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Signaling mechanism in nuclear reorientation and its functional significance in cell migration

Ph.D. Thesis

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Declaration

I hereby declare on my honor that I have written this thesis on my own under the supervision of Ing. Tomáš Vomastek, Ph.D. and that I have mentioned all sources used. This work or its major part has not been used for any other academic degree in any other university or educational establishment.

Prague, 6th April, 2016

Signature…….

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CONTENTS

LIST OF ABBREVIATIONS

ACAFAs	actin cap associated focal adhesions
ADF	actin depolymerizing factor
aPKC	atypical protein kinase C
Arp2/3	actin-related proteins 2/3
Cdc42	cell division control protein 42 homolog
Crk	v-crk avian sarcoma virus CT10 oncogene homolog
DH domain	Dbl-homology domain
DNA	deoxyribonucleic acid
DOCK180	Dedicator of cytokinesis 1
ECM	extracellular matrix
EGF	epidermal growth factor
EHNA	erythro-9-(2-hydroxy-3-nonyl)adenine
ERK	extracellular signal-regulated kinase
ESCRT	endosomal sorting complexes required for transport
GAP	GTPase-activating protein
GDI	Rho GDP dissociation inhibitors
GDP	guanosin diphosphate
GEF	guanine nucleotide exchange factor
GFP	green fluorescent protein
GPCR	G protein coupled receptor
GRAF	GTPase regulator associated with FAK
GTP	guanosine triphosphate
FAs	focal adhesions
FAK	focal adhesion kinase
FERM	F for 4.1 protein, E for ezrin, R for radixin and M for moesin
FAT domain	focal adhesion targeting domain
hALP	histone acetyltransferase
HGF	hepatocyte growth factor
IFs	intermediate filaments
KASH	Klarsicht, ANC-1, Syne Homology
LARG	leukemia-associated RhoGEF
LINC	linker of nucleoskeleton and cytoskeleton

LIS1	lissencephaly1
LPA	lysophosphatidic acid
mDia	mammalian diaphanous homolog
MEK	MAPKK, mitogen-activated protein kinase kinase
MLC	myosin light chain
MLCK	myosin light chain kinase
MTs	microtubules
MTOC	microtubule organizing center
MRCK	Myotonic dystrophy kinase-related Cdc42-binding kinase
NPC	nuclear pore complex
Cas	Crk-associated substrate
Par3/6	partitioning defective protein 3/6
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
PDZ	post synaptic density protein, Drosophila disc large tumor suppressor, zonula occludens-1 protein
PH domain	pleckstrin-homology domain
PI3K	phosphoinositide 3 kinase
PS-GAP	PH- and SH3-domain containing RhoGAP protein
Rac1	Ras-related C3 botulinum toxin substrate 1
RACK1	receptor for activated C kinase 1
Raf	rapidly accelerated fibrosarcoma
Ras	rat sarcoma
RAT2	primary rat fibroblasts
RGD	arginyl-glycyl-aspartic acid
RGS domain	regulators of G protein signaling domain
ROCK	Rho-associated protein kinase
RhoA	Ras homolog A
RTK	receptor tyrosine kinase
SH2 domain	Src-homology domain 2
SH3 domain	Src-homology domain 3
siRNA	small interfering ribonucleic acid
SUN domain	Sad/UNC-84 domain
TAN lines	transmembrane actin-associated nuclear lines

TIAM1	T-cell lymphoma invasion and metastasis-inducing protein 1
WASP	Wiskott–Aldrich Syndrome protein
WAVE	WASP family Verprolin-homologous protein

1. ABSTRACT

The establishment of cellular polarity is first critical step of directional cell migration. The process of cellular polarization requires many signaling pathways that are differently regulated at the cell front and at the rear side and enables creation of typical asymmetrical profile of migrating cell. During the polarization cell forms the leading edge and trailing rear and relocalizes the intracellular organelles to such a position that is optimal for directional movement. In many migrating cells cell nucleus is usually located at the cell rear and microtubule organizing center localizes between the nucleus and the leading edge of the cell. This cellular arrangement is prerequisite for directional cell migration. We have shown that during cell polarization cell also reorients the nucleus to the direction of migration. The nuclear reorientation is temporally restricted rotation of the cell nucleus that aligns the longer nuclear axis with the axis of migration. Nuclear reorientation promotes the establishment of cellular polarity and facilitates the movement of the cell.

The nuclear reorientation requires the physical linkage of the nucleus to cell cytoskeleton mediated by LINC (Linker of Nucleoskeleton and Cytoskeleton) complex. We have shown that LINC complex anchors the nucleus to actin stress fibers exposed above the nucleus and enables the nuclear reorientation to the direction of migration.

In migrating cells, actin forms several types of stress fibers: ventral fibers, dorsal fibers, transverse arcs and perinuclear actin fibers (perinuclear actin fibers are also referred as "perinuclear actin cap"). Ventral stress fibers are restricted to the basal side while dorsal stress fibers, transverse actin arcs and perinuclear actin cap filaments rise from the leading edge to the dorsal side of the cell, with perinuclear actin cap fibers being connected through LINC complex to the nuclear envelope. We have shown that during cell polarization, dorsal fibers, transverse arcs and perinuclear filaments form interconnected network crosslinked by actin binding protein α-actinin1. This network of actin fibers is anchored in adhesions at the cell front on one side and to the nuclear envelope on the other side thus mechanically links the nucleus with adhesions at the leading edge. Dorsal fibers and transverse arcs play central role in the actin cap assembly as they recruit preexisting peripheral stress fibers and move them to the apical side of the nucleus. Actin cap formation induces also nuclear reorientation to the direction of migration and, remarkably, actin cap promotes the actin arcs and dorsal fibers localization to the cell front. Our results thus suggest that the network of dorsal fibers, actin cap and transverse arcs functions bi-directionally to regulate both, nuclear positioning and cell front organization.

The nuclear reorientation is controlled by coordinated regulation of two signaling axis: by LPA-mediated activation of small GTPase RhoA and by activation of integrin and FAK/Src and p190A-RhoGAP signaling. LPA stimulates receptors coupled with trimeric G-proteins that activate RhoA in the whole cell. Integrin and FAK signaling is activated predominantly at the cell front and represents primary polarity signal leading to the establishment of cellular polarity. Activation of FAK/Src complex subsequently stimulates RhoA inhibitor p190A-RhoGAP and induces its recruitment to the cell front. Cooperation of these two signaling axes dynamically regulates the activity of RhoA at the leading edge that allows the cell to massively reorganize the actin cytoskeleton. RhoA stimulates proteins from the formin family and induces the formation of interconnected actin network. Subsequent RhoA mediated contractility drives the perinuclear actin cap formation and nuclear reorientation.

In addition, we have found that presence of perinuclear fibers and nuclear reorientation correlate with the shape of motile cells and with mode of migration. Fibroblasts that reorient their nucleus to the direction of migration have elongated conical shape and perinuclear fibers are aligned with the longer nuclear axis and with the axis of migration. Such cells migrate using “inchworm” manner with front protrusions followed by rear retraction. In contrast, cells that do not possess perinuclear fibers, like U2OS, display fan-like shape, reorient the nucleus perpendicular to the direction of migration and their migration is characterized by persistent protrusion and constant cell body movement without tail retraction step. These data suggest that perinuclear actin fibers and nuclear reorientation determine the mode of migration.

ABSTRAKT

Prvním důležitým krokem buněčné migrace je ustavení buněčné polarity. Na procesu polarizace buněk se podílí řada signálních drah, jejichž regulace je v přední a zadní části buňky řízena různým způsobem. To buňce umožňuje zaujmout specifický tvar s typickou předo-zadní polaritou. Během polarizace buňka formuje vedoucí lamelipodii na přední části a váznoucí konec na zadní části buňky. Zároveň dochází k takovému přeuspořádání vnitrobuněčných organel, které je pro buněčnou migraci optimální. V migrujících buňkách, jako jsou fibroblasty, se buněčné jádro nachází v zadní části buňky a mikrotubuly organizující centrum relokalizuje mezi jádro a čelo buňky. Takové uspořádání uvnitř buňky je nezbytnou podmínkou pro efektivní směrovanou migraci. V této práci jsme ukázali, že pro polarizaci buněk je nezbytná také takzvaná reorientace jádra. Reorientace jádra je rotační pohyb, ke kterému dochází v prvních hodinách buněčné polarizace a umožňuje reorientaci delší osy jádra do směru migrace. Reorientace jádra pak napomáhá ustavení buněčné polarity a usnadňuje pohyb buněk.

Aby buňka mohla reorientovat své jádro do směru migrace, je nutné, aby bylo napojeno na buněčný cytoskelet. Připojení jádra k jednotlivým komponentám cytoskeletu zprostředkovává takzvaný LINC (Linker of Nucleoskeleton and Cytoskeleton) komplex. V této práci jsme ukázali, že aktinová vlákna táhnoucí se nad buněčným jádrem jsou pomocí LINC komplexu napojena na jaderný obal a indukují reorientaci jádra do směru migrace.

V migrujících buňkách se vyskytuje několik typů aktinových stresových vláken: ventrální vlákna, dorsální vlákna, aktinová vlákna ve tvaru oblouků (příčné arcs) a perinukleární aktinová vlákna. Zatímco ventrální vlákna se vyskytují pouze na bazální straně buňky, dorsální a perinukleární vlákna a arcs vybíhají z čela buňky na její dorsální stranu. Perinukleární aktinová vlákna, která se dále pnou nad buněčným jádrem, jsou pomocí LINC komplexu napojena na jaderný obal. Naše výsledky ukazují, že během procesu buněčné polarizace dochází k formování aktinové sítě, tvořené dorsálními vlákny, arcs a perinukleárními vlákny, která mechanicky propojuje fokální adheze na předním okraji buňky s buněčným jádrem. Při tvorbě této aktinové sítě je nezbytný aktin vazebný protein α-actinin1, který propojuje jednotlivá vlákna v místech křížení aktinových vláken. Velmi důležitou roli hrají také dorsální vlákna a příčné arcs, které rekrutují aktinová vlákna z okraje buňky na dorsální stranu buňky a nad jádro a tak dávají vznik novým perinukleárním aktinovým vláknům. Pohyb aktinových vláken nad jádro buňky indukuje také reorientaci jádra do směru migrace. Překvapivě jsme také zjistili, že perinukleární vlákna zároveň vymezují lokalizaci

dorsálních vláken a arcs na přední okraj buňky. Z těchto výsledků vyplývá, že síť dorsálních a perinukleárních vláken a příčných arcs reguluje z předního okraje buňky jadernou reorientaci a zároveň fungují i opačným směrem, kdy jejich napojení na jádro reguluje organizaci lamelipodie na přední straně buňky.

Reorientace jádra je regulována dvěma signálními drahami: LPA a následnou stimulací malé GTPázy RhoA a aktivací integrínů a FAK/Src signalizačního komplexu, který aktivuje p190A-RhoGAP. LPA stimuluje receptory spřažené s trimerními G-proteiny, které následně aktivují RhoA v rámci celé buňky. K aktivaci integrínů dochází převážně na přední části migrující buňky. Tudíž integrínová signalizace představuje první signál vedoucí k ustavení buněčné polarity. Následná aktivace FAK/Src komplexu stimuluje RhoA inhibitor p190A-RhoGAP a rekrutuje ho na čelo polarizující buňky. Kooperace těchto dvou signálních drah dynamicky reguluje aktivitu RhoA na přední straně buňky, což umožňuje masivní přestavbu aktinového cytoskeletu, která dává vznik perinukleárním vláknům a reorientuje buněčné jádro do směru migrace.

Perinuklární aktinová vlákna a reorientace jádra také korelují s tvarem migrujících buněk a se způsobem jakým buňky migrují. Fibroblasty, které reorientují jádro do směru migrace, mají protáhlý tvar a perinukleární aktinová vlákna jsou zarovnána s delší osou jádra a směrem migrace. Takové buňky migrují „píďalkovitým“ způsobem, kdy protruze je následována kontrakcí přitahující zadní část buňky k tělu buňky. Naproti tomu, buňky, které nevytváří perinukleární vlákna, jako například U2OS, mají širokou lamelu, jádro orientované kolmo k ose migrace a pro jejich pohyb je charakteristické trvalé vytvoření protruze a konstantní posouvání těla buňky vpřed bez přitahování konce k tělu buňky. Tyto výsledky naznačují, že perinukleární aktinová vlákna a reorientace jádra určují, jakým způsobem budou buňky migrovat.

2. INTRODUCTION

2.1. CELL MIGRATION AND CELL POLARITY

Cell migration is an important process in mammalian biology as it plays a central role in many physiological and pathological events like embryonic development, immune response, wound healing and the dissemination of cancer. For example, epithelial cells migrate during tissue morphogenesis from basal layers and maintain the skin and intestinal barrier. Large cell sheets migrate during gastrulation and form three-layer embryo. Migration underlies also development of brain when nerve cells migrate to different parts of brain. Importantly, the migration is intimately linked also to tumor growth and metastasis. Thus, understanding the mechanism how cells migrate and invade into surrounding tissues might help to develop specific drugs interfering with the metastatic process.

Cell migration is a multistep process that is initiated in response to extracellular signals such as gradient of mitogen, chemoattractant or binding to extracellular matrix proteins. These cues induce cell polarization, protrusion formation and stabilization at the leading edge of the cell and, finally, the tail retraction as the cell move forward (Moissoglu and Schwarz, 2006; Ridley et al., 2003). Cell polarization is a crucial step in directional migration. It allows the cell to differently regulate signaling at the cell front and rear which is prerequisite for the directional cell migration (Chodniewicz and Klemke, 2004).

Individually migrating adherent cells like fibroblasts adopt conical polarized morphology with protrusions induced at the leading edge and limited at the cell edges and rear (Fig.2.1). Formation of protrusions is driven by actin polymerization and protrusion stabilization is mediated by adhesion to proteins of extracellular matrix (ECM) via transmembrane receptors of the integrin family. At the intracellular side, integrins bind through several adaptor proteins actin cytoskeleton, thus forming physical link between ECM and cell cytoskeleton. The sites of adhesion, called focal adhesions (FAs), serve as traction sites for the cell. When the cell contracts and moves forward, focal adhesions are disassembled at the cell rear and it allows tail detachment.

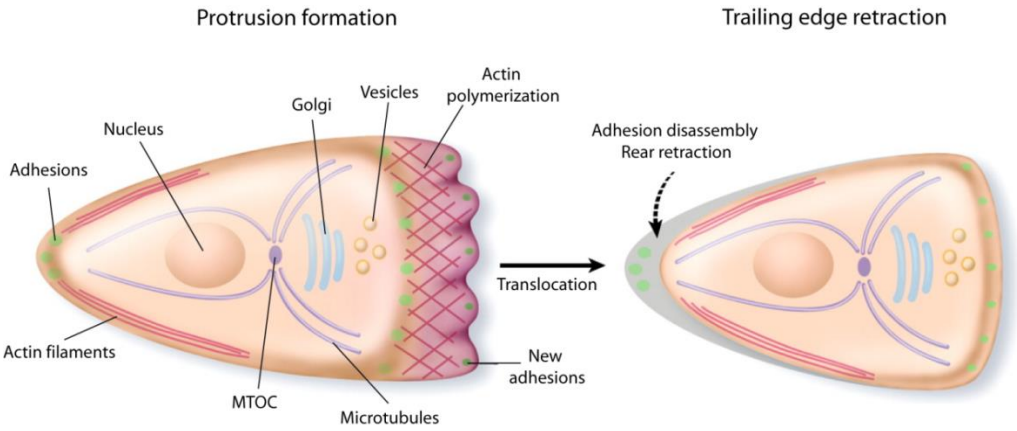

Fig. 2.1.: Polarized migrating cell with typical asymmetrical arrangement of intracellular organelles. Actin polymerization drives protrusions at the leading edge that are stabilized by new focal adhesions. During the forward movement cell translocates over the focal adhesions and they are disassembled at the cell rear allowing the tail retraction. Cell nucleus is located at the cell rear and MTOC and Golgi apparatus localize between the nucleus and the cell front. Adapted from (Ridley et al., 2003).

Polarized migrating cells display asymmetrical intracellular architecture with several typical features, namely the organization of actin, microtubules (MTs) and cell nucleus. Actin organization defines the shape of migrating cells (Mogilner and Keren, 2009; Pellegrin and Mellor, 2007). At the leading edge actin polymerization drives formation of protrusions while at the cell sides and rear the protrusions are limited and actin forms thick bundles at the periphery of the cell that demarcate nonprotruding regions. Several classes of stress fibers are also formed in the cell body, including dorsal fibers, transverse arcs and perinuclear actin fibers (Hotulainen and Lappalainen, 2006; Khatau et al., 2009). The microtubule organizing center (MTOC) and Golgi apparatus is usually localized between the leading edge and the nucleus. MTOC positioning is a prerequisite for microtubules growth from the MTOC towards the cell front where they are selectively stabilized and provide a unique track for directed vesicle trafficking toward the leading edge (Gundersen and Cook, 1999; Palazzo et al., 2004; Watanabe et al., 2005). Cell nucleus, usually located at the back part of the cell, plays important role in the establishment of the polarized, asymmetrical profile of migrating cells. Nucleus movement to the cell rear promotes MTOC localization in front of the nucleus and this nuclear-centrosomal (NC) axis has been recognized as an indicator of the migratory polarity defining the axis of migration (Gomes et al., 2005; Luxton and Gundersen, 2011). Considering the important role of the nucleus in defining the NC axis it is not surprising that the nucleus displays active movement that positions the nucleus to the specific location (Gomes et al., 2005; Luxton et al., 2010). In addition to nuclear movement to the cell rear, it was observed that during cell polarization nucleus displays also rotational movement (Houben et al., 2009; Lee et al., 2005) although the significance of nuclear rotation remains unclear.

This PhD thesis focuses on the important features of nuclear rotation and reorientation, reveals the mechanisms involved in the regulation of this process and shows the impact it has on cell polarization and migration.

2.1.1. Mediators of cellular polarity – small Rho GTPases

The process of cell migration requires precise spatiotemporal coordination of many signaling pathways that allows the cell to create a typical asymmetrical profile of a polarized cell. The crucial regulator of cell polarity and migration is family of small Rho GTPases, comprising Cdc42, Rac1 and Rho. Rho GTPases activate numerous intracellular signaling pathways, and thus they are involved in many cellular processes connected with cellular motility such as cytoskeleton dynamics, cell adhesion and directional migration, but they are implicated also in transcription regulation and cell cycle progression (Ridley et al., 2003). The activation of Rho GTPases is distributed in a highly polarized manner and by engaging the different set of effector molecules, they underlies the front-rear asymmetry.

Rho GTPases function as molecular switchers that change their conformation and activity depending on their GTP or GDP binding. GDP-GTP exchange is regulated by several GEFs (guanine nucleotide exchange factor) and GAPs (GTPase-activating protein). GEF mediated replacement of GDP with GTP induce conformational changes and activation of small GTPase that subsequently bind its downstream effector. Hydrolysis of GTP to GDP switches the small GTPase back to inactive form (Etienne-Manneville and Hall, 2002). The activity of the Rho-family GTPases is also regulated by their subcellular localization (Wennerberg and Der, 2004). Through their isoprenylated C-terminus small GTPases are inserted to the plasma membrane. GDIs (Rho GDP dissociation inhibitors), that prevent also the GDP/GTP exchange and GTP hydrolysis at small Rho GTPases, terminate the GTPase signaling at the plasma membrane and modulate their cycling between the membrane and cytosol (DerMardirossian and Bokoch, 2005; Garcia-Mata et al., 2011; Grande-Garcia et al., 2005). Although the RhoGTPase family is quite wide there are only three genes encoding RhoGDIs in mammals. In contrast, the group of GEF proteins has over one hundred members. Most of them are specific and activates one GTPase only however, some GEFs may stimulate several different GTPases. Activation of GEFs occurs after stimulation with growth factors that subsequently activates receptor tyrosine kinases (RTKs), or with mitogens that activates G-proteins coupled receptors (GPCRs).

Lysophosphatidic acid (LPA) and LPA receptor are strong activators of GPCRs that subsequently activate RhoA. RhoA activation is mediated by group of RhoA activators RGS-

GEFs (RGS – regulators of G-protein signaling) that includes p115-RhoGEF, PDZ-RhoGEF, LARG and Lbc-RhoGEF (Fukuhara et al., 2001; Fukuhara et al., 1999). These GEFs through their RGS domain interact with $G_{12,13}$ and this binding promotes RGS-GEFs activation. In contrast to RGS domain, two other domains PH (pleckstrin-homology) domain and DH (Dbl-homology) domain are common for most of GEFs. DH domain stabilizes the transition state of RhoA and enhances its loading with GTP and its activation (Siehler, 2009). The PH domain is required for the activity of GEF protein itself. In addition, it enables the anchorage of GEF to other signaling molecules, namely lipids and promotes its specific subcellular localization (Fukuhara et al., 2001; Siehler, 2009). The PDZ domain that is shared by PDZ-RhoGEF and LARG only, enables the association of GEFs with other growth factor receptors (Fukuhara et al., 2001; Siehler, 2009). RhoGEF proteins are distributed throughout the cytosol and after GPCRs activation rapidly translocate to the plasma membrane. LARG and PDZ-RhoGEF display specific localization pattern. LARG was found also along microtubules, where it contributes to establishment of cellular polarity in migrating fibroblasts (Siehler, 2009), and PDZ-RhoGEF resides also in focal adhesions and induces cell rear retraction (Iwanicki et al., 2008).

Soluble mitogens are not the only activators, as Rho-GEFs are stimulated also upon cell adhesion to the extracellular matrix proteins. Members of the RGS-GEFs family, p115-RhoGEF and LARG, are activated in fibroblasts adhering to fibronectin (Dubash et al., 2007; Guilluy et al., 2011). Other RhoGEF, p190-RhoGEF, is also activated upon cell adhesion and, similarly to PDZ-RhoGEF, localizes to focal adhesions (Iwanicki et al., 2008; Lim et al., 2008; Zhai et al., 2003). Not only GEFs but also Rho-GAPs are activated in adhering cells and their activation subsequently inhibits Rho. p190A-RhoGAP is main inhibitor of RhoA activated during cell adhesion and, intriguingly, it also translocates to focal adhesions upon activation (Tomar et al., 2009). GRAF (GTPase regulator associated with FAK) and PS-GAP (PH- and SH3-domain containing RhoGAP protein) promote the GTP hydrolysis on both, RhoA and Cdc42 (Ren et al., 2001; Taylor et al., 1999), but the extent of Rho inhibition and their significance in signaling after adhesion is not fully understood.

2.1.1.1. Cdc42 and Rac1

Cdc42 and Rac1 are active at the leading edge of the cell where they regulate the establishment of cellular polarity, actin polymerization and protrusion formation. The main regulator of cellular polarity is considered Cdc42 and its inhibition or, conversely, global

activation disrupts the directional migration (Etienne-Manneville and Hall, 2002). Activation of Cdc42 occurs in response to mitogenic stimuli such as LPA, wound induced integrin activation or fluid shear stress (Etienne-Manneville and Hall, 2001, 2002; Li et al., 2003; Tzima et al., 2003). The main downstream effector of Cdc42 involved in the control of cell polarity is Par6 polarity protein found in complex with Par3 and atypical protein kinase C (aPKC). Activation of aPKC is necessary for polarized organization of microtubules and MTOC and Golgi apparatus relocalization between the leading edge and the nucleus (Etienne-Manneville, 2004; Etienne-Manneville and Hall, 2001, 2002, 2003; Gomes et al., 2005).

In contrast to Cdc42 that is more related with polarity and directionality, Rac1 is more coupled with actin polymerization and formation of protrusions at the cell front. Similarly to Cdc42, Rac1 can be also activated by integrin engagement to the substrate (Berrier et al., 2002; Choma et al., 2007) however, LPA only scarcely activates Rac1. Rac1 activation occurs rather after stimulations with growth factors like EGF, PDGF and HGF (Berrier et al., 2002; Liu and Burridge, 2000; Mori et al., 2004).

After the activation, Rac1 induces actin polymerization and regulate the direction of protrusions and, thus enables the leading edge formation (Nobes and Hall, 1999). To stimulate the actin polymerization and lamellipodia extension, Rac1 (and Cdc42 that shares some downstream effectors with Rac1) engage the scaffold proteins of the WASP/WAVE family that subsequently activate actin nucleation complex Arp2/3 (Cory and Ridley, 2002; Pollitt and Insall, 2009). Arp2/3 bind to the sites of existing filaments, where they nucleate new actin filaments and mediate branching and formation of dendritic actin network. The dendritic actin polymerization is typical for broad lamellipodium at the leading edge and pushes the plasma membrane forward (Insall and Machesky, 2009; Pollard and Borisy, 2003). Cdc42 also activates formin mDia2 that binds to barbed ends of actin filaments and induces strong actin polymerization (Higashida et al., 2004). In contrast to Scar/Wave/Arp2/3, mDia2 induces the formation of long parallel actin bundles that push the plasma membrane and drive filopodia formation in Cdc42 dependent manner (Yang et al., 2007). Other important Cdc42 effector regulating actin cytoskeleton is MRCK (Myotonic dystrophy kinase-related Cdc42-binding kinase) (Leung et al., 1998). Cdc42-MRCK signal leads to phosphorylation of myosin light chain (MLC) and to myosin activation thus Cdc42 is involved also in acto-myosin contractility and contributes to myosin-dependent motility (Wilkinson et al., 2005).

2.1.1.2. RhoA

While Cdc42 and Rac1 are activated at the cell front, RhoA operates mainly at the sides and at the rear of the cell. At these locations RhoA regulates the actomyosin contractility and thus mediates the tail retraction during the migration cycle. RhoA drives the myosin activity by regulation of phosphorylation or dephosphorylation of the regulatory myosin light chain (Chrzanowska-Wodnicka and Burridge, 1996). The actomyosin contractility is largely controlled by RhoA effectors ROCKI and ROCKII (Rho kinase I and II) that are directly activated by binding to active RhoA. Activated ROCKs phosphorylate MLC and activate myosin directly (Amano et al., 1996) or phosphorylates and thus inhibits myosin light chain phosphatase preventing MLC dephosphorylation (Kimura et al., 1996). ROCK also activates LIM kinase that consequently inhibits severing proteins ADF/cofilin (Maekawa et al., 1999) thus Rho/ROCK signaling regulates the stability of actin fibers. Active RhoA also drive actin polymerization by activation of other important Rho effectors from the formin family such as formin mDia1 (Mammalian homolog of Diaphanous1) that stimulates the actin nucleation (Watanabe et al., 1997).

The activation of Cdc42/Rac1 and RhoA is restricted to the cell front and cell rear, respectively, which is also consistent with the notions that Rho and Cdc42/Rac1 largely antagonize each other during cell migration (Hanna and El-Sibai, 2013; Vicente-Manzanares et al., 2009). For example RhoA effector ROCK phosphorylates Par3 at the cell body and rear and this phosphorylation disrupts Par3/TIAM1/aPKC/Par6 complex. As this complex mediates Rac1 activation through TIAM1, Rac1-specific GEF, Rho negatively controls directional signaling and Rac1 activity at the cell rear (Nakayama et al., 2008). *Vice versa* Rac1 may control Rho signaling through its direct interaction with p190B-RhoGAP (Bustos et al., 2008). Cdc42 may also negatively regulate RhoA through its effector Par6 that recruits ubiquitination regulatory factor Smurf1 promoting degradation of RhoA. It inhibits actomyosin contraction and favors Cdc42- and Rac1-driven actin polymerization and protrusivity leading to establishment of front-rear polarity (Etienne-Manneville, 2008; Wang et al., 2003).

Although it is generally accepted that the activation of Cdc42/Rac1 and RhoA is restricted to the cell front and cell rear, respectively, this model seems to be too simple. Quite surprisingly, studies using biosensors have revealed that RhoA is active also at the leading edge directly at initial protrusions of migrating cells (Machacek et al., 2009; Pertz et al., 2006). The important role of RhoA at the leading edge was also demonstrated by finding that

formin mDia1, a downstream effector of Rho, stimulates polymerization of dorsal actin fibers from the leading edge of the cell (Hotulainen and Lappalainen, 2006).

2.1.2. Cell polarity

The establishment of cellular migratory polarity can be achieved by two fundamentally different ways: induced polarization and spontaneous polarization. In the first case, cells polarize in response to gradient of mitogens or growth factors and migrate to places with higher concentration of stimulants. Cell polarization is induced also after wounding the cell monolayer. Cells recognize the space created by the wound, form new protrusion and polarize toward the wound (Etienne-Manneville, 2004). In later case and in contrast to induced polarization, spontaneous polarization occurs in a uniform concentration of stimuli without any directional signal. The prototypical example of spontaneous polarization is cell spreading on the ECM proteins where the direction in which the cell polarizes and migrate is random. In spreading cells, the establishment of polarity is preceded by breaking of radial symmetry. Depending on the cell type, symmetry breaking may be induced either by establishment of the cell front characterized by high protrusivity or by retraction of prospective cell rear (Cramer, 2010). In neutrophils the symmetry breaking is initiated by actin polymerization and protrusion formation that forms the leading edge of the cell (Wong et al., 2006; Xu et al., 2003). On the contrary, fibroblasts and fish keratocytes defines the cell rear at first in actomyosin dependent manner (Mseka et al., 2007; Yam et al., 2007) . The important role of myosin has been also observed in spreading CHO cells, where activated myosin II create a region of stable actomyosin bundles anchored in stable large focal adhesions. This region is depleted on GEFs for Rac1 and Cdc42 and thus it forms stable extended rear. Cell front is then formed on the other side of the cell in zones rich in Rac and Cdc42 that drive formation of protrusions (Vicente-Manzanares et al., 2008; Vicente-Manzanares et al., 2011).

In our work we used both induced and spontaneous models of cell polarization. We observed that both symmetry breaking of radially spreading cells and cellular polarization toward the wound required adhesion and integrin mediated signaling (Maninová and Vomastek, submitted to FEBS Journal, Klimová et al., submitted to BBA-Molecular Cell Research).

2.1.3. Cell adhesion

The adhesion is mediated by family of migration-promoting proteins called integrins (Hynes, 2002; Ridley et al., 2003). Integrins are heterodimeric transmembrane receptors composed of α and β chains that through their large extracellular domains bind proteins of ECM and *via* adaptor proteins such as talin link them to the actin fibers. The association of integrins with adaptor proteins and signaling molecules dynamically regulates the affinity of integrins. These interactions induce the conformational changes of integrin extracellular domains and switch them from low to high affinity state (Carman and Springer, 2003; Kim et al., 2003). Consequent binding of ECM proteins to integrins induces other conformational changes that alter the interactions between integrins α and β chains on the cytoplasmic side (Emsley et al., 2000). It leads to integrin clustering and initiates intracellular signaling regulating adhesion, cytoskeleton dynamics and cell polarity (Geiger et al., 2001). Thus integrins have dual function: they mechanically link the actin cytoskeleton and proteins of extracellular matrix and they also convert ECM binding to intracellular signal (Geiger et al., 2001).

The structural role of integrins is manifested by formation of focal adhesions. The cell attachment to the substrate initiates the formation of small dynamic adhesions that contain structural proteins talin and paxillin. Consequently, other structural components such as α-actinin and vinculin are incorporated to these adhesions and they start to grow in size and elongate forming stable anchoring sites (Laukaitis et al., 2001). This process termed maturation of focal adhesions occurs in response to high tensional forces generated by acto-myosin contraction and, conversely, decrease in the contractility results in focal adhesions disassembly (Laukaitis et al., 2001; Zaidel-Bar et al., 2003; Gupton and Waterman-Storer, 2006; Tomar and Schlaepfer, 2009). Thus the linkage between integrins and actin cytoskeleton is crucial for the integrity of focal adhesions (Zhang et al., 2008). Adhesions could also be destabilized by phosphorylation or proteolysis of their structural protein components (Carragher et al., 2003; Franco and Huttenlocher, 2005; Franco et al., 2004, Vomastek et al., 2007; Webb et al., 2004).

2.1.3.1. Integrin signaling

In wound healing assay the integrin engagement to ECM has been suggested to represent the primary polarity signal that induces cell polarization (Etienne-Manneville, 2004; Etienne-Manneville and Hall, 2001, 2002). Adhesion to ECM and integrin clustering initiates the intracellular signals such as tyrosine phosphorylation, phosphatidylinositol signaling and activation of small GTPases. As integrins do not exhibit any inherent catalytic activity, the signals of ECM-integrin binding have to be transmitted through the activation of integrin-associated proteins (Mitra and Schlaepfer, 2006). The main component of integrin signaling is focal adhesion kinase (FAK).

FAK is evolutionary conserved protein-tyrosin kinase consisting of several distinct domains that regulate its activity and subcellular localization (Fig. 2.2). In inactive state, the N-terminal FERM domain bind centrally located catalytic tyrosine kinase domain and inhibit FAK kinase activity. FAK activity can be also controlled by binding partners that associate with FERM domain and enable the release of FERM domain from catalytic domain thus facilitating FAK activation. However, even in autoinhibited conformation the FERM domain may also associate with Arp2/3 actin nucleating complex. By this mechanism FAK promotes recruitment of Arp2/3 to adhesions, links integrins with the actin polymerization and facilitates the extension of the leading edge (Tomar and Schlaepfer, 2009). The C-terminal FAT (focal adhesion targeting) domain and proline-rich regions serve as a docking sites for other proteins that regulate FAK localization and signaling. Proteins talin and paxillin that associate with FAT domain recruit FAK to focal adhesions thus FAT domain is crucial for FAK targeting to focal adhesions (Zhao and Guan, 2011).

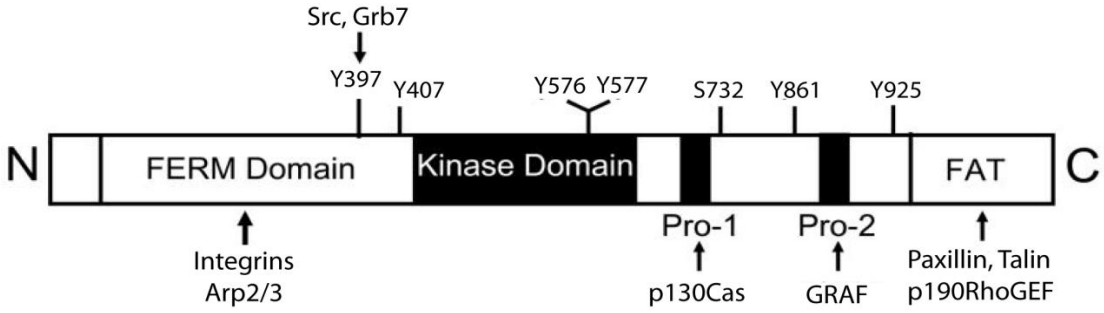

Fig. 2.2.: Molecular structure of FAK. FAK is composed of several domains (FERM, Kinase domain, FAT domain and proline rich regions) that regulate FAK activity, localization and binding with interacting partners. Several phosphorylation sites and regions which mediate binding to other proteins are indicated (adapted from (Zhao and Guan, 2011)).

The activation of FAK requires its targeting to focal adhesions where the interaction of auto-inhibitory FERM domain with β chain of integrins enables the auto-phosphorylation of FAK at tyrosine residue 397 (Schaller et al., 1995). Phosphorylation of tyrosine 397 creates docking sites for the recruitment and activation of Src family kinases which in turn phosphorylates FAK on several tyrosine residues further increasing FAK activity and creating binding sites for other FAK associated proteins (Lietha et al., 2007).

The signaling downstream of FAK is tightly connected with the regulation of activities of small Rho GTPases. By its association with GAPs and GEFs FAK facilitates spatiotemporal regulation of Rho GTPases and promote the leading edge formation, adhesions turnover, tail retraction, and also Golgi repositioning and cell polarization (Frame et al., 2010; Iwanicki et al., 2008; Tilghman et al., 2005; Tomar and Schlaepfer, 2009).

2.1.3.2. FAK/Src signaling in migrating cells

FAK/Src signaling controls the activation of many signaling pathways that regulate diverse cellular processes. However, FAK/Src mediated regulation of small GTPases Rac1, Ras and RhoA appears to be central for the regulation of cell migration.

FAK interaction with p130Cas (Cary et al., 1998; Sieg et al., 1999) mediates the increase of Rac1 activity. The mechanism involves phosphorylation of p130Cas that enables its association with SH2 domain containing protein Crk and formation of p130Cas/Crk complex. This complex then through GEF DOCK180 activates Rac1 (Cho and Klemke, 2000; Schlaepfer et al., 1999). Recruitment of Crk is mediated also by adaptor protein paxillin in the same manner as by p130Cas (Turner, 2000). Other signaling pathway leading to Rac1 activation is through PI3 kinase. PI3K and adaptor protein Grb7 has been shown to interact with FAK and cells with selective disruption of this binding by FAK mutant failed to promote cell migration (Reiske et al., 1999).

The other small GTPase targeted by FAK/Src signaling is RhoA. Active FAK regulates the activity or localization of several RhoGEFs and RhoGAPs and thus enables cycling between active and inactive state of RhoA. FAK has been shown to directly associate with Rho GEFs p190-RhoGEF (Lim et al., 2008) and PDZ-RhoGEF and LARG (Chikumi et al., 2002) that activates Rho. FAK is also in complex with Rho GAPs p190A-RhoGAP (Tomar et al., 2009), PS-GAP (Ren et al., 2001) and GRAF (Hildebrand et al., 1996) that promote Rho inactivation.

p190A-RhoGAP represents the major mechanism that downregulates the Rho activity in adhesion and FAK dependent manner. p190A-RhoGAP is recruited to focal adhesions and to FAK through its association with p120-RasGAP and both p120-RasGAP and p190-RhoGAP have been shown to regulate actin cytoskeleton dynamics in a FAK-dependent manner (Arthur et al., 2000; Roof et al., 1998; Tomar et al., 2009). The significance of GRAF and PS-GAP in Rho and cytoskeleton regulations by FAK is not fully clarified.

FAK signaling through Rho-GEFs and subsequent increase in RhoA activity and acto-myosin contractility is connected with formation of stress fibers and focal adhesions, their stabilization or turnover at the leading edge and disassembly at the trailing rear (Burridge and Wennerberg, 2004; Defilippi et al., 1999; Gupton and Waterman-Storer, 2006; Iwanicki et al., 2008; Lim et al., 2008). PDZ-RhoGEF co-localize with FAK in focal adhesions and upon LPA stimulation PDZ-RhoGEF/Rho/ROCK signaling promotes focal adhesions movement at the cell rear and the rear retraction (Iwanicki et al., 2008). p190-RhoGEF also localize to focal adhesions and together with FAK regulate focal adhesions turnover and cell migration (Lim et al., 2008; Zhai et al., 2003). The situation at the leading edge is more complex as FAK/Src signaling can elevate simultaneously RhoGAP and RhoGEF activities. Src mediated phosphorylation of FAK increases the activity of both p190A-RhoGAP and p190-RhoGEF (Huveneers and Danen, 2009; Lim et al., 2010; Lim et al., 2008; Tomar and Schlaepfer, 2009). However, recruitment of these proteins to the leading edge seems to be sequential as p190A-RhoGAP associates with FAK at initial phases of integrin engagement whereas p190-RhoGEF at later time points (Tomar and Schlaepfer, 2009). These data suggested that negative and positive Rho regulation is temporally regulated and led to the simple model that describes cycles of lamellipodia extension and its stabilization during cell migration. According to this model at early stages of cell spreading FAK and p190A-RhoGAP mediate Rho inhibition that enables actin polymerization and pushes lamellipodium forward. Subsequent activation of p190-RhoGEF and RhoA facilitates the increase of cell contractility and lamellipodium stabilization (Ren et al., 1999; Tomar and Schlaepfer, 2009).

The other target of FAK/Src signaling is small GTPase Ras that subsequently activates Raf and MEK/ERK signaling cascade (Ishibe et al., 2003; Schlaepfer et al., 1999; Slack-Davis et al., 2003). ERK regulates wide spectra of cellular processes that determine the cell fate, however, in conjunction with cell migration it is important that ERK localizes to focal adhesions (Fincham et al., 2000; Vomastek et al., 2007). In focal adhesions ERK mediates phosphorylation of p190A-RhoGAP and decreases its activity and thus increase the activity of RhoA. Higher acto-myosin activity then promotes focal adhesions maturation (Pullikuth and

Catling, 2010). ERK also phosphorylates and activates MLCK (Myosin light chain kinase) (Klemke et al., 1997). Consequent increase in the acto-myosin contractility has been shown to initiate adhesions disassembly (Webb et al., 2004). Thus, depending on the context ERK promotes both focal adhesions maturation and disassembly. ERK mediated adhesions disassembly is prominent at the leading edge where ERK drives adhesions turnover and fosters cell migration (Doan and Huttenlocher, 2007; Pullikuth and Catling, 2010; Vomastek et al., 2007; Webb et al., 2004).

Last but not least, FAK mediates the recruitment of cellular proteases such as calpains. They cleave focal adhesions constituent proteins leading to adhesion destabilization. This mechanism has been shown to facilitate retraction of trailing edge and to modulate lamellipodial dynamics at the leading edge (Carragher et al., 2003; Franco and Huttenlocher, 2005; Franco et al., 2004).

Taken together, cell adhesion to ECM stimulates integrin and FAK/Src signaling that subsequently regulates small GTPases Cdc42, Rac1, RhoA and Ras. Dynamic and spatially restricted regulation of activities of these small GTPases at the cell front and rear drives the reorganization of cell adhesions and cytoskeleton across the whole cell, and ultimately results in establishment of polarized profile that is optimal for cell migration.

2.2. CELL CYTOSKELETON IN MIGRATING CELLS

Cell cytoskeleton is the main regulator of cell motility that during the process of cell polarization and migration undergoes continuous remodeling and determines the shape and mechanical properties of the cell. Cytoskeleton reorganization during cell polarization enables to regulate the position of individual organelles within the cells and provide a path for intracellular vesicle trafficking. Both microtubules and actin cytoskeleton play significant role in cell polarization and migration in wide spectra of cells. As microtubules and IFs do not contribute significantly to nuclear reorientation in our cellular system they are discussed only in general principles how they promote cellular polarity. This thesis is focused on actin cytoskeleton.

2.2.1. Microtubules

The essential role of microtubules in cell polarity has been already shown several decades ago when interference with microtubules disrupted polarized morphology of cells and

decreased the directionality and speed of migration (Liao et al., 1995; Vasiliev et al., 1970). Microtubules consist of α and β tubulin heterodimers forming a protofilaments that then form a hollow tube. In migrating cells, microtubules minus ends are anchored in MTOC while their plus ends polymerize and grow fast to the cell periphery where they can be captured and stabilized. This arrangement gives microtubules their inherent polarity that is recognized by kinesin and dynein motor proteins that deliver cargo along the microtubules. In addition, in most migrating cells microtubules are also distributed in asymmetric manner. They extend more to the cell front then to the cell rear and promote MTOC relocalization in front of the nucleus toward the leading edge which results in polarized microtubules meshwork. In addition to MTOC, Golgi apparatus that form ribbon-like structure around MTOC also relocalizes in front of the nucleus and it facilitates cargo delivery toward the leading edge.

Microtubules are selectively captured and stabilized at cortical sites (Kaverina and Straube, 2011; Manneville et al., 2010; Watanabe et al., 2005). Stabilization of microtubules is regulated by posttranslational modifications when stable microtubules accumulate acetylated and detyrosinated tubulin that strengthens their stability (Bulinski and Gundersen, 1991; Gundersen and Bulinski, 1988; Matov et al., 2010; Westermann and Weber, 2003). Plus end motor kinesin display higher processivity toward the stable microtubules and thus stable microtubules are thought to provide a unique track for directed vesicle trafficking toward the leading edge (Watanabe et al., 2005). Directed vesicle trafficking is also necessary for microtubule stabilization suggesting that MTOC and Golgi repositioning and directed vesicle trafficking represents a positive feedback loop further increasing specific processes at the leading edge of migrating cells supporting directional cell migration (Prigozhina and Waterman-Storer, 2004).

2.2.2. Actin cytoskeleton

Similarly to microtubules actin is also asymmetrically distributed in migrating cells. Monomeric G-actin and dendritic actin meshwork is located at the leading lamellipodium whereas in the cell body actin forms stress fibers. In migrating non-muscle cells monomeric actin accumulates in dynamic protruding regions where is implicated in the formation of actin-rich pseudopodial domains and may contribute to continual formation of lamellipodia and to maintenance of directional migration (Le et al., 1998; Mounier et al., 1997; Nabi, 1999). In migrating cells actin forms a wide range of different filament assemblies. Several of them play significant role in cell motility e.g. tight parallel actin bundles push the cell

membrane and form filopodia, lamellipodial branched network of short filaments generate physical force for protrusion formation at the leading edge and several classes of contractile actin stress fibers in the cell body – ventral stress fibers, dorsal stress fibers, transverse actin arcs and perinuclear actin cap fibers generate contractile forces (Figs.2.3 and 2.4).

As already mentioned in the chapter about small Rho GTPases, branched actin network formation at the lamella is mediated by Arp2/3 complex of actin nucleating proteins that initiates new filament polymerization from the sides of preexisting filaments (Svitkina and Borisy, 1999). This dendritic actin network serves also a base for the formation of parallel actin filaments that elongate at their barbed ends and form filopodial actin bundles (Svitkina et al., 2003). The accelerated polymerization of filopodial actin filaments is facilitated by Ena/VASP proteins and by formin mDia2 (Bear et al., 2002; Higashida et al., 2004).

Actin stress fibers are composed of short filaments crosslinked by α-actinin and together with myosin both proteins are periodically distributed along the fibers. The actomyosin generated contractility is the main characteristic of many but not all stress fibers. Ventral stress fibers lie on the basal side of the cell and are attached to integrin focal adhesions at each end. They are rich in α-actinin that crosslink the actin fibers and in myosin that mediates the contractility. Ventral fibers are usually aligned with the direction of migration and regulate diverse cellular functions such as establishment of front-rear polarity (Vicente-Manzanares et al., 2008; Vicente-Manzanares et al., 2011) and cell shape on compliant substrates (Kumar et al., 2006). Ventral fibers play important role in cell adhesion and contraction as they generate the traction forces and mediate the trailing edge retraction (ChrzanowskaWodnicka and Burridge, 1996; Iwanicki et al., 2008) and may also participate in remodeling of extracellular matrix (Pellegrin and Mellor, 2007). Mechanism of ventral fibers formation is still under discussions. They can be formed by reorganization of preexisting network of dorsal fibers and transverse arcs (see below (Hotulainen and Lappalainen, 2006; Naumanen et al., 2008). Alternatively, annealing of short actin bundles anchored in focal adhesions may also generate ventral fibers (Zimerman et al., 2004). Both proposed mechanisms may be involved as it has been observed that two dorsal fibers polymerizing from opposite sides of lamella can fuse and form ventral stress fiber (Hotulainen and Lappalainen, 2006).

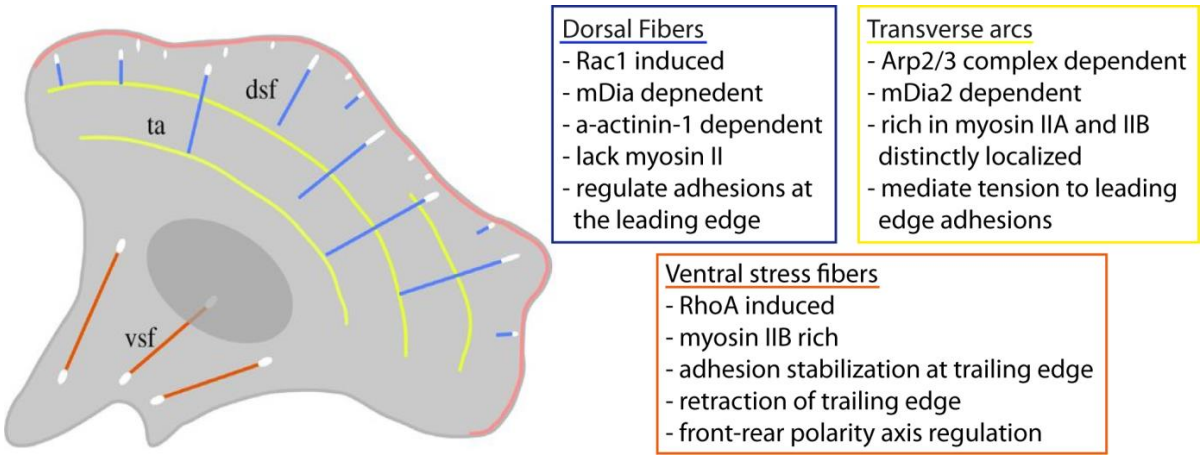

Fig. 2.3.: Schema of actin stress fibers subtypes and their anchorage to the substrate in migrating cell. Individual stress fibers are pseudocolored as indicated (dorsal fibers – dsf- blue; transverse arcs – ta- yellow and ventral stress fibers – vsf – orange). If the fibers are attached to the substrate, it is indicated by white ovals. Molecular differences between individual types of fibers and subtype-specific cellular activities are summarized in pseudocolored boxes. Adapted from (Vallenius, 2013).

While ventral fibers are confined to the basal side of the cell other types of stress fibers – dorsal fibers, transverse arcs and perinuclear actin cap fibers extend from the ventral side to the dorsal side of the cell. Dorsal fibers are anchored in focal adhesions at the leading edge and polymerize to the dorsal side where they interacts with transverse arcs at their proximal ends (Hotulainen and Lappalainen, 2006). Although it is tempting to think that dorsal fibers polymerize at their free end rising to the dorsal side of the cell it seems that their polymerization is driven in focal adhesions in a RhoA and mDia1 formin dependent manner (Hotulainen and Lappalainen, 2006). Dorsal fibers are rich in α-actinin-1 that is also necessary for their de novo formation (Kovac et al., 2013) and are devoid of myosin II (Burnette et al., 2014; Hotulainen and Lappalainen, 2006; Kovac et al., 2013). Due to absence of myosin II they represent the exception between stress fibers as they are not contractile. However, dorsal fibers can incorporate myosin usually when they associate with transverse arcs (Hotulainen and Lappalainen, 2006).

Curve-shaped transverse actin arcs are contractile actomyosin bundles that are oriented parallel with the leading lamellipodium and are not associated directly to focal adhesions. They are generated from actin filaments originating in Arp2/3 nucleated lamellipodial meshwork. When the plasma membrane undergoes cycles of extension and retraction during the protrusion, the retraction causes the association of precursor filaments with myosin to form short contractile actin bundles. These bundles fuse end-to-end and elongate, and then flow centripetally from the leading edge to the cell center in front of the nucleus where they disappear (Blanchoin et al., 2014; Burnette et al., 2011; Hotulainen and Lappalainen, 2006;

Kovac et al., 2013). Transverse arcs contain distinctly localized myosin IIA and myosin IIB with myosin IIA being dominant in lamellipodial filaments and myosin IIB occupying filaments in the lamella and cell body (Kovac et al., 2013; Vallenius, 2013). Transverse arcs contain also α-actinin that is periodically distributed in a sarcomeric-like arrangement (Burnette et al., 2014). In all probability, transverse arcs and dorsal fibers are directly connected or crosslinked (Hotulainen and Lappalainen, 2006; Tojkander et al., 2012) and they move together at the same velocity towards the cell center (Burnette et al., 2014). The mechanical linkage with dorsal fibers anchors the transverse arcs indirectly to the substrate. Concurrently transverse arcs impose the pulling forces to the dorsal fibers and promote maturation of focal adhesions and remodeling of ECM (Hotulainen and Lappalainen, 2006; Kovac et al., 2013; Skau et al., 2015). In addition, dorsal fibers anchored in focal adhesions generate resisting forces to actin arcs and these forces flatten the cell lamella at the cell front (Burnette et al., 2014).

In contrast to dorsal fibers and actin arcs that are typical for the cell front, perinuclear actin cap fibers extend from the cell front to the cell rear. Firstly observed in 1979 as an actin sheath on the dorsal side of the cell (Zigmond et al., 1979) (Fig. 2.4) it was rediscovered in 2009 and described as a highly dynamic structure composed of contractile actin fibers that emanate from focal adhesions at the leading edge, extend over the nucleus and terminate in focal adhesions at the cell rear (Khatau et al., 2009). In addition to focal adhesions, perinuclear actin cap fibers are also anchored to the nucleus *via* LINC (Linker of Nucleoskeleton and Cytoskeleton) complex that links actin fibers with nuclear lamina (Starr and Fridolfsson, 2010). Together with LINC complex, actin cap fibers are important in nucleus shaping as disruption of actin cap induces nucleus bulging (Khatau et al., 2009).

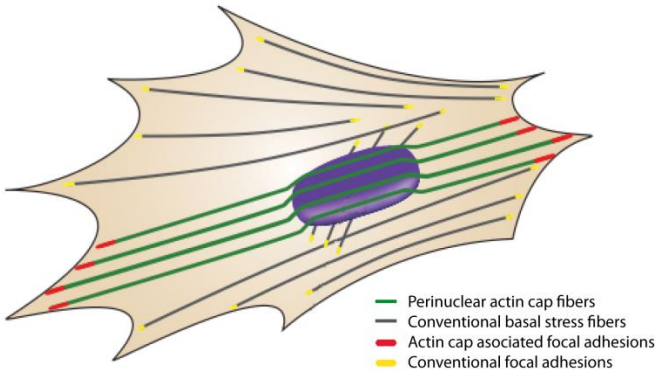

Fig.2.4.: Schematic picture of perinuclear actin cap fibers in adherent cell. Both ventral stress fibers and actin cap fibers are attached in focal adhesions at both ends. While the ventral fibers (grey) lie on the basal side of the cell, perinuclear actin cap fibers (green) emanate to the dorsal side and are exposed over the nucleus in pole-to-pole manner. Perinuclear actin cap is anchored in actin cap associated focal adhesions (red) that differ from conventional focal adhesions (yellow) in shape, dynamics and protein levels. Adapted from (Kim et al., 2013).

Actin cap fibers are attached in focal adhesions termed actin cap associated focal adhesions (ACAFAs) that are significantly larger and more elongated than conventional adhesions that anchors basal stress fibers. ACAFAs are more dynamic and are characterized by higher translocation speed than other focal adhesions (Kim et al., 2012). As perinuclear actin cap fibers are linked to the nucleus, actin cap fibers and ACAFAs are under higher tension than other focal adhesions. Higher tension acting on ACAFAs may promote their higher dynamic turnover and mediates higher mechanosensing response to changes in extracellular substrate rigidity (Kim et al., 2013; Kim et al., 2012). Moreover, actin cap fibers linking the extracellular milieu with the cell nucleus provides continual pathway for ultrafast mechanotransduction (Chambliss et al., 2013; Kim et al., 2013).

It has been proposed that perinuclear actin cap could form at the nuclear surface or that individual fibers polymerize from focal adhesions (Kim et al., 2013). However, the mechanism how perinuclear actin cap fibers are formed remains to be determined.

2.3. NUCLEUS MOVEMENT IN MIGRATING CELLS

The position and orientation of the nucleus is precisely specified and tightly regulated within the cells. It plays a critical role in the establishment of cell polarity and cell migration, as in other cellular and developmental processes such as fertilization, cell division and differentiation.

Nucleus movement and rotation was firstly observed 60 years ago in human nasal mucosa cells (Pomerat, 1953) and subsequently in other cell types as in HeLa cells (Leone et al., 1955), nerve cells (Lodin et al., 1970; Nakai, 1956) and in skeletal muscle cells (Capers, 1960). The nuclei migrated linearly through the cytoplasm or rotated around its axis clockwise or counterclockwise or perpendicular to the substrate. Initially, nuclear rotation was observed as a three-dimensional motion of chromatin domains associated with nucleoli. This observation led to the conclusion that the nuclear rotation is an expression of karyoplasmic streaming related to changes in gene expression (Fung and Deboni, 1988). Nevertheless, another reports suggested that the whole cell nucleus rotates as the nucleoli maintained the rigid pattern during the rotation (Paddock and Albrechtbuehler, 1986, 1988). This has been confirmed in a work showing that the nuclear rotation involves the movement of entire nucleus including nuclear interior as well as the inner and outer nuclear membranes (Ji et al., 2007).

There are two ways how nucleus moves within the cells. It can translocate linearly through the cytoplasm as described in neuroepithelial cells where nucleus undergo apico-basal interkinetic migration or in mammalian muscle cells. In syncytial myofibers the nuclei cluster beneath the postsynaptic membrane at neuromuscular junction (Burke and Roux, 2009; Dupin and Etienne-Manneville, 2011). Also in migrating cells the nucleus can move in such a way as nucleus moves rearward in number of cultured cell types (Dupin et al., 2011; Gomes et al., 2005; Luxton et al., 2010).

The other type of nuclear movement is its rotation. The rotational movement of the nucleus has been observed in several cell lines under different circumstances, however, its significance remained unclear. Nuclear rotation has been described as a continuous rotation sometimes exceeding 360° around or as a "jostling", "spinning" or "rocking" of the nucleus back and forth (Bard et al., 1985; Houben et al., 2009; Levy and Holzbaur, 2008; Paddock and Albrechtbuehler, 1986). The nuclear rotation that could persist for more than 360 degrees has also been observed in Swiss 3T3 fibroblasts exposed to mechanical shear stress (Lee et al., 2005) or in NIH3T3 fibroblast migrating into the wound (Levy and Holzbaur, 2008). Although we also observed such persistent nuclear rotation in NIH3T3 cells, it was evident only in a small fraction of cells migrating into the wound and such a rotation was never observed in RAT2 cells. Nuclear rotation in RAT2 fibroblasts, termed nuclear reorientation occurred only temporally during wound induced cell polarization (Maninova et al., 2013). Whether the nuclear rotation and reorientation are functionally similar remain unclear. In addition, the molecular players seem to vary according cell types. Shift from relatively static to rotating nucleus can be induced by several factors. Disruption of intermediate filaments by acrylamide induces constant nuclear spinning (Hay and Deboni, 1991). Knockdown of several genes (ACP5, ARHGAP26, CDC2L1, DMPK, NEDD9 and VEGFC) induces persistent nuclear rotation in wound-edge epithelial cells (Simpson et al., 2008), similar to that seen in cells deficient in myosin IIB or treated with myosin II inhibitor blebbistatin (Levy and Holzbaur, 2008; Vicente-Manzanares et al., 2007). In addition, uncontrolled nuclear spin was observed in cells deficient in Lamin B1 (Ji et al., 2007) or Vimentin (Gerashchenko et al., 2009). These data suggest the existence of complex "motor and brake" mechanism as postulated recently (Gerashchenko et al., 2009) that could be dynamically regulated in adherent cells.

Observations of nucleus movement within the cells also raised the question whether nucleus is moving through the cytoplasm by passive mechanism or if its movement can be actively regulated. In the model of passive translocation the cytoskeletal structures would

undergo such reorganization that the nucleus would be simply displaced. The active mechanism is based on the mechanical linkage between the cytoskeletal components and the nucleus. This model also presupposed the involvement of molecular motor proteins that are associated directly or indirectly with the nuclear envelope (Burke and Roux, 2009).

2.3.1. Nucleus-cytoskeleton mechanical coupling – LINC complex

It has been shown in many cellular systems that the nucleus movement that enables to take up a correct position and orientation is active process where connection to structural components that generate forces is necessary. Many studies showed the requirement for microtubules and microtubule motor proteins, other studies described the involvement of actin cytoskeleton, however, in neither case the mechanism(s) has not been fully clarified. The linkage to the cytoskeletal structures is mediated by nuclear envelope proteins forming transmembrane bridge termed LINC (Linker of Nucleoskeleton and Cytoskeleton) complex. Major constituents of LINC complex are Sun (Sad/UNC-84) and Nesprin (Nuclear envelope spectrin repeat) protein families that comprise proteins that span the inner and the outer nuclear membrane and connect the cytoskeleton with the nuclear lamina (Fig. 2.5.) (Crisp et al., 2006; Mellad et al., 2011; Oestlund et al., 2009; Razafsky and Hodzic, 2009; Starr and Fridolfsson, 2010).

2.3.1.1. SUN proteins and Nesprins

Mammalian cells express two SUN domain proteins, Sun1 and Sun2 (Malone et al., 1999). Both proteins localize to the inner nuclear membrane where they interact with proteins of nuclear pores and with lamin A (Crisp et al., 2006; Haque et al., 2006). Sun1 has been also shown to bind human membrane-associated histone acetyltransferase (hALP) (Chi et al., 2007). C-terminal SUN domain of Sun proteins extend to the perinuclear space and interacts with conserved KASH domain of nesprin proteins that span the outer nuclear membrane thus SUN proteins contribute to nesprins localization to the nuclear envelope (Crisp et al., 2006; Padmakumar et al., 2005). N-terminus of nesprins is exposed to the cytosolic part of the cell where nesprins interact with individual cytoskeletal structures. In this way, SUN proteins and nesprins mediate the physical connection between the cytoskeleton and the nuclear lamina (Crisp et al., 2006).

