, Venlo, Nederland
RIPA buffer (Pierce)	Thermo Scientific, Waltham, USA
Sodium chloride	Sigma, St. Louis, USA
Sodium dodecyl sulfate	Sigma, St. Louis, USA
Sodium phosphate	Sigma, St. Louis, USA
Top-Block	Lubioscience, Luzern, Switzerland
Trypsin-EDTA 1x	Sigma, St. Louis, USA
Tricine	Roth, Karlsruhe, Germany
Trizma	Sigma, St. Louis, USA
β-Mercapthoethanol	Sigma, St. Louis, USA

2. 1. 3 Equipments

Equipment	**Supplier**
Scale PM1200	Mettler Toledo, Zurich, Switzerland
PM1200 Electronic Balance	Mettler Toledo, Greifensee, Switzerland
SBA 53 balance	Scaltec science, Heiligenstadt, Germany

Thermomixer	Eppendorf, Hamburg, Germany
The Kuhner LT-X incubator shaker	Kühner AG, Basel, Switzerland
Rotamax 120 orbital platform shakers	Heidolph, Schwabach, Germany
Eppendorf thermomixer comfort	Eppendorf, Hamburg, Germany
Ultrarocker rocking platform	Bio-Rad laboratories, Hercules, USA
Roto-Torque Heavy Duty Rotators	Cole-Parmer Instrumen, Illinois, USA
Vortex genie 2 vortex mixer	Scientific industries, New York, USA
Avanti J-25 High Speed Centrifuge	Beckman Coulter, Nyon, Switzerland
MiniSpin plus	Eppendorf, Hamburg, Germany
Benchtop centrifuge	Hettich lab technology, Beverly, USA
Synergy H1 Multi-Mode Reader	Biotek, Winooski, USA
Biorad Universal hood ii Imager	Bio-Rad laboratories, Hercules, USA
Biorad Universal hood iii Imager	Bio-Rad laboratories, Hercules, USA
ELGA Prrelab dv 35 water purification	ELGA, Paris, France
Nanodrop2000	Thermo Scientific, Waltham, USA
Micro-cube ice marker	Kibernetik AG, Buchs,Switzerland
Heraeus® Heating and Drying Ovens	Thermo Scientific, Waltham, USA
Axiovert 25 Inverted Microscope	Zeiss, Oberkochen, Germany
IX81 Motorized Inverted Microscope	Olympus Corporation, Tokyo, Japan
IX50 Inverted Microscope	Olympus Corporation, Tokyo, Japan
Nikon Confocal A1	Nikon Corporation, Tokyo, Japan
Leica M80 Stereomicroscope	Leica AG, Heerbrugg, Switzerland
Heraeus HERAsafe KS Safety Cabinets	Thermo Scientific, Waltham, USA
Heraeus HERAcell 150 incubator	Thermo Scientific, Waltham, USA
Julabo sw-20c waterbath	Julabo GmbH, Seelbach, Germany
-86 ℃ freezer	Thermo Scientific, Waltham, USA
TProfessional TRIO thermo cycler	Analytik Jena, Jena, Germany
PowerPac 300 Electrophoresis Power	Bio-Rad laboratories, Hercules, USA
Mini-protean II Electrophoresis Cell	Bio-Rad laboratories, Hercules, USA
Workstation WS-120	Skan, Allschwil, Switzerland

2. 1. 4 Plasmids

Plasmids	
pEGFP-IQGAP1	CMV promoter, active in Mammalian expression
pEGFP-N1-Hax1	CMV promoter, active in Mammalian expression
pEGFP-N1-Hax1-ΔPEST	CMV promoter, active in Mammalian expression

2. 1. 5 siRNAs

siRNA	Target Sequence
SMARTpool:siGENOME Mouse Hax1 siRNA	GGAUGCGGAUUCCACGGCAA UCGAGAGAGCUAUGCGUUU GAUCUUCAGCUUUGGAUGA GAUCUUGACUCCCAGGUUU
SMARTpool:siGENOME Mouse IQGAP1 siRNA	CGAAAGCGCUAUAGAGAUC GCGAGCAGGUGGACUACUA ACUCGGCGCUGAACUCUAA CAGAUAUCUAUGACCGGAA
siGENOME Non-targeting siRNA	UAGCGACUAAACACAUCAA UAAGGCUAUGAAGAGAUAC AUGUAUUGGCCUGUAUUAG AUGAACGUGAAUUGCUCAA
HECTD1-1099	ATAGATTGTATTCGAAGTAAAGA TACCGA

2. 2 Cell biology Methods

2. 2. 1 Mouse embryos and tissue digesting

On the day of E14.5, HECTD1 heterozygote mice were sacrificed. Then their embryos were photographed with a Leica M80 Stereomicroscope and plated on clean dishes. The trunks of the embryos were cut out with sterile scissors. The tissues were transferred to clean dishes and washed thoroughly with PBS, followed by gently mincing the tissues into small clumps of cells using two sterile needles. The cell clumps were digested with 500 μl Trypsin-EDTA at 37 °C for 20 minutes. After that, the digestion was stopped by 500 μl high glucose DMEM medium with 10% FBS, pipetted up and down for 5-10 times to disperse the clumps and centrifuged at 1000 rpm at room temperature for 1 minutes. Then the supernatant was removed through aspiration. The pellets were washed with PBS and repeated centrifuged. The pellets were dispersed by pipetting and grown on new culture plates supplemented with complete medium in a humidified incubator at 37 ℃, 5% CO2. MEF cells were sub-cultured when they reached 80-90% confluence (Cheryl D et al. 1984).

2. 2. 2 Cell culture

MEF cells were maintained in high glucose DMEM medium (Hela cells in low glucose medium) with 10% FBS, 1% of Sodium Pyruvate, 1% of L-Glutaminutese and 1% of Penicillin-Streptomycin. Cells were grown in a humidified incubator at 5% CO2 at 37 ℃. For splitting the cells, the spent culture medium was removed and washed cells using PBS without calcium and magnesium. Aspirating the PBS, pre-warmed trypsin/EDTA was added into culture vessels and incubated at 37 ℃ for 2-5 minutes until detachment. To stop the trypsination, same volume of complete medium was added. Cell suspension was centrifuged at 900 rpm for 3 minutes and the pellets were resuspended in growth medium. Cells were either split at 4:1 (Hela at 3:1) or sent for counting and seeding for the following experiments.

2. 2. 3 Cell freezing

Freezing medium was prepared before cell harvestation. For MEF cells, freezing medium contained FBS and DMSO at ratio of 9:1. For Hela cells, the ratio was FBS : complete

medium : DMSO at 5 : 4 : 1. Cell pellets were collected as previous described, resuspended 1-2 $\times 10^6$ cells in 1ml freezing medium and tranferred to cryogenic storage vials. After gentle mixing the cells to a homogeneous cell suspension, the vials were put into a 4 ℃ pre-cold isopropanol chamber and stored at –80 ℃ overnight. Next day, the frozen cells were transferred to liquid nitrogen.

2. 2. 4 Cell thawing

The frozen cells were taken out of the liquid nitrogen and immediately placed into a 37 ℃ water bath, then quickly thawn by shaking the vials. Cell suspension was transferred into cell vessels with pre-warmed complete medium (1 vial per T75 flask), and the cells were cultivated in 37 ℃ overnight. Growth medium were changed the next day.

2. 2. 5 Transfection

MEF or Hela cells used for transfection were pre-seeded 24 hours in culture vessels. On the day of transfection, the confluence was 50%-80%. Transfection of MEF or Hela cells with plasmid DNA using Effectene reagent according to protocol of Qiagen. Briefly, plasmid DNA was mixed with EC buffer and Enhancer reagent, vortexed for 1 second and incubated in room temperature for 5 min. After that, effectene reagent was added to the DNA complex, vortexed for 10 seconds and incubated at room temperature for 10 min. Finally, complete medium was added to the solution and the plasmid DNA complex was dropped onto the cells and further incubated at 37 ℃ for 24 h.

2. 2. 6 Immunocytochemistry

Cells were seeded on glass coverslips pre-coated with fibronectin for defined time intervals. After that, cells were washed with PBS and fixed with 4% paraformaldehyde for 10 minutes, permeablized with 0.15% Triton-X100 in PBS for 15 minutes and blocked with 5% BSA in PBS for 1 hour at room temperature. Primary antibody diluted in PBS was added to the coverslips and incubated at 4 ℃ for overnight. After washing the cells with PBS for 5 times with PBS, the secondary antibody tagged with fluorescent dye was added and incubated for 1

hour in the dark at room temperature. After washing, cells were incubated in DAPI in PBS for 3 minutes at room temperature for counter staining. After washing, cells were mounted with Prolong® Gold Antifade Reagent and stored in 4 ℃ protected from light. The fluorescent pictures were made with the Nikon Confocal microscope.

2. 2. 7 Cell spreading assay

Cells were seeded on 6-well plate and incubated at 37 ℃ for 24 hours before serum starvation overnight. Starved cells were counted and seeded on fibronectin pre-coated 24-well plates. The plate was immediately sent to time-lapse microscopy (Nikon IX81) which was pre-warmed to 37 ℃ and the confluence of CO_2 was maintained at 5 %. Quickly adjusting the positions, the focus, the time interval and total time by CellSens software, the program was started. Duration of spreading was analyzed from attachment to formation of leading protrusion. Cell spreading area was quantified by Image J software.

2. 2. 8 Wound-healing assay

For monolayer wound-healing assays, a total 4×10^4 cells were collected and plated in 24-well plate for 24 hours. Cells were washed twice with PBS and continually cultured for 24 hours in growing medium containing 0.5% FBS, followed by starved with serum free medium supplemented with 1 μM aphidicolin for overnight. Then, cells were scratched with a 200 μl pipette tip, washed twice with PBS and placed into complete medium. The plate was immediately sent to time-lapse microscopy (Nikon IX81) which was pre-warmed to 37 ℃ and the confluence of CO_2 was maintained at 5%. Migration images were taken at 10 minutes intervals for a period of 24 hours with a 4× lens. Cell trajectories were measured by tracking the position of the cell over time using “Manual Tracking” plugin (Image J, v 2.0) and the cell velocity and straightness were determined by “Chemotaxis Tool” plugin (Image J, v 2.0). Cells that proliferate or did not migrate during the experimental period were not evaluated.

2. 3 Biochemical Methods

2. 3. 1 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

2. 3. 1. 1 Gel preparation

Separating gel (12%)	12-60 kD
1M Tris-HCl, pH 8.8	5.7 ml
30% Acr-Bis (29 : 1)	6 ml
10% SDS	150 µl
ddH2O	3 ml
10% APS	150 µl
TEMED	10 µl

Table 1. Components for 12% separating gel

Separating gel (5%)	40-150 kD
1M Tris-HCl, pH 8.8	5.7 ml
30% Acr-Bis (29 : 1)	2.5 ml
10% SDS	150 µl
ddH2O	6.5 ml
10% APS	150 µl
TEMED	10 µl

Table 2. Components for 5% separating gel

Stacking gel (5%)	
1M Tris-HCl, pH 6.8	1.25 ml
30% Acr-Bis (29:1)	1.7 ml
10% SDS	100 µl
ddH2O	6.8 ml
10% APS	100 µl
TEMED	10 µl

Table 3. Components for 5 % stacking gel

2. 3. 1. 2 Protein samples preparation

Starved cells were harvested as pellets and resuspended in serum free medium. Half of the pellets were spinned down and lysed with RIPA buffer containing protease inhibitor cocktails (Ngoka, 2008) on ice for 15 minutes followed with centrifugation (12,000 g, 5 minutes) at 4 ℃. The other half was seeded on fibronectin pre-coated plates and cultivated in 37 ℃ for

60 minutes. After that, the plates were placed on ice, washed with pre-cooled PBS and lysed with lysis buffer (as previously) for 15 minutes on ice before centrifugation. The supernatant was collected and we continued with the protein concentration assay. Protein concentration was determined with a BSA standard curve. To equation the volum of each sample, ddH2O was added to the supernatant before 1:1 mixing with 2× Laemmli sample buffer (125 mM Tris/HCl pH 6.8, 4% (w/v) SDS, 20% (v/v/) glycerol, 0.04% (w/v) bromphenol blue) and heating at 65 ℃ for 5 minutes.

2. 3. 1. 3 Western blot

Equal amounts of protein were loaded into the wells of SDS-PAGE gel, along with molecular weight markers. After running the gel at 100 V for 60-90 minutes, the protein was transferred to PVDF membrane and continued running at 300 mA for 60-80 minutes in pre-cooled transfer buffer. The blots were blocked in 5% milk in TTBS for 1 hour at room temperature followed by primary antibody incubation for overnight at 4 ℃. After 3 times washing in TTBS, the blots were incubated in secondary antibody for 1 hour at room temperature. To remove the unspecific bound antibody, the blots were washed in TTBS for 3 times. Bands were detected by ECL substrates, visualized by an infrared-based laser scanner (LiCor) and quantified using Image Lab software. Detection of GAPDH served as a loading control.

2. 3. 1. 4 Immunoprecipitation

Cell pellets were lysed with IP lysis buffer (20 mM Tris-HCl, PH 8.0, 137 mM NaCl, 1% NP40 and 2 mM EDTA supplemented with 1% protease inhibitor cocktail) on ice for 20 minutes and vortexed in between. Cellular debris was removed by centrifugation at 14,000 g for 5 minutes and the supernatant was transferred to pre-cooled fresh tubes. The protein amount was equilibrated with the IP buffer. 2 µl primary antibody was added per 500 µg protein sample and incubated for overnight at 4 ℃. The lysates were then incubated with prewashed protein A/G agarose beads (20 µl/500 µg proein) and rocked for 1 hours at 4 ℃. Beads were washed three times with IP buffer, 6000 rpm, 3 min. After washing, the beads were heated for 5 minutes at 95 ℃ in 2× Laemmli sample buffer. Target proteins were detected by western blot by using specific antibodies.

2. 3. 1. 5 *In vivo* ubiquitination

MEF cells were transfected with plasmids DNA HA-ubiquitin and GFP-IQGAP1 (or GFP-Hax1/GFP-Hax1-ΔPEST) at ratio of 1:1 24 hours after seeding. 24 hours after transfection, the cells were washed twice with PBS and changed to serum-free medium supplemented with 1 nM MG132 or DMSO, then incubated for overnight at 37 ℃. For endogenous ubiquitination assay, MEF cells were seeded for 24 hours and directly continued with starvation. Starved cells were harvested as pellets and resuspended in serum-free medium. Half of the pellets were spinned down and lysed with ubiquitination lysis buffer (50 mM Tris, pH 7.5, 1 mM EDTA, 150 mM NaCl, 0.1% Triton X-100, complete protease inhibitor cocktail, 100 μM MG132 and 100 μM N-ethylmalemide) on ice for 15 minutes followed with centrifugation (12,000 g, 5 minutes) at 4 ℃. The other half was seeded on fibronectin pre-coated plates and cultivated in 37 ℃ for 60 minutes, after that, the plates were placed on ice, washed with pre-cooled PBS and lysed with lysis buffer (as previously) 15 minutes on ice before centrifugation. The supernatant was collected and then we continued with the protein concentration assay. Equal amount of protein was immunoprecipitated with target protein and detection of ubiquitin by western blot. Ubiquitination of target proteins were normalized by the protein amount in MEF cells.

2. 4 Molecular Biology Methods

2. 4. 1 Bacterial strain

The Escherichia coli (E.coli) strains, DH5α were used for heat shock transformation. They were grown in LB (Luria-Bertani) medium for overnight and then on LB agar plates containing with the appropriate antibiotics for selection.

2. 4. 2 Competent cells

E.coli DH5α was grown in LB medium (5 g/l NaCl, 5 g/l yeast extract, 10 g/l Peptone 140) overnight (Bertani, 2004), then diluted 1:100 in 100ml LB medium. Cells were grown to Optical Density $_{600}$ to 0.5. The culture was quickly immersed in ice for 10 minutes and then harvested by centrifugation at 5000 g for 5 minutes at 4 ℃. The supernatant was poured off

and the pellet was resuspended in 24 ml ice-cold 0.1M $MgCl_2$, followed by incubation for 10 minutes on ice. After centrifugation at 5000g for 5 minutes at 4 ℃, the pellet was resuspended in 4 ml ice-cold 0.1M $MgCl_2$. Equal amounts of sterile glycerol were added to the cell suspension. Aliquots of 100 µl were snap-frozen at -80 ℃.

2. 4. 3 Transformation

Competent cells were taken out of -80 ℃ and thawed on ice for 20 minutes. Agar plates were supplemented with the appropriate antibiotic and were taken out of 4 ℃ to warm up in 37 ℃ incubator. Using DH5α bacteria, 10 µl of DNA were mixed with 20 µl competent cells and incubated on ice for 30 minutes. Then the DNA-bacteria mixture was heated at 42 ℃ for 45 seconds, followed by immediately incubating the bacteria on ice for 2 minutes followed by adding 200 µl of SOC medium (2% Tryptone, 0.5% Yeast Extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM glucose). After incubation, the mixture was shaken for 1 hour at 37 ℃ and spread on LB plates supplemented the appropriate antibiotic for selection followed by incubating at 37 ℃ for overnight.

2. 4. 4 Plasmid purification

Several bacterial clones were selected to cultivation in 4 ml LB medium containing the appropriate antibiotic at 37 ℃, 250 rmp/min overnight. To produce baterial stocks, 100% glycerol was added 1:1 to the bacterial medium and stored at -80 ℃. 1ml of the bacterial medium was add to 50 ml LB medium and cultured at 37 ℃, 250 rmp/min overnight. Bacterial pellets were harvested by centrifugation at 4 ℃, 6000 rpm/min for 15 minutes. Plasmid DNA was purified using NucleoBond® Xtra Midi kit (Macherey-Nagel) according to manufacturer's instructions. The constructs were verified by sequencing (Microsynth, Basel, Switzerland).

2. 5 Statistical analysis

All data analyses were performed using the statistical software package SPSS 13.0 for Windows 7 (SPSS Inc., Chicago, Ill, USA). Normally distributed data was analyzed for statistical differences with the t-test (paired comparisons) or ANOVA (Analysis of Variance).

For data not normally distributed, non-parametric ANOVA and the Mann-Whitney *U* test were used. All values are reported as means ± SEM. Differences are considered statistically significant with $P<0.05$, highlighted with *.

3. Results

3. 1 Result 1

3. 1. 1 Defective embryonic development in HECTD1 knockout mice

To understand the function of HECTD1 in causing or contributing disease in embryo development, the most direct way was to establish a HECTD1-mutant mouse model. This model would allow the comparison of the knockout organism to a wild-type counterpart with a similar genetic background (Martin Evans et al., 2010). Using the secretory-trap approach, we generated HECTD1-mutant mice by insertion of β-geo expression cassette between intron 26-27. The efficient translation of β-galactosidase mRNA allows the convenient analysis of HECTD1 expression by staining tissues with X-gal in heterozygous and homozygous mice. The HECTD1 knockout mice proved to be not viable at the late foetal stage. On the day of E 14.5, HECTD1 heterozygotes mouse were sacrificed, the embryos were used for further experiments The phenotype was characterized by severe neural tube defects (exnencephaly), growth retardation and impaired placental development, etc (Fig. 11). The phenotype is identical to the ENU-induced open mind (opm)-mouse which exhibit exencephaly associated with defects in head mesenchyme and dorsal-lateral hinge point formation (Zohn et al., 2007).

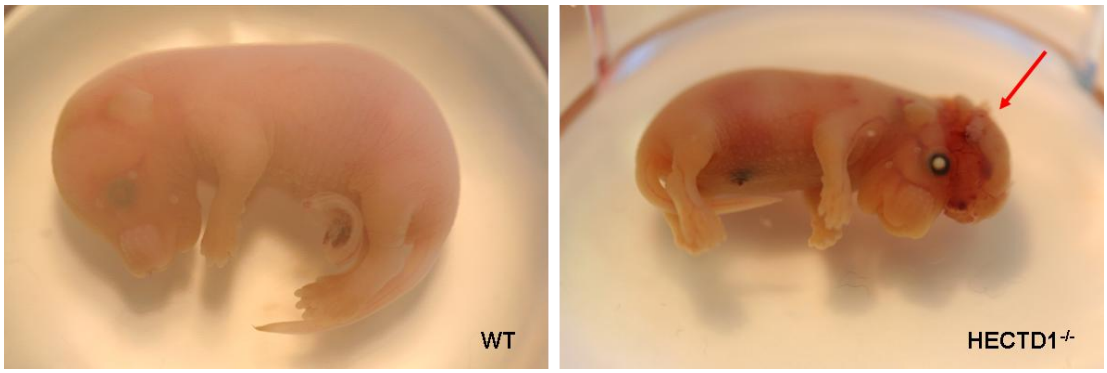


Figure 11. Mutation of HECTD1 disrupts embryonic development. Embryos of wild-type (Left panel) and HECTD1 mutant (Right panel) were imaged under stereomicroscope. Note for severe neural tube defect (red arrow).

3. 1. 2 Fibronectin is an essential extracellular matrix in cell adhesion as regulated by HECTD1

The extracellular matrix is a dynamic, physiologically active component of all living tissues. It provides a substrate for cell anchorage, serves as a tissue scaffold, guides cell migration during embryonic development and wound repair and exerts key functions during tissue morphogenesis. The extracellular matrix is also responsible for transmitting environmental signals to cells, which ultimately affects cell proliferation, differentiation and death. Several types of ECM, including FN, collagen and gelatin, they are released as 'precursor' molecules; their subsequent incorporation into the extracellular matrix is guided by the fibroblasts in accordance with the functional needs of a particular tissue (Adams, 2001; Rozario and DeSimone, 2010). To understand the specificity of the extracellular matrix in the formation of cell adhesions in the absence of HECTD1, we analyzed the cell adhesion proteins of both wild-type and HECTD1 knockout cells to extracellular matrix proteins, FN, collagen I, and gelatin. Wild-type and HECTD1 knockout cells were added to coverslips coated with the various extracellular matrix proteins. Then the cells attached to the extracellular matrix proteins were stained with paxillin or zyxin and further quantified. There was a significant difference between the adhesion of wild-type and HECTD1 knockout cells to FN, with wild-type cells being more adherent than the mutant cells. However, paxillin and zyxin were equally expressed in wild-type and HECTD1 knockout cells on collagen I, either being impaired or well-organized. Although there was different expression of paxillin in wild-type and HECTD1 knockout cells on gelatin, this difference was diminished with respect to zyxin expression (Fig. 12 A and B). This suggests that FN plays a critical role in HECTD1 involving cell adhesions expression.

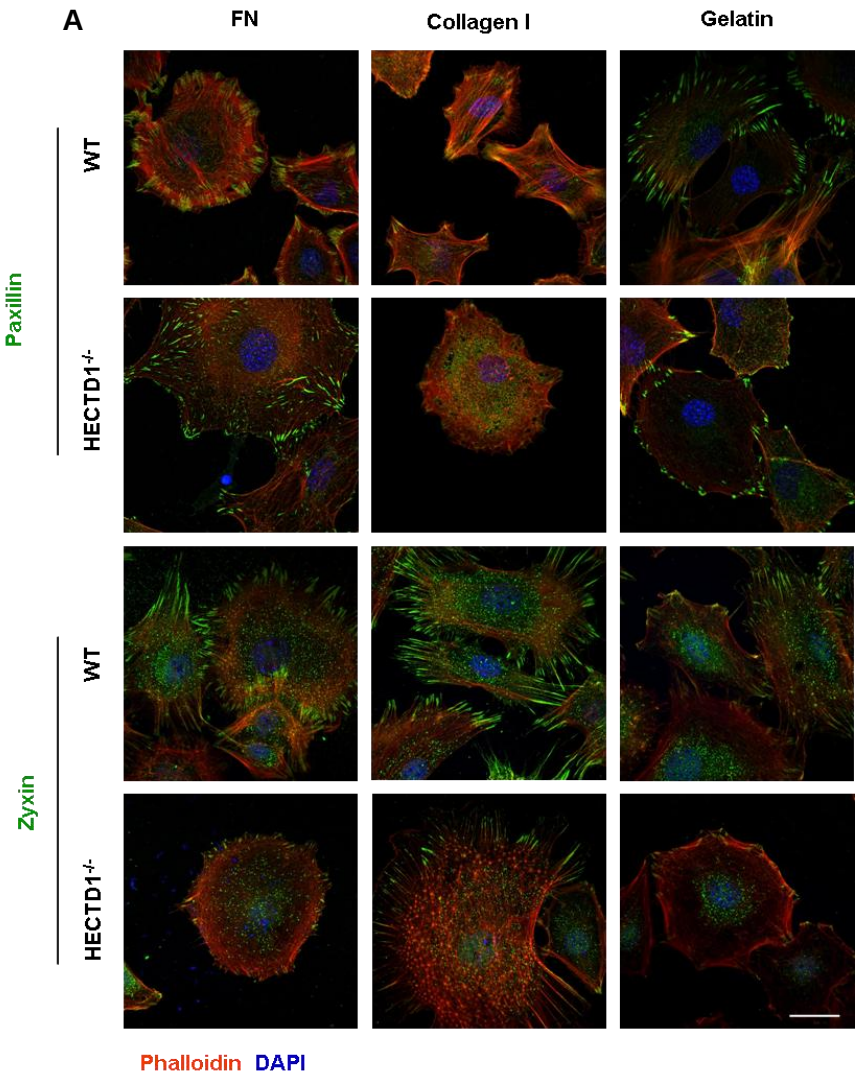
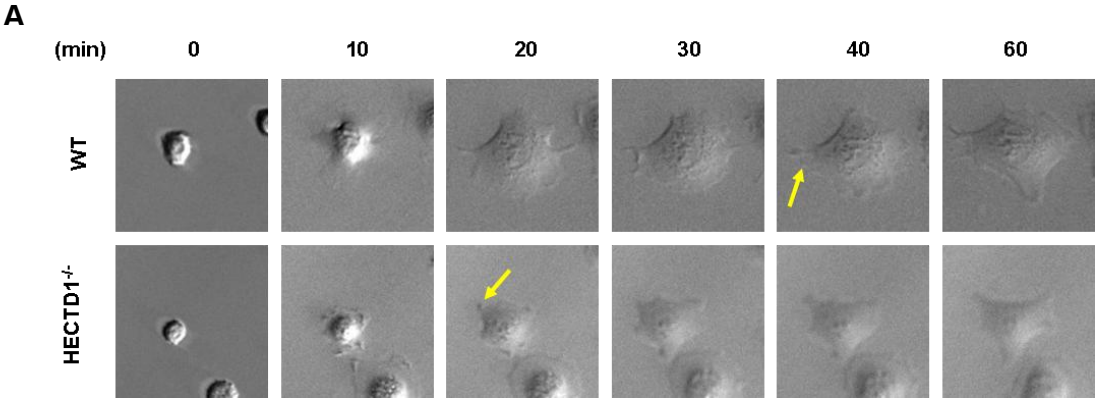

A
FN
Collagen I
Gelatin
Paxillin
WT
HECTD1-/-
Zyxin
WT
HECTD1-/-
Phalloidin DAPI
B
WT
HECTD1-/-
Size (μm2)
0
2
4
6
8
*
*
n.s.
n.s.
*
n.s.
paxillin
zyxin
paxillin
zyxin
paxillin
zyxin
FN
Collagen I
Gelatin

Figure 12. Fibronectin is a critical extracelluar matrix in HECTD1 involved in cell adhesion expression. (A) Wild-type and HECTD1 knockout cells were seeded on coverslips pre-coated with 1 μg/ml FN, 10 μg/ml collagen I or 10 μg/ml gelatin for 2 hours, followed by anti-paxillin or anti-zyxin staining. Bar, 50 μm. **(B)** Area of paxillin and zyxin were analyzed by Image J software. *, $P < 0.05$. N.S. No significance.

3. 1. 3 Loss of HECTD1 results in shorter duration and greater cell area in cell spreading

Cell spreading is the initial step before cell migration, that cell adhesion and motility depend strongly on the interactions between cells and extracellular matrix (ECM) substrates (Cuvelier et al., 2007). Since the HECTD1 knockout cells produce fewer adhesions to FN, we examined the spreading of these cells on dishes coated with FN. Wild-type cells adopted a flattened morphology and started to form leading edges around 40 minutes while this process occurred about 20 minutes earlier in HECTD1 knockout cells (Fig. 13, A and C). This is also reflected in the surface area of the two cell types: at the beginning of cell spreading, both the cells have a similar surface area, while at 30 minutes the HECTD1 knockout cells have a significantly smaller surface area. In detail, the area of wild-type cells was about 2.4 times bigger at 30 minutes respectively than 10 minutes, while in HECTD1 knockout cells, the area at 30 minutes was around 1.8 times compared as 10 minute, suggesting that the HECTD1 knockout cells have spread less than the wild-type cells. (Fig. 13, A, B and D).



