THE RHO FAMILY GEF ASEF2 REGULATES ADHESION DYNAMICS AND THEREFORE CELL MIGRATION BY MODULATING RAC AND RHO ACTIVITY

By

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To my extraordinary parents, Tom and Deanne,

who have always encouraged, supported, and loved me.

To my patient and loving husband, James, whose presence

by my side has made all of this possible.

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LIST OF ABBREVIATIONS

ABR	APC binding region
APC	Adenomatous polyposis coli
Arp	Actin related protein
Asef1	APC-stimulated exchange factor 1
Asef2	APC-stimulated exchange factor 2
Asef2Δ204	Constitutively active Asef2 lacking ABR and SH3 domains
CA	Constitutively active
Cdc42	Cell division cycle 42
DH	Dbl homology
DMEM	Dulbecco's modified Eagle's media
DN	Dominant negative
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
ERM	Ezrin/radixin/moesin
F-actin	Filamentous actin
FACS	Fluorescence-activated cell sorting
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
Fn	Fibronectin
G-actin	Monomeric actin
GAP	GTPase activating protein

GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GFP	Enhanced green fluorescent protein
GIT1	G protein-coupled receptor kinase-interacting protein 1
GSK-3	Glycogen synthase kinase-3
GTP	Guanosine triphosphate
h	Hour(s)
HEK	Human embryonic kidney
KD	Kinase dead
Min	Minute(s)
µM	micromolar
MMP	Matrix metalloprotienase
nM	nanomolar
PAK	p21 activated protein
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PH	Pleckstrin homology
PI3K	Phosphoinositide 3-kinase
PIP_3	Phosphoinositol (3,4,5)-trisphosphate
PKB	Protein kinase B
PKC	Protein kinase C
Rac	Ras-related C3 botulinum toxin substrate
Rho	Ras homolog

RNA	Ribonucleic acid
RNAi	RNA interference
Scr	Scrambled
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SH3	Src homology 3
siRNA	Short interfering RNA
Src	Sarcoma
TIRF	Total internal reflection microscopy
WASP	Wiskott-Aldrich syndrome protein
WAVE	WASP verprolin homologous protein

CHAPTER I

INTRODUCTION

Cell Motility and Migration

Cell migration is a tightly regulated process central to a number of biological functions and pathological conditions. For example, wound healing is dependent upon migration of fibroblasts and vascular endothelial cells; leukocytes migrate into injured areas to mediate an immune response; and in cancer metastasis, tumor cells migrate from the original tumor site into the blood vessels, and then back out of the vasculature and into a new location to form secondary tumors (Lauffenburger and Horwitz 1996). Cells migrate on an extracellular matrix (ECM) in a repetitive, four-step mechanical process (Lauffenburger and Horwitz 1996). The process begins with the extension of a broad lamellipodial protrusion, thought to be driven by actin polymerization (Wang 1985; Carson, Weber et al. 1986; Borisy and Svitkina 2000). The signals for actin assembly at the leading edge are generated by components of the ECM such as fibronectin (Fn) and various growth factors. Stabilization of the protrusion occurs by formation of multiple small adhesions that increase in size, organization and strength. This lends traction against the ECM to propel the cell body in sustained migration (Beningo, Dembo et al. 2001; Zaidel-Bar, Ballestrem et al. 2003). The constant assembly and disassembly, or turnover, of nascent

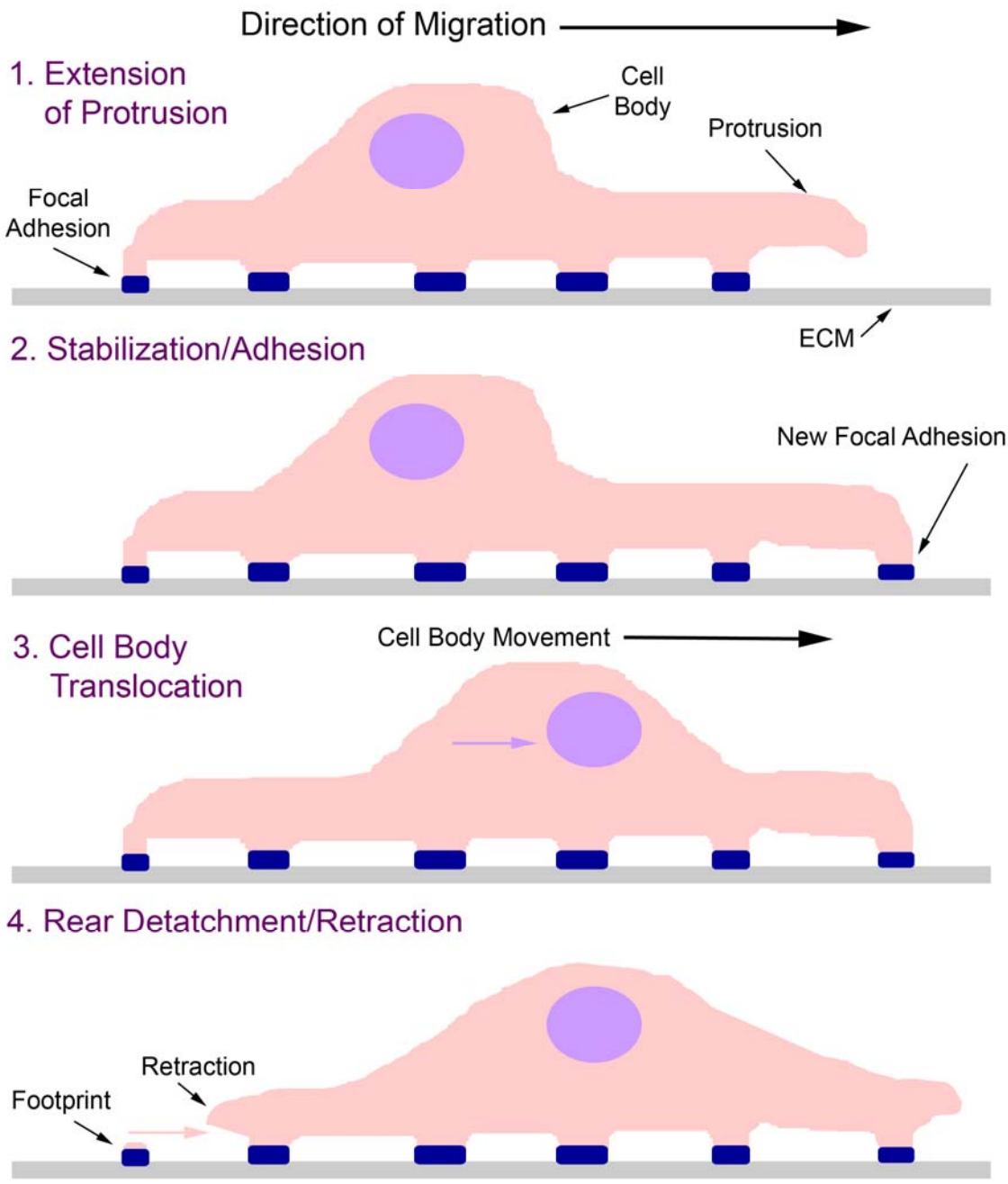

Figure 1. Cell Migration is a four-step mechanical process. Cell migration begins with the extension of a lamellipodial protrusion (1) followed by stabilization of the protrusion by the formation of adhesions between the cell membrane and the ECM (2). (3) The cell body then translocates in the direction of the protrusion and (4) the rear of the cell retracts and detaches from the ECM. Adapted from (Lodish, Berk et al. 2000).

cell-matrix adhesions within the leading edge protrusion is essential for continuous migration (Webb, Donais et al. 2004). Finally, net displacement of the cell occurs when adhesions at the trailing edge disassemble and release the substrate (Fig 1.) (Horwitz and Parsons 1999). Failure to establish adhesions at the leading edge leads to lamellipodial ruffling, an inefficient attachment to the ECM (Borm, Requardt et al. 2005). Failure to disassemble adhesions rapidly prevents the cell from disengaging the ECM, and therefore delays or disrupts the migration process. Each step of the migration process must therefore be stringently controlled for proper function.

Actin Cytoskeleton

Branched Actin of the Lamellipodium

Motile cells have a very thin leading lamellum, a sheet-like protrusion densely filled with actin filaments (Abraham, Krishnamurthi et al. 1999; Pollard and Borisy 2003). Actin is the most abundant protein in many eukaryotic cells, and by mass, filamentous actin (F-actin) is the dominant structural component of the lamellipodium. Filaments are made up of polymers of globular actin (G-actin) monomers, arranged in a helical structure with the monomers in a head-to-tail, polarized arrangement. This molecular polarity is key to the mechanism of actin assembly in cells (Pollard and Borisy 2003), and protrusion is thought to result from F-actin polymerization against the membrane (Ponti, Machacek et al. 2004). In the cell, a densely-packed network of short, stiff, branched filaments exert the