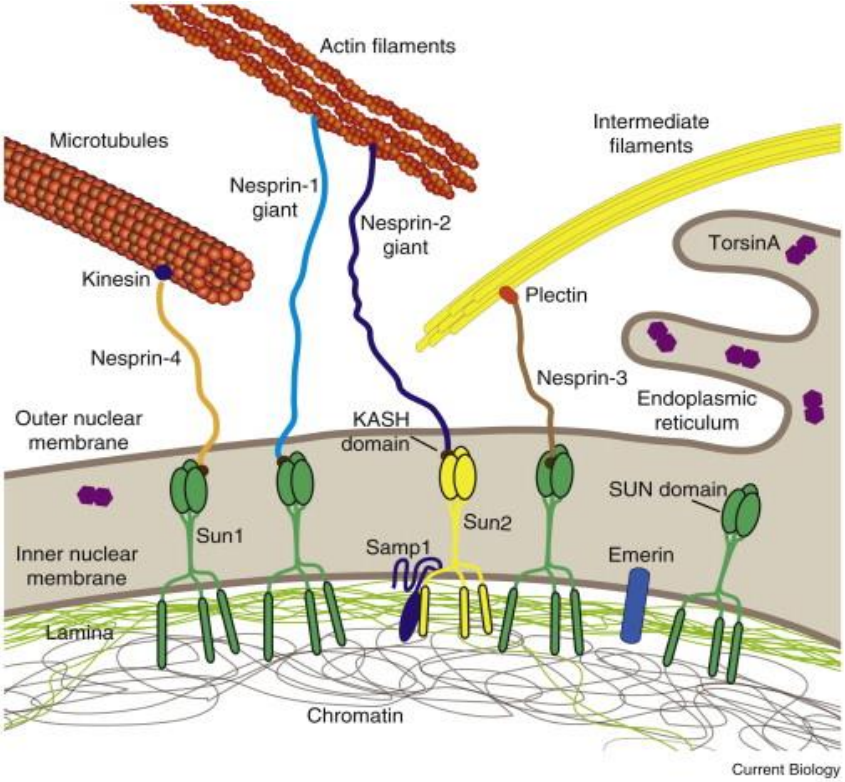

Fig. 2.5.: Schematic picture of nucleo-cytoskeleton linkage mediated by LINC complex. Sun and Nesprin proteins spanning the inner and outer nuclear membranes respectively, interact in perinuclear space. Sun proteins associate with nuclear lamina and other lamina associated proteins. Nesprin proteins interact directly or indirectly with individual cytoskeletal structures thus Nesprin and Sun proteins form mechanical connection between the nucleus and cell cytoskeleton (Isermann and Lammerding, 2013).

There are several isoforms of nesprins. They can be connected to microtubules and centrosome through kinesins and dyneins and the interaction of these motor proteins with nesprin's cytoplasmic spectrin-repeat domains (Wilhelmsen et al., 2005; Zhang et al., 2007; Zhang et al., 2009). The largest nesprin-1 and nesprin-2 isoforms (~1000 and ~800 kDa, respectively) contained calponin homology domain on their N-terminus allowing them to interact with filamentous actin in the cytoplasm (Padmakumar et al., 2004; Zhang et al., 2001; Zhen et al., 2002). Nesprin-3 is smaller than nesprin-1 and -2 (~110 kDA) and instead of actin-binding domain has a binding domain for plectin, a cytolinker that enable the interaction with intermediate filaments (Ketema et al., 2007; Wilhelmsen et al., 2005). Nesprin-4, expressed only in secretory epithelial cells, binds kinesin-1 and link the nucleus to microtubules (Roux et al., 2009; Zhang et al., 2007). Other member of LINC complex, emerin, is preferentially located in the inner nuclear membrane (Manilal et al., 1998) linking lamin A/C with nesprins (Mislow et al., 2002; Vaughan et al., 2001; Zhang et al., 2005), however, its significant fraction was also observed in the outer nuclear membrane where associates directly with centrosome (Salpingidou et al., 2007).

2.3.1.2. Lamins and nuclear movement

Together with LINC complex, nuclear lamins play key role during the nucleus movement. Lamins are intermediate filaments that form a network at the periphery of the nucleus providing mechanical support for nuclear membranes and structural stability of the nucleus. Fraction of lamins, especially lamin A/C, is also localized throughout the nucleoplasm in interphase nuclei. Major constituents are A-type lamins (lamin A and C are different splice variants encoded by one gene) and B-type lamins (lamin B1 and B2 are encoded by different genes) that has been shown to form separate filament networks with numerous contacts between them (Goldberg et al., 2008; Moir et al., 2000; Shimi et al., 2008). Nuclear lamins also associate with lamina associated sequences and chromatin domains and they are involved in chromatin organization and transcription regulation (Guelen et al., 2008; Shimi et al., 2008).

Nuclear lamins assist to LINC complex during nucleus movement. Nuclear localization of UNC84, a homolog of SUN protein in *Caenorhabditis elegans*, is lamin dependent (Lee et al., 2002) and nuclear lamina disruption impairs nuclear positioning and cell polarity establishment in photoreceptor cells in *Drosophila* eye and in oocytes (Guillemin et al., 2001; Patterson et al., 2004). Although the localization of SUN1 and nesprin-1 and -2 seems not to be affected in lamin A depleted mammalian cells (Folker et al., 2011; Haque et al., 2006), there is evidence that lamin A play role in the connection between the nucleus and cytoplasm in mammals as lamin A deficiency caused loss of nesprin-3 and emerin from the nuclear envelope (Houben et al., 2009). Lamin A depleted cells revealed delayed reorientation of the nucleus and reduced ability of these cells to polarize and to migrate to the wound (Houben et al., 2009; Lee et al., 2007; Luxton et al., 2010). Also MTOC was significantly separated from the nuclear envelope and the micromechanical properties (elasticity and viscosity) of the cytoplasm were dramatically affected in these cells (Lee et al., 2007). Lamin B has been also shown to have an effect in nucleus-cytoskeleton coupling when the absence of lamin B1 resulted in striking nuclear rotation that may be stopped by the overexpression of nesprin-1 (Ji et al., 2007). However, in nesprin-1 depleted cells nuclear rotation did not increased (Wu et al., 2011).

In conclusion, the disruption of nuclear anchorage to the cytoskeleton significantly affects the nuclear movement and cellular migration and transmission of intracellular forces (Folker et al., 2011; Houben et al., 2009; Lombardi et al., 2011; Luxton et al., 2010). As KASH-SUN proteins and their association with lamins transfer mechanical signals from the

plasma membrane directly to the chromatin the interruption of nucleus-cytoskeleton link may have far-reaching effect on cytoskeleton mediated cellular functions, tissue organization and can cause defects in development and diseases. The mechanical signaling from the extracellular environment through cell cytoskeleton and nuclear envelope to the chromatin domains is intensively studied and keeps expanding.

2.3.2. Forces involved in nuclear reorientation and rotation

Since the nucleus is by far the largest organelle in the cell, the nuclear rotation - and nuclear movement in general - it requires the force acting on the nucleus. All three types of cytoskeleton fibers, microtubules (MTs), actin filaments and intermediate filaments (IFs), seem to be implicated in nuclear rotation, although the importance of each in the process varies depending on cell type and experimental conditions used. The nucleus movement could be mediated by single component of cytoskeleton such as in the case of wound edge NIH3T3 fibroblasts where microtubules and dynein drives the nuclear rotation (Levy and Holzbaur, 2008). In other cases, interfering with the function of both MTs and actin cytoskeleton impedes nuclear rotation (Ji et al., 2007) suggesting that MTs and actin cytoskeleton may cooperate.

2.3.2.1. Actin and nucleus movement

Actin is the only component of cell cytoskeleton that interacts with the nuclear envelope directly. Actin has been shown to assist during nucleus movement in two different ways. It may act as a stabilization mechanism anchoring the nucleus in stable position or may generate forces that move the nucleus.

The linear array of transmembrane proteins nesprin2G (giant) and SUN2, called transmembrane actin-associated nuclear (TAN) lines, link the nucleus to the moving dorsal actin cables, transmit forces from actin retrograde flow and move the nucleus rearward (Luxton et al., 2010). The actin retrograde flow may be involved in nucleus positioning also in immobile cells as formation of anisotropic cell-cell contacts induces nucleus relocalization from the cell center toward cell-cell junctions (Dupin et al., 2009). The acto-myosin activity has been also shown to drive the nucleus movement in opposite direction. In neural precursors myosin II temporarily accumulates behind the nucleus (Bellion et al., 2005; Schaar and McConnell, 2005) and its activity push the nucleus forward (Schenk et al., 2009; Tsai et al.,

2007). Transmission of forces applied on integrins to the nucleus mediates also actin and these forces induce nucleus distortion (Maniotis et al., 1997b). In addition, actin coupling to the nucleus is crucial for proper mechanotransduction (Brosig et al., 2010).

As mentioned above, actin does not have to mediate only the nucleus movement but may serve as an anchoring system defending the nucleus to rotate as myosin inhibition was shown to increase nuclear rotation (Levy and Holzbaur, 2008; Vicente-Manzanares et al., 2007). Recently identified perinuclear actin cap, composed of actin bundles aligned along the longer axis of the cell, extends over the top of the cell forming a sheath. Actin cap is highly organized in nonmigrating and polarized embryonic fibroblasts and stabilize the nucleus in a proper specific location whereas the nucleus movement is mediated by microtubules and its motor protein dynein (Kim et al., 2014).

2.3.2.2. Microtubules in nucleus movement

Involvement of microtubules in nuclear positioning is well documented in several model organisms. For example, in *Caenorhabditis elegans* MTs drive the pronuclei migration in the zygote and nucleus movement in hypodermal and embryonal cells. MTs also mediate the movement of the nucleus during eye development in *Drosophila* photoreceptor cells (Burke and Roux, 2009; Dupin and Etienne-Manneville, 2011; Gundersen and Worman, 2013). First in mammalian cells, MTs have been shown to drive the nucleus relocalization after shear stress application (Lee et al., 2005). The association of MTs with the nuclear envelope is mediated by microtubule motor proteins dynein and kinesin and their interaction with nesprins that serve as recruitment factors for centrosomal MTs to the nuclear membrane (Gundersen and Worman, 2013; Starr and Fridolfsson, 2010; Tsujikawa et al., 2007; Zhang et al., 2007; Zhang et al., 2009). Dyneins are involved in nuclear movement in migrating neuronal precursors (Tsai et al., 2007; Umeshima et al., 2007) and drives nuclear rotation during fibroblast forward movement (Levy and Holzbaur, 2008). The involvement of kinesin in nucleus movement has been shown in hypodermal cells in *C. elegans* and during nuclear alignment in mammalian myotubes (Metzger et al., 2012; Meyerzon et al., 2009). Implication of both motors in nuclear movement in one system was demonstrated in neural stem cells where kinesin-3 and cytoplasmic dynein coordinate the interkinetic nucleus movement between the apical and basal surfaces (Tsai et al., 2010) suggesting that balance between individual motors and by them generated forces is necessary for precise nuclear positioning.

Question is from where these motors exert the forces that move with the nucleus. Dynein and its regulatory factors, dynactin and LIS1, are accumulated at the leading lamellipodia (Dujardin et al., 2003) and all three proteins were observed at the tips and alongside of microtubules (Vallee and Stehman, 2005). From these cortical and cytosolic anchoring sites MTs exert forces which mediate centrosome orientation. Centrosomal MTs and centrosome are, through dyneins and kinesins and their association with nesprins, anchored to the nuclear envelope (Wilhelmsen et al., 2005; Zhang et al., 2007; Zhang et al., 2009). In addition, emerin, integral protein of the inner nuclear membrane observed also in the outer nuclear membrane, has been shown to interact with β-tubulin anchoring directly the centrosome to the outer nuclear membrane (Salpingidou et al., 2007). From these findings follows one model which shows that the nucleus movement is regulated through the tightly coupled centrosome. Forces generated by MTs are transmitted through tightly connected centrosome to the nucleus and thus MT dynamic drives the centrosome reorientation and consequently the nucleus movement (Lee et al., 2005; Neujahr et al., 1998). This mechanism probably operates in neurons, where MTs form a cage-like network that converges in centrosome in front of the nucleus. Movement of centrosome forward then induces displacement of the nucleus wrapped in MTs (Solecki et al., 2004).

In another studies nuclear movement has been shown as a dynein dependent (Brodsky et al., 2007; Tsai et al., 2007) but the nucleus rotated independently on centrosome (Gerashchenko et al., 2009; Levy and Holzbaur, 2008; Tsai et al., 2007; Umeshima et al., 2007; Wu et al., 2011). The centrosome separation from the nucleus upon nocodazole or taxol treatment indicates that the link between them is much weaker, temporary or completely absent (Dupin et al., 2009; Gerashchenko et al., 2009; Tsai et al., 2007). The centrosome-independent nuclear movement require dynein at the nucleus that pull the nucleus as a huge cargo along the microtubules (Levy and Holzbaur, 2008; Wu et al., 2011). For this mechanism, strong accumulation of motor proteins and precise coordination of their activities would be required (Dupin and Etienne-Manneville, 2011). As the ability of the nucleus to rotate depends on the distance between the nucleus and the centrosome (longer distance increase the nuclear rotation) MTs may also serve as stabilizing system that fasten the nucleus in a specific position (Wu et al., 2011). Pushing forces that have been shown to move with the nucleus in actin dependent manner seem not to be possible via MTs. They play role *in vitro*, but they seem to be too weak for centrosome or nucleus movement in highly viscous living cells (Shekhar et al., 2013).

2.3.2.3. Intermediate filaments in nucleus movement

Involvement of intermediate filaments in nucleus mechanics was not so intensively studied as in the case of actin or microtubules. IFs have no motor protein thus their involvement in nuclear reorientation will be rather mechanical as they are interconnected with microtubules and actin (Wang et al., 2009). In astrocytes IFs facilitate actin-driven rearward movement of the nucleus. Actin retrograde flow induce accumulation of IFs in front of the nucleus and IFs subsequently transmit the forces to the nucleus and push it rearward (Dupin et al., 2009; Dupin and Etienne-Manneville, 2011). Involvement of IFs in nucleus positioning has been also shown during transmission of forces from ECM to the nucleus at low and high strains (Maniotis et al., 1997b). IFs may also serve as anchor for the nucleus as vimentin has been shown to inhibit the nuclear rotation (Gerashchenko et al., 2009).

Taken together, proper nucleus positioning within the cell is important for effective polarized migration. The mechanical coupling of the nucleus to the cell cytoskeleton is crucial for active nucleus movement. All three types of cytoskeletal structures seems to be involved in nucleus positioning, nevertheless, the mechanism differs across the wide spectrum of cells. This thesis is focused on nucleus rotational movement, termed nuclear reorientation, during polarization of RAT2 fibroblasts.

3. AIMS OF THE STUDY

The main goal of this thesis was to study the nuclear reorientation during polarization of RAT2 fibroblasts and describe the regulators of this process.

- The signaling pathways that regulate the establishment of cell polarity are well described however, the regulation of reorientation of cell nucleus during polarization remains to be clarified. One aim of my PhD study was to decipher the signaling pathways that control the nuclear reorientation during RAT2 fibroblasts polarization.

- In different cell types the nucleus movement is regulated by different parts of cytoskeleton. Involvement of microtubules or actin seems to be possible. Other aim of my study was to identify which part of cytoskeleton generates the forces that drive the nuclear reorientation in polarizing RAT2 cells and to describe the mechanism.

- The biological significance of nuclear reorientation is still not clear and the role of structures coupled with the nucleus, like LINC complex and perinuclear actin cap, is still intensively studied. Other goal of this thesis was to determine the significance of nuclear reorientation in migrating cells and to find out which role play the perinuclear actin fibers during nuclear reorientation and cell migration.

4. CONCISE OVERVIEW OF THE RESULTS

Parts of the results presented in this section are included in the attached manuscripts. To maintain the consistency of the content, this section contains also the unpublished results acquired during my PhD study. To present the data consistently and generally understandable, the section concerning symmetry breaking contain also results generated by my colleague PhD student Zuzana Klímová. This fact is indicated in the text where appropriate.

The reorientation of cell nucleus promotes the establishment of front-rear polarity in migrating fibroblasts (Maninová et al., 2013).

In the first manuscript we described the nuclear rotational movement termed nuclear reorientation which occurs during polarization of RAT2 fibroblasts. The nuclear reorientation occurs temporarily after the cell monolayer wounding when cells polarize and reorient their nuclei to the wound and thus to the direction of migration. In RAT2 cells the reorientation is evident as the nuclei have elliptical shape with aspect ratio (length/width) typically ranging from 1.5 – 2.0. We identified two signaling pathways that regulate the nucleus reorientation and we have shown, that the physical linkage of the nucleus to the cell cytoskeleton is necessary for the nuclear reorientation.

4.1. Nucleus reorientation in polarizing cells

To study the process of nuclear reorientation in polarizing cells we used the wound healing assay where cell polarization is induced by scratch made in confluent cell monolayer. In the monolayer, cells display non-polarized phenotype characterized by nucleus localization to the cell center and random localization of MTOC. Wounding cell monolayer by pipet tip induced synchronous formation of protrusions and spreading of the cells at the edge of the wound. In addition, cells polarizing 6 h to the wound had their nuclei close to the cell rear and MTOC was localized between the nucleus and the leading edge (Fig 4.1 B). Interestingly, in cell monolayer the orientation of longer nuclear axis was more or less random. In contrast, in polarized cells the longer nuclear axis was aligned with the axis of migration (Fig. 4.1 B). When we followed the cell polarization in living cells, we observed synchronous reorientation of nuclei towards the wound and to the direction of migration (Fig. 4.1 A). Importantly, cell nucleoli that have been shown to maintain constant position within the nucleus (Ji et al., 2007; Paddock and Albrechtbuehler, 1988) maintained the rigid pattern and rotated with the nucleus

indicating that the nucleus rotates as a whole and that nuclear reorientation is not result of mass streaming within the nucleus.

To further characterize the nuclear reorientation we defined an angle between the longer nuclear axis and the axis of the wound as a quantitative measure of nuclear reorientation (Fig. 4.1 C). Measurement of nuclear reorientation in different time points after wounding revealed that cells reorient their nuclei during first 4 hours after wounding and then maintain the orientation of the nucleus to the direction of migration and expand to the wound (Fig.4.1 D).

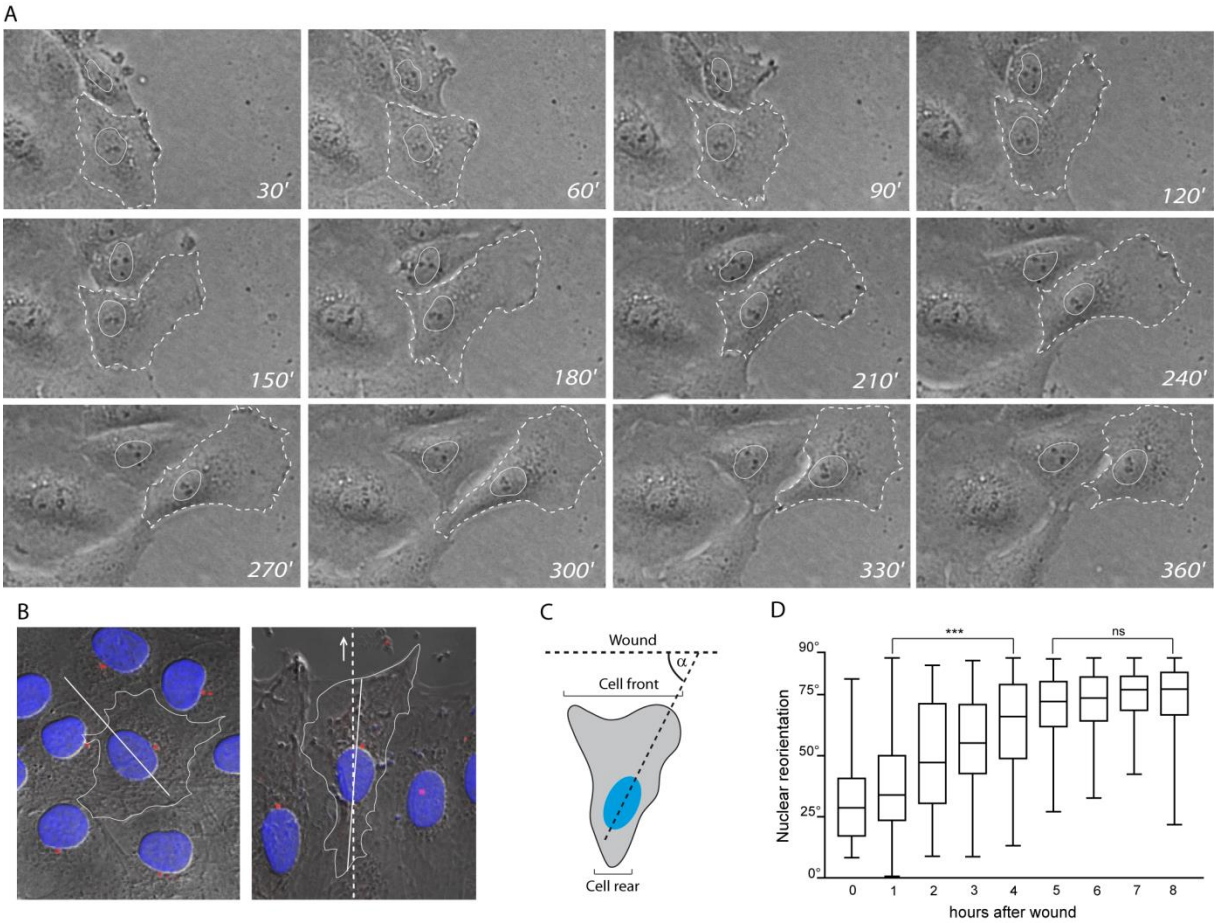

Fig. 4.1: Cell polarization and migration at the edge of the wound. (A) Still images from time-laps phase contrast microscopy recording spreading and nuclear reorientation in cells at the edge of the wound. Nuclei are indicated by white line. For better visualization the cell is outlined by dashed line. (B) Examples of typical cell shape and intracellular arrangement in non-polarized cell in cell monolayer (left panel) and in polarized cell 6 h after wounding (right panel). Cells were fixed in indicated time points and stained with γ-tubulin antibody (red) and counterstained with DAPI (blue). For better visualization cells are outlined. The longer nuclear axis is indicated by straight white line, dashed line indicates the axis of migration. (C) Schematic picture of nuclear reorientation measurement. Nuclear reorientation in cells at the edge of the wound is measured as an angle between the longer nuclear axis and the axis of the wound. (D) Time course of nuclear reorientation in cells polarizing to the wound and fixed in different time points after wounding. Data are presented in box and whiskers graph (*** $p<0.001$; ns – not significant). In each time point 100 cells was analyzed.

4.2. Nuclear reorientation is regulated by two antagonistic signaling pathways: LPA/Rho and Integrin/FAK/Src/p190A-RhoGAP signaling

It has been shown that active nucleus rearward movement during wound healing assay is stimulated by LPA (Gomes et al., 2005; Luxton et al., 2010). Thus we tested if LPA stimulation and its downstream signaling to RhoA induce also nuclear reorientation during cell polarization. We measured the nuclear reorientation in serum - deprived and LPA stimulated cells and in cells in which we interfered with Rho signaling by specific Rho inhibitor C3 exotransferase or by overexpression of constitutive active or dominant negative form of RhoA. In starved cells the nucleus reorientation was impaired and addition of LPA to media completely rescued the nucleus reorientation (Fig. 4.2 A). Also in cells treated with C3 exotransferase or transfected with dominant negative or constitutive active RhoA the nuclear reorientation was inhibited compared to control cells (Fig. 4.2 B, C). In addition, overexpression of p115-RhoGEF that can mimic the LPA activation of Rho in the absence of serum and allows cycling of active and inactive state of Rho, rescued the nuclear reorientation in starved cells (Fig. 4.2 D). Collectively, these data suggested that LPA activation of Rho and its cycling between GDP- and GTP-bound state regulates nuclear reorientation in polarizing fibroblasts.

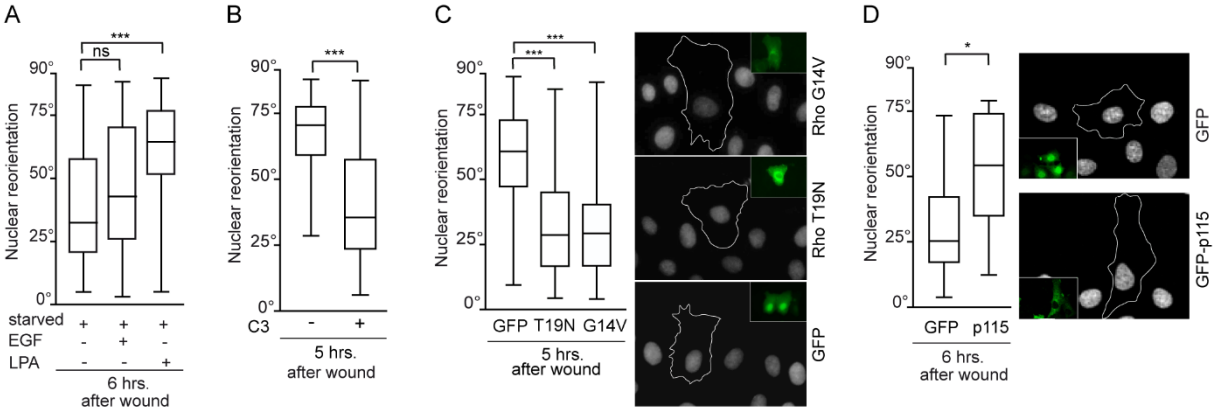

Fig. 4.2: LPA and Rho signaling are required for nuclear reorientation. (A) Nuclear reorientation is induced by LPA. Cells were starved for 5 h and then stimulated by LPA (10 μm) or by EGF (20ng/ml) as a negative control. (B) Nuclear reorientation in cells pretreated with Rho inhibitor C3 exotransferase. (C) Measurement of nuclear reorientation in cell transfected with GFP empty vector, dominant negative RhoA (RhoA T19N) or constitutive active RhoA (RhoA G14V) fused to GFP. Nuclear reorientation was determined in GFP positive cells only (outlined cells). (D) Determination of nuclear reorientation in cells transfected with GFP empty vector or p115-RhoGEF fused to GFP. Cells were starved 5 h before the wounding. In all experiments cells were fixed at indicated time points after wounding. Data are presented as box and whiskers graphs showing median and quartiles (*** p< 0,001; * p <0,05; ns, not significant). In each sample 100 cells was analyzed.

The cells actively reorient their nuclei so that the nucleus points to the new protrusions and to the newly formed leading edge. Formation of new protrusions and subsequent stabilization is coupled with integrin activation and integrin downstream signaling. Thus we

supposed that integrins may be involved in the regulation of nuclear reorientation. We interfered with integrin signaling using RGD peptide that blocks integrin binding to fibronectin and measured the nuclear reorientation. In cells where the integrin activation was blocked the nuclear reorientation was completely inhibited (Fig. 4.3 A). As integrin binding to ECM stimulates the activity of FAK/Src signaling complex (Parsons, 2003; Schaller, 2010) we further tested if FAK and Src activation and signaling affect the nuclear reorientation in polarizing cells. After monolayer wounding, during several hours strong activation of FAK occurs at focal adhesions at the leading edge (Fig. 4.3 B). When we attenuated FAK expression in cells polarizing to the wound, cells were not able to reorient the nucleus to the wound (Fig. 4.3 C) and also inhibition of Src by Src kinase family inhibitor SU6656 prevented nuclear reorientation (Fig. 4.3 D).

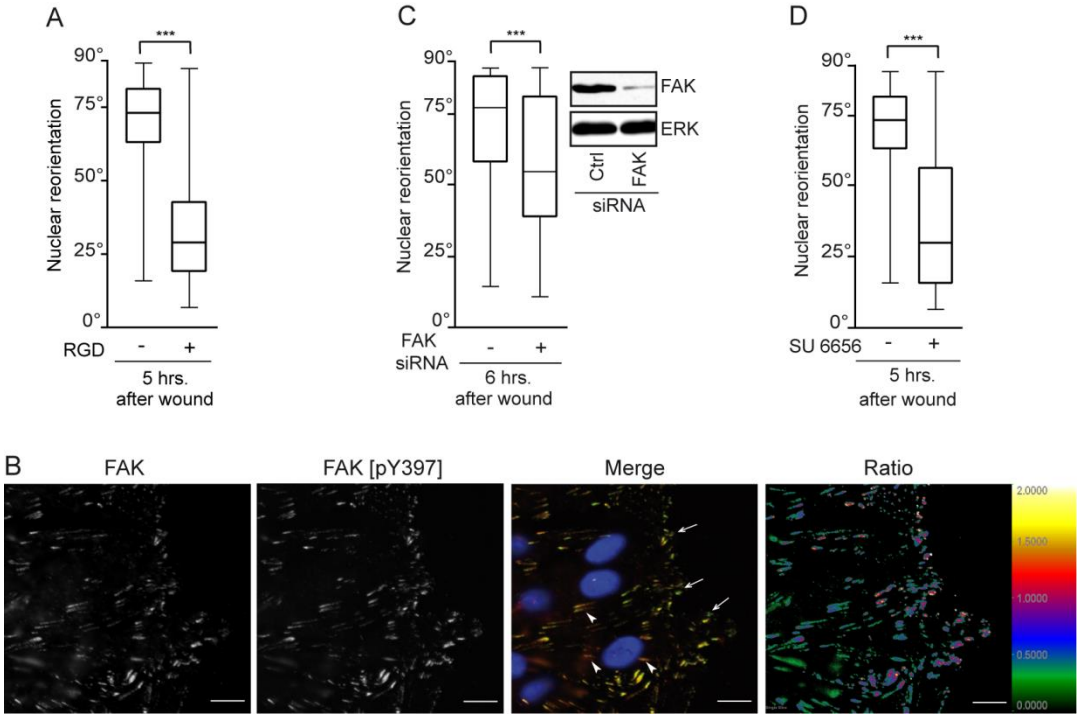

Fig. 4.3: Nuclear reorientation is regulated by integrin and FAK/Src signaling. (A) Nuclear reorientation in cells pretreated with RGD peptide (250 μM). (B) FAK activation at the leading edge in cells polarizing to the wound. Cells migrating to the wound were fixed 3 h after wounding and co-stained with FAK (red in merge chanel) and pFAK(Y397) (green) antibodies. Focal adhesions with high FAK activity are more green (indicated by arrows) and with low FAK activity are more red (arrowheads). Ratio of pFAK/FAK signal intensities is shown in the right panel. Scale bar 10 μm. (C) Measurement of nuclear reorientation in FAK depleted cells. To confirm knockdown efficiency, cell lysates were probed with FAK antibody and with ERK2 antibody to control protein loading. (D) Inhibition of Src by SU6656 (1 μM) blocks the nuclear reorientation in polarizing fibroblasts. In all experiments cells were fixed in indicated time points after wounding (*** p< 0,001). In each sample 100 cells was analyzed.

FAK/Src signaling regulates the activities of small Rho GTPases and activates also MEK/ERK signaling that regulates the focal adhesion maturation and turnover (Schaller,

2010). To find out if regulation of focal adhesions at the leading edge regulates also nuclear reorientation we inhibited MEK signaling by UO126 and measured the nuclear reorientation. Surprisingly, compared to control cells MEK signaling inhibition had no effect on nuclear reorientation (Fig. 4.4 A). We thus focused on other FAK downstream effectors. FAK associates with several GEFs and GAPs that regulate RhoA. One of them, p190A-RhoGAP has been shown to regulate the establishment of cellular polarity (Arthur et al., 2000), thus we tested if p190A-RhoGAP plays role during nuclear reorientation. At first we observed the localization of p190A-RhoGAP after cell monolayer wounding. We found that p190 accumulates at the cell front around focal adhesions at the same time when we observed strong FAK activation (Fig. 4.4 B). Measurement of nuclear reorientation in p190A-RhoGAP depleted cells revealed that p190 is involved in the regulation of nuclear reorientation (Fig. 4.4 C). In addition, attenuation of p190A-RhoGAP prevented the nuclear reorientation also in starved cells where the nuclear reorientation was rescued by overexpression of p115RhoGEF (Fig. 4.4 D) suggesting that integrin/FAK/Src and p190A-RhoGAP signaling suppress locally the activity of RhoA at the leading edge and thus regulates nuclear reorientation.

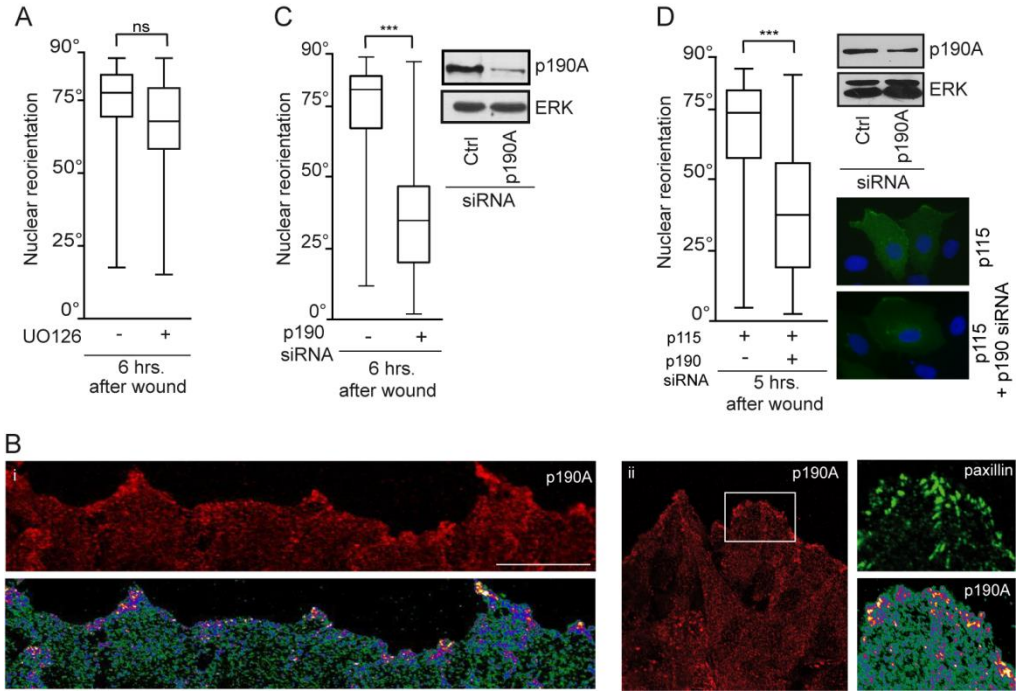

Fig. 4.4: p190A-RhoGAP localizes at the leading edge of the cell and regulates the nuclear reorientation. (A) Measurement of nuclear reorientation in cells pretreated with MEK inhibitor UO126 (20 μM). (B) Localization of p190A-RhoGAP at the leading edge in cells polarizing to the wound. Cells were fixed 3 h after wounding and stained with antibodies against p190A-RhoGAP (red) a paxillin (green). (i) Lower panel shows distribution of p190 pseudocolored according to the intensity of the staining. Scale bar 40 μm. (ii) p190 is localized around focal adhesions. Magnification of boxed area shows accumulation of p190 to focal adhesions (paxillin-upper panel). Lower panel shows distribution of p190 pseudocolored according to the intensity of the staining. Scale bar 10 μm. (C) Nuclear reorientation in p190A-RhoGAP knockdowned cells. (D) Attenuation of p190A-RhoGAP impaired the nuclear reorientation induced by p115-RhoGEF in serum deprived cells. Cells

were co-transfected with GFP-p115-RhoGEF with p190 or control siRNA and starved for 5 h. Nuclear reorientation was measured in GFP positive cells only (right panel). To confirm the knockdown efficiency and protein loading cell lysates were probed with p190A-RhoGAP and ERK antibodies, respectively. Nuclear reorientation was determined in indicated time points (*** p<0,001; ns, not significant). In each sample 100 cells was analyzed.

4.3. Nuclear reorientation facilitates the establishment of cellular polarity

As cells reorient actively their nuclei during the process of cell polarization we hypothesized that nuclear reorientation enables the establishment of cellular polarity. We interfered with individual components of signaling pathways that regulate the nuclear reorientation and measured the cell polarization to the wound by Golgi or MTOC positioning (Fig. 4.5 A). The establishment of cellular polarity in serum deprived cells was significantly decreased and stimulation of RhoA activity by p115-RhoGEF expression rescued this defect in cell polarity establishment (Fig. 4.5 B, C). Inhibition of FAK and Src signaling by specific inhibitors also prevented cell polarization (Fig.4.5 D, E) suggesting that nuclear reorientation regulated by LPA and FAK/Src signaling promotes the establishment of cellular polarity.

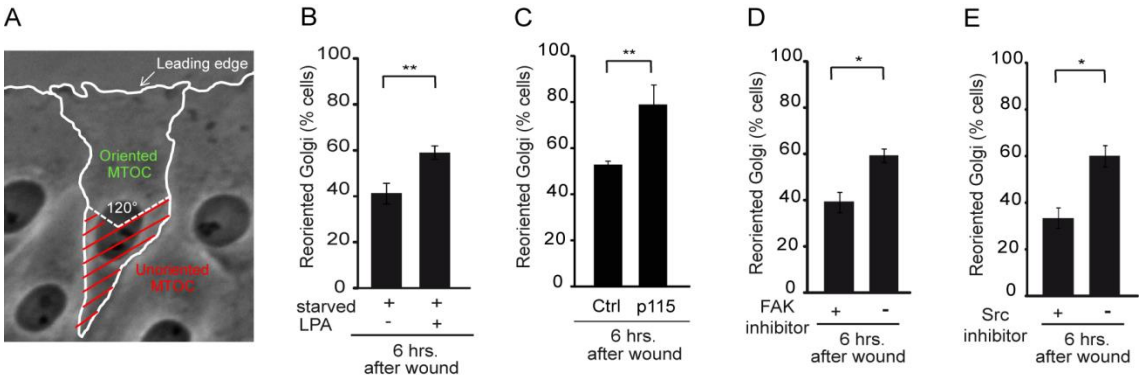

Fig. 4.5: LPA/Rho and FAK/Src signaling enables the establishment of cellular polarity. (A) Schematic picture of cellular polarity measurement in cells polarizing to the wound. Cell polarization was measured by MTOC or Golgi reorientation using 120° fork facing the wound. Cells were scored as polarized when Golgi or MTOC were localized within the fork. (B-E) Determination of cellular polarity in starved and LPA stimulated cells (B), in starved cells transfected with GFP empty vector or p115-RhoGEF fused to GFP (C) and in cell pretreated with FAK inhibitor (PF573228) (D) or Src inhibitor (SU6656) (D) before the wounding. In all experiments at least 100 cells was analyzed. Data are presented as a mean ± SD (** p< 0,01; * p< 0,05).

4.4. Nuclear reorientation requires anchorage to cell cytoskeleton through LINC complex

It has been shown that the nucleus movement requires mechanical linkage to cell cytoskeleton (Houben et al., 2009; Lee et al., 2007; Luxton et al., 2010). The mechanical coupling between the nucleus and cytoskeleton is mediated by LINC complex (Razafsky and Hodzic, 2009; Starr and Fridolfsson, 2010). Thus we further tested if LINC complex and physical link to cytoskeleton is necessary for nuclear reorientation. We disrupted the LINC

complex with either lamin A/C siRNA or by overexpression of construct coding KASH domain of nesprin 2 fused to GFP. GFP-KASH displaces endogenous nesprin proteins from LINC complex and because it lacks the cytosolic part binding directly or indirectly actin and tubulin it functions as a LINC complex dominant negative. Truncated construct GFP-KASHΔL then lacks also SUN binding part. This protein localizes to both outer nuclear membrane and endoplasmic reticulum and functions as a control (Fig. 4.6 A). In both experiments the disruption of nucleo-cytoplasmic link significantly inhibited the nuclear reorientation (Fig. 4.6 B, C) suggesting that cell cytoskeleton generates forces that reorient the nucleus during cell polarization.

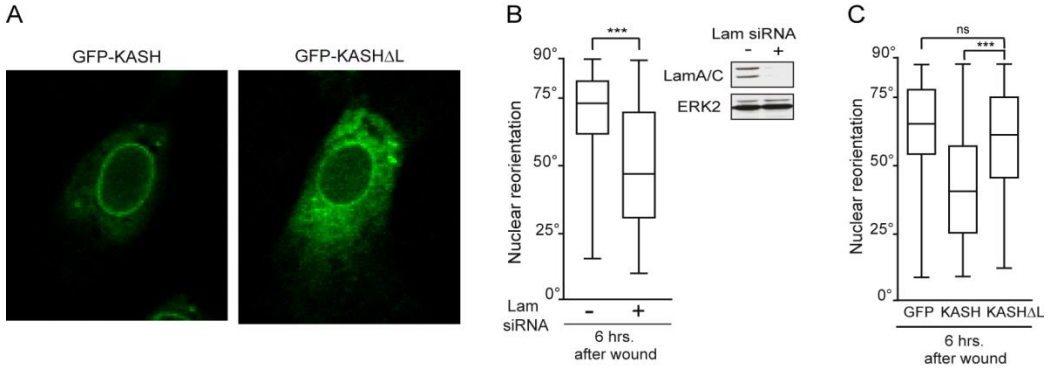

Fig. 4.6: LINC complex is important for nuclear reorientation. (A) Localization of GFP-KASH and GFP-KASHΔL proteins to nuclear membrane in transfected cells. GFP-KASH is anchored in nuclear membrane through interaction with Sun protein. GFP-KASHΔL does not bind Sun and thus significant amount is displaced from nuclear membrane to membranes of ER. (B-C) Nuclear reorientation in cells transfected with lamin A/C siRNA (B) or GFP alone, GFP-KASH and GFP-KASHΔL constructs (C) (*** $p<0,001$; ns, not significant).

4.5. Regulation of nuclear reorientation by cell cytoskeleton

Nuclear reorientation in RAT2 fibroblasts is driven by small GTPase RhoA and requires LINC complex that couples the nucleus to cell cytoskeleton. These findings strongly suggested that forces that reorient the cell nucleus during cell polarization are generated by cytoskeletal structures. We thus investigated which part of cytoskeleton moves with the nucleus in polarizing cells. As intermediate filaments do not associate with any motor proteins we omitted intermediate filaments from further testing. Microtubules are good candidate for nuclear reorientation as during cell polarization microtubules induce MTOC re-localization in front of the nucleus (Gundersen and Cook, 1999; Palazzo et al., 2004; Watanabe et al., 2005) and thus forces that reorient MTOC could also reorient the nucleus.

In cell monolayer microtubules form a meshwork that pervades the whole cell and in some cells we observed several stabilized microtubules around the nucleus (Fig 4.7 A). Wounding the cell monolayer induced cell spreading to the wound and microtubules emanated to the protrusions. In all cells at the edge of the wound stable microtubules formed a

basket-like structure around the nucleus (1 h after wounding). 6 hours after wounding the MTOC was located between the nucleus and the leading edge, stable microtubules emanated predominantly to the leading edge and only several of them localized around the nucleus (Fig. 4.7. A).

Microtubules stabilized at the leading edge have been shown to be captured by motor proteins (Watanabe et al., 2005). Using the antibody against p150Glued a subunit of dynactin, a dynein activating complex, we observed distinct foci where microtubules potentially associate with cell cortex at the leading edge where they can generate forces for MTOC re-localization and nucleus reorientation (Fig. 4.7 B). To determine the role of microtubules in nuclear reorientation during cell polarization we interfered with microtubules using microtubule polymerizing inhibitor nocodazole or inhibited the motor protein dynein by selective inhibitor EHNA hydrochloride. Low dose of nocodazole has been shown to disorganize cortically anchored microtubules only (Levy and Holzbaur, 2008; Vasquez et al., 1997) and (Fig. 4.7 C) thus we pretreated the cells with two different concentrations of nocodazole and measured the nuclear reorientation and cell polarization. As expected, nocodazole treatment and inhibition of dynein activity impaired MTOC reorientation in front of the nucleus in polarizing cells (Fig. 4.7 D, E). Nuclear reorientation in cells treated with both doses of nocodazole was blocked, however, nocodazole has been shown to stimulate Rho activity (Bershadsky et al., 1996; Lee and Chang, 2008; Liu et al., 1998) and thus stabilize actin cytoskeleton that may inhibit the nuclear reorientation. Measurement of nuclear reorientation in cells polarizing to the wound revealed that both lower dose of nocodazole and dynein inhibitor only partially impaired the nuclear reorientation (Fig. 4.7 D, E) suggesting that microtubules may participate in regulation of nuclear reorientation, however it seems that actin may be significantly involved in this process.

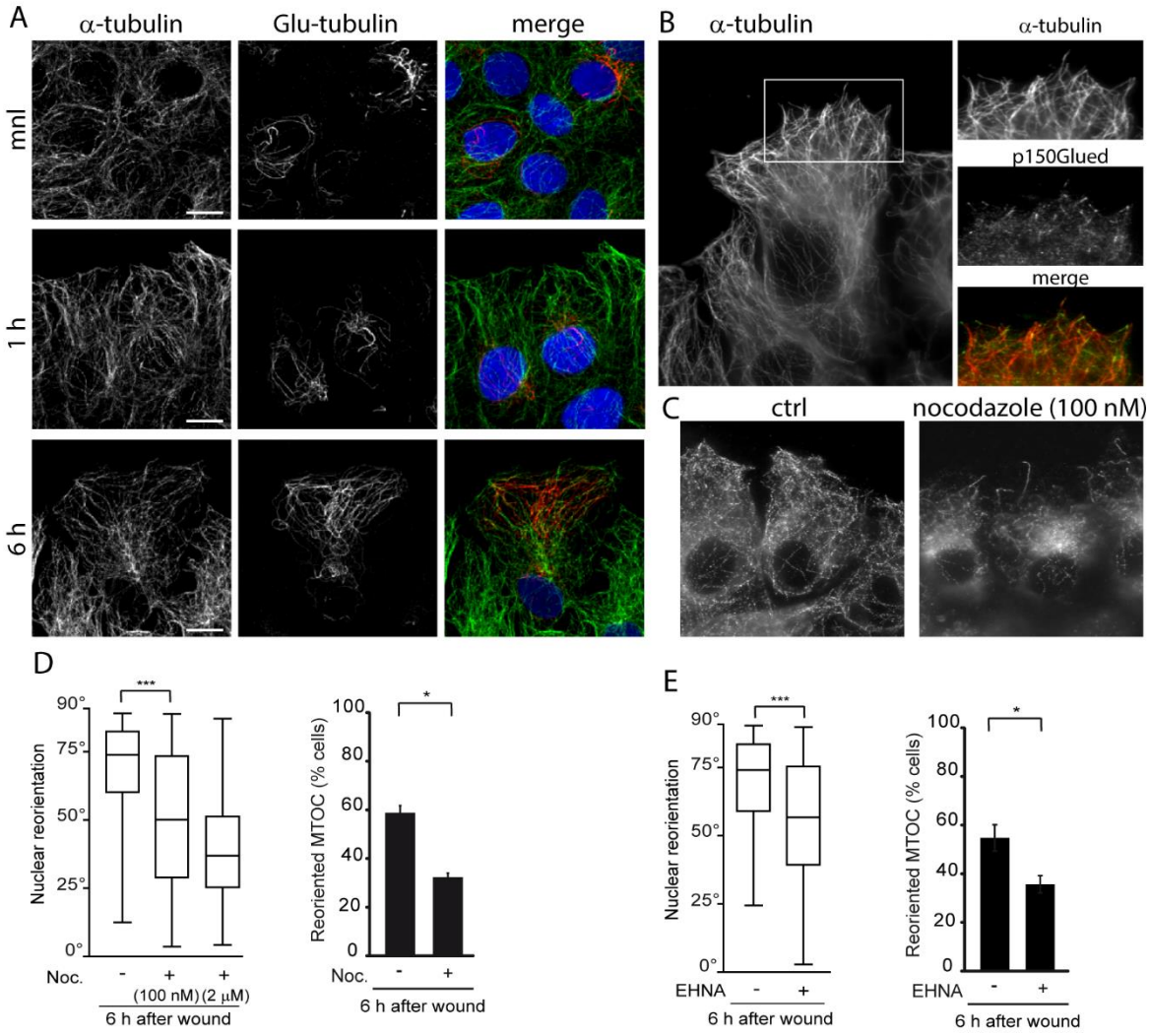

Fig. 4.7: Involvement of microtubules in nuclear reorientation. (A) Maximal projections of confocal sections show microtubule reorganization during cell polarization to the wound. Cells in a monolayer and cells polarizing to the wound were fixed at indicated time points and stained with antibodies against α-tubulin (green in merge channel) and detyrosinated (Glu-) tubulin (red) and counterstained with DAPI. (B) Cells polarizing to the wound for 4 h were fixed and stained with antibodies against α-tubulin (red in merge channel) and p150Glued (green). Magnification of boxed area shows foci of p150Glued staining where microtubules are captured at the leading edge. (C) Low dose of nocodazole disrupts microtubules at the cell periphery only. Cells pretreated with 100 nM nocodazole were fixed 6 h after wounding and stained with α-tubulin antibody. (D-E) Measurement of nuclear reorientation and MTOC repositioning in cells pretreated with nocodazole (for MTOC reorientation was used 100 nM concentration) (D) or EHNA hydrochloride (10 μM) (E). Data are presented as box and whiskers or as a mean ± SD (***p<0,001; *p<0,05).

To investigate the role of actin cytoskeleton during the nuclear reorientation we affected actin using actin polymerization inhibitor latrunculin A treatment or we blocked the myosin II activity with blebbistatin. In cells pretreated with latrunculin A actin was strongly disrupted and cells displayed only intact peripheral fibers (Fig. 4.8 A). Nuclear reorientation was completely inhibited compared to control cells and also pretreatment with myosin inhibitor blebbistatin impaired the nuclear reorientation (Fig. 4.8 B, C) suggesting that actin may drive the nuclear reorientation. Surprisingly, when we measured the cell polarization by MTOC re-localization, we found out that actin disruption had no effect on establishment of

cellular polarity (Fig. 4.8 D). These data suggest that MTOC and nuclear reorientation may be two independent processes driven by two different mechanisms.

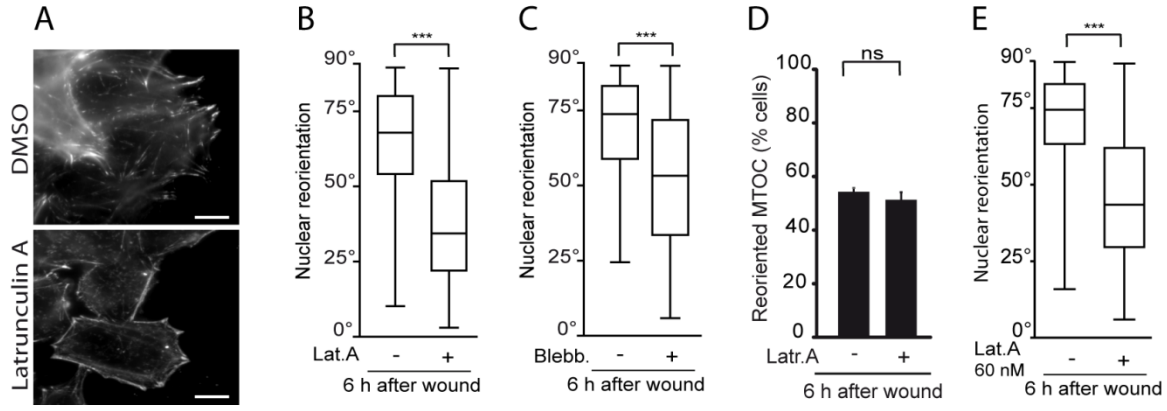

Fig. 4.8: Actin drives nuclear reorientation but not MTOC repositioning. (A) Actin staining of latrunculin A treated cells migrating to the wound. Cells were pretreated with latrunculin A (1 μM), fixed 6 h after wounding and stained with phalloidin-rhodamine. Bars 10 μm. (B-C) Measurement of nuclear reorientation in cells pretreated with latrunculin A (1 μM) (B) or blebbistatin (5 μM) (C). Data are presented as box and whiskers graph (***p<0,001). (D) MTOC reorientation in latrunculin A (1 μM) treated cells migrating to the wound. Data are presented as a mean ± SD (ns, not significant). (E) Nuclear reorientation in cells pretreated with low dose of latrunculin A (60 nM). Data are presented as box and whiskers graph (***p<0,001).

Emerging role for nuclear rotation and orientation in cell migration

(Maninová et al., 2014)

In this publication we reviewed the nucleus movement and positioning in different cell types. We proposed that two signaling pathways which converge at small GTPase RhoA regulate the nuclear reorientation to the direction of migration. We discussed three possible scenarios how actin and microtubules drive the nuclear reorientation during cell polarization and we also discussed the biological significance of nuclear reorientation in migrating cells.

4.6. Actin cytoskeleton drives nuclear reorientation

Our preliminary results suggest that nuclear and MTOC reorientations are two separate events regulated by various components of cell cytoskeleton. To further study the nuclear reorientation we focused our research on actin cytoskeleton.

In adherent migrating cells we distinguish several types of actin tress fibers. Ventral stress fibers lie on the basal side and are anchored in focal adhesions at both ends. Dorsal fibers polymerize from focal adhesions at the leading edge of migrating cell and rise to the

dorsal side of the cell where they associate with transverse arcs, curve-shaped highly contractile fibers that are not anchored in focal adhesions (Hotulainen and Lappalainen, 2006) (Fig. 4.9 A). Perinuclear actin cap fibers emanate to the dorsal side of the cell and above the nucleus and they are linked to the nuclear envelope through LINC complex (Khatau et al., 2009; Khatau et al., 2012b). Thus perinuclear actin fibers were first candidate for nuclear reorientation. Focusing on the apical side of cell we found that RAT2 fibroblasts also form perinuclear actin fibers. They extended from the cell front to the dorsal side of the cell and above the nucleus and further to the cell tail and were aligned with the longer nuclear axis (Fig. 4.9 B, C). Perinuclear actin fibers are associated with the nuclear envelope as over-expression of GFP-KASH domain of nesprin, that displace endogenous nesprins from the outer nuclear membrane, disrupted the LINC complex and subsequently also perinuclear actin fibers (Fig. 4.9 D). Next we utilized the finding that perinuclear fibers may be also impaired with low concentration of latrunculin (Chambliss et al., 2013; Khatau et al., 2009) which is true also for RAT2 cells (Fig. 4.9 E). Importantly, neither over-expression of GFP-KASH nor low dose of latrunculin A affected the ventral fibers localized under the nucleus (Fig. 4.9 D, E) or dorsal fibers and actin arcs (see below). These findings confirmed that perinuclear actin fibers are anchored through the LINC complex to the nuclear envelope and, in addition, provided us with two complementary tools how to impair perinuclear fibers. Thus we measured the nuclear reorientation in cells pretreated with 60 nM latrunculin A. Similarly to disruption of LINC complex, disruption of perinuclear fibers with low dose of latrunculin A impaired the nuclear reorientation in cells migrating to the wound (Fig. 4.8 E) indicating that perinuclear actin cap fibers drive nuclear reorientation to the direction of migration.

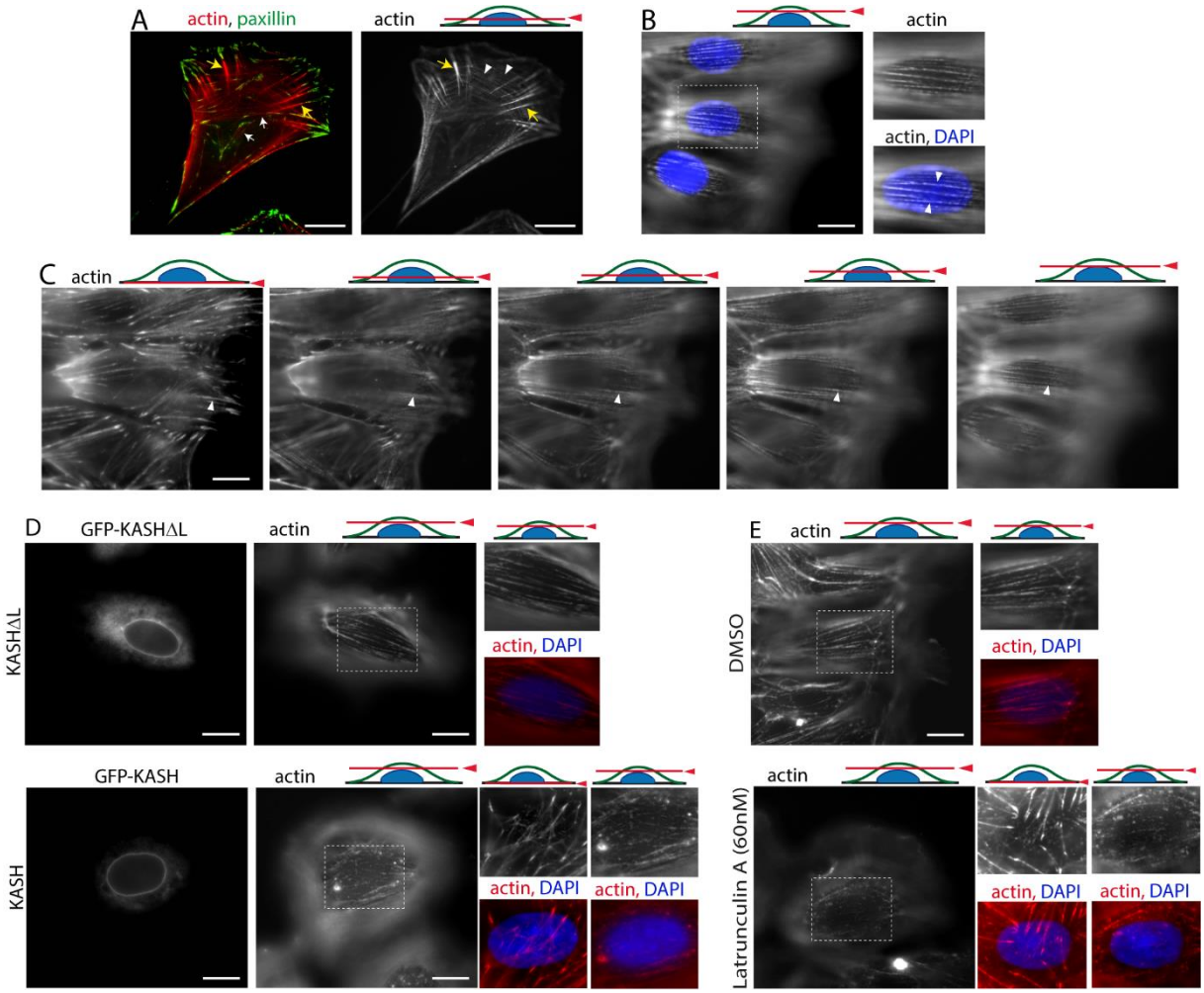

Fig. 4.9: Actin forms several different types of stress fibers in migrating cells. (A) Dorsal fibers (yellow arrows) polymerize from focal adhesions (paxillin staining, green) at the leading edge and associate with transverse arcs (white arrowheads, right panel) at midsection focal plane. Ventral fibers lying on the basal side are indicated by white arrows. Migrating cells were fixed and stained with phalloidin-rhodamine and paxillin antibody. (B) Perinuclear fibers formed above the nucleus in polarized cell. Cells migrating to the wound were fixed 6 h after wounding and stained with phalloidin-rhodamine and DAPI. (C) Actin cap fibers are exposed in pole-to-pole manner in polarized cells. Images are in order from the basal side to the top of the cell. White arrowhead follows the perinuclear actin fiber from the bottom to the top of the cell. (D) Disruption of the LINC complex affects the perinuclear fibers. Cells were transfected with GFP-KASH or GFP-KASHΔL constructs, fixed and stained with phalloidin-rhodamine and DAPI. Magnifications of boxed areas show the actin staining above or under the nucleus, respectively. (E) Low dose of latrunculin A disrupts perinuclear actin fibers. Cells were pretreated with latrunculin A (60 nM), fixed 6 h after wounding and stained with phalloidin-rhodamine and DAPI. Magnifications of boxed areas show the actin staining above or under the nucleus, respectively. Focal planes are indicated above the individual pictures. Bars, 10 μm.