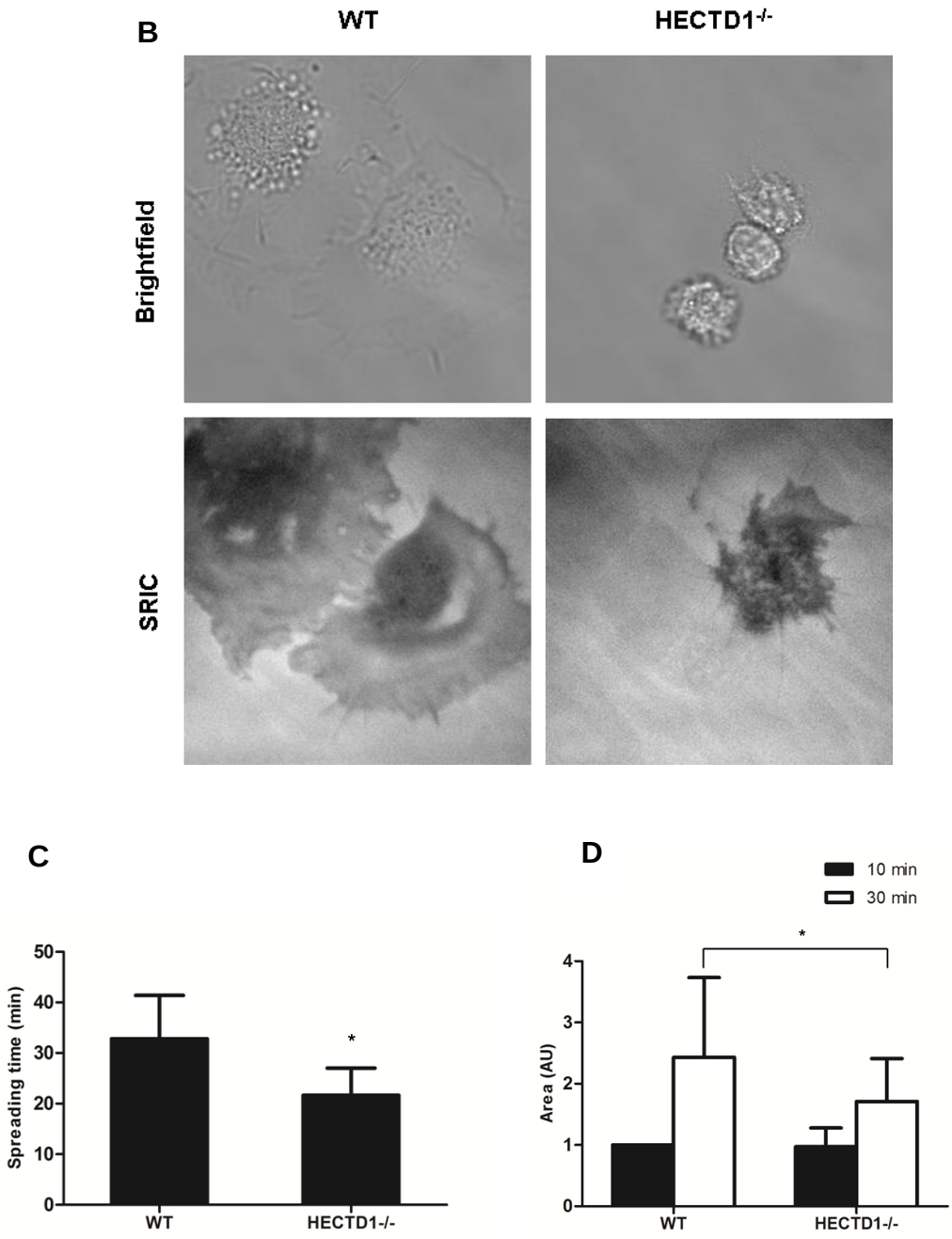


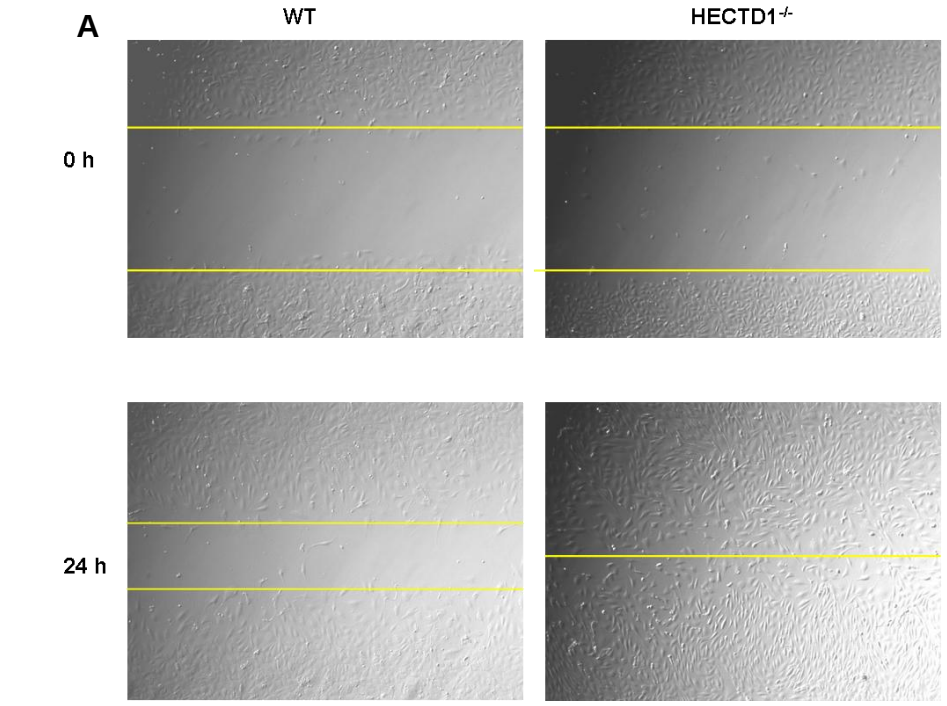
Figure 13. Loss of HECTD1 results in shorter duration time and greater area of cell spreading. Wild-type and HECTD1 knockout cells were starved overnight, then plated on FN coated plates and immediately sent to time-lapse microscopy for recording 2 hours (1 min / picture) **(A)** or TIRF (Total Internal Reflection Fluorescence) microscopy **(B).** Spreading pictures on different time points were shown. Note for cell with leading protrusion (yellow arrows). SRIC: Surface Reflective Interference Contrast. Quantification of duration of cell spreading **(C)** and spreading cell areas **(D)** by Image J software. AU, arbitrary unit. *, $P < 0.05$.

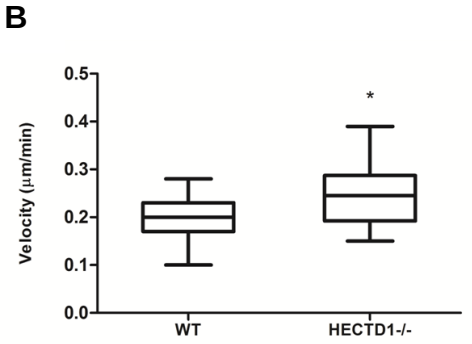
3. 1. 4 Loss of HECTD1 in MEFs accelerated cell migration and impaired directional cell migration

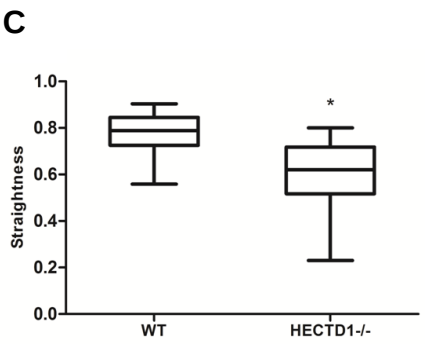
Knowing that knockout of HECTD1 promotes the speed of migration in the wound healing experiments, we then examined the directionality of cell movement in HECTD1 knockout cells and in control cells. We analyzed the effects of HECTD1 on velocity and directionality of cells in wound healing assay. Wild-type cells migrated in a cohesive fashion with little dispersion and aligned paths of displacement. In contrast, HECTD1 deficient cells lost their collective migration and dispersed as individuals, depicted by Up/down plot graphs which HECTD1 knockout cells had more scattered trajectories than wild-type cells (Fig. 14, A and D). Moreover, the velocity (total distance/time) of HECTD1 knockout cells shortened to 0.19 ± 0.05 μm/min compared to 0.25 ± 0.07 μm/min of control cells ($P<0.05$) (Fig. 14, B). The straightness (Euclidean distance/Accumulated distance) was 0.60 ± 0.14 in HECTD1 knockout cells versus 0.78 ± 0.09 in wild-type cells ($P<0.05$) (Fig. 14, C).

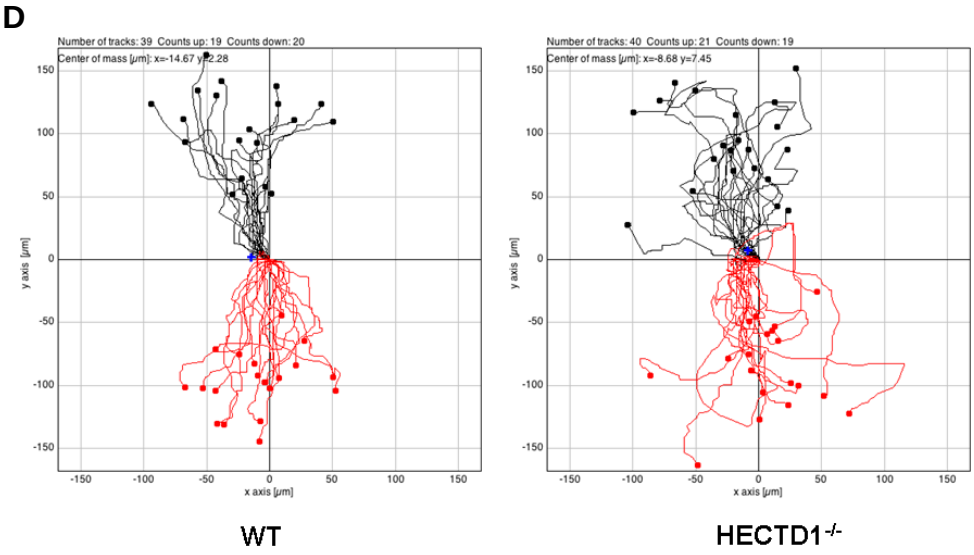
Microtubules are known to determine cell polarity in a variety of circumstances. The reorientation of the MTOC toward leading edges contributes to directional cell migration (Gundersen, 2002; Ridley et al., 2003). Furthermore, the positioning of the MTOC mostly determines the positioning of the Golgi apparatus that is known to be involved in microtubule-based motility (Thyberg and Moskalewski, 1999). A wound healing assay was also used to assess the direction of these cells by stained with Giantin and acetylated α tubulin after 12 hours of migration (Arthur and Burridge, 2001). Giantin and acetylated α tubulin were colocalized in MTOC in both wild-type and HECTD1 knockout cells (Fig. 14, E). The percent of Giantin and acetylated α tubulin oriented to the wound was 61.29 ± 15.33 % in wild-type cells, where the percent dropped to 40.67 ± 11.25 % in HECTD1 knockdown cells (Fig. 14, F).

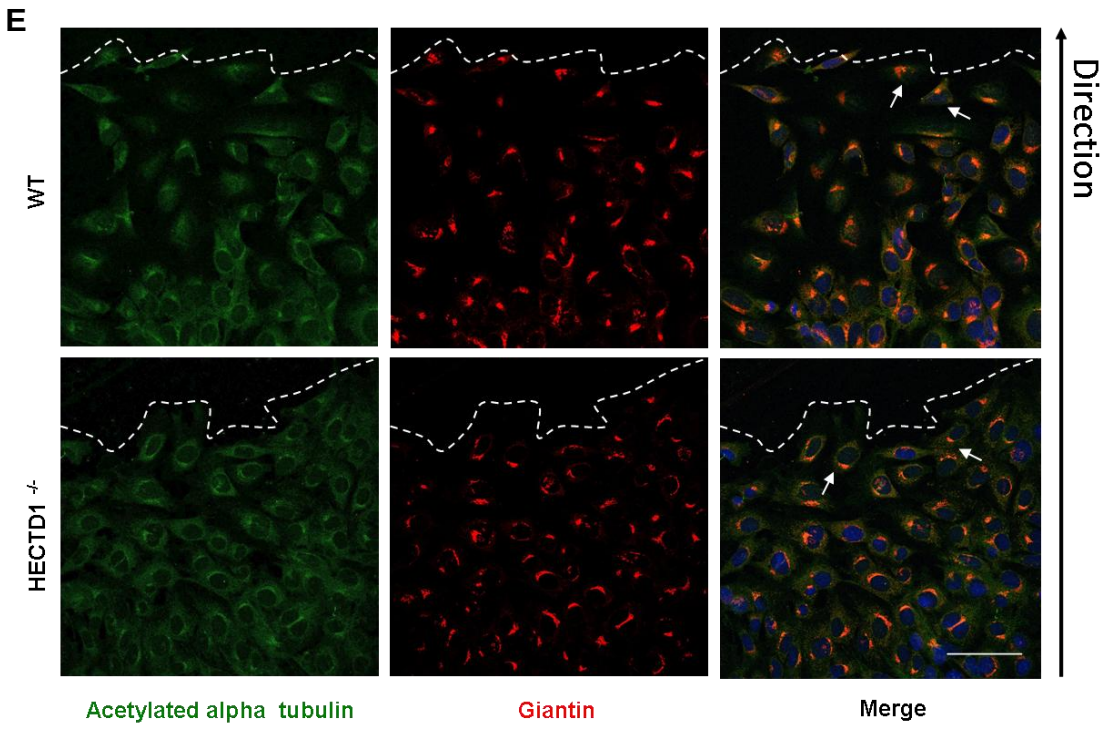
Together, our data suggest that loss of HECTD1 not only speeds up the velocity of directional cell migration, but also impairs the directionality of cell migration.


A
WT
HECTD1-/-
0 h
24 h


B
Velocity (µm/min)
0.5
0.4
0.3
0.2
0.1
0.0
*
WT
HECTD1-/-


C
Straightness
1.0
0.8
0.6
0.4
0.2
0.0
*
WT
HECTD1-/-


D
Number of tracks: 39 Counts up: 19 Counts down: 20
Center of mass [µm]: x=-14.67 y=2.28
Number of tracks: 40 Counts up: 21 Counts down: 19
Center of mass [µm]: x=-8.68 y=7.45
y axis [µm]
x axis [µm]
150
100
50
0
-50
-100
-150
WT
HECTD1-/-



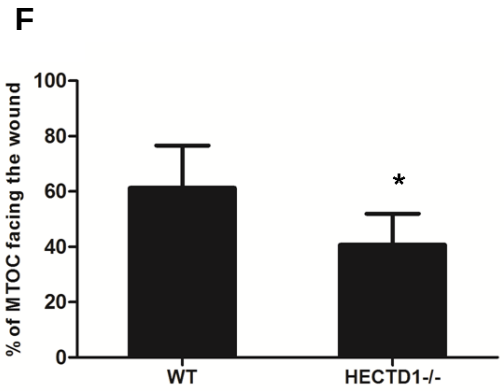


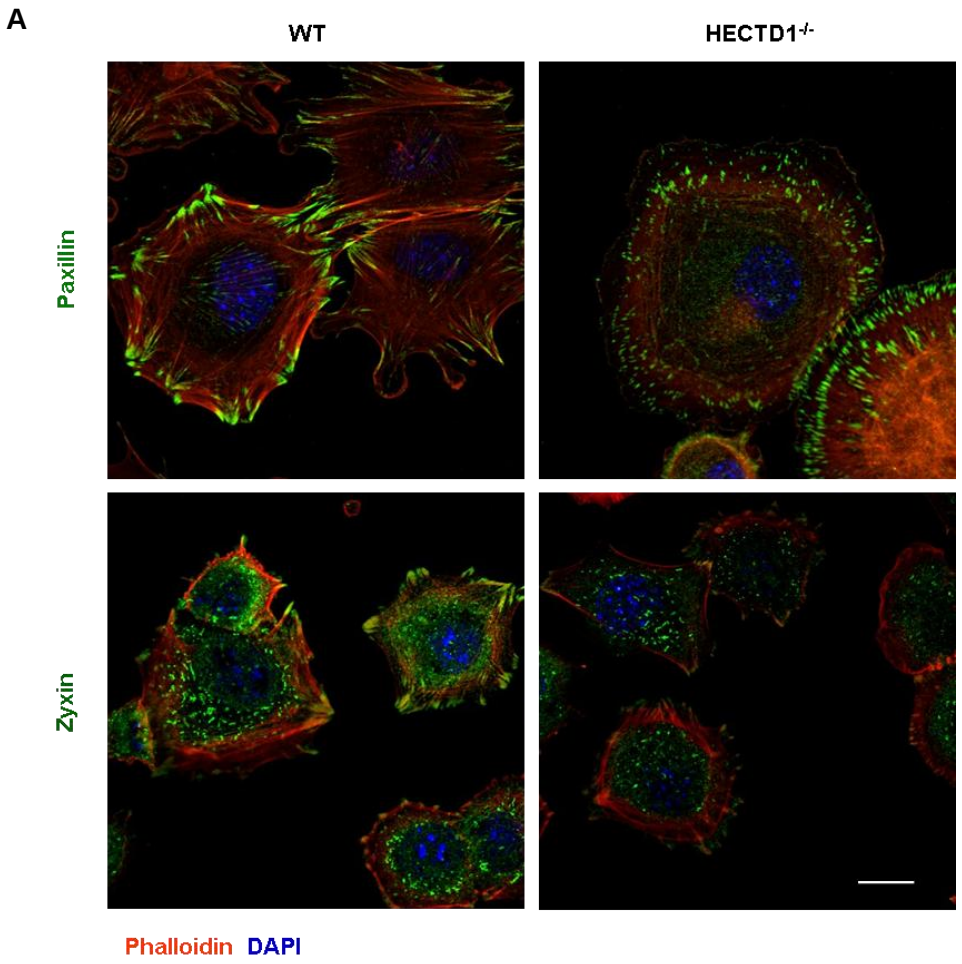
Figure 14. Loss of HECTD1 impairs directional cell migration. (A, B and C) Loss of HECTD1 impairs the velocity and straightness of directional cell migration. Equal amount of wild-type and HECTD1 knockout cells were seeded on 24 well plate for 24 hours, then the culture medium was replaced with DMEM supplemented with 0.5% FBS for 24 hours, followed by starvation overnight with 1µg/ml aphidicolin (APC). After that, wounds were created by 200µl pipette tips, cells were washed twice and replaced with complete medium. Migration images were acquired by time-lapse microscopy for 24 hours. **(D)** Up and down plots of cell migration trajectories, velocity and straightness were measured by Manual tracking and chemotaxis tool (Image J). **(E)** Loss of HECTD1 impairs the directionality of directional cell migration. Wound healing experiments were manipulated in 24 well plates with coverslips. The percentage of MTOC orientated towards the wound was determined at 10 hours post wounding. Cells were fixed with 4% paraformaldehyde then co-stained with acetylated alpha tubulin and Giantin antibodies. Bar, 50µm. **(F)** The percent of cells at the wound edge having their Golgi apparatus in the

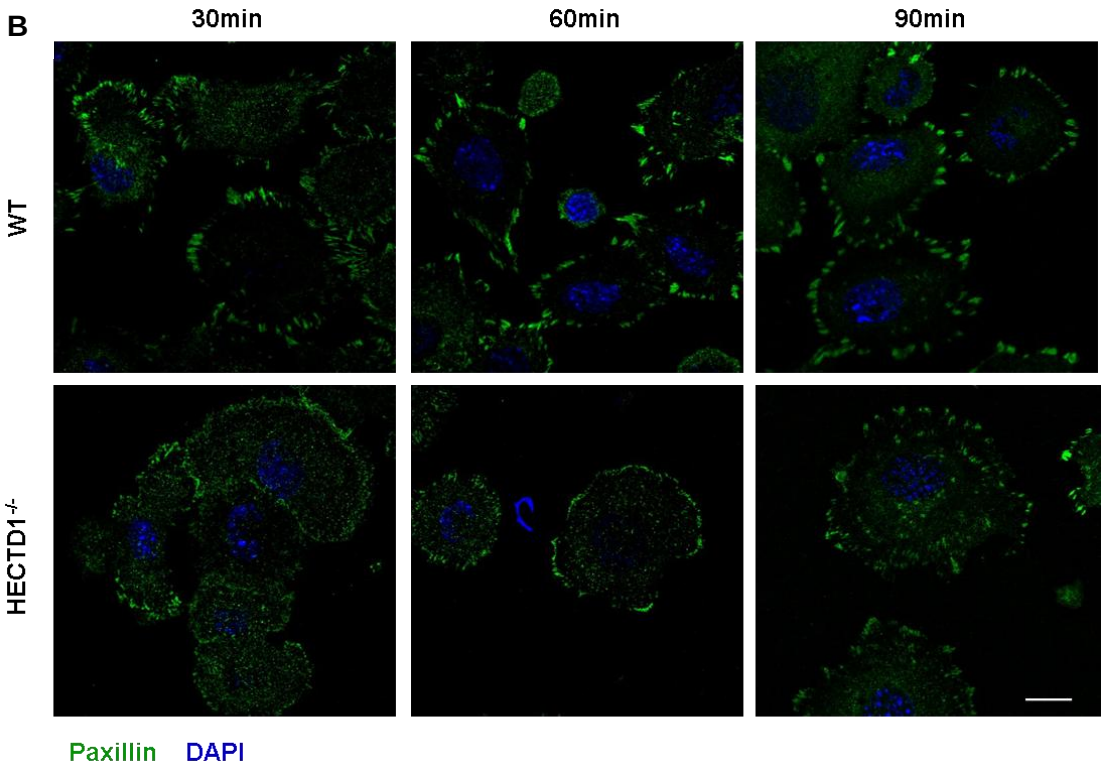
forward-facing 120° sector was measured after wounding. Over 600 cells from 3 independent experiments were analyzed. Orientation of the Golgi apparatus with respect to the wound edge corresponds to percent on the ordinate. *, $P<0.05$.

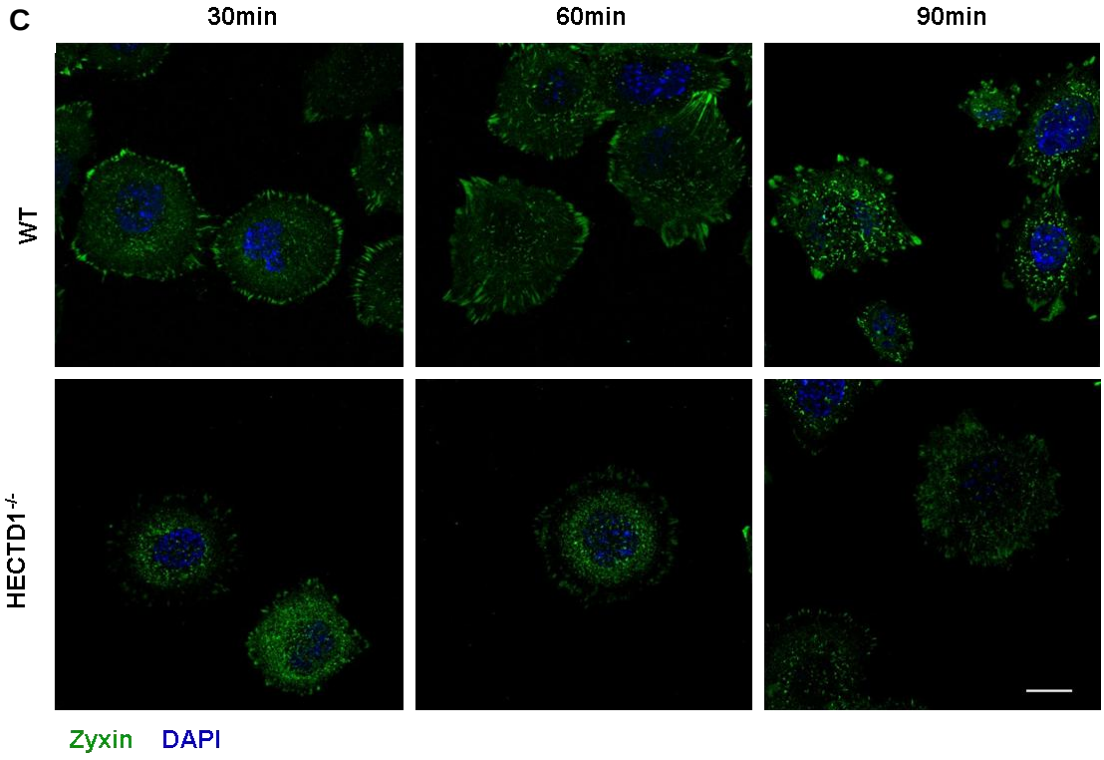
3. 1. 5 Formation of focal adhesions is impaired in HECTD1 knockout MEFs

Upon the formation of lamellipodia adhesion complexes assemble and attach to the surface allowing the cell to generate traction. We have identified the localization of adhesion proteins in this sense. Prominent paxillin and zyxin patches were clearly visible in the wild-type cells plated on FN, in contrast with fewer and smaller paxilin and zyxin patches in HECTD1 knockout cells plated on the same substrates. When cells are activated by the extracellular matrix, adhesion proteins start to aggregate at the cell leading edge and mature from nascent adhesions to focal complexes, eventually to form focal adhesions. It is believed that the dominant of each stage represents for different motile status of cell (Ilic et al., 1995). To understand the dynamics of adhesion proteins in cell spreading and migration, we immunolabled paxillin and zyxin in cells plated on FN at different time intervals. The adhesions were classified by size, localization and morphological characteristics using paxillin and zyxin as markers. Focal complexes were described as small punctate adhesions of 1-5 μm^2, lying close to the cell periphery where cortical actin located and at the leading edge of polarized cells (Fig. 5A, Right panel). Focal adhesions were classified as larger structures with sizes between 5-20 μm^2; with a more linear morphology which mostly linked with stress fibers, as well as a broader distribution throughout the cells, including the cell periphery and the cell body (Fig. 15A, Left panel). Taking paxillin as a marker, after cell spreading for 30 minutes on FN, both wild-type and HECTD1 knockout cells were dominated by focal complexes, while in contrast with wild-type, the focal complexes are significantly more than that in HECTD1 knockout cells ($P<0.05$) and focal adhesions were shown in the opposite way. However, as a late-stage adhesion marker, zyxin mainly constitutes into similar percentage of focal complexes per cell in both cell types (Fig. 15, B-E). When cells continued to migrate until 60 minutes or 90 minutes, both paxillin and zyxin showed the similar pattern, that focal adhesions make up the largest part of adhesions in wild-type cells, whereas in HECTD1 knockout cells, the dominant cell adhesions are focal complexes (Fig. 15, B-E).

Then, we evaluated the amount of focal adhesion proteins by western blot to elucidate if there were reassemble defects or express problems. The band intensity was measured by Image Lab software (Bio-Rad). The band intensity of wild-type cells of no stimulation was normalized with GAPDH as control and the other results were recorded as fold changes compared to control. Results showed there were no significant difference in protein level of paxillin and zyxin in wild-type and HECTD1 knockout cells (P>0.05) (Fig. 15, F and G). Our results suggest that the defects in assembly of focal adhesions at cell leading edges are rather caused by disability of accumulation or transportation rather than protein synthesis problems.




B
30min
60min
90min
WT
HECTD1-/-
Paxillin DAPI


C
30min
60min
90min
WT
HECTD1-/-
Zyxin DAPI

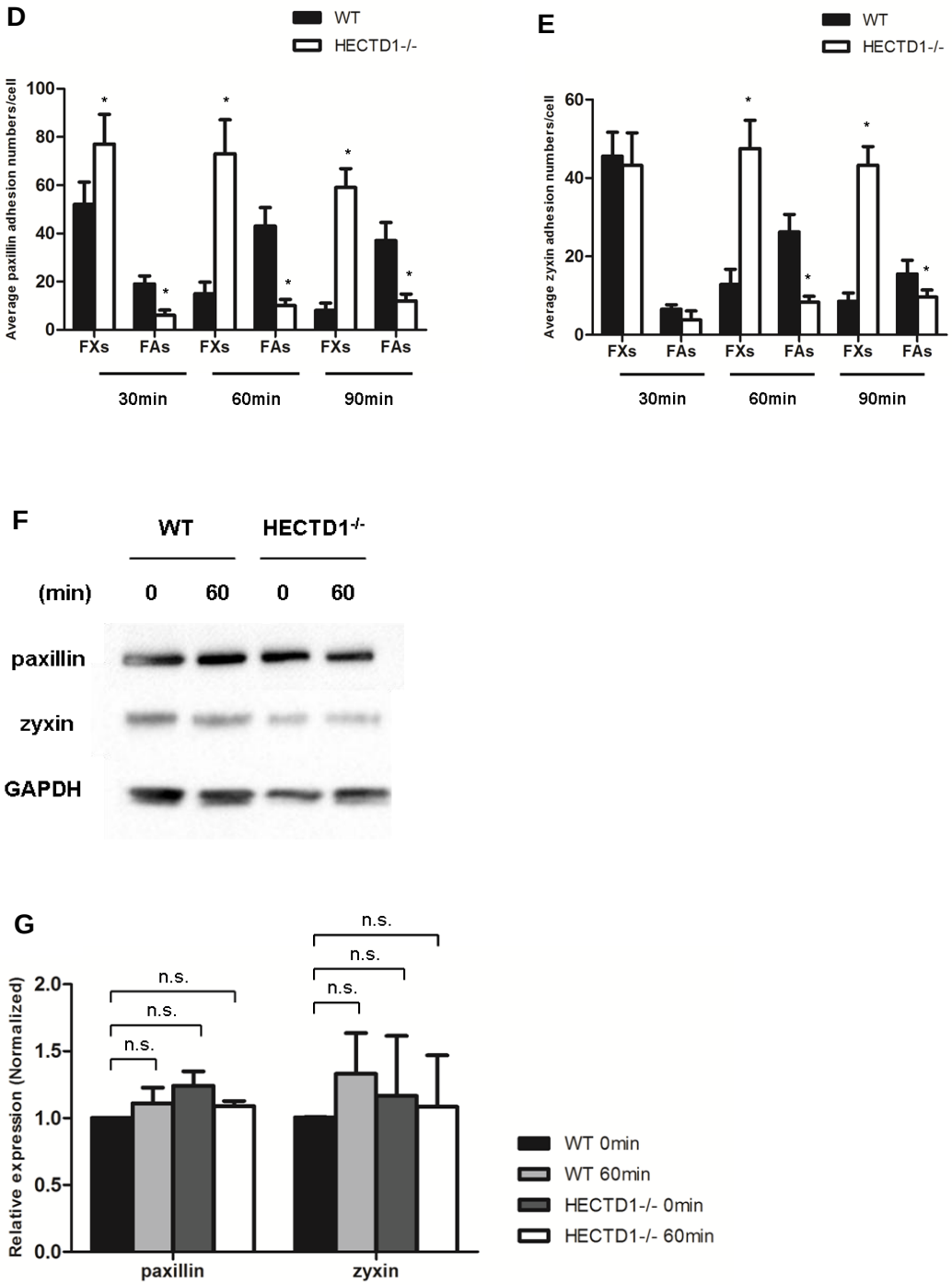


Figure 15. Loss of HECTD1 impairs formation of focal adhesions. (A-C) Fewer focal adhesions formed in HECTD1 knockout cells. Equal amounts of wild-type and HECTD1 knockout cells were seeded on culture dishes for 24 hours, followed by starvation overnight and plated on FN coated coverslips for 30, 60 or 90 minutes. The cells were fixed and co-stained with phalloidin and/or anti-paxillin/anti-zyxin, respectively, indicated by white arrow. Bar, 20μm. **(D and E)** Quantification of average adhesion numbers/cell. *, compare as wild-type cells, $P<0.05$. **(F)** Total level of adhesion proteins in wild-type and HECTD1 knockout cells. Equal amount of wild-type and HECTD1 knockout cells were seeded on culture dishes for 24 hours, after starvation

for overnight, cells were harvested immediately or after stimulation by FN for 60 minutes. The total amount of paxillin and zyxin were determined by western blot respectively. **(G)** Quantification of protein intensity by Lab Image software from 3 independent experiments. The results were first normalized with GAPDH and then with wild-type 0 minutes group. N.S. No significance.