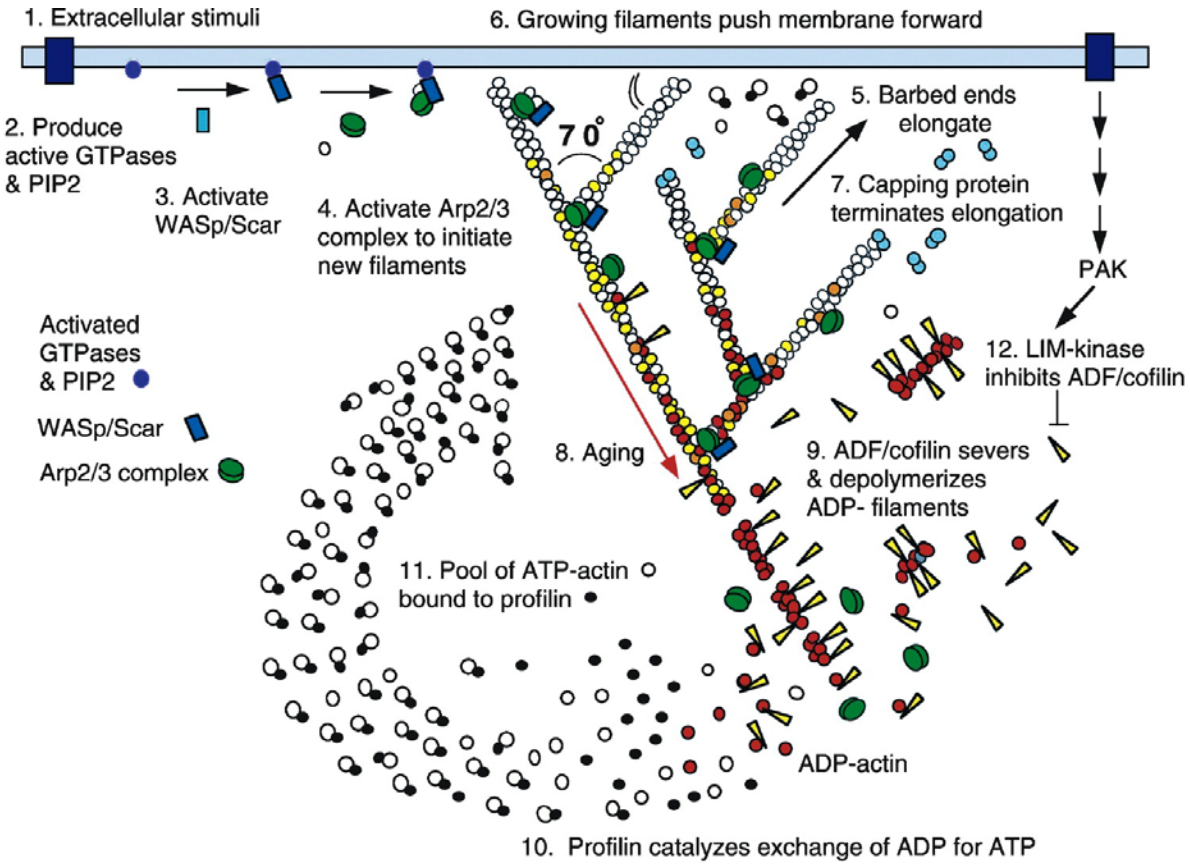

Figure 2. Treadmilling/dendritic nucleation model for leading edge protrusion. (1) Receptors are activated by extracellular signals. (2) Active Rho-family GTPases and PIP2 are produced by the associated signaling pathways, leading to (3) activation of WASp/Scar proteins. (4) Actin branches are formed when WASp/Scar proteins bring an actin monomer together with Arp2/3 complex on the side of a pre-existing filament. (5) The new branch's barbed end grows rapidly and (6) pushes the membrane forward. (7) Growth is terminated by capping protein within one or two seconds. (8) Hydrolysis of bound ATP on each actin subunit (yellow subunits) followed by γ phosphate dissociation (red subunits) causes aging. (9) Phosphate dissociation, ADP-actin filament severing and dissociation of ADP-actin from filament ends is promoted by ADF/cofilin. (10) The exchange of ADP for ATP (white subunits) is facilitated by profilin, returning subunits to (11) the profilin-bound ATP-actin pool, ready to elongate barbed ends. (12) PAK and LIM kinase are activated by Rho-family GTPases, leading to the phosphorylation of ADF/cofilin. This slows filament turnover. Reprinted from (Pollard and Borisy 2003) with permission from Elsevier.

force necessary for protrusion of lamellipodia (Fig. 2) (Borisy and Svitkina 2000; Pollard and Borisy 2003; Faix and Rottner 2006).

Filament growth is limited by depletion of the free G-actin pool within the cell, as well as by barbed end-capping proteins (Ponti, Machacek et al. 2004). Polymerization of the barbed end (toward the cell edge) and depolymerization of the pointed end (away from the cell edge) lead to an overall treadmilling effect on the actin filament that is thought to be the driving force behind protrusion of the leading edge. Treadmilling allows a strict control of actin filament stability; while capping proteins at the barbed end prevent further polymerization, depolymerization occurs at the pointed end. This leads to a replenishment of the free G-actin pool that allows the monomers to be recycled and assembled into the barbed ends of uncapped filaments, and has been suggested to be an efficient way for the cell to manage its mechanical and energetic resources (Wang 1985).

In order to maintain a pool of monomers ready to polymerize rapidly, the cell must coordinate the actions of G-actin-binding proteins and proteins that cap filament barbed ends (Pollard and Borisy 2003). More than sixty classes of actin-binding proteins are currently known, but there are a few that are considered core actin binding proteins, that seem to exhibit the minimal requirements for the very simplest motility. Among these core actin-binding proteins are ADF/cofilin, capping protein, Arp2/3 complex, an activator of Arp2/3 complex, and profilin (Pollard and Borisy 2003). Additionally, actin polymerization is regulated by the bound adenine nucleotide state of the G-actin monomers as well as actin-binding

proteins. Mg-ATP binds a cleft in monomeric G-actin and stabilizes it. In this state, the actin readily polymerizes. Hydrolysis of the bound ATP to ADP acts as a timer. As the actin ages within the filament, it becomes more likely to have hydrolyzed ATP and be bound to ADP, which induces disassembly of the branched actin and binding of ADP-G-actin to ADF/cofilin, promoting severance and depolymerization of the ADP-bound subunits from the filament pointed end. Bound ADP is exchanged for ATP on the actin monomers by a nucleotide exchange factor, profilin. This effectively returns the polymerization-competent subunits (bound to ATP and profilin) to pool, now available to be incorporated into another growing filament. p21 activated kinase (PAK), when activated by Rac, can inhibit severing by ADF/cofilin through LIM kinase, which inactivates ADF/cofilin (Pollard and Borisy 2003).

Initiation of a new filament barbed end is thought to occur predominantly through de novo nucleation. Other possible mechanisms include severing or uncapping the barbed ends of existing filaments (Condeelis 1993; Zigmond 1996; Pollard and Borisy 2003). De novo nucleation is mediated by the Arp2/3 complex, a stable complex of the actin related proteins Arp2 and Arp3, and five novel subunits. Arp2/3 achieves this nucleation by capping the pointed ends of actin subunits, then initiating assembly of actin monomers onto the barbed end. The most widely accepted model of actin assembly in the lamellipodium is the dendritic nucleation model (Pollard, Blanchoin et al. 2000). Activated by upstream signals, including those from the Rho GTPases, Rac and Cdc42, Wasp/Scar proteins bring the free actin subunit, Arp2/3 complex, and existing

actin filament together. Arp2/3 associates with the free actin and the actin incorporated into existing filaments, promoting the formation of branches on the sides of filaments, that jut out at a 70° angle (Amann and Pollard 2001; Pollard and Borisy 2003). Wasp/Scar proteins and actin filaments are co-activators of Arp2/3 complex, and cortactin binds and activates Arp2/3 while stabilizing actin branches (Pollard and Borisy 2003; Weaver, Young et al. 2003).

Filaments continue to lengthen until capped by capping protein. This serves two main purposes: it controls where the actin network exerts force on the plasma membrane to cause protrusion, and it controls filament length which is important because shorter filaments are stiffer than long ones, exerting pressure on the membrane more efficiently (Pollard and Borisy 2003). Crosslinking of actin filaments lends strength to the network, allowing a forward protrusion of the growing actin network toward the cell edge, rather than slipping backward toward the cell cortex. Filamin and the Arp2/3 complex are among the molecules thought to have crosslinking action (Pollard and Borisy 2003).

Actin in the Filopodium

Unlike the branched actin network that drives the lamellipodium, filopodia (thin, rod-like surface protrusions of the membrane) are driven by parallel, linear, bundles of F-actin. Filopodial actin bundles lack the actin branch-nucleating system seen in lamellipodia, and are thought to be controlled by a different set of regulatory molecules (Faix and Rottner 2006; Steffen, Faix et al. 2006). However, in many systems, it appears that filopodia form from the lamellipodium,

suggesting it may be a precursor structure for filopodia. In this manner, select actin filaments are elongated and bundled, and it appears that uncapping of filaments in addition to elongation are key to dynamic actin modulation (Borisy and Svitkina 2000; Faix and Rottner 2006). Formins are dimerized scaffolding molecules that may play an important role in elongation and uncapping of filaments. They are actin nucleators that remain bound to the barbed end, moving along the filament as it elongates. By doing so, formins prevent capping of the filament, thereby promoting elongation of unbranched F-actin. Some formins may also depolymerize, sever, or bundle actin (Chhabra and Higgs 2007). The actual mechanism of filopodial bundling is still unclear, but the structural and functional differences between filopodia and lamellipodia are easily seen. Filopodia are thought to serve a sensory role, and may be important in chemotactic, or directional migration (Steffen, Faix et al. 2006).

Adhesion Dynamics

Adhesion to the ECM is mediated through focal adhesions, specialized regions of the plasma membrane. There are two main, broad categories of focal adhesions described so far: focal complexes that quickly form and turn over at the leading edge of a cell within lamellipodial protusions, and focal adhesions that are larger, more stable, and more highly organized at the rear and sides of the cell (Fig. 3) (Petit and Thiery 2000; Zaidel-Bar, Ballestrem et al. 2003). Some mature adhesions degrade slightly; referred to as fibrillar adhesions, these are thought to be important for ECM modification (Broussard, Webb et al. 2008).

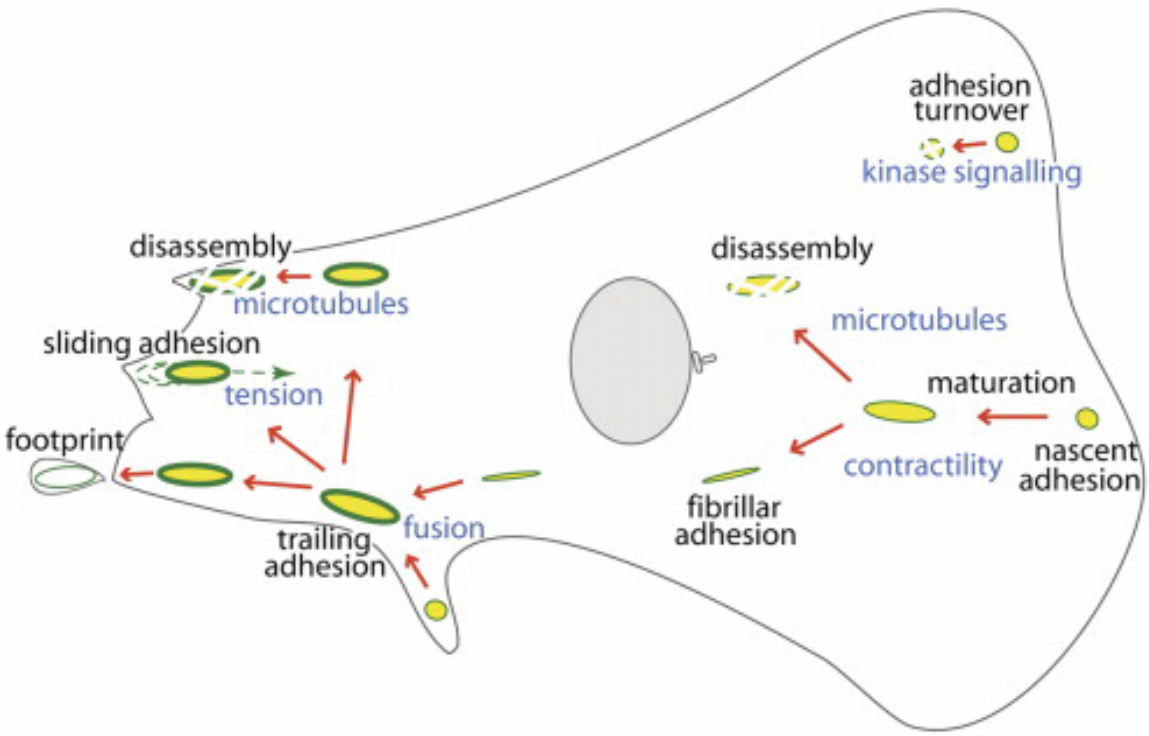

Figure 3. Adhesion dynamics model. Nascent adhesions formed at the front either undergo turnover, which is predominantly controlled by kinase signaling, or mature in response to contractile forces. Mature adhesions can disassemble in a microtubule-dependent manner or be transformed into fibrillar adhesions. Trailing adhesions arise as a result of fusion of additional nascent adhesions and remaining fibrillar adhesions. Once formed, trailing adhesions slide because of tension from attached stress fibers and either eventually disassemble or detach in the form of membrane 'footprints'. Reprinted from (Broussard, Webb et al. 2008) with permission from Elsevier.