4.7. Dorsal stress fibers, transverse actin arcs and perinuclear actin fibers form interconnected network that induces nuclear movement in polarizing fibroblasts

(Maninová and Vomastek, under review in FEBS Journal)

In this manuscript we described the dynamics of perinuclear actin fibers during polarization of RAT2 fibroblasts. We have shown that dorsal fibers and transverse arcs

participate in perinuclear actin cap assembly and that these stress fibers form an interconnected network that drives the nuclear reorientation to the direction of migration.

At first we determined how the perinuclear actin fibers are organized in cells polarizing to the wound. Perinuclear fibers were present in cells in cell monolayer and also in the majority of cells polarizing 6 h to the wound. Interestingly, 2 h after wounding when cells reorient their nuclei to the wound perinuclear fibers were disrupted or underwent significant remodeling (Fig. 4.10 A). Considering that perinuclear fibers display 3D architecture within the cells (see Fig. 4.9 C) and to get insight into the process of assembly or remodeling of perinuclear fibers we determined the actin cytoskeleton organization in different Z-sections of the cell. We found that dorsal fibers polymerizing from focal adhesions at the leading edge during initial phases of polarization are crosslinked with transverse arcs in actin rich foci (Fig. 4.10 B). Interestingly, at later time points after wounding these crosslinked actin foci accumulated in front of the nucleus and anchored also perinuclear actin fibers (Fig. 4.10 C) suggesting that dorsal fibers, transverse arcs and perinuclear actin fibers are mechanically coupled.

We have identified actin crosslinking protein α-actinin1 as a protein that crosslink dorsal fibers, transverse arcs and perinuclear fibers. α-actinin1 is present in a high amount on the dorsal fibers and is necessary for their formation (Hotulainen and Lappalainen, 2006; Kovac et al., 2013). When we stained the cells with α-actinin1 antibody we observed localization of α-actinin1 to dorsal fibers and to crosslinking spots (Fig. 4.10 D). Depletion of α-actinin1 in cells migrating to the wound impaired de novo dorsal fibers formation, formation of crosslinked foci and cells did not form perinuclear fibers (data shown in the manuscript). In addition, nuclear reorientation in α-actinin1 depleted cells was inhibited (Fig. 4.10 E) suggesting that formation of actin network crosslinked by α-actinin1 drives the nuclear reorientation.

Intriguingly, we also found that curved transverse arcs are interconnected with thin peripheral actin fibers (Fig. 4.10 F). When we followed the actin dynamics in living cells we observed that dorsal fibers and contractile transverse arcs recruit peripheral ventral fibers to the dorsal side of the cell and above the nucleus. Consequently, movement of ventral actin fibers induced nuclear rotation (proposed model in Fig. 4.10 G).

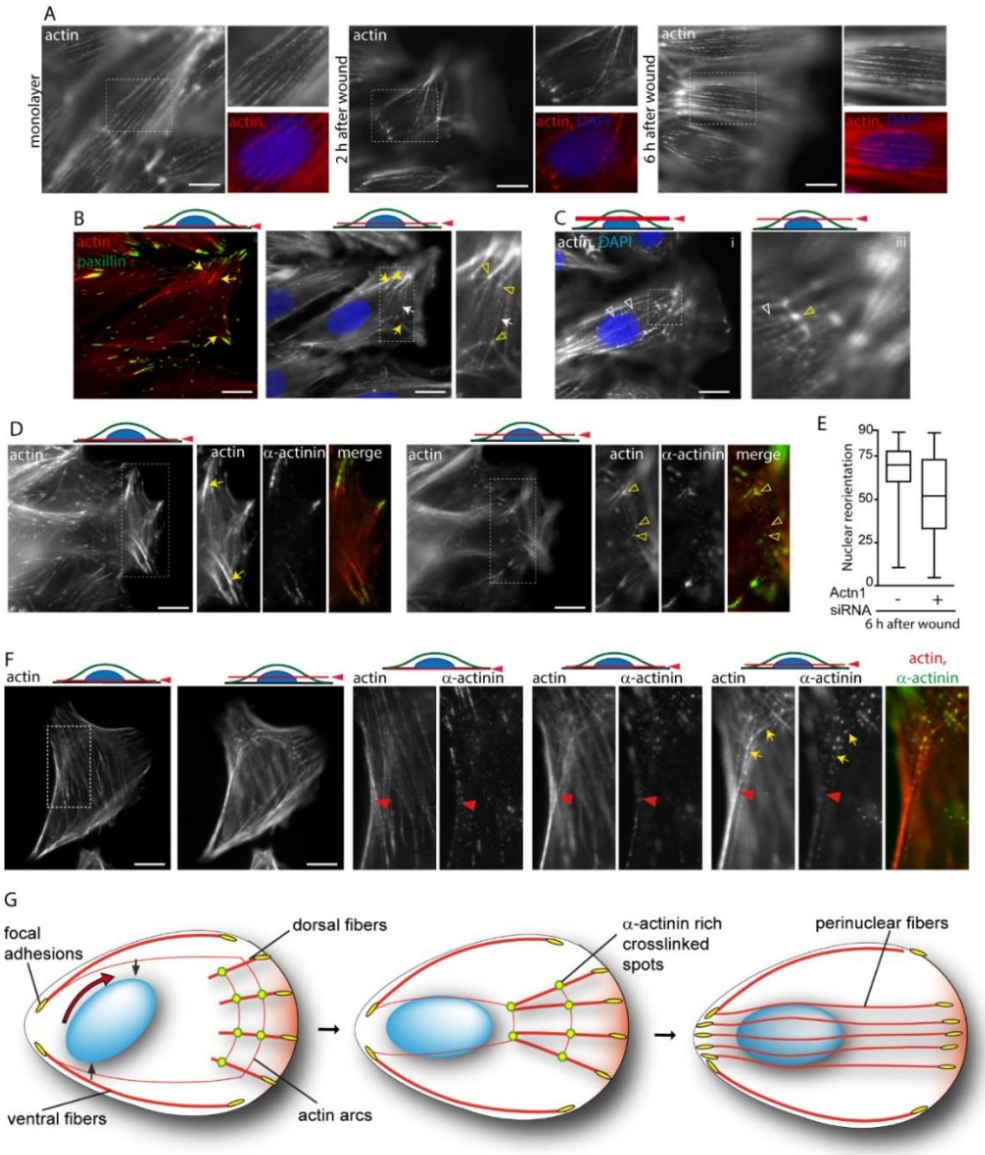

Fig. 4.10: Perinuclear actin fibers are interconnected with dorsal fibers and transverse arcs and this network drives nuclear reorientation. (A) Disassembly and reassembly of perinuclear actin fibers during cell polarization. Cells in monolayer, polarized cells (6 h after wound) and polarizing cells (2 h after wound) were fixed and stained with phalloidin and DAPI. Higher magnification of boxed areas shows actin above the nucleus (DAPI, blue). (B) Dorsal fibers (yellow arrows) and transverse arcs (white arrows) are formed in cells polarizing to the wound for 3 h. Actin rich spot appears at the dorsal fiber–arc intersection (yellow empty arrowheads, higher magnification of boxed area). (C) Perinuclear actin fibers (white empty arrowheads) terminate in actin spots in front of the nucleus during cell polarization (5 h after wound, panel i). Actin rich spots (yellow empty arrowheads) located at cell's midsection in front of the nucleus harbor dorsal fibers and multiple actin cap fibers (panel iii). In panel (i) two focal planes were overlaid to better visualize perinuclear fibers and actin spots in front of the nucleus. (D) Localization of α-actinin-1 to dorsal fibers (yellow arrows, magnification of boxed areas) and actin rich spots (yellow empty arrowheads). Images are from basal and midsection focal planes (indicated above the pictures). Cells fixed 4 h after wounding were stained with phalloidin (red in merged images) and α-actinin-1 antibody (green). (E) Measurement of nuclear reorientation in α-actinin-1 depleted cells. (F) Thick ventral stress fiber splits into thin curved actin fibers. Fixed cells were stained with rhodamine-phalloidin and α-actinin-1 antibody as indicated. Enlarged areas show the split of straight ventral fiber into curved actin fibers (red arrowheads) that extend to the dorsal side (yellow arrows) in several focal planes. (G) Proposed mechanism of perinuclear actin cap formation and nuclear reorientation. Arcs fuse with peripheral ventral fibers and their contraction drive movement of peripheral fibers toward the nucleus. Dorsal fibers crosslinked with transverse arcs ensure that the actin network moves to the dorsal side of the cell. Forces generated by the interconnected network drives perinuclear actin cap fibers formation and induce nuclear reorientation. Finally, actin network mature into perinuclear actin cap.

4.8. Integrin and LPA/Rho signaling are required for the assembly of perinuclear actin fibers and nucleus reorientation

In the first part, we have shown that the integration of LPA and integrin-FAK signaling controls nuclear reorientation. Thus, we hypothesized that the formation of dorsal fibers and perinuclear actin cap requires LPA and integrin signaling as well. To examine the role of LPA we starved the cells for 36 h and then stimulated the polarization to the wound by addition of LPA. In serum free media, cells were unable to form dorsal stress fibers and actin cap (Fig. 4.11 A) and nuclei did not reorient to the wound (Fig. 4.11 B). The formation of actin cap, dorsal fibers and nuclear reorientation in serum deprived cells was induced by the addition of LPA and also by the overexpression of GFP-p115RhoGEF, downstream effector of LPA (Fig. 4.11 A, B). In addition, latrunculin treatment of p115RhoGEF positive cells disrupted actin cap without affecting dorsal stress fibers and arcs (Fig. 4.11 A). This correlated with impaired nuclear reorientation (Fig. 4.11 B) further confirming the importance of actin cap structure during nuclear reorientation. Consistently with these results, the expression of dominant negative form of RhoA led to actin cap disruption, impaired dorsal fibers formation and nuclear reorientation. Conversely, the expression of constitutively active form of RhoA stabilized the actin cytoskeleton and actin cap structure and as a result nuclei did not rotate (Fig. 4.11 C, D). We also found that inhibition of the formin family of Rho effectors by drug SMIFH2 (Rizvi et al., 2009) blocked dorsal fibers polymerization. Formation of transverse arcs and actin cap fibers was also impaired in SMIFH2 treated cells (Fig. 4.11 E) and cells did not reorient nuclei to the wound (Fig. 4.11 F). This is consistent with previous report that formation of dorsal actin fibers is driven by the Rho effector mDia1 (Hotulainen and Lappallainen, 2006) and further reinforce the role of RhoA signaling in perinuclear fibers formation.

Second signaling pathway involved in the nuclear reorientation is integrin-FAK pathway and we thus tested if integrins and integrin signaling play role in actin cap and dorsal fibers formation. We inhibited integrin engagement to ECM by RGDS peptide. Blocking the integrin impaired the formation of dorsal fibers, actin cap as well as nuclear reorientation (Fig. 4.11 G, H). We next examined the effect of integrin downstream effector FAK on formation of actin structures. To do this, cells were wounded in the absence of serum to allow the actin cap and dorsal fibers to disassemble, cells were then pretreated with FAK inhibitor PF573228 and stimulated with LPA. Formation of dorsal fibers, actin arcs and actin cap was inhibited in PF573228 treated cells (Fig. 4.11 I) and, consistently with our previous results,

nucleus reorientation was also impaired (Fig. 4.11 J). Collectively, these data suggest, that signaling pathways that drive nuclear reorientation regulate also perinuclear actin cap fibers formation further supporting our hypothesis, that perinuclear actin cap drives nuclear reorientation.

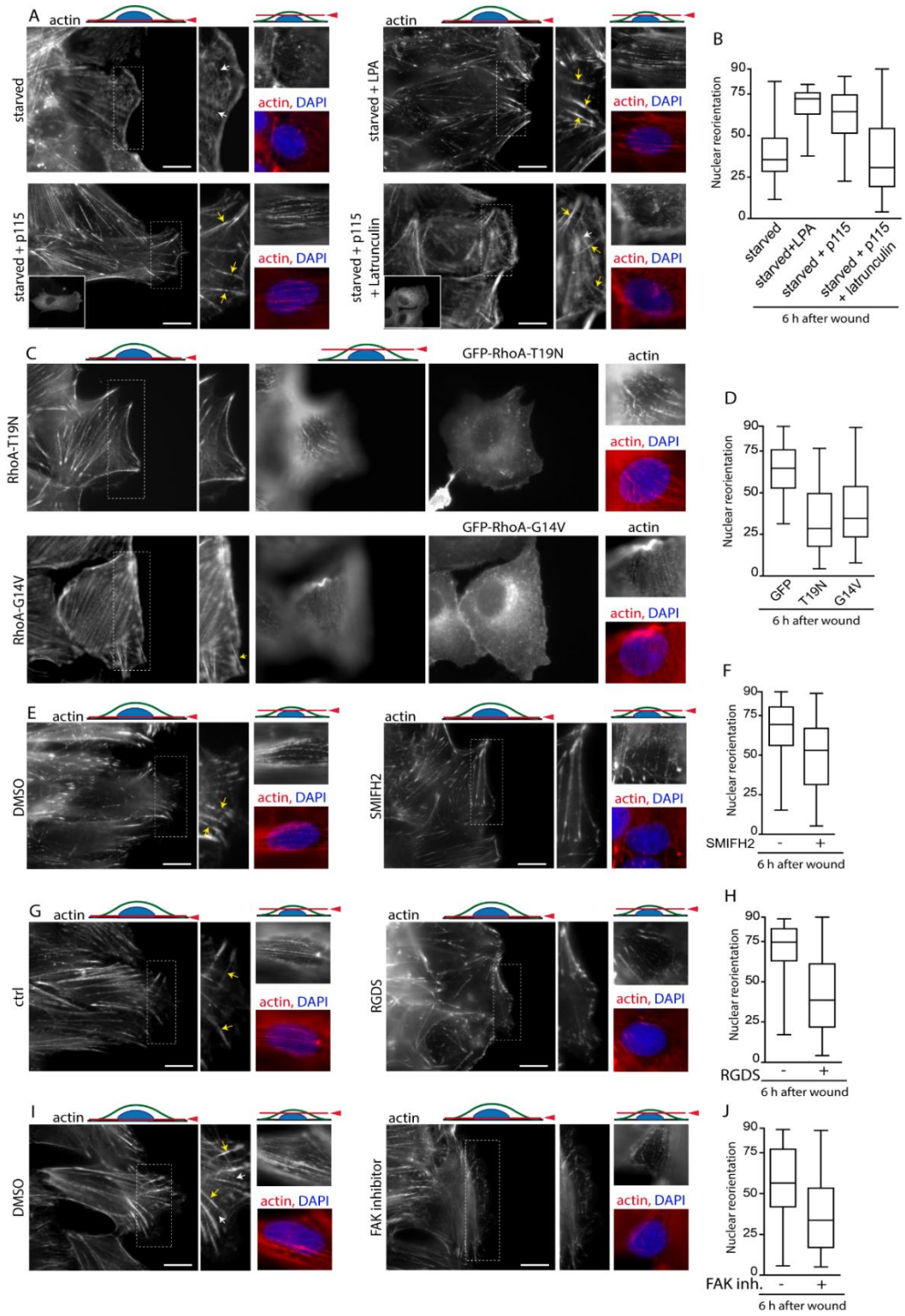

Fig. 4.11: **Integrin/FAK and LPA/Rho signaling regulate dorsal fibers and actin cap formation and nucleus reorientation.** LPA/Rho signaling induces dorsal fibers and actin cap formation. (A) Either nontransfected or p115-RhoGEF transfected RAT2 cells were starved for 36 h, fixed 6 h after wounding and

stained with phalloidin-rhodamine. For LPA treatment, cells were starved 36 h and then pretreated with LPA (10 μM) for 30 min before the wound. For actin cap disruption p115-RhoGEF transfected cells were starved and then pretreated with low concentration of latrunculin (20nM). p115-RhoGEF transfected cells were identified by GFP signal (insets). Magnification of boxed areas show, if present, formed dorsal fibers (yellow arrows) and transverse arcs (white arrows). Right panels show higher magnification of actin above the nucleus. Focal planes are indicated above the individual pictures. (B) Measurement of nuclear reorientation in cells treated as in (A) and polarizing to the wound. (C) Cells transfected with dominant negative (RhoA-T19N) or constitutively active (RhoA-G14V) form of RhoA, fixed 6 h after wounding and stained with rhodamine-phalloidin. (D) Measurement of nuclear reorientation in cells transfected as in (D). GFP empty vector was used as a positive control. (E) RAT2 cells were pretreated with formin inhibitor SMIFH2 (20 μM) for 30 min, fixed 6 h after wounding and stained with phalloidin-rhodamine. (F) Nuclear reorientation in cells treated with SMIFH2 inhibitor. (G-J) Integrin/FAK regulate dorsal fibers and actin cap fibers formation. (G) RAT2 cells were treated with RGDS peptide (250 μM) for 30 min, wounded by scratch and fixed after 6 h. Cells were stained with phalloidin-rhodamine. (H) Nuclear reorientation in RGDS peptide treated cells determined 6 h after wounding. (I) To inhibit FAK, RAT2 cells were starved for 48 h and wounded by scratch and left for 2 h. Cells were then treated with FAK inhibitor PF573228 (20 μM) for 30 min followed by LPA treatment. Cells were fixed after 6 h and stained with phalloidin-rhodamine. (J) Nuclear reorientation in cells treated as in (G) and fixed 6 h after LPA stimulation. Bars, 10 μm.

4.9. Connection of actin cables to the nucleus is required for spatial distribution of dorsal fibers and actin arcs

Our data suggested that the network of actin dorsal fibers, transverse arcs and perinuclear fibers transfers the mechanical force from the leading edge to the nucleus to induce the nuclear reorientation. We examined, whether nucleus through perinuclear actin fibers reciprocally affects the dorsal fibers – actin arcs network at the leading edge. We disrupted the perinuclear cap fibers by the expression of KASH domain of nesprin2 protein or by low concentration of latrunculin A. Both the expression of KASH domain and the latrunculin treatment impaired the formation of actin cap (Fig. 4.12 A). Interestingly, the spatial distribution of dorsal fibers and arcs was significantly affected in the absence of perinuclear actin cap and this effect was particularly pronounced in sparsely growing cells. In control cells, dorsal fibers emanated from relatively narrow leading edge while nonprotrusive regions were essentially devoid of dorsal fibers (Fig. 4.12 A, C). In cells where actin cap was disturbed dorsal fibers were dispersed around cell perimeter (Fig. 4.12 A, C). Similarly, actin arcs were also disorganized in actin cap depleted cells and they formed longer actin cables localized around cell perimeter and cell nucleus often forming concentric rings (Fig. 4.12 D). The defect in the organization of dorsal fibers and actin arcs was accompanied by the change in cell shape. The disruption of perinuclear actin fibers by KASH transfection or latrunculin A treatment resulted in cells that were rounder than the control cells as determined from the roundness index of treated cells (Fig. 4.12 E). These data suggest that crosslinking of perinuclear and dorsal fibers with perinuclear actin cap in front of the nuclear pole appear to stabilize the dorsal fibers and restrict their presence to the leading edge.

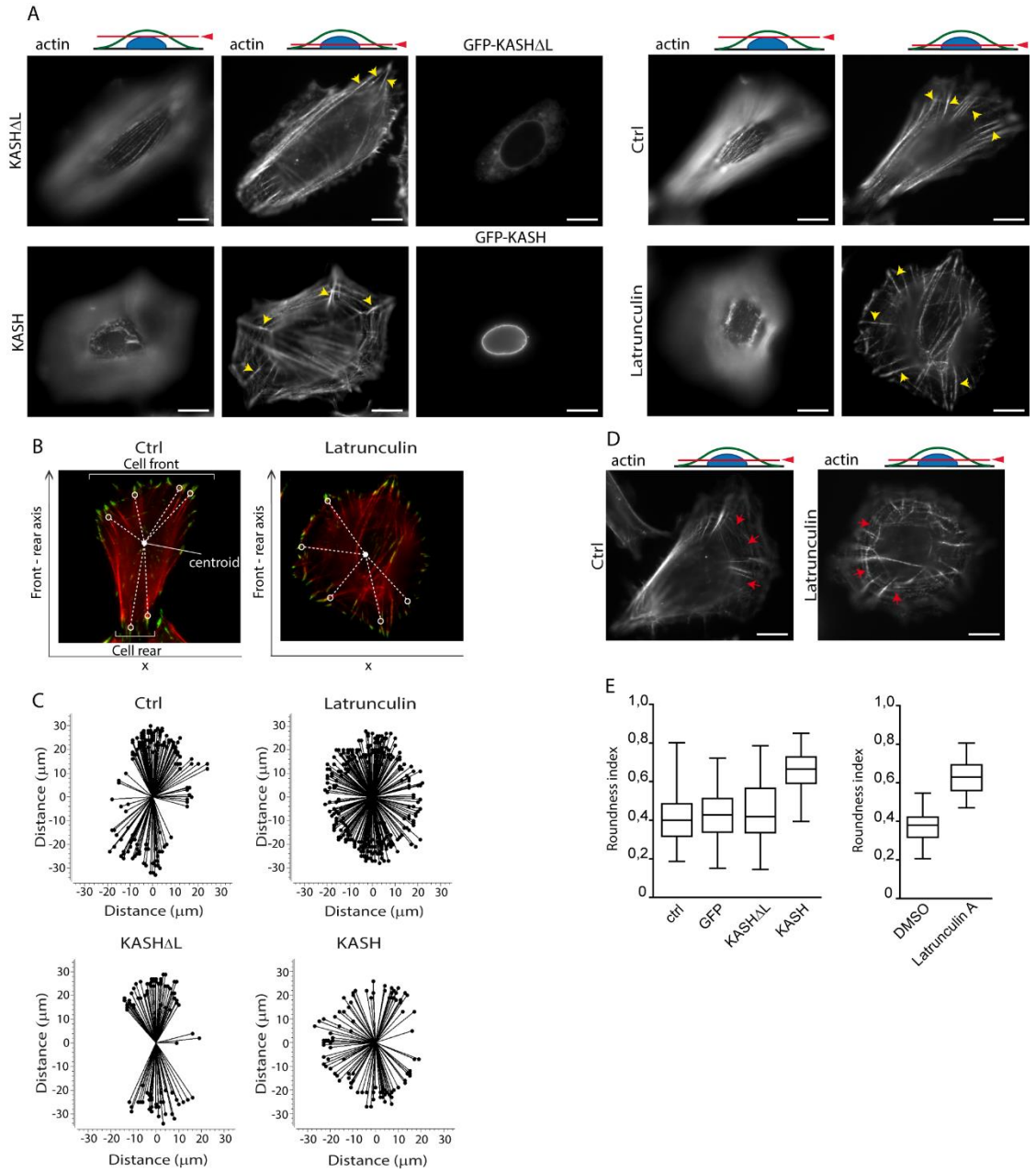

Fig. 4.12: Actin fibers organization at the leading edge requires perinuclear actin fibers. (A) Sparse cells transfected with GFP-KASH and GFP-KASHΔL constructs or treated with low dose of latrunculin (60nM, 2 h) were fixed and stained with phalloidin-rhodamine. Focal planes are indicated above the individual pictures. Yellow arrows indicate dorsal fibers. (B) Measurement of dorsal fibers distribution. Cells were stained with phalloidin-rhodamine (red) and paxillin (green) and the distance between cell centroid (white circle) and the distal end of dorsal fiber was determined (distance is indicated by dashed lines). Position of the distal end of individual dorsal fibers was determined on the borders of dorsal fibers and focal adhesions (hollow circle). To show dorsal fibers distribution the distance between the distal end of dorsal fibers and the cell centroid is plotted relative to front-rear axis (equivalent with y-axis). Note, that actin fibers anchored at focal adhesions at cell rear are not dorsal fibers but perinuclear actin fibers that terminate in focal adhesions and rise above the nucleus. However, they were included in the measurement. (C) Quantification of dorsal fibers distribution in cells transfected with GFP-KASH or GFP-KASHΔL construct or in latrunculin A treated cells. Plots show dorsal fibers distribution and distance between the cell centroid and the distal end of dorsal fibers relative to the front rear axis (equivalent with y-axis). For each plot 10 cells were scored. (D) Organization of actin arcs (red arrows) in latrunculin A treated cells. Cells were treated as in (A) and stained with phalloidin-rhodamine. (E) Quantification of cell shape in non-transfected (Ctrl) or KASH, KASHΔL and GFP transfected cells (left panel) and in latrunculin A treated cells (right panel). In each sample 110 cells was scored except latrunculin A experiment, where 20 cells were scored.

4.10. Nuclear reorientation promotes efficient cell migration to the wound

We have shown that nuclear reorientation correlates with the establishment of cellular polarity and that disruption of LINC complex impairs both nuclear reorientation and cell polarization. As cell polarization is first step of cell migration we examined next whether inhibition of nuclear reorientation influences also cell migration. Tracking the trajectories of GFP-KASH transfected cells migrating to the wound revealed that LINC complex disruption impairs also directional migration (Fig. 4.13 A) indicating that nuclear reorientation correlates with the establishment of cellular polarity and directional cell migration to the wound. Interestingly, when we analyzed the GFP-KASH transfected cells migrating to the wound we revealed two different defects in directional cell migration. Over 50% of cells showed impaired migration and these cells lagged behind non-transfected cells (Fig. 4.13 B). However, 35% of GFP-KASH transfected cells initially expanded to the wound but after a while at the edge of the wound they started to form uncoordinated multiple protrusions resulting in loss of directionality and rounder shape of the cell. Subsequently these cells stopped the movement. The loss of directionality was accompanied also by changes in nuclear orientation (Fig. 4.13 C). This observation further supports the hypothesis that proper nuclear orientation is necessary for establishment of polarity and directional migration and that perinuclear actin cap fibers are important for the leading edge formation.

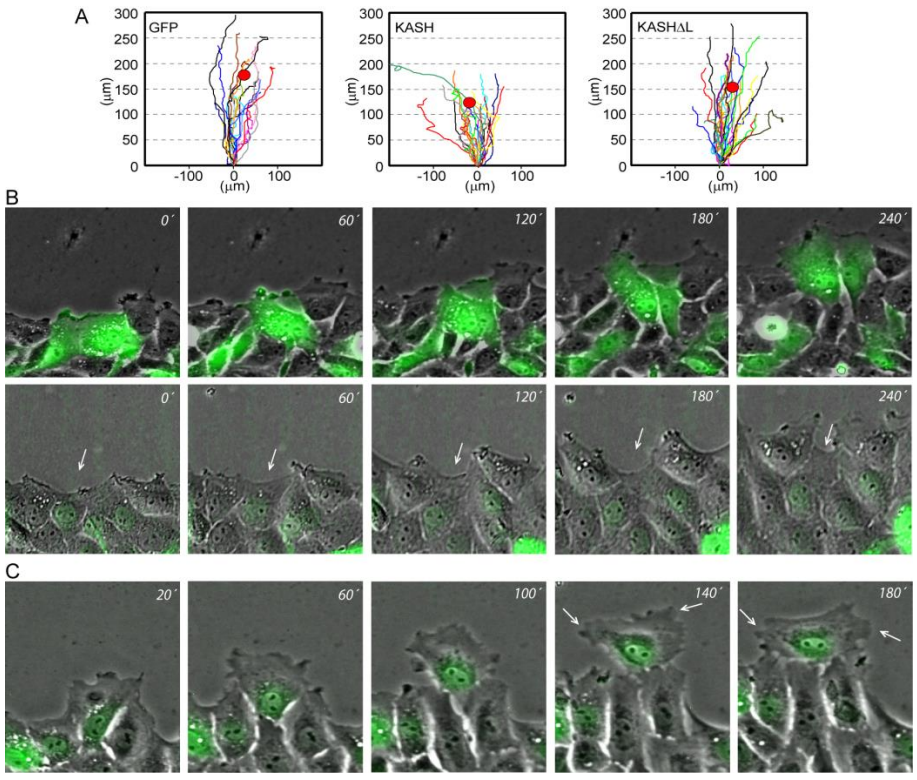

Fig. 4.13: Nuclear reorientation correlates with directional cell migration. (A) Quantification of migration of cells transfected with GFP or GFP-KASH or GFP-KASHΔL vector. GFP positive cells followed by live cell

microscopy were tracked during their migration to the wound. The plots show trajectories of 20 individual cells, red circle indicates the average migration length. (B-C) Phenotypes of GFP or GFP-KASH transfected cells. Images from time-lapse phase contrast and fluorescent microscopy of migrating GFP positive cells. (B) Migration of GFP control cells is shown in upper panel. Arrow in lower panel indicates GFP-KASH transfected cell lagging behind non-transfected cells (”lagging” phenotype). (C) GFP-KASH transfected cell that loose the directionality during the course of migration (“loss of polarity” phenotype). Arrowhead indicates cell which acquires “loss of polarity phenotype”. Arrows indicate uncoordinated multiple protrusions.

4.11. Presence or absence of perinuclear actin fibers correlates with different modes of migration

It has been shown that U2OS cells and also other cancer cells do not form perinuclear actin cap fibers (Kim et al., 2012; Lin et al., 2015). To further prove that perinuclear actin fibers drive the nuclear reorientation we quantified the presence of actin cap and measured the nuclear reorientation in U2OS cells. Although U2OS cells form other actin stress fibers, ventral, dorsal fibers and transverse arcs, majority of U2OS cells do no assemble perinuclear actin cap fibers (Fig. 4.14 A, B). Intriguingly, U2OS cells do not reorient their nuclei to the wound (Fig. 4.14 C) supporting our findings that perinuclear actin cap fibers are central to nuclear reorientation to the direction of migration. As U2OS cells do not reorient the nucleus we further studied the difference between U2OS and RAT2 cell migration. Solitary migrating U2OS cells usually display broad leading edge and their nucleus is oriented perpendicular to the direction of migration and they adopt crescent like shape. In clear contrast, RAT2 cells display elongated conical shape, form slender lamella and they align the nucleus to the direction of migration (Fig. 4.14 D). Following the migration of U2OS and RAT2 cells using live cell microscopy we found that these cells utilize different migration mode. RAT2 cells are moving using typical “inchworm manner” with clearly separated cyclical protrusion and retraction steps (Fig. 4.14 E). In contrast, these steps are largely diminished in U2OS cells and cells moves forward with continuous cell front protrusion and continuous cell rear inward movement. These observations suggest that nucleus orientation regulated by perinuclear actin cap fibers correlates with different mode of migration.

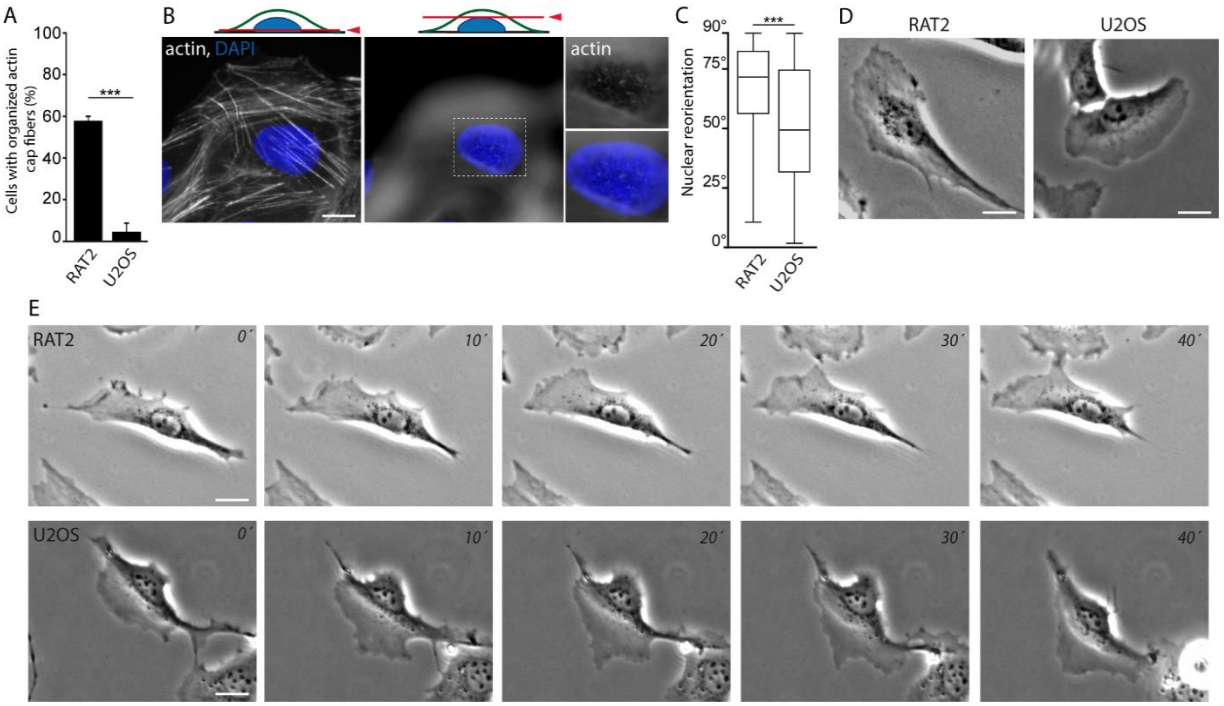

Fig. 4.14: Perinuclear actin fibers and nuclear orientation determine the mode of migration. (A) Quantification of perinuclear actin fibers in RAT2 and U2OS cells. RAT2 and U2OS cells polarizing to the wound were fixed 6 h after wounding. (B) Actin cytoskeleton in U2OS cells. Left panel shows ventral and dorsal fibers and transverse arcs on the basal side of U2OS cell. Right panel and magnification of boxed area shows actin staining on the dorsal side above the nucleus. Cells fixed 6 h after wounding were stained with phalloidin-rhodamine and DAPI. Focal planes are indicated above the pictures. Bar, 10 µm. (C) Nuclear reorientation in U2OS and RAT2 cells fixed 6 h after wounding. Data are presented as box and whiskers (*** p<0,001). (D-E) Images from time laps phase contrast microscopy recording the migration of RAT2 and U2OS cells. (D) Differences in shape of migrating RAT2 and U2OS cells. (E) RAT2 and U2OS cells use different migration mode. Bars, 20 µm.

4.12. Symmetry breaking in spreading fibroblasts requires RACK1 that integrates FAK, p190A-RhoGAP and ERK2 signaling

(Klímová et al., under review in BBA-MCR)

The establishment of cellular polarity is usually initiated by external signal such as chemotactic gradient, however there is growing body of evidence that establishment of cellular polarity may occur spontaneously without external cues. This publication shows that radial symmetry breaking of spreading fibroblasts and establishment of the cellular polarity is regulated by ERK scaffold protein RACK1 that upon adhesion promotes activation of ERK. ERK locally suppress p190A-RhoGAP by its depletion from the plasma membrane and thus cells form non-protruding cell rear. Therefore, RACK1 and ERK represent mechanism by which cells transform uniform extracellular signals to spatially distributed cellular signaling.

During the study of cellular polarity we found that attenuation of scaffold protein RACK1 resulted in dramatic changes of cell morphology. Compared to control cells that displayed typical front-rear polarity axis, RACK1 depletion resulted in loss of polarity characterized by rounded shape of cells. To determine the role of RACK1 protein in symmetry breaking the RACK1 siRNA transfected cells were plated on fibronectin. During adhesion, control cells formed protrusive leading edge and trailing rear with non-protruding regions whereas RACK1 depleted cells did not break the radial symmetry and maintained round shape (Fig. 4.15 A). The quantification utilizing cell's roundness index (Fig. 4.15 B) revealed that RACK1 deficient cells are significantly rounder that control cells (Fig. 4.15 C) reflecting the inability of RACK1 depleted cells to break the radial symmetry during adhesion. Not surprisingly, we observed that the changes in cells shape were accompanied with the changes in actin cytoskeleton organization. Actin staining in control cells showed typical thick peripheral actin bundles that underlied concave non-protruding regions at the cell sides and rear. In contrast, cells without RACK1 protein were largely devoid of concave non-protruding regions and thick peripheral actin bundles (Fig. 4.15 D). Moreover, time-laps microscopy of fluorescently labeled actin revealed that RACK1 depleted cells protruded along the whole periphery (data not shown).

Since cell adhesion activates FAK/p190A-RhoGAP signaling and since we observed accumulation of p190A-RhoGAP at the leading edge of migrating cells (see Fig. 4.4 B), we examined the role of FAK/p190A-RhoGAP signaling in symmetry breaking. Both knockdowns of FAK or p190A-RhoGAP reverted RACK1 phenotype suggesting that RACK1 opposes FAK-p190A-RhoGAP signaling (data shown in the manuscript). In addition, in

RACK1 depleted cells the localization of p190A-RhoGAP was altered. In contrast to control cells where p190A-RhoGAP localized at the leading edge and concave regions were devoid of p190A-RhoGAP, in cells transfected with RACK1 siRNA was p190A-RhoGAP localized around the whole cell periphery (Fig. 4.15 D). In addition, p190A-RhoGAP localization was altered in the same manner in ERK2 defective cells. As RACK1 promotes the activation of ERK we suggested that activated ERK2 regulates p190A-RhoGAP localization (data shown in the manuscript). As symmetry breaking preceded the polarity establishment we examined also perinuclear actin fibers. We found, that RACK1 deficient cells did not form actin cap, compared to control cells suggesting that perinuclear actin cap fibers may be other marker of cellular polarity in fibroblasts (Fig. 4.15 E).

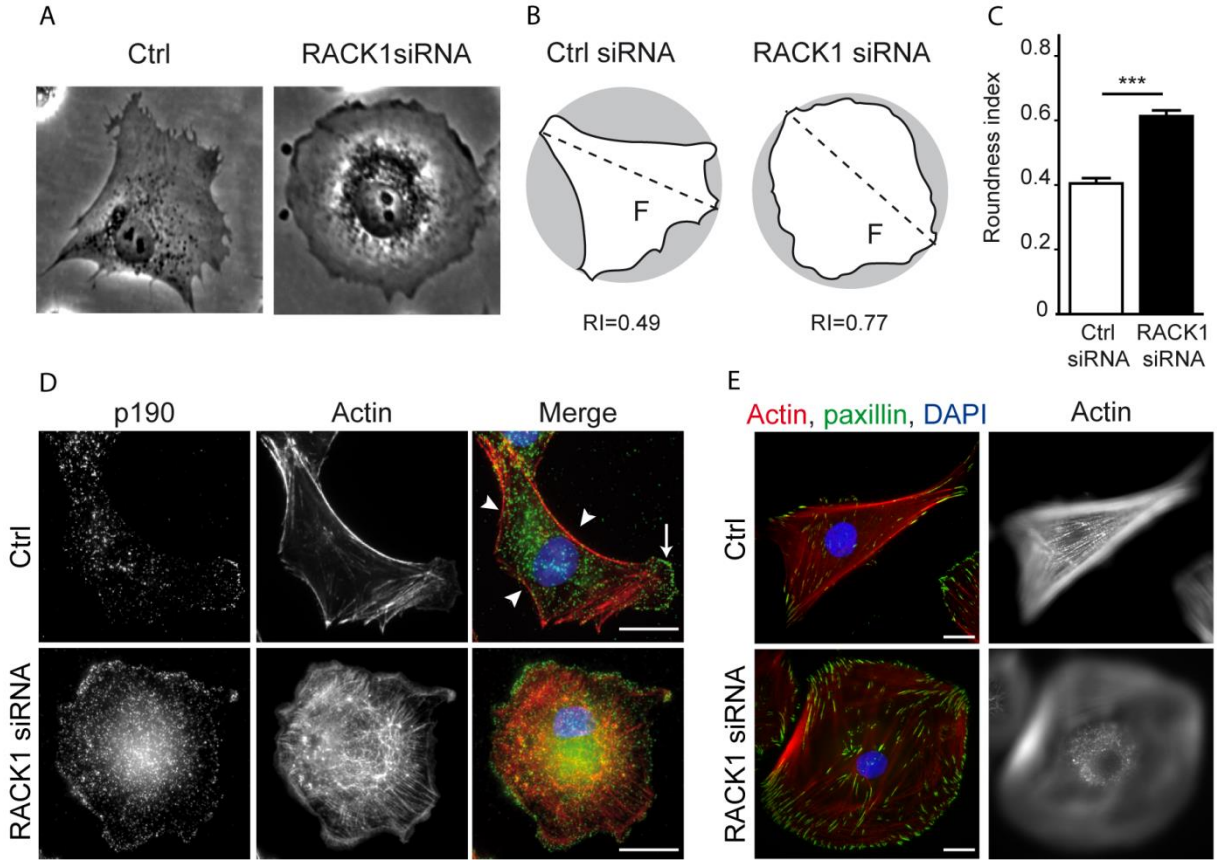

Fig. 4.15: RACK1 is required for symmetry breaking in spreading fibroblasts. (A) Representative images of cells transfected with control or RACK1 siRNA and plated on fibronectin (10 μg/ml). (B) Schema of determination of roundness index in control and RACK1 depleted cells. Shape of cells transfected with control or RACK1 siRNA is indicated. Parameters used for roundness index calculation are indicated by dashed line (ferret`s diameter, F), area of circle calculated based on ferret`s diameter by grey circle and cell area by white color. Roundness index was calculated according to formula RI=cell area/(ferret/2)$^2\pi$. Examples of values of roundness index are indicated under the pictures. Value closer to 1 indicates round phenotype. (C) Measurement of roundness index in control and RACK1 depleted cells. Data are presented as a mean ± SEM, ***p< 0.001 (n = 100 cells). (D) Localization of p190A-RhoGAP in control and RACK1 depleted cells. Cells transfected with control or RACK1 siRNAs for 48 h were plated on fibronectin (10 μg/ml) and fixed after 60 min. Cells were stained with p190A-RhoGAP antibody (green) and phalloidin-rhodamine (red). White arrow indicates localization of p190 in protruding areas in control cells. Arrowheads indicate non-protrusive concave regions formed during cell spreading. Bars, 20 μm. (E) RACK1 deficient cells do not form perinuclear actin cap fibers.

Images of control and RACK1 depleted cells plated on fibronectin. Cells were stained with paxillin antibody (green in merge channel) and phalloidin-rhodamine (red). Left panel shows control and RACK1 deficient cells on the basal side to present differences in cell shape. Right panel shows actin staining above the nucleus. Bars, 10 μm. Results in panels A-C were generated by PhD student Zuzana Klímová.

4.13. Ultrastructural localization of actin and actin-binding proteins in the nucleus

(Dingová et al., 2009)

Actin, one of the main components of cell cytoskeleton has been also found in the nucleus where has been shown to participate in the regulation of chromatin organization, transcription, RNA processing and transport from the nucleus (Visa and Percipalle, 2010). As in the cytoplasm also in the nucleus actin associates with actin binding proteins that bridge the actin with other nuclear components. In this manuscript we described ultrastructural distribution of actin and actin binding proteins in the cell nucleus.

Using the transmission electron microscopy and immunogold labeling on ultrathin sections we followed the localization of actin and several actin binding proteins in cell nucleus. To evaluate the distribution and clustering of immunogold labeling, we used special plugins developed for Ellipse program that map significantly labeled compartments in the whole cell. Actin clusters localized in areas of decondensed chromatin and in nucleolus and significant amount was present at the nuclear periphery under the nuclear envelope (Fig. 4.16). Localization of actin binding proteins spectrin and filamin was interesting as they formed dense meshwork throughout the whole nucleus. Especially their prominent localization under the nuclear membrane suggested that spectrin and filamin, that anchors plasma membrane to actin cytoskeleton in the cytoplasm, may play similar role also in the nucleus.

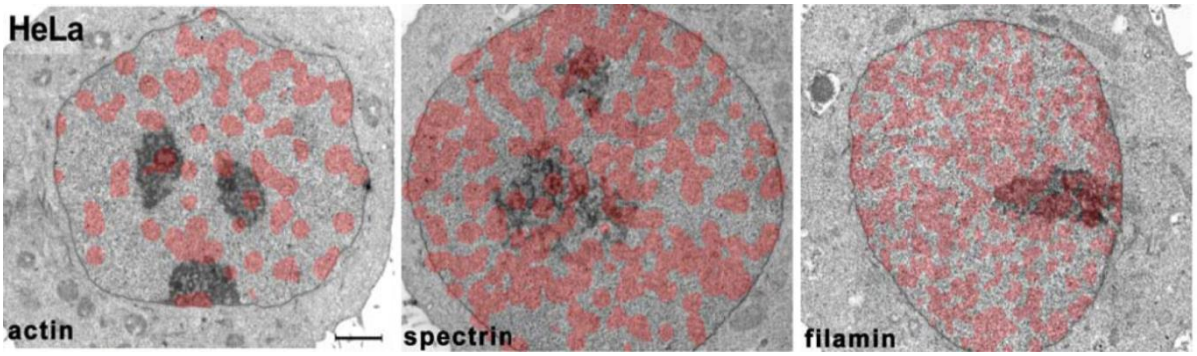

Fig. 4.16: Ultrastructural localization of actin, spectrin and filamin in nucleus of HeLa cells. Ultrathin sections of HeLa cells immunolabeled with antibodies against actin, spectrin and filamin and secondary antibodies conjugated with gold particles. The areas of high density labeling are highlighted with red color. Bar 1 μm.

5. LIST OF METHODS

1) Cell cultures
2) Plasmid and siRNA transfection
3) The polymerase chain reaction (PCR)
4) Plasmid construction
5) Live cell and fluorescence microscopy
6) Cell polarity and migration assays,
7) Replating assay
8) Determination of cell migration – live cell tracking
9) Determination of cell polarity
10) Determination of nuclear reorientation
11) Determination of roundness index
12) Western blotting, immunodetection
13) Transmission electron microscopy – immunogold labeling, negative staining
14) Isolation of nuclei – preparation of cytosolic and nuclear fractions

6. LIST OF PUBLICATIONS

Klímová Z., Bráborec V., Maninová M., Čáslavský J., Weber M.J. and Vomastek T. **Symmetry breaking in spreading Rat2 fibroblasts requires the MAPK/ERK pathway scaffold RACK1 that integrates FAK, p190A-RhoGAP and ERK2 signaling.**
(manuscript submitted to BBA-MCR; manuscript send back to authors for major revisions)
Author of this thesis contributes with results of p190A-RhoGAP localization.

Maninová M. and Vomastek T. **Dorsal stress fibers, transverse actin arcs and perinuclear actin fibers form interconnected network that induces nuclear movement in polarizing fibroblasts.**
(manuscript submitted to FEBS Journal; manuscript send back to authors for major revisions)
Author of this thesis as a first author elaborated all experiments and together with TV coordinated the project and prepared the manuscript.

Maninová M., Iwanicki M., Vomastek T. **Emerging role for nuclear rotation and orientation in cell migration.**
Cell Adh Migr 2014; 8(1): 42–48. IF (2014) 4.505
Author of this thesis together with TV prepared the manuscript. Parts of this manuscript were rewritten to this thesis.

Maninová M., Klímová Z., Parsons J.T., Weber M.J., Iwanicki M., Vomastek T. **The reorientation of cell nucleus promotes the establishment of front-rear polarity in migrating fibroblasts.**
J Mol Biol 2013; 425(11):2039-55. IF (2013) 3.959
Author of this thesis as a first author generated major part of results, participated in manuscript preparation and submission.

Dingová H., Fukalová J., Maninová M., Philimonenko V., Hozák P. **Ultrastructural localization of actin and actin-binding proteins in the nucleus.**
Histochem Cell Biol 2009; 131: 425-434. IF (2009) 3.021
The author of this thesis contributed with results from TEM (filamin, actinin and tropomyosin immunogold staining) and prepared cytosolic and nuclear fractions.

7. DISCUSSION

7.1. Nucleus reorientation and its regulation by signaling pathways

Taken together, we have shown that nuclear rotational movement, termed nuclear reorientation, is important component of directional cell migration. Cells polarizing to the wound reorient their nuclei perpendicular to the wound and then maintain the nucleus orientation aligned with the direction of migration as cells expand to the wound. LINC complex connecting the nucleus to actin cytoskeleton is essential for nuclear reorientation as it transfers cytoskeleton generated forces to the nucleus inducing nuclear reorientation. We have demonstrated that nuclear reorientation is regulated by two signaling events that converge at small GTPase RhoA. One signaling pathway is initiated by LPA leading to global cellular activation of RhoA. Other signaling is initiated at the leading edge by integrin engagement to ECM. Integrin activation induces formation of FAK/Src signaling complex that subsequently activates p190A-RhoGAP and inhibits RhoA. Dynamic spatiotemporal regulation of RhoA activity within the cell enables the reorientation of the nucleus allowing cell polarization and rearrangement of intracellular organelles. This intracellular organization is optimal for efficient directional migration.

LPA stimulation of trimeric G protein receptors activates the small GTPases Cdc42 and RhoA, and the activation of Cdc42 is believed to be central for the establishment of cellular polarity and nuclear movement (Gomes et al., 2005; Palazzo et al., 2001). Similarly, Cdc42 regulates the nucleus movement in 3T3 cells exposed to shear stress (Lee et al., 2005). In RAT2 fibroblasts model, the LPA stimulation of cells induced nuclear reorientation and cell polarization pointing to Cdc42 and RhoA as regulators of nuclear reorientation. However, interfering with RhoA activity blocked nuclear reorientation and, conversely, overexpression of RhoA specific GEF p115RhoGEF in serum deprived cells rescued the inhibition of nuclear reorientation. These data indicate that Cdc42 does not affect nuclear reorientation in RAT2 fibroblasts and that this nuclear movement is predominantly regulated by RhoA.

Cells migrating to the wound recognize the space created by the scratch and integrin binding to fibronectin at the front of the cell induce primary signal inducing cellular polarization (Etienne-Manneville and Hall, 2001). As integrins are activated differently at the cell front and at the back part of the cell, integrin activation provides a first signaling platform that facilitates the establishment of front-back polarity (Moissoglu and Schwarz, 2006).

Binding of integrins to proteins of ECM activates tyrosine kinase FAK that regulates cell polarity (Serrels et al., 2010; Tilghman et al., 2005; Tomar et al., 2009). Our results showed that FAK kinase is massively activated at the cell front and its downstream effector p190A-RhoGAP accumulates at the leading edge. It suggests that integrin and FAK/p190A-RhoGAP activation at the leading edge initiates first asymmetrical signaling event in RAT2 cells polarizing to the wound. Given that GTPase activating protein p190A-RhoGAP inhibits RhoA activity and that FAK transiently inhibits RhoA (Ren et al., 2000) we hypothesize that dynamic regulation of RhoA activity at the cell front results in cytoskeleton remodeling that enables the reorientation of the nucleus to the direction of migration. This is also in agreement with our other experimental system that shows that during the symmetry breaking of spreading fibroblasts p190A-RhoGAP localizes only to protruding areas and is absent in non-protruding concave regions with thick peripheral actin bundles.

7.2. Regulation of nuclear reorientation by actin cytoskeleton

To move with the nucleus it needs to be linked to cell cytoskeleton. This is mediated by LINC complex that anchors all three types of cytoskeletal filaments to the nuclear envelope (Razafsky and Hodzic, 2009; Starr and Fridolfsson, 2010). Our experiments where we interfered with microtubules showed that microtubules are not the main regulator of nuclear reorientation in RAT2 fibroblasts. Dynein inhibition impaired the nuclear reorientation only partially and 6 hours after wounding the nuclear reorientation was rather random. In addition, it has been shown that nocodazole treatment activates RhoA (Bershadsky et al., 1996; Lee and Chang, 2008; Liu et al., 1998). In cells pretreated with nocodazole we observed a large number of actin stress fibers including perinuclear fibers that can “lock” the nucleus and block nuclear reorientation. Nevertheless, the microtubules cannot be completely excluded as they can cooperate with actin or may serve as an additional mechanism supporting actin driven nuclear reorientation. The mechanisms are discussed in our second publication (Maninova et al., 2014).

We found that in our cellular system the nuclear reorientation is regulated by actin stress fibers. Actin fibers and LINC complex associate together directly (Padmakumar et al., 2004; Zhang et al., 2001; Zhen et al., 2002) and actomyosin forces are strong enough to move the nucleus (Luxton et al., 2010). We have shown for the first time that the perinuclear actin fibers present above the nucleus are required for nuclear movement. Perinuclear actin fibers are present in polarized cells and in cells in cell monolayer and are significantly disrupted

during cell polarization and nuclear reorientation to the wound. Thus it is tempting to consider perinuclear fibers as anchoring structure that stabilize the nucleus in a specific position. However, our data suggest that perinuclear actin fibers undergoes significant remodeling during cell polarization and that they are involved in the regulation of nuclear reorientation as cells in which we interfered with perinuclear actin fibers are not able to reorient their nuclei to the direction of migration. Intriguingly, the ventral actin fibers present under the nucleus seems not to be anchored to the nuclear envelope as the LINC complex disruption did not affect the arrangement of these fibers.

The assembly of perinuclear actin fibers is preceded by the formation of interconnected network composed of dorsal fibers, contractile transverse arcs and peripheral actin fibers. Dorsal fibers polymerization is driven by RhoA and mDia1 (Hotulainen and Lappalainen, 2006) and is α-actinin1 dependent (Kovac et al., 2013). Consistently, we observed that inhibition of RhoA-formin signaling axis or depletion of α-actinin1 affects the formation of dorsal fibers. Simultaneously, formation of perinuclear actin fibers was also blocked indicating that dorsal fibers are required for perinuclear fibers assembly. Significance of dorsal fibers for perinuclear actin fibers formation is also shown in our study of symmetry breaking where cells depleted in scaffold protein RACK1 displayed prolonged p190A-RhoGAP peripheral localization and presumably low Rho activity. As a consequence these cells do not form dorsal and perinuclear actin fibers. Inhibition of actomyosin contractility also impairs formation of perinuclear fibers and we suppose that contractility generated by transverse arcs is indispensable for perinuclear fibers formation. Taken together, all these experiments showed that interfering with dorsal fibers polymerization, perinuclear fibers formation and arcs contraction impedes the nuclear reorientation supporting our hypothesis that perinuclear fibers formation induces nucleus rotational movement.

Our work at least partly resolved the question how are the perinuclear fibers formed. It has been hypothesized that perinuclear actin cap fibers polymerize at focal adhesions or that they are formed at the nuclear surface (Kim et al., 2013). It was also suggested, by the same group, that new actin cap fibers are formed from ventral fibers. The pressure generated by actin cap fibers can displace the nucleus from its original position and moves it under neighboring basal stress fibers (Kim et al., 2012). We propose that perinuclear actin fibers develop from thick peripheral actin bundles that are recruited to the dorsal side and above the nucleus. We have shown that α-actinin1 crosslinked network of dorsal fibers and transverse arcs fuse with preexisting peripheral actin fibers. When fused to transverse arcs contraction

generated by transverse arcs mediates movement of these fibers toward the nucleus and crosslinking with dorsal fibers then mediates the dorsal orientation of the whole actin network.

Dorsal fibers polymerizing from the cell front associate with transverse arcs and they move together to the dorsal side with the same velocity (Hotulainen and Lappalainen, 2006; Tojkander et al., 2012), however, if these fibers are crosslinked remains unknown. Crosslinking of actin fibers by α-actinin1 was already observed several decades ago in spreading cells (Lazarides, 1975, 1976). In the same way, we observed the actin and α-actinin rich spots at the intersection of dorsal fibers and transverse arcs. Time laps fluorescent microscopy showed that these spots are moving from the leading edge toward the dorsal side and toward the nucleus further supporting the idea that these two types of stress fibers are mechanically coupled. Interestingly, we observed that crosslinking spots are formed only temporarily during cell polarization. In already polarized cells the crosslinking spots largely disappear and only perinuclear actin cap fibers directly linking adhesions with the nucleus are present. Thus it is tempting to speculate that perinuclear fibers crosslinked with dorsal fibers ultimately fuse and mature into perinuclear actin cap fibers that link focal adhesions with the nuclear envelope.

The mechanical actomyosin pulling forces generated by stress fibers has been shown to mediate the maturation of adhesion complexes and this process is accompanied by recruitment of several adhesion protein markers like vinculin or α-actinin (Galbraith et al., 2002; Laukaitis et al., 2001; Riveline et al., 2001). Perinuclear actin cap fibers are also contractile and they exert forces on the nucleus from the apical side thus reducing the high of the nucleus (Khatau et al., 2009). These forces could be seen as the indentation sites of the nucleus where actin cap fibers press on the nuclear envelope with such a force that causes deformation of the nuclear envelope and plunge of the perinuclear actin fiber to the nucleus ((Versaevel et al., 2014) and our unpublished observation). In addition, perinuclear actin fibers induce force-dependent recruitment of LINC complex proteins on the apical side of the nucleus that stabilize the fibers anchored to the nuclear envelope (Versaevel et al., 2014). Thus we hypothesize that perinuclear fibers exert forces on the nuclear envelope that induce LINC complex recruitment and maturation of anchorage sites where the perinuclear actin fibers are attached. Thus LINC complex functionally mimics the focal adhesions at the cell periphery as both these structures are stabilized under tension. Such an anchorage then may stabilize the nucleus in a specific position, maintaining the cellular polarity and promoting directional cell migration. Highly dynamic perinuclear fibers observed during the

establishment of cellular polarity probably reflect only temporary attachment of perinuclear fibers to LINC complexes. We speculate that dynamic perinuclear fibers move above the nucleus where they may disassemble or they form thick bundles (Gay et al., 2011a; Gay et al., 2011b) after the nucleus is reoriented.

Dorsal fibers are thought to associate with plasma membrane on the dorsal side of lamella providing a dorsal localization also for transverse arcs (Burnette et al., 2014). As perinuclear actin cap fibers, anchored to the nuclear envelope, are highly contractile (Khatau et al., 2009) and they are crosslinked with dorsal fibers and transverse arcs it is likely that they pull on dorsal fibers and actin arcs further promoting dorsal orientation of the whole interconnected actin network.

7.3. Biological significance of nuclear reorientation

7.3.1. Nuclear reorientation facilitates cell polarization

Why do cells reorient the nucleus during cell migration? One obvious reason for specific nuclear orientation is that it may promote cell polarization. During cell polarization nucleus moves rearward allowing centrosome and Golgi apparatus to reposition in front of the nucleus (Gomes et al., 2005). Our results also indicate that nuclear reorientation promotes the establishment of cellular polarity and subsequently cell motility as disruption of LINC complex affects MTOC/Golgi polarization and nucleus reorientation. However, our results indicate that the nuclear reorientation and MTOC/Golgi polarization are two separate events regulated by two different mechanisms – microtubules drive MTOC polarization independently of nuclear reorientation and *vice versa.* Moreover, our dominant negative KASH construct disrupts the anchorage of both actin and microtubules to the nucleus and provide us very little information about the requirements for specific cytoskeletal structures. The expression of nesprin proteins mutated in binding sites for microtubules or actin – when these sites are known - will shed a light on the mechanism how nucleus is moved and how it participates in cell polarization and migration.

7.3.2. Nuclear reorientation promotes cell migration

This work shows that disruption of nucleo-cytoskeletal linkage and also perinuclear fibers results in defects in directional cell migration to the wound. Interestingly, we observed two phenotypes in defective migration. Majority of cells did not reorient the nucleus to the wound and lagged behind other cells (lagging phenotype). However, over 30% of cells

initially moved faster to the wound but suddenly they collapsed at the edge of the wound, lost the directionality and stopped the movement (collapsed migration phenotype). Closer examination revealed that at the onset of migration nucleus in these cells was properly aligned with the direction of migration. We hypothesize that the proper nuclear orientation is a due to random orientation of nucleus that is also mechanically supported by neighboring cells. When the mechanical support of surrounding cells is lost nucleus became arranged perpendicular to the direction of migration and cells were not able to maintain the directional migration. This morphology was strikingly similar to the morphology in sparse cells with disrupted perinuclear fibers. These cells were not able to form leading and trailing edge and the distribution of dorsal fibers and transverse arcs was not restricted to the leading edge. These cells had round or crescent like phenotype like cells that collapsed at the edge of the wound. These results indicates that perinuclear fibers restricts the dorsal fibers and transverse arcs at the leading edge thus promote the conical cellular shape, reorient the nucleus toward the leading edge and contribute to the establishment of polarity and to the directional migration.