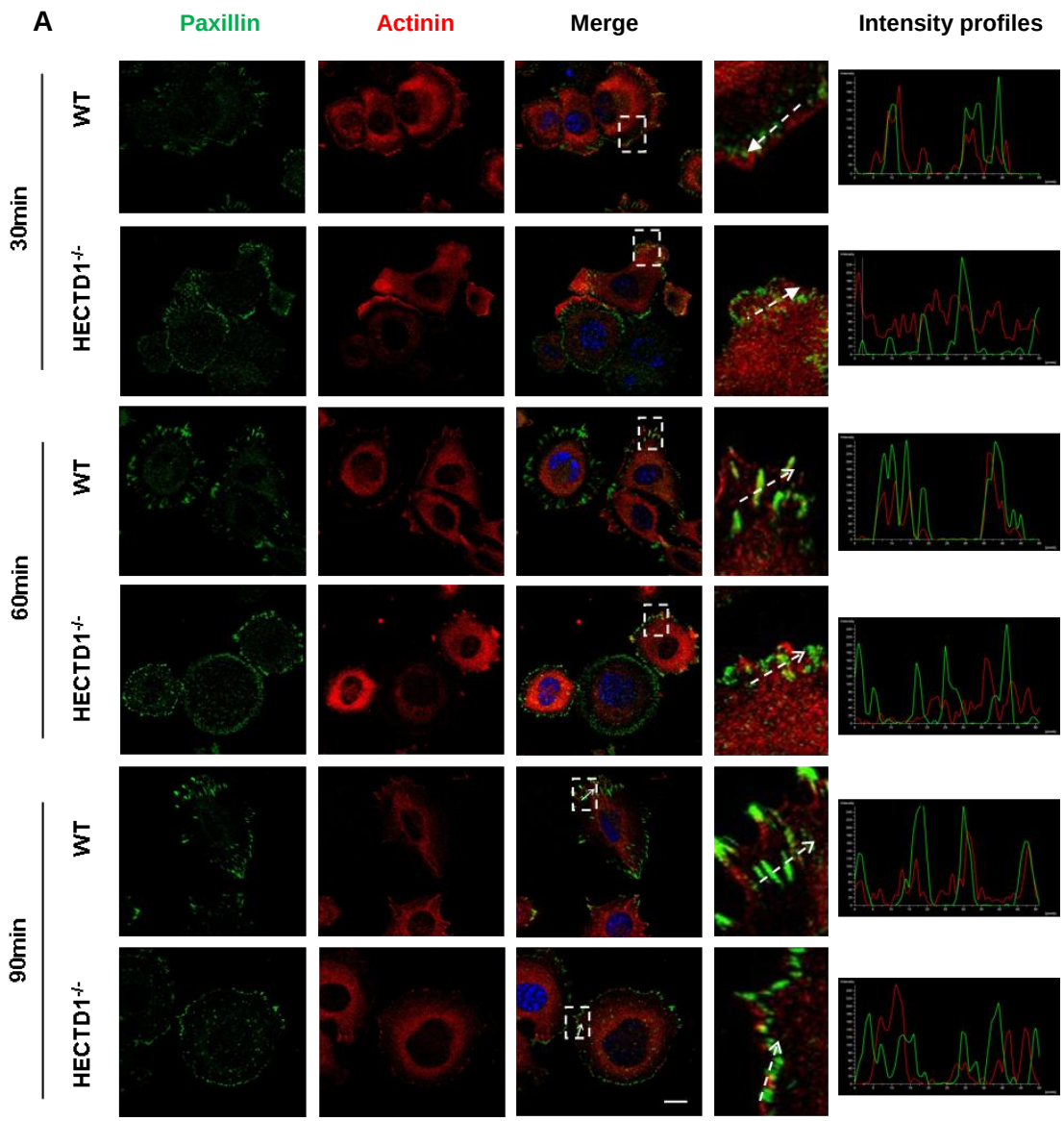
3. 1. 6 Loss of HECTD1 impairs the subcellular localization of adhesion proteins

Another adhesion protein, α-actinin is an actin filament-binding protein, which connects with actin cytoskeleton (Maruyama and Ebashi, 1965). It is mainly localized at the leading edge of cells and acts as an important component of adhesion modules (Knight et al., 2000). To further investigate the location of paxillin and zyxin and its relationship between actin cytoskeleton in HECTD1 knockout cells, we double-immunolabled α-actinin and paxillin/zyxin in cells plated on FN for different time intevals.

Like paxillin and zyxin, the maturation of α-actinin in HECTD1 knockout cells was inhibited compared to wild-type cells, that after 30 minutes' cell spreading, we could barely detect patches of α-actinin at the cell periphery; as the the cells continuing to migrate, a few patches of α-actinin could be seen, but still less than wild-type control (Fig. 16, A and B).

At the cell leading edges, α-actinin was mainly colocalized with paxillin and zyxin in wild-type cells activated by FN for 30, 60 and 90 minutes, while this interaction was lost in HECTD1 knockout cells. The location relationship of α-actinin with paxillin or zyxin were shown in fluorescence intensity profiles (Fig. 16, A and B).

Our findings revealed that in HECTD1 knockout cells the acting binding protein, α-actinin, was inhibited during formation of focal adhesions, as well as the mislocalized with respect to paxillin and zyxin. It is suggested that the α-actinin may act as a bridge to mediate the link between paxillin, zyxin and the actin cytoskeleton.


A
Paxillin
Actinin
Merge
Intensity profiles
30min
WT
HECTD1-/-
60min
WT
HECTD1-/-
90min
WT
HECTD1-/-

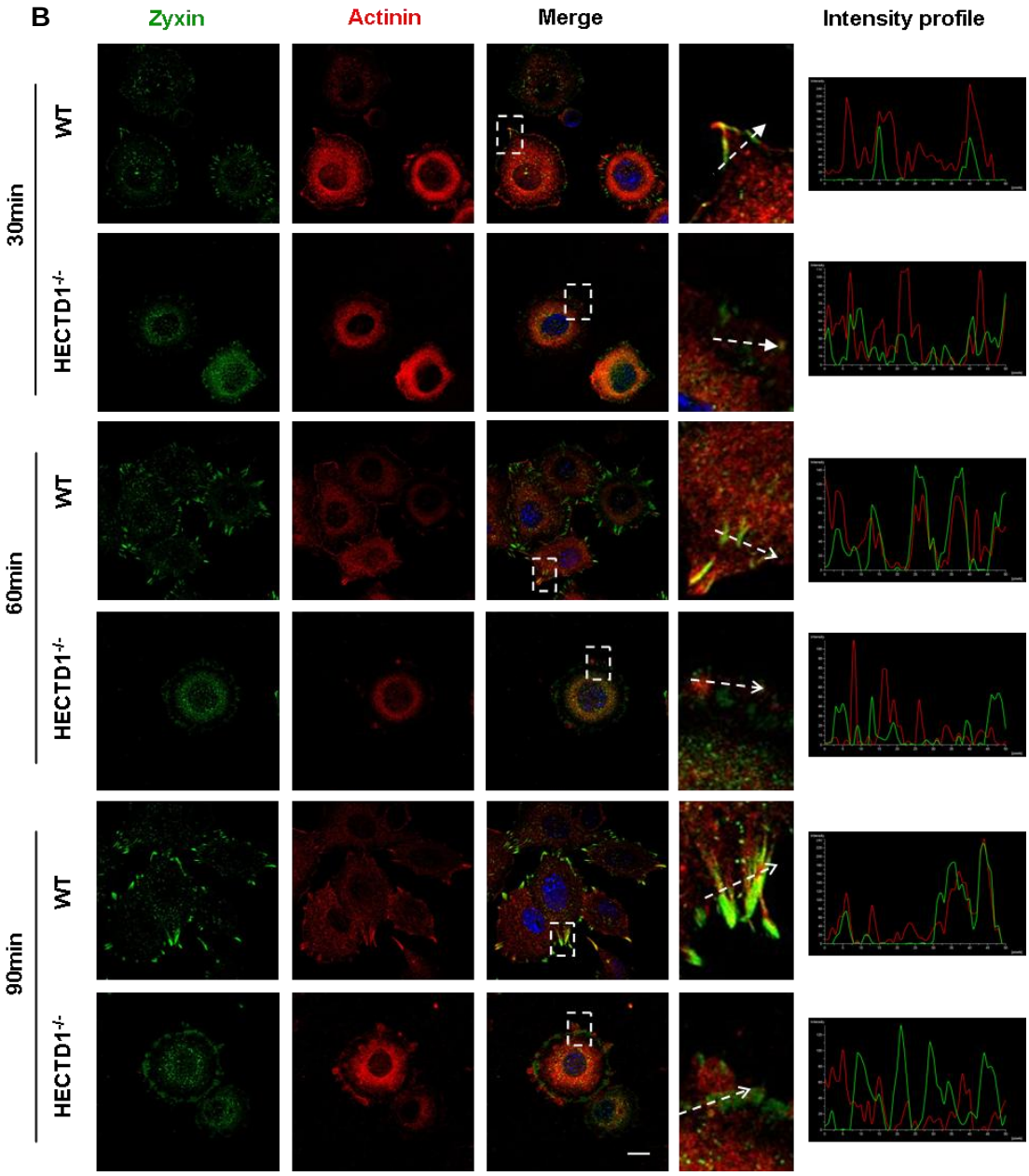
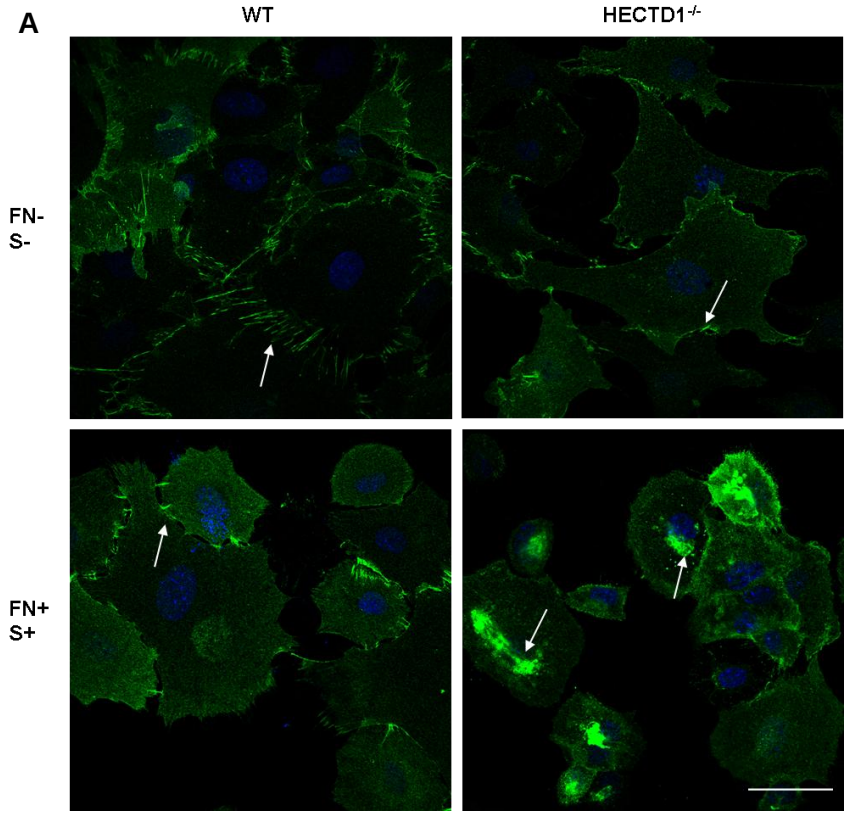


Figure 16. Loss of HECTD1 leads to mislocalization of α-actinin and paxillin/zyxin. Equal amounts of wild-type and HECTD1 knockout cells were seeded on culture dishes for 24 hours, followed by starvation overnight and plated on FN coated coverslips for 30, 60 or 90 minutes. The cells were fixed and co-stained with anti-α-actinin and anti-paxillin **(A)** or anti-zyxin **(B)**, respectively. The dotted frame was zoomed out at the right panel, and the colocalization of two proteins across the dashed line was shown in the fluorescence intensity profiles. Bar, 20μm.

3. 1. 7 HECTD knockout reduces β-catenin accumulation at sites of adherens junctions

Adherens junctions are important complexes that regulating the cell-cell contact and collective cell movement. β-catenin is one of the protein complexes that link cadherins to the cytoskeleton in neighbouring cells. Since we demonstrated that the velocity and directionality of cell migration were altered in HECTD1 mutated cells, we asked whether the expression of β-catenin contributed to these findings. Thus, we compared the localization of β-catenin in HECTD1 knockout and wild-type cells. In wild-type cells, β-catenin robustly accumulated to the connection of two adjacent cells, unlike β-catenin expressed less and weaker in HECTD1 knockout cells. When both cells were starved overnight and seeded on FN-coated plate for 6 hours, β-catenin was still concentrated at the adherens junctions, just weaker compared to normal situation, while β-catenin dominantly located in the cytoplasm of HECTD1 knockout cells instead of the adherens junctions (Fig. 17, A and B).



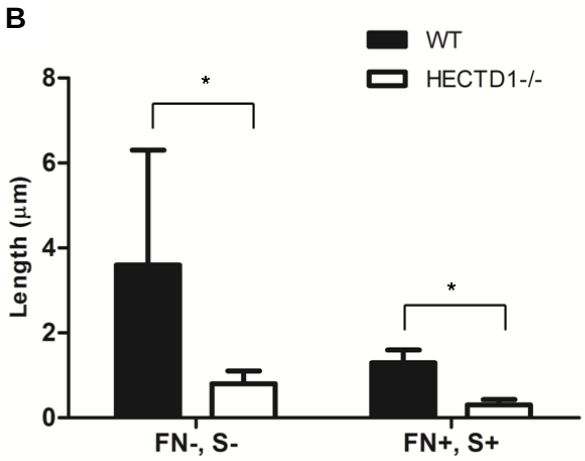


Figure 17. Loss of HECTD1 impairs the location of β-catenin. (A) Location of β-catenin. Equal amounts of wild-type and HECTD1 knockout cells were seeded on culture dishes for 6 hours before staining with anti-β-catenin (upper panels). Equal amount of wild-type and HECTD1 knockout cells were seeded on culture dishes for 24 hours, followed by starvation overnight and plated on FN coated coverslips for 6 hours. The cells were fixed and co-stained with β-catenin, (lower panels). The location of accumulated β-catenin was indicated by a white arrow. Bar, 50 μm. **(B)** Quantification of average β-catenin length by Image J software. *, $P < 0.05$.

3. 2 Result 2

3. 2. 1 Loss of HECTD1 alters tyrosine phosphorylation of paxillin

Focal adhesion (FA) assembly and turnover has been shown to be intrinsically involved in cell spreading and migration (Burridge and Chrzanowska-Wodnicka, 1996). One of the mechanisms believed to regulate these processes is tyrosine phosphorylation of FA proteins such as focal adhesion kinase (FAK) and paxillin (Panetti, 2002). These proteins undergo phosphorylation upon integrin engagement with the extracellular matrix (Bockholt and Burridge, 1993); during spreading (Panetti, 2002); after serum stimulation (Barry and Critchley, 1994); or upon the formation of focal adhesions (Ridley and Hall, 1994) (Zaidel-Bar et al., 2007). Phosphorylation of paxillin at Y118 has been implicated in the regulation of adhesion dynamics and protrusion (Choi et al., 2011). To examine whether phosphorylation process contributes to the impaired expression of focal adhesions in HECTD1 knockout cells, we studied the focal adhesions phosphorylation using paxillin Y118 as an example. First, we detected the location of paxillin-Y118 in wild-type and HECTD1 knockout cells after FN stimulation.

In wild-type cells Paxillin-Y118 is similarly located as paxillin. After migrating for 90 minutes, obvious focal adhesions could be seen, which were associated with stress fibers. As the cells continued to migrate for 4 hours, paxillin-Y118 constituted mainly as focal adhesions. However, in HECTD1 knockout cells, the expression of paxillin-Y118 was mostly located at the cell leading edges as focal complexes with disperse distribution in cytoplasm (Fig. 18 A).

To further understand the possible reason for the mislocalization of paxillin-Y118 we next checked the total amount of paxillin-Y118. Hela cells, stably transfected with control siRNA and HECTD1 siRNA 1099, were starved overnight. Total protein lysates were harvested for the detection of the amount of paxillin-Y118 (Fig. 18 B). 60 minutes after stimulation with FN the amount of paxillin-Y118 was dramatically elevated in HECTD1 siRNA 1099 transfected cells, when compared to control siRNA treated cells ($P<0.05$) (Fig. 18 C).

Taken all data together, high phosphorylation and mislocalization of paxillin-Y118 in

HECTD1 knockout cells indicate that paxillin-Y118 is actively involved in focal adhesion dynamics.

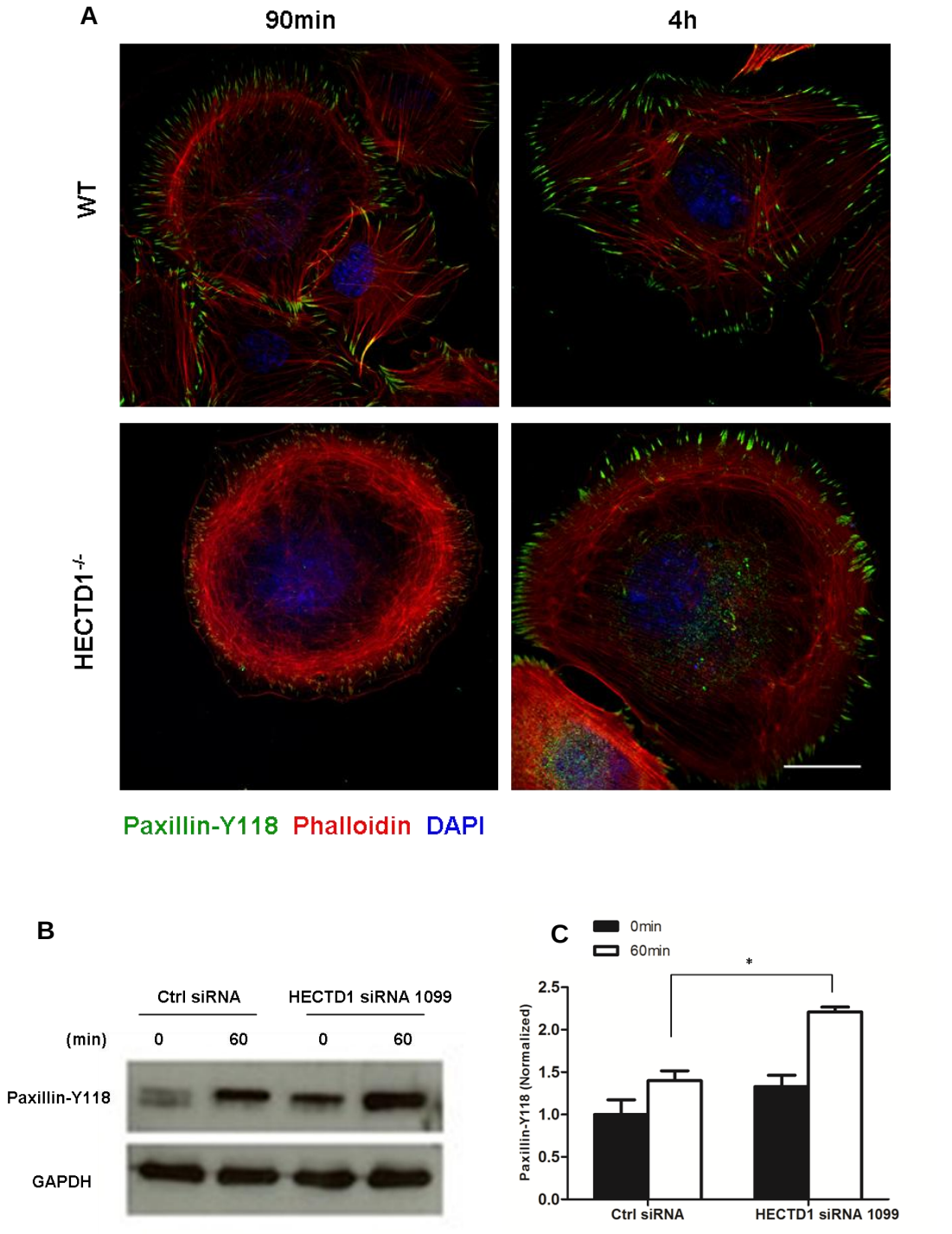


Figure 18. Phosphorylation of paxillin at site Y118 was altered in HECTD1 deficient cells. (A) Mislocalization of paxillin-Y118 in HECTD1 knockout cells. Equal amount of wild-type and HECTD1 knockout cells were seeded on culture dishes for 24 hours, followed by starvation overnight and plated on FN coated coverslips for 90 minutes or 4 hours. The cells were fixed and

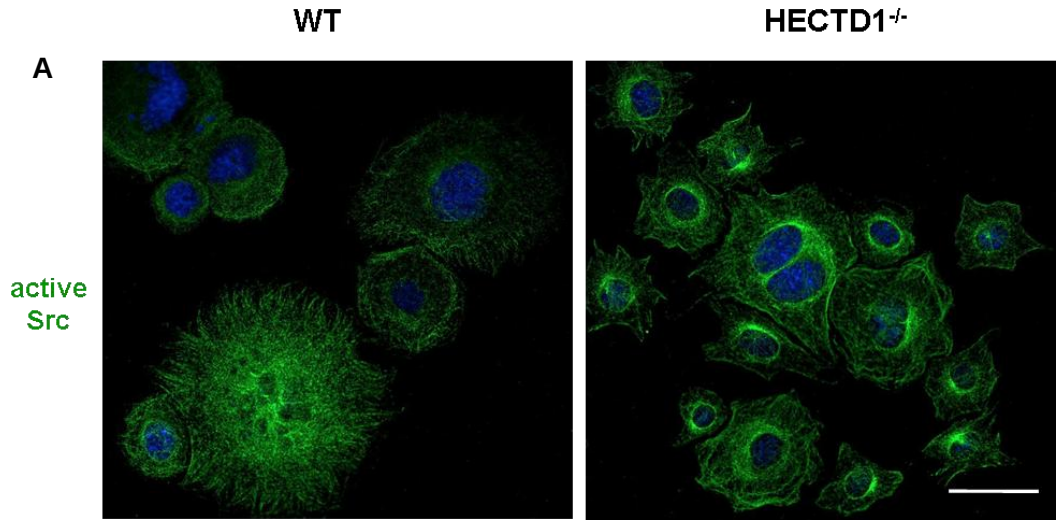
co-stained with phalloidin and anti-paxillin-Y118, respectively. Bar, 20 μm. **(B)** Total level of paxillin-Y118. Hela cells stably expressing control siRNA or HECTD1 siRNA 1099 were seeded on culture dishes for 24 hours. After starvation for overnight, cells were harvested immediately and plated on FN-coated plates for 60 minutes. The total amount of paxillin-Y118 was determined by Western blot respectively. Quantification of paxilin-Y118 **(C)** protein intensity by Lab Image software from 3 independent experiments. The results were first normalized with GAPDH. *, $P<0.05$.

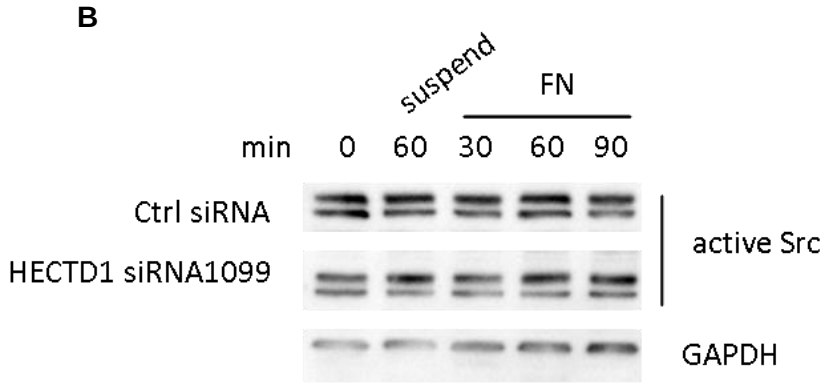
3. 2. 2 Mislocalization of c-Src and increased PIP5K1A in HECTD1 knockout MEFs

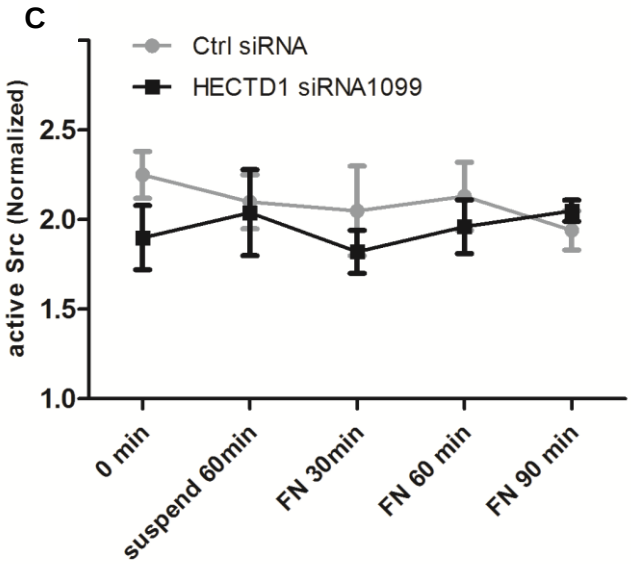
The main kinases believed to be responsible for tyrosine phosphorylation of adhesion molecules are Src and FAK (Frame, 2004; Schlaepfer and Mitra, 2004) and that of paxillin was demonstrated by a Src substrate (Iwasaki et al., 2002). The activity of Src family members is regulated by tyrosine-protein kinase CSK at a conserved C-terminal site (Chong et al., 2005; Nada et al., 1991; Nada et al., 1993). During cell migration, Src kinase is known to cycle in and out of focal adhesions and to regulate rearrangement of actin structures. To understand whether active Src is involved in phosphorylation of cell adhesions regulated by HECTD1, we tested the localization and protein level of active Src. In wild-type cells, active Src distributed averagely through the cytoplasm in a radial pattern or concentrated staining adjacent to the membrane after 60 minutes of FN stimulation. In contrast, loss of HECTD1 causes a striking disorganization of active Src, which became accumulated in a perinuclear pattern (Fig. 19 A). Then, we detected the level of active Src after FN stimulation by western blot. However, as can be seen in the results, there was no significant difference between HECTD1 knockout and wild-type cells ($P>0.05$) (Fig. 19 B and C).

It is well known that phosphatidylinositol 4-phosphate (PI4P) 5-kinase (PIP5K) is a precursor in phosphoinostitide signaling, directly modulating the activity of signaling proteins and cellular processes. To date, three mammalian PIP5K isozymes, α, β and γ, have been identified. Upon cell stimulation by agonists, such as hormones, extracellular matrix, and growth factors, PIP5K is activated (Kanaho et al., 2007). Knowing that tyrosine phosphorylation of paxillin in HECTD1-deficient cells was enhanced, we further detected the expression of PIP5K in HECTD1-deficient cells. Our results showed protein levels of PIP5K1A ($P<0.05$), rather than PIP5K1B or PIP5K1C ($P>0.05$) was increased as in contrast

to control cells after FN stimulation for 60 minutes (Fig. 19 D and E), indicating that PIP5K1A plays a role in phosphorylation of cell adhesions.







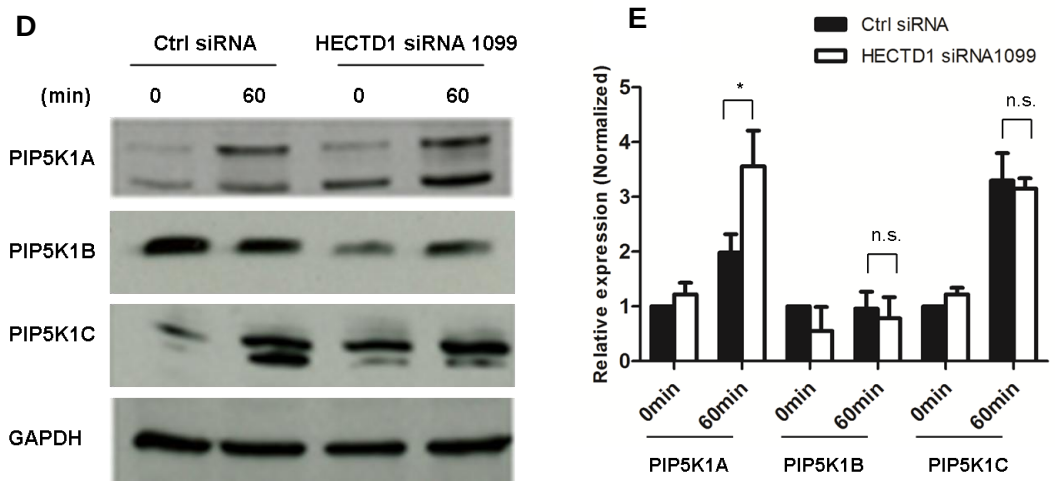


Figure 19. Mislocalization of c-Src and increased PIP5K1A in HECTD1-deficient cells. (A) Mislocalization of active Src. Wild-type and HECTD1 knockout cells were seeded on culture plates for 24 hours and then starved for overnight. Cells were plated on FN coated coverslips for 60 minutes and fixed for further staining with anti-Src (active). Bar, 50 μm. **(B)** Unchanged level of active Src after HECTD1 knocking down compared to control. Hela cells stably expressing control or HECTD1 1099 siRNA were seeded on culture plastic for 24 hours. After being starved for overnight, cell lysates were either harvested immediately as 0 minute control, suspended in culture medium at 37 ℃ for 60 minutes or plated on FN coated culture dishes for 30, 60 and 90 minutes at 37 ℃. Lysates from Hela control siRNA and Hela HECTD1 1099 siRNA were analyzed by anti-Src (active) and GAPDH blotting. **(C)** Quantification of c-Src intensity by Image J software based on three independent experiments. Src intensity was normalized by GAPDH. **(D)** Hela cells stably expressing control or HECTD1 1099 siRNA were starved overnight. Cells were either collected immediately as 0 minute control, or plated on FN coated dishes for 60 minutes at 37 ℃. Lysates were detected by anti-PIP5K1A, anti-PIP5K1B, anti-PIP5K1C and GAPDH, respectively. **(E)** Quantification of protein intensity by Image J software based on three independent experiments. Src intensity was normalized by GAPDH. *, $P<0.05$. N. S. No significant.

3. 2. 3 The activity of PP2A was increased in HECTD1 knockout cells

Protein phosphatase 2A (PP2A) is abundantly and ubiquitously expressed in eukaryotes and plays an important role in regulating cell motility and invasion, cytoskeleton dynamics, etc (Jackson et al., 1997). PP2A is a major serine/threonine phosphatase, but there are some studies showing that PP2A is also a regulator for protein tyrosine phosphatase activity (Jackson and Young, 2003). Taken together, PP2A acts as a regulator of the balance between phosphorylation and dephosphorylation of serine and tyrosine residues.

In our study, there was no significant difference of the total level of PP2A between

wild-type cells and HECTD1 knockout cells (Fig. 20 A and B), but the activity of PP2A in HECTD1 knockout cells was evidently stronger as compared to wild-type counterpart (Fig. 20 C).

Thus, in our study conditions, higher activity of PP2A in HECTD1 mutant cells indicates that PP2A would not directly be involved in dephosphorylating proteins such as paxillin.

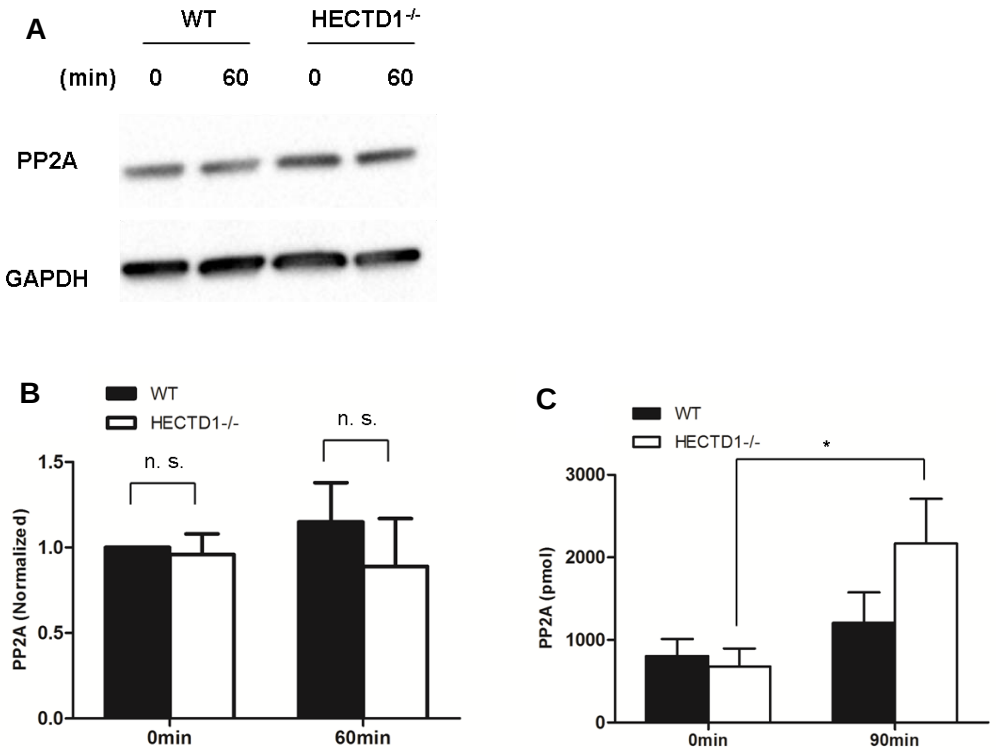


Figure 20. Activity of PP2A was elevated in HECTD1 knockout cells. (A) Total amount of PP2A in wild-type and HECTD1 knockout cell. **(B)** Quantification of PP2A intensity by Image J software. PP2A intensity was normalized by GAPDH. N. S. No significant. **(C)** Wild-type and HECTD1 knockout cells were seeded on culture plastic for 24 hours. After being starved for overnight, cell lysates were harvested immediately or plated on FN coated culture dishes for 90min at 37℃. The activity of PP2A was determined by PP2A Immunoprecipitation Phosphatase Assay (Millipore). *, $P<0.05$.