In this dissertation, I will refer generally to focal adhesions, though those in this study tended to form within 1-2 μm of the cell edge and turn over rapidly. The primary transmembrane components found in focal adhesions are integrins. These function in adhesion and signaling to the cytoskeletal matrix from multiple ECM components via a large multimolecular complex of structural proteins, such as α-actinin, vinculin, and filamin and regulatory proteins, such as gelsolin, tensin, and zyxin, that can affect actin nucleation, capping, and crosslinking (Petit and Thiery 2000). Although the mechanism by which adhesions assemble is poorly understood, it appears that adhesion molecules assemble sequentially, individually, or in small complexes, rather than as a large, pre-assembled complex recruited to the adhesion nucleation site (Webb, Parsons et al. 2002).

Initiation of adhesion assembly is thought to be triggered by aggregation of integrins at the plasma membrane, likely as a result of an extracellular stimulus (Fig. 4). Upon formation, new focal complexes contain the regulatory molecule, talin, and focal adhesion kinase (FAK) —both responsible for adhesion disassembly. Ligand binding and/or integrin aggregation is required to recruit FAK, tensin, α-actinin, vinculin, and talin (Webb, Parsons et al. 2002). New adhesions contain the adapter molecules, vinculin and paxillin, that act to bind signaling molecules and actin cytoskeletal components at the plasma membrane. Studies using GFP-tagged proteins have shown that paxillin and α-actinin join the adhesion sequentially (Webb, Parsons et al. 2002). Paxillin contains multiple protein-protein binding sites, including proline-rich residues and a Src homology 3 (SH3)-binding domain that is regulated by tyrosine phosphorylation

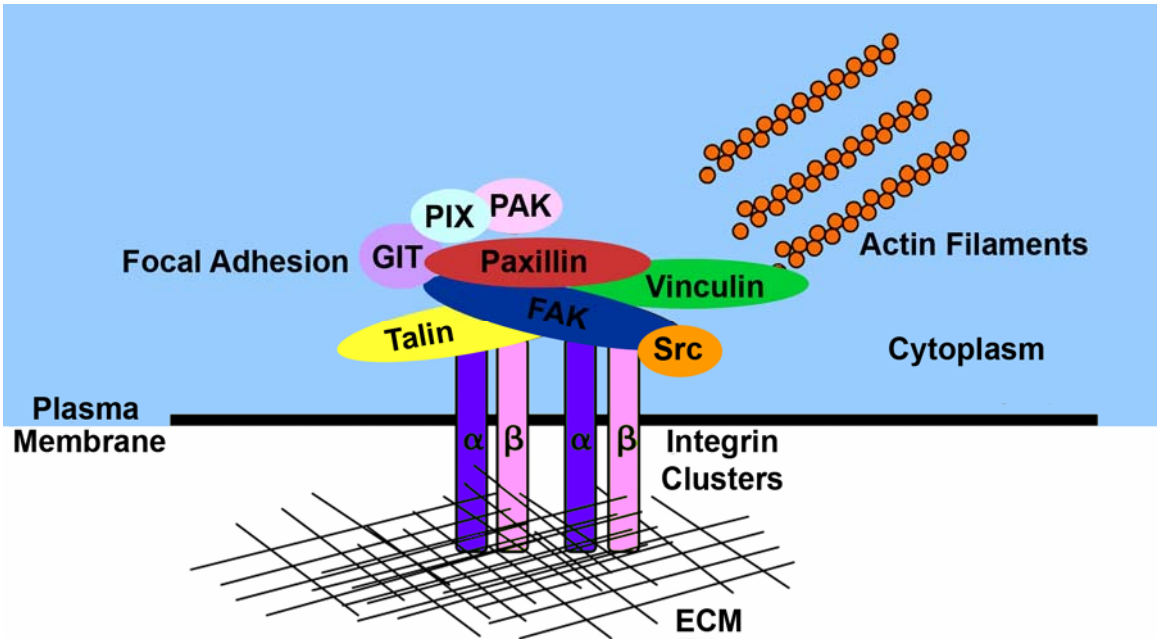

Figure 4. Focal adhesion. Focal adhesions are composed of more than 125 structural and signaling proteins (only a few examples are shown here) that link the ECM to the actin cytoskeleton. Following extracellular stimulation of αβ-integrin heterodimers, integrins become active, and molecules are recruited individually and in small pre-formed complexes to the forming adhesion site. Adapted from (Deakin and Turner 2008).

(Petit and Thiery 2000). Interestingly, paxillin also contains multiple leucine-rich motifs, each of which have shown some selective protein binding, and interact with important adhesion-regulating molecules such as FAK, GIT1, vinculin, and the Rac GEF, PIX (Nayal, Webb et al. 2006). Binding of GIT1 to PIX, and then PIX to PAK, forms a multimolecular signaling complex. PIX is upstream of PAK, which is known to regulate adhesion disassembly (Turner, Brown et al. 1999). There is evidence suggesting paxillin, PAK, and PIX assimilate into the growing adhesion as a small, pre-formed complex (Webb, Parsons et al. 2002). Paxillin binds GIT1 in a phosphorylation-dependent manner, then targets it to the leading edge, where GIT1 is thought to regulate protrusion and promote adhesion disassembly. GIT1 targets the complex to the leading edge and adhesion sites in multiple cells types, including epithelial cells and fibroblasts (Nayal, Webb et al. 2006). Once assembled, adhesions may stabilize protrusions and serve as traction for the cell as it propels its body in the direction of migration, but these adhesions must also disassemble for the cell to effectively continue the migration process.

Adhesions assemble sequentially, but disassembly is more than a simple reversal of the assembly process. For example, paxillin and α-actinin disassemble from adhesions simultaneously, whereas during assembly, they are recruited sequentially (Laukaitis, Webb et al. 2001; Webb, Parsons et al. 2002). In addition, it is clear that a number of adhesion components are essential for proper disassembly. Phosphorylation of paxillin is thought to be important in the formation of focal adhesions; however, it has also been implicated in adhesion

disassembly, through recruitment of FAK (Zaidel-Bar, Milo et al. 2007). Studies have shown that FAK over-expression leads to enhanced mobility (Cary, Chang et al. 1996; Petit and Thiery 2000), and that FAK knockdown cells spread poorly, migrate more slowly, and have more focal adhesions than controls (Ilic, Furuta et al. 1995; Petit and Thiery 2000). These data indicate an essential role for FAK in adhesion disassembly, and is supported by a more recent study showing that FAK is indeed required for adhesion disassembly (Tilghman, Slack-Davis et al. 2005). PAK is also an important regulator of adhesion disassembly, although its mechanism remains to be clarified. PAK's downstream effectors, MLCK and LIM Kinase, are known regulators of the cytoskeleton, and are possible players in PAK-mediated adhesion disassembly (Webb, Parsons et al. 2002). The role of talin in adhesion disassembly is an interesting one; it mediates connectivity of integrins and the actin cytoskeleton. Proteolysis of talin by the calcium-dependent protease, calpain, is a rate limiting step in adhesion disassembly, as seen in NIH 3T3 cells (Franco, Rodgers et al. 2004). Furthermore, disassembly of paxillin, vinculin, and zyxin from adhesions are dependent upon calpain-mediated cleavage of talin at the adhesion site. Recent studies have indicated adhesion turnover is dependent on coordination of microtubules, the actin cytoskeleton, signaling and structural proteins, and integrins (Franco, Rodgers et al. 2004; Broussard, Webb et al. 2008). Despite the importance of adhesion assembly and disassembly for effective migration, adhesion turnover is poorly understood on a molecular level.

Rho Family GTPases

Rho family GTPases represent a large portion of the Ras superfamily of small GTPases, with an average size of approximately 21 kD (Rossman, Der et al. 2005). Reorganization and polymerization of the actin cytoskeleton is modulated by members of the Rho family of small GTPases, of which there are approximately twenty currently known in mammals (Cain and Ridley 2009). The best studied of these twenty are Rho, Rac, and Cdc42. GTPases undergo conformational changes as they cycle between an inactive (GDP-bound) and an active (GTP-bound) state (Hall 1998; Bishop 2000; Ridley 2001; Etienne-Manneville 2004). The cycling of these molecules between an active and inactive state is regulated by guanine nucleotide exchange factors (GEFs), which activate GTPases by facilitating the exchange of GDP for GTP, and GTPase-activating proteins (GAPs) that stimulate intrinsic GTPase activity, promoting GTP hydrolysis and thereby returning the GTPase to its inactive state. In the active conformational state, GTPases can interact with downstream targets, or effector molecules, to elicit a biological response. In effect, GTPases act as molecular switches in signaling cascades (Cerione and Zheng 1996; Aspenström 1999).