The observation that perinuclear fibers and nuclear reorientation determine the mode of cellular migration is completely unexpected discovery. Nucleus frequently displays ovoid or elliptical shape and the longer nuclear axis is aligned with the axis of migration in RAT2 cells. However, some cell types like human osteosarcoma U2OS or fish keratocytes have their elliptical nucleus oriented perpendicular to the axis of migration. These cells when migrate have broad fan-like shaped lamellipodial protrusions at the cell front and their movement is described as rolling of the body together with the cell nucleus (Anderson et al., 1996). Thus the nucleus rolling may ease the migration and help to overcome the blockage of cytoplasmic granules accumulated in front of the nucleus as has been shown during *C. elegans* development (Fridolfsson and Starr, 2010). In contrast, polarizing fibroblasts reorient the nucleus to the wound and to the direction of migration. The oval shaped nucleus may move forward like a “torpedo” through the cytoplasm and facilitate nucleus translocation as cells move. This nucleus orientation may represent another way how to overcome the obstacles in front of the nucleus and facilitates the cell migration.

Cells that form perinuclear fibers have elongated shape and the actin fibers are aligned with the longer nuclear axis and the longer axis of the cell. This predicts the cells for high persistentency of migration with slow change of direction. U2OS cells have also the longer nuclear axis aligned with the longer axis of the cell but in contrast to RAT2 cells they do not form perinuclear fibers. Their migration seems to be similar to migration of fish keratocytes, however, we observed that U2OS often formed protrusion at the side of the cell to explore the

area around and frequently change the direction of migration with low persistency of migration. U2OS are in fact cancer cells and their behavior during the migration is similar to that of cancer cells in the tissue when they search the path through the tissue or adapt to a chemical gradient of chemokines or growth factors. As we show that perinuclear fibers are regulated by Rho signaling, we hypothesize, that the loss of perinuclear fibers in U2OS cells is besides a consequence of deregulated RhoA signaling that favors these cells during the movement through tissues. Our unpublished results show that overexpression of p115RhoGEF in U2OS cells induce the formation of perinuclear fibers and it leads to changes in cell shape that is more similar to the shape of RAT2 fibroblasts. Nevertheless, this idea needs to be more tested and migrational assays with measurement of persistency of migration are necessary. These experiments are now in progress.

Nuclear shape can have a significant impact also in 3D migration, in addition to migration on flat 2D substrates. In 2D environment cells are usually spreading on greater area thus their nuclei are also larger in diameter and not much bulged in height. In contrast, in 3D matrices the cell nucleus has ovoid shape thus it is smaller in diameter. When the cells squeeze through the narrow pores the higher plasticity of the cell body allows the cell to pass through whereas much stiffer cell nucleus that is mechanically stabilized by structural proteins under the membrane represents obstacle for migration through 3D environment. The cells have several options how to pass through: they can degrade the environment by metalloproteinases and thus widen the path or they deform the cell body and the nucleus to fit into the pore constriction or they can find another way where they can go through easier (Friedl et al., 2011). Although, the cell nucleus displays quite high deformability the nuclear reorientation during the migration in 3D matrices could be important event that enables the cell to minimize the cell width. The actin cap-like structure was observed also in cells migrating in 3D matrices and the disruption of nucleo-cytoskeletal linkage dramatically affected the actin organization. In these cells actin collapsed around the nucleus resulting in loss of protrusivity and impaired migration (Khatau et al., 2012a). In contrast, other study shows that decrease of lamin A/C increases the deformability of cell nucleus and cell with reduced level of lamin A/C migrated faster through the narrow constrictions (Davidson et al., 2014) despite of presumably weakened LINC complex.

Deregulated level of lamin A/C is often sign of cancer cell and lamin A/C deficient cells often displays transient ruptures of nuclear envelope (Davidson et al., 2014; Denais and Lammerding, 2014). In these cancer cells, when they migrate in 3D lattice and squeeze the nucleus through the narrow constrictions, the nuclear envelope ruptures and is possible to

observe chromatin herniations to the cytoplasm. This recruits proteins of nuclear envelope integrity repair system ESCRT-III (Burke, 2015) that again close the nuclear envelope (Denais et al., 2016). This repetitive event then may contribute to genomic instability of cancer cells that further supports cancer progression (Denais and Lammerding, 2014). Thus the presence of perinuclear actin cap fibers is somewhat counterintuitive to cancer progression. We hypothesize, that it could represents mechanism that facilitates orientation of the nucleus to the direction of migration that enables to go through the narrow constriction in regulated manner with minimum damage.

7.3.3. Can be the nucleus itself polarized?

The fact that nucleus reorient during cell polarization leads us to the hypothesis that the nucleus reorientation is actually nucleus "polarization" inside the cell. Chromatin association with lamins determines its spatial positioning within the nucleus which plays important role in gene regulation. Keeping the gene in the specific location within the nucleus and thus within the cell may promote specific transcripts to be delivered to the specific locations. Indeed, it has been demonstrated that specific transcripts that promote migration are preferentially delivered to the leading edge of migrating cells (Mili et al., 2008).

Nuclear envelope itself can be polarized as nesprin-4 accumulates asymmetrically at the pole of the nucleus distally to the centrosome. Interestingly, overexpression of nesprin-4 leads to the polarization of other nuclear envelope components, including lamins and NPC proteins (Roux et al., 2009).

Polarization of the cell nucleus or nuclear envelope has been documented also in response to the formation of perinuclear actin cap fibers. Perinuclear actin fibers anchored to the apical side of the nucleus cause a tension that is through the LINC complex transferred to the nucleus and induce redistribution of lamin A/C to the apical side of the nucleus (Kim and Wirtz, 2015). There lamin A/C should serve as an absorber of tensional forces applied on the nucleus (Dahl et al., 2004). On the contrary, another study shows that higher mobility of lamin proteins is induced after the perturbation of perinuclear actin organization and formation of perinuclear actin strengthens the lamin A and chromatin association (Toh et al., 2015). Either way, lamin association with chromatin plays role in gene expression and evidence began to emerge that the perinuclear actin fibers may also play significant role. Lamin A/C as a component of nuclear matrix controls the level of histon deacetylation by regulation of the activity of histon deacetylases (Li et al., 2011). Accumulation of lamin A/C

on the apical side of the nucleus, induced by perinuclear fibers, leads to higher occurrence of hyper-acetylated histones under lamin A/C on the apex of the nucleus (Kim and Wirtz, 2015).

Perinuclear fibers may also act with such a force that it causes the indentations of nuclei leading to reversible high level of chromatin condensations (Versaevel et al., 2014). As perinuclear actin cap fibers directly link the nucleus with proteins of ECM they can transfer the mechanical signals from ECM directly to the nucleus and to the chromatin. Indeed, it has been previously shown that pulling on integrins lead to actin fibers reorganization and redistribution of subnuclear compartments that induce chromatin remodeling activating mechanosensitive genes (Maniotis et al., 1997a; Maniotis et al., 1997b). But it is still not clear whether the activation of mechanosensitive genes is the result of mechanical forces acting on the nucleus or whether the activation is mediated by signaling initiated in the cytoplasm.

7.4. Actin and actin binding proteins are present under the nuclear envelope

Actin, a well-known cytosolic protein implicated in many cellular functions, has been demonstrated also in the cell nucleus during several last few decades. This observation, originally considered rather controversially, lead to the understanding that actin may play important role also in the nucleus in many processes such as structural organization, DNA replication and transcription, chromatin remodeling and DNA repair (Castano et al., 2010; Kapoor and Shen, 2014; Miyamoto and Gurdon, 2013; Visa and Percipalle, 2010). Together with actin, several actin binding proteins were identified to locate within the nucleus (Gettemans et al., 2005). We know the ultrastructural localization of actin and several actin binding proteins in the cell nucleus, however, their role remain largely unclear. Actin binding proteins may assist in conformational changes of actin, but they also may be implicated in nuclear processes independently of actin. The localization of actin and actin binding proteins, spectrin and filamin, under the nuclear membrane lead to the hypothesis that these proteins may be involved in arrangement of nuclear periphery or that they may anchor nuclear structures to the nuclear envelope and nuclear pore complexes. It has been shown that lamin A has a potential to bind nuclear actin (Zastrow et al., 2004) thus it is possible that also nuclear actin may play role in mechanical signaling to mechanosensitive genes through the nuclear envelope. In the cytoplasm, spectrin and filamin anchors the plasma membrane and transmembrane proteins to actin cytoskeleton. Their localization under the nuclear membranes

suggests that spetrin and filamin may play similar role also in the nucleus linking the nuclear actin structures to the nuclear membrane. We hypothesize that actin and actin related proteins may transfer signals from ECM through the cytoplasm and nuclear envelope directly to the nucleus to cellular chromatin.

8. Conclusions

- nuclear reorientation is important component of migratory polarity that enables specific spatial organization of intracellular organelles that is optimal for effective cell migration;
- nuclear reorientation is regulated by two signaling axes LPA/p115RhoGEF and integrin/FAK/Src and p190A-RhoGAP signaling that converge at small GTPase Rho;
- dynamic spatio-temporal regulation of RhoA allows such a reorganization of cytoskeleton that induces movement of the nucleus;
- nuclear reorientation depends on LINC complex that connects the nucleus to perinuclear actin fibers;
- perinuclear fibers are formed from peripheral actin fibers that are recruited above the nucleus by dorsal fibers and contractile transverse arcs;
- dorsal fibers, transverse actin arcs and perinuclear actin fibers forms interconnected network that drives nuclear reorientation to the direction of migration;
- perinuclear actin fibers restrict the presence of dorsal fibers at the leading edge of the cell and facilitates the formation of the cell front;
- perinuclear actin fibers represent novel marker of cellular polarity in fibroblasts and their disruption results in loss of polarity;
- nuclear reorientation promotes the establishment of cellular polarity and correlates with efficient directional migration;
- presence or absence of perinuclear actin fibers correlate with different modes of migration;

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10. Appendices

The reorientation of cell nucleus promotes the establishment of front-rear polarity in migrating fibroblasts

(Maninová et al., 2013)

jmb Article

The Reorientation of Cell Nucleus Promotes the Establishment of Front–Rear Polarity in Migrating Fibroblasts

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Abstract

The establishment of cell polarity is an essential step in the process of cell migration. This process requires precise spatiotemporal coordination of signaling pathways that in most cells create the typical asymmetrical profile of a polarized cell with nucleus located at the cell rear and the microtubule organizing center (MTOC) positioned between the nucleus and the leading edge. During cell polarization, nucleus rearward positioning promotes correct microtubule organizing center localization and thus the establishment of front–rear polarity and directional migration. We found that cell polarization and directional migration require also the reorientation of the nucleus. Nuclear reorientation is manifested as temporally restricted nuclear rotation that aligns the nuclear axis with the axis of cell migration. We also found that nuclear reorientation requires physical connection between the nucleus and cytoskeleton mediated by the LINC (linker of nucleoskeleton and cytoskeleton) complex. Nuclear reorientation is controlled by coordinated activity of lysophosphatidic acid (LPA)-mediated activation of GTPase Rho and the activation of integrin, FAK (focal adhesion kinase), Src, and p190RhoGAP signaling pathway. Integrin signaling is spatially induced at the leading edge as FAK and p190RhoGAP are predominantly activated or localized at this location. We suggest that integrin activation within lamellipodia defines cell front, and subsequent FAK, Src, and p190RhoGAP signaling represents the polarity signal that induces reorientation of the nucleus and thus promotes the establishment of front–rear polarity.

Introduction

Nuclear movement and positioning have been implicated in many cellular and developmental processes such as yeast division, formation of multi-nucleated hypodermal syncytium in *Caenorhabditis elegans*, nuclei anchorage in muscular fibers and at neuromuscular junction, and migration of neuroepithelial cells.[1–3] Nucleus movement and anchoring to the specific intracellular location require physical linkage between the nucleus and cytoskeleton. The LINC (linker of nucleoskeleton and cytoskeleton) complex has been shown to function as a bridge that connects nuclear lamina with cytoskeleton by virtue of its ability to span both outer and inner nuclear membranes and to interact with nucleoplasmic A- and B-type lamins and cytoplasmic cytoskeleton. Proteins of the Sun (Sad1/UNC-84) and Nesprin (Nuclear envelope spectrin repeat) families are the major constituents of the LINC complex.[2,4] Both Sun and Nesprin proteins are transmembrane proteins that span the inner nuclear membrane and the outer nuclear membrane, respectively. Sun proteins interact with lamins at the nucleoplasmic side whereas Nesprin proteins associate either directly or indirectly with all three types of cytoskeletal filaments at the cytoplasmic side. Sun proteins associate with the KASH (Klarsicht/ANC-1/Syne homology) domain of Nesprin proteins in the perinuclear space linking

nuclear lamina to cytoskeleton. Depletion of A-type lamins, depletion of Sun proteins, or disruption of Sun–Nesprin interaction by the expression of KASH domain of Nesprin results in redistribution of endogenous Nesprins from the outer nuclear membrane to the endoplasmic reticulum, suggesting that Sun–Nesprin interaction is essential for LINC complex integrity.[5–9] Importantly, recent findings provide evidence that the association of Sun and Nesprin proteins and the functional LINC complex plays a central role in the transmission of intracellular forces between cytoskeleton and nucleus.[10] Consequently, forces exerted by cytoskeletal filaments and associated motor proteins regulate nuclear movement and positioning in several cell types.[2,3]

In polarized cells, the relative position of nucleus and microtubule organizing center (MTOC) defines an asymmetrical profile of polarized cells and it is important for efficient directional migration.[11,12] In adherent cells such as fibroblasts, the positioning of the nucleus is important in the establishment and maintaining of cellular asymmetry. Majority of adherent cell types adopt polarized morphology with nucleus located at the cell rear and MTOC localized between the leading edge and the nucleus. There are notable exceptions such as rat kangaroo Ptk cells and leukocytes migrating toward chemoattractant that position MTOC behind the nucleus.[13,14] In addition, the positioning of the MTOC may also vary within the same cells and it is dictated by the geometrical constraints applied to the cell.[15]

The establishment of cell polarity requires precise spatiotemporal coordination of signaling pathways that control nucleus and MTOC movement to the proper location. The evidence suggests that MTOC movement between the leading edge and the nucleus during cell polarization is a microtubule-dependent process and that it requires the activity of minus-end-directed microtubule motor protein dynein.[16,17] On the other hand, the movement and positioning of the nucleus in polarizing cells are less well understood. The nuclear rearward movement or positioning is predominantly dependent on actomyosin or actin-intermediate filament networks.[18,19] In addition, nuclear rotation has been observed in many cell types. Depending on the cell type, microtubules, intermediate filaments and actomyosin cytoskeleton have been implicated in the regulation of the rotational movement of the nucleus.[20–23] The evidence indicates the importance of the interaction of the LINC complex with cytoskeletal filaments in cell polarization. The functional inhibition of the LINC complex by depletion of Lamin A/C or by the expression of the KASH domain of Nesprin prevents nuclear movement and also MTOC polarization in response to shear stress or in cells polarizing toward the wound made in cell confluent monolayer.[9,10,24,25] These data point to the direct role of nucleus in the establishment of cell polarity.

Previous studies showed that during the polarization of NIH3T3 fibroblasts, the MTOC and nucleus coordinately but independently move to the cell centroid and cell rear, respectively. Nucleus movement to the cell rear is crucial for polarization as blocking the nuclear rearward movement impairs cell polarization and directed migration.[18,25] Here, we demonstrate that in RAT2 fibroblasts, nucleus positioning to the cell rear is not sufficient for cells to polarize. We show that active nucleus movement, which we refer to as nucleus reorientation, is also required for cell polarization. The nucleus reorientation is the temporally restricted rotation of nucleus that promotes alignment of the nucleus' longer axis with the axis of migration. Disruption of the nucleus–cytoskeleton linkage results in impaired nuclear reorientation, cell polarization, and directional migration. Nucleus reorientation requires coordinated activity of two different signaling pathways, lysophosphatidic acid (LPA)-mediated activation of GTPase Rho and the integrin/FAK (focal adhesion kinase)/Src/p190RhoGAP pathway.

Results

The reorientation of nucleus in polarizing cells

To analyze the role of the nuclear movement in cell polarization, we used wound healing assay to induce the polarization of RAT2 fibroblasts. Consistently with previous results,[26] we found that wounding of the RAT2 cell monolayer induced cell polarization in the cells at the wound edge. The establishment of cell polarity was characterized by nucleus localization at the cell rear and the MTOC located close to the cell center between the nucleus and leading edge (Fig. 1a). Moreover, the nuclei in polarized cells displayed specific and uniform orientation. In polarized cells, the longer nuclear axis was aligned with the axis of migration perpendicularly to the wound edge. In contrast, cells in a confluent monolayer displayed a non-polarized phenotype with random distribution of MTOC and random orientation of the longer nuclear axis (Fig. 1a).

To gain further understanding into the process of nuclear movement, we followed cells by live cell microscopy. In the cell monolayer, nuclei displayed only random oscillatory movements (Supplementary Movie S1). Wounding the cell monolayer in the presence of serum induced rapid spreading of the edge cells (Fig. 1b and Supplementary Movie S2). The analysis of the nucleus position relative to the cell center showed that, in the confluent monolayer, the nucleus is located close to the cell center but relocalized to the cell rear as cell polarized and advanced into the wound (Fig. 1b and c). Tracking the movement of the nucleus after wounding

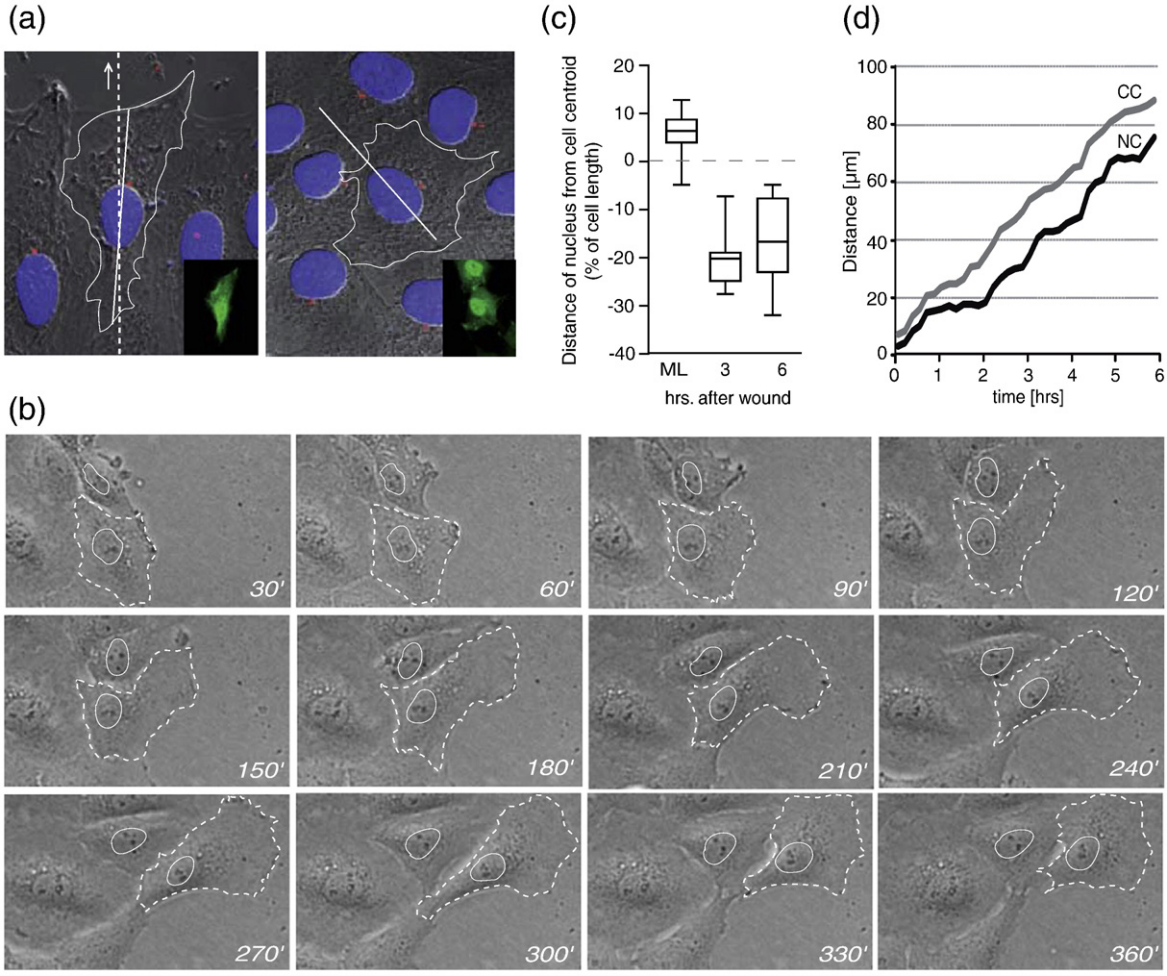

Fig. 1. Cell polarization and nuclear reorientation in wounded cell monolayer. (a) Cells at the edge of the wound (left panel) or cells in monolayer (right panel) were transfected with GFP, stained with γ-tubulin antibody, and counterstained with DAPI to visualize cell boundary, MTOC, and nucleus, respectively. Cell boundary is outlined; the axis of migration is indicated by a broken line; the longer nuclear axis is indicated by a continuous line. The arrow indicates the direction of migration. Insets show cells transfected with GFP. (b) Images from time-lapse phase contrast microscopy recording the dynamics of nuclear movement at the edge of the wound. The positions of nuclei are indicated by white lines; the cell is outlined by a broken line. Still images are extracted from Supplementary Movie 2. (c) Distance of nucleus from cell centroid during extension into the wound. Nucleus positioning in the polarizing cells was measured in cells recorded by time-lapse phase contrast microscopy. The data show the nucleus position relative to the cell centroid in cells in monolayer (ML) and at 3 or 6 h after wounding (n = 10). (d) Representative example of nucleus forward movement in the cell at the edge of the wound. The graph shows the time course of nucleus (NC) and cell (CC) centroids distance from the position of respective centroids at time = 0. Series of images from time-lapse phase contrast microscopy corresponding to Supplementary Movie 2 were used for centroids tracking.

revealed that the nucleus moved forward in the direction of the wound without significant rearward movement that could contribute to the nucleus positioning to the cell rear (Fig. 1d). Thus, nucleus relocalization to the cell rear is a result of the fast extension of cell into the wound and the slower phase of nucleus forward movement (Fig. 1d). Along with the rearward positioning, we observed that the wound made in the cell monolayer induced synchronous rotation of nuclei in cells at the wound edge (Fig. 1b and Supplementary Movie 2). Wound-induced nuclear rotation was temporal; nuclear rotation was dampened approximately 4 h after wounding and then moved only toward the wound as cells migrated (Fig. 1b). The termination of nuclear rotation coincided with the alignment of the longer nuclear axis with the axis of migration—we refer to this process of nucleus rotation and alignment of nuclear axis with the axis of migration as “nuclear reorientation”. The rotational movement

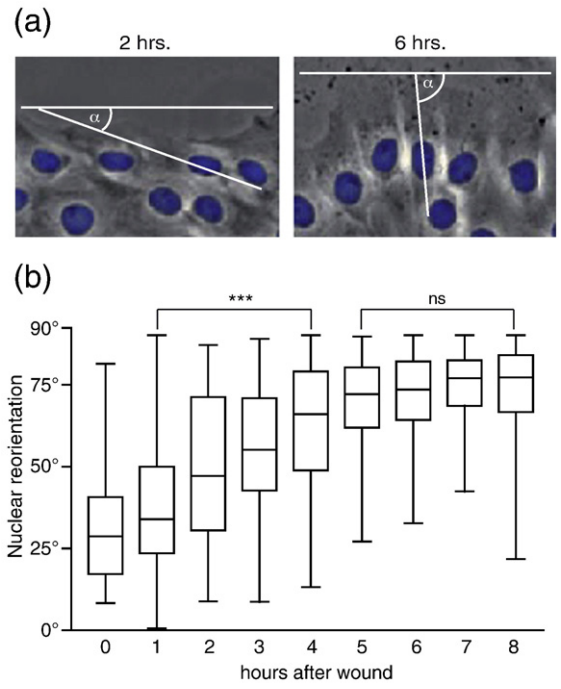

Fig. 2. Quantification of nuclear reorientation. (a) Measurement of nuclear reorientation. Cells fixed 2 h and 6 h after wounding were imaged by DIC microscopy and counterstained with DAPI. Nucleus reorientation is measured as an angle (α) between the longer nuclear axis and the wound. (b) Time course of nuclear reorientation. RAT2 cells were grown until confluency and wounded by pipette tip, fixed at different time points after wounding, and stained with DAPI; nuclear reorientation was determined as described in (a). Data are represented in box-and-whiskers plot with units in degrees. $^{***}p < 0.001$; ns, not significant. At least 100 cells per time point were analyzed.

encompassed the movement of the nucleoli, suggesting that the nucleoplasm is also rotating (Supplementary Fig. S1).

To quantitatively measure the time course of nuclear reorientation in wound healing assay, we calculated the angle between the wound and the longer nuclear axis as described in Materials and Methods (Fig. 2a). Time course of nuclear reorientation in cells fixed at different time points after wounding showed that at early time points after wounding, the nuclei were oriented more parallel to the wound as a consequence of wounding (Fig. 2b). However, at later time points, nuclei changed their orientation toward the wound. Similarly to nuclear reorientation observed in live cells, the reorientation of the nuclei occurred during the first 4 h and cells maintained this orientation for several hours (Fig. 2b). These data suggest the hypothesis that in RAT2 cells, wound-induced polarization includes spatial organization of the cell that involves both nuclear reorientation and nuclear relocalization to the cell rear.

Nuclear reorientation requires the LINC complex

In several cell systems, the nuclear movement and anchoring to specific location require linkage of the cell nucleus to the cell cytoskeleton mediated by the LINC complex.[2,3] Thus, we next determined whether nucleus reorientation requires the LINC complex. First, we attenuated Lamin A/C expression level by RNAi. We found that the depletion of Lamin A/C prevented nuclear reorientation (Fig. 3a). Since the disruption of Lamin A/C affects the morphology of the nucleus, we disrupted the LINC complex by the expression of the KASH domain of Nesprin-2 fused to green fluorescent protein (GFP). The KASH domain binds Sun proteins and prevents Sun–Nesprin interactions, thus functioning as a dominant negative form of the LINC complex.[6–8,10] The GFP-KASH protein localized to the nuclear envelope without altering the morphology of nuclei. Importantly, the expression of GFP-KASH efficiently blocked nucleus reorientation as compared to nuclear reorientation in GFP-transfected cells (Fig. 3b). Expression of control construct KASHΔL that is unable to bind Sun proteins did not affect nuclear reorientation although GFP-KASHΔL still localized to the nuclear envelope (Fig. 3b). Surprisingly, disruption of the LINC complex did not block the localization of the nucleus to the cell rear (Fig. 3c). These data indicate that in RAT2 cells, nuclear reorientation specifically requires the functional LINC complex and that the LINC complex is dispensable for the positioning of the nucleus to the cell rear.

Nuclear reorientation requires LPA and Rho signaling

Treatment of serum-deprived cells with LPA has been shown to induce active rearward movement of nucleus in wound healing assay.[18,25] Serum-deprived RAT2 cells were wounded and either left untreated or treated with LPA to determine whether LPA also regulates nuclear reorientation. We found that nuclear reorientation was inhibited in serum-deprived cells and that LPA addition rescued nuclear reorientation (Fig. 4a). LPA-induced rotation was similar to that induced by fetal bovine serum (unpublished results); however, the treatment of cells with epidermal growth factor (EGF) had minimal effect on nuclear reorientation (Fig. 4a).

Since LPA potently activates Rho GTPase,[27] the role of RhoA in nucleus reorientation was examined next. The cells were transfected with either constitutively active or dominant negative RhoA constructs fused to GFP and nuclear reorientation was determined in GFP-positive cells at the wound edge. In the presence of serum, the expression of constitutively active RhoA inhibited the nuclear reorientation compared to control cells transfected with GFP alone (Fig. 4b). In addition, nuclear reorientation was also inhibited by expression of dominant negative RhoA (Fig. 4b). To further confirm

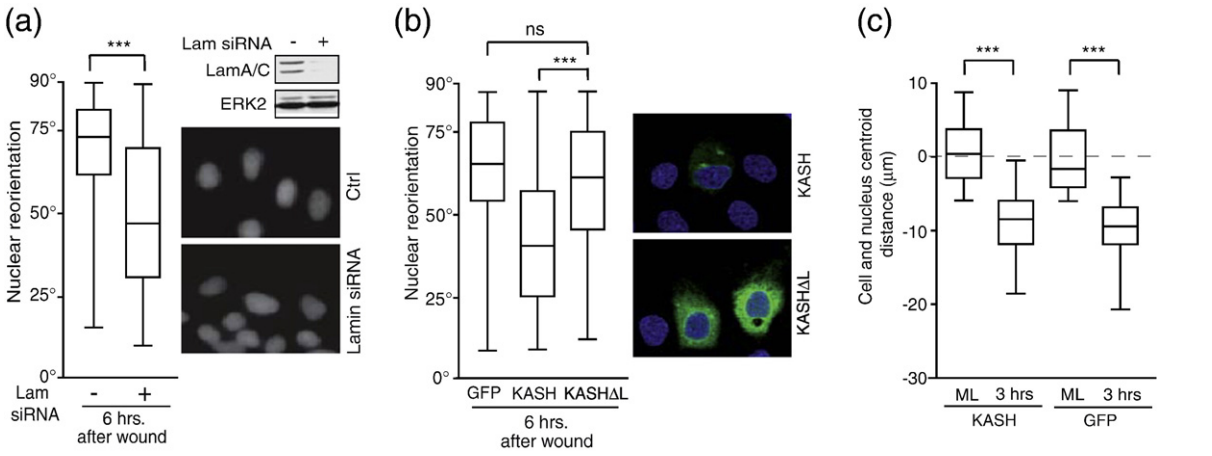

Fig. 3. Nuclear reorientation requires the LINC complex. (a) Nuclear reorientation in mock- or Lamin A/C siRNA-transfected cells. RAT2 cells were transfected as indicated, wounded, and fixed 6 h after wounding. Nuclear reorientation was determined as described in Fig. 2a. Cell lysates were probed with Lamin A/C and ERK antibodies to confirm knockdown efficiency and equal loading of proteins, respectively. Since Lamin A/C depletion alters nuclear morphology, only nuclei with oval shape were analyzed. (b) Nuclear reorientation in cells transfected with GFP, GFP-KASH, and GFP-KASHΔL. RAT2 cells were transfected as indicated, fixed 6 h after wounding, and counterstained with DAPI. (c) Nucleus rearward positioning in GFP- and GFP-KASH-transfected cells. Distance of nucleus centroid from cell center was measured in cells in monolayer (ML) or cells fixed 3 h after wounding (3 h). Data are represented in the box-and-whiskers plot. At least 35 cells were analyzed for each condition. $^{***}p < 0.001$; ns, not significant.

that RhoA signaling is necessary for nuclear reorientation, cells were treated with cell-permeable C3 exotransferase that specifically inhibits Rho proteins without affecting the activity of GTPases Rac and Cdc42. Inhibition of Rho by C3 exotransferase resulted in inhibition of nuclear reorientation (Fig. 4c).

LPA regulation of Rho can be attributed to the activation of guanine exchange factors (GEFs), and the family of RGS-RhoGEFs, p115-RhoGEF, PDZ-RhoGEF, and LARG, serve as effectors of activated LPA receptor and activate Rho.[28] Ectopic overexpression of RGS-RhoGEFs is sufficient to activate Rho in the absence of LPA[29] and allows cycling between Rho-GDP- and Rho-GTP-bound states. We thus determined whether expression of RGS-RhoGEFs can bypass the requirement for LPA and restore nuclear reorientation in serum-deprived cells. Cells were transfected with either GFP-p115RhoGEF or empty control vector, serum deprived for 5 h, and wounded in the absence of serum. We found that the expression of GFP-p115RhoGEF is sufficient to restore nuclear reorientation (Fig. 4d). These data indicate that LPA-mediated Rho GTP/GDP cycling is required for nucleus reorientation and that the increase in RhoA loading mediated by p115RhoGEF can bypass the requirement of LPA.

Integrin–FAK–Src–p190RhoGAP signaling pathway regulates nuclear reorientation

Actin polymerization at the leading edge drives lamellipodial protrusions that are stabilized by binding of integrins to the extracellular matrix (ECM) proteins. Integrin activation and clustering lead to the recruitment of structural and signaling proteins and the formation of focal adhesions.[30] Since we observed that the nucleus actively changes the position to point toward the leading edge and lamellipodial protrusion, we asked if integrin activation and signaling were important for nuclear reorientation. We found that blocking the function of integrins with RGD peptide or blocking antibodies against integrin β1 and β3 subunits inhibited nuclear reorientation (Fig. 5a and Supplementary Fig. S2).

Integrin engagement to ECM stimulates tyrosine phosphorylation and activation of the non-receptor tyrosine kinase FAK. FAK activation leads to recruitment and activation of Src.[31,32] We therefore tested the requirement of integrin signaling components FAK and Src. We used RNAi to attenuate the expression level of FAK protein. As shown in Fig. 5b, cells treated with FAK small interfering RNA (siRNA) were unable to reorient the nucleus. In addition, blocking the kinase activity of FAK by the pharmacological inhibitor PF573228[33] largely prevented nuclear reorientation (Supplementary Fig. S3). To determine the effect of Src on nuclear reorientation, we pretreated the cells with the Src family kinase inhibitor SU6656. Similarly to FAK knockdown, Src inhibition prevented nuclear reorientation (Fig. 5c). In addition, SYF fibroblasts, which are deficient of Src family protein kinases Src, Yes, and Fyn,[34] were also unable to reorient the nucleus in the wound assay and this defect was rescued by re-expressing Src (Supplementary Fig.

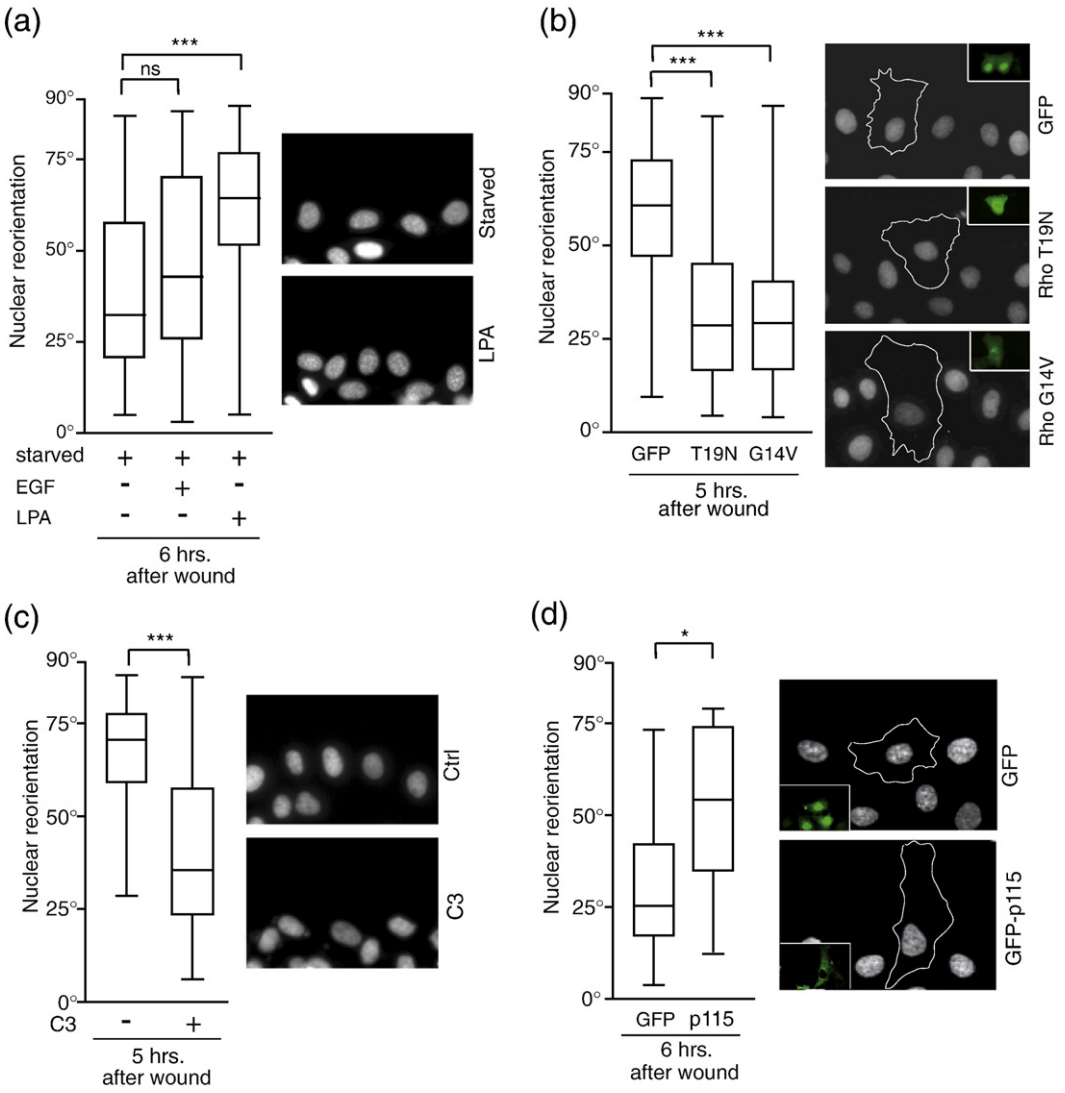

Fig. 4. Nuclear reorientation requires LPA and Rho signaling. (a) LPA induces nucleus reorientation. Confluent cells were serum deprived for 5 h, treated with LPA (10 μM) or EGF (20 ng/ml), and wounded. Nuclear reorientation was determined 6 h after wounding as described in Fig. 2a. (b) Nuclear reorientation in cells transfected with constitutively active or dominant negative RhoA. Cells were transfected with either dominant negative (RhoA-T19N) or constitutively active (RhoA-G14V) RhoA constructs fused to GFP. Nuclear reorientation was determined in GFP-positive cells fixed 5 h after wounding. Outlined cells in the right panel show the nucleus orientation in GFP-positive cells (insets). (c) Nuclear reorientation in C3-exotransferase-treated cells. Nuclear reorientation was determined in RAT2 cells pretreated with cell-permeable C3 exotransferase for 4 h and fixed 5 h after wounding. (d) Nuclear reorientation was determined in cells transfected with either GFP (Ctrl) or GFP-p115RhoGEF, fixed 6 h after wounding, and stained with DAPI.

S4). Thus, integrins, FAK, and Src are required for nuclear reorientation.

Src phosphorylates FAK on several tyrosine residues, creating binding sites for FAK-associated proteins and phosphorylated FAK associates with several proteins involved in the regulation of Rho family GTP.[31,35] Among them, Rho GTPase-activating protein (GAP) p190A-RhoGAP is a plausible candidate for nuclear reorientation as p190RhoGAP is activated by the FAK/Src signaling complex and regulates front–rear polarity.[36–40] To test whether p190RhoGAP regulates nuclear reorientation, we attenuated p190RhoGAP expression by siRNA treatment. We found that depletion of p190RhoGAP prevented nuclear reorientation (Fig. 5d). The organization of cell–matrix adhesions, actin cytoskeleton, and the phosphorylation of focal adhesion components FAK and paxillin were largely unaffected by p190RhoGAP knockdown (Fig. 5e and Supplementary Fig. S5), suggesting that p190RhoGAP acts downstream of adhesion and integrin signaling.

Given that integrins, FAK, and p190RhoGAP can suppress the activity of RhoA,[27,38,39,41] these findings imply that nuclear reorientation requires integrin-mediated downregulation of RhoA activity. Taken together with our previous experiment showing that GTP loading of Rho was required for LPA-receptor-mediated nuclear reorientation, these data suggest that integrin, FAK/Src, and p190RhoGAP signaling intervenes with LPA- and RGS-RhoGEF-dependent RhoA activation to regulate nuclear reorientation. We tested this hypothesis by knockdown of p190RhoGAP and simultaneous

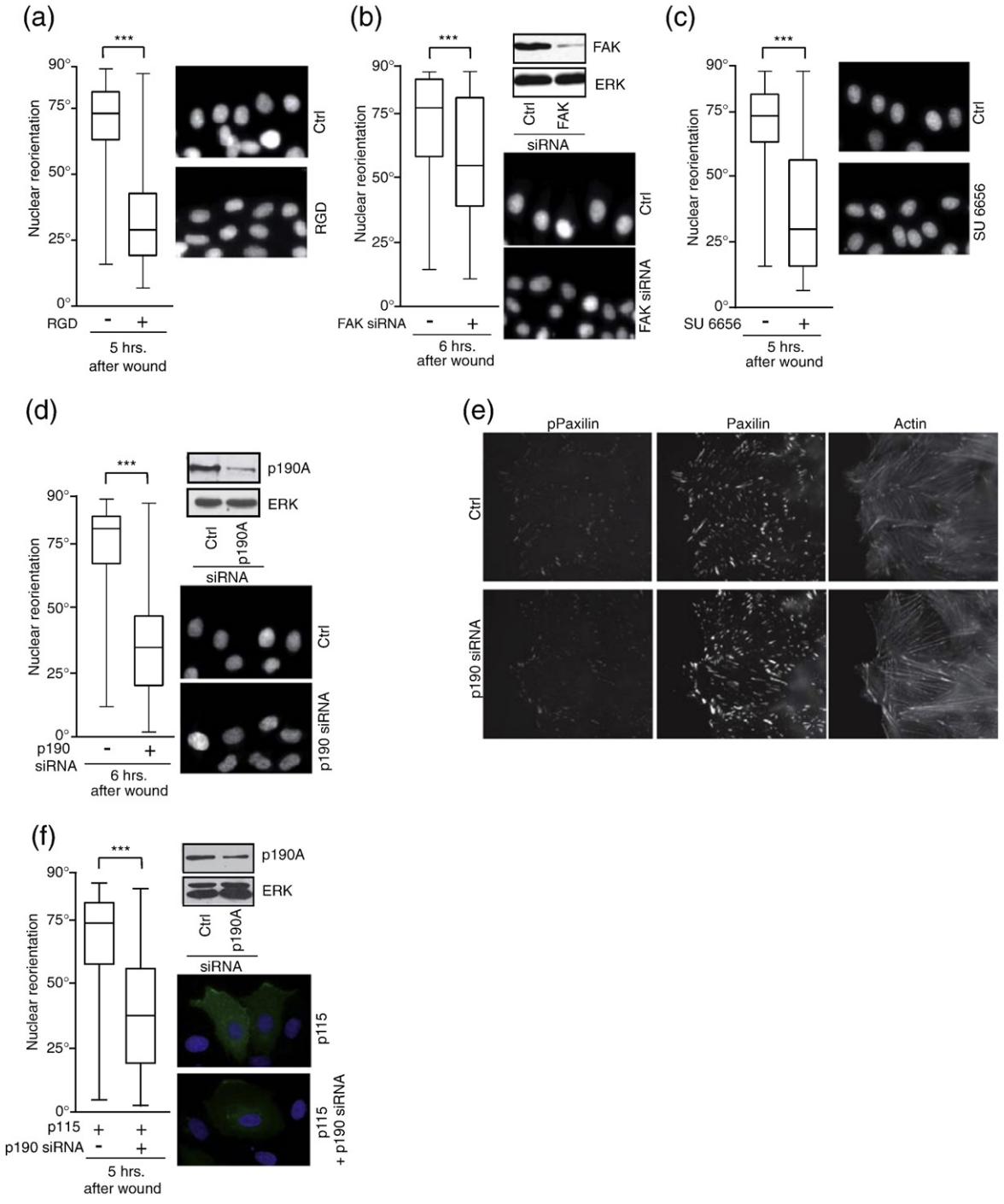

Fig. 5 (*legend on next page*)

expression of p115-RhoGEF. We found that the nuclear reorientation induced by p115-RhoGEF in serum-deprived cells was abolished by p190RhoGAP knockdown (Fig. 5f).

Spatiotemporal regulation of FAK and p190RhoGAP

The observation that nuclear reorientation requires both LPA/Rho and integrin/FAK/Src/p190RhoGAP signaling raised the question how these signals are integrated. We hypothesized that acute integrin signaling at the cell front induces FAK–p190RhoGAP activation that locally suppress RhoA activity. Since integrin engagement to ECM stimulates FAK phosphorylation on tyrosine residue 397, we examined the intracellular localization of active FAK. Wounded cells were co-stained with antibodies recognizing total FAK and active FAK phosphorylated on Tyr397 (Fig. 6a). In cells analyzed 3 h after wounding, we detected the distinct profile of FAK activation within the cell. Active FAK was predominantly localized within the edge and front of the cell while lower levels of active FAK were found at the cell rear (Fig. 6a). Consistently, ratio imaging of active FAK to total FAK showed that the high ratio of active FAK to total FAK was found at the leading edge of cells migrating into the wound and decreased toward the cell rear (Fig. 6b). On the contrary, this spatial profile of FAK activation was either undetectable in cell in the confluent monolayer or largely diminished in cells stained 1 h after wounding (Fig. 6a and b). These findings are in agreement with the idea that acute integrin engagement at the cell front results in localized FAK activation in this location.

Since cellular adhesion also induces p190RhoGAP recruitment to cell periphery,[40,42,43] we next examined the localization of p190RhoGAP. We found that the wound in the cell monolayer induced the p190RhoGAP accumulation at the leading edge (Fig. 6c). Similarly to FAK, p190RhoGAP localization at the leading edge was time dependent. High amount of p190RhoGAP at the leading edge was detected in cells fixed 3 h after wounding, whereas at earlier time points, negligible amount of p190RhoGAP was detected (Fig. 6c). We conclude that integrin, FAK, and Src signaling at the cell front promotes the recruitment of p190RhoGAP to transiently suppress Rho activity at this location.

Nuclear reorientation is required for cell polarization

Next, we investigated the biological significance of nuclear reorientation. Our observations that a longer nuclear axis aligns with the axis of migration and that it correlates with the establishment of cell polarity motivated the hypothesis that nuclear reorientation plays a role in cell polarization. Since nuclear rotation is controlled by the activity of two different signaling pathways, LPA-mediated activation of Rho-GTPase and the integrin/FAK/Src/p190RhoGAP signaling, we first determined whether manipulating with these pathways affects the establishment of cell polarity. Serum-deprived cells were wounded and left untreated or treated with LPA to test the effect of LPA. We found that LPA addition to serum-deprived cells induced cell polarization as determined by Golgi positioning between the leading edge and nucleus (Fig. 7a), consistent with previous results.[17] In addition, expression of GFP-p115RhoGEF substituted the requirement of LPA and restored cell polarization in serum-deprived cells (Fig. 7b). To interfere with the integrin/FAK/Src/p190RhoGAP signaling pathway, we examined the requirement of FAK and Src signaling for cell polarization. We found that in RAT2 cells, both FAK inhibition by PF573228 and Src inhibition by SU6656 prevented Golgi polarization (Fig. 7c and d).

Fig. 5. Nuclear reorientation requires integrin, FAK, Src, and p190RhoGAP signaling. (a) Blocking integrins impairs nuclear reorientation. Confluent monolayer of RAT2 cells was preincubated with RGD peptide (250 μM) for 30 min and wound-induced nuclear reorientation was determined 5 h after wounding. (b) Knockdown of FAK blocks nuclear reorientation. RAT2 cells were either mock or FAK siRNA transfected and fixed 6 h after wounding. Nuclear reorientation was determined as described in Fig. 2a. Cell lysates were probed with antibody directed against FAK and ERK2 to confirm knockdown efficiency and equal loading of proteins, respectively (right panel). (c) Inhibition of Src family kinases blocks nuclear reorientation. Confluent monolayer of RAT2 cells was pretreated with Src inhibitor SU6656 (1 μM) for 10 min. Nuclear reorientation was determined in cells fixed 5 h after wounding. (d) Knockdown of p190RhoGAP blocks nuclear reorientation. RAT2 cells were either mock or p190RhoGAP siRNA transfected and fixed 6 h after wounding. Cell lysates were probed with antibody directed against p190A-RhoGAP and ERK2 to confirm knockdown efficiency and equal loading of proteins, respectively. (d) Focal adhesion staining after downregulation of p190-RhoGAP. RAT2 cells were transfected with p190RhoGAP siRNA, wounded, and fixed 6 h after wounding. Cells were co-stained with antibodies recognizing the phosphorylated form of paxilin (pPaxilin Y-118) and paxilin. Actin was visualized with rhodamine phalloidin. (e) Knockdown of p190RhoGAP blocks nuclear reorientation induced by GFP-p115RhoGEF. RAT2 cells were co-transfected with mock or p190RhoGAP siRNA with GFP-p115RhoGEF and fixed 6 h after wounding. Cell lysates were probed with antibody directed against p190A-RhoGAP and ERK2 to confirm knockdown efficiency and equal loading of proteins, respectively. Data are represented in the box-and-whiskers plot. $^{***}p < 0.001$.

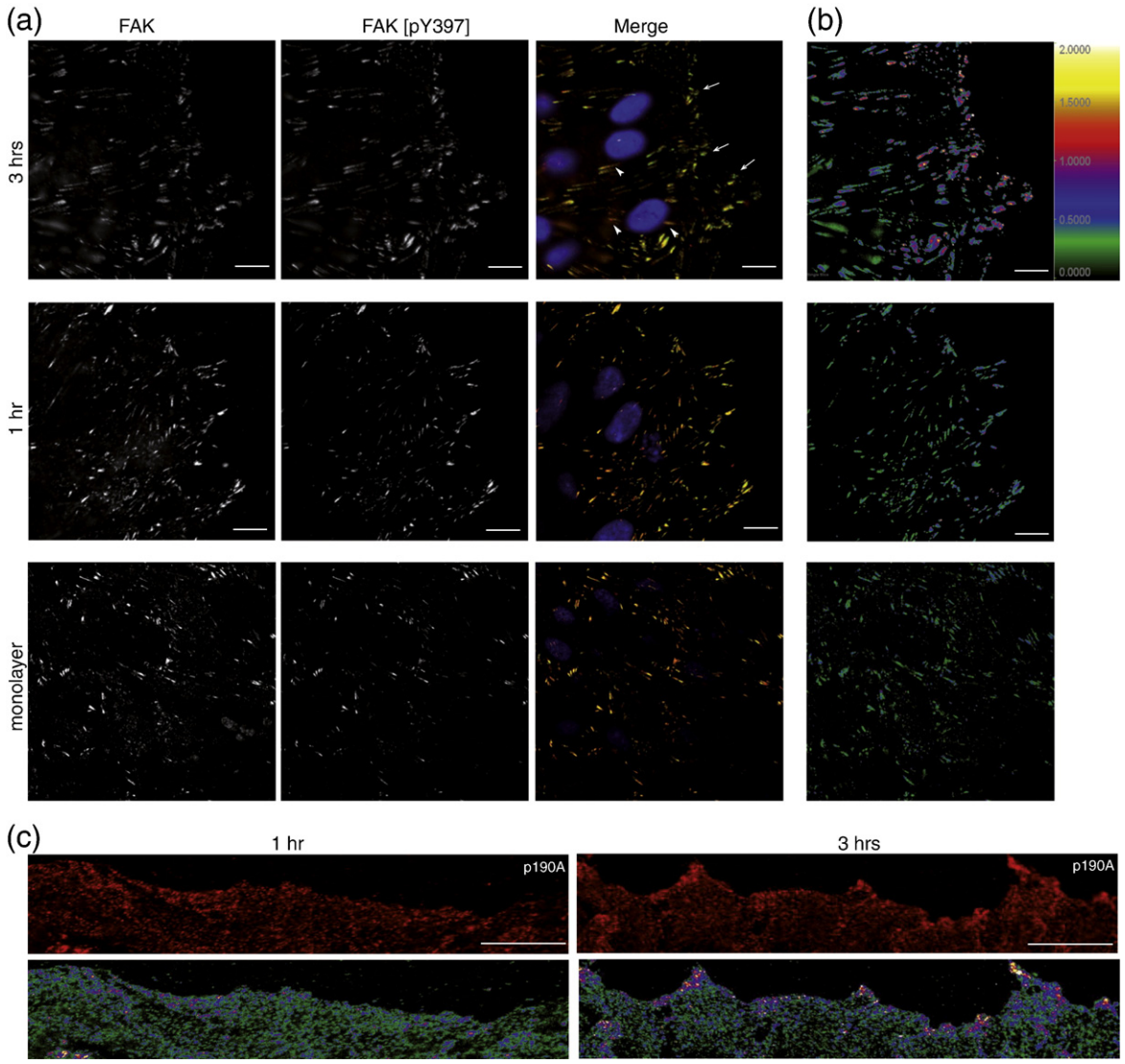

Fig. 6. Spatiotemporal regulation of FAK and p190RhoGAP. (a) Time course of FAK activation at the leading edge. Confluent monolayer of RAT2 cells was wounded, fixed at indicated time points, and co-stained with antibodies recognizing the active form of FAK (FAK-pY397, green), total FAK (red), and counterstained with DAPI (blue). Arrows in the merged image point to areas with high FAK activation that appear as green. Arrowheads point to areas with low FAK activation that appear as red. The scale bar represents 10 μm. (b) Ratio of FAK-pY397 to total FAK fluorescence signals. Immunofluorescence signals from images shown in Fig. 5a were pseudocolored to display the FAKpY397/FAK ratio. High active FAK/FAK ratio is yellow/white and low ratio is black/green. The scale bar represents 10 μm. (c) Recruitment of p190RhoGAP to the leading edge. RAT2 cells were wounded, fixed at indicated time points, and stained with p190RhoGAP antibody (red, upper panel). Lower panels show p190RhoGAP distribution pseudocolored according to fluorescence signal intensity to accentuate leading edge localization. The scale bar represents 40 μm.

To further confirm the role of nucleus reorientation in cell polarization, we examined whether disrupting the LINC complex affects wound-induced cell polarization. Lamin A/C attenuation prevented nuclear reorientation (see Fig. 3a) and Lamin A/C knockdown also blocked cell polarization as determined by Golgi and MTOC positioning (Fig. 8a and Supplementary Fig. S6). In addition, the expression of the KASH domain but not KASHΔL or GFP-alone control construct efficiently blocked cell polarization (Fig. 8b). We also found that the functional LINC complex is important for p115RhoGEF-mediated nuclear reorientation and cell polarization. The expression of the KASH domain of Nesprin-2 blocked both cell polarization and nuclear reorientation in p115RhoGEF-transfected cells (Fig. 8c and d). Taken together with our observations that the expression of KASH domain specifically blocks

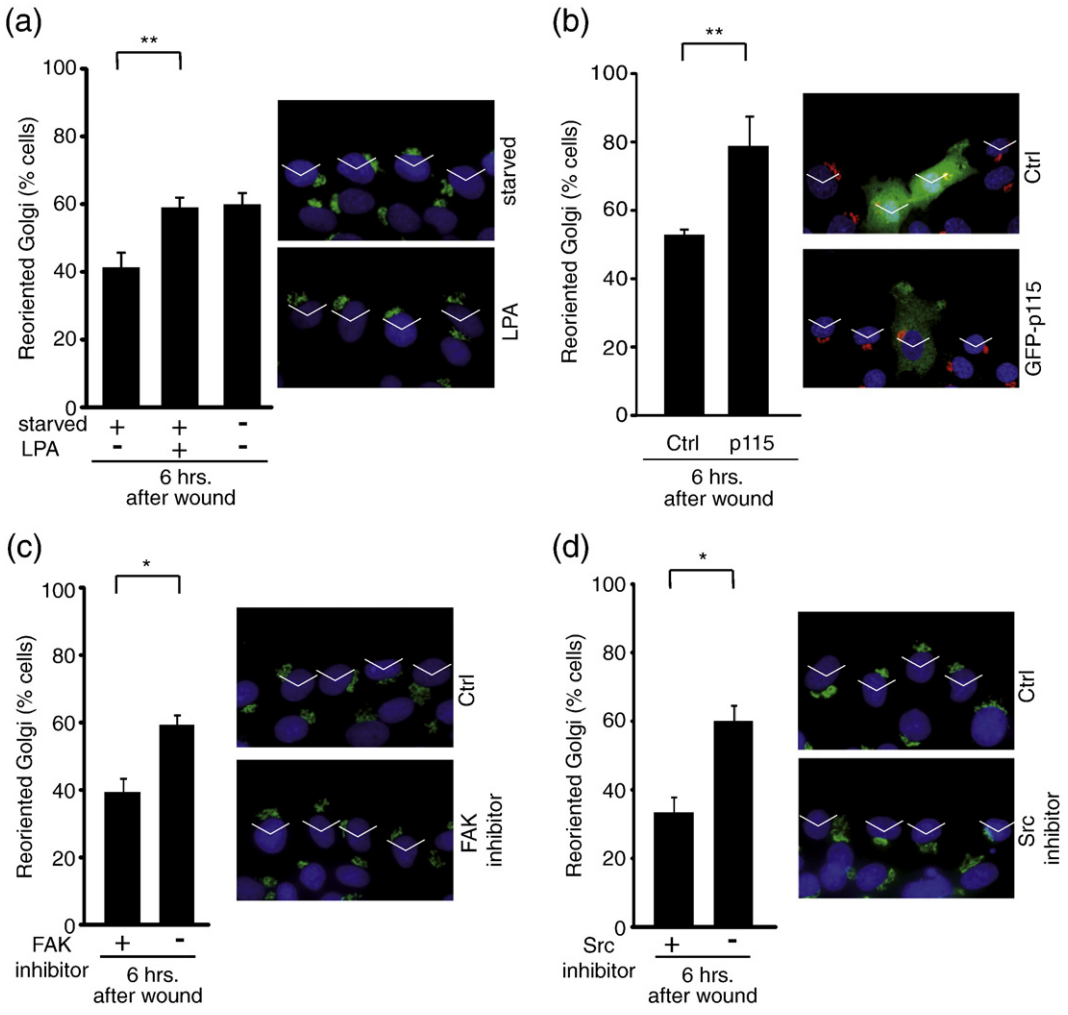

Fig. 7. LPA and FAK-Src signaling are required for the establishment of cell polarity. (a) LPA induces cell polarization. Confluent cells were starved for 5 h, treated with LPA, and fixed 6 h after wounding. Cells were stained for Golgi (GM130, green) and nucleus (DAPI, blue). (b) Cell polarization determined in cells transfected with p115RhoGEF fused to GFP, fixed 6 h after wounding, and stained for Golgi (red) and nucleus (blue). (c and d) FAK and Src inhibitor treatment blocks cell polarization. Confluent cells were treated with FAK inhibitor PF573228 (c) or Src inhibitor SU6656 (d), fixed 6 h after wounding, and stained for Golgi (green) and nucleus (blue).

nuclear reorientation but not nuclear localization to the cell rear, these data suggest that the positioning of the nucleus to the cell rear is not sufficient for cell polarization. Thus, in RAT2 cells, the establishment of front–rear polarity is tightly coupled to nuclear reorientation.

Nuclear reorientation correlates with efficient cell migration into the wound

Since the disruption of the LINC complex inhibits nucleus reorientation and the establishment of cell polarity, we examined whether the impaired polarization and nuclear reorientation also affect directional cell migration of RAT2 cells. To measure directional migration, we used wound healing assay and measured the migration by live cell microscopy followed by tracking the trajectories of individual cells migrating into the wound. We found that the expression of GFP-KASH resulted in impaired directional migration compared to control cells (Fig. 9a–c and Supplementary Movies S3 and S4), consistent with previous results.[10,25] Collectively, these observations show that nuclear reorientation correlates with the establishment of front–rear polarity and efficient cell migration into the wound.