3. 2. 4 Activity of Rac1 and RhoA are elevated in HECTD1 knockout MEFs

Rho GTPases family members act as "molecular switches" in regulating cytoskeletal dynamics, cell movement and other cellular functions. RhoA and Rac1 belong to the family of Rho GTPases and regulate a variety of biological response pathways including cell motility, cell division, gene transcription, and cell transformation. Importantly, paxillin-Y118

phosphorylation mediates the interaction with Crk and p120RasGAP (Schaller and Parsons, 1995; Tsubouchi et al., 2002). These interactions are associated with cytoskeletal regulation through modulation of the Rho GTPases, Rac1 and RhoA respectively, and appear to perform cell specific roles in regulating integrin signaling and migration (Tumbarello et al., 2005).

We have validated that loss of HECTD1 affects cell spreading and migration in the FN-integrin pathway. In consequence we were curious whether this pathway would also activate Rac1 and RhoA. Using the Rac1 and RhoA activation assay, we found that the activities of both Rac1 and RhoA were significantly ($P<0.05$) enhanced in HECTD1 knockout cells 60 minutes after FN stimulation as compared that of wild-type cells, in which the total level of Rac1 and RhoA were not significant altered (Fig. 21 A, B and C).

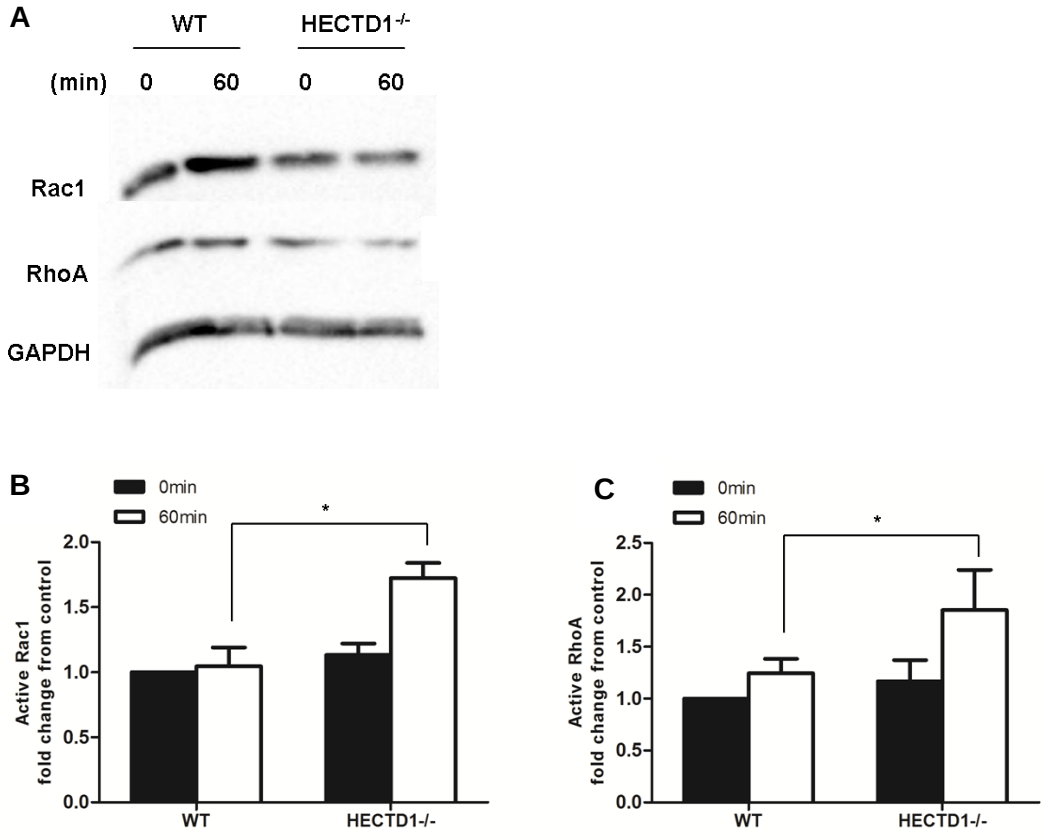


Figure 21. Activities of Rac1 and RhoA are enhanced in HECTD1 knockout cells. (A) Wild-type and HECTD1 knockout cells were seeded on culture dishes for 24 hours. After being starved for overnight, cell lysates were harvested immediately or plated on FN-coated culture dishes for 60 min at 37 ℃. From protein collection, all the steps were manipulated on ice and snap frozen in liquid nitrogen. Activity of Rac1**(B)** and RhoA **(C)** were recorded as fold change from wild-type 0 min group based on three independent experiments *, $P<0.05$.

3. 3 Result 3

3. 3. 1 Overexpression of IQGAP1 and Hax1 in HECTD1 deficient cells

In order to investigate the interacting proteins of HECTD1, previously we performed Mass Spectrum analysis and the yeast two-hybrid screening test. Among the list of potential binding partners of HECTD1, we found IQGAP1 and Hax1, both widely reported to be involved in regulating focal adhesions dynamics and cell migration (Fig. 22 A). IQGAP1 has been reported to be a scaffold protein localized in the leading edge and cell-cell contacts. As an effector of Rac1 and cdc42, IQGAP1 binds to APC, actin, β-catenin and E-cadherin then regulates cell polarization, directed migration and cell-cell adhesion (Choi et al., 2013; Fukata et al., 2001; Kuroda et al., 1998; Noritake et al., 2005).

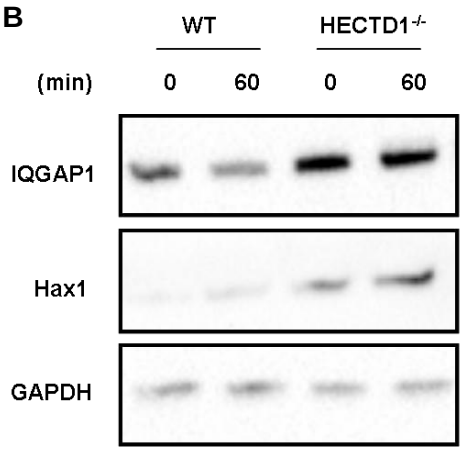
Hax1 has been reported to associate with HS-1, a substrate of Src family of tyrosine kinases. Mutations in Hax1 result in congenital neutropenia. Hax1 has known to be involved in a large range of cellular processes, including cell migration, cell proliferation, cytokinesis, by regulating integrin β6, cortactin, integrin linking kinase (ILK), and so on (Gallagher et al., 2000; Radhika et al., 2004; Ramsay et al., 2007). The PEST sequence in Hax1 is responsible for its poly-ubiquitination and rapid degradation, resulting in short intracellular half life (Li et al., 2012).

To test whether IQGAP1 and Hax1 would take part in cell adhesions dynamics and cell movement in HECTD1 knockout cells, we detected the protein level in wild-type and HECTD1 knockout cells by western blot. Interestingly, the protein level of IQGAP1 was about three times in HECTD1 knockout cells than wild-type cells ($P<0.05$), while IQGAP1 was two times more than wild-type cells in HECTD1 knockout cells ($P<0.05$) (Fig. 22 B, C and D), indicates IQGAP1 and Hax1 could act as important effectors regulated by HECTD1.

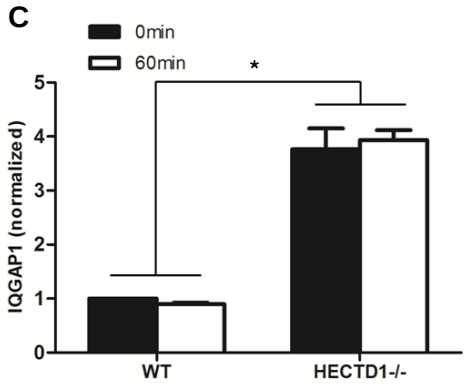
A

Short list of proteins that were in the complex with Hectd1 in **Mass spectrum**	Short list of proteins that interact with Hectd1 in **yeast two hybrid**
•Arpc2 (Actin-related protein 2/3 complex subunit 2)	•AGR2
•Cand1	•APPL1
•Ddx39	•BMI
•Dusp3	•CDK9
•Exoc2	•Ddx56
•IQGAP1 (IQ motif containing GTPase activating protein 1)	•Hax1 (HS1 associated protein X-1)
•Jmjd6	•HDAC10
•Jup	•IGSF1
•Mapk15	•Lmo4
•Mapksp1	•POLR2A
•Rab2a	•Ppp5c
•Rabex-5	•Rorb
•SIRT1	•SOX6
•Sgta	•Znf622
•Snx5	
•Spin1	
•Tnpo3	
•Tubb3	
•Ube2o	
•Wdr43	
•Zfp646	

B



C



D

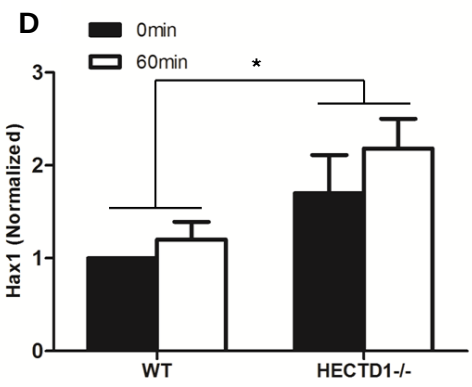


Figure 22. Overexpression of IQGAP1 and Hax1 in HECTD1 knockout cells. (A) IQGAP1 and Hax1 were two potential binding partners of HECTD1. List for binding partners of HECTD1 resulted from Mass spectrum and yeast two hybrid assay. **(B)** Level of IQGAP1 and Hax1 were increased in HECTD1 knockout cells. Wild-type and HECTD1 knockout cells were seeded on culture plastic for 24 hours. After being starved for overnight, cell lysates were harvested immediately or plated on FN coated culture dishes for 60min at 37℃. Lysates from wild-type and HECTD1 knockout cells were analyzed by anti-IQGAP1, anti-Hax1 and GAPDH blotting. Quantification of IQGAP1 **(C)** and Hax1 **(D)** by Image Lab software. Level of IQGAP1 and Hax1 were recorded as fold change from wild-type 0 min group based on three independent experiments *, $P<0.05$.

3. 3. 2 Protein-protein interaction of IQGAP1, Hax1 and HECTD1

3. 3. 2. 1 Immunoprecipitation of IQGAP1, Hax1 and HECTD1

To confirm the interaction between HECTD1 and IQGAP1, we transfected GFP-IQGAP1 plasmid DNA into HEK293 cells for immunoprecipitation. Immunoprecipitation of endogenous HECTD1 resulted in the co-immunoprecipitation of GFP-IQGAP1. We further extended these interaction studies with co-immunoprecipitation of GFP-IQGAP1 and detection of the endogenous PIP5K1A and β-catenin, which were demonstrated to interact with GFP-IQGAP1, and the interaction enhanced by 60 minutes stimulation of FN (Fig. 23 A).

In order to further investigate whether Hax1 forms a complex with IQGAP1 and HECTD1, and the role of PEST sequence in the turnover of Hax1, we transfected either GFP-Hax1-WT or GFP-Hax1-ΔPEST into Hela cells stably expressing His-HECTD1-1131 (Fig. 23 B). IP results revealed that Hax1 interacts with both HECTD1 and IQGAP1, while this interaction was much weaker in cells transfected with GFP-Hax1-ΔPEST than that of wild-type (Fig. 23 C, D). Thus, our data suggests the PEST sequence in Hax1 plays an essential role in interaction with HECTD1 and IQGAP1.

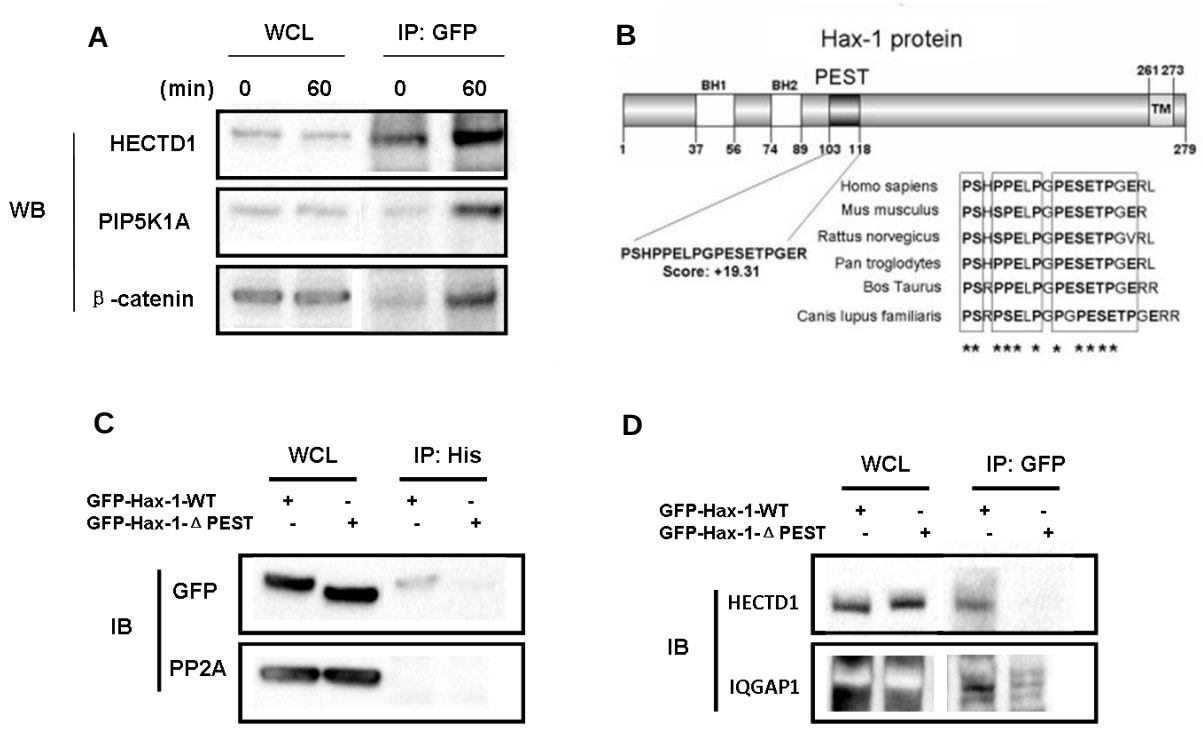


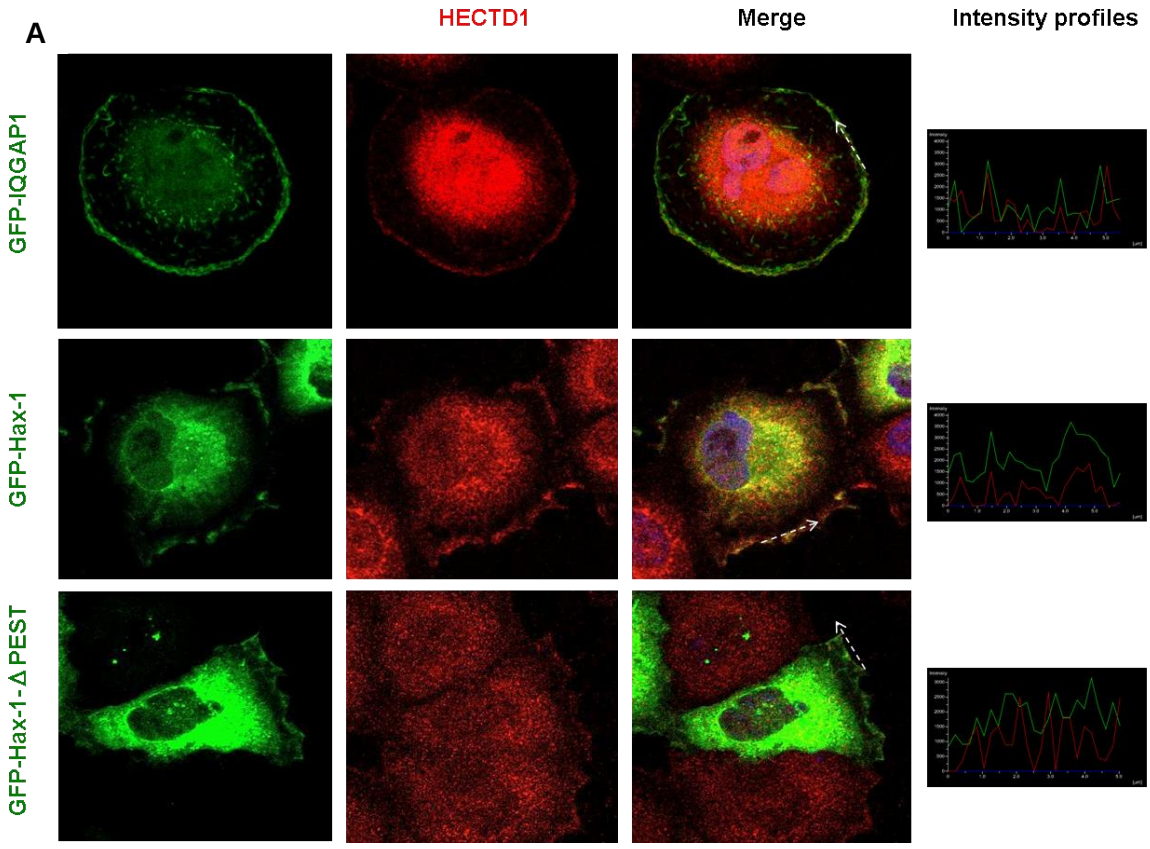
Figure 23. IQGAP1 and Hax1 interact with HECTD1. (A) IQGAP1 interacts with HECTD1, PIP5K1A and β-catenin. HEK293 cells were stably transfected with GFP-IQGAP1 and cells were starved overnight, seeded on FN-coated dishes for 60 min, protein lysates were harvested and immunoprecipitated (IP) by GFP antibody. The lP lysates and whole cell lysates were used for detecting HECTD1, PIP5K1A and β-catenin by western blot. **(B)** Schematic representation of a PEST sequence in Hax1 protein. The PEST sequence in Hax-1 is conserved among different mammals **(C, D)** Interaction of Hax1 with HECTD1 and IQGAP1 requires the PEST sequence. Hela cell stably expressing His-HECTD1-1131 was transiently transfected with GFP-Hax1-WT or GFP-Hax1-ΔPEST for 24 hours. Protein lysates were harvested and precipitated by His/GFP antibody. Target proteins were analyzed by GFP/HECTD1, IQGAP1 blotting. PP2A levels were used as negative control.

3. 3. 2. 2 Co-localization of IQGAP1, Hax1 and HECTD1

Next, we performed colocalization assay to support the protein-protein interaction between IQGAP1, Hax1 and HECTD1. Hela cells expressing His-HECTD1-1131 were transfected with GFP-IQGAP1, GFP-Hax1-WT or GFP-Hax1-ΔPEST and starved in serum free medium for overnight, then plated on FN-coated plates for 60 min. After that, we immunolabled subcellular HECTD1 and checked the colocalization by confocal microscopy. Our results revealed that both IQGAP1 and Hax1 displayed mainly cytoplasmic localization and was

enriched at the leading edge of cells. The Pearson's correlation coefficient of GFP-IQGAP1 and HECTD1 at the cell leading edge was 0.65 ± 0.19. However, although HECTD1 showed a similar localization as Hax1 in GFP-Hax1-WT positive cells with a Pearson's correlation coefficient of 0.52 ± 0.13 at cell leading edge, the enrichment of HECTD1 was much weaker than Hax1 at the leading edge of GFP-Hax1-ΔPEST positive cells, resulting in a lower Pearson's correlation coefficient of 0.38 ± 0.09 ($P<0.05$). The colocalization of the HECTD1 between GFP-IQGAP1, GFP-Hax1-WT or GFP-Hax1-ΔPEST across the white arrows at the leading edge were also shown in fluorescence intensity profiles. (Fig. 24 A, B).

Therefore, the colocalization of HECTD1 and IQGAP1/Hax1 lend support to the interaction between these proteins. For Hax1, the PEST sequence plays an essential role in colocalization with HECTD1.



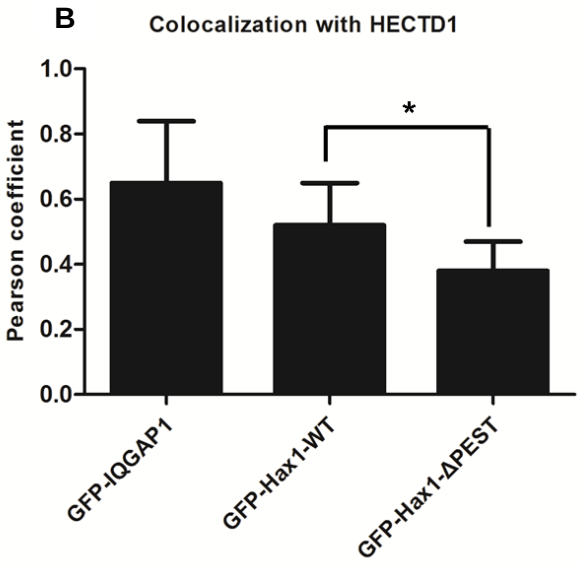


Figure 24. Colocalization of HECTD1 with IQGAP1 and Hax1 in cell leading edge. (A) Hela cell stably express His-HECTD1-1131 was transiently transfected with GFP-IQGAP1, GFP-Hax1-WT or GFP-Hax1-ΔPEST for 24 hours. Cells were starved overnight and plated on FN-coated slides for 60 min, followed by fixation and staining with HECTD1. Note the site of colocalization shown in intensity profiles (white arrows). **(B)** Pearson's correlation coefficient were analyzed by Image J software. *, $P<0.05$.

3. 3. 3 Ubiquitination of IQGAP1 and Hax1 is regulated by HECTD1

To test whether IQGAP1 and Hax1 are ubiquitinated by HECTD1, first we examined the ubiquitination level of IQGAP1 and Hax1. We treated cells with proteasome inhibitor MG132 to block the ubiquitin-proteasome degradation pathway. In general, the overall IQGAP1 ubiquitination level was increased after MG132 treatment comparing to DMSO treated control cells. After MG132 treatment, we could detect increased IQGAP1 ubiquitination after FN stimulation for 60 min, while this increase was greater in wild-type cells than HECTD1 knockout cells (Fig. 25, A). However, in contrast with IQGAP1, ubiquitination of Hax1 was more pronounced in HECTD1 knockout cells than in wild-type cells, but less than in cells transfected with GFP-Hax1-ΔPEST (Fig. 25, B). The ubiquitination results of IQGAP1 support the notion that IQGAP1 functions as a direct substrate of HECTD1, whereas the contrasting results of Hax1 failed to provide the evidence that Hax1 is a director effector of HECTD1. However, we confirm that the PEST sequence of Hax1 is responsible for

degradation of Hax1 by ubiquitination, which is consistent with the previous report (Li et al., 2012) .

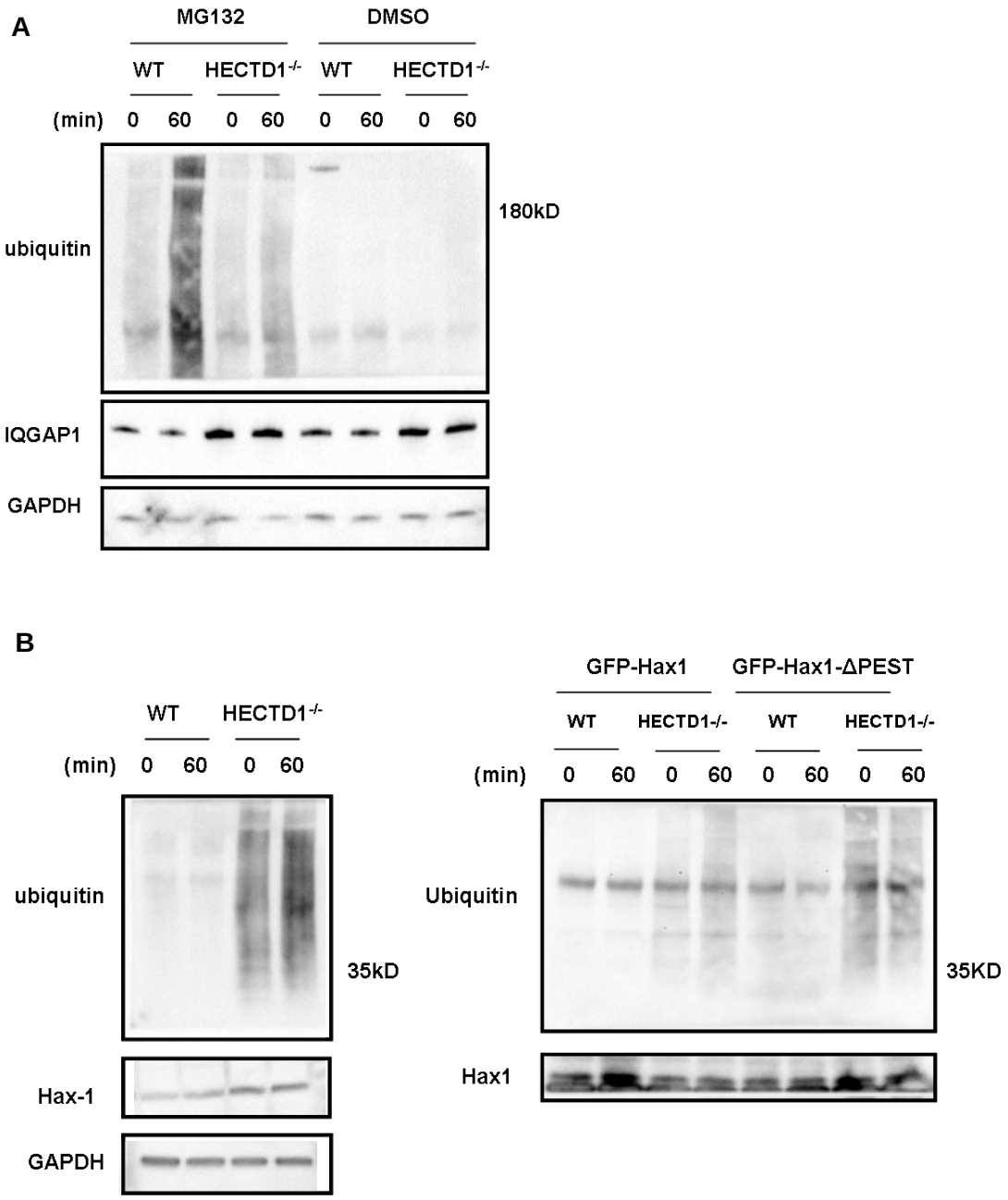


Figure 25. Ubiquitination of IQGAP1 and Hax1 are regulated by HECTD1. **(A)** Endogenous ubiquitination of IQGAP1 and Hax1. Wild-type and HECTD1 knockout cells were treated with the proteasome inhibitor MG132 1 μg/ml or DMSO in serum free medium for overnight. Cell pellets were harvested and lysed immediately or seeded on FN coated dishes for 60 min. The ubiquitination of IQGAP1/Hax1 was further verified by immunoprecipitating IQGAP1/Hax1 and detecting with an anti-ubiquitin antibody. **(B)** Ubiquitination of Hax1 after transfection. Wild-type and HECTD1 knockout cells were transfected with GFP-IQGPA1/GFP-Hax1 and

GFP-Hax1-ΔPEST together with HA-ubiquitin for 24 hours. Cells were treated with MG132 1μg/ml or DMSO in serum starvation medium for overnight. Cell pellets were harvested and lysed immediately or seeded on FN coated dishes for 60min. The ubiquitination of Hax1 was further identified by immunoprecipitating Hax1 and detecting with an anti-ubiquitin antibody.

3. 3. 4 Increase of half-life of IQGAP1 and Hax1 in HECTD1 knockout cells

As we understand HECTD1 plays a regulatory role in ubiquitination of IQGAP1 and Hax1, our next question was whether the half-life of IQGAP1 and Hax1 changes accordingly. We tested the degradation profile of IQGPA1 and Hax1 using a cycloheximide (CHX) chase experiment in both wild-type and HECTD1 knockout cells. The CHX chase experiments showed that the IQGAP1 level remained largely unchanged up to 30 hours in HECTD1 knockout cells, whereas in wild-type cells this level decreased to near 50% within 12 hours (Fig. 26 A). Similarly, Hax1 level in wild-type cells rapidly decreased to < 50% in 2 hours in contrast with almost no change to 6 hours in HECTD1 knockout cells (Fig. 26 B). Our results show that IQGAP1 and Hax1 protein are slowly degraded in HECTD1 mutant cells, suggesting HECTD1 is necessary for proper degradation of IQGAP1 and Hax1.