Rho GTPases are well established as regulators of adhesion formation (Hall 1994; Turner, Brown et al. 1999; Petit and Thiery 2000) and other cytoskeletal modulation (Fig. 4). Rac stimulates extension of the initial lamellipodial protrusion and promotes the formation of associated adhesions (Ridley and Hall 1992; Hotchin and Hall 1995; Petit and Thiery 2000). Rho is associated with maturation of nascent adhesions as well as the formation of

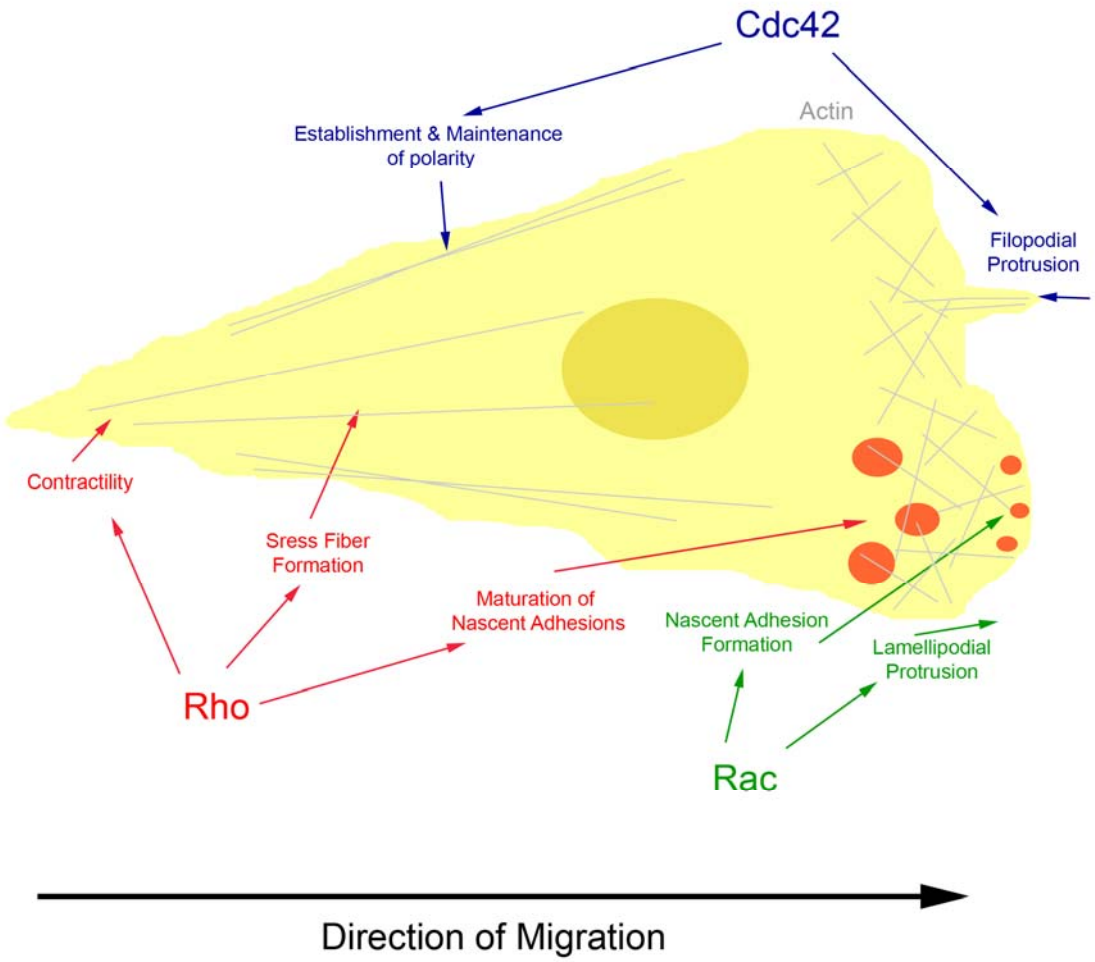

Figure 5. Rac, Rho, and Cdc42 are key regulators of cell migration. Rac activation stimulates protrusion of the lamellipodium and the formation of nascent adhesions that stabilize the protrusion. Rho activity promotes stress fiber formation and generation of contractile forces that allow detachment and retraction of the rear of the migrating cell. Rho also promotes maturation of nascent adhesions. Cdc42 stimulates the formation of filopodial protrusions and promotes the establishment and maintenance of cell polarity, which is essential for directional migration.

stress fibers, which are thought to impede migration (Ridley and Hall 1992; Hotchin and Hall 1995; Chrzanowska-Wodnicka and Burridge 1996; Rottner, Hall et al. 1999; Petit and Thiery 2000). Cdc42 regulates filopodium formation and promotes the establishment and maintenance of cell polarization, which is essential for directional migration (Nobes and Hall 1995). While the role of these Rho Family GTPases in regulating cell migration and adhesion dynamics is well established, much less is known about the regulatory molecules that contribute to these processes and the mechanisms by which they function.

Interactions and Crosstalk Between Rho GTPase Pathways

The Rho family GTPases must coordinate their activity in order to effectively control migratory behavior. Therefore, it is not surprising that much crosstalk occurs among Rac, Rho, and Cdc42 pathways. One pathway consists of Cdc42 activation at the leading edge of a cell promoting filopodial formation and Rac activation, leading to protrusion of the leading edge and finally stimulating Rho activation which promotes the necessary contractile forces for effective migration (Kozma, Ahmed et al. 1995; Nobes and Hall 1995; Burridge and Wennerberg 2004). In particular, the protrusive activity of Rac is frequently considered to act as an opposing force to the stabilizing stress fiber and focal adhesion formation and contractile forces generated in a Rho-dependent manner. It follows that Rac and Rho must coordinate their activation states so that they compliment one another, rather than hindering the other's actions. This frequently occurs via crosstalk in the form of positive or negative regulation of

one GTPase by another. Although there are several examples of Rac acting upstream of Rho to activate it, interestingly, there are several cases showing that Rac can also function to inactivate Rho, or vice versa (Sander, ten Klooster et al. 1999; Burridge and Wennerberg 2004). In Swiss 3T3 cells, the Rac GEF Tiam1 activates Rac, causing a decrease in Rho activation following stimulation with PDGF. However, it was noted that basal levels of Rho activation are necessary to maintain Rac-mediated morphology in these cells, which supports the idea that a positive feedback loop exists between Rho and Rac (Sander, ten Klooster et al. 1999). Others have found, with the use of FRET biosensors, that Rac activation leads to a localized decrease in active Rho at the leading edge of migrating HEK293T cells in response to stimulation with PDGF (Pertz, Hodgson et al. 2006). Clearly, cell shape and motility are controlled by a delicate balance between Rac and Rho activation.

This crosstalk can be mediated in a number of ways. One GTPase may stimulate activation of another by activating a GEF. Conversely, one GTPase may inhibit activation of another by activating a GAP. One example of this GAP-mediated crosstalk is Rac-mediated activation of p190 RhoGAP, which leads to Rho down-regulation (Nimnual, Taylor et al. 2003; Burridge and Wennerberg 2004; Bustos, Forget et al. 2008). Interestingly, this can occur through multiple mechanisms. One group found the isoform p190B RhoGAP binds directly to active Rac in COS7 cells in order to decrease Rho activation and thereby regulate cell shape (Bustos, Forget et al. 2008). Another group has shown reactive oxygen species production can be stimulated by Rac activation, which

then inhibits low-molecular-weight protein tyrosine phosphatases, leading to an increase of p190 RhoGAP phosphorylation and activation. This ultimately leads to a decrease in Rho activation, and is required for the promotion of Rac-induced membrane ruffling and integrin-mediated cell spreading in HeLa cells (Nimnual, Taylor et al. 2003; Burridge and Wennerberg 2004). GTPases may also affect one another's activity indirectly, through interaction of their downstream effectors, circumventing direct regulation of downstream GTPases. For example, when constitutively active, the Rac and Cdc42 effector, PAK, causes the dissolution of focal adhesions and stress fibers. It has been suggested that it does so by phosphorylating and thereby inhibiting MLCK, a downstream effector of Rho (Burridge and Wennerberg 2004). Because there are multiple signaling pathways mediated by Rho GTPases, several different GEFs and GAPs, and a wide variety of downstream effectors, it is clear that crosstalk is an important and complex mechanism of signal coordination regulating the steps of cell migration.

Regulation of Rho Family GTPases

The activation of Rho family GTPases is tightly controlled by guanine nucleotide exchange factors (GEFs). Currently, there are approximately 80 known GEFs (Cain and Ridley 2009). All Rho family GEFs contain a Dbl-homology (DH) domain. This domain catalyzes the dissociation of GDP (Sjoblom, Jones et al. 2006), while stabilizing the nucleotide-free intermediary conformation (Kaibuchi, Kuroda et al. 1999), in which the released GDP is preferentially replaced by GTP because of a favorable intracellular ratio of GTP to GDP

(Schmidt and Hall 2002; Rossman, Der et al. 2005). GTPase specificity is thought to be determined by the interface between the GTPase and non-conserved regions of the DH domain. Biochemical studies by several labs have verified the importance of these non-conserved regions, and often a single amino acid change terminates normal GTPase recognition and allows for recognition of another Rho GTPase not typically affected by the GEF (Manser, Loo et al. 1998; Karnoub AE 2001; Rossman, Der et al. 2005).

Adjacent, and C-terminal to the DH domain, is a pleckstrin homology (PH) domain. The PH domain is known for lipid, phosphoinositide, and protein-binding, and is thought to be responsible for membrane-targeting. However, it may also affect the activity of the DH domain, since both domains together are typically the minimum functional unit essential for GEF activity (Rameh, Arvidsson et al. 1997; Schmidt and Hall 2002; Jaffe, Hall et al. 2005) and both may play a role in GTPase binding. Cellular localization, interaction of phosphoinositides with the PH domain, tyrosine (Tyr) phosphorylation, oligomerization, and other protein-protein interactions via the PH domain frequently regulate Rho family GEFs (Aghazadeh, Lowry et al. 2000; Das, Shu et al. 2000; Bi, Debreceni et al. 2001; Russo, Gao et al. 2001; Kubiseski, Culotti et al. 2003; Schiller, Chakrabarti et al. 2006; Itoh, Kiyokawa et al. 2008).

In addition, some GEFs also have Src homology (SH2 and SH3) domains, which mediate protein-protein interactions and are commonly found in molecules involved in signal transduction. Approximately one-third of all known human Rho family GEFs contain at least one SH3 domain, and some studies indicate a

possible role for them in GEF activity regulation (Schiller, Chakrabarti et al. 2006; Hamann, Lubking et al. 2007; Kawasaki, Sagara et al. 2007; Mitin, Betts et al. 2007). For example, the SH3 domain of the RhoA/Cdc42 GEF, Ost, appears to inhibit its cellular transforming activity (Lorenzi MV 1999; Schiller, Chakrabarti et al. 2006) by promoting normal Rho GTPase activation (Rossman, Der et al. 2005). The SH3 domains of the Rac/Cdc42 GEFs, Asef1 and Asef2, are necessary for the auto-inhibition of these molecules (Hamann, Lubking et al. 2007; Kawasaki, Sagara et al. 2007; Murayama, Shirouzu et al. 2007). Additionally, the Rho GEF, Trio, which contains two SH3 domains, requires the presence of its most N-terminal SH3 domain to exert an active effect on downstream effectors (Estrach, Schmidt et al. 2002; Schiller, Chakrabarti et al. 2006). More commonly, the SH3 domain is required for docking with other proteins, such as scaffolds and effectors (Petit and Thiery 2000).