Discussion

Taken together, the data presented here suggest that nuclear reorientation is an important component

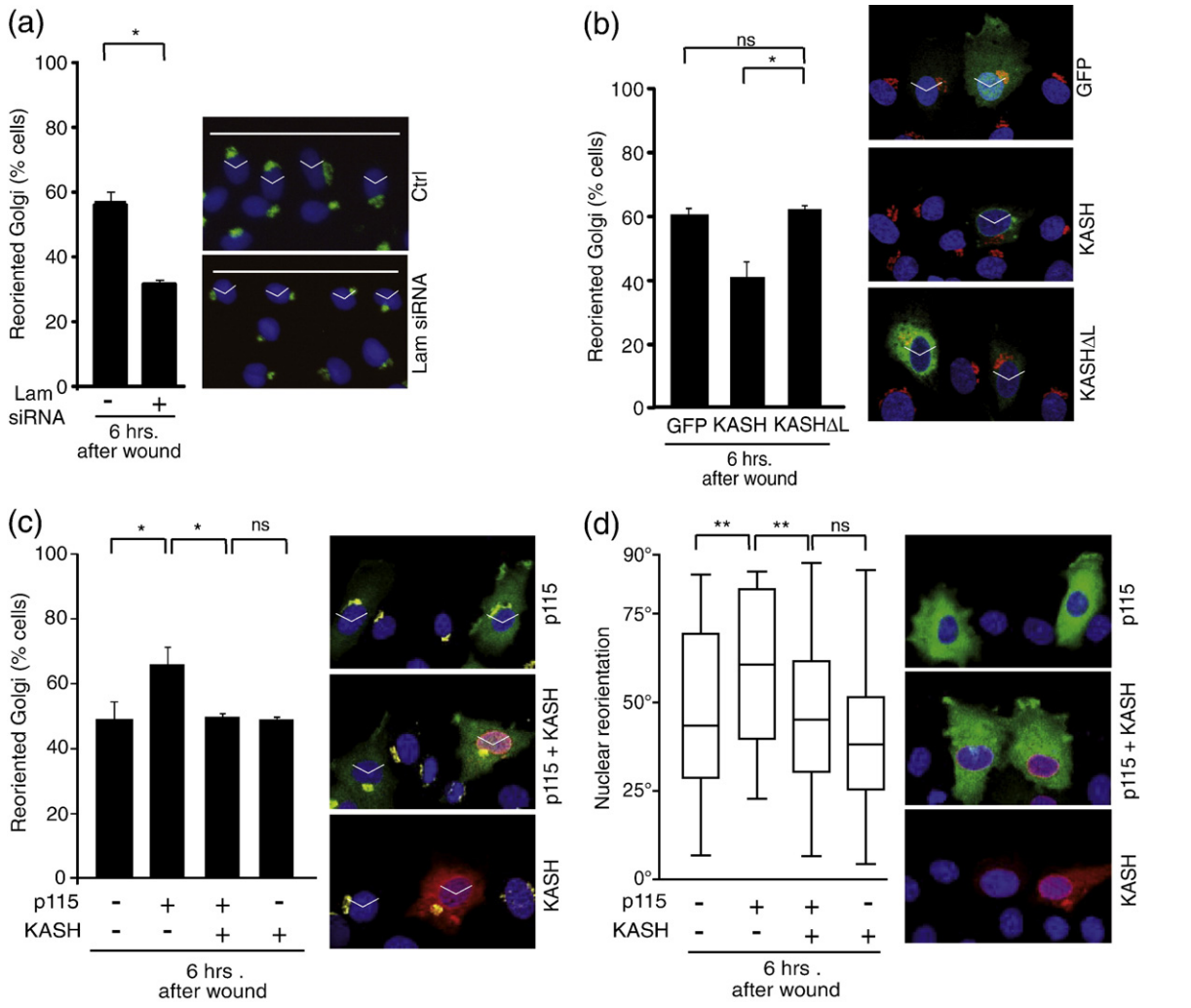

Fig. 8. The LINC complex is required for the establishment of cell polarity. (a) Cell polarization in mock- or Lamin A/C siRNA-transfected cells. Cells were fixed 6 h after wounding and stained with GM130 antibody (green) and counterstained with DAPI (blue). (b) Golgi polarization (red) in cells transfected with GFP, GFP-KASH, and GFP-KASHΔL fixed at 6 h after transfection. (c) Cell polarization in cells co-expressing GFP-p115RhoGEF (green) and tdT-KASH (red), fixed at 6 h after wounding, and stained for Golgi (yellow). In (a) to (c), cell polarization was determined as described in Materials and Methods. Data are represented as mean ± SD. $^{*}p < 0.05$; ns, not significant. (d) Nuclear reorientation in cells co-expressing GFP-p115RhoGEF (green) and tdT-KASH (red), fixed at 6 h after wounding. Nuclear reorientation was determined as described in Materials and Methods. Data are represented in the box-and-whiskers plot. $^{**}p < 0.01$; ns, not significant.

of migratory polarity. Nuclear reorientation requires two signaling events that converge at small GTPase Rho. The first signal is provided by LPA that globally stimulates Rho. The second signal is provided by the acute integrin engagement to ECM proteins within the leading edge that activates the FAK/Src/p190RhoGAP signaling pathway. The coordination of LPA and integrin signaling induces the alignment of nuclear axis with MTOC and direction of migration. This spatial organization of migrating cell appears to be optimal for cell migration.

The localization of nucleus to the cell rear promotes correct MTOC positioning in wounded fibroblasts as well as in astrocytes and in epithelial and endothelial cells plated on microfabricated matrices.[18,44] In NIH3T3 fibroblasts, the rearward movement is dependent on the LINC complex that associates with the actin cytoskeleton on the dorsal side of the nucleus;[25] however, in astrocytes, the nuclear rearward positioning is LINC complex independent.[19] Using the KASH domain of Nesprin-2, we were able to dissect nuclear reorientation from rearward positioning, showing that in RAT2 cells, the functional LINC complex is specifically required for nuclear reorientation while it is dispensable for nuclear rearward positioning. Thus, the observed defect in MTOC polarization is likely to

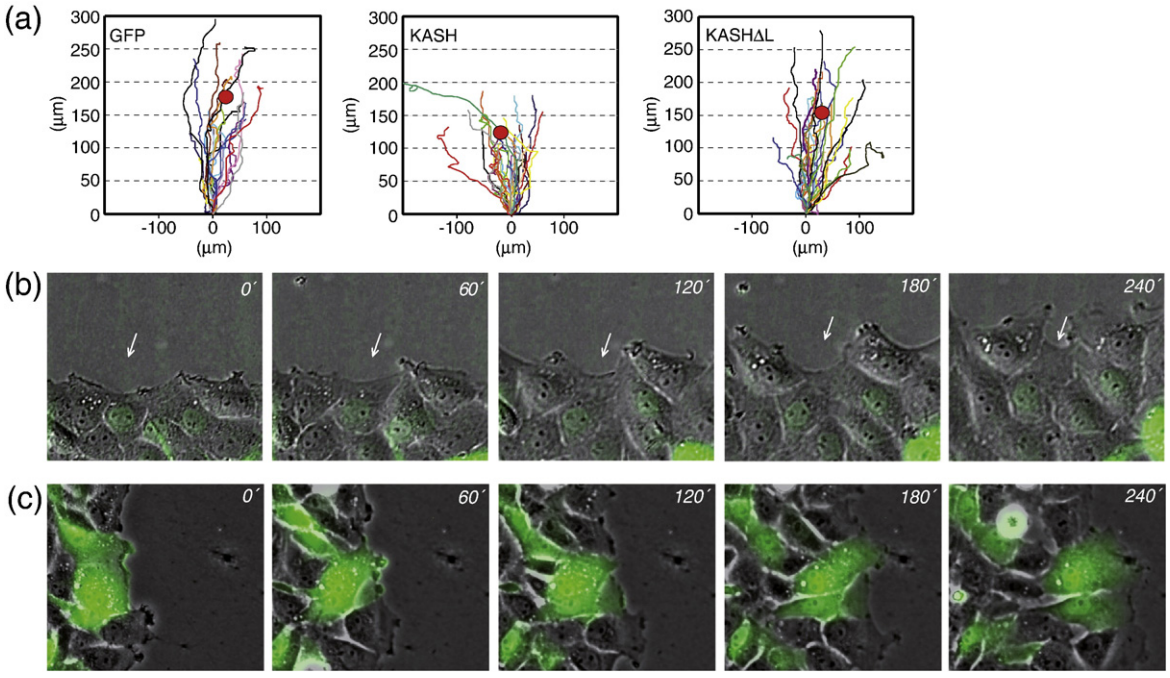

Fig. 9. The LINC complex disruption impairs directional cell migration. (a) Quantification of cell migration. RAT2 cells transfected with GFP, GFP-KASH, and GFP-KASHΔL were grown until confluency and wounded, and the migration of GFP-positive cells was followed by live cell microscopy. The plot shows trajectories of 20–30 individual cells; the red circle indicates the average migration length. (b and c) Images from time-lapse phase contrast and fluorescent microscopy recording the movement of GFP-KASH-transfected (b) and GFP-transfected (c) cells. The arrow in (b) indicates GFP-KASH-transfected cell lagging behind non-transfected cells.

be a consequence of impaired nuclear reorientation rather than nuclear rearward positioning.

In many cell types, the MTOC positioning between the nucleus and the cell leading edge is important in maintaining and propagating the polarized state of the cell. Microtubules emanating from MTOC are arranged in radial arrays pointing preferentially toward the leading edge and they support directed delivery of growth factor receptors, lipids, integrins, and actin polymerization machinery components.[45–48] In addition, microtubules at the cell front induce focal adhesions, endocytosis, and disassembly.[49,50] These events increase specific processes at the leading edge of migrating cells and support directional migration. The disruption of the LINC complex causes migration defect in cells migrating in planar two-dimensional conditions as well as in three-dimensional ECM.[10,24,25,51] Impaired MTOC polarization in KASH-transfected cells is likely causing the impaired directional migration linking cell migration to nuclear reorientation.

We have identified LPA and integrin pathways as a main signaling component involved in the regulation of nuclear reorientation. In the wound healing assay, integrins recognize the space created by the scratch, and their activation represents a primary polarity signal that induces cell polarization.[16] Because integrin activation differs between cell, front and cell rear, integrins provide a signaling platform for generating front–back polarity.[52] Integrin engagement to ECM stimulates the activation of tyrosine kinase FAK, and FAK signaling has been shown to regulate cell polarization.[26,40,53] We observed that both FAK activation and localization of FAK downstream effector p190RhoGAP are enriched at the cell front. Thus, the acute integrin, FAK, and p190RhoGAP activation or localization at the cell front establishes an initial asymmetrical signaling event in cell polarization.

The second signal in nuclear reorientation is provided by the LPA receptor. LPA signaling has been shown to regulate cell polarization and cell movement through the activation of small GTPases Cdc42[17,18] and Rho.[54–56] In RAT2 cells, the LPA requirement during nuclear reorientation and cell polarization could be substituted by direct activation of RhoA. These data suggest that in RAT2, the activation of Cdc42 by LPA is dispensable for nuclear reorientation and cell polarization although we cannot rule out that Cdc42 can be activated by different means, for example, by integrins.[16,57] In addition, our data point to Rho as a node integrating

FAK and LPA signaling during nuclear reorientation. Integrin signaling suppresses the activity of RhoA only transiently,[27,41] indicating that the dynamic regulation of Rho and cytoskeleton remodeling at the cell front promote nuclear reorientation.

The forces that regulate nuclear reorientation remain to be characterized. In fibroblasts, contractile actin bundles associate with the nucleus through the LINC complex on the apical side of the nucleus.[25,58,59] Apical actin fibers but not basal stress fibers connect the nucleus to a specific subset of focal adhesions at the cell periphery.[58,60] The inhibition of myosin II activity by RNA interference or by blebbistatin stimulates nuclear rotation.[21,23] These data raise the possibility that nucleus-associated apical actin fibers and focal adhesions restrict nuclear rotation, similarly to intermediate filaments.[22,61,62]

We found that nuclear reorientation is impaired by nocodazole (unpublished results), implicating microtubule-mediated forces in nuclear reorientation. Consistently, it has been shown that in fibroblasts, microtubules drive nuclear rotation.[20–22,62] We envisage two not mutually exclusive models that could be involved in the nuclear reorientation. Microtubule motor proteins dynein and kinesin localize to the nuclear envelope and they are both involved in nuclear rotation.[21,22,63,64] Syne/Nesprin-2 proteins interact with both dynein and kinesin, indicating that the LINC complex attaches nucleus to microtubules.[63] As a result, nuclear reorientation may be driven by microtubule motor proteins associated with the LINC complex at the nuclear envelope. This also raises the possibility that actin or intermediate filaments dampen nuclear rotation as suggested previously.[21,22] Alternatively, nuclear reorientation may be induced by MTOC and involve also cortically anchored dynein. Cortical dynein exerts pulling forces on the microtubules and moves MTOC close to the cell center between the leading edge and the nucleus.[65–68] Dynein is enriched at the leading edge of polarizing cells and moves MTOC forward to maintain its centrality as cells expand into the wound.[69] Since MTOC is closely attached to the nucleus, MTOC movement to the cell center during cell polarization may exert torque forces on the nucleus, thus inducing nuclear reorientation.

In conclusion, our findings suggest that nuclear reorientation plays an active role in MTOC centration and thus in cell polarization. The precise mechanism by which nuclear reorientation promotes MTOC centration remains unknown. Much evidence point to microtubule pulling forces exerted by cortically anchored dynein that move the MTOC, and these forces became balanced when the MTOC reaches the geometrical center of the cell.[11,70–72] Since the nucleus is by far the largest organelle in the cell, the association of centrosomal microtubules and dynein with the nuclear envelope most likely plays an important role in MTOC orientation. It is tempting to speculate that microtubule motor proteins acting from the nuclear envelope cooperate with cortically exerted forces to promote MTOC centration and consequently define the spatial organization of migrating cell.

Materials and Methods

Materials

RGD peptide, LPA, and FAK inhibitor PF573228 were purchased from Sigma. SU6656 was from Calbiochem, and EGF was from Upstate Biotechnology. C3 cell-permeable transferase was from Cytoskeleton Inc. Integrin β1 (clone HMb1-1) and β3 (clone 2C9.G3) blocking antibodies were from eBioscience Inc. Lamin A/C, phospho-Paxillin (Tyr118), and p190A-RhoGAP rabbit polyclonal antibodies were from Cell Signaling. The following antibodies were used for immunofluorescence staining: anti-p190A-RhoGAP, GM130 (mouse, BD Transduction Laboratories), anti-γ-tubulin (mouse, ExBio Prague), and anti-paxillin (mouse, Upstate Biotechnology). Fluorescent secondary goat anti-mouse IgG and goat anti-rabbit IgG antibodies labeled with Alexa Fluor 488 and 546 were from Invitrogen. Cy5-labeled anti-rabbit IgG was from Jackson ImmunoResearch. Horseradish-peroxidase-conjugated goat anti-mouse IgG and goat anti-rabbit IgG antibodies were from Sigma.

Cell culture and plasmid transfection

RAT2 cells were maintained in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% fetal bovine serum (Gibco). Cells were transfected with plasmid DNA using Lipofectamine 2000 (Invitrogen). GFP-Rho constructs (RhoA-G14V and RhoA-T19N) were a gift from Prof. Ingo Just. GFP-p115RhoGEF was obtained from Dr. Phillip Wedegaertner.

For LPA and EGF stimulation experiments, confluent cells were serum deprived for 5 h and either LPA (10 μM) or EGF (20 ng/ml) was added just before wounding. To determine nuclear reorientation in C3-transferase-treated cells, we pretreated the confluent monolayer with cell-permeable C3 transferase in serum-free medium for 4 h and changed the culture medium to a medium containing 10% fetal bovine serum before wounding. For the FAK and Src inhibition experiments, the confluent cells were preincubated with FAK inhibitor PF573228 and Src inhibitor SU6656 for 10 min before wounding.

Small interfering RNA

A double-stranded siRNA against rat p190A-RhoGAP targeted the 19-nt sequence GGTGGTGACGATCTGGGCT, and control siRNA targeted the AGGTAGTGTAATCGCCTTG sequence. The sequences of FAK (GCTAGTGACGTATGGATGT) and Lamin A/C (GGTGGTGACGATCTGGGCT) siRNA oligonucleotides have been described previously.[26,73] All siRNA oligonucleotides

were synthesized with 3′TT overhangs. Control, p190A-RhoGAP, and Lamin A/C siRNAs were from Eurofins MWG Operon and FAK siRNA was obtained from Dharmacon Research. Mock control cells received transfection reagent only. siRNA oligonucleotides were transfected using calcium phosphate as described previously.[26,74]

Plasmid construction

KASH constructs were prepared essentially as described previously.[25] The KASH domain of Nesprin-2 was amplified from HeLa cDNA using nested PCR with 5′-primer 5′-AGATCTCGAGTCTCAGAGAGTGACGCCGATG-3′, 3′-primer 5′-AAAAGGATCCTATGTGGGGGGTGGCCC-3′ in the first round of PCR and 5′-primer 5′-AGATCTCGAGGAACTACAGAAGGCGAGGAGGAGACA-3′, 3′-primer 5′-AAAAGGATCCTATGTGGGGGGTGGCCC-3′ in the second step. For KASHΔL amplification, PCR primers were 5′-AGATCTCGAGGAACTACAGAAGGCGAGGAGGAGACA-3′ and 5′-AAAAGGATCCTAGCAGCTGTAGTCTTCTTCGGAGGA-3′. PCR fragments were inserted into pEGFP-C1 vector using BamHI/XhoI. GFP from pEGFP-KASH was replaced with tdTomato from ptdTomato-C3 vector using NheI/BsrGI to generate tdTomato-KASH constructs.

Live cell and fluorescence microscopy

Live cell microscopy was performed at 37 °C using Olympus CellR imaging station (Olympus IX81 inverted microscope, MT20 illumination system, and Olympus FV2T CCD camera). Phase contrast or epifluorescence images were captured with CellR software and analyzed with ImageJ software. Cells and nuclei were tracked using the MTrackJ plugin for ImageJ.

For immunostaining, cells were grown on glass coverslips coated with fibronectin (1 μg/ml) and fixed with 2% paraformaldehyde in phosphate-buffered saline (PBS) for 30 min and permeabilized with 0.5% Triton X-100 in PBS for 5 min. Coverslips were blocked with 20% normal goat serum in PBS and stained with indicated antibodies. Fluorescent images were acquired using either Olympus FluoView-1000 confocal microscope with Hamamatsu EM-CCD camera or epifluorescence microscope Olympus IX81.

FAK imaging

Cells were co-stained with antibodies against FAK (mouse monoclonal, Upstate Biotechnology) and phospho-FAK-Y397 (rabbit polyclonal, Invitrogen). Images were acquired using the Olympus FluoView-1000 confocal microscope system with Hamamatsu EM-CCD camera. Sample background was eliminated by manual thresholding and the ratio of pFAK/FAK was displayed as a heat map.

Nuclear reorientation and cell polarity assays

Cells were grown on dishes or glass coverslips coated with fibronectin (1 μg/ml) and cell monolayer was wounded by pipette tip. To quantitatively measure nuclear reorientation, we stained cell nuclei with DAPI and calculated the angle between the wound and the longer nuclear axis using ImageJ. It allows measuring the nuclear reorientation, from the nucleus parallel to the wound (nuclear axis angle, 0°) to the nucleus perpendicular to the wound (nuclear axis angle, 90°). In all experiments, nuclei with round or irregular shape were excluded from analysis. The values were plotted as box-and-whiskers graph with the median and quartiles, and statistical analyses [Mann–Whitney non-paired (not assuming Gaussian distribution) *t* test] were performed using Prism software (GraphPad Software, Inc.). At least 100 nuclei were analyzed in each experiment.

Nucleus and cell centroid distance was determined with ImageJ software and plotted as a distance of the nucleus centroid relative to the cell centroid. Cell polarization was measured by MTOC and Golgi reorientation. Cells were scored as polarized when MTOC or Golgi was within the 120° fork facing the wound. In each sample, at least 100 cells were analyzed.

Western blotting

Cells were lysed in 200 μl of RIPA buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, and 1 mM ethylenediaminetetraacetic acid, pH 8.0) and clarified by centrifugation at 10,000***g*** for 15 min. Clarified lysates were boiled in 1× sample buffer for 5 min, resolved by SDS-PAGE, and transferred to Optitran nitrocellulose membrane (Whatman). Membranes were blocked with 5% bovine serum albumin in PBS–0.1% Tween 20 for 1 h at room temperature and incubated overnight at 4 °C with primary antibodies. Membranes were subsequently probed with secondary antibodies conjugated with horseradish peroxidase and developed using Super-Signal WestPico enhanced chemiluminescent substrate (Pierce).

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Supplementary Data

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Abbreviations used:
MTOC, microtubule organizing center; LPA, lysophosphatidic acid; LINC, linker of nucleoskeleton and cytoskeleton; FAK, focal adhesion kinase; ECM, extracellular matrix; GFP, green fluorescent protein; EGF, epidermal growth factor; GEF, guanine exchange factor; siRNA, small interfering RNA; GAP, GTPase-activating protein; PBS, phosphate-buffered saline.

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Supplementary Figures

Fig. S1

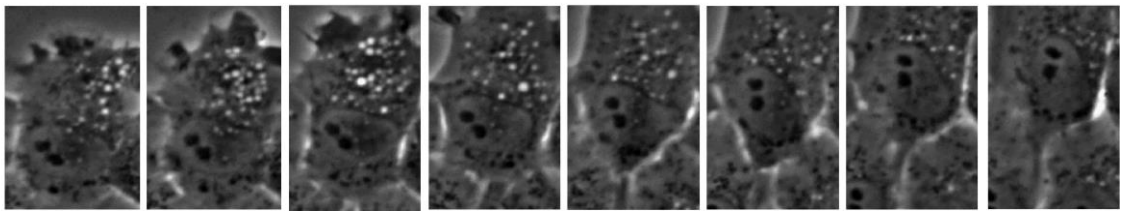

Figure S1. Rotation of nucleoplasm in wounded cell monolayer. Images from time-lapse phase contrast microscopy recording the nuclear movement and movement of nucleoplasm including nucleoli in cell at the edge of the wound.

Fig. S2

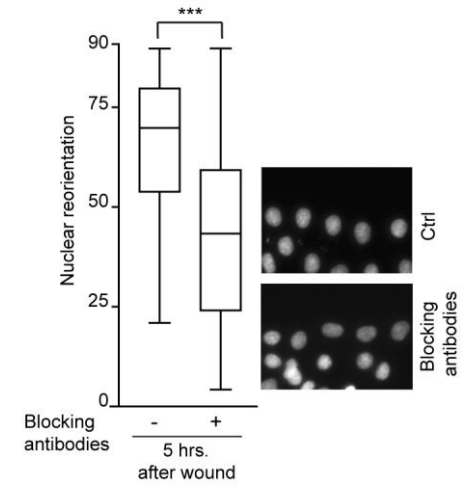

Figure S2. Integrin blocking antibodies impair nuclear reorientation. Confluent monolayer of RAT2 cells was wounded and then treated with anti-β1 and anti-β3 integrin blocking antibodies.

Fig. S3

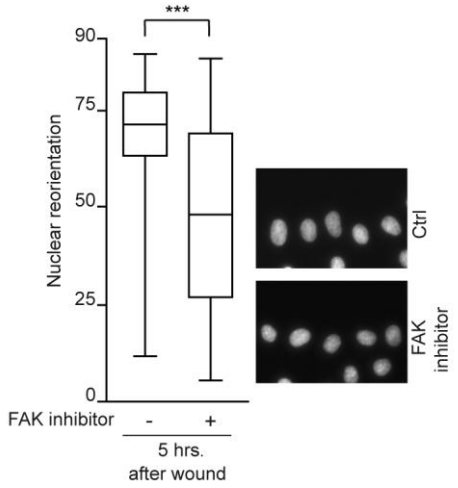

Figure S3. Pharmacological inhibition of FAK blocks nuclear reorientation. RAT2 cells were pretreated with FAK inhibitor PF573228 (3μM) for 10 min.

Fig. S4

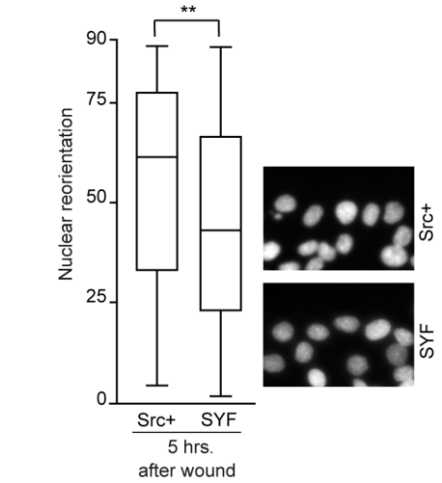

Figure S4. Family of Src kinases regulates nuclear reorientation. Nuclear reorientation was determined in Src/Yes/Fyn deficient fibroblasts (SYF) and SYF cells re-expressing Src (Src+). In all panels cells were allowed to migrate into the wound for 5 hrs, fixed and nuclear reorientation was determined as described in Materials and Methods. Data are represented in the box and whiskers plot. ** $p < 0.01$, *** $p < 0.001$.

Fig. S5

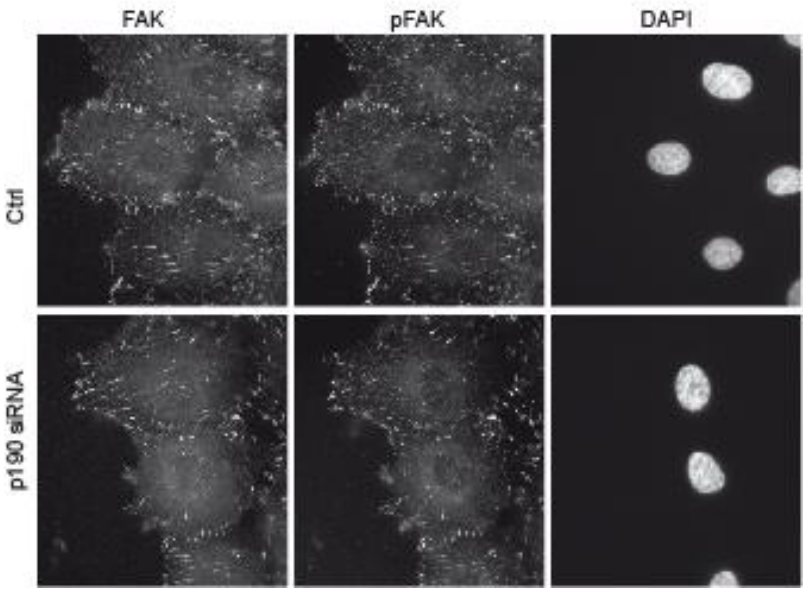

Fig S5. Focal adhesion staining after downregulation of p190RhoGAP. RAT2 cells were transfected with p190RhoGAP siRNA, wounded and fixed 5 hrs after wound. Cells were co-stained with antibodies recognizing phosphorylated form of FAK (pFAK Y-397), FAK and counterstained with DAPI.

Fig. S6

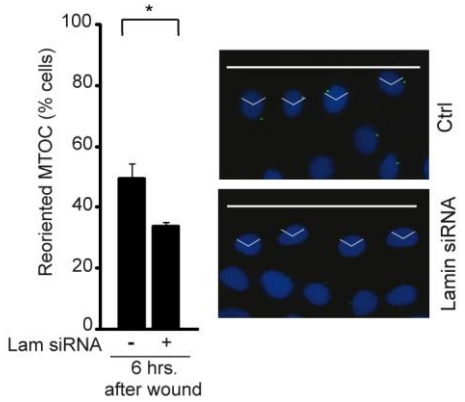

Figure S6. Lamin A/C knockdown blocks MTOC polarization. RAT2 cells were either mock or Lamin A/C siRNA transfected, fixed 6 hrs. after wound and stained with γ-tubulin antibody to visualize MTOC (green). Cell polarization was measured as described in Materials and methods. Values are mean ± SD. * $p < 0.05$

Emerging role for nuclear rotation and orientation in cell migration

(Maninová et al., 2014)

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Emerging role for nuclear rotation and orientation in cell migration

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Nucleus movement, positioning, and orientation is precisely specified and actively regulated within cells, and it plays a critical role in many cellular and developmental processes. Mutation of proteins that regulate the nucleus anchoring and movement lead to diverse pathologies, laminopathies in particular, suggesting that the nucleus correct positioning and movement is essential for proper cellular function. In motile cells that polarize toward the direction of migration, the nucleus undergoes controlled rotation promoting the alignment of the nucleus with the axis of migration. Such spatial organization of the cell appears to be optimal for the cell migration. Nuclear reorientation requires the cytoskeleton to be anchored to the nuclear envelope, which exerts pulling or pushing torque on the nucleus. Here we discuss the possible molecular mechanisms regulating the nuclear rotation and reorientation and the significance of this type of nuclear movement for cell migration.

Keywords: cell polarity, migration, nuclear reorientation, LINC, actin, microtubules, focal adhesions, FAK, dynein, myosin

Abbreviations: ECM, extracellular matrix; FAK, focal adhesion kinase; LINC, linker of nucleoskeleton and cytoskeleton; KASH, Klarsicht, ANC-1, Syne Homology, LPA, lysophosphatidic acid; MTOC, microtubule organizing center; SUN, Sad1p, UNC-84; TAN lines, transmembrane actin-associated nuclear lines

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Introduction

Nucleus contributes to the establishment of the polarized, asymmetrical profile of migrating cells. During migration, nucleus positions to the cell's rear and promotes microtubule organizing center (MTOC) localization close to the cell center between the leading edge and the nucleus. MTOC positioning in front of the nucleus is a prerequisite for polarized microtubule growth from the MTOC toward the leading edge. Microtubules are selectively stabilized at the leading edge and they are thought to provide a unique track for directed vesicle trafficking toward the leading edge (**Fig. 1A**). The stereotypical localization of the nucleus to the cell's rear and MTOC close to the cell center has been recognized as an indicator of the migratory polarity defining the axis of migration.[1]

More recently, it was observed that during cell polarization the nucleus undergoes synchronous and temporally restricted rotational movement. This reorientation of the nucleus is characterized by the alignment of the longer nuclear axis with the direction of migration.[2,3] Nuclear reorientation further promotes the establishment of bilateral symmetry characteristic for migrating fibroblasts (**Fig. 1**).

Nuclear reorientation is propelled by the cytoskeleton attached to the nucleus. Microtubules are the prime candidates for nuclear rotation as they have been shown to control nuclear movement in several cell types. However, recent identification of perinuclear actin cap[4] and actin associated with TAN (transmembrane actin-associated nuclear) lines,[5] two different actomyosin structures anchored to the nucleus, suggests that specific types of actin filaments may promote the establishment of migratory polarity and cellular locomotion (**Fig. 1B**). Here, we highlight the important features and mechanisms involved in the regulation of nuclear rotation and nuclear reorientation.

Nuclear rotation and reorientation in adherent cells

Nucleus movement and rotation was first observed 60 years ago in human nasal mucosa cells,[6] and subsequently, in other cell types.[7-9] These studies showed that

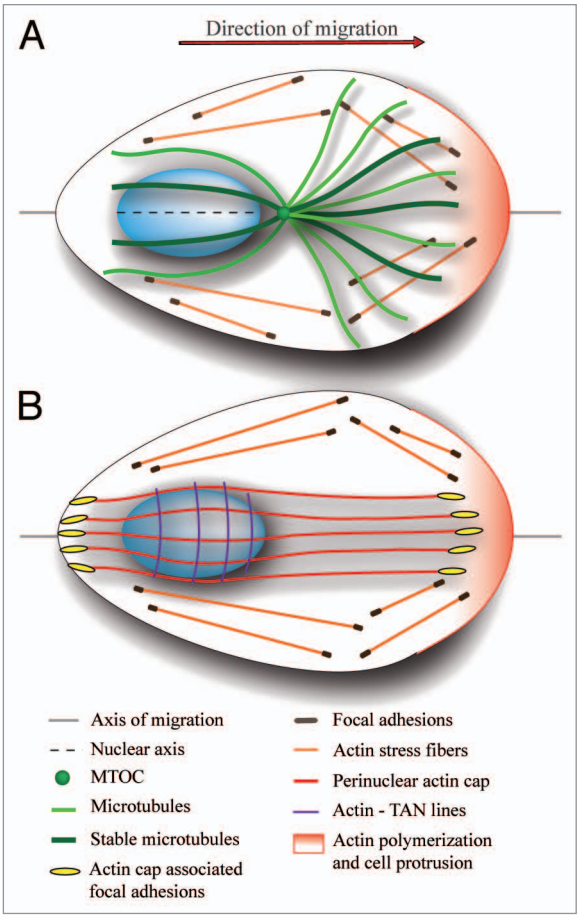

Figure 1. Schematic representation of an adherent migrating cell with front-rear polarity. Polarized cell displays conical shape with actin polymerization induced at the leading edge (pink) and limited at the cell rear. Cells are attached to the substrate through cell–matrix adhesion, such as focal contacts and adhesions, which connects extracellular matrix to cellular cytoskeleton. (**A**) In polarized cells, the oval-shaped nucleus localizes to the cell rear, MTOC in front of the nucleus close to the cell center, and microtubules are preferentially oriented toward the leading edge and they are stabilized at this location. The relative position of the nucleus and MTOC is an important marker of cell migration polarity defining the axis of migration. In addition, the longer nuclear axis is aligned with the axis of migration. (**B**) Specific types of actin filaments anchored to the dorsal side of the nucleus contribute to the establishment of asymmetric profile of the migrating cell. TAN lines are arranged perpendicular to axis of migration and drive the rearward movement of the nucleus during cell polarization. Perinuclear actin cap fibers span the nucleus and link the nucleus to subset of focal adhesions at cell periphery. Actin cap filaments are aligned with the axis of migration and longer nuclear axis and they probably stabilize nuclear orientation. It is not clear whether perinuclear actin cap and TAN lines co-exist in the same cell.

nuclei migrated linearly through the cytoplasm or rotated around the axis clockwise or counterclockwise, or occasionally perpendicular to the substrate. Nuclear rotation was observed as a three-dimensional motion of chromatin domains associated with nucleoli, leading to the conclusion that the nuclear rotation is in fact karyoplasmic streaming.[10] Nevertheless, other reports suggested that the whole cell nucleus rotates as the nucleoli maintained the rigid pattern during the rotation.[11,12] Recent experiments using fluorescence labeling of discrete nuclear compartments have conclusively shown that the nuclear rotation involves the movement of an entire nucleus, including nuclear interior as well as the inner and outer nuclear membranes.[13]

Nuclear rotation is a nuclear movement around the nucleus axis perpendicular to the substratum. Nuclear rotation ranges from stochastically or oscillatory rotation of the nucleus back and forth within a few degrees only to more sustained and directional rotation that changes the nuclear orientation.[2,3,13-17] Sustained nuclear rotation could be induced by diverse stimuli such as mechanical shear stress[16] or cyclical stretches of the substrate.[18] Nuclear rotation is also induced in two-dimensional migration models, where cells polarize and migrate into the wound made in a confluent monolayer of cells.[2,3,17] In the wound healing model the nucleus appears to be relatively static in non-polarized cells present in the cell monolayer and in polarized cells migrating into the wound. Nuclear rotation, termed nuclear reorientation, occurs only temporally during the wound-induced cell polarization. We defined nuclear reorientation as controlled nuclear rotation allowing the nucleus to rotate in the "xy" plane until its longer axis is aligned with the axis of migration, i.e., perpendicular to the wound.[3] Therefore, both rotation and reorientation are functionally similar, although the molecular players and the precise mechanism that control the rotation may vary according to the cell type.

Sustained nuclear rotation could be continuous, sometimes exceeding 360°.[17-19] We suppose that constant nuclear rotation is a consequence of deregulated reorientation and lack of control over nuclear reorientation. To support this, continuous nucleus rotation can be experimentally induced by disruption of intermediate filaments[19,20] and actomyosin contractility.[17,21] Continuous nuclear rotation was also observed in cells deficient in lamin B1[13] or in wound-edge epithelial cells deficient in heterogeneous group of genes.[22] These data suggest the existence of a complex molecular mechanism that regulates nuclear reorientation.

Nuclear reorientation and rotation requires the LINC complex

All three types of cytoskeleton, microtubules, actin filaments, and intermediate filaments, have been shown to associate with the components of nuclear envelope and to regulate nuclear movement including nuclear rotation. The key role in nuclear movement plays the nuclear lamina, particularly lamin A/C, which provides mechanical support and structural stability to the nucleus. Tight coupling of the nuclear lamina to the cytoskeleton

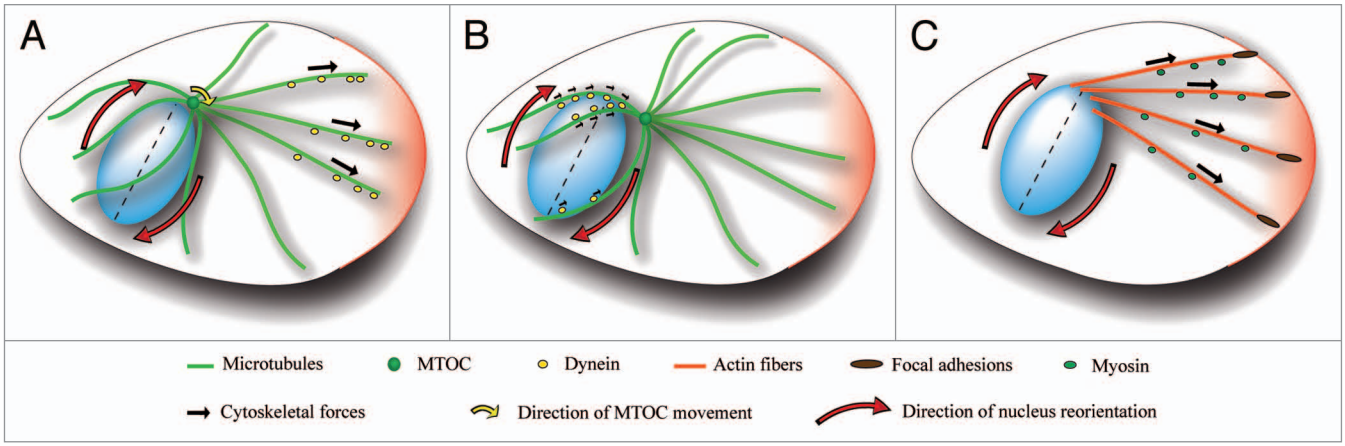

Figure 2. Schematic representation of hypothetical nuclear reorientation models. Forces exerted and transmitted by the cell cytoskeleton (black arrows) are transferred to the nucleus through LINC complex (not shown) to induce nucleus rotation and reorientation (red arrows). (**A and B**) Microtubules induce nuclear reorientation by forces exerted by microtubule-associated motor protein dynein. (**A**) Dynein pulls at the tips and alongside of microtubules to induce MTOC re-positioning close to the cell center (yellow arrow). Because MTOC associates with the nucleus, MTOC movement also induces nucleus reorientation (red arrows). (**B**) Dynein, through its interactions with nesprins, links microtubules to the nuclear envelope and pulls the nucleus as a huge cargo toward minus end of microtubules mediating nuclear reorientation. The asymmetric distribution of microtubules associated with nucleus is required to induce torque on the nucleus. (**C**) Actin cap fibers reorientate the nucleus. Actin cap fibers emanating from the focal adhesions at the leading edge associate with LINC complex at the nuclear envelope, predominantly at one pole of the nucleus. Nuclear reorientation is induced by actomyosin contractile forces between the leading edge and the nucleus.

allows the forces exerted by the cytoskeleton to move the nucleus (for a review. see refs. 23–26). Connection between the cell cytoskeleton and nuclear lamina is mediated by the LINC (Linker of Nucleoskeleton and Cytoskeleton) complex that passes both through the outer and inner nuclear membranes. The LINC complex is composed of SUN proteins that span the inner nuclear membrane and their N terminus interacts with nuclear lamina. The conserved SUN domain of SUN proteins localized within the perinuclear space interacts with the KASH domain of Syne/nesprin family of proteins that span the outer nuclear membrane. At the cytoplasmic side Syne/nesprin proteins interact with cytoskeletal components linking the nuclear lamina with the cell cytoskeleton. Actin filaments are the only components of the cell cytoskeleton known so far to interact directly with the LINC complex. The association is mediated by nesprin-1 and -2 and their calponin homology domains. Nesprin-1 and -2 also interact with microtubule motor proteins dynein and kinesin, which capture centrosomal microtubules, while nesprin-3 interacts with plectin, which links the LINC complex to intermediate filaments (for a review, see refs. 26 and 27). Kinesin also links microtubules to nesprin-4; nevertheless, the expression of nesprin-4 is restricted to epithelial secretory cells.[28]

The integrity of nuclear lamina and LINC complex is essential for the nuclear rotation, and deficiency of lamin A/C or disruption of the LINC complex by dominant negative versions of Sun or nesprin proteins impairs nuclear rotation and reorientation.[2,3,18,29] The evidence also suggests the important role of the LINC complex and nucleus movement in the regulation of cell migration as disruption of nucleo-cytoskeletal link impairs cell migration.[2,3,5,29-31] However, it should be noted that the nucleus–cytoskeleton association and nucleus movement could also be LINC-independent. For example, microtubules have been shown to interact with the nuclear pore complex components,[32] nuclear envelope protein emerin,[33] and nucleus rearward positioning could be LINC-independent in some cell types.[3,34]

Proposed mechanisms for nuclear reorientation

Nuclear rotation requires forces acting on the nucleus that are mediated by the cytoskeleton and cytoskeleton-associated motor proteins. The nuclear rotation in some cells is exclusively driven by microtubules and microtubule motor dynein, such as in wound edge NIH3T3 fibroblasts.[17] In other cases, the inhibition of myosin motor proteins and actomyosin contractility revealed that actin is required for nuclear rotation[18] or, conversely, serves as an anchoring system preventing nuclear rotation.[17,19,21] Microtubules and actin could also have non-redundant functions because interfering with the function of either microtubules or actin cytoskeleton impedes nuclear rotation in lamin B1-deficient cells.[13] In addition, intermediate filaments (IFs) are also implicated in nuclear rotation despite the fact that IFs have no motor protein.[19,20] Thus, it appears that the requirements for the specific cytoskeletal structures vary depending on the cell type and experimental conditions used.

We propose three mechanisms based on microtubules and actin-associated motor proteins, although these mechanisms may cooperate in the nucleus reorientation. Dynein is accumulated at the leading lamellipodia of migrating cells and at the tips and alongside of microtubules. From these cortical and cytosolic anchoring sites dynein exerts pulling forces on microtubules that induce MTOC centration (for a review, see ref. 35). MTOC is connected to the nucleus e.g., by association of emerin with MTOC[33] or by centrosomal microtubules captured by the LINC

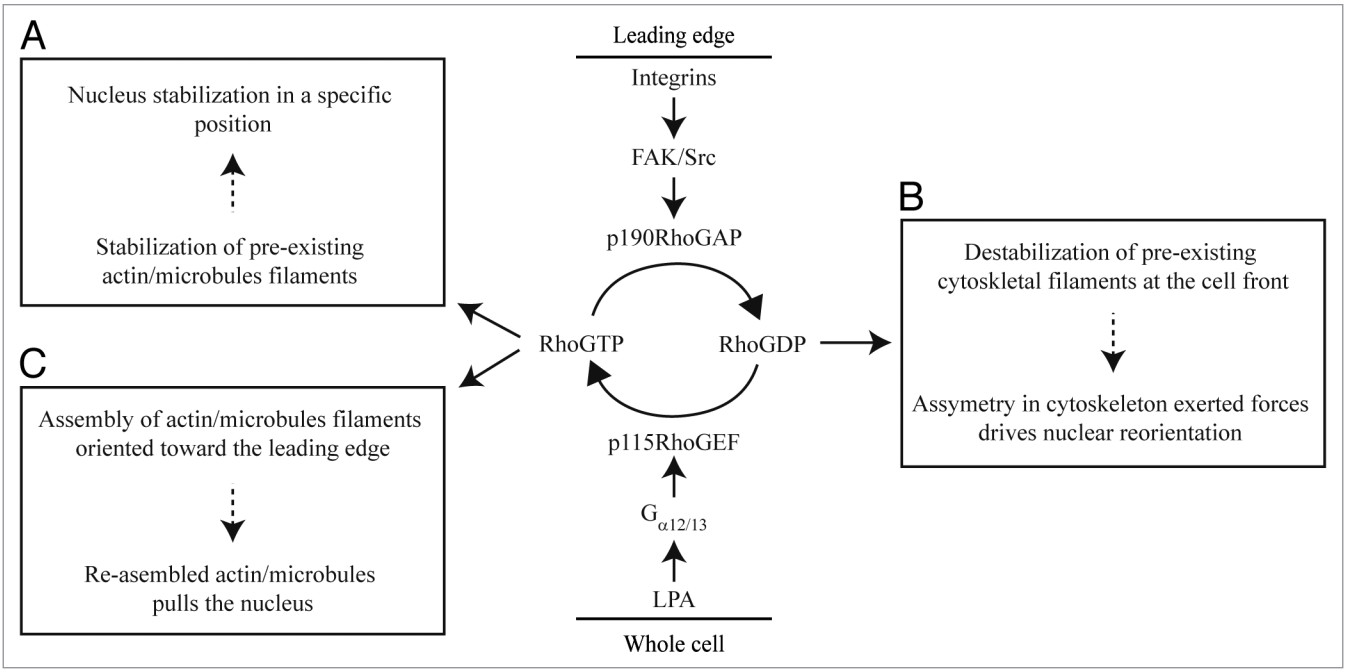

Figure 3. Signaling pathways involved in the regulation of nuclear reorientation. Two signaling pathways that converge at small GTPase Rho regulate cycling between active (GTP-bound) and inactive (GDP-bound) state of Rho allowing the cytoskeleton remodeling and subsequently nuclear reorientation. LPA signaling induces the activation of heterotrimeric G proteins, and consequently, RGS-RhoGEF, such as p115–RhoGEF, which directly activates Rho. Since LPA is a soluble mitogen it presumably activates Rho within cell uniformly. Active Rho regulates the stabilization of pre-existing cytoskeletal filaments that anchor the nucleus in immobile state (box **A**). Acute integrin engagement to ECM at the cell front activates FAK/Src signaling complex that recruits Rho inhibitor p190RhoGAP to the leading edge. Transient Rho inhibition at the leading edge leads to the destabilization of cytoskeletal filaments and consequent asymmetry in cytoskeletal forces may induce nucleus reorientation (box **B**). Since integrin-mediated Rho inactivation is transient, new cycle of Rho activation allows re-assembly and stabilization of new cytoskeletal filaments oriented toward the leading edge that may pull the nucleus and contribute to the nucleus reorientation (box **C**).

complex.[26,27] In first model, cortical and lengthwise pulling forces move MTOC, which consequently induces nuclear rotation (**Fig. 2A**). Nevertheless, we observed that during the cell polarization MTOC moves toward the leading edge without significant nucleus movement (our unpublished data). Also in other studies the nucleus was shown to rotate independently of MTOC, indicating that the link between the nucleus and MTOC is weak or reversible[16,17,19,36,37] and that the forces exerted by cortically and cytoplasmically anchored dynein are not sufficient to induce nuclear rotation.

Cortically and lengthwise exerted pulling forces can be supported by the microtubule motor proteins, particularly dyneins, which are associated with the nuclear envelope through the LINC complex.[37] In this model (**Fig. 2B**), dynein pulls the nucleus as a huge cargo along the microtubules toward the minus end of microtubules emanating from MTOC.[17,37] Transient asymmetric distribution of microtubules associated with the nucleus would create net torque on the nucleus, thus inducing its rotation. The nuclear rotation would be terminated when the asymmetry of the microtubular network is reversed.[37]

Recently identified perinuclear actin cap represents an additional player that may be involved in the regulation of nuclear reorientation. Actin cap is composed of contractile actomyosin filaments that interact with the LINC complex on the dorsal side of the nucleus and with focal adhesions at the cell periphery. Since actin cap fibers extend over the nucleus in a pole-to-pole manner and they are aligned with the longer nuclear axis and the axis of migration,[4,38] it is possible that the actin cap serves as an anchoring structure stabilizing the nucleus in specific orientation. Consistently, we observed that the actin cap is disrupted directly above the nucleus during cell polarization and then reassembled when the cells are polarized, allowing the nucleus to rotate (our unpublished results). We also observed that during reassembly of the actin cap, actin fibers attach predominantly to one pole of the nucleus (our unpublished data). Since the actin cap is anchored at focal adhesions at the leading edge, it is tempting to speculate that actin cap fibers also induce nuclear rotation (**Fig. 2C**). The potential mechanism involves the attachment of actin cap fibers newly formed from the leading edge to the pole region of the nucleus. Consequently, the nucleus reorientation may be induced by actin-mediated forces (**Fig. 2C**).

Is nuclear reorientation regulated by the distant signaling at the leading edge?

Nuclear rotation and reorientation is likely a consequence of cytoskeleton rearrangement. Because cytoskeleton is to a large extent regulated by Rho family GTPases it is likely that Rho GTPase signaling also regulates nuclear rotation. Indeed, Cdc42 was shown to control nucleus rotation in cells exposed to shear stress.[16]

In our previous study[3] we described that nucleus reorientation requires two signaling axes, LPA-mediated Rho activation and integrin/FAK/p190RhoGAP signaling leading to Rho inactivation (**Fig. 3**). Adhesion and LPA signaling are coordinated in order to induce nuclear reorientation. After wounding the cell monolayer, integrin engagement to ECM activates FAK and active FAK localizes with its downstream effector p190RhoGAP at the leading edge. Adhesion and integrin/FAK/p190RhoGAP signaling inhibits Rho transiently,[39] suggesting that LPA-mediated Rho activation is inhibited at the leading edge. Thus, integration of adhesion and LPA stimuli at Rho constitutes a gradient-forming mechanism with the capability to differentially regulate Rho at the cell front and rear.

We suppose that signaling induced at the leading edge provides the molecular framework for the mechanism underlying nuclear reorientation. The actin fibers or microtubules and associated motor proteins remain anchored to the cell cortex and ECM structures on one side and to the nucleus on the other side.[23,40-43] Therefore, through the cytoskeletal elements nucleus remains under isometric tension and it is relatively immobile in stationary, non-polarized cells (**Fig. 3**, box A). In response to migratory polarity cues like wounding, transient decrease of Rho activity at the cell front allows actin and microtubule disassembly promoting partial relaxation of the isometric tension. The relaxation of isometric tension could be sufficient for nuclear reorientation as it generates asymmetric distribution of cytoskeletal elements associated with nucleus to induce torque on the nucleus (**Fig. 3**, box B; see also **Fig. 2B**). In addition, it is possible that actin cap fibers that are attached to focal adhesions at the leading edge[44] respond to Rho inactivation and their disassembly allows nucleus to rotate. Since integrin-mediated Rho inactivation is transient, Rho induces the re-assembly of cytoskeleton that may represent additional mechanism controlling nuclear rotation. De novo polymerized actin cap fibers and microtubules are oriented toward the leading edge (**Fig. 3**, box C). Actin or microtubule-mediated forces would then rotate the nucleus (**Fig. 2A and C**). Once the cells have polarized, formation of the actin-myosin fibers and stabilization of the microtubules restores isometric tension that put the break on nucleus rotation. Therefore, we speculate that signal-dependent changes in actin and microtubule dynamics constitute the molecular mechanisms that control nuclear rotation (**Fig. 3**).

Functional significance of nuclear orientation in migrating cells

There is growing body of evidence that the disruption of LINC complex impairs cell polarization and migration suggesting that the attachment of cytoskeleton to the nucleus plays an important role in cell motility.[2,3,5,29-31] Nevertheless, the role of nuclear shape and nuclear orientation in cell migration has not been extensively studied. The nucleus frequently displays ovoid or elliptical shape with characteristic orientation in different cell types. For example, migrating fish keratocytes are fan-like shaped with broad lamellipodial protrusion at the cell front and elliptical nucleus oriented perpendicular to the axis of migration. On the contrary, in conically shaped fibroblasts the nucleus is oriented parallel to the axis of migration. This raises the question why cells orientate the nucleus and how nuclear orientation affects cellular functions, notably cell migration.

One obvious reason for specific nuclear orientation is that it may promote cell polarization, and thus, cell motility, as we proposed recently.[3] It was found that disruption of the LINC complex specifically affects nucleus reorientation, MTOC and/or Golgi polarization, and cell migration.[2,3,29] It indicates that the defect in MTOC/Golgi polarization and cell migration is a consequence of impaired nuclear reorientation.

Alternatively, specific nucleus orientation may help overcome the physical constraints facing the migrating cells. During keratocyte movement, the cell body is rolling together with the nucleus[45] and the nuclear oval shape and its orientation perpendicular to the direction of migration may ease the nucleus rolling and cell locomotion. Nucleus rolling may also help to overcome the blockage of cytoplasmic granules accumulated in front of the nucleus, as described in *C. elegans* development.[46] Such rolling is not probable in migrating fibroblasts because the longer nuclear axis is aligned with the direction of migration. Nevertheless, the oval-shaped nucleus may move forward like a "torpedo" through the cytoplasm to facilitate nucleus translocation as cells move. Similarly to fibroblasts migrating in 2D environment, cells migrating in 3D environment reorient the nucleus toward the direction of migration. It has been suggested that the nuclear reorientation helps the cells passage through the narrow pores in the collagen lattice.[47]

The characteristic orientation of the cell nucleus in motile cells allows us to speculate whether the nucleus itself is a polarized organelle. It has been described that the nuclear envelope is polarized as nesprin-4 accumulates asymmetrically at the pole of the nucleus distally to MTOC. Interestingly, overexpression of nesprin-4 leads to polarization of other nuclear components, including lamins and nuclear pore complex proteins.[28] Given that chromatin interacts with lamins, the spatial positioning of chromatin within the nucleus could be regulated. Keeping the gene in specific location within the nucleus, and thus, within the cell, may promote specific transcripts to be delivered to the specific locations. To support this speculation, it has been demonstrated that migration-specific transcripts are preferentially delivered to the leading edge of migrating cells.[48]

Conclusion and Perspectives

A large body of recent work shows that precise nucleus location inside the cell is important for correct cellular functioning. In respect of motile cells, it is interesting that the cells possess the mechanism(s) that purposely move and orientate the nucleus in order to facilitate their motility. In particular, the rearward positioning of the nucleus and nuclear reorientation emerged to be important for the establishment of cell polarity and optimal for cellular migration. The regulatory mechanism(s) that move the nucleus could also be employed by cells migrating in 3D environment to facilitate their invasion, suggesting the possible role of the nucleus mechanics in the patho-physiological processes such as cancer cell invasion. Clearly,

additional studies are required to understand the functional significance of the nucleus orientation in motile cells and to decipher the basic molecular mechanisms controlling this process.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Dorsal stress fibers, transverse actin arcs and perinuclear actin fibers form interconnected network that induces nuclear movement in polarizing fibroblasts.

(Maninová and Vomastek, submitted manuscript)

Dorsal stress fibers, transverse actin arcs and perinuclear actin fibers form interconnected network that induces nuclear movement in polarizing fibroblasts

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Abstract

Motile cells display 3D organization of stress fibers with ventral fibers at the basal side of the cell and dorsal fibers, transverse actin arcs and perinuclear actin cap extending at the cell front from the ventral to the dorsal side of the cell. Perinuclear cap fibers further rise above the nucleus and are connected to the nuclear envelope. We show that dorsal fibers, actin arcs and perinuclear fibers are transiently interconnected in spots rich in α-actinin and that this network links the nucleus with adhesions at the leading edge. During cell polarization, dorsal fibers and transverse arcs recruit the preexisting ventral stress fibers, move them above the nucleus and promote actin cap assembly. Actin cap is required for the nucleus movement as perinuclear actin fibers induce nuclear reorientation to the direction of migration. These results suggest that the network of dorsal fibers, transverse arcs and perinuclear fibers transfers mechanical signal between the focal adhesions and nuclear envelope that regulates the nuclear reorientation in migrating cells.

Introduction

In migrating cells actin forms morphologically diverse types of stress fibers [1-3]. Contractile ventral or basal stress fibers lie along the base of the cell usually parallel to the direction of migration and they are attached to the focal adhesions at both ends. Ventral stress fibers are rich in α-actinin and myosin that crosslink the actin fibers and mediate their contractility. Ventral stress fibers regulate diverse cellular functions such as maturation of adhesions, establishment of front-rear polarity [4-6], generation of traction forces, retraction of the trailing edge [7;8] and cell shape on compliant substrates [9]. Contractile stress fibers also participate in remodeling of extracellular matrix [10].

In contrast to ventral stress fibers that are confined to the basal side of the cell, an array of dorsal fibers, transverse actin arcs and perinuclear actin cap fibers extend from the ventral to the dorsal side of the cell. Dorsal fibers polymerize from focal adhesions at the leading edge and rise toward the dorsal side of cell lamella. Their formation requires actin bundling protein α-actinin and α-actinin depletion by siRNA impacts the dorsal fibers without affecting ventral stress fibers and transverse arcs [11-13]. Dorsal fibers are devoid of myosin II [12;14;15] and, in addition, some tropomyosin isoforms are also absent or display specific localization pattern [16;17]. Orthogonal to the dorsal fibers are transverse actin arcs, curve-shaped contractile actomyosin bundles oriented parallel with the leading edge that flow centripetally from the leading edge to the cell center where they disassemble [12;14;18;19]. Transverse arcs contain MyosinII with MyosinIIB as a predominant isoform present in actin arcs in lamella closer to the nucleus [3;12]. Transverse arcs contain also α-actinin that is periodically distributed along actin filaments in sarcomeric-like arrangement [15]. Actin arcs are presumably crosslinked with dorsal fibers [2] and, accordingly, arcs and dorsal fibers move at the same velocity toward the cell center [15]. Arcs impose pulling forces to the dorsal fibers that promote adhesions maturation and ECM remodeling [12;20]. Anchoring of dorsal fibers to adhesions also generates resisting force to actin arcs and this force flattens the lamella in migrating U2OS cells [15].

While actin arcs and dorsal fibers are typical for the cell front, contractile perinuclear actin cap fibers emanate from focal adhesions at the leading edge, extend over the nucleus and terminate in adhesions at the cell rear forming dome-like structure [21]. In addition to adhesions, central part of perinuclear actin cap is attached to the nucleus *via* LINC (Linker of Nucleoskeleton and Cytoskeleton) complex. LINC complex composed of Sun and Nesprin proteins spans nuclear envelope and interacts with nuclear lamina on nucleoplasmic side and thus mechanically couples nucleus with actin [22]. Perinuclear actin cap fibers and LINC complex have been shown to be important in the regulation of nuclear shape [21;23;24] and in mechanosensing and mechanotransduction [25-27]. Dissimilarly to perinuclear stress fibers, the ventral stress fibers are not directly attached to the nucleus and LINC complex [25;26;28;29]. Interestingly, the assembly of perinuclear actin cap is impaired in several cancer cells such as U2OS [25;27].

The mechanical coupling of the nucleus to the actin cytoskeleton is well suited for the regulation of nuclear movement by extracellular mechanical stimuli [30-32]. Indeed, nuclear movement and repositioning to the cell rear during fibroblast polarization has been shown to depend on actin fibers of TAN (transmembrane actin-associated nuclear) lines that are arranged above the nucleus and associate with LINC complex [33]. Furthermore, it has been

shown that in adherent cells, pulling on integrins by fibronectin coated beads, cyclic stretches of substratum or cell adhesion restricted to micropatterned substrates induce nuclear movement and rotation that requires actomyosin contractility [34-36]. In contrast, perinuclear actomyosin fibers could serve as an anchoring system preventing the nucleus rotation [37] in agreement with the observations that the inhibition of actomyosin contractility induces nuclear rotation [38;39].

Previously, we have shown that during the polarization of RAT2 fibroblasts the nucleus undergoes temporal rotational movement which aligns the nuclear axis with the axis of migration. Nuclear reorientation requires both integrin attachment to extracellular matrix and LINC complex suggesting that adhesions associated cytoskeleton and LINC complex transmit force from substratum to the nucleus [40]. We have hypothesized that contractile actin fibers linking the nucleus with adhesions at the leading edge could drive the nuclear reorientation [41]. Here we show that nuclear reorientation is driven by the interconnected network of dorsal fibers, transverse arcs and perinuclear actin cap fibers that links adhesions at the leading edge with the nucleus. Mechanistically, dorsal fibers and arcs at the cell front recruit preexisting ventral stress fibers and move them to the apical side of the nucleus inducing nuclear rotation. Furthermore, perinuclear fibers, arcs and dorsal fibers are transiently connected in spots rich in α-actinin-1 suggesting that α-actinin-1 crosslinks individual actin fibers.

RESULTS AND DISCUSSION:

Perinuclear actin fibers in RAT2 cells.

To examine the role of perinuclear actin fibers in the nuclear reorientation we initially determined their presence in RAT2 cells either by confocal (Fig. 1A) or wide-field microscopy (Movie S1). Both confocal and wide-field microscopy revealed long parallel actin fibers above the nucleus that were arranged in a pole-to-pole manner. These fibers extended to the cell front and the leading edge where they were anchored at focal adhesions. We next examined whether perinuclear fibers are also attached to the LINC complex and nuclear envelope, in addition to focal adhesions. To this end, we over-expressed the KASH domain of Nesprin2 (GFP-KASH) that displaces endogenous Nesprin proteins from nuclear envelope and consequently disrupts LINC complex [33;35;42]. The expression of GFP-KASH impaired the perinuclear fibers suggesting that they are linked to the nuclear envelope (Fig. 1B).