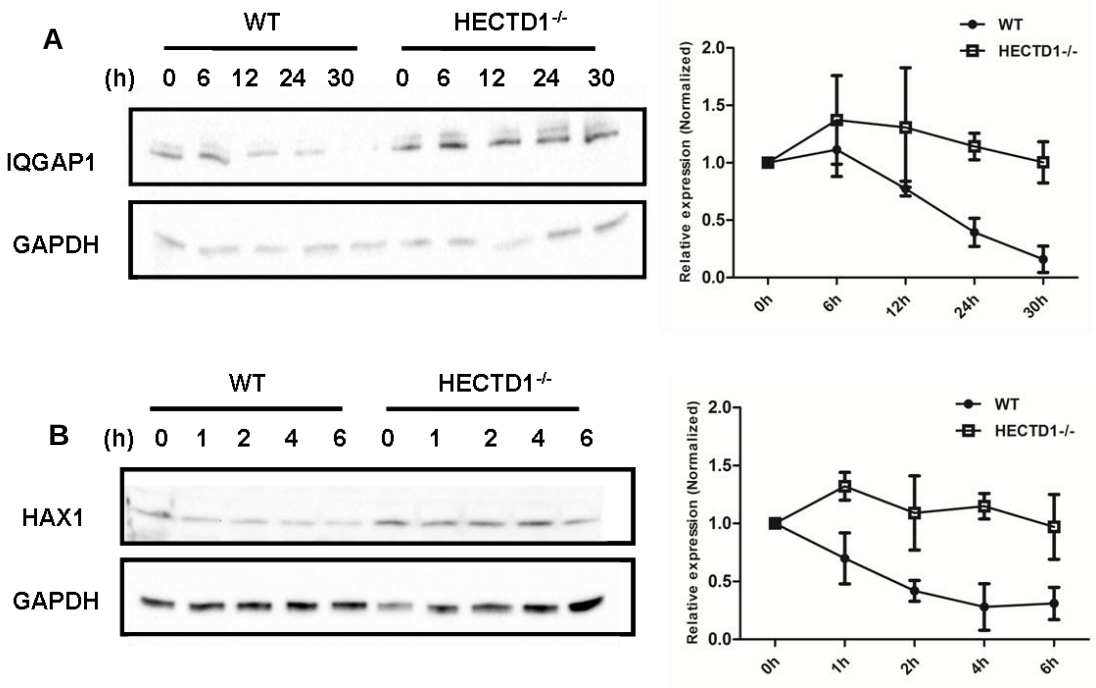


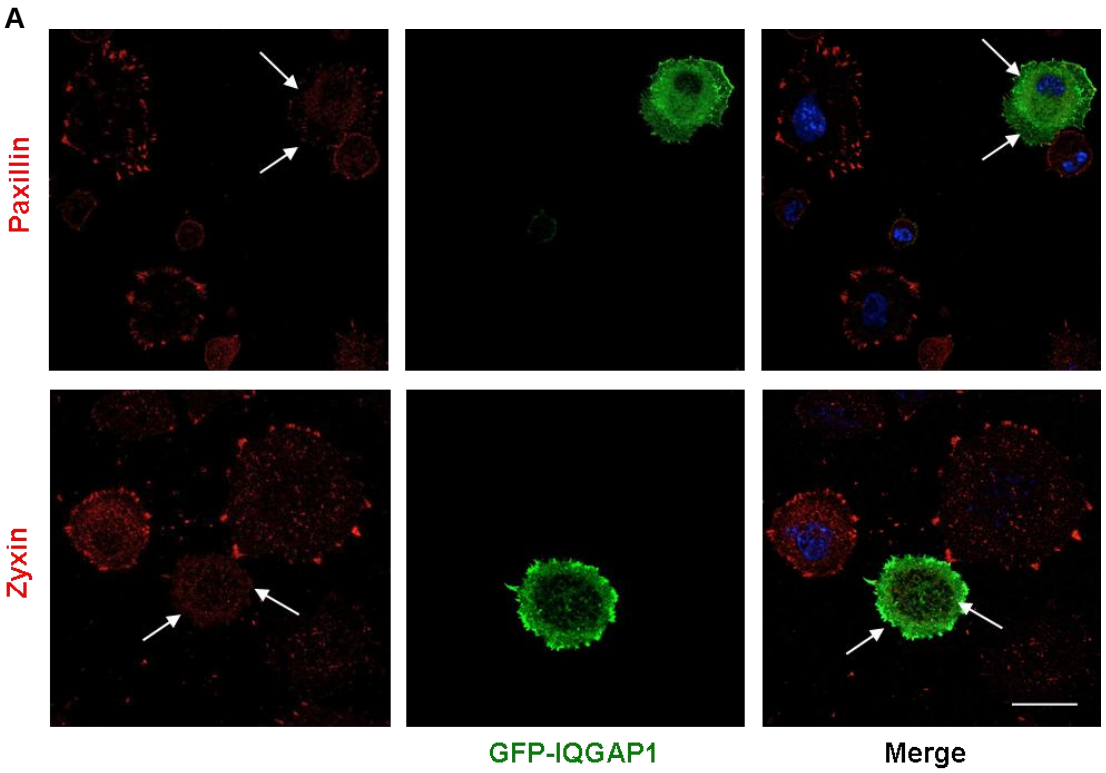
Figure 26. Half-life of IQGAP1 and Hax1 is increased in HECTD1 knockout cells. Equal amounts of cells were plated on 100 mm dishes for 24 hours and treated with 100 μg/ml

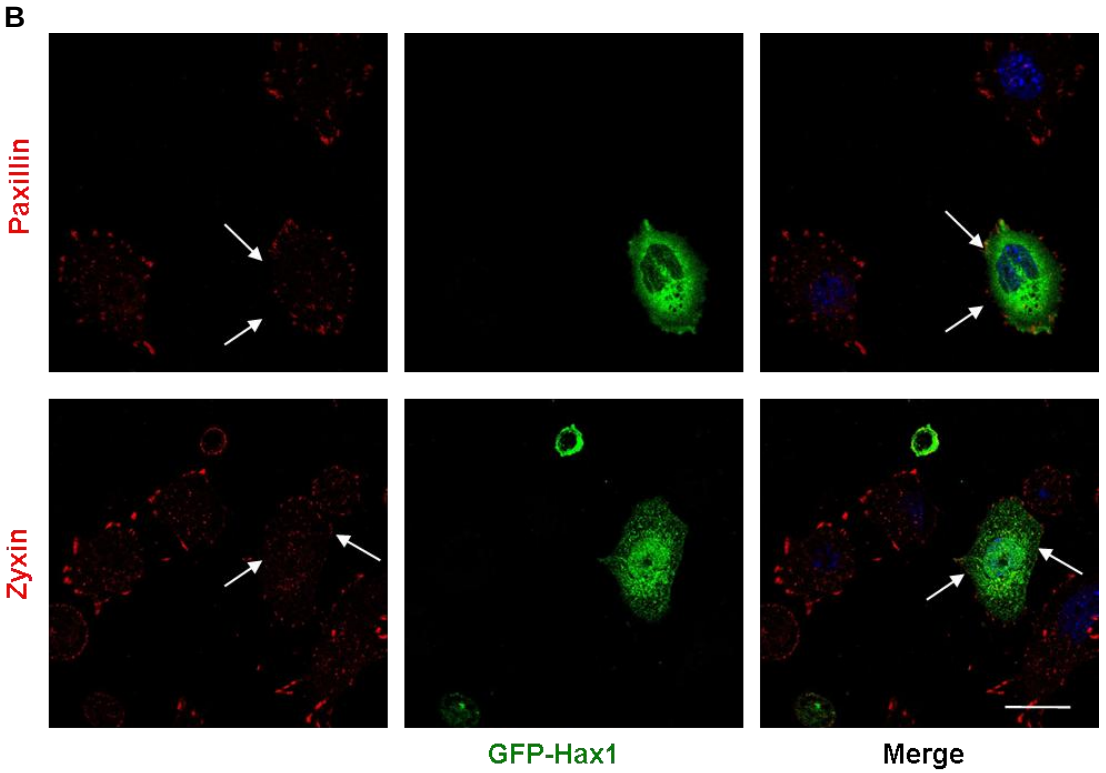
Cycloheximide (CHX) for 6h, 12h, 24h and 30h **(A)** or 1h, 2h, 4h and 6h **(B)**. Cell pellets were harvested and expression of IQGAP1 **(A)** and Hax1 **(B)** were detected by Western blot.

3. 3. 5 Overexpression of GFP-IQGAP1 and GFP-Hax1 in wild-type cells induces defects in formation of focal adhesions

We found that IQGAP1 is ubiquitinated and degraded by HECTD1, and IQGAP1 has been reported to regulate focal adhesion and cell migration (Noritake et al., 2005). Increased ubiquitination and longer half-life of Hax1 in HECTD1 knockout cells were reminiscent of Hax1 playing a role in cell movement. Thus, we speculated that elevated protein level of IQGAP1 and Hax1 were the direct causes of impaired formation of focal adhesions in HECTD1 knockout cells.

In order to examine this, first we overexpressed GFP-IQGAP1/GFP-Hax1 in wild-type cells, performed immunostaining for paxillin and zyxin, then measured the average focal complexes and focal adhesions per cell for different time points by using paxillin or zyxin as markers. Interestingly, irrespective of paxillin or zyxin as the respective marker used, after GFP-IQGAP1 transfection, the expression of focal adhesions was dramatically decreased when compared to non-transfected wild-type cells. Similar results were shown in GFP-Hax1 overexpressed cells: the focal adhesions were mostly replaced by focal complexes. In detail, wild-type cell contains approximately two times focal adheisons than focal complexes, while the ratio of focal adhesions to focal complexes decreased to around 1/8 in GFP-IQGAP1 overexpression cells and 1/3 in GFP-Ha1 overexpression cells (Fig. 27 A-D). Overall, our results note that overexpression of IQGAP1 and Hax1 leads to defects in maturation of focal adhesions, suggest that IQGAP1 and Hax1 are essential for their assembly.


A
Paxillin
Zyxin
GFP-IQGAP1
Merge


B
Paxillin
Zyxin
GFP-Hax1
Merge

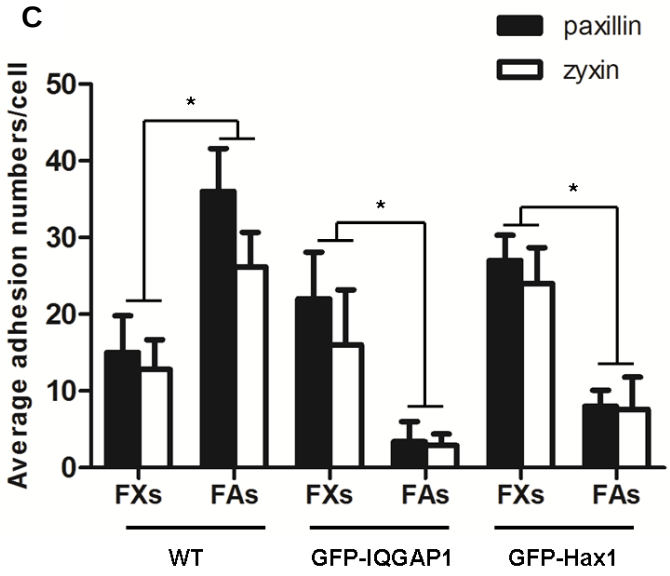


Figure 27. Overexpression of IQGAP1 and Hax1 affects focal adhesions formation. Wild-type cells were transfected with GFP-IQGAP1 **(A)** /GFP-Hax1 **(B)** for 24 hours. Then, cells were starved overnight and seeded on FN coated plate for 60 minutes and were subsequently fixed and stained with paxillin and zyxin, respectively. Non-transfected cells were used as control. Note impaired formation of focal adhesions in GFP-IQGAP1/GFP-Hax1 transfected cells (white arrows) compared with the control cells. **(C)** Quantification of average adhesion numbers/cell. Bar, 20μm. *, P<0.05.

3. 3. 6 IQGAP1 and Hax1 knockdown in HECTD1 knockout cells rescue the dynamics of focal adhesion, duration of cell spreading and directional cell migration

3. 3. 6. 1 siRNA silence of IQGAP1 and Hax1

Previously, loss of HECTD1 was shown to inhibit the formation of focal adhesions, accelerate the speed of cell spreading and alter the directional cell migration. To further test our hypothesis that overexpression of both IQGAP1 and Hax1 are involved in causing dysfunctional cell adhesion, spreading and migration in HECTD1 knockout cells, we transfected IQGAP1, Hax1 or control siRNA to HECTD1 knockout cells (Fig. 28). siRNA-downregulation of IQGAP1 reduced around 1/3 protein expression as compared to control siRNA, resulting in proteins level of IQGAP1 in HECTD1-knockout cells close to

wild-type cells (1.6 : 1). Additionally, silence of Hax1 siRNA slightly reduced the expression of Hax1 in HECTD1 knockout cells as compare to control siRNA treated cells (1.8 : 2.3).

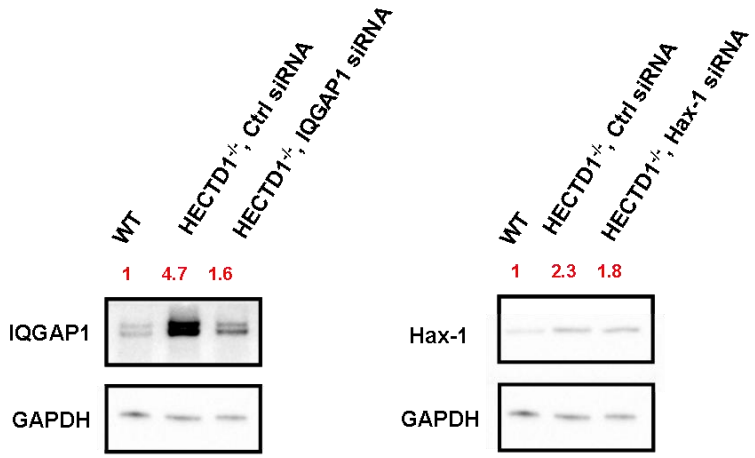


Figure 28. Silence of IQGAP1 and Hax1 in HECTD1 knockout cells. HECTD1 knockout cells were treated with control siRNA, IQGAP1 siRNA or Hax1 siRNA respectively. 36 hours after transfection, cell pellets were harvested and the level of IQGAP1 and Hax1 were detected by western blot. GAPDH was taken as endogenous control.

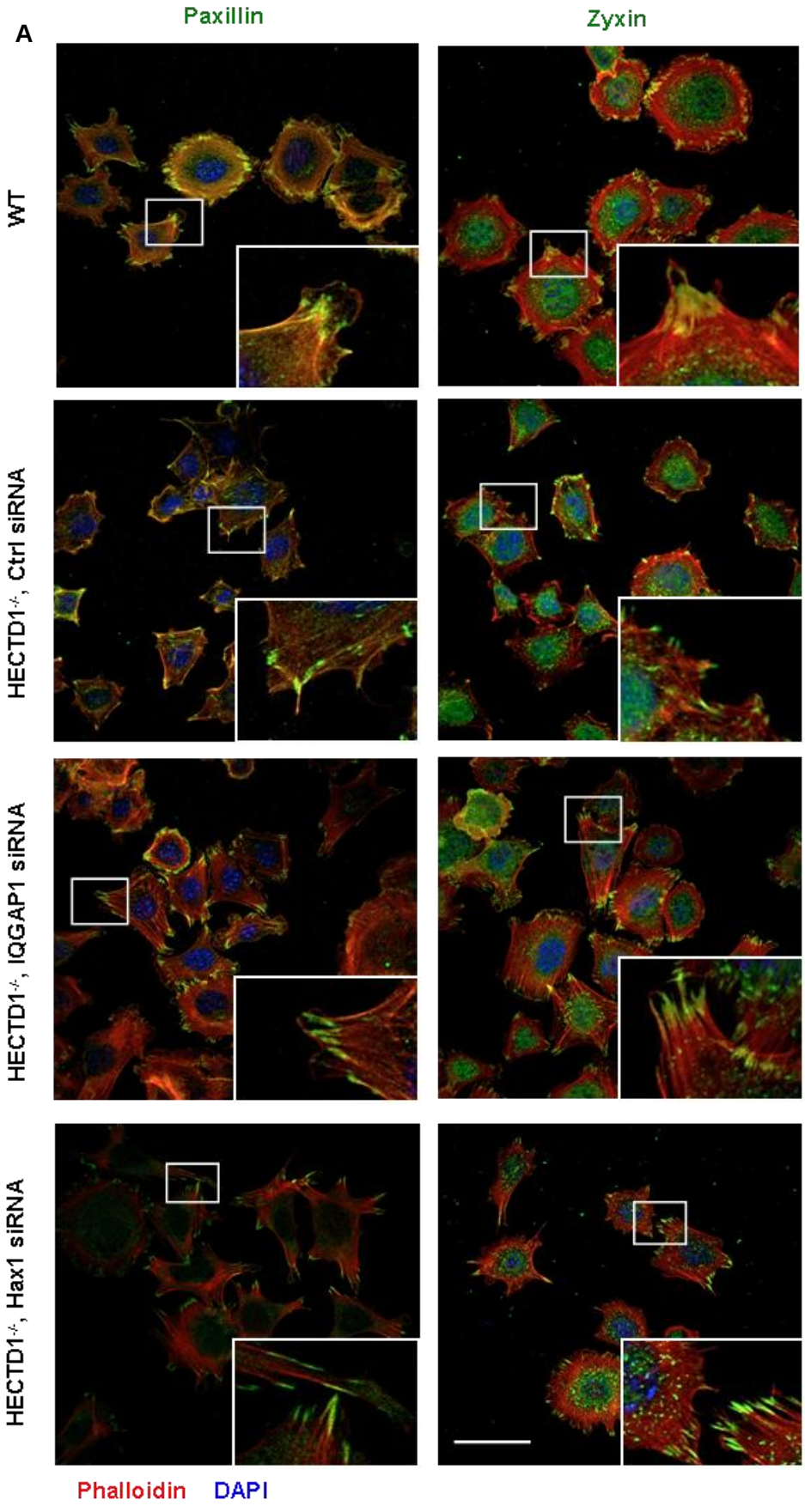
3. 3. 6. 2 Silence of IQGAP1 and Hax1 rescues the formation of focal adhesion proteins

To address how the IQGAP1 and Hax1 contribute to cell migration, we analyzed cytoskeleton and focal adhesions by immunostaining for actin, paxillin and zyxin. In wild-type cells, different structures of actin could be clearly distinguished, including stress fibers in the cell body, cortical F-actin enriched at the periphery and well-organized lamellipodia structures at the leading edge. In contrast, in control siRNA treated HECTD1 knockout cells, stress fibers became less visible than in wild-type cells and lamellipodia were difficult to detect with uniform flat edge. Importantly, the formation of actin could be rescued by down-regulation of IQGAP1 and Hax1 siRNA in HECTD1-knockout cells (Fig. 29 A, B).

In line with our previous results, taking paxillin and zyxin as cell adhesion markers, the average number of focal adhesions was about two times more than focal complexes per cell in wild-type cells, while in control siRNA treated HECTD1 knockout cells, focal complexes constituted the largest part of cell adhesions, that the average focal adhesions/focal complexes

switched to around 1/3. Intriguingly, the expression of focal complexes and focal adhesions could be rescued by IQGAP1 and Hax1 siRNA knockdown in HECTD1 knockout cells, where the focal adhesions accounted for the majority of cell adhesions again, and the average focal adhesions were three times than focal complexes per cell (Fig. 29 A, B).

Combining the data of the GFP-IQGAP1 and GFP-Hax1 overexpression experiments, our current findings prove the hypothesis that overexpression of IQGAP1 and Hax1 in HECTD1 knockout cells are regulators of adhesion proteins, paxillin and zyxin, controlling the adhesion dynamics by inhibiting maturation of focal adhesions from focal complexes.


A
Paxillin
Zyxin
WT
HECTD1-/-; Ctrl siRNA
HECTD1-/-; IQGAP1 siRNA
HECTD1-/-; Hax1 siRNA
Phalloidin
DAPI

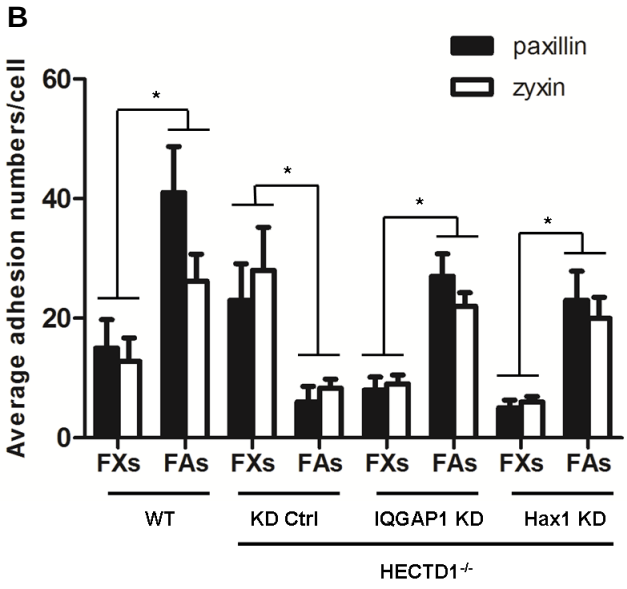


Figure 29. Knockdown of IQGAP1 and Hax1 rescue focal adhesion formation. (A) Knockdown of IQGAP1/Hax1 rescued the formation of cell adhesions. HECTD1 knockout cells were transfected with control, IQGAP1 or Hax1 siRNA, respectively. After 36 hours, cells were starved overnight and plated on FN coated coverslips for 60min, followed by fixing and phalloidin and paxillin/zyxin costaining. **(B)** Quantification of average cell adhesion numbers per cell. Bar, 50μm. *, P<0.05.

3. 3. 6. 3 Silence of IQGAP1 and Hax1 rescues the area and duration of cell spreading

To understand the exact duration of spreading of different cells on FN substrates, we analyzed the time period from cell attachment to cell polarization, which is an indication for cell migration. Similar with the results of spread cell area, the spreading duration time shortened to (29.03 ± 4.48 min) in HECTD1 knockout control group in contrast with (41.80 ± 10.19 min) in wild-type group. Moreover, the duration of spreading time in IQGAP1 and Hax1 siRNA silencing groups (37.23 ± 6.60 min and 34.60 ± 8.50 min, respectively) was partly rescued as compared to control siRNA cells (P<0.05, P<0.05) (Fig. 30 A and B).

To examine whether IQGAP1 and Hax1 silencing could the recovery of spread cell area, wild-type and HECTD1 knockout cells down-regulated of control, IQGAP1 and Hax1 siRNA were serum-free starved for overnight, followed by seeding on FN coated plate and the cell spreading was recorded by time lapse microscopy. Spread cell areas were measured at 10 min and 30 min after plating. We set the area of wild-type cells at 10 min as control, the other

results were normalized to control. Cell area was maximal in wild-type group (1 ± 0 AU at 0 min, 2.32 ± 1.08 AU at 30 min) and minimal in HECTD1 knockout control group (0.86 ± 0.22 AU at 0 min, 1.45 ± 0.9 AU at 30 min) at both 10 min and 30 min. As expected, the cell areas observed in IQGAP1 and Hax1 siRNA silencing groups (0.92 ± 0.37 AU at 10 min, 2.18 ± 1.5 AU at 30 min and 1.09 ± 0.38 AU at 10min, 1.93 ± 1.3 AU at 30 min, respectively) were partly rescued as compared to control siRNA cells ($P<0.05$, $P<0.05$) (Fig. 30 C).

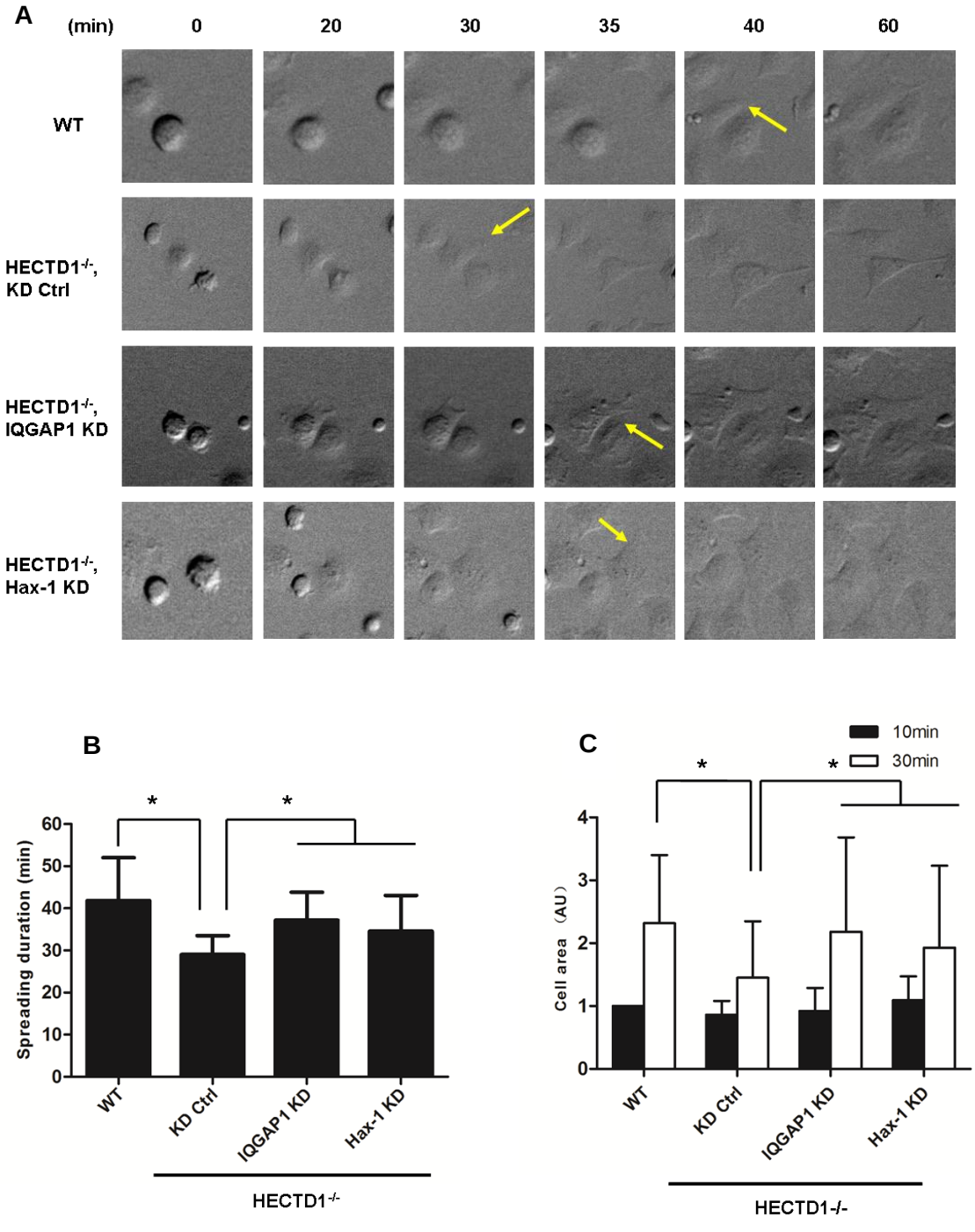
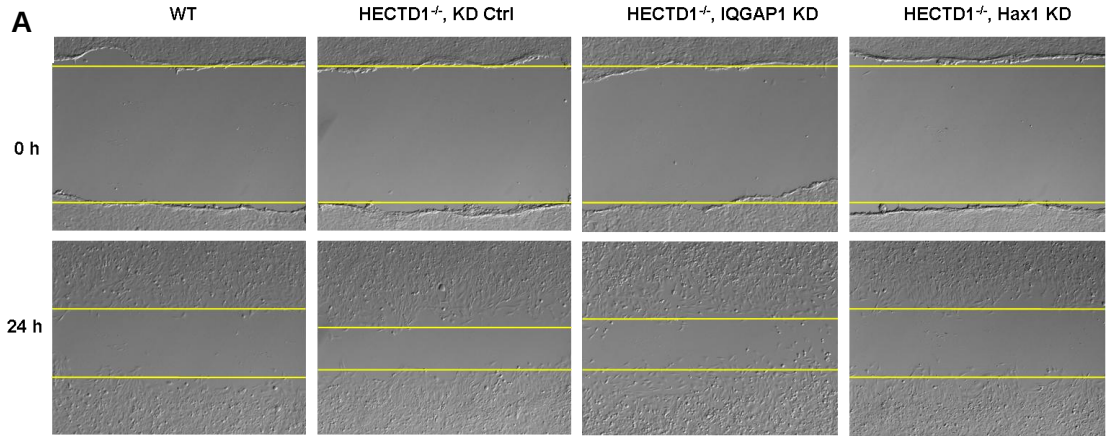


Figure 30. Knockdown of IQGAP1/Hax1 rescue duration time and area of cell spreading. (A) HECTD1 knockout cells were transfected with control siRNA, IQGAP1 siRNA and Hax1 siRNA, respectively. After 36 hours, cells were starved overnight and plated on FN-coated cell culture dishes and immediately sent to time-lapse assay for continued recording for 2 hours (1min/picture). Spreading pictures at different time points were shown. Note for cells with leading protrusion (yellow arrows). Quantification of duration of cell spreading **(B)** and spreading cell areas **(C)**. AU, arbitrary unit. *, P<0.05.

3. 3. 6. 4 Silence of IQGAP1 and Hax1 rescues the velocity of cell migration

Next, in order to further investigate whether down-regulation of IQGAP1 and Hax1 in HECTD1 knockout cells could also affect directional cell migration, confluent cell layers of wild-type and HECTD1 knockout cells down-regulated of control, IQGAP1 and Hax1 siRNA were scratched and wound closure was recorded by time lapse microscopy. The migration speed of control siRNA treated cells was 0.97 ± 0.14 μm/min, as compared with 0.86 ± 0.17 μm/min of wild-type cells, which was consistent with our previous results. The migration defect could be rescued by down-regulation of IQGAP1 (0.94 ± 0.14 μm/min) or Hax1 (0.79 ± 0.17 μm/min) (Fig. 31 A and B).



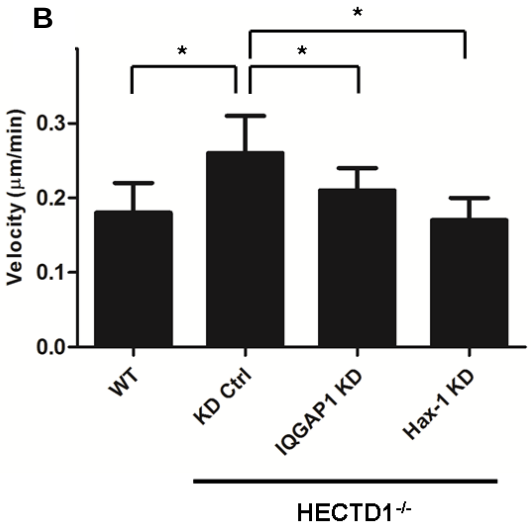


Figure 31. Knockdown of IQGAP1/Hax1 rescue speed of cell migration. (A) 24 hours after siRNA transfection, the cell medium was replaced with DMEM supplemented with 0.5% FBS for 24 hours, followed by starvation overnight with 1μg/ml aphidicolin (APC). Then, to create a wound in each well with 200 μl pipette tips, cells were washed twice and replaced with complete medium. Migration images were acquired by time-lapse microscopy for 24 hours. **(B)** The images were analyzed quantitatively by Image J software. *, $P<0.05$.

3. 3. 7 Activity of RhoA is enhanced in HECTD1 knockout cells

Rho GTPases family acts as "molecular switches" in regulating cytoskeletal dynamics, cell movement and other cellular functions. These protrusive events are tightly regulated by the small GTPases Rac1 and Rho, which trigger polymerization of actin in the leading edge and actomyosin contractility, respectively (Ballestrem et al., 2001; de Rooij et al., 2005; Wittmann et al., 2003). Reports have proved that activation of Rac1 induces reinforcement and stabilization of newly expanded protrusions, lamellipodia extension and cell spreading (Machacek and Danuser, 2006; Machacek et al., 2009; Wu et al., 2009). Several observations indicate the requirement of the small GTP binding protein RhoA in integrin signaling (Defilippi et al., 1997; Schwartz et al., 1996), and RhoA activation is required for the assembly of microfilament bundles and of adhesion plaques (Ridley and Hall, 1992).

Previously, we found the activity of Rac1 was significantly ($P<0.05$) increased in HECTD1 knockout cells as compared to wild-type controls. To check how RhoA is involved in this process, we tested RhoA activity in both wild-type and HECTD1 knockout cells by

using the RhoA activation assay. Similar as Rac1, activity of RhoA was also evidently increased in control siRNA HECTD1 knockout MEFs after FN stimulation for 60min ($P<0.05$), whereas RhoA activity could be significantly inhibited by IQGAP1 siRNA scilencing in HECTD1 knockout MEFs ($P<0.05$) (Fig. 32). These results suggest that in the absence of HECTD1, the activation of RhoA correlated with increased protein levels of IQGAP1.