Asef Family Guanine Nucleotide Exchange Factors

Asef1

Asef (now Asef1) is a Rho Family GEF that activates Rac1 and Cdc42, originally identified in a two-hybrid screening of a human fetal brain library, using the armadillo binding repeat of APC as bait. Like all known Rho GEFs, it contains conserved DH and PH domains, and an SH3 domain which is found in some, but not all Rho GEFs. Interestingly, Asef1 activity appears to be stimulated by APC binding which has been shown to occur by APC-mediated relief of Asef1's auto-

inhibitory conformation in which the SH3 domain interacts with a C-terminal region of the molecule, thereby blocking the catalytic DH domain and simultaneously making interaction with other proteins via the SH3 domain energetically unfavorable (Kawasaki, Senda et al. 2000; Mitin, Betts et al. 2007). Asef1 contains two conserved Tyr residues, one of which (Tyr 104) is located within the APC-binding region (ABR). Asef1 appears to be translocated to the plasma membrane in a PH domain-dependent manner, then phosphorylated at Tyr 104 and activated in a Src-dependent manner (Itoh, Kiyokawa et al. 2008).

Specificity of Asef1 for Cdc42 or Rac1 activation is thought to be cell-type specific, in at least some cases. In MDCK cells, APC and Asef1 co-localize in the cytoplasm and concentrate within lamellipodial ruffles at the leading edge, and Asef1 expression is associated with an increase in Rac-mediated morphology including increased protrusion and ruffling (Kawasaki, Senda et al. 2000). Others have found in mouse fibroblasts that Asef1 expression coincides with filopodial formation; morphology characteristic of Cdc42 activation. Moreover, they have rescued this Asef1-mediated phenotype by blocking Cdc42 with expression of the Cdc42/Rac Interacting Binding (CRIB) domain of Cdc42 effector, N-Wasp, which effectively acts as a Cdc42 dominant negative (Mitin, Betts et al. 2007). Because Asef1 activates Cdc42 and Rac1, and its expression increases the morphology associated with these important regulators of the actin cytoskeleton, Asef1 is thought to be an important regulator of cell migration.

Collybistin

Collybistin is an Asef family GEF highly expressed in brain and found in very low levels in heart and skeletal muscle with no evidence of expression in other tissues. It was first identified in a yeast two-hybrid screening for molecules that interacted with gephyrin, a scaffolding protein thought to organize postsynaptic inhibitory glycine receptor clusters and γ-aminobutyric acid type A receptors ($GABA_ARs$) and anchor these receptors to the cytoskeleton in neurons (Kins, Betz et al. 2000). Collybistin induces gephyrin clustering and has been shown to be necessary in hippocampal neurons for proper localization and maintenance of gephyrin and gephyrin-dependent $GABA_ARs$ in the postsynaptic membrane (Papadopoulos, Eulenburg et al. 2008). Although collybistin is a Dbl family GEF, having the requisite DH and PH domains, it lacks the ABR found in the other Asef family GEFs (Kins, Betz et al. 2000; Mitin, Betts et al. 2007). Additionally, only some isoforms include an SH3 domain (Kins, Betz et al. 2000). Collybistin is a Cdc42-specific GEF, and its binding to gephyrin appears to act as a negative regulator of Cdc42 activation (Xiang, Kim et al. 2006; Papadopoulos, Eulenburg et al. 2008). This has led some researchers to speculate that collybistin plays an important role in the initial stages of synapse formation, but must be turned off when no longer required (Papadopoulos, Eulenburg et al. 2008). Mutations in collybistin have been linked to epileptic pathology, further supporting a role for this GEF in synaptogenesis (Harvey, Duguid et al. 2004).

Asef2

The Rho Family GEF, Asef2, was identified in a database search for molecules with homology to Asef1. Because it has only recently been described, very little is known about Asef2. Most studies to date have focused on the biochemical nature and structure of the molecule. Like Asef1, it activates Cdc42 and Rac1, and comprises a DH, PH, and SH3 domain, with an ABR adjacent and N-terminal to the SH3 domain (Hamann, Lubking et al. 2007; Kawasaki, Sagara et al. 2007). The ABR in Asef2 shares 50% homology with that of Asef1. Interestingly, yeast two-hybrid assays determined that the Asef2 ABR alone (or SH3 domain alone) is not enough to bind APC, but rather, the SH3 domain is also required for effective APC binding (Kawasaki, Sagara et al. 2007). APC binding stimulates Asef2 activity, and deletion of the N-terminus of the molecule including the ABR and SH3 domain activates Asef2 without need of APC stimulation, apparently relieving auto-inhibition caused by an intra-molecular interaction between a C-Terminal region of Asef2 and the SH3 domain, with the ABR acting to stabilize this interaction. Similar to the Rho Family GEF, Vav, Asef2 depends on it's C-Terminal tail for proper function—deletion of this region does not render Asef2 constitutively active like deletion of the N-Terminus (including the ABR and SH3 domain) does, instead it prevents proper binding of Cdc42 (Hamann, Lubking et al. 2007). Surprisingly, Asef2 lacks the Tyr phosphorylation required for activity of Asef1, and therefore Asef2 activity appears to be Src-independent (Itoh, Kiyokawa et al. 2008).

Asef2 seems to activate Cdc42 and Rac1 specifically in a cell-type specific manner in many cases. Expression of activated Asef2 in MDCK cells increased Rac1-type morphology including increased lamellipodial ruffles. In contrast, activated Asef2 in HeLa cells increased filopodia but not ruffling, associated with Cdc42 activation. In trans-well assays used to assess cell motility, MDCK cells expressing full-length Asef2 showed increased motility, and cells expressing constitutively active Asef2 had further enhanced motility, quantified by the number of cells able to migrate through pores in a membrane separating two wells. Further, in SW480 colorectal cancer cells expressing a truncated APC associated with pathology and, specifically, with increased Asef1-mediated motility, shRNA targeting Asef2 lead to decreased migration in a trans-well. This implies full-length Asef2 and mutant APC are important for promotion of SW480 cell migration, and suggests a role for Asef2 in colorectal cancer pathology (Kawasaki, Sagara et al. 2007).

Little is known about Asef2's downstream effector and other associated molecules and the molecular mechanism by which it regulates cell migration. Interestingly, Asef2 and APC associate with the scaffolding protein, Neurabin2. In HeLa cells, Neurabin2 and Asef2 co-localize and translocate from the cytoplasm to the cell periphery when stimulated by HGF. Knockdown of Neurabin2 significantly reduces the ability of HeLa cells to migrate in a trans-well assay after HGF stimulation and decreases the number of filopodia, though Neurabin2 has no impact on Asef2-mediated Cdc42 or Rac1 activation (Sagara, Kawasaki et al. 2009). This indicates that Neurabin2 associates with Asef2, but does not act to

regulate migration and actin modulation directly through Asef2 activation. Much is left to discover about the molecules with which Asef2 interacts, both directly and indirectly, and how these molecules cooperate to regulate cell migration.

Role of PI3K and its Phosphoinositide Products in Cell Migration

PI3Ks act by phosphorylating phosphoinositides at the 3' position that go on to bind and regulate downstream effectors, thereby acting as second messengers. There are three classes of PI3K family lipid kinases which are characterized by their sequence homology and substrate specificity. Class I PI3Ks are the most closely studied group, and can be activated by tyrosine phosphorylation, G-protein coupled receptor (GPCR) signaling and Ras signaling. Class I PI3Ks and their lipid products, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3, have been shown to regulate cell polarity and migration (Carpenter and Cantley 1996; Cain and Ridley 2009). Class I PI3Ks are frequently studied with the use of the chemical inhibitors, wortmannin and LY294002. Class II PI3Ks have also been implicated in cell adhesion and migration, but are poorly understood at this time due to a lack of specific chemical inhibitors. Only one Class III PI3K has been identified in mammals, but has not been shown to play any role in migration (Carpenter and Cantley 1996).

A complex relationship exists between the Rho GTPases and PI3K and its products. Rac and Cdc42 have been shown to bind and activate PI3K. Interestingly, Rac activity stimulates PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 synthesis (Tolias, Cantley et al. 1995) and actin uncapping in vitro and in vivo (Cain and

Ridley 2009). Additionally, active Rac, PtdIns(3,4)P_2, and PtdIns(3,4,5)P_3 are concentrated at the leading edge and in ruffles of migrating cells (Tolias, Cantley et al. 1995; Kraynov, Chamberlain et al. 2000). PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 binding regulates activity of many Rho Family GEFs and GAPs, such as pRex1 and ArhGAP15, implying a possible positive feedback loop between Rho GTPases, such as Rac, and PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 synthesis, which could lead to a rapid response to extracellular stimuli, such as chemokines (Kraynov, Chamberlain et al. 2000; Cain and Ridley 2009). While Rho has been implicated in PtdIns(3,4)P_2 synthesis in fibroblasts, and Arp2/3-driven actin polymerization can be stimulated by phosphoinositide activity occurring downstream of Rac, Rac and Rho can also be activated downstream of phosphoinositide signaling (Kraynov, Chamberlain et al. 2000; Cain and Ridley 2009).

Phosphoinositides are key regulators of actin-modulating proteins, and as such, play an important role in regulating cell migration, adhesion, polarity and chemotaxis (Fenteany 2003; Cain and Ridley 2009). PI3K-mediated production of PtdIns(3,4,5)P_3 is sufficient to recruit WAVE2 to the plasma membrane, where it binds to and is subsequently activated by PtdIns(3,4,5)P_3. This activation of WAVE2 occurs downstream of Rac activation, and leads to rapid Arp2/3-mediated polymerization of the actin cytoskeleton, promoting protrusion of the lamellipodium (Oikawa, Yamaguchi et al. 2004). The ERM protein, ezrin, binds PI3K and regulates its cellular activity that leads to Akt activation (Yin and Janmey 2003). In addition, the lipid product of PI3K is thought to directly bind

and activate Akt through its PH domain following PI3K-stimulation by platelet derived growth factor (PDGF) (Carpenter and Cantley 1996).

Role of Akt in Cell Migration

Akt, also known as Protein Kinase B (PKB), was originally identified as a homologue to the transforming oncogene, v-Akt. It is a serine/threonine kinase and a downstream effector of PI3K that is recruited to the plasma membrane via interaction of its PH domain with the PI3K lipid products, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3, which, along with other molecules such as 3-phosphoinositide kinase 1 (PKD1), activate Akt (Kim, Kim et al. 2001). The Akt/PKB family of kinases includes three structurally homologous isoforms, Akt1, -2, and -3. Isoforms -1 and -2 are expressed ubiquitously, whereas Akt3 expression is limited, and is the predominant isoform found in the brain (Irie, Pearline et al. 2005). Once activated, Akt may affect a number of different responses in cells, and has been implicated in regulation of cell survival and glucose metabolism, as well as promoting chemotaxis (Kim, Kim et al. 2001). When Akt signaling is enhanced or deregulated, as it frequently is in many human cancers (Bellacosa, Kumar et al. 2005), it can play an important role in promoting cancer cell invasion and metastasis (Irie, Pearline et al. 2005).