It has been shown that low concentration of actin polymerization inhibitor latrunculin A selectively disrupts perinuclear actin cap without affecting basal stress fibers [21;26;37]. In agreement, we found that low dose of latrunculin A significantly impaired perinuclear fibers (Fig. 1C) . In addition, the inhibition of actomyosin contractility disrupted perinuclear fibers (Fig. 1D). To quantify the presence of the perinuclear actin fibers we used the method described previously [26;37;43] where perinuclear actin fibers were distinguished as well-organized, disrupted or absent (Fig. 1D). The quantification of perinuclear actin confirmed that the expression of KASH domain (Fig. 1E) or pretreatment of cells with low concentration of Latrunculin A or blebbistatin (Fig. 1F) significantly reduced the number of cells with perinuclear actin fibers. These data suggest that in polarized RAT2 cells contractile actin

fibers present above the nucleus are actin cap fibers that link focal adhesions to the LINC complex and nuclear envelope.

Dynamics of perinuclear actin fibers during cell polarization

Previously we showed that polarization of RAT2 cells toward the wound induces temporal rotation of cell nuclei that aligns the longer nuclear axis with the axis of migration. The nuclear reorientation occurs between 2-4 h after the wound [40] (See also Fig. 2C). Given that perinuclear fibers are attached to the nucleus through LINC complex we next examined whether the perinuclear actin fibers rotate with the nucleus or if they disassemble and then reassemble during nuclear rotation. Perinuclear actin fibers were present in cells in confluent monolayer, however they were significantly disrupted two hours after wounding when nuclei started to reorient toward the wound (Fig. 2A,B). Six hours after wounding nuclei were oriented perpendicular to the wound and well-organized parallel perinuclear fibers were observed above the nucleus (Fig. 2A,B). Parallel perinuclear fibers were invariantly aligned with the longer nuclear axis and their orientation followed the orientation of the nucleus (Fig. 2D). The measurement of the perinuclear fibers orientation in respect to the wound revealed that the orientation of these perinuclear fibers was random in cell monolayer, however, in polarized cells they were aligned with the direction of migration (Fig. 2E).

To investigate the mechanism underlying the formation of the perinuclear actin cap during cell polarization we transfected the cells with RFP-LifeAct and followed perinuclear actin fibers in real time. We observed that during actin cap formation ventral fiber at the basal side of the cell raised vertically above the nucleus creating new actin cap fiber (Fig. 2F and Movie S2). Surprisingly, perinuclear actin fibers that formed above the nucleus terminated in actin rich foci in front of the nucleus at the dorsal side of the cell (Fig. 2F and Movie S2). Noteworthy and similarly to our observations, it has been shown that actin stress fibers could be mechanically linked in actin rich foci [44;45], therefore, we next determined the relationship of the actin spots with perinuclear actin cap, dorsal fibers and transverse actin arcs during cell polarization.

Dorsal fibers, transverse arcs and actin cap fibers are coupled by α-actinin-1

The dorsal fibers and transverse arcs can be characterized by their orientation and molecular contents. Dorsal fibers are anchored at focal adhesions at the leading edge and rise toward the dorsal side of cell lamella. They contain alpha-actinin but not myosin [12;14;15]. On the other hand, transverse arcs are parallel to the leading edge, display centripetal flow toward the nucleus and contain preferentially myosin IIB [3;12;14]. Consistently, we observed that wounding cell monolayer induced the formation of dorsal actin fibers that emanated from the subset of focal adhesions at the leading edge and elongated towards the dorsal side of the cell and toward the nucleus (Fig. 3A,B). These fibers were enriched in α-actinin-1 and devoid of myosin IIB (Fig. 4A,B,C see also Fig. 5B,C and Movie S3). We also observed thin actin fibers oriented perpendicularly to dorsal fibers (Fig. 3A,B). These fibers resembled transverse arcs as they moved toward the nucleus (Movies S2,S3) and contained predominantly myosin IIB while α-actinin-1 was periodically distributed along actin filaments (see Fig. 4D).

Remarkably, we observed actin rich spots that appeared at the sites where dorsal fibers crossed transverse arcs-like fibers (Fig. 3A,B). Spots close to the basal side localized close to the cell front while spots at the cell's midsection were close to the nucleus (Fig. 3B,C). Interestingly, we found that perinuclear actin fibers were anchored to midsection spots with multiple perinuclear actin fibers anchored to single actin rich spot (Fig. 3C). We also observed that actin rich spots were almost completely absent in cells present in confluent monolayer. Wounding cell monolayer induced the occurrence of the actin spots in cells at the wound edge, peaking 4 h after wounding. Their number decreased at later time points where cells already polarized and reoriented the nucleus (Fig. 3D). These data suggest that transverse arcs, dorsal and actin cap fibers are linked and that the appearance of the actin rich spots coincided with the formation of actin cap fibers.

α-actinin-1 localized to actin fluorescent foci in rat embryo cells [44;45] and is present both in dorsal stress fibers and in transverse arcs [12;14]. We thus supposed that α-actinin-1 may be enriched in actin rich spots in RAT2 fibroblasts. In polarizing cells α-actinin-1 strongly decorated dorsal actin fibers and faintly also actin arcs and perinuclear actin cap (Fig. 4A,B,C). Importantly, α-actinin-1 localized to individual actin spots at cell midsection (Fig. 4A). Specificity of the staining was confirmed by the expression of GFP-α-actinin-1 which also localized to actin foci (Fig. 4B). In contrast to α-actinin-1 staining, myosin IIB that is found in actin arcs and actin cap fibers but absent from dorsal fibers was excluded from the crosslinked spots (Fig. 4D).

It has been shown that α-actinin-1 knockdown impairs the formation of dorsal fibers without affecting ventral stress fibers and transverse arcs [11-13]. We thus depleted α-actinin-1 by siRNA to determine the role of dorsal fibers in the formation of perinuclear actin fibers (Fig. 5A). Consistently with previous results, depletion of α-actinin-1 impaired the formation of dorsal fibers while transverse arcs and ventral stress fibers were maintained (Fig. 5B). In addition to dorsal fibers, depletion of α-actinin-1 also reduced the formation of perinuclear fibers and actin rich spots (Fig. 5B). Re-expression of siRNA resistant GFP-α-actinin-1 restored dorsal fibers and perinuclear actin fibers as well as the occurrence of crosslinking spots confirming the specificity of siRNA (Fig. 5C,D). Moreover, during cell polarization perinuclear actin fibers became oriented perpendicular to the leading edge similarly to control cells (Fig. 5E). These observations suggest that α-actinin-1 and dorsal fibers are important for actin cap formation. They also suggest that dorsal fibers, transverse arcs and actin cap fibers are coupled by α-actinin-1.

Transverse actin arcs and dorsal fibers participate in perinuclear actin cap assembly.

The observations that perinuclear actin fibers originate in ventral stress fibers and form continuous actin network with dorsal fibers and arcs raised the possibility that dorsal fibers and actin arcs take part in the formation of actin cap. To address the mechanism, we initially examined the actin network in fixed cells. We often observed that multiple thin curved actin fibers that extended to the dorsal side were linked to thick peripheral ventral stress fiber (Fig. 6A). We hypothesized that actin arcs recruit the preexisting ventral fibers and move them to the dorsal side above the nucleus to form perinuclear fibers.

To further explore this hypothesis we followed the dynamics of actin structures in real time in cells transfected with GFP-α-actinin-1 and mCherry-LifeAct. Focal planes were changed during the time-lapse microscopy to observe different sections of the cell. We observed dynamic actin polymerization at the cell front that was separated from nonprotruding areas with bundled actin filaments (Movie S3, yellow arrowhead). In cells migrating to the wound actin arcs were formed at the cell front and moved centripetally to the cell center, and dorsal fibers polymerized from the leading edge to the dorsal side and toward the nucleus (Fig. 6B,C and Movie S3). In keeping with previous results, GFP-α-actinin-1 rich spots at the end of dorsal fibers moved to the dorsal side toward the nucleus (Fig. 6C, yellow empty arrowheads, and Movies S3,S4). Concurrently, at the interface of protruding and nonprotruding areas ventral stress fibers moved laterally from the cell periphery toward the nucleus and they moved upwards along the nucleus to the dorsal side of the cell (Fig. 6D, yellow arrowheads, and Movie S4). These newly formed perinuclear actin fibers were anchored to the GFP-α-actinin-1 rich spots in front of the nucleus (Movie S4). We also observed that moving fibers originated in thick ventral fiber that can either move as a whole or split up into several thin fibers that continued to the front of the cell (Fig. 6E and Movie S4). Remarkably, single nascent actin cap fiber contained α-actinin-1 pattern typical for both actin arcs (periodically but distantly spaced GFP-α-actinin-1 bands), and ventral fibers (uniform GFP-α-actinin-1 staining) thus it is tempting to speculate that transverse arcs fused with preexisting ventral fibers (Fig. 6F and Movie S4). These results suggest that during cell polarization the dorsal fibers - actin arcs – ventral stress fibers assemble into interconnected contractile actin network that coordinately moves toward the nucleus and serves as a platform from which perinuclear actin cap is formed.

Perinuclear fibers induce nuclear reorientation

Formation of perinuclear fibers was paralleled by the nuclear rotational movement that reoriented the nucleus to the direction of migration (Fig. 7A and Movie S5). Nuclear reorientation required dorsal fibers at the leading edge as the absence of dorsal fibers in α-actinin-1 depleted cells resulted in impaired nuclear reorientation despite the presence of actin arcs. The re-expression of GFP-α-actinin-1 rescued dorsal fibers and also nuclear reorientation (Fig. 7B). Nuclear reorientation was also dependent on the formation of actin cap and its attachment to the nucleus as the disruption of perinuclear actin fibers with KASH construct or by low level of latrunculin A blocked nuclear reorientation (Fig. 7C,D). In addition the inhibition of myosin II by blebbistatin impaired nuclear reorientation indicating that this process requires actomyosin contractility (Fig. 7E).

To further prove that the nuclear reorientation requires perinuclear fibers we examined nuclear reorientation in osteosarcoma U2OS cells. Cancer cells including U2OS cells do not assemble perinuclear actin cap [25;27]. In agreement, we found that only minority of U2OS cells assembled perinuclear actin fibers despite the presence of dorsal fibers and transverse arcs (Fig. 8A,B). Interestingly, U2OS cells do not reorient their nucleus to the wound (Fig. 8C).

In conclusion, we suggest that in polarized RAT2 cells perinuclear actin fibers physically link nuclear envelope to focal adhesions as they are anchored in focal adhesions at the cell front at one side and to the nucleus through LINC complex on the other side. Similarly to previous reports [21;26] we found that these fibers are contractile and dynamic fibers sensitive to low concentration of latrunculin A and blebbistatin. Previously, we suggested that perinuclear actin cap fibers could serve as an anchoring structure stabilizing nucleus in a specific position [41]. In support of this idea we found that during cell polarization to the wound perinuclear actin cap is disrupted. This is also in agreement with report that the perinuclear actin cap promotes directional cell migration and that dissolution of actin cap allows the nuclear reorientation during cell repolarization [37]. However, our data suggest that the formation of perinuclear actin cap is also required for nuclear reorientation.

The formation of perinuclear actin cap correlates with the transient formation of contractile actin network that connects different types of stress fibers encompassing transverse actin arcs, dorsal and perinuclear fibers. The interconnected actin network contracts in coordinated manner which suggests mechanical coupling of individual stress fibers. Accordingly, we detected α-actinin-1 in distinct actin fluorescent spots where individual stress fibers intersect each other or where they are joined, similarly to what has been described four decades ago [44;45]. These spots move from the leading edge toward the dorsal side and toward the nucleus along with polymerizing dorsal fibers and centripetally moving transverse arcs. This coordinated movement further supports the idea that these stress fibers are crosslinked. Actin and α-actinin-1 spots largely disappear in polarized cells where perinuclear fibers extend directly from the nucleus to the adhesions at cell front. We thus hypothesize that nascent perinuclear actin fibers mature into classical perinuclear actin cap fibers that directly link focal adhesions with the nucleus [21]. The mechanism of actin cap maturation is not known but it is tempting to hypothesize that nascent perinuclear fibers fuse with dorsal fibers as they are often joined in actin and α-actinin-1 enriched spots in front of the nucleus.

Based on our data we propose a model in which ventral fibers, dorsal fibers and transverse arcs assemble into contractile actin network that promotes the formation of perinuclear actin fibers during cell polarization (Fig. 8D). We hypothesize that the formation of perinuclear actin fibers is driven by ventral–arcs fibers that are anchored in focal adhesions at one end while their distal ends are crosslinked by α-actinin-1 with dorsal fibers. This network moves toward the cell center and to the dorsal side due to the contraction and shortening of actin arcs. At the perinuclear region dorsal fibers and arcs pull the ventral fibers on the top of the nucleus to create perinuclear actin structure. Perinuclear fibers on the apical side of the nucleus are stabilized by LINC complex recruitment [21;46] thus mechanically linking adhesions with the nucleus. Importantly, contractile forces these fibers impose on oval-shaped nucleus induce nuclear reorientation that consequently adopts sterically favored orientation with the nuclear axis aligned with the direction of migration.

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Author contribution

M.M and T.V. designed experiments; M.M. performed experiments; M.M and T.V. analyzed data and wrote the paper.

Materials and Methods

Materials

Blebbistatin was purchased from Sigma-Aldrich. Latrunculin A was from Santa Cruz Biotechnology Inc. Phalloidin-rhodamine conjugate was from Life Technologies. For immunofluorescence staining following antibodies were used: α-actinin-1 mouse monoclonal antibody (clone BM-75.2, Sigma-Aldrich), FAK mouse antibody (clone 4.47, Millipore), paxillin mouse antibody (clone 5H11, Upstate Biotechnology) and rabbit polyclonal myosin IIB antibody (Cell Signaling Technology). ERK2 antibody was generous gift from M. J. Weber and described elsewhere [47]. Secondary antibodies goat anti mouse and anti rabbit IgGs conjugated with Alexa Fluor 488 and 546 were from Life Technologies. Horseradish-peroxidase conjugated antibodies goat anti mouse IgG and goat anti rabbit IgG were from Sigma-Aldrich.

Cell culture, plasmids and transfection

RAT2 and U2OS cells were cultivated in Dulbecco´s modified Eagle´s medium with 10% fetal bovine serum (Gibco) at 37°C and under atmosphere of 5% CO_2. Transient DNA transfections were performed using Lipofectamine 2000 (Invitrogen). GFP-KASH and GFP-KASHΔL (domain lacking the luminal region unable to bind SUN proteins that still localizes to the nuclear envelope) constructs were described previously [40]. GFP-α-actinin-1 construct was generous gift from J.T. Parsons, mCherry-LifeAct expression vector was generous gift from D. Rosel and RFP-LifeAct was provided by M.P. Iwanicki.

siRNA and siRNA transfection

siRNA oligonucleotides were transfected using calcium phosphate precipitation method as described previously [40;47]. A double-stranded siRNA against rat α-actinin-1 targeted the sequence CACUUAUCUUCGACAAUAA and control siRNA targeted the sequence AGGTAGTGTAATCGCCTTG. Both siRNAs were obtained from Eurofins MWG Operon. For α-actinin-1 rescue experiments, co-transfection of siRNA and rescue construct was performed using Lipofectamine 2000.

Live cell and fluorescence microscopy

For live cell fluorescence microscopy cells were plated on glass bottom dishes (In vitro scientific) coated with 1 μg/ml of human fibronectin (Millipore) and live cell microscopy was performed at 37° C using Olympus CellR imaging station (Olympus IX81 inverted microscope, MT20 illumination system and Olympus FV2T CCD camera) using 100x oil objective NA 1,3. Time lapse phase contrast or fluorescence images were captured with CellR software and processed using ImageJ software. For better visualization of actin fibers in mCherry-LifeAct transfected cells (Fig. 2F and Fig.6B,C and 6E,F) the fluorescent signal in still images was converted to grayscale mode and inverted.

For immunofluorescence staining, cells were cultivated on glass coverslips coated with 1 μg/ml of fibronectin, fixed with 2% paraformaldehyde in PBS for 30 min and permeabilized with 0,5 % Triton X-100 in PBS for 4 min. Coverslips were blocked with 20% normal goat serum in PBS, stained with indicated antibodies and mounted into Vectashield mounting medium containing DAPI (Vector Laboratories). Fluorescent images were acquired using Olympus Fluoview-1000 confocal microscope (Fig. 1A) or epifluorescence microscope Olympus BX43 with Hamamatsu digital camera Orca R2 using 100x oil objective NA 1,40. Images shown in Fig. 3A,B, Fig. 5C nad Fig. 6A were acquired using DeltaVision microscope equipped with a NA 1.40, 60× oil immersion objective (Applied Precision) and CoolSNAP HQ2camera (Photometrics). When indicated, z-series of 0.25-μm stacks were deconvolved using Huygens software (Scientific Volume Imaging). Images were processed using ImageJ software.

Wound healing assays and quantification of perinuclear actin cap fibers and nuclear reorientation

Cells plated on glass coverslips coated with 1 μg/ml of fibronectin, were grown till confluency and wounded by pipette tip. In the picture with fixed cells wound is always on the right side. To determine the nuclear reorientation we measured the angle between the wound and the longer nuclear axis as described previously [40] and in Fig. 2C. In each experiment at least 100 nuclei were analyzed. To determine the orientation of perinuclear actin fibers we measured the angle between the wound and the line parallel with the perinuclear actin fibers. Both nuclear reorientation and perinuclear fibers orientation were determined using ImageJ software and values were plotted as box and whiskers graph with the median and quartiles using Prism software (GraphPad Software, Inc.). Representative results from three independent experiments are shown.

To quantify the presence of the perinuclear actin fibers we used the previously described method [26;37;43] where perinuclear actin fibers were distinguished as well-organized, disorganized or disrupted, or completely absent. Only cells with well-organized perinuclear fibers were scored as positive.

The statistical analyses (t-test (assuming Gaussian distribution)) were done using Prism software (GraphPad Software, Inc). Data are presented as means $\pm$ SD from at least two independent experiments except Fig. 5D which is mean from one experiment $\pm$ SEM.

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Figures:

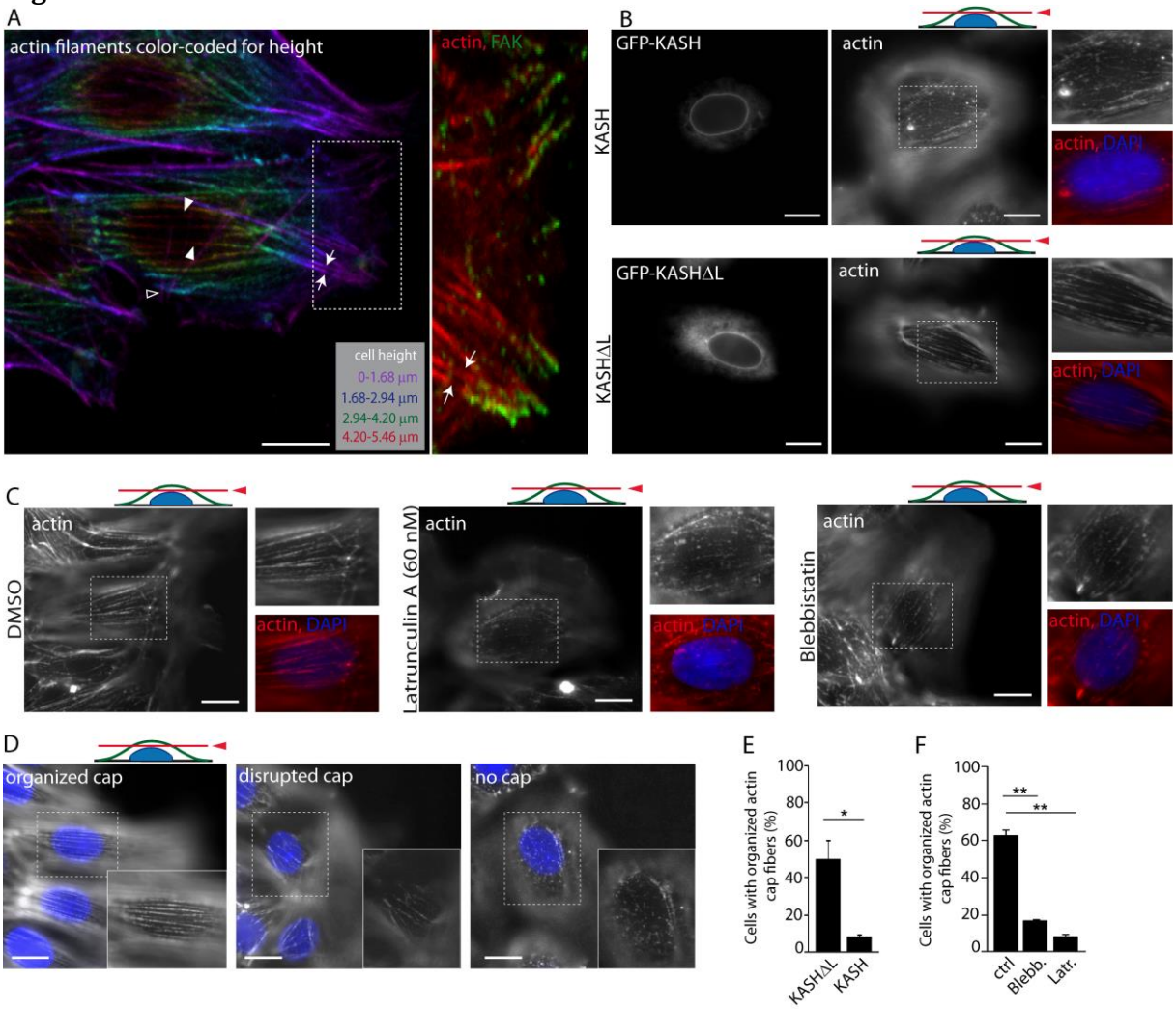

Figure 1. Organization of perinuclear actin fibers in RAT2 cells. (A) Perinuclear actin fibers are arranged in a pole-to-pole manner in polarized cells and emanate from focal adhesions. Cells were fixed 6 h after the monolayer wounding, stained with phalloidin-rhodamine and visualized by confocal microscopy. Overlay of confocal sections was pseudocolored purple (ventral section), blue-green (middle sections) and red (top sections). Example of ventral fiber is indicated by empty arrowhead. Perinuclear actin fibers extend from the leading edge (arrows) above the nucleus (arrowheads). Boxed area shows that these fibers (red) are anchored in focal adhesions (FAK, green) at the leading edge. (B) LINC complex disruption affects perinuclear actin fibers. Cells were transfected with GFP-KASH or control GFP-KASHΔL constructs, stained with phalloidin and DAPI, and imaged by conventional wide-field microscopy focusing on the apical side of the nucleus. Right panels show higher magnification of actin (red) above the nucleus (blue). The KASH domain of Nesprin2 (GFP-KASH) binds SUN protein disrupting LINC complex. GFP-KASHΔL unable to bind SUN proteins is used as a control. (C) Organization of perinuclear actin in cells pretreated with either low dose of latrunculin A (60 nM) or blebbistatin (5 μM) for 20 min and wounded by scratch. After 6 h cells were fixed and stained with phalloidin and DAPI. Higher magnifications show actin above the nucleus (DAPI, blue). (D) Examples of well-organized perinuclear actin fibers (left panel), disrupted perinuclear fibers (middle panel) or absent perinuclear actin fibers (right panel) above the nucleus (DAPI, blue). Images were obtained by wide-field microscopy focusing at apical side of the nucleus. Boxed areas show higher magnification of actin above the nucleus. (E-F) Quantification of perinuclear actin fibers in cells transfected with GFP-KASH or GFP-KASHΔL constructs (E) or in cells pretreated with either latrunculin A or blebbistatin (F) as described in Materials and methods (mean $\pm$ SD; n>100; ** $p<0.005$; * $p<0.05$). In all figures, focal planes are indicated above the pictures. Bars, 10 μm.

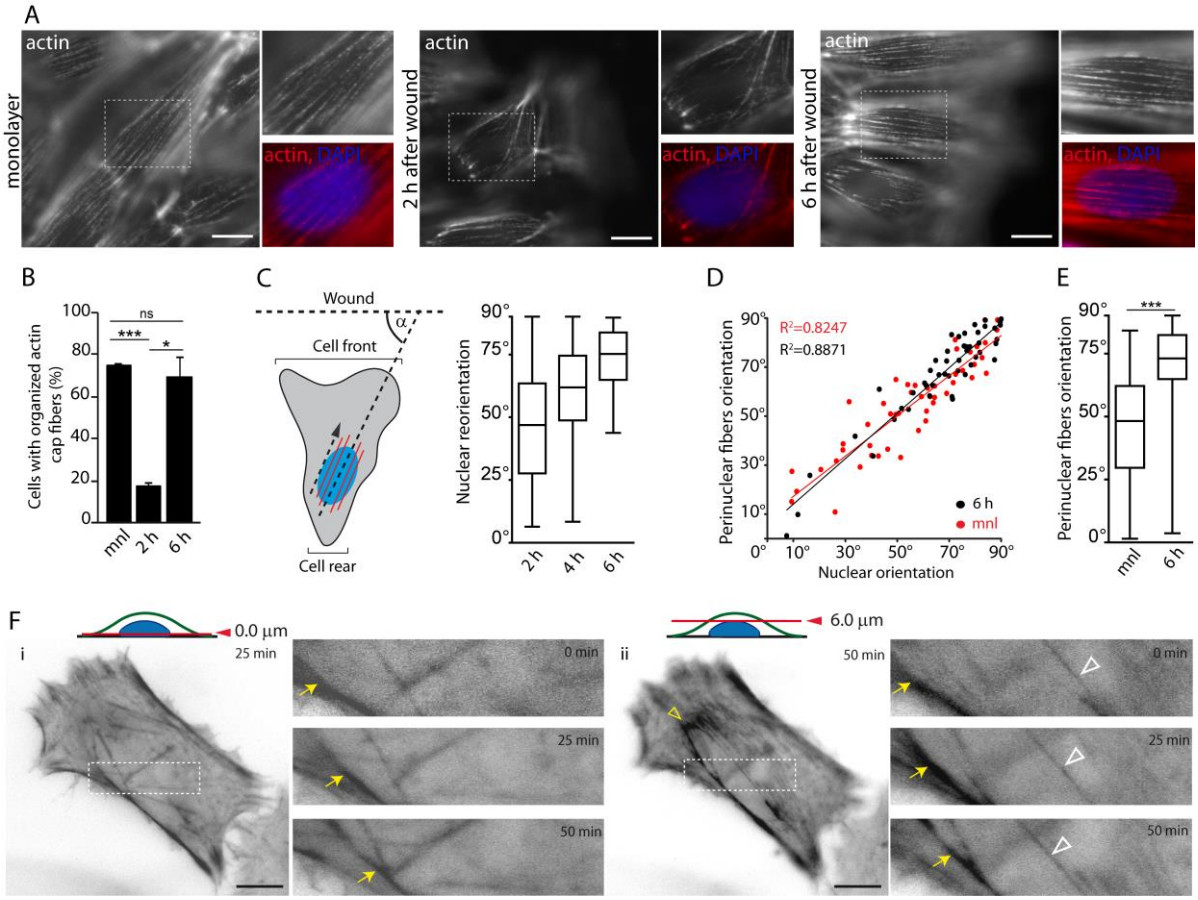

Figure 2. Dynamics of perinuclear actin fibers in polarizing cells. (A) Disruption of perinuclear actin fibers during cell polarization. Cells in monolayer, polarized cells (6 h after wound) and polarizing cells (2 h after wound) were fixed and stained with phalloidin and DAPI. Higher magnification of boxed areas shows actin above the nucleus (DAPI, blue). Images were obtained by conventional wide-field microscopy focusing on the apical side of the nucleus. (B) Quantification of perinuclear actin fibers in cell monolayer (mnl), in cells polarizing to the wound (2 h after wounding) and in polarized cells (6 h after wounding) as described in Materials and methods and Fig. 1E (mean ± SD; n>100; *** $p<0.0005$; * $p<0.05$; ns $p>0.05$). (C) Schema of nuclear reorientation and perinuclear actin fibers orientation measurement (left panel). Nuclear orientation is measured as an angle between the longer nuclear axis (dashed line) and the wound. Perinuclear actin fibers orientation is measured as an angle between the line (line arrowhead) parallel with perinuclear actin fibers (red lines) and the wound. Right panel shows time course of nuclear reorientation in cells fixed in indicated time points after wounding. (D) Perinuclear actin fibers orientation correlates with reorientation of the nucleus. Nuclear reorientation and perinuclear actin fibers orientation in each cell was measured in cell monolayer (mnl – red dots) and 6 h after wounding (black dots). Trendlines are indicated by black and red lines. (E) Perinuclear actin fibers orientation in cell monolayer (mnl) and in polarized cells (6 h after wounding). Orientation of perinuclear actin fibers was measured as described in Materials and methods and in Fig. 2C (n=100; *** $p<0.0005$). Note that the perinuclear actin fibers orientation was not measured in cells 2 h or 4 h after wounding as in these cells actin cap is disrupted or undergoes significant remodeling. (F) Grayscale inverted images from time-lapse fluorescent microscopy recording actin dynamic at the bottom of the cell (panel i) and above the nucleus (panel ii). Yellow arrow indicates actin fiber moving from the basal side above the nucleus, white empty arrowhead indicates actin fiber that moves laterally above the nucleus. Yellow empty arrowhead indicates actin structure where perinuclear actin fibers terminate. Distance of focal plane from the basal side is indicated above the pictures. Bars, 10 µm.

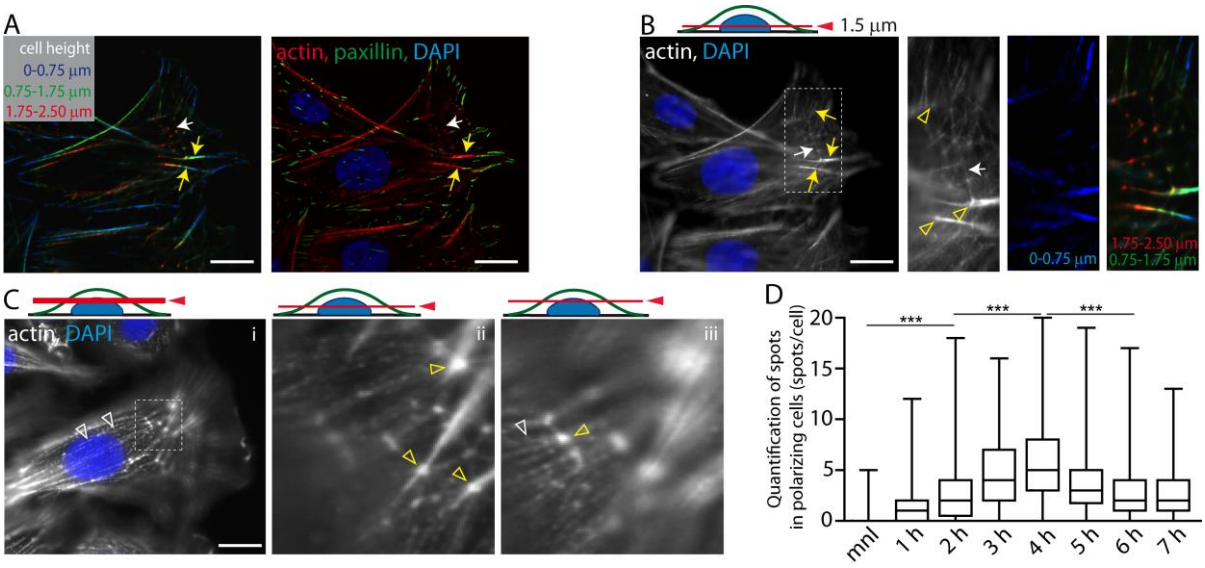

Figure 3. Dorsal fibers, transverse arcs and actin cap fibers are coupled in actin rich foci. (A) Dorsal fibers (yellow arrows) and transverse arcs (white arrows) in cells polarizing to the wound for 3 h and stained with phalloidin-rhodamine and paxillin. Left panel shows overlaid deconvolved z-projection of actin fibers from focal planes 0.0 – 2.5 µm pseudocolored for height. Dorsal fibers rise from the basal side (blue) of cell front edge toward the low-middle section (green) and middle section (red) close to the nucleus. These dorsal fibers are anchored in focal adhesions (paxillin, green) at cell front (right panel, overlaid actin z-projections shown in red). (B) Actin rich spots appear at the dorsal fiber–arc intersection (yellow empty arrowheads in boxed area). Localization of dorsal fiber – transverse arc intersections relative to the nucleus (DAPI, blue) is shown in greyscale panels (focal plane 1.5 µm above the bottom; unprocessed images are shown to better visualize thin arcs and actin foci). Deconvolved and pseudocolored panels show that spots at cell's low-middle section (green) are closer to the cell front while spots at midsection (red) are closer to the nucleus. The spots are largely absent from basal side (pseudocolored blue). (C) Perinuclear actin fibers (white empty arrowheads) terminate in actin spots in front of the nucleus (5 h after wound, panel i). Actin rich spots (yellow empty arrowheads) located at cell's midsection in front of the nucleus harbor dorsal fibers and multiple actin cap fibers (panel iii). Actin rich spots closer to basal side are located more to cell front (panel ii). In panel (i) two focal planes were overlaid to better visualize perinuclear fibers and actin spots in front of the nucleus. (D) Quantification of actin rich spots in cell monolayer and in cells polarizing to the wound. Cells were fixed at indicated time points and spots were quantified as described in Materials and methods (n>80; *** $p<0.0001$). Note that in monolayer (mnl) 75% of cells did not have detectable actin spots. Bars, 10 µm.

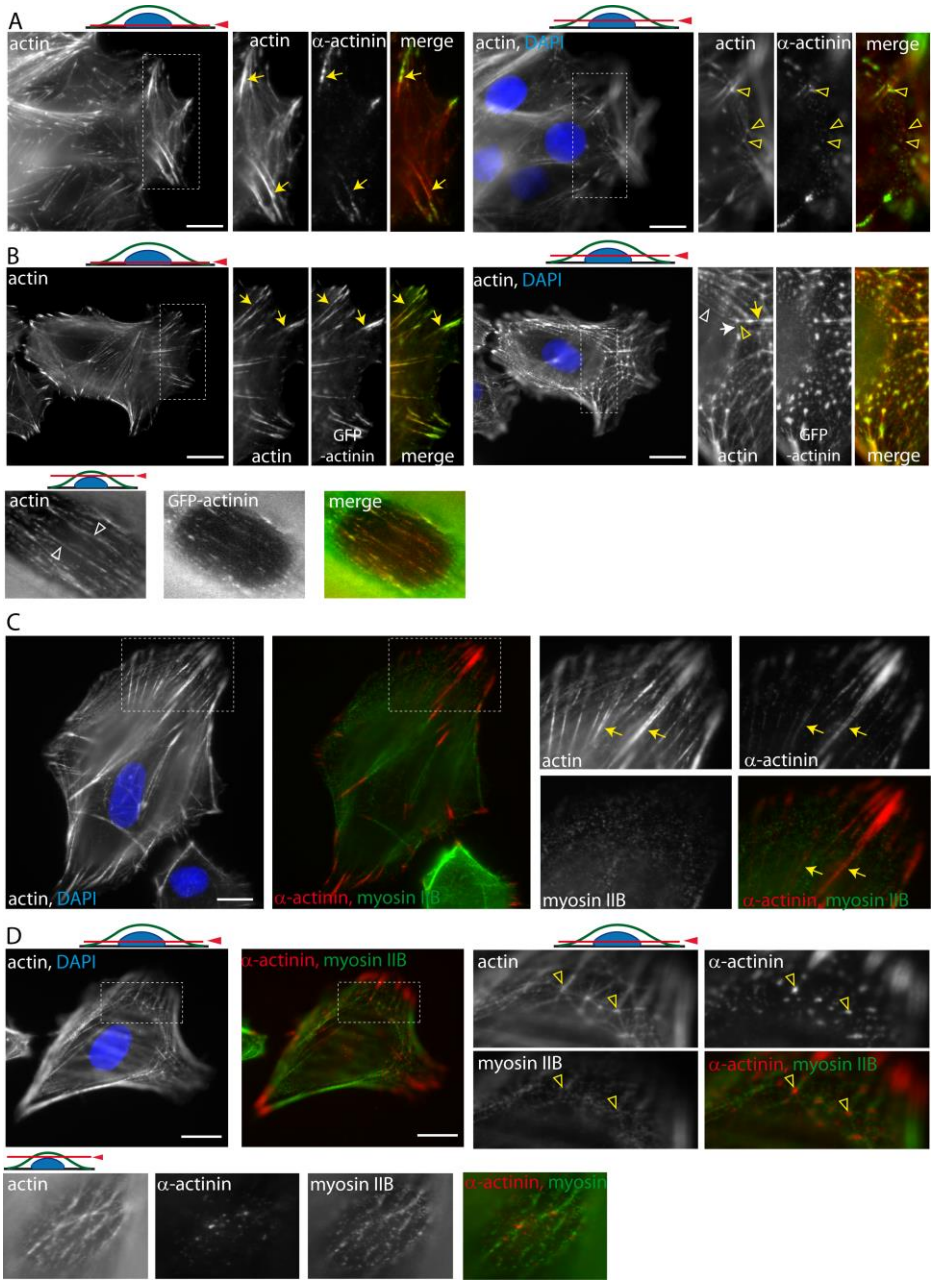

Fig. 4. Localization of α-actinin-1 to dorsal fibers and actin rich spots. (A) Non-processed images from basal and midsection focal planes (indicated above the pictures). Higher magnification of boxed areas shows that α-actinin-1 localizes to dorsal fibers (yellow arrows) and crosslinked spots (yellow empty arrowheads). Cells fixed 4 h after wounding were stained with phalloidin (red in merged images) and α-actinin-1 antibody (green). (B) Localization of GFP-α-actinin-1 to dorsal fibers, transverse arcs, perinuclear fibers and actin spots. GFP-α-actinin-1 transfected cells were fixed 4 h after the wound and stained with phalloidin. GFP-α-actinin-1 (green in merged images) and actin (red) were observed at basal side (left panel), at cell's midsection (right panel) and above the nucleus (bottom panel). Dorsal fibers are indicated by yellow arrows, transverse arcs by white arrows, perinuclear actin by white empty arrowheads and crosslinked spots by yellow empty arrowheads. (C) Dorsal fibers contain α-actinin-1 but not myosin IIB. Fixed cells were co-stained with antibodies against α-actinin-1 (red in merged images) and myosin IIB (green). Dorsal fibers are indicated in boxed areas by yellow arrows. (D) Localization of α-actinin-1 and myosin IIB to perinuclear actin fibers (bottom panels). Upper panels show that α–actinin-1 but not myosin IIB is present in crosslinked spots (yellow empty arrowheads in boxed area). Fixed cells were co-stained with antibodies against α-actinin-1 (red in merged images) and myosin IIB (green). Bars, 10 μm.

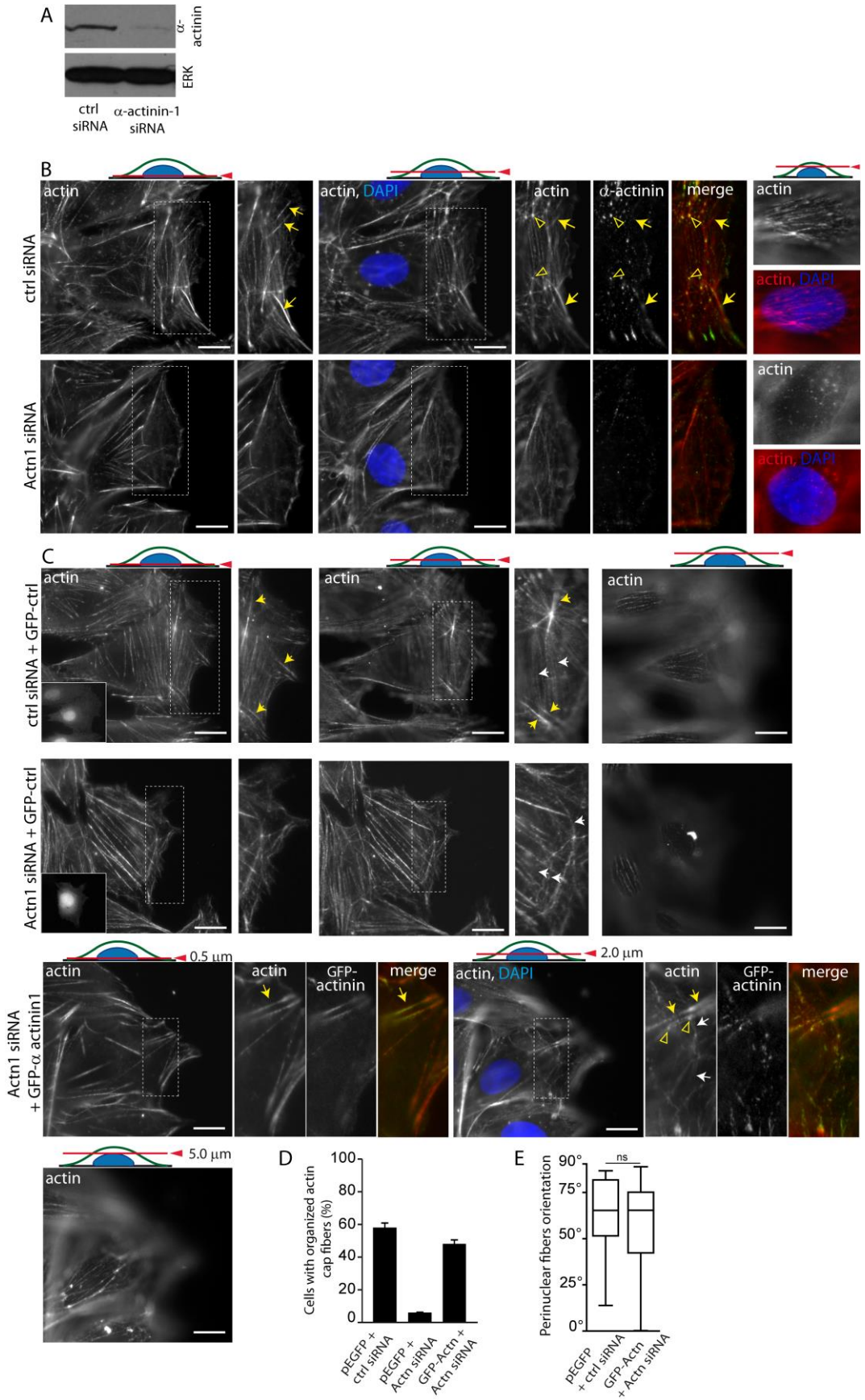

Figure 5. α-actinin-1 knockdown impairs the formation of dorsal and perinuclear actin fibers. (A) α-actinin-1 level in cells transfected with α-actinin-1 or control siRNAs. ERK2 was used as loading control. (B) siRNA transfected cells were fixed 6 h after the wound and stained with

phalloidin (red), α-actinin-1 antibody (green) and DAPI (blue). Non processed images from basal, midsection and top focal planes (indicated above the individual pictures) shows dorsal fibers (yellow arrows) and crosslinked spots (yellow empty arrowheads) indicated in higher magnification of boxed areas. Actin staining above the nucleus is shown in right panels. (C) Re-expression of siRNA resistant chicken α-actinin-1 in α-actinin-1 depleted cells rescued dorsal fibers and actin cap formation. Cells were co-transfected with α-actinin-1 or control siRNA and GFP-α-actinin-1 (green in merged images) or GFP alone construct, fixed 6 hours after wounding and stained with phalloidin-rhodamine (red). Dorsal fibers (yellow arrows), actin arcs (white arrows) and dorsal fibers - arcs crosslinked spots (yellow empty arrowheads) are indicated. Bars, 10 μm. (D) Quantification of actin cap fibers in cells transfected as described in (D). Actin cap was quantified 6 h after wounding as described in Materials and methods and Fig. 1D (mean $\pm$ SEM). (E) Perinuclear actin fibers orientation. Note that the perinuclear actin fibers orientation was not measured in cells co-transfected with α-actinin-1 siRNA and GFP-empty construct as these cells do not form actin cap. Perinuclear actin fibers orientation was measured as described in Materials and methods and Fig. 2C ($n>20$; ns $p>0.05$).

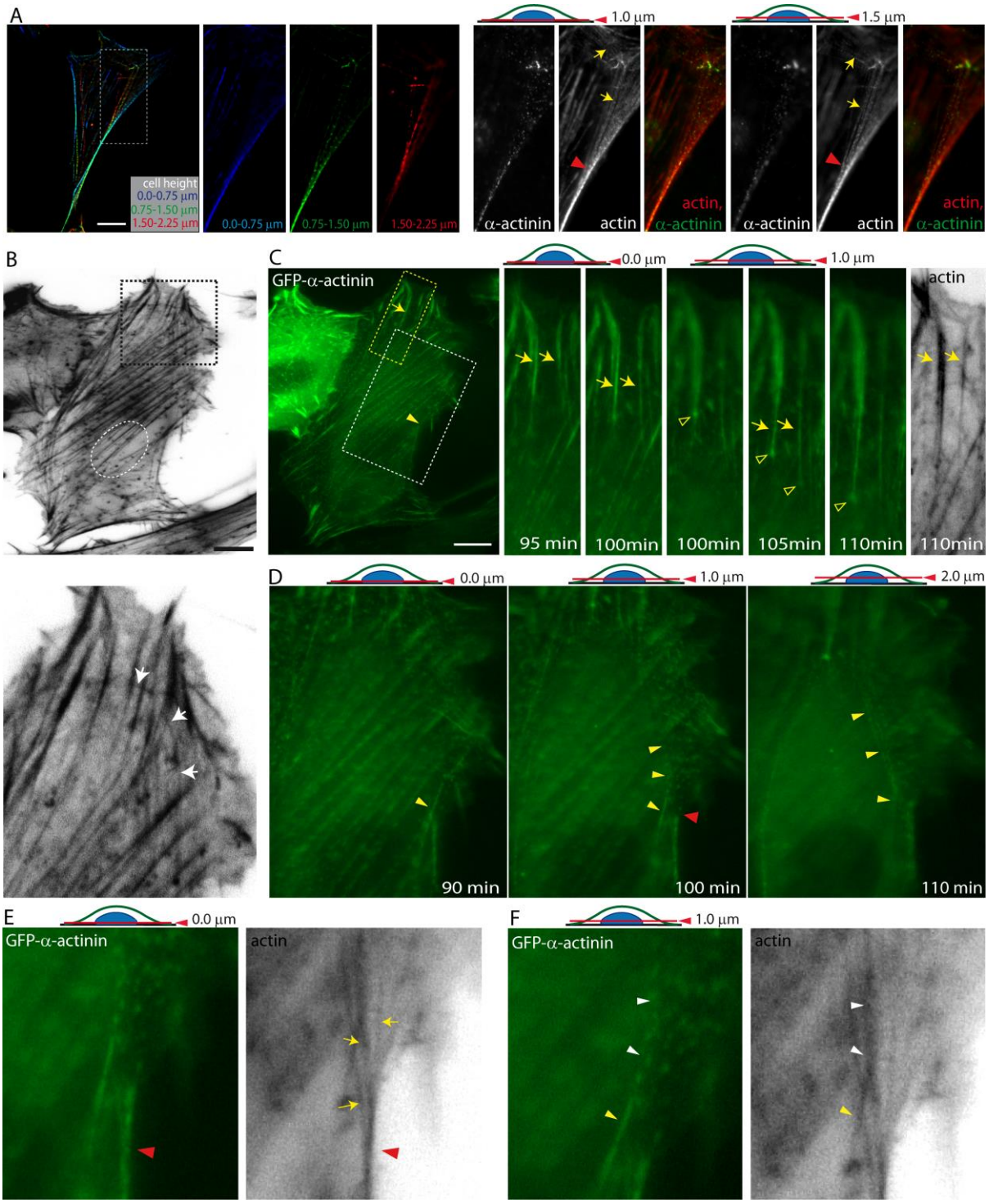

Figure 6. Dorsal fibers and transverse arcs serve as a platform for perinuclear fibers formation. (A) Thin curved actin fibers are linked to ventral stress fiber. Fixed cells were stained with phalloidin and α-actinin-1 antibody. Deconvolved images were pseudocolored as indicated to visualize actin fibers position in z-axis (left panels). Magnifications of boxed areas show the split of straight ventral fiber into curved actin fibers (red arrowheads) that extend to the dorsal side (yellow arrows) in several focal planes (magnified images in right panel are non-deconvolved images). (B-D) Time-lapse fluorescent images of GFP-α-actinin-1 and mCherry-LifeAct transfected cell at the edge of the wound. (B) Inverted grayscale images of curved actin arcs at the protrusive leading edge visualized by mCherry-LifeAct (white arrows in magnification of boxed area, bottom panel). White dashed oval

represents the position of the nucleus. (C) Dorsal fibers (yellow arrows in enlarged area marked by dashed yellow line) visualized by GFP-α-actinin-1 (green) polymerize from the leading edge to the dorsal side of the cell. Yellow empty arrowheads indicate α-actinin rich spots. Grayscale image shows dorsal fibers visualized by mCherry-LifeAct. (D) At the interface of protruding and non-protruding areas (red arrowhead) straight ventral fiber (yellow arrow) fuses with curved transverse arcs and raise towards the dorsal side of the cell (see also Movies S3 and S4). Enlarged area is marked by dashed white line in panel C. (E) Perinuclear fibers originate in thick ventral fiber (red arrowhead) that split up into several thin fibers (yellow arrows). (F) Actin fiber shown in (D) contains α-actinin-1 patterns typical for both ventral stress fibers (uniform GFP-α-actinin-1 staining, yellow arrowheads) and actin arcs (periodically but distantly spaced GFP-α-actinin-1 bands, white arrowheads). Bars, 10 μm.

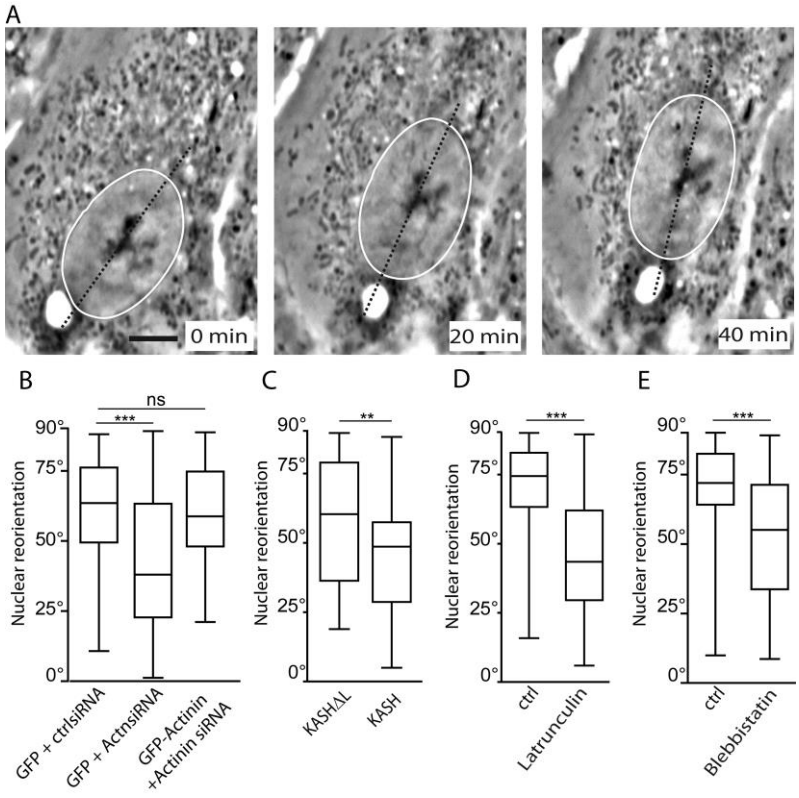

Figure 7. Perinuclear fibers are required for nuclear reorientation. (A) Time lapse phase contrast images of nuclear reorientation during actin cap assembly. Cell nucleus is indicated by white line and the nuclear axis indicating the orientation of the nucleus by black dashed line. Bar, 5 μm. (B-E) Nuclear reorientation requires dorsal fibers, actin cap and actomyosin contractility. Nuclear reorientation was determined in cells transfected with control or α-actinin-1 siRNA altogether with siRNA resistant GFP-α-actinin-1 or empty GFP construct (B), in cells transfected with GFP-KASH or GFP-KASHΔL construct (C), in cells pretreated with latrunculin A (60 nM) (D) or blebbistatin (5 μM) (E) 20 min before wounding. In all experiments nuclear reorientation was measured in cells fixed 6 h after wounding as described in materials and methods and Fig. 2C ($n>100$; in α-actinin-1 rescue experiment $n>45$; *** $p<0.0001$; ** $p<0.005$; ns $p>0.05$).

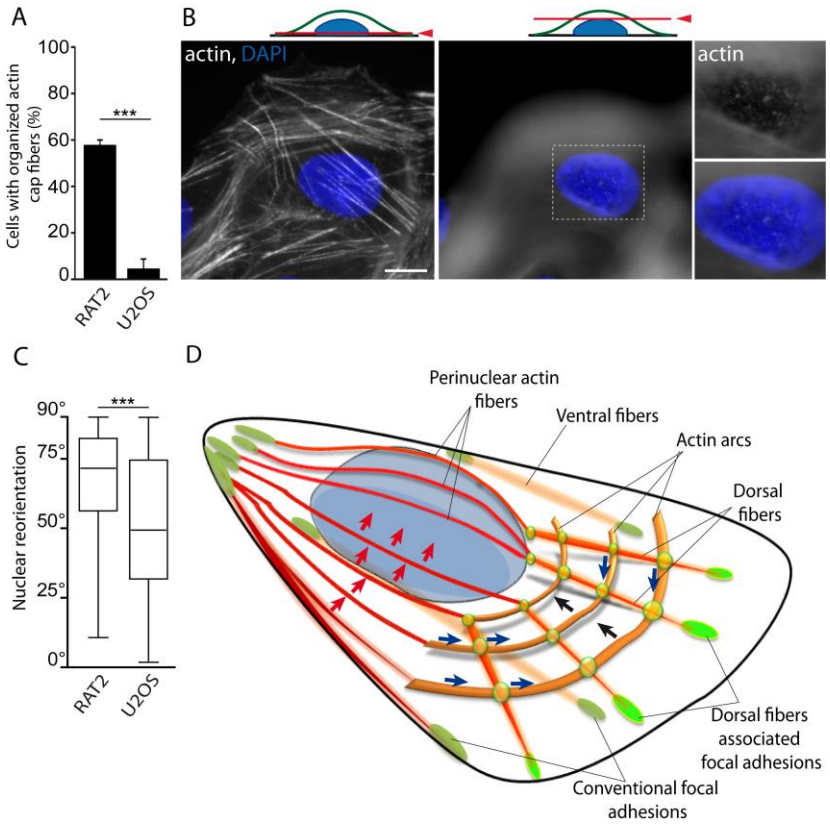

Figure 8. U2OS cells are impaired in perinuclear actin fibers formation and nuclear reorientation. (A) Quantification of perinuclear actin fibers in RAT2 and U2OS cells. RAT2 and U2OS cells polarizing to the wound were fixed 6 h after wounding. Presence of perinuclear actin fibers was quantified as described in Materials and methods and Fig. 1D (mean ± SD; n>100; *** $p<0.0001$). (B) Actin cytoskeleton in U2OS cells fixed 6 h after wounding. Left panel shows dorsal fibers and transverse arcs on the basal side of U2OS cell. Right panel and magnification of boxed area shows actin staining on the apical side above the nucleus. Bar, 10 μm. (C) Nuclear reorientation in RAT2 and U2OS cells. Nuclear reorientation was measured 6 h after wounding as described in materials and methods and Fig. 2C (n>100; *** $p<0.0001$). (D) Hypothetical model of actin cap formation. Dorsal fibers are crosslinked with contractile transverse actin arcs in spots rich in α-actinin (yellow spots). At the cell periphery, arcs become linked to one end of ventral stress fibers. Actomyosin contraction mediates centripetal flow of the actin network while the dorsal fibers ensure that the network moves to the cell's dorsal side (black arrows). Arcs contraction pulls the crosslinking spots as well as the ventral fibers in front of the nucleus (blue arrows). Finally, dorsal fibers and arcs pull the ventral fibers on the top of the nucleus (red arrows) to create perinuclear actin structure.

Symmetry breaking in spreading Rat2 fibroblasts requires the MAPK/ERK pathway scaffold RACK1 that integrates FAK, p190A-RhoGAP and ERK2 signaling.

(Klímová et al., submitted manuscript)

Symmetry breaking in spreading RAT2 fibroblasts requires the MAPK/ERK pathway scaffold RACK1 that integrates FAK, p190A-RhoGAP and ERK2 signaling

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Abstract

The spreading of adhering cells is a morphogenetic process during which cells break spherical or radial symmetry and adopt migratory polarity with spatially segregated protruding cell front and non-protruding cell rear. The organization and regulation of these symmetry-breaking events, which are both complex and stochastic, is not fully understood. Here we show that in radially spreading cells, symmetry breaking commences with the development of discrete non-protruding regions characterized by large but sparse focal adhesions and long peripheral actin bundles. Establishment of this non-protruding static region specifies the distally oriented protruding cell front and thus determines the polarity axis and the direction of cell migration. The development of non-protruding regions requires ERK2 and the ERK pathway scaffold protein RACK1. RACK1 promotes adhesion-mediated activation of ERK2 that in turn inhibits p190A-RhoGAP signaling by reducing the peripheral localization of p190A-RhoGAP. We propose that sustained ERK signaling at the prospective cell rear induces p190RhoGAP depletion from the cell periphery resulting in peripheral actin bundles and cell rear formation. Since cell adhesion activates both ERK and p190RhoGAP signaling this constitutes a spatially confined incoherent feed-forward signaling circuit.

INTRODUCTION

The establishment of cell polarity plays an essential role in many physiological and pathophysiological processes including cell motility, embryonic development, morphogenesis, immune response as well as the migration and dissemination of cancer cells. Polarized cells display asymmetrical distributions of protein complexes, signaling components and cytoskeletal networks that allow the spatial segregation and regulation of distinct intracellular processes (Drubin and Nelson, 1996; Macara and Mili, 2008; Goehring and Grill, 2013). Cell polarity also plays a critical role in motile adherent cells where it promotes directional migration. In migrating cells the polarity axis develops between a functionally and morphologically distinct protruding cell front and a retracting cell rear. The presence of a branched F-actin filament network and abundant dynamic adhesions are typical for the cell front. Dendritic actin polymerization and cellular protrusivity are limited at the cell sides and the cell rear where actin predominantly forms long actomyosin bundles that terminate in less abundant but large stable adhesions (Small et al., 1998;Ridley et al., 2003). The spatial differences in the organization of the actin cytoskeleton and focal adhesions at the cell front and rear are reflected in asymmetrical cell shapes that range from conical or crescent to more irregularly shaped cells (Mogilner and Keren, 2009; Mullins, 2010).

The establishment of a front-rear axis and the direction of migration are generally thought to be determined by external directional signals such as chemotactic gradients or mechanical stimuli and physical constraints (Etienne-Manneville, 2004; Roca-Cusachs et al., 2013). Nevertheless, evidence suggests that the establishment of cell polarity and directional migration can be a self-organizing process that can occur spontaneously in the absence of external cues. Depending on the cell type, symmetry breaking can be induced either by the establishment of a single cell front with a localized increase in protrusion, or by the retraction of the cell rear (Cramer, 2010). Polarizing neutrophils break the symmetry by localized actin polymerization and protrusion formation that define the leading edge and precede the formation of cell rear (Xu et al., 2003;Wong et al., 2006). On the contrary, in spontaneously polarizing fibroblasts and fish keratocytes the symmetry breaking is initiated by localized cell edge retraction that results in the establishment of cell rear (Mseka et al., 2007;Yam et al., 2007).

Spontaneous symmetry breaking and cell polarization generally is believed to be initiated by stochastic fluctuations in the cellular environment that are amplified and stabilized by positive feedback signaling (Lauffenburger and Horwitz, 1996;Mullins, 2010;Wu and Lew, 2013). It is presumed that symmetry breaking could also be elaborated by the competition of locally activated signaling with globally exerted inhibition. Such an antagonistic signaling circuit composed of a short range activator - long range inhibitor system is sufficient for the generation of a signaling gradient from spatially homogenous conditions (Meinhardt and Gierer, 2000;Goehring and Grill, 2013;Wu and Lew, 2013;Hart and Alon, 2013, Verkhovsky, 2015). An important question in understanding the symmetry breaking is, what is the signaling mechanism that governs spatially restricted intracellular signaling from uniform external cues.