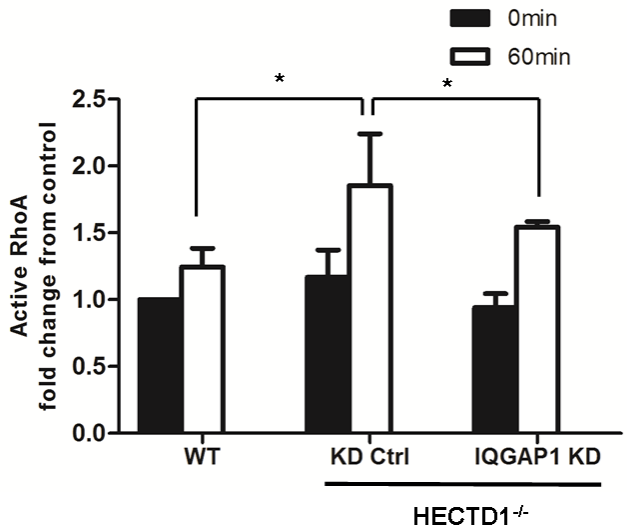


Figure 32. Activity of RhoA is enhanced in HECTD1 knockout cells. Wild-type and HECTD1 knockout cells were seeded on culture dishes for 24 hours, and then HECTD1 knockout cells were transfected with IQGAP1 siRNA and control siRNA respectively. After 36 hours, cells were starved overnight and plated on FN coated culture dishes for 60 min. Protein lysates were harvested on ice and snap freezed in liquid nitrogen. The activity of RhoA was measured by RhoA G-LISA Activation Assay Kit. *, $P<0.05$.

4. Discussion

Although HECTD1 has been found to play a crucial role in mouse embryogenesis, including neural tube formation, placenta formation and embryonic growth, limited studies have been shown to uncover the regulatory mechanisms involved in the organogenesis during embryonic development. Moreover, the role of HECTD1 in cell migration has remained so far largely unclear. In the current work, we observed that loss of HECTD1 resulted in earlier cell spreading and enhanced cell migration through controlling IQGAP1 and adhesion proteins. Our study proposes a new mechanism of HECTD1 in maintaining accurate cell movement during embryogenesis.

4. 1 The phenotype of the HECTD1 knockout embryo

In our research, we generated HECTD1 mutant mice by insertion of β-geo expression cassette between intron 26-27 using the secretory-trap approach. The HECTD1 knockout phenotype of E14.5 embryos was characterized by severe neural tube defects (anencephaly), growth retardation and impaired placental development, as well as eye malformation. Zohn and coworkers have demonstrated that the defect of neural tube closure in opm mutant embryos is combined with a failure in the formation of dorsal-lateral hinge point and an unusual organization of the head mesenchyme surrounding the neural tube. The opm mutation has been identified in HECTD1 by positional cloning, and further confirmed by genetrap allele ($HECTD1^{XC}$) in which the HECT domain was disrupted. Interestingly, embryos heterozygous for the opm allele display a low frequency (5%) neural tube defects, while the embryos heterozygous for the gene trap allele ($HECTD1^{XC}$) display a higher frequency (20%) of neural tube defects. It has been shown that mutation of a conserved cysteine in the HECT domain results in a dominant-negative protein by maintaining the interaction of the ligase with the substrate but preventing ubiquitination (Huibregtse et al., 1995; Talis et al., 1998). Therefore, the different frequency of neural tube defects in opm allele and XC heterozygotes suggests that posttranslational modification of an unknown substrate protein(s) is required for normal development of the head mesenchyme (Zohn et al., 2007). The similar phenotype of opm

mutants, HECTD1^{XC} mutants and our HECTD1 knockout embryos together confirm the critical role of HECTD1 in embryonic development, especially neural tube formation.

4. 2 Function of ECM in cell movement

The adhesion of cells to the ECM is an important step of mammalian physiology and critically regulates a variety of cellular functions, such as cell spreading and migration, proliferation and survival (Hynes, 2009). When binding to the ECM, complex networks of intracellular signaling pathways are activated, as demonstrated by adhesion of cells onto the ECM and cell spreading. While certain signaling proteins that react to attachment rely on different cell types and the composition of ECM. The ECM consists of a cross-linking network of fibrous proteins (e.g., FN, collagen, laminin and elastin) and various proteoglycans (Rozario and DeSimone, 2010). The macromolecules combine together to provide the ECM with structural integrity and form adhesive substrates. The complexity of the molecular signaling which are responsible for ECM selective guidance is associated with various ligand-binding possibilities with integrin subtypes (Barczyk et al., 2010; Levental et al., 2009). Several types of ECM, including FN, collagen and gelatin, are released as 'precursor' molecules; their subsequent incorporation into the ECM is leaded by the fibroblasts in accordance with the functional requires of a specific tissue. To understand the specificity of ECM in the formation of cell adhesions in the absence of HECTD1, we analyzed the adhesion proteins of both wild-type and HECTD1 knockout cells on various ECM proteins, such as FN, collagen I, and gelatin. Upon attaching to different substrates, the expression of adhesion proteins varied in both cell types. Based on staining of paxillin and zyxin, we detected that there was a significant difference of these two proteins between wild-type and HECTD1 knockout cells on FN, with more focal adhesions in wild-type cells and more focal complexes in mutant cells. However, on collagen I and gelatin, the different expression of adhesion proteins between two cell types is not as evident as on FN. Based on previous studies, it is wildly accepted that different ECM proteins are bond to different integrin subtypes. FN has been described to bind $\alpha_5\beta_1$, $\alpha_4\beta_1$, and $\alpha_v\beta_3$ integrins (Lobert et al., 2010; Wary et al., 1996) and for collagen I, $\alpha_1\beta_1$ and $\alpha_2\beta_1$ are the two common integrin

binding motifs (Lowell and Mayadas, 2012). For the adhesion proteins, there is also a specific binding relationship between integrins. For instance, paxillin is normally binds with $\alpha_5\beta_1$, $\alpha_4\beta_1$, $\alpha_5\beta_3$ and $\alpha_4\beta_3$ integrins (Crowe and Ohannessian, 2004; Laukaitis et al., 2001; Liu et al., 1999) (Schaller and Parsons, 1995), while zyxin prefers subtype $\alpha_5\beta_1$ and $\alpha_5\beta_5$ (Bianchi-Smiraglia et al., 2013; Mise et al., 2012) . Besides, the integrin motifs expressing in different cell types vary a lot, while in fibroblasts $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$ and $\alpha_5\beta_1$ integrins were identified (Mineur et al., 2005). Overall, combining the previous studies with our data, we suggest that FN plays a critical role in the involvement of HECTD1 in focal adhesion expression in fibroblasts, probably through coordinating with $\alpha_5\beta_1$ integrin subtypes.

4. 3 Role of HECTD1 in cell spreading

Cell movement is the process which coordinates ECM sensing, formation of cell adhesions, polymerization of actin filaments and adhesion-mediated signaling, that locally and globally regulates cell spreading and migration. Cell spreading is the initial step of a cell contacting with the surface and starting to move, at the end of cell spreading, polarity of cell forms and followed by cell migration.

In our research, loss of HECTD1 results in altered spreading duration, which shortens to around 30 minutes as compared to 40 minutes in wild-type cells, together with smaller spreading areas than in wild-type counterparts. Therefore, we set 30 minutes as the differential time for cell spreading and cell migration in our experimental setting: we defined as cell spreading the time interval from cell attachment to 30 minutes. After the first 30 minutes, we defined the process as cell migration. Besides the duration and area of spreading, in contrast with wild-type cells, adhesion proteins (paxillin) in HECTD1 knockout cells changed in size, number and localization. Here, we stained paxillin or zyxin in both HECTD1 knockout and wild-type cells after 30 minute spreading on FN-coated slides and analyzed the number, length and area of paxillin or zyxin patches. When comparing to wild-type cells, we observed in HECTD1-knockout cells that paxillin was more abundant while down-regulated in size, in length and in area, whereas the expression of zyxin in both cell lines remained similar. These results are partly supported by one study from Ilic and coworkers, who

demonstrated that an increased number and size of focal-adhesion-like structures are associated with a less-motile phenotype, whereas a decrease in the number and size of adhesions is associated with a more motile phenotype (Ilic et al., 1995). Furthermore, another research group came to similar conclusions by investigating the role of dystroglycan in cell adhesion. As an ubiquitously expressed cell adhesion protein, deficient dystroglycan increases the number and size of adhesions, based on staining for vinculin, and further enhanced the cell motility, whereas overexpression decreases the number of adhesions together with inhibited cell motility (Thompson et al., 2010). Although our results showed that knockout of HECTD1 results in increased number but decreased size of paxillin, we speculate that it is because in HECTD1 knockout cells focal complexes including paxillin failed to reassemble and to constitute mature focal adhesions, which are bigger in size than focal complexes. Since the presence of focal complexes and nascent adhesions are markers of highly motile cells, their quick appearance and turnover correlates directly with high velocities of protrusion and cell movement. The increased number of small paxillin patches in HECTD1 knockout cells could be the reason of high motility and faster spreading. However, we didn't find any significant difference in number, length or area of zyxin patches in both cell lines. In motile cells, the recruitment of the adhesion proteins into focal complexes occurs sequentially, so that the composition of specific proteins relies on their age. Moreover, using double color staining and time-lapse imaging, one study demonstrated that the transition from paxillin-rich focal complexes to zyxin-containing focal adhesions, takes place after the leading edge stops advancing or retracts (Zaidel-Bar et al., 2003). Generally, zyxin has been thought to be a component of focal adhesion plaques and is absent in focal complexes (Zaidel-Bar et al., 2003; Zaidel-Bar et al., 2004). Although these three types of adhesion are distinguishable, there is always a continuum between types and many of the same adhesion proteins have been identified in each (Parsons et al., 2010). Based on our classification, we detected zyxin at the site of focal complexes. Taken together with the previous studies and our results, we propose that 30 minute spreading is too early for recruitment of abundant zyxin into focal adhesions, so that the presence of zyxin is not sufficient to distinguish the difference in HECTD1 knockout and wild-type cells.

Another adhesion protein, α-actinin, is an actin filament binding protein, which connects

with the actin cytoskeleton (Maruyama and Ebashi, 1965). It is mainly localized at the leading edge of cells and acts as an important component of adhesion modules (Knight et al., 2000). It is suggested that α-actinin creates a scaffold that affords stability. Actin crosslinking acts as a bridge between cytoskeleton and signaling pathways and is involved in the maturation of focal adhesions (Choi et al., 2008). In addition, previous studies demonstrated that α-actinin interacts with zyxin, which interaction is required for zyxin-dependent α-actinin recruitment and proper zyxin localization in living cells. Thus, it is indicated that the colocalization of α-actinin and zyxin exhibits a biologically relevant role in coordinating membrane-cytoskeleton interactions and focal adhesion maturation (Feng et al., 2013; Smith et al., 2010; Zaidel-Bar et al., 2003). Moreover, paxillin appeared to reassemble from older to newer adhesions at the leading edge when a new protrusion formed, followed by α-actinin entering adhesions, which translocated toward the cell center, and inhibited paxillin turnover (Laukaitis et al., 2001).

We co-stained α-actinin with paxillin or zyxin to demonstrate the colocalization of these proteins. Then, we found that α-actinin is colocalized with paxillin or zyxin at cell leading edges in wild-type cells, but not in HECTD1 knockout cells. We acknowledge that at the back of lamellipodia, the nascent adhesions either disassemble or mature in length and area. Adhesions maturation occurs along an α-actinin–actin template that elongates centripetally from nascent adhesions. The formation of the template and organization of adhesions associated with filamentous actin is mediated by α-actinin (Choi et al., 2008). Since the colocalization of paxillin and zyxin with α-actinin was lost, the association of paxillin and zyxin into focal adhesions may be inhibited while this process is promoted by α-actinin. Consistent with our findings, in highly motile cells, such as melanoma cells, glioma cells and growing neurons (Gatlin et al., 2006), the dynamic adhesions which are most similar to focal complexes are enriched in the leading edge of cells and act as common features of rapid cell movement (Estrada-Bernal et al., 2009). Therefore, our results suggest that the accumulation of paxillin and zyxin in lamellipodia during the formation of focal complexes is a typical feature of highly motile cells.

4. 4 Role of HECTD1 in cell migration

During cell migration, the velocity of the cells is affected by the dynamics of cell adhesion formation, actomyosin-based contractility, activity of small GTPases, as well as the rigidity of the ECM. In our study, we used the wound healing assay to investigate role of HECTD1 in directional cell migration. After 24 hour migrating on FN coated plate, in contrast to wild-type cells, we observed that the velocity of HECTD1 knockout cells was increased, while the directionality was impaired. To further understand the molecular signaling that modulates cellular migration, we studied the formation of adhesions, their phosphorylation, the effects of the ECM and the activity of small GTPases.

When cells move forward, the small and most dynamics focal complexes grow in size to form stress fiber-associated focal adhesions, which continue to mature into fibrillar adhesions. Fibrillar adhesions are integrin-involved large, linear structures located in the center of cells. Increased numbers of the adhesions are accompanied with a less-motile migration status (Lu et al., 2001; Nagano et al., 2012). Here, we show that the dynamics of cell adhesion is responsible for the velocity of cell migration.

To examine the role of cell adhesions in cell migration regulated by HECTD1, we took paxillin and zyxin as markers of cell adhesions. Depending on the size, localization and morphological characteristics, we classified the cell adhesions into focal complexes and focal adhesions. Interestingly, when compared to wild-type cells, the average total adhesion number in HECTD1 knockout cells is increased. Moreover, irrespective of paxillin or zyxin as markers, focal complexes are evidently more abundant while significantly fewer focal adhesions were found in HECTD1 cells than in their wild-type counterparts. Based on our observation, the most likely explanation for the observed changes in migration speed, is that focal complexes in HECTD1 knockout cells fail to recruit sufficient adhesion proteins to mature into focal adhesions. Therefore, the alteration in number and/or size of focal complexes is expected to influence cell motility. Consistent with our results, Ilic and coworkers have demonstrated that an increased size and number of focal-adhesion-like structures are combined with a less-motile phenotype, while a decrease in the size and number of adhesions is related to a more motile phenotype (Ilic et al., 1995). In addition, using

Dystrglycan deficient or overexpression fibroblasts as tool, they already suggested that the decreased size of focal adhesions relates to higher velocity and impaired directionality of cells, and vice versa (Thompson et al., 2010).

Li and coworkers have shown that knockdown of HECTD1 reduces the velocity of cell migration to a minor extent, without reaching statistical significance (Li et al., 2013), which is inconsistent with our findings. One explanation could be that while we use knockout MEF cells they used knockdown breast cancer cells.

Our directional cell migration experiments could also mimic the collective cell migration. As an important protein component in adherens junctions, β-catenin expresses weaker at site of cell-cell contacts in HECTD1 knockout cells than wild-type control. Since strong adherens junctions function in maintaining the stability of cell-cell contacts, weakened or lost adherens junctions promotes collective cell migration (Zhang et al., 2015) (Guo et al., 2007; Hage et al., 2009). Thus, the impaired expression of β-catenin in adherens junctions may act as another reason for the increased velocity of collective cell migration.

4. 4. 1 Tyrosine phosphorylation of paxillin in cell migration

The ability of paxillin to modulate the dynamics of focal adhesions and cellular motility depends on tyrosine phosphorylation. Two of the main kinases discovered in focal adhesions are FAK and Src, which bind to different substrates to regulate focal adhesion dynamics and cell behavior (Wozniak et al., 2004). Tyrosine phosphorylation allows paxillin to interact with a great number of signaling molecules: tyrosine residues (Y) 31 and 118 being especially predominant targets of phosphorylation by kinases (Iwasaki et al., 2002; Sen et al., 2011; Zaidel-Bar et al., 2007). To examine the role of paxillin tyrosine phosphorylation in regulating cell adhesions, we double stained wild-type and HECTD1 knockout cells with paxillin Y118 antibody as well as phalloidin, which specifically recognizes actin. After migrating on FN coated plates for 90 minutes, paxillin Y118 in wild-type cells is expressed both in focal complexes and in focal adhesions. After up to 4 hours migration, paxillin Y118 is observed in the classical focal adhesions, largely associated with actin stress fibers. However, in HECTD1 knockout cells, regardless of the duration of migration (90 minutes or 4 hours), paxillin Y118

remains lying proximal to the cell periphery and at the cell leading edge, concentrates in lamellipodia around cortical actin and remains in the forming focal complexes, as well as aggregates in the cell center as small dots. Moreover, paxillin Y118 at the focal complexes in HECTD1 knockout cells is around two times more abundant than focal adhesions in wild-type cells. Besides the immnocytochemistry results, using Hela cell line as a tool, we found that paxillin Y118 is significantly more abundant in HECTD1 siRNA knockdown than in control siRNA treated cells after FN stimulation for 60 minutes. Consistent with the results of N term paxillin, the high proportion of paxillin Y118 in focal complexes in HECTD1 knockout cells afford another support for the high motility after loss of HECTD1. Moreover, as the active form of adhesions, overexpression of paxillin Y118 might also be a stimulating factor for the increased velocity of HECTD1 knockout cells. Together, our findings are supported by the previous findings, focal adhesion formation and cellular adhesion are accompanied by tyrosine phosphorylation of paxillin, and phosphorylation of paxillin on tyrosine-118 is not essential for the recruitment of paxillin to sites of focal adhesions (Turner, 2000; Zaidel-Bar et al., 2007). In contrast, breakdown of focal adhesions and loss of cellular adherence are accompanied by tyrosine dephosphorylation of paxillin (Blackstone et al., 2015; Qin et al., 2015; Turner, 2000). Therefore, we provide the evidence that loss of HECTD1 induces the overexpression and mislocalization of paxillin-Y118, which in turn inhibits the formation of focal adhesion and further effects on cell migration.

4. 4. 2 Role of protein phosphatase 2 in cell migration

In our understanding, PP2A is a major serine/threonine phosphatase, while there are some studies showing PP2A is also a regulator for protein tyrosine phosphatase activity. PP2A is a ubiquitous and conserved phosphatase with broad substrate specificity and diverse cellular functions, such as regulating cell motility and invasion and cytoskeletal dynamics (Jackson and Young, 2003). In cell adhesions, PP2A is an important regulator of FAK/Src/paxillin complexes. In our study, although there was no significant difference of total level of PP2A between wild-type and HECTD1 knockout cells, the activity of PP2A in HECTD1 knockout cells was evidently enhanced as compared to wild-type counterpart. In addition to the

increased levels of paxillin Y118, increased activity of PP2A is connected with overexpression of paxillin Y118 in HECTD1 knockout cells, but the process of tyrosine dephosphorylation is not directly mediated by PP2A.

Previous studies showed that inhibition of PP2A results in the disorganization of focal adhesion sites in different cell types, coupled with increased serine phosphorylation and reduced tyrosine phosphorylation of paxillin (Jackson and Young, 2003). Moreover, blocking of PP2A also induces the dissolution of FAK/Src/paxillin complexes and reduced adherence (Young et al., 2003). However, the regulation of tyrosine phosphorylation by PP2A is indirect, and may involve tyrosine phosphatases, such as Shp-2. Together with these earlier studies our results also suggest that that enhanced PP2A contributes to the tyrosine phosphorylation of paxillin, while the precise mechanism still remains vague.

4. 4. 3 Role of small GTPases in cell migration

The Rho GTPases family has been shown to regulate various aspects of intracellular events, including cell movement, cytoskeletal dynamics, organelle development and so on. All G proteins are acting as “molecular switches” and are cyclically regulated by GEFs and GAPs. During cell migration, intensive studies have been focused on the role of Rac1 and RhoA. For example, in the development of cell adhesions, activation of Rac enables the formation of focal complexes, whereas Rho activity induces the induction and growth of focal adhesions (Nobes and Hall, 1995)(Khalil et al., 2014; Machacek et al., 2009) and enhances the formation of fibrillar adhesions (Chrzanowska-Wodnicka and Burridge, 1996; Zaidel-Bar et al., 2007). In phosphorylation-dependent signaling, the recruitment of adhesion proteins requires modulation of activity of Rac and Rho, and finally regulate adhesion dynamics and protrusion extension (Choi et al., 2011; Schaller and Parsons, 1995)(Lawson and Burridge, 2014). Here, we show that activity of Rac1 and RhoA promotes cell migration, as demonstrated by increased activity of Rac1 and RhoA and leading to higher velocity rates of HECTD1 knockout cells. When compared to wild-type cells and using the Rac1 and RhoA activation assay, we found that the activity of both Rac1 and RhoA was significantly increased in HECTD1 knockout cells after FN stimulation for 60 minutes, whereas the total

level of Rac1 and RhoA was not significantly different. Consistent with our findings, the activity of small GTPases Rac1 dynamically regulates cell migration by promoting cytoskeletal re-organization and membrane protrusion (Ridley, 2006). Disruption of Rac1 induces impaired of cell directionality on fibrillar matrices (Bass et al., 2007; Pankov et al., 2005) and inhibited wound healing *in vivo* (Tscharntke et al., 2007). Furthermore, overexpression of active Rac1(V12) impairs cell-cell adhesion and enhances directed cell motility and migration, while dominant negative Rac1(N17) induces the opposite effects (Hage et al., 2009).

As we understand that RhoA activity has been shown to maintain an inverse relationship with cell motility and cell migration. On one hand, active RhoA stimulates the formation of stress fibers and focal adhesions (Ridley and Hall, 1992) and precise regulation of RhoA is critical for efficient cell migration. On the other hand, RhoA activity could inhibit cell movement, for instance, localized RhoA inactivation by p190RhoGAP promotes efficient cell spreading and migration by enhancing membrane protrusion and cell polarity as well (Arthur and Burridge, 2001).

For the relationship of Rac1 and RhoA, the published data were varied. There was study shows that both Rac1 and RhoA are active with spatial and temporal distinctions at the leading edge of migrating cells (Hodgson et al., 2010). However, Rac1 and RhoA have also been shown in migrating cells to exhibit mutual antagonism, suggesting that the activity of Rac1 and RhoA must be balanced to regulate cell shape and morphogenesis (Burdisso et al., 2013; Chauhan et al., 2011). In general, under our experiment conditions, it is most likely that increased activity of Rac1 and RhoA contribute together to promote cell migration, probably via controlling the dynamics of adhesion proteins. However, the precise relationships between Rac1 and RhoA require more experiments to be answered.

4. 4. 4 MTOC network in directional cell migration

The MTOC is located in a perinuclear site and comprises the negative ends of microtubules when the positive ends grow quickly towards the edge of the cell. The Golgi apparatus reorients along with the MTOC, and together induce the cell to seemingly form a polarized

signal (Sancho et al., 2002)(Murata et al., 2015). In the present study, we examined MTOC polarization in the wound healing experiments, using microtubule marker (acetylated α-tubulin) and Golgi membrane marker (giantin) in MTOC networks. Therefore, although the velocity of HECTD1 knockout cells is increased, the directionality during directional cell migration is impaired, suggesting that cell migration is less efficient after loss of HECTD1, which may be the one of the important reasons for defects in embryonic development.

4. 5 HECTD1 interacting partners

Zohn and coworkers have demonstrated the neural tube defects in HECTD1 mutants during embryonic development. In their further search for the substrates regulated by HECTD1, they first identified Hsp90 as a physically interactor of HECTD1 in both yeast two-hybrid screen and liquid chromatography-mass spectrometry (LC-MS) approach. Then, in HECTD1 mutant cranial mesenchyme (CM) cells, decreased ubiquitination leads to high levels of Hsp90, which is responsible for the abnormal migratory behavior of CM (Sarkar and Zohn, 2012). Then, during their search for interactors and substrates of deubiquitylating enzyme (DUB) Trabid, Tran and coworkers identified HECTD1 as a specific Trabid-associated protein by Immunoprecipitation-Mass Spectrometry (IP-MS) experiments. They identified HECTD1 as a E3 ubiquitin ligase that modifies adenomatous polyposis coli protein (APC) with Lys-63 polyubiquitin. Knockdown of HECTD1 down-regulated APC ubiquitination, destroyed the APC-Axin interaction, and enhanced Wnt3a-induced β-catenin stabilization and signaling. Their results suggest that HECTD1 promotes the APC-Axin interaction to negatively regulate Wnt signaling (Tran et al., 2013). Moreover, HECTD1 has been shown to be responsible for PIPKIγ90 ubiquitination in breast cancer cells (MDA-MB-231) as an E3 ubiquitin ligase homologous to Smurf1. Partly (D6) or completely (D9) knockdown of HECTD1 results in increased PIPKIγ90 levels induced by proteasome inhibitors. Since E3 ubiquitin ligases have been implicated in regulating FA dynamics, they demonstrated that depletion of endogenous HECTD1 significantly inhibited both FA assembly and disassembly rates taking DsRed-paxillin as a marker. Furthermore, they found that the net distance and directionality were suppressed in MDA-MB-231 cells expressing HECTD1 shRNA (D6 or D9), without

difference in velocity (Li et al., 2013).

Previously, by using Mass Spectrum analysis and yeast two-hybrid screen, we have identified a list of potential binding partners of HECTD1, among them, we found IQGAP1 and Hax1 that has been widely reported to be involved in regulating focal adhesion dynamics and cell migration. Then, we confirmed the interaction and colocalization between HECTD1 and IQGAP1/Hax1 by co-immunoprecipitation and double-labeled immunocytochemistry respectively. As an E3 ubiquitin ligase, we tested whether IQGAP1 and Hax1 are downstream substrates of HECTD1 by half-life and *in vivo* ubiquitination assay. We observed that after FN stimulation for 60 minutes, loss of HECTD1 induces overexpression of IQGAP1, decreased ubiquitination of IQGAP1, associated with a prolonged half-life.

Taken these results together, it is strongly indicated that IQGAP1 serves as a substrate and which degradation is regulated by HECTD1. However, we obtained different results in Hax1, in line with IQGAP1, the total amount and half-life of Hax1 is increased in HECTD1 knockout cells. The ubiquitination of cells transfected with Hax1-WT is more than control cells, whereas less than cells transfected with GFP-Hax1-ΔPEST. Based on the ubiquitination and half-life results of Hax1, we confirm the function of PEST sequence in Hax1, as it is responsible for its poly-ubiquitination and rapid degradation, resulting in short intracellular half life (Li et al., 2012). Nevertheless, we could not conclude directly that Hax1 is a unique substrate of HECTD1.

Under the condition of our study, there are two possible reasons: first, loss of HECTD1 induces the compensatory overexpression of other E3 ubiquitins, which may function together with HECTD1 on Hax1 and result in increased ubiquitination of Hax1. Another possibility is that compared with wild-type cells, the increased level of Hax1 ubiquitination is lower than the overexpresssion level of Hax1 total amount in HECTD1 knockout cells. To check the first possibility, it is better to examine the activity of other E3 ubiquitin ligases. For the second possible reason, quantification of *in vitro* Hax1 ubiquitination amount may provide a solving method.

4. 6 Role of IQGAP1 and Hax1 in regulating adhesion dynamics

Accumulating evidences have shown to emphasis the role of IQGAP1 in regulating cell migraiton via affecting on cell adhesion, small Rho GTPases or actin cytoskeleton (Kozlova et al., 2012). Similarly, Hax1 is also widely reported to involve in cell migration through interacting with different proteins. Overexpression of IQGAP1 and Hax1 in HECTD1 knockout cells raised the hypothesis that elevated protein level of IQGAP1 and Hax1 were the direct causes of impaired formation of focal adhesions in HECTD1 knockout cells. By overexpression IQGAP1 and Hax1 in wild-type cells, we mimicked the expression of paxillin and zyxin in HECTD1 knockout cells. Moreover, siRNA knockdown of IQGAP1 and Hax1 in HECTD1 knockout cells could compensate the defects in the formation of cell adhesions, cell spreading and cell migration. Therefore, our studies suggest the conclusion that both IQGAP1 and Hax1 are important regulators of cell adhesion and through them control cell spreading and migration.

These results show that in the absence of HECTD1, the activation of RhoA correlates with increased protein level of IQGAP1, indicating a feedback loop between IQGAP1 and small Rho GTPases.

4. 7 Model for the role of HECTD1 in regulating cell movement

Based on the evidence presented in our study and the background knowledge of cell movement, I would like to suggest the following model for the role of HECTD1 in cell movement (Fig. 33). During cell movement the cells receive stimulating signals of the ECM, such as FN, which activates small GTPases Rac1/RhoA and IQGAP1/Hax1. Then, IQGAP1/Hax1 are responsible for recruiting paxillin and zyxin to focal adhesions or to focal contacts sites and associate paxillin and zyxin with actin filaments through the regulation of tyrosine phosphorylation. At last, by proper dynamics of adhesion proteins, paxillin and zyxin, cell could migrate in an accurate speed and direction during embryogenesis. However, as an E3 ubiquitin ligase, loss of HECTD1 effects on the ubquitination of IQGAP1 and Hax1, induces the prolonged half-life and overexpression of this two proteins. Overexpression of IQGAP1 exerts a feedback loop between Rac1 and RhoA, results in elevated Rac1 and RhoA activity, which thereby causes the increased expression and mislocalization of paxillin Y118.

Consequently, paxillin and zyxin at the focal complex sites are failed to mature into focal adhesions, and are responsible for high cell motility during cell movement.

Whereas the suggested model explains most of the results that have been discussed in our study, several questions remain unanswered needing further research. What is the precise mechanism of Rac1 and RhoA in regulating cell adhesion dynamics? How do IQGAP1 and small GTPases influence each other? How does phosphorylation affect adhesion assemble and disassemble? How does HECTD1 mediate the ubiquitination of Hax1?

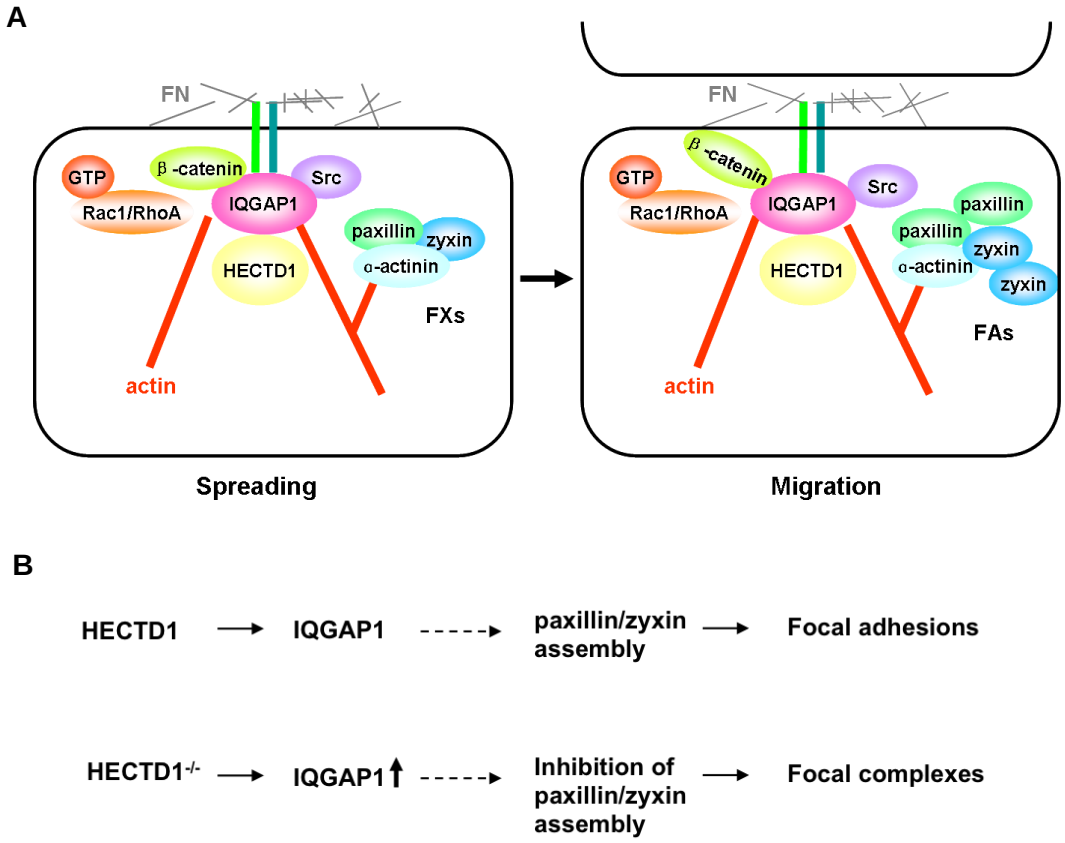


Figure 33. Model for the role of HECTD1 in cell movement. (**A**) Model for recruitment of adhesion proteins in cell spreading and migration. After FN stimulation, intracellular cell signaling is activated through integrins. The activation signal is then transferred to IQGAP1 and small GTPases, finally results in recruitment of adhesion proteins during cell migration. (**B**) Loss of HECTD1 reduced the ubiquitination of IQGPA1, results in increased IQGAP1 protein expression. The involvemnt of higher activity rates of small GTPases, the positive feedback between IQGAP1 and small GTPases commonly lead to the turnover of adhesion proteins.