Akt can promote invasion and metastasis by promoting an epithelial–mesenchymal transition (EMT) and by stimulating the secretion of metalloproteinases (Kim, Kim et al. 2001; Bellacosa, Kumar et al. 2005). The EMT is an important part of normal embryological development. It essentially

consists of a series of morphological changes that occur in epithelial-like cells that have three membrane domains: apical, lateral, and basal; have tight junctions between apical and lateral domains; are cohesive, forming cell layers; have a polarized arrangement of organelles and cytoskeletal components, oriented apicobasally; and they lack individual motility within their local environment. During this transition, epithelial cells become more like mesenchyme. They loosen their cell-cell contacts and no longer form continuous sheets of cells. They lose their apicobasal membrane distribution, and their organelles and cytoskeletal components are no longer oriented in this manner. Importantly, after undergoing EMT, cells become motile and possess invasive properties (Larue and Bellacosa 2005). It is not surprising that tumor cells might undergo a similar process allowing them to separate from a tumor mass during metastasis.

Another important role Akt plays in invasion and metastasis is promotion of metalloproteinase secretion. In particular, the matrix metalloproteinases (MMPs) are important enzymes that degrade the ECM in a zinc-dependent manner, and are thought to play a critical role in tumor invasion. In order for a tumor cell to invade the surrounding tissue, it must be able to break through biological barriers such as basement membranes, in addition to expressing changes in adhesion and motility. This is facilitated by matrix degradation mediated by MMPs. MMP-9 expression appears to be regulated by Akt via activation of the transcription factor, nuclear factor κB (NF-κB) (Kim, Kim et al. 2001). This suggests Akt promotes invasion through an MMP-9-mediated

mechanism. Thus, Akt appears to promote invasion and metastasis by enabling cells to dissociate from confluent, tightly-knit layers, and transform into a less rigidly organized morphology that is motile and can increase secretion of MMPs that aid the cell in breaking and maneuvering through biological barriers.

In migrating HT-1080 human fibrosarcoma cells, a highly metastatic, invasive cancer cell line, GFP-Akt localizes to the leading edge membrane of migrating cells. Using a GFP-tagged PH domain of Akt as an indicator of PI3K activity, one research group also showed similar localization of active PI3K in migrating cells, and Akt localization was abrogated by the use of PI3K chemical inhibitors, suggesting PI3K acts locally on Akt to promote protrusion and migration. With the use of dominant negative, kinase dead, and lipid-binding defective mutants, it was determined that while Akt expression increases the rate of migration in a trans-well assay, Akt-mediated promotion of migration is dependent upon both Akt's kinase activity and proper membrane translocation. Possible downstream effectors of Akt that would link it to actin cytoskeleton rearrangement are filamin and PAK. Filamin binds to Akt in vivo, localizes to the leading edge of migrating cells, and is essential for migration. Akt is also capable of stimulating PAK independently of Rac activation (Kim, Kim et al. 2001).

Summary and Hypothesis

Cell migration is a complex process that can be broken down into four mechanical steps: protrusion of the leading edge, stabilization of the protrusion with cell-matrix adhesions, translocation of the cell body in the direction of the

protrusion, and finally, a contraction of the cell rear that leads to a release of the extracellular matrix. Migration is thought to be driven primarily by actin dynamics. The actin cytoskeleton is tightly regulated by the Rho family of small GTPases, including Rac, Rho, and Cdc42, that act as molecular switches regulated by their guanine nucleotide-bound state. GTPases are activated by GEFs, and inactivated by GAPs. In the active state they can interact with downstream effectors to elicit a biological response. The Rho GTPases are fairly well described in the literature, however very little is known about the individual GEFs that regulate their activity or the molecular mechanisms by which they function. Here, we investigate the role of a recently-described Rho family GEF, Asef2 and its molecular mechanism in regulating cell migration and its underlying processes. Asef2 is a member of the Asef family of GEFs, which typically activate Rac and/or Cdc42, and have been implicated in promoting migration and the protrusion of actin cytoskeletal structures. We hypothesize Asef2 acts through Rac, Rho, and/or Cdc42 to promote cell migration and adhesion turnover in a mechanism that may be dependent on other regulators of migration, such as kinases including PI3K and Akt/PKB. This enhanced Asef2-mediated migration may underlie a mechanism of cancer metastasis, which is linked to poor prognosis.

CHAPTER II

MATERIALS AND METHODS

Generation of stable cell lines via retroviral transduction

Cell lines that stably express GFP (for use as control) or GFP-Asef2 were generated using a retroviral transduction system developed in the Nolan lab at Stanford University (Fig. 6). GFP-Asef2 was inserted into the LZRS^{neo} retroviral vector following PCR-mediated cloning from a C1 vector containing GFP-Asef2 that attached EcoRI and BamHI restriction sites to the construct. GFP was inserted into the LZRS^{neo} vector similarly. Once GFP-Asef2-LZRS^{neo} and GFP-LZRS^{neo} were generated, purified, and the DNA sequences verified, a calcium chloride delivery system was used to transfect Phoenix 293 packaging cells. Briefly, Phoenix cells were plated at approximately 80% confluency in a T75 culture flask and incubated at 37°C overnight. 5-10 min prior to transfection, 4 µl chloroquine (25 mM) was added to the media on the Phoenix cells. A cocktail containing 62 µl calcium chloride, 8 µl (1 mg/ml) DNA, and 430 µl ddH_2O was added gently to the cells, followed by 500 µl HBS (pH 7.0). Cells were incubated at 37°C for 7-9 h, and the media refreshed. Phoenix cells were drug selected with 5 µg/ml puromycin for one week. In order to harvest virus from the Phoenix cells, they were plated at medium density in a T75 flask for 24 h (one flask GFP, one flask GFP-Asef2), where they were cultured in 12 ml DMEM supplemented

with 10% heat-inactivated FBS and 1% penicillin/streptomycin. Meanwhile, 300,000 HT-1080 target cells were plated in each of two T75 flasks where they were cultured in 12 ml DMEM supplemented with 10% heat-inactivated FBS and 1% penicillin/streptomycin. All cells were incubated overnight at 37°C. Virus was harvested by removing the supernatant from the Phoenix cells and filtering it with a 0.45μm-pore syringe-end filter, collecting the filtered supernatant in a vial. To each vial of filtered virus supernatant, polybrene was added for a final concentration of 4 μg/ml and gently mixed. Media from the target cells was aspirated and replaced with the virus/polybrene mixture. The target cells were incubated at 37°C for 9 h, then the media was aspirated and refreshed with DMEM supplemented with 10% FBS and 1% penicillin/streptomycin and the cells are incubated at 37°C overnight. Cells were drug selected with 400 μg/ml G418 for 10 days. The resulting stably-expressing GFP and GFP-Asef2 HT-1080 cells were sorted by FACS to yield homogenous populations based on expression level, as determined by fluorescence intensity. GFP and GFP-Asef2 cell populations that fluoresced with comparable intensity were chosen for experimentation.

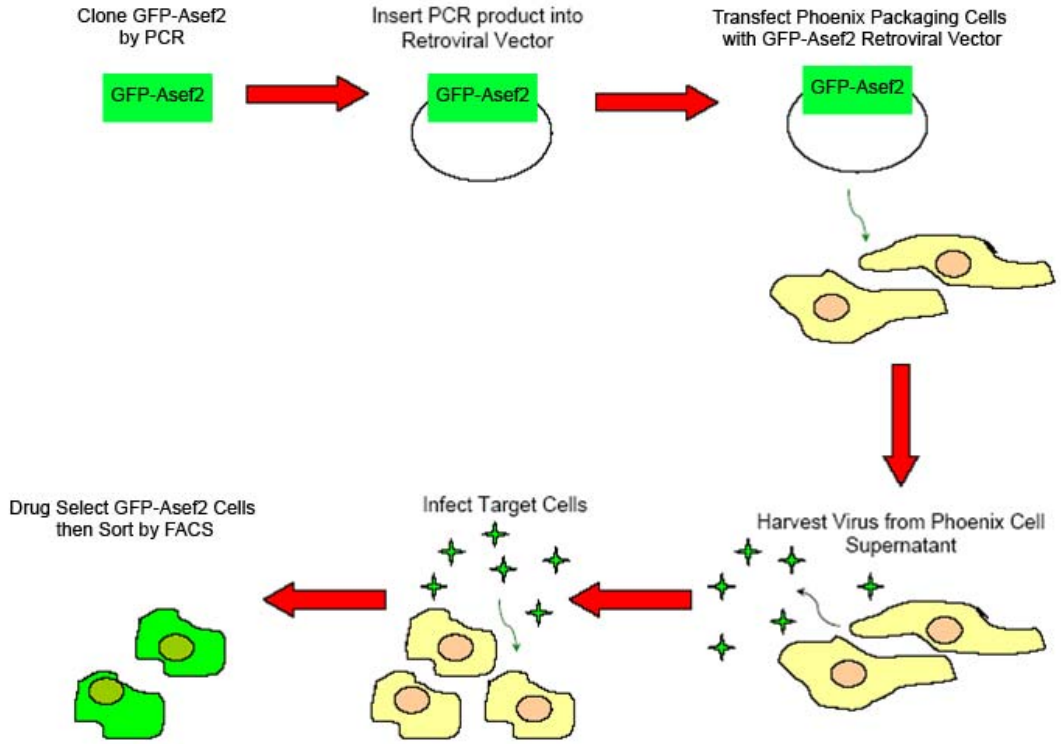

Figure 6. HT-1080 cells stably-expressing GFP or GFP-Asef2 were generated by a retroviral transduction system. Stable cell lines expressing GFP or GFP-Asef2 were generated as follows: GFP or GFP-Asef2 was cloned from the C1 vector by PCR, then the PCR product was inserted into the LZRS^{neo} retroviral vector. Phoenix 293 packaging cells were transfected with the LZRS vector containing the construct, then drug selected with puromycin, and virus was harvested from the transfected packaging cells. Harvested virus was applied to the HT-1080 target cells and stably-expressing GFP or GFP-Asef2 cells were selected with G418. Populations were sorted according to expression level by FACS.