Scaffold/adaptor proteins, because they are capable of promoting the activation of a signaling pathway in a specific subcellular compartment, represent one mechanism by which external or stochastically generated signals can be directed to specific locations (Kolch, 2005). We have previously shown that the scaffold protein RACK1 specifically controls activation of the Extracellular Signal-Regulated Kinase (ERK) MAP Kinase and its localization to focal adhesions during integrin dependent adhesion (Vomastek et al., 2007). Here, we show that the Extracellular Signal-Regulated Kinase (ERK) pathway scaffold RACK1 regulates symmetry breaking in radially spreading RAT2 fibroblasts by enabling cells to form a non-protruding cell rear. RACK1 promotes adhesion-mediated activation of ERK that in turn locally suppresses p190A-RhoGAP (hereafter p190) signaling by depletion of p190 from the cell periphery. We show that knockdown of RACK1 or ERK2 enables a uniform p190 cellular distribution and a uniform, non-polar spreading without symmetry breaking. Since both ERK and p190 are activated by cell adhesion we hypothesize that adhesion induces a signaling circuit termed "incoherent feed-forward loop" (Alon, 2007; Hart and Alon, 2013). In this signaling circuit spatially restricted and sustained ERK signaling opposes globally activated p190 to induce cell rear formation.

RESULTS

RACK1 regulates migratory cell polarity.

To determine whether RACK1 plays a role in cell polarization we attenuated RACK1 expression level by siRNA in RAT2 fibroblasts and evaluated the ability of these cells to form leading and trailing edges (Fig. 1A). The majority of control cells displayed a front-rear polarity which is typical for migrating fibroblasts. It is characterized by well-defined leading and trailing edges with long concave sides which give the cells a prolonged conical shape (Fig. 1B and 1C). Some of the control cells also displayed a triangular or more irregular shape and a less well defined leading edge (Fig. 1B and 1C). Only a small fraction of control cells had an oval shape. In contrast, knockdown of RACK1 induced dramatic changes in cell morphology. RACK1 deficient cells displayed radial symmetry presented by a rounded or ovoid shape (Fig. 1B and 1C).

To quantify the change in cell morphology, we determined the roundness index and cell area of individual cells (Fig. 1D). The roundness index is an appropriate indicator of cellular shape as it reflects the shape of a cell with value of 1 assigned for a perfect circle and lower values for differently shaped objects (Schober et al., 2009). The quantification showed that RACK1 deficient cells display a significantly rounder cell shape and, in addition, they spread over a larger area than control cells (Fig. 1E and F). The changes in cell morphology could be observed 36 h after siRNA transfection and RACK1 depleted cells regained normal morphology when the effect of siRNA was worn out (data not shown).

RACK1 is required for symmetry breaking in spreading RAT2 fibroblasts.

Since our data indicated that RACK1 plays a significant role in the regulation of cell shape we investigated the function of RACK1 protein in cells spreading on fibronectin. Upon adhesion to fibronectin, control cells established distinct protruding front and rear with concave non-protruding areas or they displayed more irregular shapes with concave edges (Fig. 2A). The morphological changes and the adoption of polarized phenotype were observed regardless of the presence or absence of fetal bovine serum (FBS) although the control cells plated in the presence of FBS were larger and more often developed well-defined leading edges. On the contrary, RACK1 deficient cells failed to break radial symmetry which resulted in significant increases of both cell area and cell roundness compared to control cells (Fig. 2B and C). Similar results were obtained with additional RACK1 siRNA duplexes targeting different sequences of the RACK1 gene (Supplementary Fig. S1).

To characterize the phenotypic changes in time we imaged cells adhering to fibronectin (Fig. 2D and Supplementary Movie 1). We found that at the initial phase of spreading, cells formed a discoid or ovoid shape with dynamically formed protrusions and retractions around the entire cell periphery. The initiation of symmetry breaking became apparent approximately 15 min after plating as we observed that there was a significant change of protrusivity in a discrete cell region (indicated by white arrow in Fig. 2D). This cellular region became relatively static and progressively adopted an inward oriented concave profile of the cell periphery. About 30 min after plating, cells developed the cell rear in this location (Fig. 2D, white arrow) and protrusive cell front on the distal cell edge. The polarization of control cells was completed within 1 h after plating and it was generally followed by cell migration (Fig. 2E and Supplementary Movie 2). A majority of the cells (~50 %) developed cell rear by the formation of a single dominant concave region while approximately one third of the cells developed more concave regions that together formed an extended tail (Fig. 2E and Supplementary Movie 2). The establishment of the non-protruding static region followed by the formation of distally oriented protruding cell front determined the polarity axis and the direction of migration of the cell (Fig. 2E and Supplementary Movie 2). On the contrary, RACK1 deficient cells did not undergo breaking of radial symmetry. When plated on fibronectin these cells spread with radially symmetric ovoid or discoid shape and protrusions formed omnidirectionaly around the cell perimeter (Fig. 2D).

Since the changes of cell shape reflect the remodeling of actin cytoskeleton we next examined the actin dynamics in adhering cells. To visualize changes in the actin structure, cells were transfected with fluorescently tagged actin constructs and followed by time-lapse fluorescence microscopy. During the initial phase of spreading, cells displayed dynamic actin polymerization along the cell periphery. Symmetry breaking and appearance of a stable quiescent concave region correlated with reduced actin polymerization, dampened protrusions and formation of bundled actin filaments (Fig. 3A and Supplementary Movie 3). During the appearance of a non-protruding concave region the remaining part of the cell periphery showed continued actin polymerization and fast protrusion that eventually ceased as cells

became fully spread. On the other hand, formation of two or more stable concave non-protruding areas defined the extended cell rear (Supplementary Fig . S2). In striking contrast to control cells, the spreading of RACK1 depleted cells was dominated by constant actin polymerization and protrusions along the entire cell periphery. In addition, RACK1 deficient cells formed predominantly circumferential concentric actin arcs and non-parallel actin bundles and were devoid of peripheral concave actin bundles (Fig. 3A, 3D and Supplementary Movie 4).

We next analyzed the dynamics of cell edge protrusion in spreading cells. First, we generated cell outlines for each time point using an electrostatic contour migration method (Tyson et al., 2010) and QuimP11 toolbox as described in Material and Methods. The superimposed time sequence of control cell outlines during spreading showed that the development of non-protruding regions is the first visual break in cell symmetry and allows formation of the prospective cell rear (black arrows, Fig. 3B and Supplementary Fig. S2B). In RACK1 deficient cells the specification of cell rear was impaired, and cells continuously protruded along the entire cell periphery (Fig. 3B). Subsequently, we quantified the protrusion and retraction rates as well as the curvature of cell edge along the cell periphery. The cell outlines for each time point were used to generate convexity and motility maps. The convexity map represents the changes of cell perimeter curvature in time with concave regions visualized in blue and convex regions in yellow-red. Similarly, the motility map shows cell edge movement rate along the cell perimeter in time with protrusions indicated in yellow-red and retractions indicated in blue. In control cells we identified dominant concave regions corresponding to the non-protruding regions and to the prospective cell rear (Fig. 3C and Supplementary Fig. S2C). In RACK1 deficient cells we did not identify such stable and large concave regions, as these cells spread with constant protrusion rate and minimal retractions along the cell perimeter. Although RACK1 deficient cells occasionally also developed concave regions, in contrast to control cells, these regions were smaller, persisted for shorter periods of time and displayed a significant protrusive rate (Fig. 3B, 3C and Supplementary Fig. S3).

In addition to the changes in actin structure, staining of fixed cells for the focal adhesion marker paxillin revealed an altered pattern of focal adhesions in RACK1 deficient cells (Fig. 3D and Supplementary Fig. S4). In control cells, focal adhesions localized asymmetrically and small peripheral focal adhesions decorated exclusively broad lamellipodia-like structures, presumably the protrusive cell front. Less abundant, large focal adhesions localized to the cell rear and cell edge corners (Fig. 3D and Supplementary Fig. S4). Peripheral actin bundles terminated in elongated focal adhesions that often displayed a triangular shape, indicating that these adhesions are under high tensional stress exerted from different directions (Fig. 3D and Supplementary Fig. S4). RACK1 depleted cells contained predominantly small dot-shaped focal adhesions that were evenly localized on the cell edge, and arc-shaped concentric actin lines that were not visibly anchored in the focal adhesions. Large focal adhesions and peripheral actin bundles were absent and numerous short disorganized focal adhesions were randomly spread across the whole cell body (Fig. 3D and Supplementary Fig. S4). These data are in agreement with our previous finding that during

cell spreading RACK1 depletion inhibits disassembly of focal adhesions (Vomastek et al., 2007).

These data indicate that symmetry breaking in RAT2 fibroblasts spreading on fibronectin is characterized by the development of non-protruding, concavely oriented cell regions accompanied by the formation of thick peripheral actin bundles terminating in large focal adhesions. The formation of these regions specifies the distally oriented cell front and the direction of migration. RACK1 depletion inhibited the formation of cell front and rear and the assembly of peripheral actin bundles. In addition and consistently with the previous report (Serrels et al., 2010), RACK1 depleted cells neither reorient Golgi nor polarize to a wound (data not shown).

RACK1 and FAK are involved in the regulation of symmetry breaking.

Since our results suggested that cell adhesion is sufficient to induce symmetry breaking of spreading cells, we sought to explore the signaling events downstream of adhesion that control this process. Cell adhesion leads to the integrin dependent activation of FAK (Owen et al., 1999), a protein tyrosine kinase involved in cytoskeleton remodeling and in the formation and disassembly of cell adhesion structures (Chang et al., 1995;Parsons et al., 2000). RACK1 has been shown to interact with both integrins and FAK (Liliental and Chang, 1998;Kiely et al., 2009;Serrels et al., 2010) suggesting that RACK1 could modulate FAK activation or FAK signaling induced by integrin adhesion to extracellular matrix. We found that upon adhesion RACK1 knockdown did not affect the activity of FAK as judged by the phosphorylation of FAK on tyrosine 397 (Fig. 4A). We next attenuated FAK expression in RAT2 fibroblasts by siRNA to determine whether FAK signaling regulates symmetry breaking in adhering cells (Fig. 4B). FAK deficient cells were elongated and had a larger area than control cells, consistent with previous results that FAK knockdown in adherent cells induces cell elongation (Iwanicki et al., 2008). Simultaneous silencing of FAK and RACK1 led to suppression of the round phenotype characteristic of RACK1 depleted cells: FAK-RACK1 double deficient cells were able to form concave cell regions and to establish a leading edge and tail (Fig. 4E). Because FAK knockdown rescues the effects of RACK1 knockdown, it demonstrates that FAK is epistatic to RACK1 in symmetry breaking, and thus the two proteins are on the same signaling pathway. Interestingly, although the knockdown of FAK in RACK1 attenuated cells led to a significant decrease in the roundness index, it only slightly reverted the changes in cell area (Fig. 4C and 4D). The changes in cell morphology were paralleled by changes in actin cytoskeleton and focal adhesions as FAK and RACK1 double negative cells were able to form thick peripheral actin bundles terminated in large focal adhesions localized on the edges of concave regions (Fig. 4E). We hypothesize that during cell adhesion RACK1 intervenes with integrin signaling downstream of FAK to reduce radial spreading and to promote symmetry breaking of adhering cells.

p190 silencing suppresses the RACK1 round phenotype.

The recruitment of GTPase activating protein p190 to FAK has been implicated in the temporal inactivation of the small GTPase Rho and actin remodeling during cell spreading on fibronectin. Since FAK has also been shown to control p190 activity upon cellular adhesion (Arthur and Burridge, 2001;Tomar et al., 2009) and RACK1 affects p190 signaling (Miller et al., 2004) we silenced p190 expression by siRNA to examine the role of p190 in the regulation of symmetry breaking in RAT2 cells (Fig. 5A). Depletion of p190 suppressed the RACK1 round phenotype during cell spreading (Fig. 5D). Quantitative analysis showed that p190 and RACK1 double negative cells have similar roundness index and cell area as control cells (Fig 5B and C). The depletion of p190 also reverted changes in actin cytoskeleton and focal adhesions induced by RACK1 knockdown (Fig. 5D). Simultaneous depletion of RACK1 and p190 induced the formation of thick peripheral actin bundles that paralleled the concave regions of cells and terminated in large adhesions, as well as the formation of oriented actin filament bundles aligned along the front-rear axis (Fig. 5D).

p190 localization is altered in RACK1 deficient cells.

Previous reports have suggested that p190 activity is determined in large measure by its recruitment to the cell periphery (Bradley et al., 2006;Bass et al., 2008;Tomar et al., 2009; Pullikuth and Catling, 2010). In agreement with these findings, in polarized cells p190 localizes to the cell periphery and to the protruding areas in cells migrating into the wound (Tomar et al., 2009;Maninova et al., 2013). Consistently, in polarized migrating RAT2 cells a fraction of p190 localized to the cell leading edge (Fig. 6A). Notably, p190 was absent from non-protruding concave regions that contain peripheral actin bundles (Fig. 6A). For these reasons we examined the localization of p190 in control and RACK1 deficient cells. During the spreading of control cells, the establishment of an asymmetrical cell profile and development of concave edges with peripheral actin bundles resulted in a distinct localization pattern of p190. As with continuously adherent cells, p190 was absent from non-protruding concave edges and the peripheral p190 localization was detectable only in protruding areas (Fig. 6B). In contrast, in RACK1 deficient cells a significant fraction of p190 remained localized evenly along the entire cell periphery (Fig. 6B). Importantly, the suppression of RACK1 did not significantly affect the tyrosine phosphorylation of p190 (Fig. 6C). These data are consistent with the hypothesis that RACK1 regulates p190 localization during cell spreading and that peripheral localization of p190 is crucial for maintaining the radial symmetry of spreading cells.

ERK regulates p190 localization and symmetry breaking in spreading fibroblasts.

To delineate the mechanism for RACK1-mediated suppression of p190 signaling during symmetry breaking in adhering cells, we focused on the ERK pathway. We have previously shown that RACK1, in addition to integrins and FAK, also associates with core protein kinases of the ERK pathway and promotes adhesion-induced ERK activation and

localization to focal adhesions (Vomastek et al., 2007). Both RACK1 and ERK promote disassembly of focal adhesions (Webb et al., 2004;Doan and Huttenlocher, 2007;Vomastek et al., 2007) and, conversely, ERK mediated phosphorylation of p190 promotes the maturation of focal adhesions (Pullikuth and Catling, 2010). We reasoned that ERK inhibition during adhesion would block both disassembly of small dynamic focal adhesions and adhesion maturation, ultimately yielding RACK1 phenotype. To test this hypothesis we first confirmed that RACK1 knockdown reduces the active ERK level in adhering RAT2 cells (Supplemenatry Fig. S5). Next, we silenced individually ERK1 and ERK2 isoforms with siRNA duplexes targeting either ERK1 or ERK2 (Fig. 7A). The knockdown of ERK2 resulted in the hyperactivation of ERK1; similarly the knockdown of ERK1 hyperactivated ERK2 (Fig. 7A) suggesting that both ERK1 and ERK2 are involved in the feedback regulation of the ERK pathway as demonstrated previously (Lefloch et al., 2008). ERK2 depletion largely diminished the phosphorylated, active ERK signal in focal adhesions (Fig. 7I) suggesting that ERK2 is a dominant isoform in this location. Importantly, we found that knockdown of ERK2 leads to prominent changes in the shape of spreading cells. ERK2 deficient cells failed in radial symmetry breaking, in the development of concave regions and in the establishment of the front-rear axis (Fig 7B). In addition, ERK2 deficient cells significantly increased the roundness index and cell area (Fig. 7C and D). On the contrary, ERK1 depletion led to statistically significant but only intermediate changes in cell shape (Fig. 7B-D). We next determined whether ERK2 depletion affects the actin cytoskeleton. As with RACK1 knockdown cells, ERK2 deficient cells contained non-oriented stress fibers and circumferential actin bundles; however, they also formed short concave peripheral actin bundles (Fig. 7G). The pattern of focal adhesions in ERK2 deficient cells was similar to RACK1 knockdown cells as the appearance of large focal adhesions was diminished and replaced by short peripheral structures. Importantly, the defect in cell shape and actin organization induced by ERK2 knockdown was largely rescued by simultaneous silencing of p190 (Fig. 7E and 7F). ERK2 and p190 simultaneous knockdown led to a significant decrease of roundness index, however, the area of these cells was higher (Fig. 7E and 7F). ERK2-p190 deficient cells formed long concave regions with organized peripheral actin bundles terminated in large focal adhesions (Fig. 7G). Finally, we examined whether ERK2 regulates p190 localization. We found that ERK2 depletion by siRNA led to a significant increase in peripheral localization of p190 that localized evenly along the entire cell periphery (Fig. 7H). These data suggest that ERK2 regulates p190 function by control of p190 localization.

Active ERK is enriched at focal adhesions in both spreading and adhering cells (Fincham et al., 2000;Slack-Davis et al., 2003;Vomastek et al., 2007). We examined the pattern of active ERK localization during spreading of RAT2 cells. We found that the localization pattern of active ERK substantially differed between early and late time points of cell spreading. During the early phases of cell spreading, cells formed small focal adhesions arranged uniformly along the cell periphery and active ERK localized to these focal adhesions (Fig. 8A). At later time points, small focal adhesions containing active ERK decorated only convex, presumably protrusive regions of the cell. Importantly, active ERK was also present in less abundant large adhesion plaques. These adhesions localized to cell rear and cell edge

corners where concave non-protruding regions emanate (Fig. 8A). Since large focal adhesions persist for longer period of time than short-lived small adhesions we hypothesize that localization of active ERK to stable focal adhesions provides a platform for ERK signaling that is both sustained and locally restricted.

DISCUSSION

In this study we demonstrate that the scaffold protein RACK1 is an important regulator of symmetry breaking which occurs during spreading of RAT2 fibroblasts. Symmetry breaking of radially protruding cells is initiated by the establishment of a non-protruding cell rear delineated by long thick peripheral actin bundles that terminate in distantly spaced large focal adhesions. In RACK1 deficient cells, the development of the cell rear is compromised and these cells display a round shape, with focal adhesions spaced evenly on the cell periphery and actin fibers that are predominantly circumferential. We have identified specific signaling components that contribute to symmetry breaking in spreading fibroblasts. RACK1 promotes adhesion-mediated activation of ERK that in turn suppresses p190 signaling by reducing p190 localization to cell periphery. The depletion of p190 from cell periphery occurs in a localized manner: at the prospective cell rear it spatially coincides with reduced lamellipodial protrusivity and formation of large focal adhesions and thick peripheral actin bundles.

Cell spreading is a morphogenetic process in which cells break the radial symmetry and acquire the shape typical for motile cells (Mseka et al., 2007;Dubin-Thaler et al., 2008;Prager-Khoutorsky et al., 2011). Adhesion of RAT2 fibroblasts is characterized by initial radial cell spreading accompanied by dynamic actin polymerization at the cell periphery. The establishment of a quiescent non-protruding region is the first occurrence of asymmetry during radial cell spreading. Dynamic protrusions are oriented distally to the non-protruding region suggesting that the formation of the cell rear specifies the position of the cell front and subsequently the direction of migration. Our findings are in agreement with data showing that in fish keratocytes and chick fibroblasts the development of cell rear represents the first obvious sign of symmetry breaking. However, these cells develop cell rear as a consequence of cell edge retraction and inward movement that is driven by actin retrograde flow and actin depolymerization, respectively (Mseka et al., 2007;Yam et al., 2007;Mseka and Cramer, 2011). Although we also observed cell edge retraction and inward movement in spreading RAT2 fibroblasts, most cells define cell rear by establishing a static cell edge that appears as a tail or as an inward curved arc. This mechanism is altered in RACK1 deficient cells as during spreading they protrude continuously along the cell perimeter without specifying the cell rear.

The specification of a non-protruding cell rear correlates with the occurrence of an asymmetrical pattern of focal adhesions. The radially oriented short-lived small adhesions that continuously turnover are typical for early phases of cell spreading and also for the protrusive cell front in polarized cells. At the cell sides and rear, a small fraction of dynamic adhesions is stabilized and matures into large focal adhesions while a majority of adhesions disassemble. Consequently, the cell rear and sides adopts the shape of long inward curved non-adhesive cell edges underlined by thick, long stress fibers anchored in these distant mature adhesions. The assembly of these large actomyosin bundles is important for cell polarization as they determine the rear of the cell (Vicente-Manzanares et al., 2008; Vicente-Manzanares et al., 2011). In addition, non-adhesive cell areas lacking adhesion sites are incapable of stimulating Rac1 activity (Xia et al., 2008) which is consistent with with our observations that non-

adhesive cell edges are incompatible with dynamic actin polymerization and lamellipodia based protrusions. Together with the findings that adhesion geometry governs cell polarization (Thery et al., 2006;Prager-Khoutorsky et al., 2011), these data suggest that the maturation of a subset of focal adhesions at the cell side and rear is fundamental for symmetry breaking of spreading cells as it determines the protruding and non-protruding cellular regions. Symmetrical localization of adhesions in RACK1 deficient cells and omnidirectional protrusivity are consistent with this hypothesis.

Our study suggests p190 as a hub through which adhesion-dependent signaling pathways control the symmetry breaking in spreading cells. This function of p190 is likely dependent on p190 localization. We found that p190 localizes to protruding but not to non-protruding areas, in agreement with previous reports that p190 localizes to the cell periphery upon cellular adhesion to fibronectin (Bradley et al., 2006;Bass et al., 2008;Tomar et al., 2009; Pullikuth and Catling, 2010). In addition, p190 promotes membrane protrusion (Arthur and Burridge, 2001) and p190 persistent activation and peripheral localization promote omnidirectional protrusivity, formation of circumferential actin fibers, symmetrical focal adhesions and overall roundness of the cell (Grande-Garcia et al., 2007;Pullikuth and Catling, 2010), features also typical of the RACK1 knockdown phenotype. The recruitment of p190 to the cell periphery is associated with tyrosine phosphorylation (Nakahara et al., 1998;Arthur et al., 2000;Hernandez et al., 2004;Bradley et al., 2006;Bass et al., 2008;Tomar et al., 2009). On the other hand, p190 peripheral localization and activity is inhibited by ERK mediated phosphorylation of p190 serine/threonine residues close to the C-terminus (Shen et al., 2008;Pullikuth and Catling, 2010). We found that RACK1 knockdown did not affect p190 tyrosine phosphorylation while the suppression of RACK1 or ERK2 increased the p190 localization to the cell periphery. Given that RACK1 promotes ERK activation in response to adhesion (Vomastek et al., 2007) these data suggest that RACK1 and ERK are required for spatio-temporal control of p190 function in spreading cells.

RACK1 has been reported to associate with several focal adhesion proteins, namely integrins, Src and FAK, and to localize to focal adhesions (Liliental and Chang, 1998;Chang et al., 1998;Cox et al., 2003;Kiely et al., 2009;Serrels et al., 2010). RACK1 interaction with FAK recruits several proteins involved in the regulation of cell shape, such as cAMP phosphodiesterase PDE4D5 in squamous cell carcinoma cells (Serrels et al., 2010) and Arf-GTPase AGAP2 in PC12 cells (Dwane et al., 2014) where they regulate cell polarization and neurite outgrowth, respectivelly. We identified RACK1 in the screen for binding partners of MP1, a small ERK/MEK scaffold, and we showed that RACK1 is a scaffold for the ERK pathway as it forms complexes with client proteins Raf, MEK and ERK (Vomastek et al., 2004;Vomastek et al., 2007). Thus, RACK1 has the capacity to pull together diverse signaling elements, including active ERK, in order to coordinate their action and transform stochastic inputs into deterministic, localized outputs.

Intriguingly, the activation of the ERK pathway upon adhesion is FAK dependent (Schlaepfer et al., 1994;Schlaepfer and Hunter, 1996;Slack-Davis et al., 2003) and adhesion-dependent activation of p190 also requires FAK (Arthur and Burridge, 2001;Tomar et al., 2009). Thus, it appears that adhesion and FAK signaling induce both positive and negative

signaling toward p190 (Fig. 8B). This circuit resembles incoherent feed-forward loop where one pathway activates its downstream effector and simultaneously represses it (Alon, 2007; Hart and Alon, 2013). In this circuit RACK1 specifically functions as a co-activator of the ERK signaling axis as we found that RACK1 knockdown impairs ERK activation upon adhesion without affecting the tyrosine phosphorylation of p190 (Fig. 8B).

It is presumed that antagonistic signaling circuits, such as incoherent feed-forward loops, can generate stable and robust signaling patterns if they are composed of a diffusible inhibitor and a localized activator (Meinhardt and Gierer, 2000;Goehring and Grill, 2013;Hart and Alon, 2013). ERK is a candidate for locally enhanced signaling as it is enriched at focal adhesions in both spreading and adherent cells (Fincham et al., 2000;Slack-Davis et al., 2003;Vomastek et al., 2007) and RACK1 ensures active ERK localization to focal adhesions (Vomastek et al., 2007). Important implication of ERK targeting to focal adhesions is that it would allow the formation of a dynamic spatio-temporal pattern of ERK signaling. Focal adhesions are largely stationary structures with their lifetime ranging from ~1 min for small dynamic adhesions, to several minutes for large and stable adhesions (Parsons et al., 2010). Active ERK localizes to both dynamic and stable focal adhesions and, accordingly, ERK signaling from stable adhesions will be sustained while ERK signaling from short-lived dynamic adhesions will be only transient. We hypothesize that localization of active ERK to small adhesions fosters their disassembly resulting in transient ERK signaling that is insufficient to induce depletion of p190 from plasma membrane. On the other hand ERK localization to large adhesion plaques induces sustained ERK signaling and p190 depletion. The depletion of p190 increases RhoA activity which in turn stabilizes remaining adhesions. This model is in good agreement with observations that ERK can induce both disassembly and maturation of focal adhesions (Webb et al., 2004;Doan and Huttenlocher, 2007;Vomastek et al., 2007; Pullikuth and Catling, 2010).

In summary, we hypothesize that symmetry breaking of spreading fibroblasts requires ERK localization to stable but scarce focal adhesions that stochastically develop in later phases of cell spreading. In this way, the pool of active ERK associated with stabilized long-lived focal adhesions provides a platform for spatially restricted and prolonged ERK signaling. Localized and sustained ERK signaling in turn induces permanent depletion of p190 from the cell periphery resulting in the formation of actin bundles, reduced protrusivity and cell rear formation. The finding that symmetry breaking requires the inhibition of p190 present at the cell periphery by locally sustained ERK signaling supports the hypothesis that morphogenetic processes could be regulated by antagonistic signaling circuits composed of a long-range inhibitor and a short-range activator.

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METHODS

Antibodies and materials

Following antibodies were used for immunofluorescence staining: anti-p190A-RhoGAP (mouse, BD Transduction Laboratories), anti-paxillin (clone 5H11, mouse, Upstate Biotechnology), anti-phospho ERK (rabbit, Cell Signaling) and fluorescent secondary goat anti mouse IgG labeled with Alexa Fluor 488 (Invitrogen). Avidin-Biotin blocking kit, biotinylated goat anti rabbit IgG and TexasRed-avidin were from Vector Laboratories. Actin was stained with rhodamine-phalloidin (Invitrogen) and nucleus was visualized by DAPI. Immunoblotting was performed with following antibodies: anti-RACK1 (clone B3, mouse, Santa Cruz), anti-p190A-RhoGAP (mouse, BD Transduction Laboratories), anti-FAK (clone 4.47; mouse, Upstate Biotechnology), anti-FAK (pY397) (rabbit, Invitrogen), anti-phosphotyrosine (clone 4G10, mouse, Millipore), anti-ERK1/2 (clone 3A7, mouse, Cell Signaling), anti-p120RasGAP (mouse, ECM Biosciences). Anti-ERK2 (mouse) and p-ERK (rabbit) antibodies were described elsewhere (Vomastek et al., 2007). Secondary HRP conjugated goat anti-mouse IgG and goat anti-rabbit antibodies were from Sigma-Aldrich. Glass-bottom dishes for life cell imaging were obtained from In Vitro Scientific.

Cell culture, plasmid and siRNA transfection

RAT2 cell lines were maintained in Dulbecco's Modified Eagle Medium (Gibco) supplemented with 10% fetal bovine serum (Gibco).

RAT2 cell line stably expressing mCherry-LifeAct was established by transfection of RAT2 cells with mCherry-LifeAct plasmid (kindly provided by Dr. D. Rosel) and by puromycin selection (3.5 μg/ml). RAT2-mCherry-LifeAct cells were maintained in DMEM with 10% FBS and puromycin (1 μg/ml). pcDNA5-mRFP-Actin (Iwanicki et al., 2008) was transiently transfected using Lipofectamine 2000.

The siRNAs oligonucletides targeting FAK (GCTAGTGACGTATGGATGT), p190A-RhoGAP (GGTGGTGACGATCTGGGCT), RACK1#1 (AAGGTGTGGAATCTGGCTAAC) and RACK1#2 (GCTAAAGACCAACCACATTTT) were described previously (Tilghman et al., 2005;Vomastek et al., 2007;Maninova et al., 2013). Additional siRNAs targeting RACK1 RACK1 #4 (CTGTCCAGGATGAGAGTCA), RACK1#5 (TCTGGCTAACTGCAAGCTA) as well as Non-Specific control (AGGTAGTGTAATCGCCTTG) were used. Where indicated, RACK1 oligonucleotides were used as a pool of RACK1#1, RACK1#2 and RACK1#4 siRNAs. The sequence for the siRNA oligonucleotides targeting ERK1 and ERK2 are as follows: ERK1 #1 (GACCGGATGTTAACCTTTA), ERK1#2 (GAAACTACCTACAGTCTCT), ERK2 #1 (AGTTCGAGTTGCTATCAAG), ERK2#2 (GGTGCCATGGAACAGGTTG). To silence ERK1 and ERK2 expression RAT2 cells were transfected by siRNA pools for ERK1 (ERK1#1and ERK1#2) and ERK2 (ERK2#1 and ERK2#2). All siRNA oligonucleotides were synthesized with 3'TT overhangs by Eurofins MWG Operon. Specific siRNAs were transfected into RAT2 cells using the Calcium phosphate protocol as described previously (Tilghman et al., 2005;Vomastek et al., 2007) and analyzed 48 h post-transfection.

Replating assay

To determine roundness index and cell area, cells were plated 2 days before in DMEM with 10% FBS to reach 60-80% confluency on the day of replating experiment. Cells were detached by trypsin, treated with trypsin inhibitor (1μg/ml; Sigma-Aldrich), washed with serum free DMEM and kept in suspension at 37° C for 60 min. Cells were then plated on fibronectin (10 μg/ml) coated dishes for indicated times. For quantification of cell shape phase contrast images were acquired from cells fixed with 2% paraformaldehyde.

Live cell microscopy and immunocytochemistry

For live cell fluorescence microscopy cells were replated on fibronectin (10 μg/ml) coated glass bottom dishes and cell live microscopy was performed at 37° C using Olympus CellR imaging station (Olympus IX81 inverted microscope, MT20 illumination system and Olympus FV2T CCD camera). Time lapse phase contrast or epifluorescence images were captured with CellR software.

For phase contrast microscopy and immunostaining cells were kept in suspension for 60 min in medium with 2% FBS and replated on glass coverslips coated with fibronectin (10 μg/ml) for 60 min. Cells were fixed with 2% paraformaldehyde in PBS for 25 min and permeabilized with 0.5% Triton X-100 in PBS for 5 min. Coverslips were blocked with 20% normal goat serum in PBS, stained with indicated antibodies and mounted with Vectashield mounting medium containing DAPI (Vector Laboratories). Active ERK was visualized following modified Avidin-Biotin amplification protocol (Fincham et al., 2000;Vomastek et al., 2007). Briefly, cells were fixed in 1% paraformaldehyde in PHEM buffer (60 mM Pipes, 25 mM Hepes, 10 mM EGTA, 4 mM $MgSO_4$, pH6.9) supplemented with 0.2 mM vanadate and 50 mM β-glycerophosphate for 20 min and extracted with 1% CHAPS in PHEM buffer for 5 min. Cells were blocked in 20% normal goat serum in MBST (50 mM MOPS, 150 mM NaCl, 0.05% Tween 20, pH7.4) for 1 h and for additional 30 min with 20% normal goat serum supplemented with Avidin. Cells were then incubated for 1 h with rabbit polyclonal anti-pERK antibody in 5% normal goat serum supplemented with Biotin followed by the incubation with biotinylated secondary goat anti-rabbit antibody conjugated to biotin (Vector laboratories) for 1 h, and by incubation with Texas Red-avidin for 30 min. Paxillin was detected with mouse monoclonal anti-Paxillin antibody and visualized with goat anti mouse IgG antibody labeled with Alexa Fluor 488. Fluorescent images were acquired by epifluorescence microscope Olympus IX81, MT20 illumination system and Olympus FV2T CCD camera.

Western blotting, immunodetection and immunoprecipitation

Cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 1 mM EDTA pH 8.0) and clarified by centrifugation at 14 000 rpm for 20 min. Lysates were boiled in 1x sample buffer for 5 min, resolved by SDS-PAGE and transferred to Optitran nitrocellulose membrane (Whatman). Membranes were blocked with 5% BSA in PBS-0.5% Tween 20 for 1 hour at room temperature and incubated overnight at 4 °C with primary antibodies. Membranes were subsequently probed with HRP-

conjugated secondary antibodies and developed using SuperSignal WestPico enhanced chemiluminescent substrate (Pierce).
For immunoprecipitation cells were replated on fibronectin (10μg/ml) coated dishes for 20 min, lysed in RIPA buffer supplemented with phosphatase (Serva) and protease (Sigma) inhibitor cocktails and clarified by centrifugation at 14 000 rpm for 15 min. Cell lysates (500-1 000 μg) were incubated with 1μg of anti-p190A-RhoGAP antibody (mouse, BD Transduction Laboratories) overnight at 4 °C. Antibody-lysate mixture was then incubated with protein A/G ultralink Resin (Thermoscientific) for 2 h at 4 °C. Immune complexes were twice washed with immunoprecipitation buffer (25mM Tris, 150mM NaCl, pH 7.2), boiled in 1x sample buffer for 10 min, resolved by SDS-PAGE and transferred to Optitran nitrocellulose membrane (Santa-Cruz). Immunodetection was performed as described above.

Quantification of cell shape

All microscopic images were analyzed and quantified using ImageJ software. Roundness index (RI) was calculated according to the formula: $RI = cell\ area\ /\ \Pi * (feret's\ diameter/2)^2$ where feret's diameter is the longest straight distance between two points on cell perimeter. Cell area as well as feret´s diameter were obtained from phase contrast images of fixed cells in ImageJ by manual marking of the cell periphery. Data are presented as a mean ± the standard error of the mean and statistical analyses (Mann-Whitney non paired t-test) were done by using Prism software (GraphPad Software, Inc).

Cell edge velocity and curvature maps

For the tracking of cell edge velocity and cell edge curvature, RAT2 cells stably expressing LifeAct-mCherry were plated on fibronectin coated glass-bottom dishes in the presence of 10% FBS and time-lapse fluorescence images were acquired every 10 s using CellR imaging station with a 60x NA 1.25 Apo and 100x NA 1.30 objectives, respectively. Fluorescence images were pre-processed in ImageJ software and analysed using QuimP11 toolbox for ImageJ available from: http://go.warwick.ac.uk/quimp (Dormann et al., 2002;Bosgraaf et al., 2009;Tyson et al., 2010). QuimP11 software was used as follows: cell outlines were extracted from each frame of image sequence using BOA plugin, then movement of outlines between individual frames were mapped using ECMM Mapping plugin and data analysis was performed by the Q Analysis plugin. Data were visualized in the form of three spatiotemporal maps. Cell Track Map shows time sequence of the cell outlines (with frame increment 2) and it was pseudocolored in Adobe Illustrator. Motility Map represents the movement of each node on the cell outline in time and Convexity Map (with parameters of sum over 2 μm and average over 6 μm) shows the changes in the curvature of cell edge. To reduce the pixelation of the cell edge velocity and curvature maps, the maps were further processed in Adobe Photoshop using Gaussian blur filter (diameter 3.0 pixels).

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FIGURES

Figure 1.

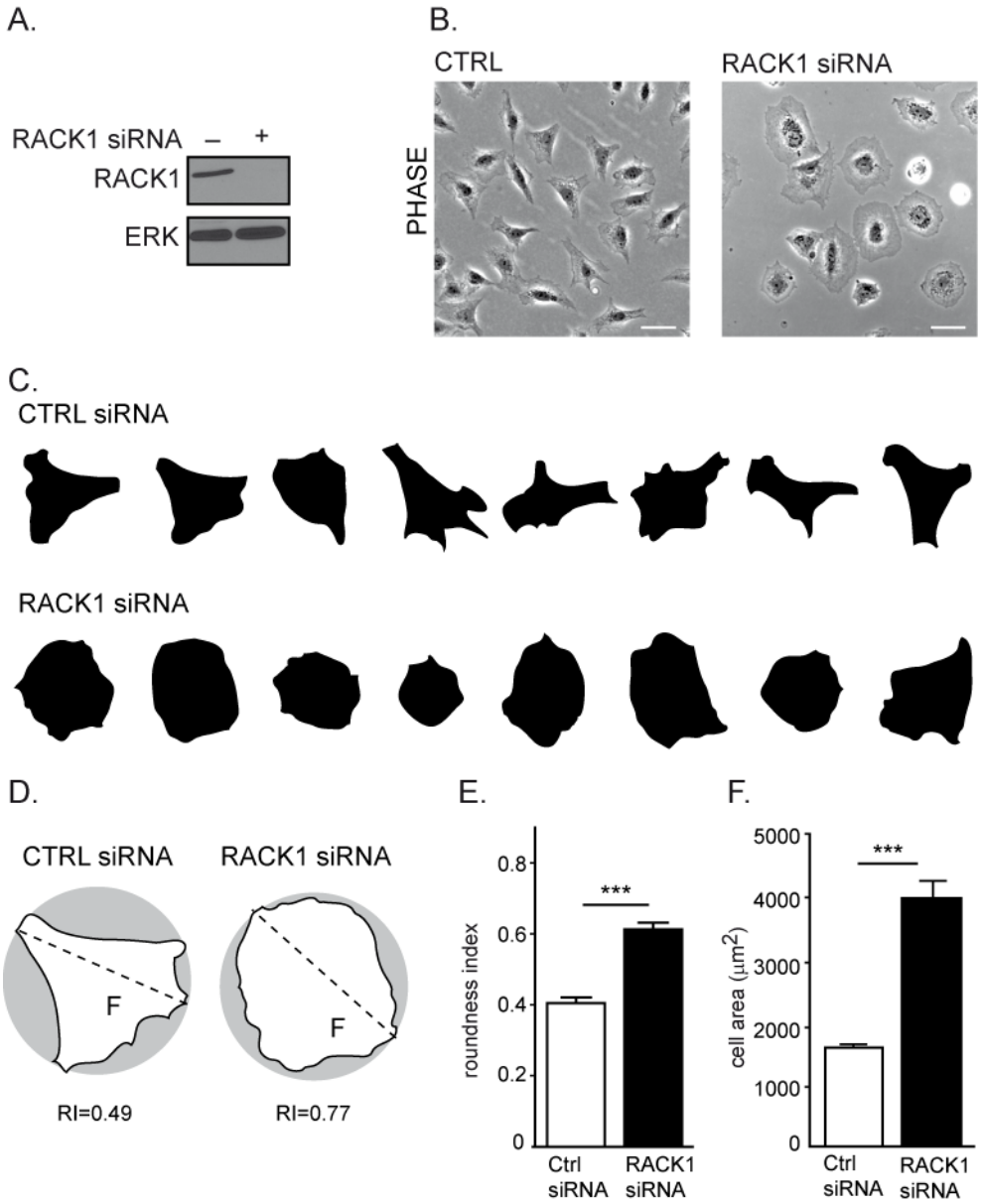


Figure 1. RACK1 regulates cell shape in adherent RAT2 fibroblasts. Cells were transfected with control or RACK1 siRNA pool and analyzed 48 h after transfection. (A) RACK1 silencing in RAT2 cells. Cell lysates were probed with RACK1 antibody to determine knockdown efficiency and with ERK2 antibody to confirm equal protein loading. (B) Representative phase contrast images of control and RACK1 deficient RAT2 cells. Panel (C) shows black-and-white representation of phase contrast images to accentuate the shape of individual RAT2 cells. (D) Determination of roundness index and cell area. Schematic representation of cells with indicated feret's diameter (F, dashed line), area of circle (gray) calculated based on feret's diameter superimposed on the cell area (white). These parameters were used for determination of roundness index (RI) as described in the material and methods; examples of roundness index are indicated. (E-F) Quantification of roundness index (E) and cell area (F) in control and RACK1 transfected cells. At least100 cells were analyzed for each condition. Data are presented as a mean ± SEM, ***p< 0.001.

Figure 2.

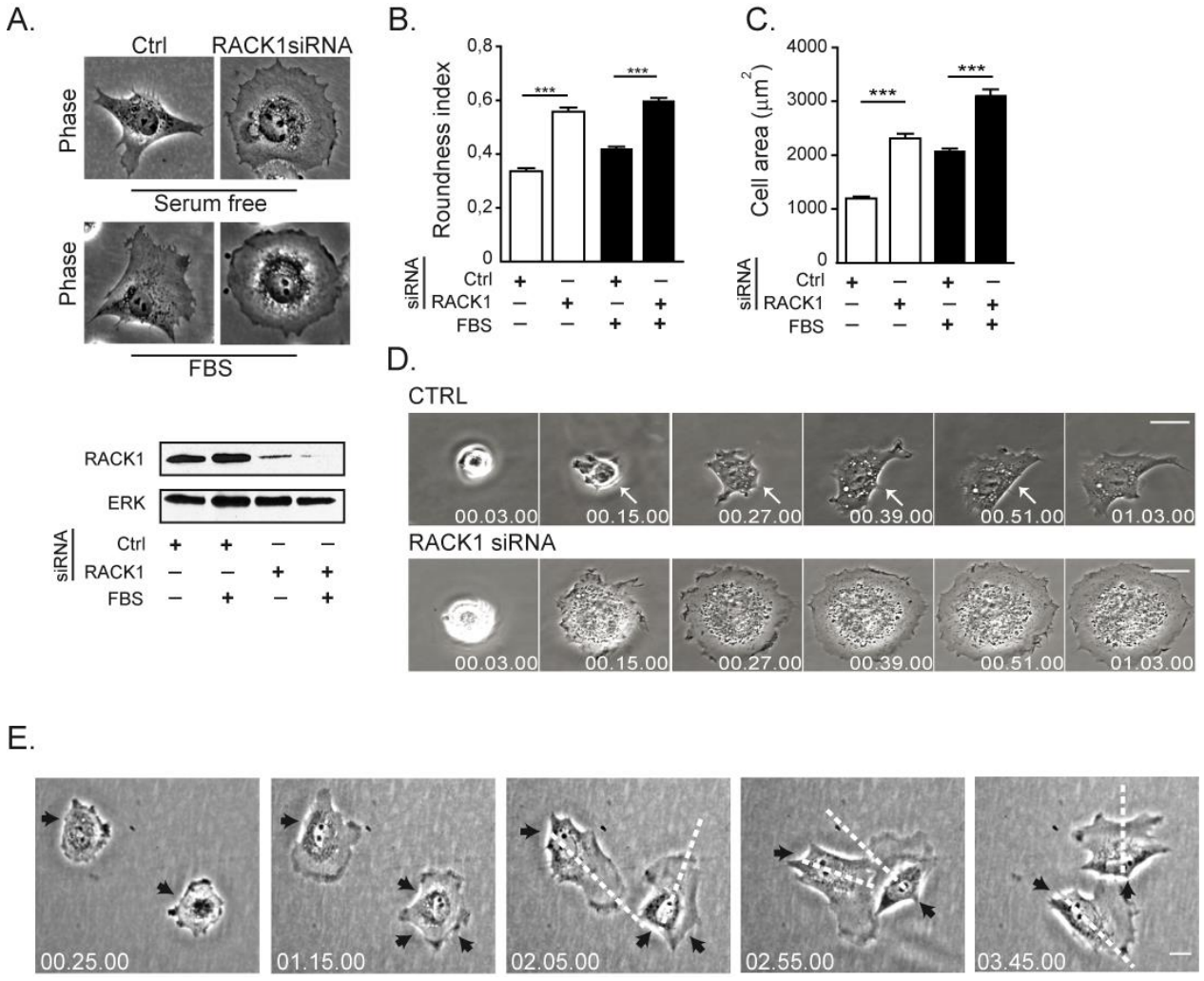


Figure 2. RACK1 regulates symmetry breaking in spreading cells. (A) Representative phase contrast images of control and RACK1 deficient cells replated in the presence or absence of 10% FBS. Cells were transfected with control or RACK1 siRNA pool for 48 h, trypsinized and kept in suspension for 60 min with or without FBS. Cells were then replated on fibronectin coated dishes (10 μg/ml) for 90 min and fixed. The efficiency of RACK1 knockdown (lower panel) was determined as in Fig. 1A. (B-C) Quantification of roundness index (B) and cell area (C) in control and RACK1 transfected cells spreading in the presence or absence of FBS. Data are presented as a mean ± SEM, *** $p < 0.001$ (n = 100 cells). (D) Series of phase contrast images of control (upper panel) and RACK1 deficient (lower panel) cells spreading in serum free media on fibronectin, time is indicated in h.min.s. White arrows in control cell indicate region with decreased protrusivity and the formation of prospective cell rear. (E) Series of phase contrast images of RAT2 cells spreading on fibronectin. Cells were plated for 48 h, trypsinized and kept in suspension for 60 min with 10% FBS. Cells were then replated on fibronectin coated dishes and recorded for 4 h with images acquired every 5 min. During spreading cells form static stable regions (indicated by black arrows). Cell on the left develops one dominant concave region that is later converted into elongated tail. Cell on the right forms multiple concave non-protruding regions, two of them form elongated cell tail. The establishment of cell rear specifies distally located cell front and direction of migration (dashed white lane).

Figure 3.

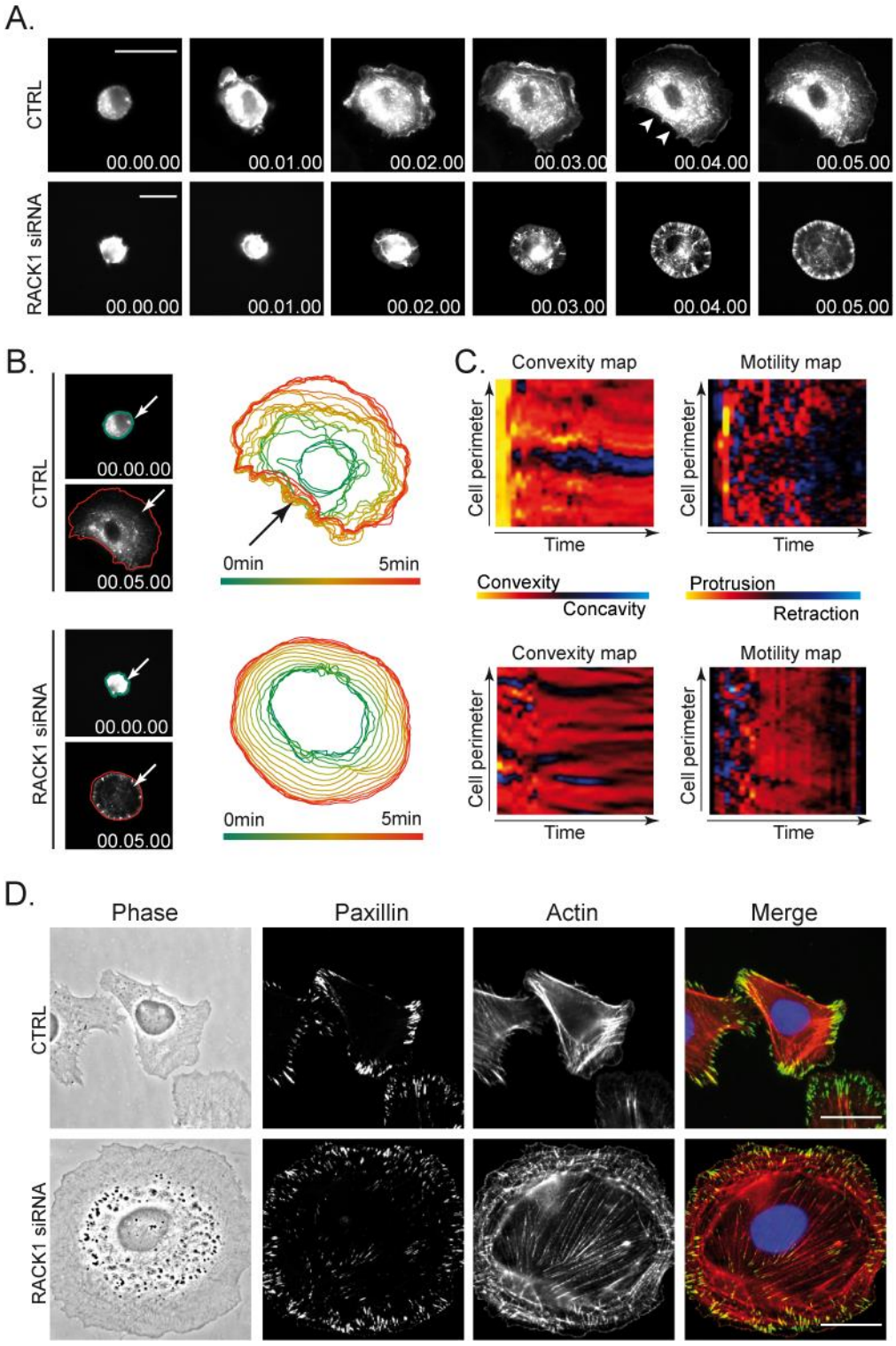


Figure 3. RACK1 regulates actin remodeling, focal adhesions formation and cell rear development in spreading cells. (A) Series of images from time-lapse fluorescence microscopy

showing the actin dynamics in control (upper panel) and RACK1 knockdown (lower panel) cells during spreading. RAT2 cells expressing mRFP-Actin or mCherry-LifeAct were transfected with control or RACK1 siRNAs pool, respectively, and replated on fibronectin (10 μg/ml) in 10% FBS. Time is indicated in (h.min.s). White arrowheads in control cells (upper panel) indicate establishment of long peripheral concave actin bundle. Scale bars = 10 μm. (B) Development of stable cell rear is compromised in RACK1 deficient cells. Left panel shows the first and last images of spreading cells with outlined cell edges, white arrows indicate the start point of the cell outline. Right panel shows the time sequence of the cell outline during spreading of control and RACK1 deficient cells superimposed from first image to last image. Black arrow in the superimposed cell boundaries panel indicates the establishment of stable non-protrusive concave region. (C) Convexity (left) and motility (right) maps of the cell edges shown in Fig. 3A. Convexity map represents the changes in the curvature of cell edge with negative, concave regions shown in blue and positive, convex regions shown in red/yellow. Motility map represents the movement of the cell outline in time. Red and yellow colors indicate expanding regions, blue colors contracting regions. (D) Representative phase contrast and fluorescence images of control and RACK1 deficient cells. RAT2 cells were transfected as in Fig. 3A and replated on fibronectin coated coverslips for 60 min, fixed and stained for paxillin (green) and actin (red) to visualize changes in the architecture of actin bundles and focal adhesions.

Figure 4.

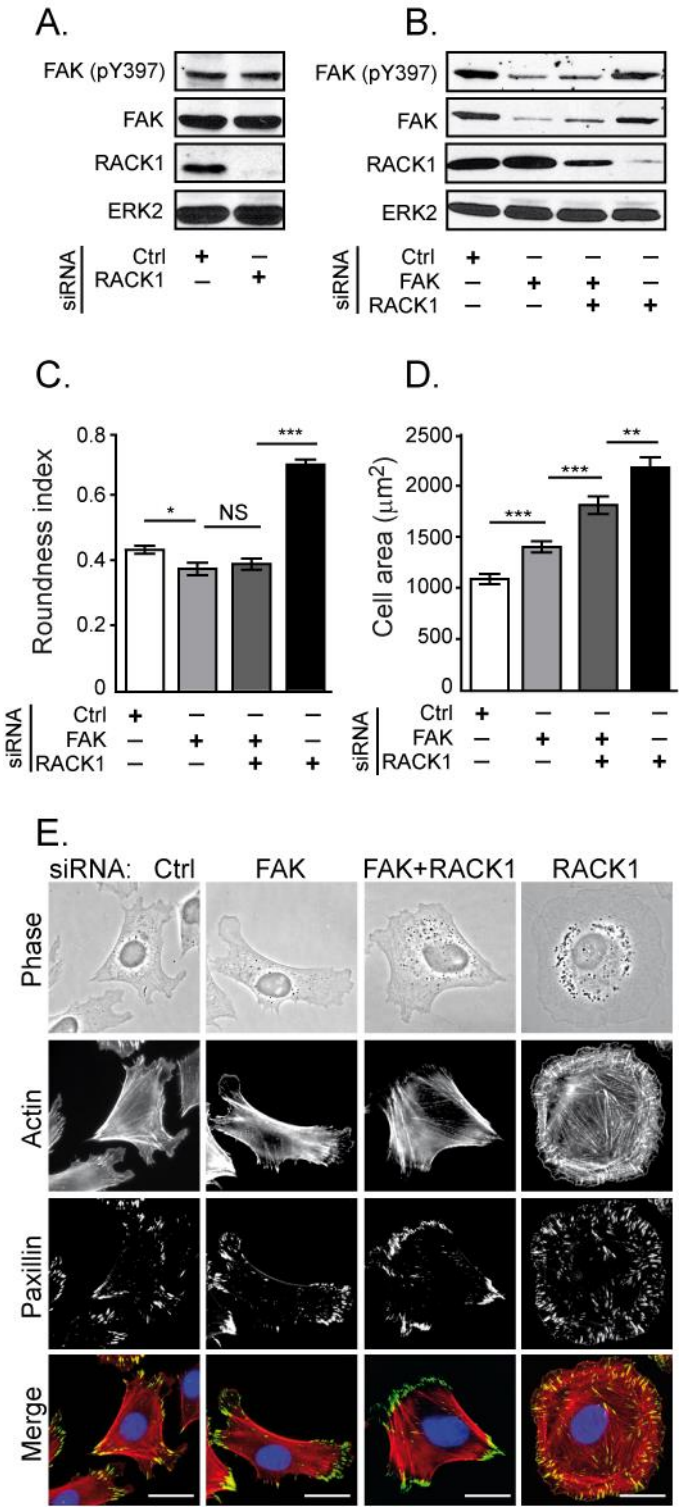


Figure 4. **FAK knockdown suppresses defect in symmetry breaking in RACK1 deficient cells.** (A) RACK1 knockdown does not affect the FAK activity in cells adhering to fibronectin. Cells were

transfected with control or RACK1 siRNA#1, cultivated for 48 h and replated on fibronectin coated dishes for 90 min. Cell lysates were probed with pFAK (Y397) antibody, RACK1 antibody was used to confirm knockdown efficiency, FAK and ERK2 antibodies were used to confirm equal loading of proteins. (B) RACK1 and FAK expression in cells transfected with control, FAK and RACK1#1 siRNAs. Cells were transfected for 48 h as indicated and replated on fibronectin coated dishes for 90 min. Cell lysates were probed with RACK1 and FAK antibodies to confirm knockdown efficiency, pFAK (Y397) antibody was used to determine the phosphorylation level of FAK, and ERK2 antibody was used to confirm equal loading of proteins. (C-D) Quantification of roundness index (C) and cell area (D) of control, RACK1, FAK and FAK-RACK1 deficient cells. Data are presented as a mean ± SEM, * $p < 0.05$, *** $p < 0.001$, NS – not significant ($n > 50$ cells). (E) FAK knockdown suppresses changes in actin cytoskeleton and focal adhesions. Representative phase contrast and fluorescence images of control, FAK, FAK-RACK1 and RACK1 deficient cells 60 min after replating on fibronectin. Cells were transfected as in Fig. 4B, fixed and stained for paxillin (green) and actin (red) to visualize the focal adhesions and actin cytoskeleton. Scale bars = 20 μm.

Figure 5.

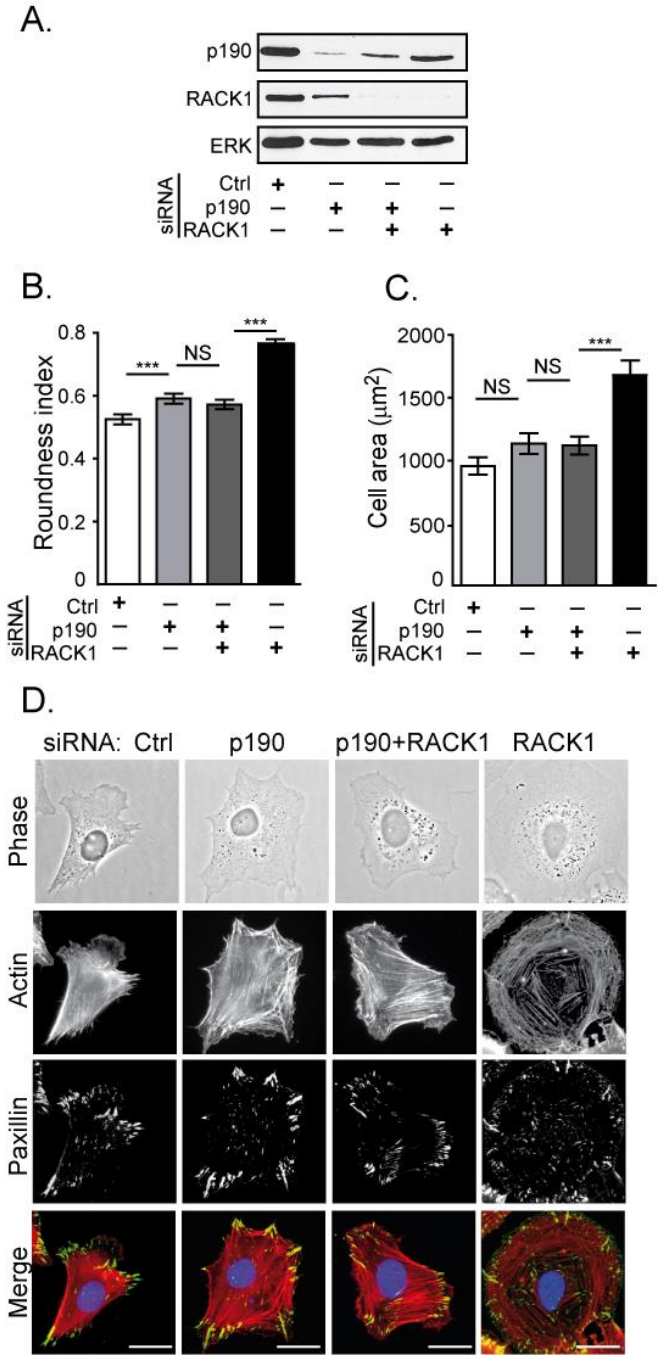


Figure 5. **p190 knockdown suppresses defect in symmetry breaking in RACK1 deficient cells.** (A) RACK1 and p190 expression in cells transfected with control, p190 and RACK1#1 siRNAs. Cell lysates were probed with RACK1 and p190 antibodies to confirm knockdown efficiency and with ERK2 antibody to confirm equal protein loading. (B-C) Quantification of roundness index (B) and cell area (C) of control, RACK1, p190 and p190/RACK1 deficient cells. Cells were transfected for 48 h as indicated and replated on fibronectin coated dishes for 90 min. Data are presented as a mean ± SEM, *** $p<0.001$, NS – not significant ($n > 50$ cells). (D) p190 knockdown suppresses changes in actin cytoskeleton and focal adhesions. Representative phase contrast and fluorescence images of control, p190, p190/RACK1 and RACK1 deficient cells fixed 60 min after plating. Cells were transfected as in Fig. 5B, replated on fibronectin coated coverslips for 60 min and stained for paxillin (green) and actin (red) to visualize the focal adhesions and actin. Scale bars = 20 μm.

Figure 6.