Reference

Abercrombie, M., and Dunn, G.A. (1975). Adhesions of fibroblasts to substratum during contact inhibition observed by interference reflection microscopy. Exp Cell Res *92*, 57-62.

Adams, J.C. (2001). Cell-matrix contact structures. Cell Mol Life Sci *58*, 371-392.

Alexandrova, A.Y., Arnold, K., Schaub, S., Vasiliev, J.M., Meister, J.J., Bershadsky, A.D., and Verkhovsky, A.B. (2008). Comparative dynamics of retrograde actin flow and focal adhesions: formation of nascent adhesions triggers transition from fast to slow flow. PLoS One *3*, e3234.

Aman, A., and Piotrowski, T. (2008). Wnt/beta-catenin and Fgf signaling control collective cell migration by restricting chemokine receptor expression. Dev Cell *15*, 749-761.

Anselme, K. (2000). Osteoblast adhesion on biomaterials. Biomaterials *21*, 667-681.

Ardley, H.C., and Robinson, P.A. (2005). E3 ubiquitin ligases. Essays Biochem *41*, 15-30.

Arrigo, A.P., Tanaka, K., Goldberg, A.L., and Welch, W.J. (1988). Identity of the 19S 'prosome' particle with the large multifunctional protease complex of mammalian cells (the proteasome). Nature *331*, 192-194.

Arthur, W.T., and Burridge, K. (2001). RhoA inactivation by p190RhoGAP regulates cell spreading and migration by promoting membrane protrusion and polarity. Mol Biol Cell *12*, 2711-2720.

Ballestrem, C., Hinz, B., Imhof, B.A., and Wehrle-Haller, B. (2001). Marching at the front and dragging behind: differential alphaVbeta3-integrin turnover regulates focal adhesion behavior. J Cell Biol *155*, 1319-1332.

Banno, A., Goult, B.T., Lee, H., Bate, N., Critchley, D.R., and Ginsberg, M.H. (2012). Subcellular localization of talin is regulated by inter-domain interactions. J Biol Chem *287*, 13799-13812.

Barczyk, M., Carracedo, S., and Gullberg, D. (2010). Integrins. Cell Tissue Res *339*, 269-280.

Barry, S.T., Flinn, H.M., Humphries, M.J., Critchley, D.R., and Ridley, A.J. (1997). Requirement for Rho in integrin signalling. Cell Adhes Commun *4*, 387-398.

Ballestrem, C., Hinz, B., Imhof, B.A., and Wehrle-Haller, B. (2001). Marching at the front and dragging behind: differential alphaVbeta3-integrin turnover regulates focal adhesion behavior. J Cell Biol *155*, 1319-1332.

Bass, M.D., Roach, K.A., Morgan, M.R., Mostafavi-Pour, Z., Schoen, T., Muramatsu, T., Mayer, U., Ballestrem, C., Spatz, J.P., and Humphries, M.J. (2007). Syndecan-4-dependent Rac1 regulation determines directional migration in response to the extracellular matrix. J Cell Biol *177*, 527-538.

Bebington, C., Doherty, F.J., and Fleming, S.D. (2000). Ubiquitin and ubiquitin-protein conjugates are present in human cytotrophoblast throughout gestation. Early Pregnancy *4*, 240-252.

Bellis, S.L., Perrotta, J.A., Curtis, M.S., and Turner, C.E. (1997). Adhesion of fibroblasts to fibronectin stimulates both serine and tyrosine phosphorylation of paxillin. Biochem J *325 (Pt 2)*, 375-381.

Beningo, K.A., Dembo, M., Kaverina, I., Small, J.V., and Wang, Y.L. (2001). Nascent focal adhesions are responsible for the generation of strong propulsive forces in migrating fibroblasts. J Cell Biol *153*, 881-888.

Bertani, G. (2004). Lysogeny at mid-twentieth century: P1, P2, and other experimental systems. J Bacteriol *186*, 595-600.

Bianchi-Smiraglia, A., Kunnev, D., Limoge, M., Lee, A., Beckerle, M.C., and Bakin, A.V. (2013). Integrin-beta5 and zyxin mediate formation of ventral stress fibers in response to transforming growth factor beta. Cell Cycle *12*, 3377-3389.

Blackstone, B.N., Li, R., Ackerman, W.E.t., Ghadiali, S.N., Powell, H.M., and Kniss, D.A. (2015).

Myoferlin depletion elevates focal adhesion kinase and paxillin phosphorylation and enhances cell-matrix adhesion in breast cancer cells. Am J Physiol Cell Physiol *308*, C642-649.

Borisy, G.G., and Svitkina, T.M. (2000). Actin machinery: pushing the envelope. Curr Opin Cell Biol *12*, 104-112.

Botero-Velez, M., Curtis, J.J., and Warnock, D.G. (1994). Brief report: Liddle's syndrome revisited--a disorder of sodium reabsorption in the distal tubule. N Engl J Med *330*, 178-181.

Boureux, A., Vignal, E., Faure, S., and Fort, P. (2007). Evolution of the Rho family of ras-like GTPases in eukaryotes. Mol Biol Evol *24*, 203-216.

Brill, S., Li, S., Lyman, C.W., Church, D.M., Wasmuth, J.J., Weissbach, L., Bernards, A., and Snijders, A.J. (1996). The Ras GTPase-activating-protein-related human protein IQGAP2 harbors a potential actin binding domain and interacts with calmodulin and Rho family GTPases. Mol Cell Biol *16*, 4869-4878.

Brown, M.D., and Sacks, D.B. (2006). IQGAP1 in cellular signaling: bridging the GAP. Trends Cell Biol *16*, 242-249.

Burdisso, J.E., Gonzalez, A., and Arregui, C.O. (2013). PTP1B promotes focal complex maturation, lamellar persistence and directional migration. J Cell Sci *126*, 1820-1831.

Burger, A.M., and Seth, A.K. (2004). The ubiquitin-mediated protein degradation pathway in cancer: therapeutic implications. Eur J Cancer *40*, 2217-2229.

Burnette, D.T., Manley, S., Sengupta, P., Sougrat, R., Davidson, M.W., Kachar, B., and Lippincott-Schwartz, J. (2011). A role for actin arcs in the leading-edge advance of migrating cells. Nat Cell Biol *13*, 371-381.

Burnicka-Turek, O., Kata, A., Buyandelger, B., Ebermann, L., Kramann, N., Burfeind, P., Hoyer-Fender, S., Engel, W., and Adham, I.M. (2010). Pelota interacts with HAX1, EIF3G and SRPX and the resulting protein complexes are associated with the actin cytoskeleton. BMC Cell Biol *11*, 28.

Burridge, K., and Chrzanowska-Wodnicka, M. (1996). Focal adhesions, contractility, and signaling. Annu Rev Cell Dev Biol *12*, 463-518.

Bustelo, X.R., Sauzeau, V., and Berenjeno, I.M. (2007). GTP-binding proteins of the Rho/Rac family: regulation, effectors and functions in vivo. Bioessays *29*, 356-370.

Cai, Y., Biais, N., Giannone, G., Tanase, M., Jiang, G., Hofman, J.M., Wiggins, C.H., Silberzan, P., Buguin, A., Ladoux, B., *et al.* (2006). Nonmuscle myosin IIA-dependent force inhibits cell spreading and drives F-actin flow. Biophys J *91*, 3907-3920.

Campbell, I.D., and Ginsberg, M.H. (2004). The talin-tail interaction places integrin activation on FERM ground. Trends Biochem Sci *29*, 429-435.

Cavalcanti-Adam, E.A., Volberg, T., Micoulet, A., Kessler, H., Geiger, B., and Spatz, J.P. (2007). Cell spreading and focal adhesion dynamics are regulated by spacing of integrin ligands. Biophys J *92*, 2964-2974.

Cavnar, P.J., Berthier, E., Beebe, D.J., and Huttenlocher, A. (2011). Hax1 regulates neutrophil adhesion and motility through RhoA. J Cell Biol *193*, 465-473.

Chamaraux, F., Ali, O., Keller, S., Bruckert, F., and Fourcade, B. (2008). Physical model for membrane protrusions during spreading. Phys Biol *5*, 036009.

Chau, V., Tobias, J.W., Bachmair, A., Marriott, D., Ecker, D.J., Gonda, D.K., and Varshavsky, A. (1989). A multiubiquitin chain is confined to specific lysine in a targeted short-lived protein. Science *243*, 1576-1583.

Chauhan, B.K., Lou, M., Zheng, Y., and Lang, R.A. (2011). Balanced Rac1 and RhoA activities

regulate cell shape and drive invagination morphogenesis in epithelia. Proc Natl Acad Sci U S A *108*, 18289-18294.

Chen, C., and Matesic, L.E. (2007). The Nedd4-like family of E3 ubiquitin ligases and cancer. Cancer Metastasis Rev *26*, 587-604.

Chen, F., Zhu, H.H., Zhou, L.F., Wu, S.S., Wang, J., and Chen, Z. (2010). IQGAP1 is overexpressed in hepatocellular carcinoma and promotes cell proliferation by Akt activation. Exp Mol Med *42*, 477-483.

Cheryl D. Helgasonk., Cindy L. Miller (1984). Basic cell culture protocols. Third edition. Edited.

Choi, C.K., Vicente-Manzanares, M., Zareno, J., Whitmore, L.A., Mogilner, A., and Horwitz, A.R. (2008). Actin and alpha-actinin orchestrate the assembly and maturation of nascent adhesions in a myosin II motor-independent manner. Nat Cell Biol *10*, 1039-1050.

Choi, C.K., Zareno, J., Digman, M.A., Gratton, E., and Horwitz, A.R. (2011). Cross-correlated fluctuation analysis reveals phosphorylation-regulated paxillin-FAK complexes in nascent adhesions. Biophys J *100*, 583-592.

Choi, S., Thapa, N., Hedman, A.C., Li, Z., Sacks, D.B., and Anderson, R.A. (2013). IQGAP1 is a novel phosphatidylinositol 4,5 bisphosphate effector in regulation of directional cell migration. EMBO J *32*, 2617-2630.

Chong, Y.P., Mulhern, T.D., and Cheng, H.C. (2005). C-terminal Src kinase (CSK) and CSK-homologous kinase (CHK)--endogenous negative regulators of Src-family protein kinases. Growth Factors *23*, 233-244.

Chrzanowska-Wodnicka, M., and Burridge, K. (1996). Rho-stimulated contractility drives the formation of stress fibers and focal adhesions. J Cell Biol *133*, 1403-1415.

Cilenti, L., Soundarapandian, M.M., Kyriazis, G.A., Stratico, V., Singh, S., Gupta, S., Bonventre, J.V., Alnemri, E.S., and Zervos, A.S. (2004). Regulation of HAX-1 anti-apoptotic protein by Omi/HtrA2 protease during cell death. J Biol Chem *279*, 50295-50301.

Copp, A.J. (2005). Neurulation in the cranial region--normal and abnormal. J Anat *207*, 623-635.

Copp, A.J., Greene, N.D., and Murdoch, J.N. (2003). The genetic basis of mammalian neurulation. Nat Rev Genet *4*, 784-793.

Cramer, L.P., Siebert, M., and Mitchison, T.J. (1997). Identification of novel graded polarity actin filament bundles in locomoting heart fibroblasts: implications for the generation of motile force. J Cell Biol *136*, 1287-1305.

Crowe, D.L., and Ohannessian, A. (2004). Recruitment of focal adhesion kinase and paxillin to beta1 integrin promotes cancer cell migration via mitogen activated protein kinase activation. BMC Cancer *4*, 18.

Cukierman E, Pankov R, Stevens DR, Yamada KM. Taking cell-matrix adhesions to the third dimension. Science 2001;294:1708 – 12.

Cukierman E, Pankov R, Yamada KM. Cell interactions with threedimensional matrices. Curr Opin Cell Biol 2002;14:633 – 9.

Cuvelier, D., Thery, M., Chu, Y.S., Dufour, S., Thiery, J.P., Bornens, M., Nassoy, P., and Mahadevan, L. (2007). The universal dynamics of cell spreading. Curr Biol *17*, 694-699.

Darribere, T., and Schwarzbauer, J.E. (2000). Fibronectin matrix composition and organization can regulate cell migration during amphibian development. Mech Dev *92*, 239-250.

de Martino, M., Taus, C., Wessely, I.S., Lucca, I., Hofbauer, S.L., Haitel, A., Shariat, S.F., and Klatte, T. (2015). The T309G murine double minute 2 gene polymorphism is an independent prognostic factor for patients with renal cell carcinoma. DNA Cell Biol *34*, 107-112.

de Rooij, J., Kerstens, A., Danuser, G., Schwartz, M.A., and Waterman-Storer, C.M. (2005). Integrin-dependent actomyosin contraction regulates epithelial cell scattering. J Cell Biol *171*, 153-164.
Del Pozo, M.A., and Schwartz, M.A. (2007). Rac, membrane heterogeneity, caveolin and regulation of growth by integrins. Trends Cell Biol *17*, 246-250.
Defilippi, P., Venturino, M., Gulino, D., Duperray, A., Boquet, P., Fiorentini, C., Volpe, G., Palmieri, M., Silengo, L., and Tarone, G. (1997). Dissection of pathways implicated in integrin-mediated actin cytoskeleton assembly. Involvement of protein kinase C, Rho GTPase, and tyrosine phosphorylation. J Biol Chem *272*, 21726-21734.
Di Lullo, G.A., Sweeney, S.M., Korkko, J., Ala-Kokko, L., and San Antonio, J.D. (2002). Mapping the ligand-binding sites and disease-associated mutations on the most abundant protein in the human, type I collagen. J Biol Chem *277*, 4223-4231.
Discher, D.E., Janmey, P., and Wang, Y.L. (2005). Tissue cells feel and respond to the stiffness of their substrate. Science *310*, 1139-1143.
Dobereiner, H.G., Dubin-Thaler, B., Giannone, G., Xenias, H.S., and Sheetz, M.P. (2004). Dynamic phase transitions in cell spreading. Phys Rev Lett *93*, 108105.
Dobereiner, H.G., Dubin-Thaler, B.J., Hofman, J.M., Xenias, H.S., Sims, T.N., Giannone, G., Dustin, M.L., Wiggins, C.H., and Sheetz, M.P. (2006). Lateral membrane waves constitute a universal dynamic pattern of motile cells. Phys Rev Lett *97*, 038102.
Doherty, G.J., and McMahon, H.T. (2008). Mediation, modulation, and consequences of membrane-cytoskeleton interactions. Annu Rev Biophys *37*, 65-95.
Dong, P.X., Jia, N., Xu, Z.J., Liu, Y.T., Li, D.J., and Feng, Y.J. (2008). Silencing of IQGAP1 by shRNA inhibits the invasion of ovarian carcinoma HO-8910PM cells in vitro. J Exp Clin Cancer Res *27*, 77.
Dubin-Thaler, B.J., Giannone, G., Dobereiner, H.G., and Sheetz, M.P. (2004). Nanometer analysis of cell spreading on matrix-coated surfaces reveals two distinct cell states and STEPs. Biophys J *86*, 1794-1806.
Dubin-Thaler, B.J., Hofman, J.M., Cai, Y., Xenias, H., Spielman, I., Shneidman, A.V., David, L.A., Dobereiner, H.G., Wiggins, C.H., and Sheetz, M.P. (2008). Quantification of cell edge velocities and traction forces reveals distinct motility modules during cell spreading. PLoS One *3*, e3735.
Egelman, E.H. (1985). The structure of F-actin. J Muscle Res Cell Motil *6*, 129-151.
Engler, A., Bacakova, L., Newman, C., Hategan, A., Griffin, M., and Discher, D. (2004). Substrate compliance versus ligand density in cell on gel responses. Biophys J *86*, 617-628.
Ezratty, E.J., Partridge, M.A., and Gundersen, G.G. (2005). Microtubule-induced focal adhesion disassembly is mediated by dynamin and focal adhesion kinase. Nat Cell Biol *7*, 581-590.
Feng, Y., Ngu, H., Alford, S.K., Ward, M., Yin, F., and Longmore, G.D. (2013). alpha-actinin1 and 4 tyrosine phosphorylation is critical for stress fiber establishment, maintenance and focal adhesion maturation. Exp Cell Res *319*, 1124-1135.
Folkman, J., and Moscona, A. (1978). Role of cell shape in growth control. Nature *273*, 345-349.
Frantz, C., Stewart, K.M., and Weaver, V.M. (2010). The extracellular matrix at a glance. J Cell Sci *123*, 4195-4200.
Friedl, P. (2004). Prespecification and plasticity: shifting mechanisms of cell migration. Curr Opin Cell Biol *16*, 14-23.
Friedl, P., and Gilmour, D. (2009). Collective cell migration in morphogenesis, regeneration and cancer. Nat Rev Mol Cell Biol *10*, 445-457.
Friedl, P., and Wolf, K. (2010). Plasticity of cell migration: a multiscale tuning model. J Cell Biol *188*,

11-19.
Fukata, M., Watanabe, T., Noritake, J., Nakagawa, M., Yamaga, M., Kuroda, S., Matsuura, Y., Iwamatsu, A., Perez, F., and Kaibuchi, K. (2002). Rac1 and Cdc42 capture microtubules through IQGAP1 and CLIP-170. Cell *109*, 873-885.
Gallagher, A.R., Cedzich, A., Gretz, N., Somlo, S., and Witzgall, R. (2000). The polycystic kidney disease protein PKD2 interacts with Hax-1, a protein associated with the actin cytoskeleton. Proc Natl Acad Sci U S A *97*, 4017-4022.
Ganguly, A., Yang, H., Sharma, R., Patel, K.D., and Cabral, F. (2012). The role of microtubules and their dynamics in cell migration. J Biol Chem *287*, 43359-43369.
Geiger, B., and Bershadsky, A. (2001). Assembly and mechanosensory function of focal contacts. Curr Opin Cell Biol *13*, 584-592.
Gerards, W.L., de Jong, W.W., Boelens, W., and Bloemendal, H. (1998). Structure and assembly of the 20S proteasome. Cell Mol Life Sci *54*, 253-262.
Giannone, G., Dubin-Thaler, B.J., Rossier, O., Cai, Y., Chaga, O., Jiang, G., Beaver, W., Dobereiner, H.G., Freund, Y., Borisy, G., *et al.* (2007). Lamellipodial actin mechanically links myosin activity with adhesion-site formation. Cell *128*, 561-575.
Gilbert, Scott F. (2010). Developmental biology (9th ed. ed.) Sunderland, Mass.: Sinauer Associates.
Gumbiner, B.M. (1996). Cell adhesion: the molecular basis of tissue architecture and morphogenesis. Cell *84*, 345-357.
Gundersen, G.G. (2002). Evolutionary conservation of microtubule-capture mechanisms. Nat Rev Mol Cell Biol *3*, 296-304.
Guo, R., Sakamoto, H., Sugiura, S., and Ogawa, M. (2007). Endothelial cell motility is compatible with junctional integrity. J Cell Physiol *211*, 327-335.
Hage, B., Meinel, K., Baum, I., Giehl, K., and Menke, A. (2009). Rac1 activation inhibits E-cadherin-mediated adherens junctions via binding to IQGAP1 in pancreatic carcinoma cells. Cell Commun Signal *7*, 23.
Hall, A. (2005). Rho GTPases and the control of cell behaviour. Biochem Soc Trans *33*, 891-895.
Hamblet, N.S., Lijam, N., Ruiz-Lozano, P., Wang, J., Yang, Y., Luo, Z., Mei, L., Chien, K.R., Sussman, D.J., and Wynshaw-Boris, A. (2002). Dishevelled 2 is essential for cardiac outflow tract development, somite segmentation and neural tube closure. Development *129*, 5827-5838.
Han, Y., Chen, Y.S., Liu, Z., Bodyak, N., Rigor, D., Bisping, E., Pu, W.T., and Kang, P.M. (2006). Overexpression of HAX-1 protects cardiac myocytes from apoptosis through caspase-9 inhibition. Circ Res *99*, 415-423.
Hart, M.J., Callow, M.G., Souza, B., and Polakis, P. (1996). IQGAP1, a calmodulin-binding protein with a rasGAP-related domain, is a potential effector for cdc42Hs. EMBO J *15*, 2997-3005.
Hayashi, H., Nabeshima, K., Aoki, M., Hamasaki, M., Enatsu, S., Yamauchi, Y., Yamashita, Y., and Iwasaki, H. (2010). Overexpression of IQGAP1 in advanced colorectal cancer correlates with poor prognosis-critical role in tumor invasion. Int J Cancer *126*, 2563-2574.
Heasman, S.J., and Ridley, A.J. (2008). Mammalian Rho GTPases: new insights into their functions from in vivo studies. Nat Rev Mol Cell Biol *9*, 690-701.
Hinck, L., Nathke, I.S., Papkoff, J., and Nelson, W.J. (1994). Dynamics of cadherin/catenin complex formation: novel protein interactions and pathways of complex assembly. J Cell Biol *125*, 1327-1340.
Hirata, H., Tatsumi, H., and Sokabe, M. (2007). Dynamics of actin filaments during tension-dependent formation of actin bundles. Biochim Biophys Acta *1770*, 1115-1127.

Hodgson, L., Shen, F., and Hahn, K. (2010). Biosensors for characterizing the dynamics of rho family GTPases in living cells. Curr Protoc Cell Biol *Chapter 14*, Unit 14 11 11-26.

Horwitz, A.R., and Parsons, J.T. (1999). Cell migration--movin' on. Science *286*, 1102-1103.

Hotchin, N.A., and Hall, A. (1995). The assembly of integrin adhesion complexes requires both extracellular matrix and intracellular rho/rac GTPases. J Cell Biol *131*, 1857-1865.

Hotulainen, P., and Lappalainen, P. (2006). Stress fibers are generated by two distinct actin assembly mechanisms in motile cells. J Cell Biol *173*, 383-394.

Hou, Y.C., and Deng, J.Y. (2015). Role of E3 ubiquitin ligases in gastric cancer. World J Gastroenterol *21*, 786-793.

Huibregtse, J.M., Scheffner, M., Beaudenon, S., Howley, P.M., 1995. A family of proteins structurally and functionally related to the E6-AP ubiquitinprotein ligase. Proc. Natl. Acad. Sci. U. S. A. 92, 5249.

Hynes, R.O. (2009). The extracellular matrix: not just pretty fibrils. Science *326*, 1216-1219.

Ikeda, F., and Dikic, I. (2008). Atypical ubiquitin chains: new molecular signals. 'Protein Modifications: Beyond the Usual Suspects' review series. EMBO Rep *9*, 536-542.

Ilic, D., Furuta, Y., Kanazawa, S., Takeda, N., Sobue, K., Nakatsuji, N., Nomura, S., Fujimoto, J., Okada, M., and Yamamoto, T. (1995). Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK-deficient mice. Nature *377*, 539-544.

Ilina, O., and Friedl, P. (2009). Mechanisms of collective cell migration at a glance. J Cell Sci *122*, 3203-3208.

Insall, R.H., and Machesky, L.M. (2009). Actin dynamics at the leading edge: from simple machinery to complex networks. Dev Cell *17*, 310-322.

Iwasaki, T., Nakata, A., Mukai, M., Shinkai, K., Yano, H., Sabe, H., Schaefer, E., Tatsuta, M., Tsujimura, T., Terada, N., *et al.* (2002). Involvement of phosphorylation of Tyr-31 and Tyr-118 of paxillin in MM1 cancer cell migration. Int J Cancer *97*, 330-335.

Jackson, J., Meisinger, J., Patel, S., Lim, Z.C., Vellody, K., Metz, R., and Young, M.R. (1997). Protein phosphatase-2A associates with the cytoskeleton to maintain cell spreading and reduced motility of nonmetastatic Lewis lung carcinoma cells: the loss of this regulatory control in metastatic cells. Invasion Metastasis *17*, 199-209.

Jackson, J.L., and Young, M.R. (2003). Protein phosphatase-2A regulates protein tyrosine phosphatase activity in Lewis lung carcinoma tumor variants. Clin Exp Metastasis *20*, 357-364.

Jaffe, A.B., and Hall, A. (2005). Rho GTPases: biochemistry and biology. Annu Rev Cell Dev Biol *21*, 247-269.

Jarvelainen, H., Sainio, A., Koulu, M., Wight, T.N., and Penttinen, R. (2009). Extracellular matrix molecules: potential targets in pharmacotherapy. Pharmacol Rev *61*, 198-223.

Jean, L., Majumdar, D., Shi, M., Hinkle, L.E., Diggins, N.L., Ao, M., Broussard, J.A., Evans, J.C., Choma, D.P., and Webb, D.J. (2013). Activation of Rac by Asef2 promotes myosin II-dependent contractility to inhibit cell migration on type I collagen. J Cell Sci *126*, 5585-5597.

Kanaho, Y., Kobayashi-Nakano, A., and Yokozeki, T. (2007). The phosphoinositide kinase PIP5K that produces the versatile signaling phospholipid PI4,5P(2). Biol Pharm Bull *30*, 1605-1609.

Katoh, H., and Negishi, M. (2003). RhoG activates Rac1 by direct interaction with the Dock180-binding protein Elmo. Nature *424*, 461-464.

Kaunas, R., Nguyen, P., Usami, S., and Chien, S. (2005). Cooperative effects of Rho and mechanical stretch on stress fiber organization. Proc Natl Acad Sci U S A *102*, 15895-15900.

Kellogg, D.R., Mitchison, T.J., and Alberts, B.M. (1988). Behaviour of microtubules and actin

filaments in living Drosophila embryos. Development *103*, 675-686.
Khalil, B.D., Hanna, S., Saykali, B.A., El-Sitt, S., Nasrallah, A., Marston, D., El-Sabban, M., Hahn, K.M., Symons, M., and El-Sibai, M. (2014). The regulation of RhoA at focal adhesions by StarD13 is important for astrocytoma cell motility. Exp Cell Res *321*, 109-122.
Kibar, Z., Vogan, K.J., Groulx, N., Justice, M.J., Underhill, D.A., and Gros, P. (2001). Ltap, a mammalian homolog of Drosophila Strabismus/Van Gogh, is altered in the mouse neural tube mutant Loop-tail. Nat Genet *28*, 251-255.
Kim, D.H., and Wirtz, D. (2013). Focal adhesion size uniquely predicts cell migration. FASEB J *27*, 1351-1361.
Knight, B., Laukaitis, C., Akhtar, N., Hotchin, N.A., Edlund, M., and Horwitz, A.R. (2000). Visualizing muscle cell migration in situ. Curr Biol *10*, 576-585.
Kirchner, J., Kam, Z., Tzur, G., Bershadsky, A.D., and Geiger, B. (2003). Live-cell monitoring of tyrosine phosphorylation in focal adhesions following microtubule disruption. J Cell Sci *116*, 975-986.
Koestler, S.A., Auinger, S., Vinzenz, M., Rottner, K., and Small, J.V. (2008). Differentially oriented populations of actin filaments generated in lamellipodia collaborate in pushing and pausing at the cell front. Nat Cell Biol *10*, 306-313.
Kohno, T., Urao, N., Ashino, T., Sudhahar, V., Inomata, H., Yamaoka-Tojo, M., McKinney, R.D., Fukai, T., and Ushio-Fukai, M. (2013). IQGAP1 links PDGF receptor-beta signal to focal adhesions involved in vascular smooth muscle cell migration: role in neointimal formation after vascular injury. Am J Physiol Cell Physiol *305*, C591-600.
Koyama, H., Raines, E.W., Bornfeldt, K.E., Roberts, J.M., and Ross, R. (1996). Fibrillar collagen inhibits arterial smooth muscle proliferation through regulation of Cdk2 inhibitors. Cell *87*, 1069-1078.
Kozlova, I., Ruusala, A., Voytyuk, O., Skandalis, S.S., and Heldin, P. (2012). IQGAP1 regulates hyaluronan-mediated fibroblast motility and proliferation. Cell Signal *24*, 1856-1862.
Kuo, B.R., and Erickson, C.A. (2010). Regional differences in neural crest morphogenesis. Cell Adh Migr *4*, 567-585.
Kuroda, S., Fukata, M., Kobayashi, K., Nakafuku, M., Nomura, N., Iwamatsu, A., and Kaibuchi, K. (1996). Identification of IQGAP as a putative target for the small GTPases, Cdc42 and Rac1. J Biol Chem *271*, 23363-23367.
Kuroda, S., Fukata, M., Nakagawa, M., Fujii, K., Nakamura, T., Ookubo, T., Izawa, I., Nagase, T., Nomura, N., Tani, H., *et al.* (1998). Role of IQGAP1, a target of the small GTPases Cdc42 and Rac1, in regulation of E-cadherin- mediated cell-cell adhesion. Science *281*, 832-835.

Lauffenburger, D.A., and Horwitz, A.F. (1996). Cell migration: a physically integrated molecular process. Cell *84*, 359-369.
Laukaitis, C.M., Webb, D.J., Donais, K., and Horwitz, A.F. (2001). Differential dynamics of alpha 5 integrin, paxillin, and alpha-actinin during formation and disassembly of adhesions in migrating cells. J Cell Biol *153*, 1427-1440.
Lawson, C.D., and Burridge, K. (2014). The on-off relationship of Rho and Rac during integrin-mediated adhesion and cell migration. Small GTPases *5*, e27958.
Le Clainche, C., and Carlier, M.F. (2008). Regulation of actin assembly associated with protrusion and adhesion in cell migration. Physiol Rev *88*, 489-513.
Lecaudey, V., and Gilmour, D. (2006). Organizing moving groups during morphogenesis. Curr Opin Cell Biol *18*, 102-107.

Lee, J.M., Dedhar, S., Kalluri, R., and Thompson, E.W. (2006). The epithelial-mesenchymal transition: new insights in signaling, development, and disease. J Cell Biol *172*, 973-981.

Leigh, P.N., Whitwell, H., Garofalo, O., Buller, J., Swash, M., Martin, J.E., Gallo, J.M., Weller, R.O., and Anderton, B.H. (1991). Ubiquitin-immunoreactive intraneuronal inclusions in amyotrophic lateral sclerosis. Morphology, distribution, and specificity. Brain *114 (Pt 2)*, 775-788.