Trans-well Migration Assay

For trans-well migration assays, MCF-7 human carcinoma cells were transiently transfected with either GFP (control) or GFP-Asef2 with Lipofectamine™ 2000 according to the manufacturer's instructions. 50,000 transfected MCF-7 cells were added to a 24-well trans-well plate (includes 12 inserts) with 80 μm pores previously coated with Fn. The cells were incubated in the trans-well plate in serum-free DMEM at 37°C for 3 h. The remaining cells were swabbed from the inside of the insert and the membrane was fixed in methanol for 15 min at room temperature. Membranes were then stained with 0.1% crystal violet for 15 min before washing three times in PBS. Crystal violet stain was eluted from the membranes with 10% acetic acid and the number of cells that passed through the pores of the insert was determined by measuring absorbance at 600 nm with a spectrophotometer.

Scratch-wound Migration Assay

For scratch-wound assays, 5 x 10^5 GFP-expressing or GFP-Asef2-expressing HT-1080 cells were plated on 35 mm dishes previously coated with 10 μg/ml Fn in PBS for 1 h at 37°C. The cells were allowed to adhere to the substrate while incubated overnight at 37°C in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. The resulting monolayer was then scratched with a 1 ml pipette tip, and refreshed with CCM1 with 2% FBS immediately prior to imaging. Cell migration data were generated from time-lapse images collected at 5 min intervals for 12 h, and used to calculate the wound-edge velocity, which

was determined by dividing the mean net displacement of the wound edge by the time interval.

CHAPTER III

THE RHO FAMILY GEF ASEF2 ACTIVATES RAC TO MODULATE ADHESION AND ACTIN DYNAMICS AND THEREBY REGULATE CELL MIGRATION

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Abstract

Asef2 is a recently identified Rho family guanine nucleotide exchange factor (GEF) that has been implicated in the modulation of actin, but its function in cell migration and adhesion dynamics is not well understood. In this study, we show that Asef2 is an important regulator of cell migration and adhesion assembly and disassembly (turnover). Asef2 localizes with actin at the leading edge of cells. Knockdown of endogenous Asef2 impairs migration and significantly slows the turnover of adhesions. Asef2 enhances both Rac1 and Cdc42 activity in HT-1080 cells, but only Rac1 is critical for the Asef2-promoted increase in migration and adhesion turnover. Phosphoinositide 3-kinase (PI3K) and the serine/threonine kinase Akt are also essential for the Asef2-mediated effects on migration and adhesion turnover. Consistent with this, Asef2 increases the amount of active Akt at the leading edge of cells. Asef2 signaling leads to an overall decrease in Rho activity, which is critical for stimulating migration and adhesion dynamics. Thus, our results reveal an important new role for Asef2 in promoting cell migration and rapid adhesion turnover by coordinately regulating the activities of Rho family GTPases.

Introduction

Cell migration is a highly coordinated, tightly regulated process that begins with the extension of a protrusion, which is most likely driven by the polymerization of the actin cytoskeleton at the leading edge of cells (Wang 1985; Carson, Weber et al. 1986; Lauffenburger and Horwitz 1996; Borisy and Svitkina 2000). The formation of cell-matrix adhesions, which consist of transmembrane integrins along with cytoplasmic signaling and structural proteins, such as paxillin and vinculin, stabilize the protrusion and provide traction for translocation of the cell body forward (Turner, Glenney et al. 1990; Miyamoto, Teramoto et al. 1995; Beningo, Dembo et al. 2001; Zaidel-Bar, Ballestrem et al. 2003). The constant assembly and disassembly of nascent adhesions, which is termed adhesion turnover, within leading edge protrusions is essential for continuous migration to occur (Webb, Donais et al. 2004), but is poorly understood on a molecular level. Emerging data indicate that the actin cytoskeleton plays a critical role in the formation, dynamics, and turnover of leading edge adhesions (Alexandrova, Arnold et al. 2008; Choi, Vicente-Manzanares et al. 2008; Lim, Lim et al. 2008), which underscores the importance of actin regulation in cell migration and its underlying processes.

Polymerization and reorganization of actin are modulated by members of the Rho family of small GTPases, which includes Rho, Rac, and Cdc42. Rho GTPases, like many small GTPases, function as molecular switches and cycle between an inactive GDP-bound form and an active GTP-bound state (Hall 1998; Ridley 2001). The cycling of these molecules is tightly controlled by GEFs, which

serve to activate the GTPases by promoting the exchange of GDP for GTP, and GTPase-activating proteins (GAPs) that increase their intrinsic GTPase activity and return them to an inactive state. Once activated, the GTPases can convert upstream molecular signals into coordinated rearrangements of the actin cytoskeleton by modulating the activity of downstream effectors, which can ultimately contribute to the regulation of cell migration and adhesion dynamics (Ridley 2001).

Rac can promote migration by stimulating the initial extension of the leading edge protrusion and by subsequently inducing the formation of nascent adhesions within this region (Ridley and Hall 1992; Nobes and Hall 1995; Rottner, Hall et al. 1999). Rho activity is associated with the maturation of nascent adhesions into larger, more mature focal adhesions and with the formation of stress fibers, which are thought to impede cell migration (Ridley and Hall 1992; Chrzanowska-Wodnicka and Burridge 1996; Rottner, Hall et al. 1999). Cdc42 can regulate the formation of filopodia as well as promote the establishment and maintenance of cell polarity, which is essential for directed migration (Nobes and Hall 1995; Etienne-Manneville and Hall 2003). While the role of these GTPases in regulating migration and adhesion dynamics is well established, much less is known about the specific GEFs that contribute to these processes and the mechanisms by which they function.

Asef2 is a recently identified Rho family GEF that is composed of a N-terminal APC binding region (ABR), which interacts with the tumor suppressor *adenomatous polyposis coli* (APC), an adjacent Src homology 3 (SH3) domain, a

central Dbl homology (DH) domain, and a Pleckstrin homology (PH) domain (Kawasaki, Sagara et al. 2007). The binding of APC dramatically enhances the GEF activity of Asef2 by relieving it from an autoinhibitory conformation in which the ABR/SH3 domains are associated with the C-terminus of the molecule (Hamann, Lubking et al. 2007). Following activation, Asef2 significantly increases the level of active Rac1 and Cdc42 in epithelial cells, indicating it has GEF activity toward these GTPases (Kawasaki, Sagara et al. 2007). Asef2 is thought to regulate actin dynamics possibly through its interaction with the F-actin binding protein Neurabin2 (Sagara, Kawasaki et al. 2009). It is also implicated in the modulation of cell migration (Sagara, Kawasaki et al. 2009), but the molecular mechanisms by which it contributes to this process are currently unknown.

In this study, we show Asef2 promotes cell migration and the rapid turnover of adhesions. It increases both Rac1 and Cdc42 activity, but only Rac is essential for the Asef2-mediated effects on migration and adhesion turnover. Asef2 regulates migration and adhesion dynamics through a mechanism that is dependent on PI3K and Akt. Asef2 also decreases the amount of active Rho, which is critical for Asef2-promoted migration. Thus, our results reveal an important function for Asef2 in regulating migration and adhesion turnover via a previously unknown mechanism, involving Rac, PI3K, Akt, and Rho.

Materials and Methods

Antibodies and reagents

A rabbit polyclonal antibody against Asef2 was generated by 21st Century Biochemicals (Marlboro, MA) using the N- and C-terminal peptides MTSASPEDQNAPVGC and AEPKRKSSLFWHTFNRLTPFRK, respectively, as antigen. Myc 9E10 polyclonal antibody, Cdc42-specific monoclonal antibody (clone B8), Rac1 C-14 polyclonal antibody, and phospho-Akt (Thr308) polyclonal antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Alexa Fluor® 488 and 555 anti-rabbit, Alexa Fluor® 488 and 555 anti-mouse, Alexa Fluor® 680 anti-mouse, and Alexa Fluor® 647-phalloidin were purchased from Molecular Probes (Eugene, OR). IRDye® 800 anti-mouse and 800 anti-rabbit were from Rockland Immunochemicals (Gilbertsville, PA). Wortmannin was purchased from VWR. Fibronectin, β-actin (clone AC-15) monoclonal antibody, FLAG M2 monoclonal antibody, α-tubulin antibody, vinculin (clone VIN-11-5) monoclonal antibody, and TRITC-phalloidin were from Sigma. Paxillin monoclonal antibody was obtained from BD Bioscience Pharmingen (San Diego, CA). Y-27632 and calyculin A were purchased from CALBIOCHEM®. LY294002 was from Cell Signaling (Beverly, MA). Glutathione Sepharose beads came from Amersham. CCM1 was purchased from Hyclone. Secramine A was generously provided by the laboratories of Tomas Kirchhausen (Harvard Medical School, Cambridge, MA) and Gerald Hammond (University of Louisville, Louisville, KY). It was synthesized by Bo Xu and G.B. Hammond.

Plasmids

The full length Asef2 cDNA was generated by reverse transcription of HEK-293 cell RNA followed by amplification using the SuperScript™ One-Step RT-PCR kit (Invitrogen) with the primers: 5'-ATGACTTCTGCCAGCCCTGAAGACC-3' (forward) and 5'-TTTCCGGAAGGGGGTGAGCCTGTTG-3' (reverse). The Asef2 cDNA was then sequenced and cloned into pEGFP-C3 vector (CLONTECH Laboratories). Small interfering RNA (siRNA) constructs were prepared for Asef2, Rac1, Cdc42 and Akt by ligating 64-mer oligonucleotides into pSUPER vector as previously described (Zhang and Macara 2008). The siRNA oligos contained the following 19-nucleotide target sequences: Asef2 #1, 5'-TTGCGCAGCTAGCCACTAT-3'; Asef2 #2, 5'-TTCGTCAGATTGCGAGTGA-3'; and Cdc42 #2, 5'-AAAGTGGGTGCCTGAGATA-3'. Both Rac1 and Akt target sequences and Cdc42 target sequence #1 have been previously described (Katome, Obata et al. 2003; Chan, Coniglio et al. 2005; Degtyarev, De Maziere et al. 2008; Wegner, Nebhan et al. 2008). DN-Akt (Akt T308A/S473A) and KD-Akt (Akt K179A/T308A/S473A) were kindly provided by Brian Hemmings (Friedrich Miescher Institute, Basel, Switzerland) and Jeffrey Field (University of Pennsylvania, Philadelphia, PA). mCherry-paxillin was a generous gift from Steve Hanks (Vanderbilt University, Nashville, TN). Flag-tagged Asef2Δ204 was generously provided by Daniel Billadeau (Mayo Clinic, Rochester, MN). Wild-type Rac1 and Cdc42, CA-Rho (RhoA V14), and GST-tagged PAK binding

domain were kindly provided by Alan Hall (Memorial Sloan-Kettering Cancer Center, NY). Myc-tagged wild-type RhoA and GST-tagged rhotekin binding domain were a generous gift from Sarita Sastry (University of Texas Medical Branch, Galveston, Texas).