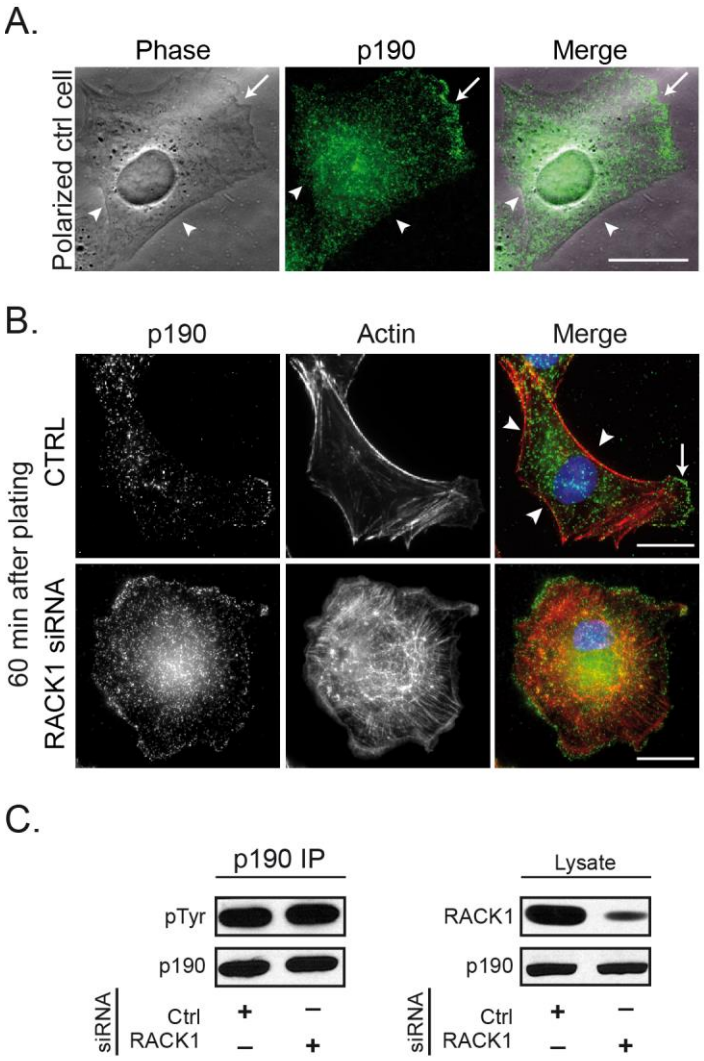


Figure 6. RACK1 regulates p190 localization during cell spreading. (A) p190 localizes to protruding areas in migrating RAT2 fibroblasts. Representative fluorescence and phase contrast image of migrating cell stained with antibody recognizing p190 (green). Arrow points to leading, protrusive region of cell enriched with p190, arrowheads indicate non-protrusive concave regions. (B) Localization of p190 in control and RACK1 deficient cells spreading on fibronectin. Cells were transfected with control and RACK1#1 siRNAs for 48 h and replated on fibronectin for 60 min. Cells were stained with antibody recognizing p190 (green) and rhodamine - phalloidin (red). White arrow indicates localization of p190 in protruding areas of control cells, arrowheads indicate non-protrusive concave regions that developed during cell spreading. (C) Phosphorylation level of p190 in spreading control and RACK1 deficient cells. Cells were transfected with control and RACK1 pool siRNAs for 48 h and replated on fibronectin for 20 min and p190 was immunoprecipitated as desribed in Materials and Methods. Immunoprecipitates were probed with anti-phosphotyrosine antibody and reprobed with p190 antibody. Cell lysates were probed with RACK1 and p190 antibodies to confirm knockdown efficiency and equal protein loading, respectively. Scale bars = 20 μm.

Figure 7.

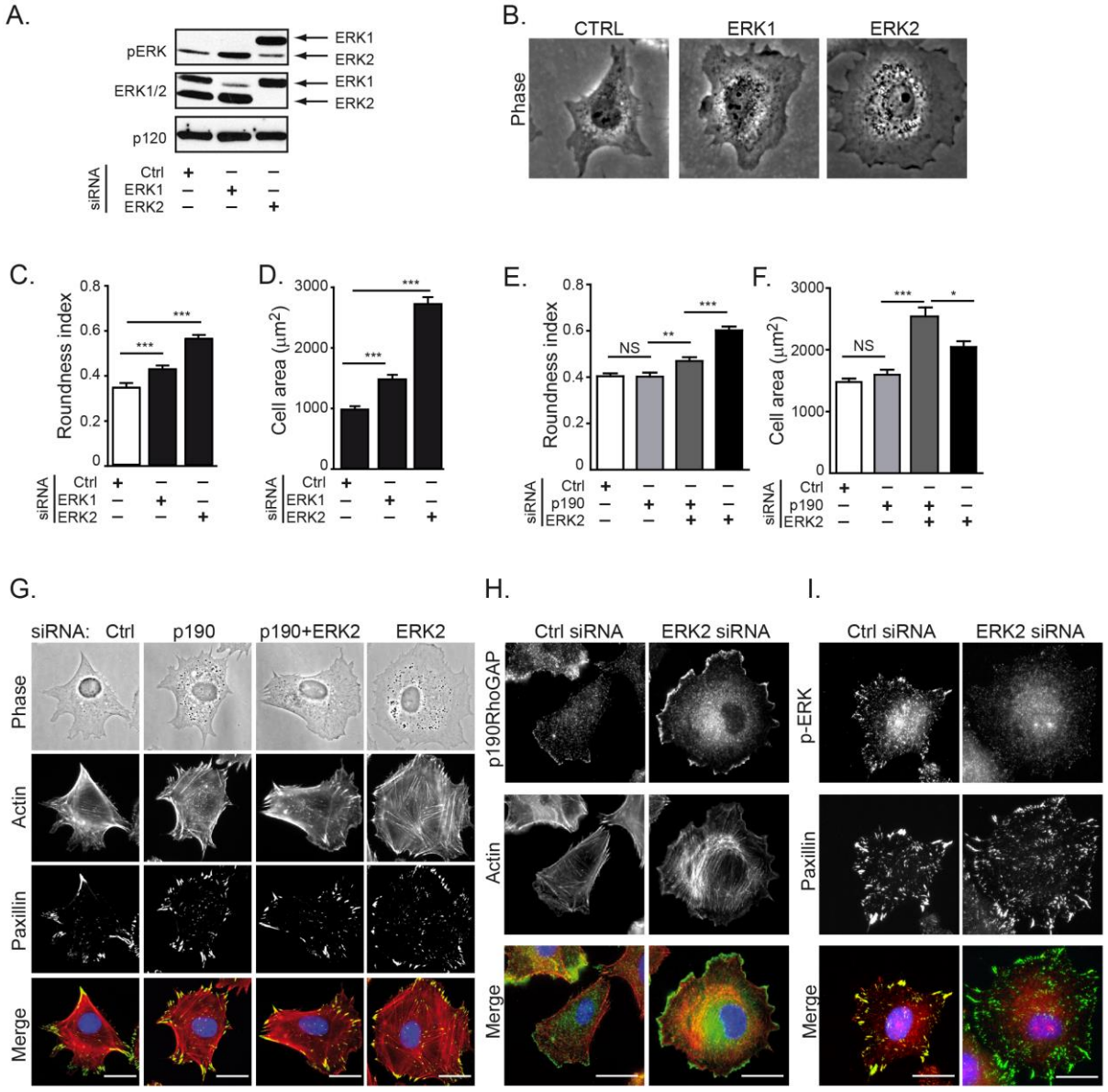


Figure 7. ERK2 knockdown mimics RACK1 knockdown phenotype. (A) Knockdown of ERK1 and ERK2 in cells adhering to fibronectin. Cells were transfected with control, ERK1 and ERK2 siRNAs for 48 h and replated on fibronectin coated dishes for 90 min. Cell lysates were probed with antibodies recognizing active, doubly phosphorylated ERK (pERK), ERK1 and ERK2 (ERK1/2) to determine activation level and knockdown efficiency of individual ERK isoforms. p120RasGAP (p120) antibody was used as a loading control. Noteworthy, knockdown of ERK1 induced the hyperactivation of remaining isoform ERK2 and *vice versa.* (B) Representative phase contrast images of control, ERK1 and ERK2 transfected cells 60 min after plating on fibronectin coated coverslips. (C-D) Quantification of roundness index (C) and cell area (D) of control, ERK1 and ERK2 deficient cells fixed 60 min after plating on fibronectin. Data are presented as a mean ± SEM (n > 100 cells), *** $p < 0.001$. (E-F) p190 knockdown suppresses defect in symmetry breaking in ERK2 deficient cells. Quantification of roundness index (E) and cell area (F) in control, p190, ERK2 and p190-ERK2 deficient cells. Data are presented as a mean ± SEM (n = 100 cells), * $p < 0.05$, ** $p < 0.01$, *** $p <$

0.001, NS – not significant. (G) p190 knockdown suppresses changes in actin cytoskeleton and focal adhesions in ERK2 deficient cells. Representative phase contrast and fluorescence images of control, p190, ERK2 and p190-ERK2 deficient cells fixed 60 min after plating on fibronectin coated coverslips. Cells were stained with antibody recognizing paxillin (green) and rhodamine - phalloidin (red) to visualize the reversion of cytoskeletal/adhesions architecture in p190-ERK2 deficient cells. (H) Localization of p190 in control and ERK2 deficient cells spreading on fibronectin. Cells were transfected with control and ERK2 siRNAs for 48 h and replated on fibronectin for 60 min. Cells were stained with antibody recognizing p190 (green) and rhodamine - phalloidin (red). (I) Depletion of ERK2 inhibits active ERK localization to focal adhesions in response to adhesion. RAT2 cells were transfected with siRNA for 48 hours, suspended and replated on fibronectin for 60 minutes. Cells were stained for active ERK (red in merged images) and paxillin (green). Scale bars = 20 μm.

Figure 8.

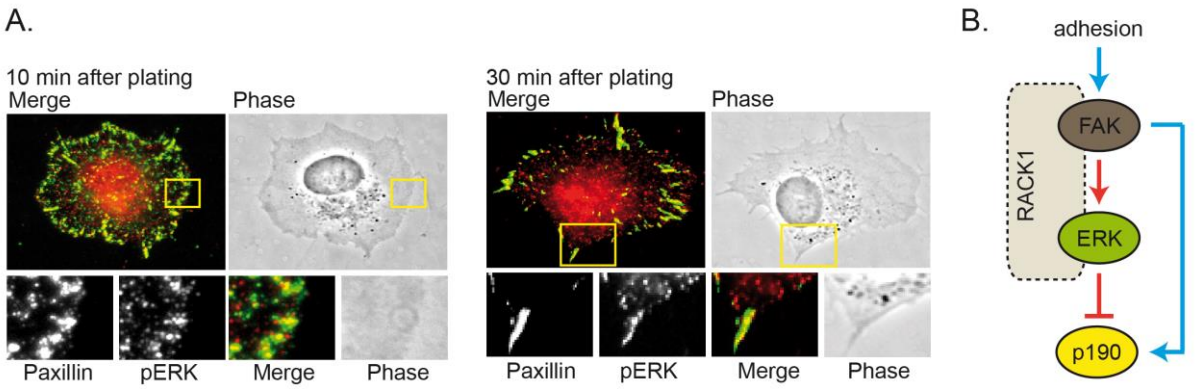
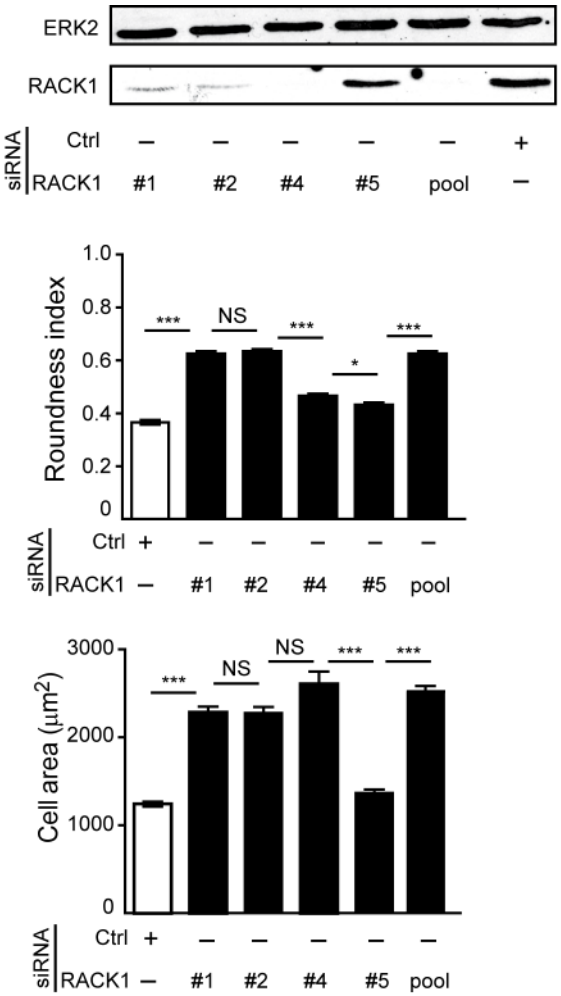


Figure 8. Hypothetical model of symmetry breaking of spreading RAT2 cells that involves ERK localization to stable adhesions. (A) Active ERK localizes to both small and large focal adhesions in spreading cells. Cells adhering on fibronectin for 10 or 30 min were stained for active ERK (red) and paxillin (green). In early phase of cell spreading, small, round and symmetrically arranged focal adhesions predominate (left panel). In later time points large focal adhesions appear at cell rear and sides along with concave non-adhesive cell edges (right panel). The inset shows a higher magnification of boxed areas. **(B)** Hypothetical model of incoherent feed forward loop that regulates symmetry breaking of spreading RAT2 fibroblasts. In this system adhesion promotes the p190 activation and recruitment to cell periphery via FAK dependent mechanism (blue line). Simultaneously, adhesion and FAK activates the ERK pathway that imposes inhibitory signaling on p190 (red lines) and causes p190 depletion from cell periphery. Scaffold protein RACK1 (indicated by grey rectangle) associates with integrins, FAK and the ERK pathway components, and promotes adhesion dependent ERK activation. In the absence of RACK1 or ERK, the inhibitory signaling is diminished resulting in sustained p190 localization to cell periphery.

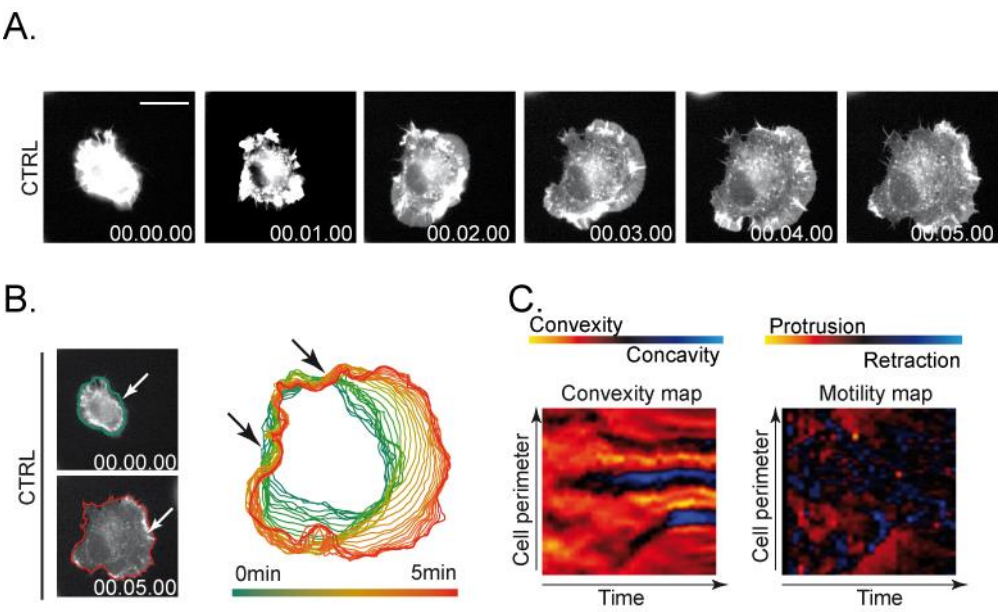
SUPPLEMENTARY FIGURES

Figure S1.



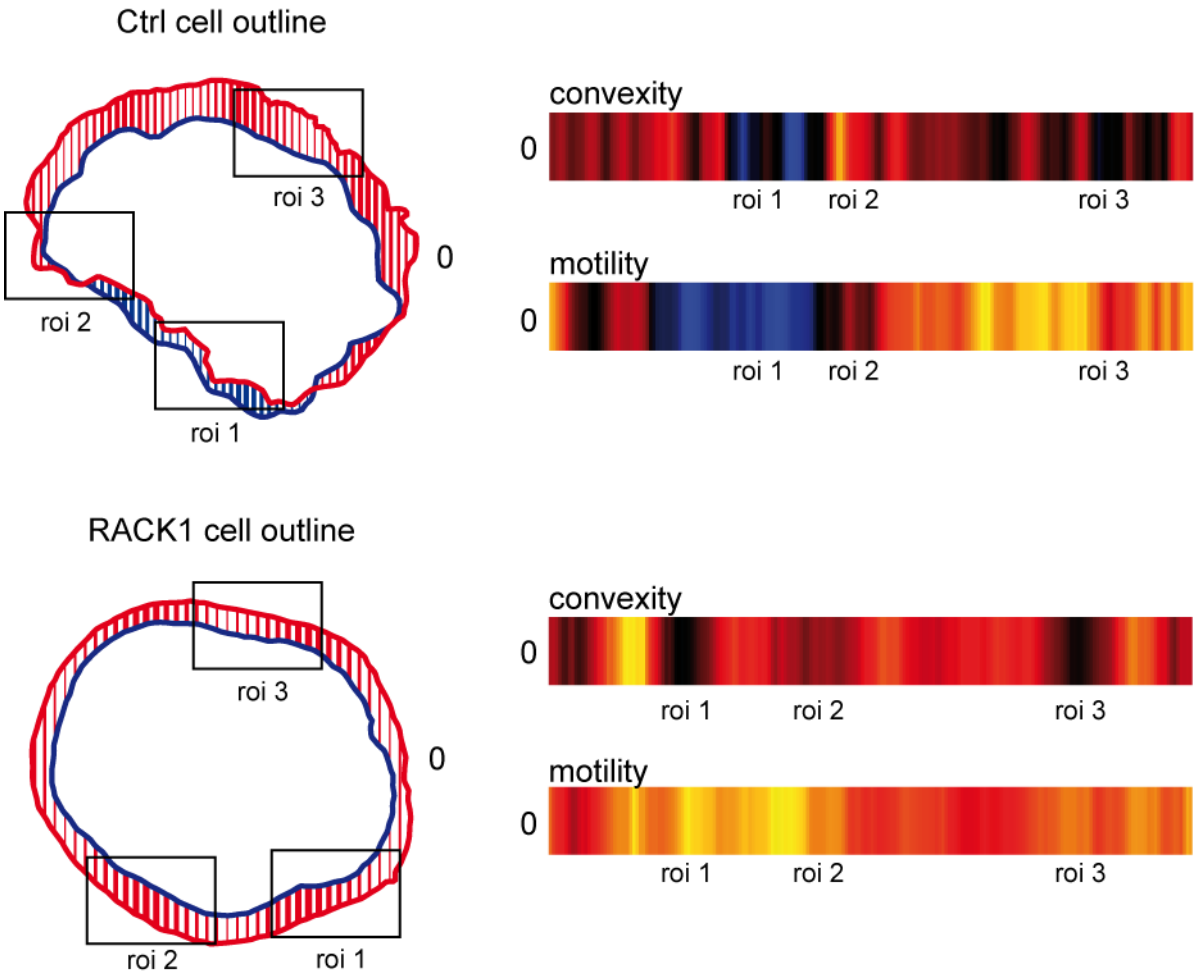
Supplementary Figure S1. RACK1 siRNAs targeting different sequences of RACK1 gene regulate symmetry breaking in adhering cells. Cells were transfected with control or individual RACK1 siRNAs (#1,#2,#4,#5) and analyzed for silencing efficiency 48 h after transfection. (A) The efficiency of RACK1 silencing in RAT2 cells. Cell lysates were probed with RACK1 antibody to determine knockdown efficiency and with ERK2 antibody to confirm equal protein loading. As RACK1 siRNA # 5 did not significantly change the expression level of RACK1 protein we used pool of siRNAs #1, 2 and 4 in majority of experiments. (B) Quantifications of roundness index and cell area in control and RACK1 transfected cells. Data are presented as a mean ± SEM, ***p< 0.001, * p < 0.05, NS – not significant (n = 100 cells).

Figure S2.



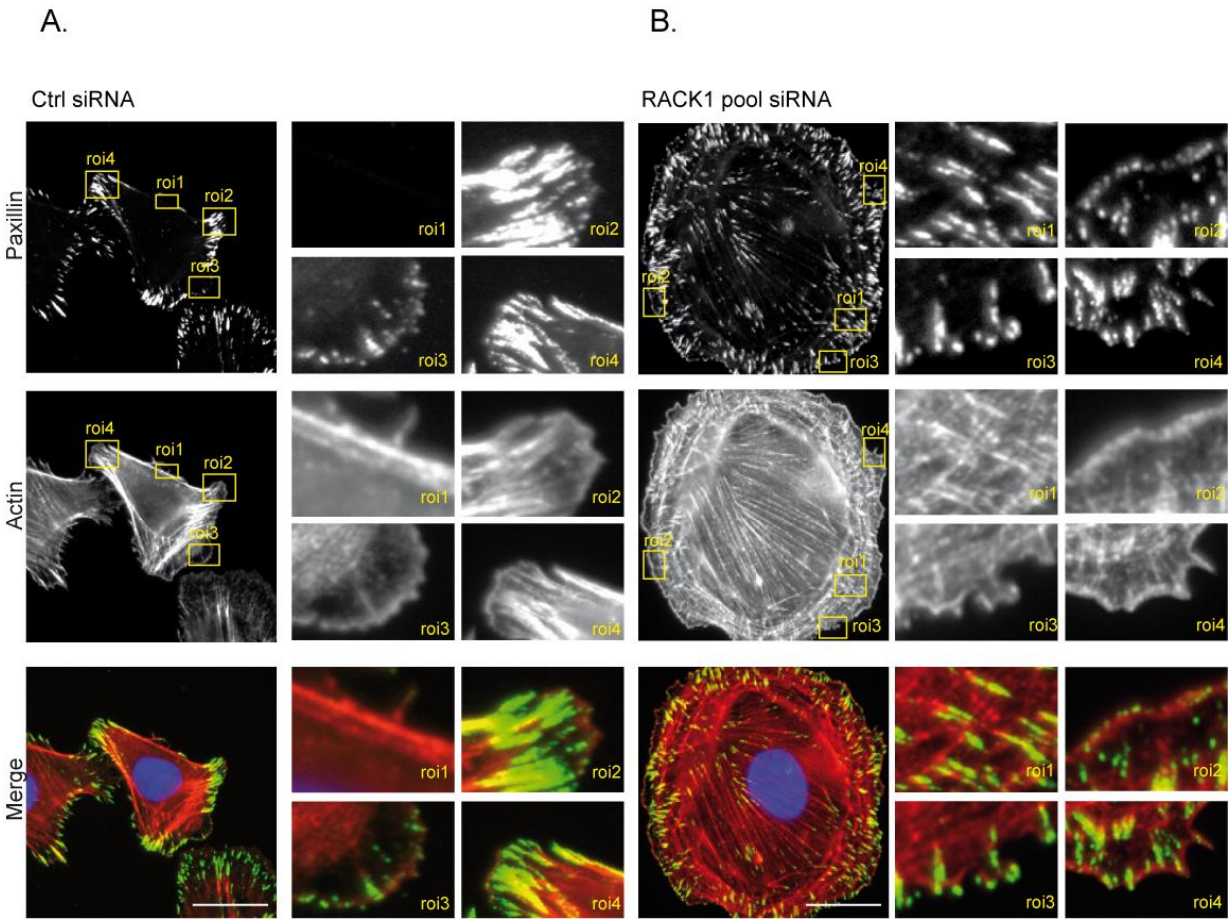
Supplementary Figure S2. Actin and cell edge dynamics in spreading cell developing extended cell rear. (A) Series of images from time-lapse fluorescence microscopy showing the actin dynamics in control cells during spreading. RAT2 cells stably expressing LifeAct-RFP were replated on fibronectin (10 μg/ml) in 10% FBS. Time is indicated in h.min.s, scale bar = 20 μm. (B) Cell edges outlined in the first and last image of spreading cell shown in (A) (left panel), white arrows in the left panels indicate the start point of the cell outline. Right panel shows the time sequence of the cell outline during spreading of control cell superimposed from the first image to the last image. Black arrows in the superimposed cell boundaries panel indicate the establishment of stable non-protrusive concave regions. (C) Convexity (left) and motility (right) maps of the cell edges shown in (A). Convexity map represents the changes in the curvature of cell edge with negative, concave regions shown in blue and positive, convex regions shown in red/yellow. Motility map represents the movement of each node on the cell outline in time. Red and yellow colors indicate expanding regions, blue colors contracting regions. Start point of the cell outline is indicated by white arrows in the left panels of (B).

Figure S3.



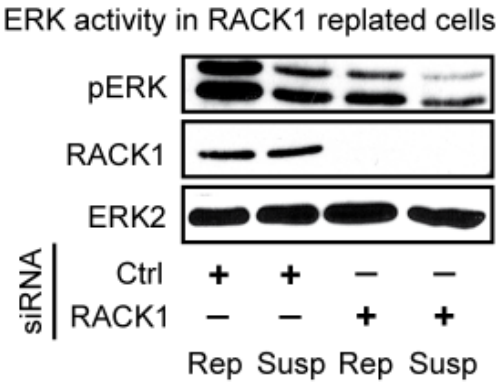
Supplementary Figure S3. Short concave regions in RACK1 deficient cells are part of dynamically protruding areas. The movement of the cell periphery between two time frames (time gap 60 seconds) of cell outlines extracted from Fig. 3. Upper panel shows the movement of the cell periphery of control cell. Cell outlines were obtained from cells spreading for 150 seconds (blue outline) and 210 seconds (red outline), zero indicates the origin of cell outline. Three regions are indicated: ROI1 is a concave region (marked by blue color in convexity map), ROI2 is a convex region (marked by yellow color in convexity map), and ROI3 displays both convex and straight shape region. Cell edge motility analysis depicted regions shows that convex region ROI2 displays dynamic protrusion while mixed region displays retraction (red and blue colors, respectively), and that long concave region ROI1 remains static or slightly retracting (black and blue colors). Lower panel shows the movement of cell periphery of RACK1 deficient cell, 150 seconds (blue outline) and 210 seconds (red outline) after replating; zero indicates the origin of cell outline. Three regions are indicated: ROI1 is a short concave region (marked by dark color in convexity map), ROI2 is a convex region (marked by red color in convexity map) and ROI3 is a straight region (marked by black color in convexity map). The same regions are indicated in motility map and they display continuous protrusion irrespectively of cell edge curvature (marked by red and yellow colors).

Figure S4.



Supplementary Figure S4. Different structure and distribution of focal adhesions in control and RACK1 deficient cells. (A) Images of control cell from Fig. 3D stained for focal adhesion marker paxillin and actin (in merged image colored green and red, respectively). ROI1 indicates region of the cell which is absent from focal adhesions, ROI2 shows focal adhesions at the cell edge corner, ROI3 shows short newly formed focal adhesions underlying the protrusive area of the cell edge and ROI4 shows focal adhesions on the cell tail. Focal adhesions at cell corner and cell tail are elongated, often of triangular shape, as they anchor long concave actin bundles and they are under actomyosin tension. (B) Images from RACK1 deficient cell stained as in panel (A). ROI1 shows focal adhesions which are randomly distributed in inner region of the cell, ROI2-4 show uniformly distributed focal adhesions which are on the edge of the cell periphery. These focal adhesions could be elongated but short and centripetally oriented (ROI4), or dot shaped (ROI2). These two types of focal adhesions could be also intermixed (ROI3).

Figure S5.



Supplementary Figure S5. RACK1 is required for efficient adhesion-induced ERK activation in RAT2 cells. Cells were transfected with control or RACK1 siRNA for 48 h, suspended and replated for 30 min. Equal amounts of proteins from cells in suspension (Susp) and from replated cells (Rep) were probed with antibodies recognizing active, doubly phosphorylated ERK (pERK) and RACK1. ERK2 level was used as a loading control (ERK2 protein level was determined from gel run in parallel).

Ultrastructural localization of actin and actin-binding proteins in the nucleus.

(Dingová et al., 2009)

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SHORT COMMUNICATION

Ultrastructural localization of actin and actin-binding proteins in the nucleus

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Abstract Nuclear actin plays an important role in such processes as chromatin remodeling, transcriptional regulation, RNA processing, and nuclear export. Recent research has demonstrated that actin in the nucleus probably exists in dynamic equilibrium between monomeric and polymeric forms, and some of the actin-binding proteins, known to regulate actin dynamics in cytoplasm, have been also shown to be present in the nucleus. In this paper, we present ultrastructural data on distribution of actin and various actin-binding proteins (α-actinin, filamin, p190RhoGAP, paxillin, spectrin, and tropomyosin) in nuclei of HeLa cells and resting human lymphocytes. Probing extracts of HeLa cells for the presence of actin-binding proteins also confirmed their presence in nuclei. We report for the first time the presence of tropomyosin and p190RhoGAP in the cell nucleus, and the spatial colocalization of actin with spectrin, paxillin, and α-actinin in the nucleolus.

Keywords Nuclear actin · Ultrastructure · Actin-binding proteins

Introduction

Actin, which is best known as a cytoskeletal component, has important functions also in the cell nucleus. Nuclear actin has been implicated in diverse nuclear processes such as chromatin remodeling, transcriptional regulation, RNA processing, and nuclear export (Vartiainen et al. 2007; Percipalle et al. 2006; for reviews, see de Lanerolle et al. 2005; Grummt 2006; Percipalle and Visa 2006). Actin is also required for transcription of all three classes of eukaryotic RNA polymerases (Smith et al. 1979; Egly et al. 1984; Scheer et al. 1984; Percipalle et al. 2003; Fomproix and Percipalle 2004; Hu et al. 2004; Philimonenko et al. 2004; Kukalev et al. 2005; Ye et al. 2008).

Actin in cytoplasm exists in equilibrium between monomers (globular- or G-actin) and polymers (filamentous- or F-actin). The variety of cytoplasmic functions of actin cytoskeleton is based on its ability to adapt to diverse structures depending on the ion conditions and interaction with specific proteins. Actin accessory proteins include monomer-binding, capping, severing, nucleating actin filament polymerization and actin filament side-binding and stabilizing proteins (for reviews, see Higaki et al. 2007; Uribe and Jay 2007; Pak et al. 2008). The precise conformation of nuclear actin is not known so far (for reviews, see Jockusch et al. 2006; Pederson and Aebi 2005; Pederson 2008; Schleicher and Jockusch 2008). It has been doubted for a long time that actin filaments exist in the nucleus because of the absence of intranuclear phalloidin stained-structures in most cell types in physiological conditions. However, several studies have demonstrated that polymeric actin is needed for nuclear export of RNAs and proteins in *Xenopus* oocytes (Hofmann et al. 2001), for intranuclear movement of Herpes simplex virus-1 capsid (Forest et al. 2005), for nuclear envelope assembly (Krauss et al. 2003), for a long-range directional movement of an interphase chromosome site (Chuang et al. 2006), and for transcription by RNA polymerase I (Ye et al. 2008). Moreover, a recent study using FRAP analysis has shown that about 20% of the total nuclear actin pool is in the polymeric state (McDonald et al. 2006). The results of this study, along with other reports

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using conformation-specific anti-actin antibodies (Gonsior et al. 1999; Schoenenberger et al. 2005) have indicated that polymeric forms of actin in the nucleus and cytoplasm are fundamentally different. Nevertheless, the dynamic equilibrium between nuclear actin monomers, oligomers and polymers suggests a controlled regulation as is the case in cytoplasm. Indeed, a number of actin-binding and actin-related proteins have been identified in the nucleus. For some of them, participation in nuclear functions such as transcriptional regulation, splicing, chromatin remodeling, and DNA repair have been suggested (reviewed in Olave et al. 2002; Gettemans et al. 2005). However, the questions of whether these proteins control nuclear processes on their own or in association with actin and how they participate in nuclear actin regulation remain largely unanswered.

In the present study, we have analyzed ultrastructural localization of actin and six actin-binding proteins: α-actinin, filamin, paxillin, p190RhoGAP, spectrin, and tropomyosin, in nuclei of HeLa cells and resting human lymphocytes. We show that each of these proteins occupy specific compartments of the cell nuclei and/or nucleoli. Moreover, we found that spectrin, paxillin, and α-actinin spatially colocalize with actin in nucleolus. Our data support previous reports on the presence of actin-binding proteins in the nucleus and provide more detailed data of their intranuclear localization. We demonstrate for the first time the presence of tropomyosin and p190RhoGAP in the cell nucleus. The precise functions of nuclear actin-binding proteins and their role in nuclear actin regulation should be a subject of future research.

Results and discussion

Resting human lymphocytes were isolated according to standard procedure (Boyum 1968), fixed in 2% paraformaldehyde and 0.25% glutaraldehyde in SB for 1 h; HeLa cells were fixed in 3% paraformaldehyde and 0.1% glutaraldehyde for 40 min at 0°C. Cells were dehydrated in a series of ethanol solutions, embedded in LR White resin, and 80 nm sections were immunolabeled according to standard procedures (Hozák et al. 1994). The specificity of the antibodies used was always controlled by Western blot and by the typical labeling in the cytoplasm. The specimens were observed in F. E. I. Morgagni electron microscope equipped with a CCD Mega View II camera (SIS, Germany). For spatial statistics of immunogold labeling patterns, we used special plug-ins (for details, see Philimonenko et al. 2000; Schöfer et al. 2004; and http://nucleus.img.cas.cz/gold) developed for Ellipse program (ViDiTo, Slovakia). They allow one to evaluate clustering and colocalization patterns detected by immunogold labeling, and also map significantly labeled cellular compartments over the surface of immunogold-labeled ultrathin sections. Gold particles on the image are detected as points and their density is estimated using kernel density estimate method with conical kernel function. Then threshold method is used for segmentation of the image to delineate the areas with density of immunogold particles over the background values (Schöfer et al. 2004). Detection of labeled compartments was performed on composite images produced by stitching of 12–25 images per nucleus taken at high magnification. This allows both to obtain high resolution needed for the detection of gold particles, and to perform the detection on the images of entire nuclei thus not loosing the overall view.

Actin-binding proteins have characteristic localization patterns in nuclei of HeLa cells and resting human lymphocytes

The results of ultrastructural mapping of actin and actin-binding proteins in HeLa cells and resting human lymphocytes are presented in Figs. 1 and 2, and summarized in Table 1.

Actin labeling (mouse monoclonal anti-actin antibody, Amersham N350, clone JLA20; Lin 1981) in HeLa cells is localized preferentially in decondensed chromatin and in the nucleolus, where fibrillar centers (FCs) along with neighboring dense fibrillar component (DFC) and granular component (GC) are labeled. In resting human lymphocytes, clusters of actin labeling are located mainly in decondensed chromatin at the border of heterochromatin blocks and in the FCs of nucleoli (Fig. 1, Table 1). This localization of actin is consistent with the reported roles for actin in transcription and chromatin remodeling (for reviews see Rando et al. 2000; Olave et al. 2002; Grummt 2006; Percipalle and Visa 2006).

Small Rho GTPases and activating proteins (RhoGAPs) are involved in regulation of actin filaments nucleation via WASP family proteins and Arp2/3 complex (Machesky et al. 1999; Rohatgi et al. 1999; Sagot et al. 2002; for reviews, see Zigmond 2004; Pollard 2007). On ultrathin sections of HeLa cells labeled with polyclonal rabbit anti-p190 RhoGAP antibody (Chang et al. 1995), the labeling is distributed throughout nucleoplasm in areas of heterogeneous size (70–140 nm in diameter). RhoGAP is localized both in condensed and decondensed chromatin area, as well as in the nucleolus over GC. In resting human lymphocytes, RhoGAP is localized in fewer areas of larger size as compared to HeLa cells. The labeling is observed preferentially in the condensed chromatin close to nuclear envelope or on the border of condensed and decondensed chromatin. Clusters of interchromatin granules and the GC of nucleoli are also labeled (Fig. 1, Table 1).

So far, only two RhoGAP domain-containing proteins have been identified in the nucleus. Nuclear translocation of

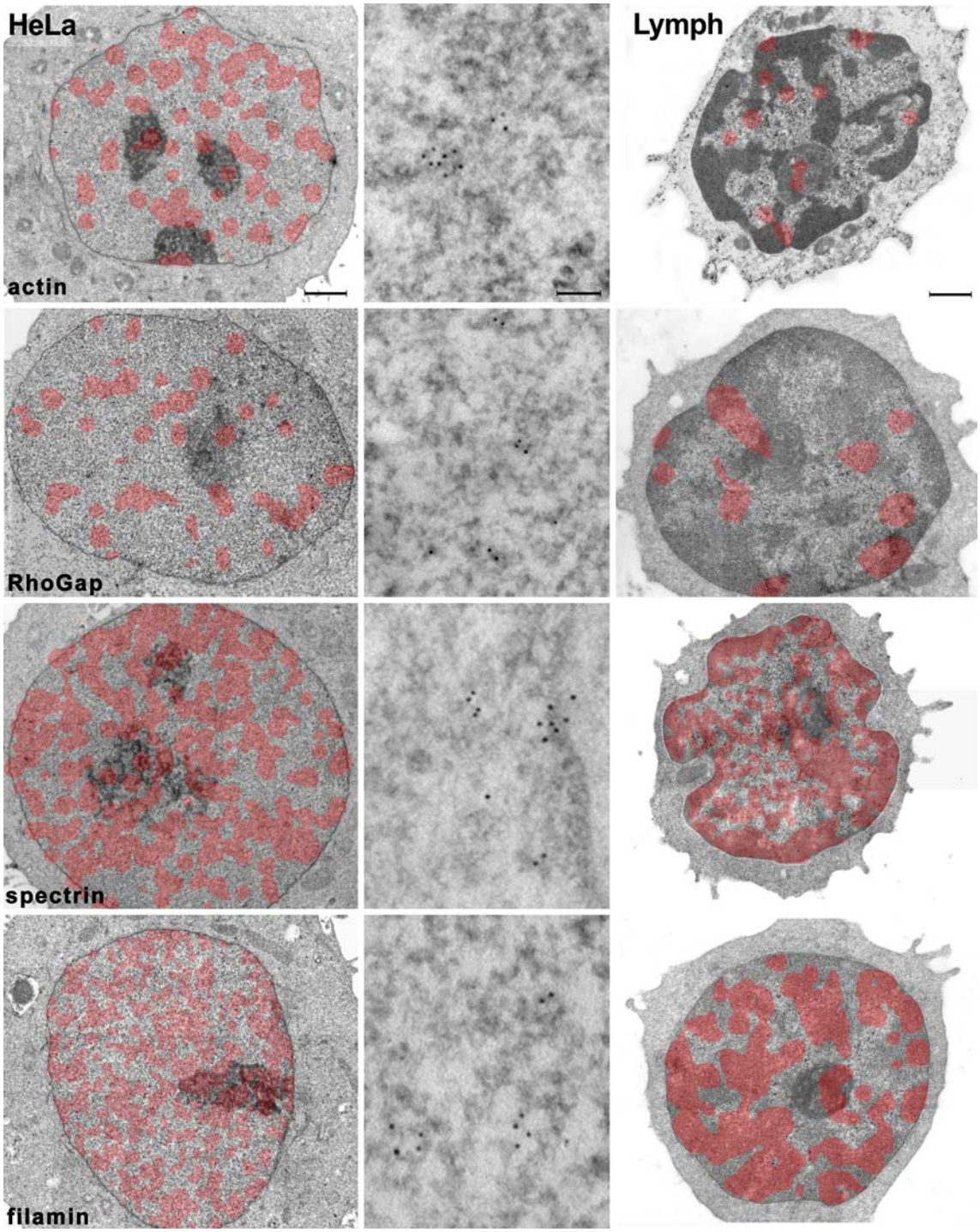

Fig. 1 Ultrastructural mapping of actin, p190RhoGAP, spectrin and filamin in HeLa cells and resting human lymphocytes. Ultrathin sections of HeLa cells (left column, HeLa; central column, high magnification) and resting human lymphocytes (right column, Lymph) were gold-immunolabeled with antibodies to actin and actin-binding proteins, as indicated always in the left column. The areas of increased density labeling in the nucleus are highlighted with *red color*. Labeling in cytoplasm is not depicted. *Bar* 1 μm (left and right column); 100 nm (central column)

DLC1 tumor suppressor protein induced apoptosis in human non-small cell lung carcinoma cells (Yuan et al. 2007). An ARHGAP19 protein contains a bipartite nuclear localization signal and is expressed in the nucleus in several human adult and embryonic tissues (Lv et al. 2007). Our results represent the first evidence for nuclear localization of p190RhoGAP. Interestingly, the appearance of RhoGAP-positive areas differs in HeLa cells and resting

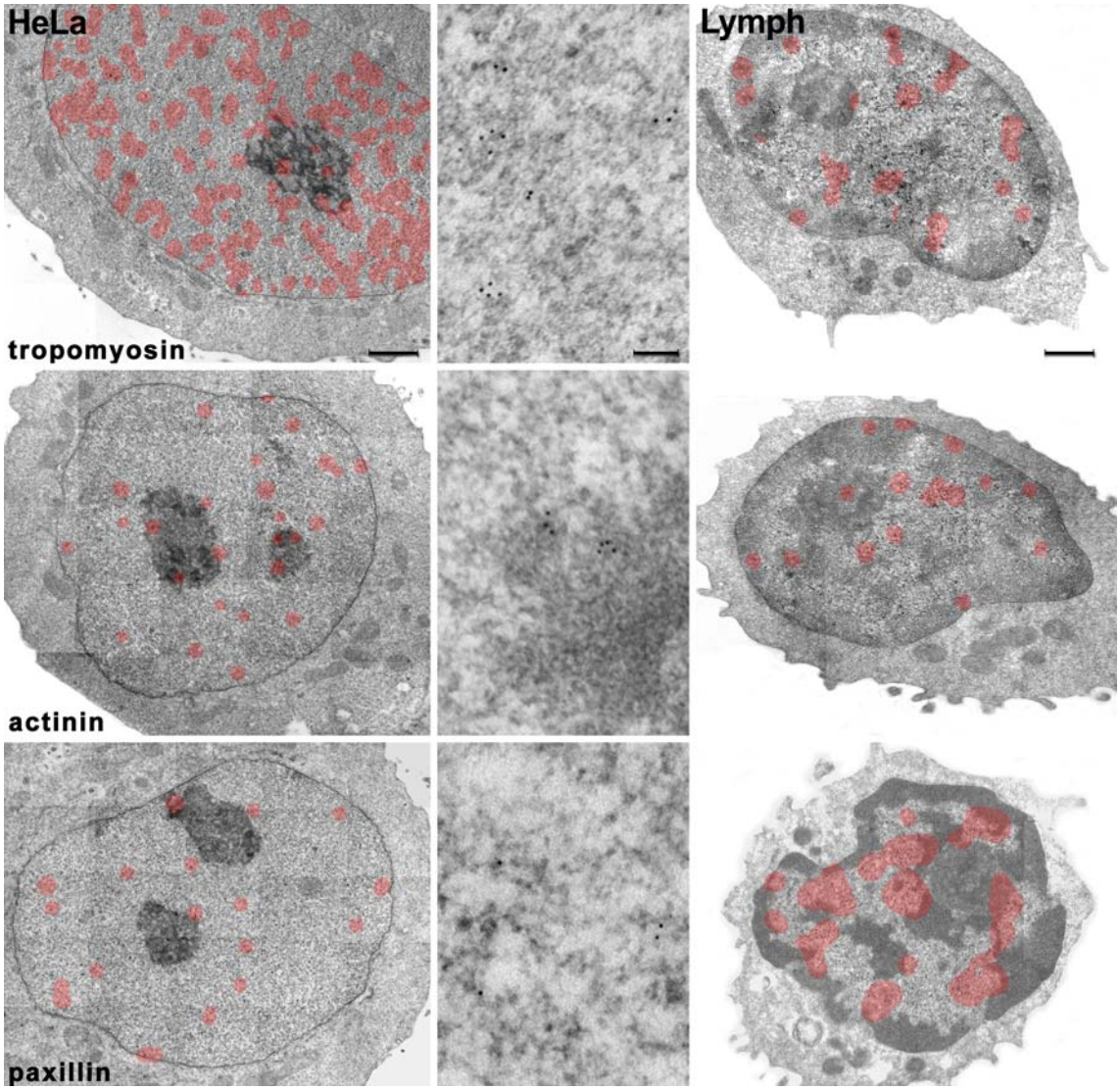

Fig. 2 Ultrastructural mapping of tropomyosin, α-actinin and paxillin in HeLa cells and resting human lymphocytes. Ultrathin sections of HeLa cells (left column, HeLa; central column, high magnification) and resting human lymphocytes (right column, Lymph) were gold-immunolabeled with antibodies to actin and actin-binding proteins, as indicated always in the left column. The areas of increased density labeling in the nucleus are highlighted with *red color*. Labeling in cytoplasm is not depicted. Bar, 1 μm (left and right column); 100 nm (central column)

human lymphocytes, which might be connected with different level of transcriptional activity or that of chromatin organization in these two cell types.

Spectrins, first described in the erythrocyte (Marchesi and Steers 1968), are found in all or almost all cells (Burridge et al. 1982; Repasky et al. 1982; Glenney and Glenney 1983). Spectrin links actin filamentous network to cell membrane via association with membrane proteins and is important for maintaining membrane structural integrity and generating distinct membrane protein domains (for a review, see Bennett 1990). In a complex with 4.1 protein and tropomodulin, spectrin forms a capping system for pointed ends of actin filaments (for a review see Schafer and Cooper 1995). Spectrin clusters in HeLa cells (immunolabeling with rabbit polyclonal antibody, Sigma) are grouped in elongated branched patches that occupy a large area in the nucleoplasm over both condensed and decondensed chromatin. The labeling is excluded from interchromatin granules. In many cases spectrin clusters are located at the nuclear envelope. Intense labeling is observed in nucleoli, predominantly over DFC and GC (Fig. 1, Table 1). Spectrin in resting human lymphocytes is mainly associated with condensed chromatin and is present virtually along the whole length of the nuclear envelope. In lymphocyte nucleoli, spectrin is localized in the FC, in contrast to HeLa cells (Fig. 1, Table 1).

Spectrins have been previously identified in nuclei of various cell types at the nuclear envelope and associated

Table 1 Nuclear localization of actin and actin-binding proteins in resting human lymphocytes and HeLa cells

	Nuclear compartments				
	Heterochromatin		Decond. chromatin	Interchromat. granules	Nucleolus
	Bulk	Peripheral			
HeLa cells					
Actin	ND	±	+	+	+
p190RhoGAP	ND	+	+	+	+
Spectrin	ND	+	+	−	+
Filamin	ND	+	+	±	+
Tropomyosin	ND	+	+	+	+
α-Actinin	ND	±	+	−	+
Paxillin	ND	−	+	−	+
Human lymphocytes					
Actin	±	±	+	+	+
p190RhoGAP	+	+	±	+	+
Spectrin	±	+	+	−	+
Filamin	+	+	+	±	+
Tropomyosin	−	±	+	+	−
α-Actinin	±	+	±	+	+
Paxillin	+	−	+	+	+

ND not detected (because of heterochromatin lack in HeLa cells)

with intranuclear granules, PML bodies, and nucleoli (Bachs et al. 1990; Vendrell et al. 1991; Tse et al. 2001). Strong evidence exists that the αSpecII isoform of spectrin plays a role in mediating DNA repair, possibly acting as a scaffolding protein for DNA repair proteins (McMahon et al. 2001; Sridharan et al. 2003, 2006). Our data on ultrastructural localization of spectrin are in agreement with previous reports showing spectrin in the nucleus. Especially interesting is the prominent localization of spectrin along the nuclear envelope of both HeLa cells and human lymphocytes. According to its function in linking cytoskeleton to plasma membrane, spectrin may play similar role in the nucleus providing anchoring of internal nuclear actin structures to the nuclear membrane. Other nuclear spectrin-repeat proteins have been described in the nucleus, including syne/nesprin, mAKAP and Bpag1, that bind both emerin and lamins A/C and link the nucleoskeleton to inner and outer nuclear membrane and to actin cytoskeleton (Holaska et al. 2004; for a review see Young and Kothary 2005).

Filamins organize filamentous actin in networks and stress fibers. Filamins anchor various transmembrane proteins to the actin cytoskeleton and provide a scaffold for a wide range of cytoplasmic signaling proteins (reviewed by van der Flier and Sonnenberg 2001).

Filamin labeling (goat polyclonal antibody, Sigma) in HeLa cells is intense and appears as a dense mesh throughout the whole nucleus, including nucleoli. In human lymphocytes, filamin clusters fuse together to form large labeled areas that occupy a significant proportion of condensed chromatin and perichromatin area but are mainly excluded from interchromatin granules. In nucleoli, filamin is apparently present in all three components (FC, DFC and GC) (Fig. 1, Table 1). Several recent reports have linked filamin to transcriptional regulation (Ozanne et al. 2000; Loy et al. 2003) and DNA repair (Yuan and Shen 2001; Meng et al. 2004; reviewed by Uribe and Jay 2007). Our results provide further evidence that filamin is abundant in the cell nucleus.

Tropomyosin is an actin-binding protein responsible for stabilizing the actin microfilament system in the cytoskeleton of non-muscle cells and is involved in processes such as growth, differentiation, and polarity of neuronal cells (Vrhovski et al. 2003). Tropomyosin in HeLa cells (immunolabeling with rabbit polyclonal antibody, Sigma) is concentrated in numerous small clusters and distributed over the whole nucleoplasm and nucleolus, without obvious preference to any distinct compartment (Fig. 2, Table 1). In resting human lymphocytes, a few tropomyosin clusters about 70 nm in diameter are observed, mainly in the region of interchromatin granules and on decondensed chromatin at the border of heterochromatin areas. This is for the first time that tropomyosin presence in the nucleus is shown. Relative abundance of tropomyosin in the nucleus suggests functional nuclear roles for this protein, which should be subject of future research.

α-Actinin is a member of spectrin superfamily of proteins that cross-links F-actin into bundles and networks (Meyer and Aebi 1990; Pelletier et al. 2003) and is an essential component of adherence junctions and focal adhesion

junctions (reviewed by Djinovic-Carugo et al. 2002). α-Actinin in HeLa cells (immunolabeling with rabbit polyclonal antibody, Sigma) is concentrated in small spots about 30 nm in diameter spread sparsely in the regions of decondensed chromatin in nucleoplasm and in the GC of nucleoli (Fig. 2, Table 1). In resting human lymphocytes, α-actinin clusters of roughly the same size as in HeLa cells (20–50 nm in diameter) are localized mainly at interchromatin granules clusters in the vicinity of heterochromatin. Interestingly, considerable proportion of α-actinin is found adjacent to nuclear envelope. Similarly to HeLa cells, α-actinin is also present in the GC of nucleoli in resting human lymphocytes (Fig. 2, Table 1).

There are two muscle-specific and two ubiquitously expressed isoforms of α-actinin (reviewed by Otey and Carpen 2004). α-actinin-4 was found in the nucleus of certain cell types where it accumulates in nuclei in response to extracellular stimuli (Honda et al. 1998). α-actinin-4 has been shown to associate with transcription factors (Babakov et al. 2004; Poch et al. 2004) and participate in transcriptional regulation by MEF2 by antagonizing histone deacetylase 7 (Chakraborty et al. 2006). Our results present further evidence for specific localization of α-actinin in nuclei of HeLa cells and resting human lymphocytes. Interestingly, α-actinin colocalizes with actin in nucleoli (see below).

Paxillin is a focal adhesion adapter protein that participates in the integrin-mediated signaling (Ogawa et al. 2003; for a review see Schaller 2004). Paxillin labeling (mouse monoclonal antibody, a kind gift of Prof. M. Way, Heidelberg) in the nuclei of HeLa cells is distributed sparsely in small spots (about 40 nm in diameter) that tend to locate around interchromatin granules; some labeling is also associated with the border of nucleoli In resting human lymphocytes, paxillin is concentrated in nucleoplasm in larger areas in comparison to HeLa cells; interchromatin granules and the GC of nucleoli are labeled (Fig. 2, Table 1).

So far, only two groups have shown nuclear localization of paxillin by immunohistochemistry and Western blot. Ogawa et al. (2001) reported phosphorylation-dependent nuclear translocation of paxillin in Xenopus kidney epithelial cell line. In mouse fibroblasts, paxillin was shown to undergo nucleocytoplasmic shuttling and participate in nuclear export of poly(A)-binding protein 1 (PABP1) in CRM1-exportin dependent manner (Woods et al. 2002; Woods et al. 2005). Here we demonstrate for the first time the nuclear distribution of paxillin in unaffected cells on ultrastructural level.

Actin-binding proteins are detected in HeLa cells nuclear extracts

HeLa cells growing in suspension were spun down, washed with ice-cold PBS, and resuspended in hypotonic buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 5 mM DTT, 2 mM PMSF). The suspension was homogenized through 23G needle (>90% cells lysed as judged by microscopic observation) and centrifuged at 1,000 g (15 min, 4°C). Supernatant was saved for cytoplasmic extract. Sedimented nuclei were washed with ice-cold PBS, lysed in RIPA buffer (20 min on ice) and nuclear extract was cleared by centrifugation at 16,000*g* (15 min, 4°C). Saved supernatant was supplemented with extraction buffer (300 mM HEPES, pH 7.9, 1.4 M KCl, 30 mM $MgCl_2$; 0.11 of the supernatant volume) and centrifuged at 39,000*g* (1 h, 4°C). Supernatant was dialysed (2 h and then overnight, 4°C) in dialysis buffer (20 mM HEPES, pH 7.9, 20% glycerol, 100 mM KCl, 2 mM EDTA, 5 mM DTT) and resulting cytoplasmic extract was cleared by centrifugation at 16,000*g* (30 min, 4°C). Proteins were separated on 10 or 7% SDS-PAGE and analyzed on Western blots. For signal detection, secondary antibodies conjugated with IRDye® 800CW Odyssey (LI-COR) and Odyssey Scanner (Infrared Imaging System, LI-COR) were used. The results are presented in Fig. 3.

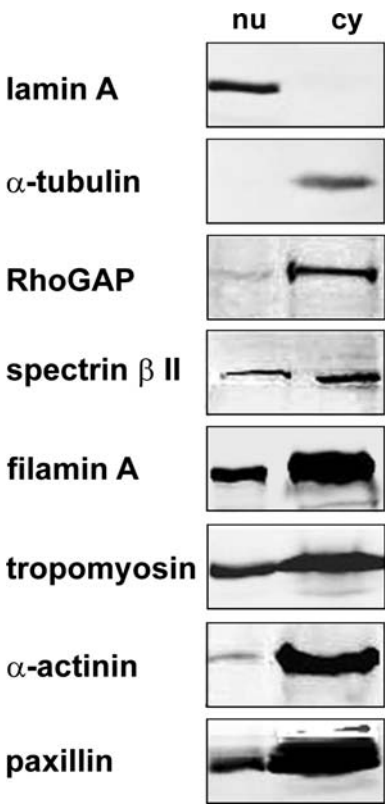

Fig. 3 Detection of actin-binding proteins in nuclear and cytoplasmic extracts of HeLa cells. Proteins of nuclear (left column, nu) and cytoplasmic (right column, cy) extracts were analyzed on Western blots using antibodies to actin-binding proteins, as indicated. Anti-lamin A and anti-tubulin α antibodies were used for controlling the purity of the fractions

Table 2 Colocalization of actin with actin-binding proteins in the nucleolus

	Immunogold particles		Statistical significance of colocalization for various intervals of distances between particles			Nucleolar component where labels colocalize
	6 nm	12 nm	50–100 nm	100–150 nm	150–200 nm	
HeLa cells	Spectrin	Actin	$p < 0.01$	NS	NS	DFC, GC
	Paxillin	Actin	$p < 0.01$	$p < 0.01$	NS	GC
Human lymphocytes	Spectrin	Actin	NS	$p < 0.01$	NS	FC
	α-Actinin	Actin	NS	$p < 0.01$	$p < 0.01$	GC

NS statistically non-significant

As cross-contamination of nuclear and cytoplasmic extracts is a common problem, we controlled the purity of the fractions using anti-lamin A mouse monoclonal antibody (kindly provided by Y. Raymond) and anti-tubulin α mouse monoclonal antibody (kindly provided by P. Dráber). A prominent band of lamin A is observed only in nuclear extract while tubulin α is exclusively cytoplasmic (Fig. 3). This result is consistent with the known localization of these two proteins and demonstrates that our extracts are free of major cross-contamination.

Detection of actin-binding proteins was performed using following antibodies: rabbit polyclonal anti-p190 RhoGAP (Chang et al. 1995), rabbit polyclonal anti-β spectrin II (Santa Cruz), mouse monoclonal anti–filamin A (Chemicon), mouse monoclonal anti-tropomyosin (Sigma), mouse monoclonal anti-actinin (Chemicon), rabbit monoclonal anti-paxilin (Upstate). All proteins under study are detected in cytoplasmic extract and are also present to various extent in nuclear extract (Fig. 3). These results strongly support our ultrastructural data showing nuclear localization of actin-binding proteins.

Colocalization of actin and actin-binding proteins in nuclei of resting human lymphocytes and HeLa cells

Despite numerous reports of localization and functions of actin-binding proteins in the cell nucleus, no information is available about their interaction with nuclear actin. Only profilin has been shown to be a cofactor of actin export from the nucleus mediated by a novel nuclear export receptor exportin 6 (Stuven et al. 2003). We therefore analyzed colocalizations of the various actin-binding proteins with actin in nuclei of HeLa cells and resting human lymphocytes. The significance of colocalization of two kinds of immunogold labels was evaluated as described previously (Philimonenko et al. 2000). For each variant, random digital images of nucleoplasm and nucleoli were taken from 20 cells. The intervals of distances at which colocalization of two labels is statistically significant reflect the size of nuclear structures where the two detected proteins are located together. The results are presented in the Table 2. Surprisingly, statistically significant colocalizations were found only in nucleoli, and not in the nucleoplasm.

Spectrin statistically significantly ($p < 0.01$) colocalized with actin in nucleoli of both cell types analyzed (Table 2). In resting human lymphocytes, spectrin is together with actin in FC of nucleoli, known as storage site of proteins necessary for transcription and processing of RNA. In nucleoli of HeLa cells, which are more transcriptionally active, spectrin colocalized with actin at the border of DFC and predominantly in GC, where rRNA processing takes place. Colocalization of paxillin with actin was found in the GC in the nucleoli of HeLa cells, but not in human lymphocytes (Table 2). α-Actinin colocalized with actin in resting human lymphocytes in the GC of nucleoli (Table 2).

FCs are storage sites of RNA polymerase I and transcription factors, and they form structural cores of nucleoli (Schwarzacher et al. 1978; Hozák et al. 1994). We show colocalization of actin and spectrin in the FCs. As a recent work shows that F-actin participates in transcription of ribosomal genes (Ye et al. 2008), it is tempting to speculate that actin in complex with spectrin may anchor the transcription machinery on the surface of FCs. Colocalization of actin with paxillin or α-actinin in GC in different cell types may reflect the differences in actin regulation in transcriptionally active and inactive cells.

Taken together, we have shown at the ultrastructural level specific distribution of actin and six actin-binding proteins in the cell nucleus. HeLa cells and resting human lymphocytes differ dramatically by the level of cellular transcription. For most proteins analyzed, their localization was rather different in HeLa cells and in lymphocytes relative to nuclear compartments. This suggests their involvement in regulation of nuclear functions, and/or in nuclear architecture reorganization connected with various level of transcription. For tropomyosin and p190RhoGAP, we show for the first time their presence in the cell nucleus. Surprisingly, we have not found colocalization of actin with any of actin-binding proteins in the nucleoplasm. Possibly, actin interacting with actin-binding proteins has a specific conformation which is not recognized by the antibodies used, or the epitopes on actin

are masked. There is still little data on nuclear actin-binding proteins. Their precise functions in the nucleus and modes of regulation of nuclear actin should be thoroughly studied as they seem to be connected with important nuclear processes.

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