Letourneau, P.C., Condic, M.L., and Snow, D.M. (1992). Extracellular matrix and neurite outgrowth. Curr Opin Genet Dev *2*, 625-634.

Levental, K.R., Yu, H., Kass, L., Lakins, J.N., Egeblad, M., Erler, J.T., Fong, S.F., Csiszar, K., Giaccia, A., Weninger, W., *et al.* (2009). Matrix crosslinking forces tumor progression by enhancing integrin signaling. Cell *139*, 891-906.

Li, B., Hu, Q., Xu, R., Ren, H., Fei, E., Chen, D., and Wang, G. (2012). Hax-1 is rapidly degraded by the proteasome dependent on its PEST sequence. BMC Cell Biol *13*, 20.

Li, W., Bengtson, M.H., Ulbrich, A., Matsuda, A., Reddy, V.A., Orth, A., Chanda, S.K., Batalov, S., and Joazeiro, C.A. (2008). Genome-wide and functional annotation of human E3 ubiquitin ligases identifies MULAN, a mitochondrial E3 that regulates the organelle's dynamics and signaling. PLoS One *3*, e1487.

Li, X., Zhou, Q., Sunkara, M., Kutys, M.L., Wu, Z., Rychahou, P., Morris, A.J., Zhu, H., Evers, B.M., and Huang, C. (2013). Ubiquitination of PIPKIgamma90 by HECTD1 regulates focal adhesion dynamics and cell migration. J Cell Sci.

Liu, C., Billadeau, D.D., Abdelhakim, H., Leof, E., Kaibuchi, K., Bernabeu, C., Bloom, G.S., Yang, L., Boardman, L., Shah, V.H., *et al.* (2013). IQGAP1 suppresses TbetaRII-mediated myofibroblastic activation and metastatic growth in liver. J Clin Invest *123*, 1138-1156.

Liu, S., Thomas, S.M., Woodside, D.G., Rose, D.M., Kiosses, W.B., Pfaff, M., and Ginsberg, M.H. (1999). Binding of paxillin to alpha4 integrins modifies integrin-dependent biological responses. Nature *402*, 676-681.

Lobert, V.H., Brech, A., Pedersen, N.M., Wesche, J., Oppelt, A., Malerod, L., and Stenmark, H. (2010). Ubiquitination of alpha 5 beta 1 integrin controls fibroblast migration through lysosomal degradation of fibronectin-integrin complexes. Dev Cell *19*, 148-159.

Loeser, R.F., Sadiev, S., Tan, L., and Goldring, M.B. (2000). Integrin expression by primary and immortalized human chondrocytes: evidence of a differential role for alpha1beta1 and alpha2beta1 integrins in mediating chondrocyte adhesion to types II and VI collagen. Osteoarthritis Cartilage *8*, 96-105.

Loosli, Y., Luginbuehl, R., and Snedeker, J.G. (2010). Cytoskeleton reorganization of spreading cells on micro-patterned islands: a functional model. Philos Trans A Math Phys Eng Sci *368*, 2629-2652.

Lu, K., Yin, X., Weng, T., Xi, S., Li, L., Xing, G., Cheng, X., Yang, X., Zhang, L., and He, F. (2008). Targeting WW domains linker of HECT-type ubiquitin ligase Smurf1 for activation by CKIP-1. Nat Cell Biol *10*, 994-1002.

Lowell, C.A., and Mayadas, T.N. (2012). Overview: studying integrins in vivo. Methods Mol Biol *757*, 369-397.

Lu, Z., Jiang, G., Blume-Jensen, P., and Hunter, T. (2001). Epidermal growth factor-induced tumor cell invasion and metastasis initiated by dephosphorylation and downregulation of focal adhesion kinase. Mol Cell Biol *21*, 4016-4031.

Machacek, M., and Danuser, G. (2006). Morphodynamic profiling of protrusion phenotypes. Biophys J *90*, 1439-1452.

Machacek, M., Hodgson, L., Welch, C., Elliott, H., Pertz, O., Nalbant, P., Abell, A., Johnson, G.L., Hahn, K.M., and Danuser, G. (2009). Coordination of Rho GTPase activities during cell protrusion. Nature *461*, 99-103.

Machesky, L.M. (2008). Lamellipodia and filopodia in metastasis and invasion. FEBS Lett *582*, 2102-2111.

Martel, V., Racaud-Sultan, C., Dupe, S., Marie, C., Paulhe, F., Galmiche, A., Block, M.R., and Albiges-Rizo, C. (2001). Conformation, localization, and integrin binding of talin depend on its interaction with phosphoinositides. J Biol Chem *276*, 21217-21227.

Martin Evans,Mario Capecchi, Oliver Smithies, **The 2007 Nobel Prize in Physiology or Medicine - Advanced Information**. Nobelprize.org. Retrieved 26 June 2010.

Martin, P. (1997). Wound healing--aiming for perfect skin regeneration. Science *276*, 75-81.

Maruyama, K., and Ebashi, S. (1965). Alpha-actinin, a new structural protein from striated muscle. II. Action on actin. J Biochem *58*, 13-19.

Mateer, S.C., Wang, N., and Bloom, G.S. (2003). IQGAPs: integrators of the cytoskeleton, cell adhesion machinery, and signaling networks. Cell Motil Cytoskeleton *55*, 147-155.

McBeath, R., Pirone, D.M., Nelson, C.M., Bhadriraju, K., and Chen, C.S. (2004). Cell shape, cytoskeletal tension, and RhoA regulate stem cell lineage commitment. Dev Cell *6*, 483-495.

McGrath, J.L. (2007). Cell spreading: the power to simplify. Curr Biol *17*, R357-358.

McNeill, H., Ryan, T.A., Smith, S.J., and Nelson, W.J. (1993). Spatial and temporal dissection of immediate and early events following cadherin-mediated epithelial cell adhesion. J Cell Biol *120*, 1217-1226.

Mekkawy, A.H., Morris, D.L., and Pourgholami, M.H. (2012). HAX1 Augments Cell Proliferation, Migration, Adhesion, and Invasion Induced by Urokinase-Type Plasminogen Activator Receptor. J Oncol *2012*, 950749.

Melino, G., Gallagher, E., Aqeilan, R.I., Knight, R., Peschiaroli, A., Rossi, M., Scialpi, F., Malatesta, M., Zocchi, L., Browne, G., *et al.* (2008). Itch: a HECT-type E3 ligase regulating immunity, skin and cancer. Cell Death Differ *15*, 1103-1112.

Meyer, A.S., Hughes-Alford, S.K., Kay, J.E., Castillo, A., Wells, A., Gertler, F.B., and Lauffenburger, D.A. (2012). 2D protrusion but not motility predicts growth factor-induced cancer cell migration in 3D collagen. J Cell Biol *197*, 721-729.

Mineur, P., Guignandon, A., Lambert Ch, A., Amblard, M., Lapiere Ch, M., and Nusgens, B.V. (2005). RGDS and DGEA-induced [Ca2+]i signalling in human dermal fibroblasts. Biochim Biophys Acta *1746*, 28-37.

Mise, N., Savai, R., Yu, H., Schwarz, J., Kaminski, N., and Eickelberg, O. (2012). Zyxin is a transforming growth factor-beta (TGF-beta)/Smad3 target gene that regulates lung cancer cell motility via integrin alpha5beta1. J Biol Chem *287*, 31393-31405.

Mitchison, T.J., and Cramer, L.P. (1996). Actin-based cell motility and cell locomotion. Cell *84*, 371-379.

Murata, S.I., Warigaya, K., Matsuzaki, I., Itonaga, M., Shimizu, Y., and Shuto, M. (2015). Microtubule-organizing center-mediated nuclear polarity in various normal and neoplastic human tissues. Virchows Arch.

Nabeshima, K., Shimao, Y., Inoue, T., and Koono, M. (2002). Immunohistochemical analysis of IQGAP1 expression in human colorectal carcinomas: its overexpression in carcinomas and association with invasion fronts. Cancer Lett *176*, 101-109.

Nada, S., Okada, M., MacAuley, A., Cooper, J.A., and Nakagawa, H. (1991). Cloning of a complementary DNA for a protein-tyrosine kinase that specifically phosphorylates a negative regulatory site of p60c-src. Nature *351*, 69-72.

Nada, S., Yagi, T., Takeda, H., Tokunaga, T., Nakagawa, H., Ikawa, Y., Okada, M., and Aizawa, S. (1993). Constitutive activation of Src family kinases in mouse embryos that lack Csk. Cell *73*, 1125-1135.

Nagano, M., Hoshino, D., Koshikawa, N., Akizawa, T., and Seiki, M. (2012). Turnover of focal adhesions and cancer cell migration. Int J Cell Biol *2012*, 310616.

Nagasaki, A., Kanada, M., and Uyeda, T.Q. (2009). Cell adhesion molecules regulate contractile ring-independent cytokinesis in Dictyostelium discoideum. Cell Res *19*, 236-246.

Nakayama, K.I., and Nakayama, K. (2006). Ubiquitin ligases: cell-cycle control and cancer. Nat Rev Cancer *6*, 369-381.

Ngoka, L.C. (2008). Sample prep for proteomics of breast cancer: proteomics and gene ontology reveal dramatic differences in protein solubilization preferences of radioimmunoprecipitation assay and urea lysis buffers. Proteome Sci *6*, 30.

Nimnual, A.S., Taylor, L.J., and Bar-Sagi, D. (2003). Redox-dependent downregulation of Rho by Rac. Nat Cell Biol *5*, 236-241.

Nishimura, T., Honda, H., and Takeichi, M. (2012). Planar cell polarity links axes of spatial dynamics in neural-tube closure. Cell *149*, 1084-1097.

Nishiya, N., Kiosses, W.B., Han, J., and Ginsberg, M.H. (2005). An alpha4 integrin-paxillin-Arf-GAP complex restricts Rac activation to the leading edge of migrating cells. Nat Cell Biol *7*, 343-352.

Nishizaka, T., Shi, Q., and Sheetz, M.P. (2000). Position-dependent linkages of fibronectin-integrin-cytoskeleton. Proc Natl Acad Sci U S A *97*, 692-697.

Nobes, C.D., and Hall, A. (1995). Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. Cell *81*, 53-62.

Nobes, C.D., and Hall, A. (1999). Rho GTPases control polarity, protrusion, and adhesion during cell movement. J Cell Biol *144*, 1235-1244.

Noritake, J., Watanabe, T., Sato, K., Wang, S., and Kaibuchi, K. (2005). IQGAP1: a key regulator of adhesion and migration. J Cell Sci *118*, 2085-2092.

Olofsson, B. (1999). Rho guanine dissociation inhibitors: pivotal molecules in cellular signalling. Cell Signal *11*, 545-554.

Pankov R, Cukierman E, Katz BZ, Matsumoto K, Lin DC, Lin S, et al. Integrin dynamics and matrix assembly: tensin-dependent translocation of alpha(5)beta(1) integrins promotes early fibronectin fibrillogenesis. J Cell Biol 2000;148:1075–90.

Pankov, R., Endo, Y., Even-Ram, S., Araki, M., Clark, K., Cukierman, E., Matsumoto, K., and Yamada, K.M. (2005). A Rac switch regulates random versus directionally persistent cell migration. J Cell Biol *170*, 793-802.

Pankov, R., and Yamada, K.M. (2002). Fibronectin at a glance. J Cell Sci *115*, 3861-3863.

Parsons, J.T., Horwitz, A.R., and Schwartz, M.A. (2010). Cell adhesion: integrating cytoskeletal dynamics and cellular tension. Nat Rev Mol Cell Biol *11*, 633-643.

Peglion, F., Llense, F., and Etienne-Manneville, S. (2014). Adherens junction treadmilling during collective migration. Nat Cell Biol *16*, 639-651.

Pleines, I., Dutting, S., Cherpokova, D., Eckly, A., Meyer, I., Morowski, M., Krohne, G., Schulze, H.,

Gachet, C., Debili, N., *et al.* (2013). Defective tubulin organization and proplatelet formation in murine megakaryocytes lacking Rac1 and Cdc42. Blood *122*, 3178-3187.
Pollard, T.D., and Borisy, G.G. (2003). Cellular motility driven by assembly and disassembly of actin filaments. Cell *112*, 453-465.
Pollard, T.D., and Cooper, J.A. (2009). Actin, a central player in cell shape and movement. Science *326*, 1208-1212.
Ponti, A., Machacek, M., Gupton, S.L., Waterman-Storer, C.M., and Danuser, G. (2004). Two distinct actin networks drive the protrusion of migrating cells. Science *305*, 1782-1786.
Qin, R., Schmid, H., Munzberg, C., Maass, U., Krndija, D., Adler, G., Seufferlein, T., Liedert, A., Ignatius, A., Oswald, F., *et al.* (2015). Phosphorylation and turnover of paxillin in focal contacts is controlled by force and defines the dynamic state of the adhesion site. Cytoskeleton (Hoboken) *72*, 101-112.
Radhika, V., Onesime, D., Ha, J.H., and Dhanasekaran, N. (2004). Galpha13 stimulates cell migration through cortactin-interacting protein Hax-1. J Biol Chem *279*, 49406-49413.
Ramsay, A.G., Keppler, M.D., Jazayeri, M., Thomas, G.J., Parsons, M., Violette, S., Weinreb, P., Hart, I.R., and Marshall, J.F. (2007). HS1-associated protein X-1 regulates carcinoma cell migration and invasion via clathrin-mediated endocytosis of integrin alphavbeta6. Cancer Res *67*, 5275-5284.
Ren, X.D., Kiosses, W.B., and Schwartz, M.A. (1999). Regulation of the small GTP-binding protein Rho by cell adhesion and the cytoskeleton. EMBO J *18*, 578-585.
Rhodes, D.R., Yu, J., Shanker, K., Deshpande, N., Varambally, R., Ghosh, D., Barrette, T., Pandey, A., and Chinnaiyan, A.M. (2004). ONCOMINE: a cancer microarray database and integrated data-mining platform. Neoplasia *6*, 1-6.
Ridley, A.J. (2006). Rho GTPases and actin dynamics in membrane protrusions and vesicle trafficking. Trends Cell Biol *16*, 522-529.
Ridley, A.J., and Hall, A. (1992). The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. Cell *70*, 389-399.
Ridley, A.J., Schwartz, M.A., Burridge, K., Firtel, R.A., Ginsberg, M.H., Borisy, G., Parsons, J.T., and Horwitz, A.R. (2003). Cell migration: integrating signals from front to back. Science *302*, 1704-1709.
Rorth, P. (2007). Collective guidance of collective cell migration. Trends Cell Biol *17*, 575-579.
Rotin, D., and Kumar, S. (2009). Physiological functions of the HECT family of ubiquitin ligases. Nat Rev Mol Cell Biol *10*, 398-409.
Rottner, K., Hall, A., and Small, J.V. (1999). Interplay between Rac and Rho in the control of substrate contact dynamics. Curr Biol *9*, 640-648.
Rottner, K., and Stradal, T.E. (2011). Actin dynamics and turnover in cell motility. Curr Opin Cell Biol *23*, 569-578.
Rozario, T., and DeSimone, D.W. (2010). The extracellular matrix in development and morphogenesis: a dynamic view. Dev Biol *341*, 126-140.
Sarkar, A.A., Nuwayhid, S.J., Maynard, T., Ghandchi, F., Hill, J.T., Lamantia, A.S., and Zohn, I.E. (2014). Hectd1 is required for development of the junctional zone of the placenta. Dev Biol *392*, 368-380.
Sarkar, A.A., and Zohn, I.E. (2012). Hectd1 regulates intracellular localization and secretion of Hsp90 to control cellular behavior of the cranial mesenchyme. J Cell Biol *196*, 789-800.
Sattler, M., Pisick, E., Morrison, P.T., and Salgia, R. (2000). Role of the cytoskeletal protein paxillin in oncogenesis. Crit Rev Oncog *11*, 63-76.

Schaefer, L., and Schaefer, R.M. (2010). Proteoglycans: from structural compounds to signaling molecules. Cell Tissue Res *339*, 237-246.
Schaller, M.D., and Parsons, J.T. (1995). pp125FAK-dependent tyrosine phosphorylation of paxillin creates a high-affinity binding site for Crk. Mol Cell Biol *15*, 2635-2645.
Schlie-Wolter, S., Ngezahayo, A., and Chichkov, B.N. (2013). The selective role of ECM components on cell adhesion, morphology, proliferation and communication in vitro. Exp Cell Res *319*, 1553-1561.
Schwartz, M.A., Toksoz, D., and Khosravi-Far, R. (1996). Transformation by Rho exchange factor oncogenes is mediated by activation of an integrin-dependent pathway. EMBO J *15*, 6525-6530.
Schulman, B.A., and Harper, J.W. (2009). Ubiquitin-like protein activation by E1 enzymes: the apex for downstream signalling pathways. Nat Rev Mol Cell Biol *10*, 319-331.
Sen, S., Tewari, M., Zajac, A., Barton, E., Sweeney, H.L., and Discher, D.E. (2011). Upregulation of paxillin and focal adhesion signaling follows Dystroglycan Complex deletions and promotes a hypertensive state of differentiation. Eur J Cell Biol *90*, 249-260.
Serrels, B., Serrels, A., Brunton, V.G., Holt, M., McLean, G.W., Gray, C.H., Jones, G.E., and Frame, M.C. (2007). Focal adhesion kinase controls actin assembly via a FERM-mediated interaction with the Arp2/3 complex. Nat Cell Biol *9*, 1046-1056.
Shekaran, A., and Garcia, A.J. (2011). Extracellular matrix-mimetic adhesive biomaterials for bone repair. J Biomed Mater Res A *96*, 261-272.
Small, J.V., Kaverina, I., Krylyshkina, O., and Rottner, K. (1999). Cytoskeleton cross-talk during cell motility. FEBS Lett *452*, 96-99.
Small, J.V., Stradal, T., Vignal, E., and Rottner, K. (2002). The lamellipodium: where motility begins. Trends Cell Biol *12*, 112-120.
Smith, M.A., Blankman, E., Gardel, M.L., Luettjohann, L., Waterman, C.M., and Beckerle, M.C. (2010). A zyxin-mediated mechanism for actin stress fiber maintenance and repair. Dev Cell *19*, 365-376.
Stegemann, J.P., Hong, H., and Nerem, R.M. (2005). Mechanical, biochemical, and extracellular matrix effects on vascular smooth muscle cell phenotype. J Appl Physiol (1985) *98*, 2321-2327.
Stieren, E.S., El Ayadi, A., Xiao, Y., Siller, E., Landsverk, M.L., Oberhauser, A.F., Barral, J.M., and Boehning, D. (2011). Ubiquilin-1 is a molecular chaperone for the amyloid precursor protein. J Biol Chem *286*, 35689-35698.
Sudol, M. (1998). From Src Homology domains to other signaling modules: proposal of the 'protein recognition code'. Oncogene *17*, 1469-1474.
Takaishi, K., Sasaki, T., Kato, M., Yamochi, W., Kuroda, S., Nakamura, T., Takeichi, M., and Takai, Y. (1994). Involvement of Rho p21 small GTP-binding protein and its regulator in the HGF-induced cell motility. Oncogene *9*, 273-279.
Takemoto, H., Doki, Y., Shiozaki, H., Imamura, H., Utsunomiya, T., Miyata, H., Yano, M., Inoue, M., Fujiwara, Y., and Monden, M. (2001). Localization of IQGAP1 is inversely correlated with intercellular adhesion mediated by e-cadherin in gastric cancers. Int J Cancer *91*, 783-788.
Talis, A.L., Huibregtse, J.M., Howley, P.M., 1998. The role of E6AP in the regulation of p53 protein levels in human papillomavirus (HPV)-positive and HPV-negative cells. J. Biol. Chem. 273, 6439–6445.
Tang, T., Song, X., Yang, Z., Huang, L., Wang, W., and Tan, H. (2014). Association between murine double minute 2 T309G polymorphism and risk of liver cancer. Tumour Biol *35*, 11353-11357.
Teixeira, L.K., and Reed, S.I. (2013). Ubiquitin ligases and cell cycle control. Annu Rev Biochem *82*,

387-414.
Theveneau, E., Marchant, L., Kuriyama, S., Gull, M., Moepps, B., Parsons, M., and Mayor, R. (2010). Collective chemotaxis requires contact-dependent cell polarity. Dev Cell *19*, 39-53.
Theveneau, E., and Mayor, R. (2012). Neural crest delamination and migration: from epithelium-to-mesenchyme transition to collective cell migration. Dev Biol *366*, 34-54.
Thompson, O., Moore, C.J., Hussain, S.A., Kleino, I., Peckham, M., Hohenester, E., Ayscough, K.R., Saksela, K., and Winder, S.J. (2010). Modulation of cell spreading and cell-substrate adhesion dynamics by dystroglycan. J Cell Sci *123*, 118-127.
Thyberg, J., and Moskalewski, S. (1999). Role of microtubules in the organization of the Golgi complex. Exp Cell Res *246*, 263-279.
Tojkander, S., Gateva, G., and Lappalainen, P. (2012). Actin stress fibers--assembly, dynamics and biological roles. J Cell Sci *125*, 1855-1864.
Tomaic, V., and Banks, L. (2015). Angelman syndrome-associated ubiquitin ligase UBE3A/E6AP mutants interfere with the proteolytic activity of the proteasome. Cell Death Dis *6*, e1625.
Tran, H., Bustos, D., Yeh, R., Rubinfeld, B., Lam, C., Shriver, S., Zilberleyb, I., Lee, M.W., Phu, L., Sarkar, A.A., *et al.* (2013). HectD1 E3 ligase modifies adenomatous polyposis coli (APC) with polyubiquitin to promote the APC-axin interaction. J Biol Chem *288*, 3753-3767.
Trebinska, A., Rembiszewska, A., Ciosek, K., Ptaszynski, K., Rowinski, S., Kupryjanczyk, J., Siedlecki, J.A., and Grzybowska, E.A. (2010). HAX-1 overexpression, splicing and cellular localization in tumors. BMC Cancer *10*, 76.
Tscharntke, M., Pofahl, R., Chrostek-Grashoff, A., Smyth, N., Niessen, C., Niemann, C., Hartwig, B., Herzog, V., Klein, H.W., Krieg, T., *et al.* (2007). Impaired epidermal wound healing in vivo upon inhibition or deletion of Rac1. J Cell Sci *120*, 1480-1490.
Tumbarello, D.A., Brown, M.C., Hetey, S.E., and Turner, C.E. (2005). Regulation of paxillin family members during epithelial-mesenchymal transformation: a putative role for paxillin delta. J Cell Sci *118*, 4849-4863.
Turner, C.E. (1998). Paxillin. Int J Biochem Cell Biol *30*, 955-959.
Turner, C.E. (2000). Paxillin and focal adhesion signalling. Nat Cell Biol *2*, E231-236.
Tzu, J., and Marinkovich, M.P. (2008). Bridging structure with function: structural, regulatory, and developmental role of laminins. Int J Biochem Cell Biol *40*, 199-214.
Upadhya, S.C., and Hegde, A.N. (2007). Role of the ubiquitin proteasome system in Alzheimer's disease. BMC Biochem *8 Suppl 1*, S12.
Vafiadaki, E., Arvanitis, D.A., Pagakis, S.N., Papalouka, V., Sanoudou, D., Kontrogianni-Konstantopoulos, A., and Kranias, E.G. (2009). The anti-apoptotic protein HAX-1 interacts with SERCA2 and regulates its protein levels to promote cell survival. Mol Biol Cell *20*, 306-318.
Vallotton, P., and Small, J.V. (2009). Shifting views on the leading role of the lamellipodium in cell migration: speckle tracking revisited. J Cell Sci *122*, 1955-1958.
Van Aelst, L., and D'Souza-Schorey, C. (1997). Rho GTPases and signaling networks. Genes Dev *11*, 2295-2322.
van Wijk, S.J., and Timmers, H.T. (2010). The family of ubiquitin-conjugating enzymes (E2s): deciding between life and death of proteins. FASEB J *24*, 981-993.
Verstrepen, L., and Beyaert, R. (2012). No ubiquitin anchors and fully RIGged. Immunity *36*, 897-899.
Vucic, D., Dixit, V.M., and Wertz, I.E. (2011). Ubiquitylation in apoptosis: a post-translational

modification at the edge of life and death. Nat Rev Mol Cell Biol *12*, 439-452.
Wakatsuki, T., Wysolmerski, R.B., and Elson, E.L. (2003). Mechanics of cell spreading: role of myosin II. J Cell Sci *116*, 1617-1625.
Wang, X.X., Li, X.Z., Zhai, L.Q., Liu, Z.R., Chen, X.J., and Pei, Y. (2013). Overexpression of IQGAP1 in human pancreatic cancer. Hepatobiliary Pancreat Dis Int *12*, 540-545.
Wang, X.X., Wang, K., Li, X.Z., Zhai, L.Q., Qu, C.X., Zhao, Y., Liu, Z.R., Wang, H.Z., An, Q.J., Jing, L.W., *et al.* (2014). Targeted knockdown of IQGAP1 inhibits the progression of esophageal squamous cell carcinoma in vitro and in vivo. PLoS One *9*, e96501.
Wang, Y., Guo, N., and Nathans, J. (2006). The role of Frizzled3 and Frizzled6 in neural tube closure and in the planar polarity of inner-ear sensory hair cells. J Neurosci *26*, 2147-2156.
Wang, Y.L. (1985). Exchange of actin subunits at the leading edge of living fibroblasts: possible role of treadmilling. J Cell Biol *101*, 597-602.
Wary, K.K., Mainiero, F., Isakoff, S.J., Marcantonio, E.E., and Giancotti, F.G. (1996). The adaptor protein Shc couples a class of integrins to the control of cell cycle progression. Cell *87*, 733-743.
Watanabe, T., Noritake, J., and Kaibuchi, K. (2005). Regulation of microtubules in cell migration. Trends Cell Biol *15*, 76-83.
Watanabe, T., Wang, S., Noritake, J., Sato, K., Fukata, M., Takefuji, M., Nakagawa, M., Izumi, N., Akiyama, T., and Kaibuchi, K. (2004). Interaction with IQGAP1 links APC to Rac1, Cdc42, and actin filaments during cell polarization and migration. Dev Cell *7*, 871-883.
Webb, D.J., Parsons, J.T., and Horwitz, A.F. (2002). Adhesion assembly, disassembly and turnover in migrating cells -- over and over and over again. Nat Cell Biol *4*, E97-100.
Weijer, C.J. (2009). Collective cell migration in development. J Cell Sci *122*, 3215-3223.
Wittmann, T., Bokoch, G.M., and Waterman-Storer, C.M. (2003). Regulation of leading edge microtubule and actin dynamics downstream of Rac1. J Cell Biol *161*, 845-851.
Woodhouse, E.C., Chuaqui, R.F., and Liotta, L.A. (1997). General mechanisms of metastasis. Cancer *80*, 1529-1537.
Wozniak, M.A., Modzelewska, K., Kwong, L., and Keely, P.J. (2004). Focal adhesion regulation of cell behavior. Biochim Biophys Acta *1692*, 103-119.
Wu, Y.I., Frey, D., Lungu, O.I., Jaehrig, A., Schlichting, I., Kuhlman, B., and Hahn, K.M. (2009). A genetically encoded photoactivatable Rac controls the motility of living cells. Nature *461*, 104-108.
Yamaguchi, H., and Condeelis, J. (2007). Regulation of the actin cytoskeleton in cancer cell migration and invasion. Biochim Biophys Acta *1773*, 642-652.
Yeung, T., Georges, P.C., Flanagan, L.A., Marg, B., Ortiz, M., Funaki, M., Zahir, N., Ming, W., Weaver, V., and Janmey, P.A. (2005). Effects of substrate stiffness on cell morphology, cytoskeletal structure, and adhesion. Cell Motil Cytoskeleton *60*, 24-34.
Yoshigi, M., Hoffman, L.M., Jensen, C.C., Yost, H.J., and Beckerle, M.C. (2005). Mechanical force mobilizes zyxin from focal adhesions to actin filaments and regulates cytoskeletal reinforcement. J Cell Biol *171*, 209-215.
Zaidel-Bar, R., Ballestrem, C., Kam, Z., and Geiger, B. (2003). Early molecular events in the assembly of matrix adhesions at the leading edge of migrating cells. J Cell Sci *116*, 4605-4613.
Zaidel-Bar, R., Cohen, M., Addadi, L., and Geiger, B. (2004). Hierarchical assembly of cell-matrix adhesion complexes. Biochem Soc Trans *32*, 416-420.
Zaidel-Bar, R., Itzkovitz, S., Ma'ayan, A., Iyengar, R., and Geiger, B. (2007a). Functional atlas of the integrin adhesome. Nat Cell Biol *9*, 858-867.

Zaidel-Bar, R., Milo, R., Kam, Z., and Geiger, B. (2007b). A paxillin tyrosine phosphorylation switch regulates the assembly and form of cell-matrix adhesions. J Cell Sci *120*, 137-148.
Zamir E, Katz M, Posen Y, Erez N, Yamada KM, Katz BZ, et al. Dynamics and segregation of cell-matrix adhesions in cultured fibroblasts. Nat Cell Biol 2000;2:191 – 6.
Zhang, P., Bai, H., Fu, C., Chen, F., Zeng, P., Wu, C., Ye, Q., Dong, C., Song, Y., and Song, E. (2015). RacGAP1-driven focal adhesion formation promotes melanoma transendothelial migration through mediating adherens junction disassembly. Biochem Biophys Res Commun *459*, 1-9.
Zohn, I.E., Anderson, K.V., and Niswander, L. (2007). The Hectd1 ubiquitin ligase is required for development of the head mesenchyme and neural tube closure. Dev Biol *306*, 208-221.

Abbreviations

℃	celsius
Acr-Bis	acrylamide-bisacrylamide
ADP	adenosindiphosphate
AMP	adenosine monophosphate
APS	ammonium persulfate
ATP	adenosintriphosphate
BSA	bovine serum albumin
Ca^{2+}	Calcium
c-Src	tyrosine-protein kinase CSK
CO_2	carbon dioxide
ddH2O	double-distilled water
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethylsulfoxid
DNA	deoxyribonucleic acid
dNTP	deoxynukleosidtriphosphate
DTT	dithiothreitol
E2	ubiquitin-conjugating enzyme
E3	ubiquitin protein ligase
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
FBS	fetal bovine serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	green fluorescent protein
GTPase	guanine triphosphatease
HAX-1	HCLS1 associated protein X-1
HA-epitop	hemagglutinin-epitop
HECT	homologous to the E6-AP carboxyl terminus
HECTD1	HECT domain containing E3 ubiquitin protein ligase 1

HRP	horseradish peroxidase
IQGAP1	IQ motif containing GTPase activating protein 1
Kan	kanamycin
kD	kilo Dalton
l	liter
LB	Luria-Bertani
MEF	mouse embryonic fibroblast
MG132	Z-Leu-Leu-Leu-al
mg	milligram
ml	milliliter
mM	millimolar
NaCl	sodium chloride
OD	optical density
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PIP5K1A	Phosphatidylinositol-4-phosphate 5-kinase type-1 alpha
PP2A	Protein phosphatase 2
PVDF	polyvinylidene fluoride
RFP	red fluorescent protein
RIPA	Radioimmunoprecipitation assay buffer
RT	room temperature
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
TBST	Tris-buffered saline
TEMED	tetramethylethylenediamine
UPS	ubiquitin proteasome system
V	volt
µl	microliter
µM	micromolar