Cell culture and transfection

HEK-293 and HT-1080 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Hyclone) and penicillin/streptomycin (Invitrogen). Phoenix 293 packing cells (from Gary Nolan, Stanford University) were cultured in DMEM with 10% heat-inactivated FBS and penicillin/streptomycin and selected as previously described (Bryce, Clark et al. 2005). Stable HT-1080 cells expressing GFP or GFP-Asef2 were prepared by retroviral transduction as previously described (Bryce, Clark et al. 2005) and selected for stable expression by incubation with 400 μg/ml G418 (Fisher) for 10 days. Cells were sorted into populations based on expression level by fluorescence activated cell sorting (FACS). HT-1080 cells were transiently transfected with Lipofectamine 2000 (Invitrogen) according to instructions from the manufacturer.

Microscopy and image analysis

Images were collected using an inverted Olympus IX71 microscope (Melville, NY) with a Retiga EXi CCD camera (QImaging, Surrey, BC) and a 10X objective (NA 0.3) or a PlanApo 60X OTIRFM objective (NA 1.45). Image

acquisition was controlled using MetaMorph software (Molecular Devices, Sunnyvale, CA), which was interfaced to a Lambda 10-2 automated controller (Sutter Instruments, Novato, CA). An Endow GFP Bandpass filter cube (excitation HQ470/40, emission HQ525/50, Q495LP dichroic mirror) (Chroma, Brattleboro, VT) was used for EGFP and Alexa Fluor® 488. For mCherry and Alexa Fluor® 555, a TRITC/Cy3 cube (excitation HQ545/30, emission HQ610/75, Q570LP dichroic mirror) was used. For three color imaging, a far red filter cube (excitation HQ620/60, emission HQ700/75, Q660LP dichroic mirror) was used. Red fluorescent TIRF images were obtained by exciting with the 543 nm laser line of a HeNe laser (Prairie Technologies, Inc., Middleton, WI).

To quantify the enrichment of GFP-Asef2 and phospho-Akt Thr308, and total Akt at the leading edge of HT-1080 cells, images were collected and analyzed with Metamorph software. The integrated fluorescence intensity was determined for a region of interest at the cell edge and the background fluorescence intensity was subtracted from these values. The fluorescence intensity was then normalized to the unit area.

Immunocytochemistry

Cells were plated on coverslips for 1 h at 37°C and fixed with 4% paraformaldehyde/4% glucose in phosphate buffered saline (PBS) for 5 min. Prior to cell plating, the coverslips were pre-incubated with 10 μg/ml fibronectin (Fn). Following fixation, cells were then permeabilized with 0.2% (v/v) Triton X-100 for 5 min at room temperature. Following each step, cells were washed

three times with PBS. Cells were then blocked with 20% goat serum in PBS for 1 h and incubated with primary antibody for 1 h followed by fluorescently-conjugated secondary antibody for 1 h. Antibodies were diluted in PBS with 5% goat serum. After each step, cells were washed three times with PBS. Coverslips were mounted with Aqua Poly/Mount (Poly-sciences, Inc., Warrington, PA). For total and phospho-Akt staining, cells were treated with a phosphatase inhibitor cocktail containing 1 mM peroxovanadate (Fisher) and 10 nM calyculin A for 15 min at 37°C in the dark prior to fixation.

Migration Assay

For random migration assays, cells were plated at low density on culture dishes, pre-incubated with 10 μg/ml Fn, and allowed to adhere for 1 h at 37°C. Cells were then imaged in phase with a 10X objective every 5 min for 12 h. During microscopy, cells were maintained at 37^{o}C in CCM1 with 2% FBS at pH 7.4. Cell migration data were generated from time-lapse images and used to calculate the migration velocity, which was determined by dividing the mean net displacement of the cell centroid by the time interval. Wind-Rose plots were generated by plotting the XY-coordinates of 4-5 individual cells and transposing cell tracks to a common origin.

Adhesion Turnover Assay

Wild-type and stably expressing GFP or GFP-Asef2 HT-1080 cells were transfected with 0.5 μg mCherry-paxillin cDNA and incubated at 37°C for 24 h. In

some experiments, cells were co-transfected with 0.5 µg mCherry-paxillin cDNA and 2.5 µg Asef2 siRNA #1, Rac1 siRNA #1, Akt siRNA #1, pSUPER vector, scrambled siRNA, or KD-Akt cDNA. Transfected cells were then seeded at low density on glass-bottom 35 mm Fn-coated microscopy dishes and incubated at 37°C for 1 h. Fluorescent time-lapse images were obtained at 15-30 sec intervals and the $t_{1/2}$s for adhesion assembly and disassembly were determined as previously described (Webb, Donais et al. 2004) using Metamorph software.

Rho family GTPase activity assays

HT-1080 stable cell lines were transfected with 3 µg of myc-RhoA, FLAG-Cdc42, or FLAG-Rac1 cDNA and incubated at 37°C for 24 h. In some experiments, cells were co-transfected with 2 µg of Rho GTPase (RhoA, Rac1, or Cdc42) cDNA and 4.5 µg of Asef2 siRNA #1, pSUPER vector, or scrambled siRNA cDNA. Cell lysates were collected and assayed as previously described (Ren 1999). Active GTPase assays were performed in HEK-293 cells as described above except in these experiments, cells were co-transfected with 1 µg GFP or GFP-Asef2 cDNA along with the tagged GTPases.

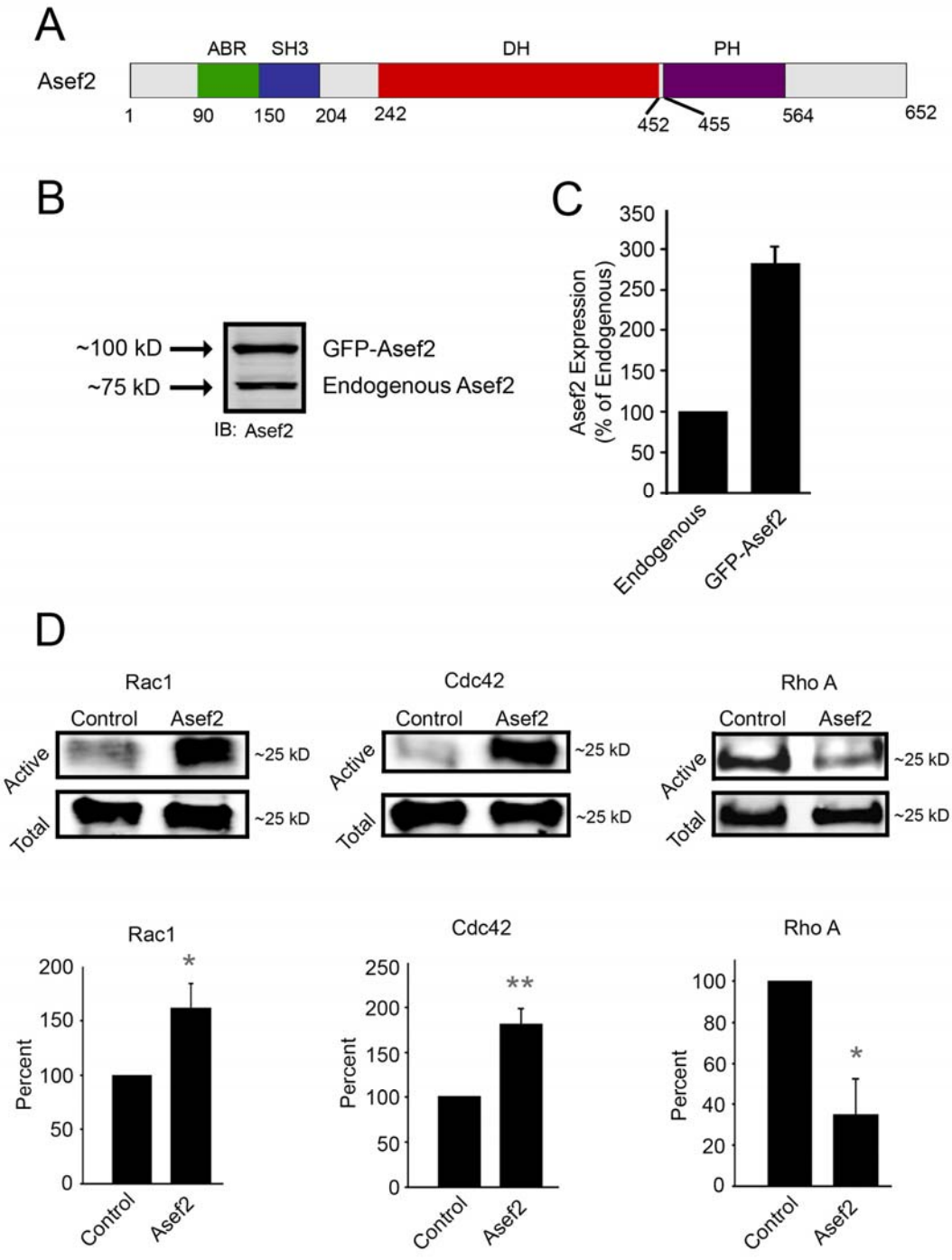
Results

Asef2 increases the activity of Rac and Cdc42, but decreases the amount of active Rho

We recently developed a proteomics-based screen to search for proteins that regulate migration and actin dynamics (Mayhew, Webb et al. 2006). One of the molecules that we detected was a 652 amino acid protein, Asef2 (Fig. 7A), which is reported to have GEF activity for Rac1 and Cdc42 (Kawasaki, Sagara et al. 2007). Since the function of Asef2 in cell migration is not well understood, this provided us with an opportunity to study the role of this protein in regulating migration. We began by examining the effects of Asef2 on Rho family GTPases by assaying for active Rac1, Cdc42, and RhoA in HEK-293 cells expressing GFP or GFP-tagged Asef2. In these assays, GST-tagged binding domains from effectors are used to detect the active form of the GTPases. As shown in Fig. 8, GFP-Asef2 expression increased the level of active Rac and Cdc42 by two-fold and 3-fold, respectively, which is consistent with Asef2 having GEF activity toward these GTPases (Kawasaki, Sagara et al. 2007). Interestingly, RhoA activity was decreased by approximately two-fold in GFP-Asef2 expressing cells. These results indicate that Asef2 significantly affects the amount of active Rac, Cdc42, and RhoA in cells.

To study the function of Asef2 in regulating actin dynamics and cell migration, we generated HT-1080 cell lines stably expressing low levels of GFP-Asef2 or GFP as a control. In these cells, the level of GFP-Asef2 expression was

Figure 7. Asef2 increases the amount of active Rac1 and Cdc42, but decreases Rho activity in HT-1080 cells. (A) Schematic of the domain structure of Asef2. APC binding region (ABR), Src homology 3 (SH3), Dbl homology (DH), and Pleckstrin homology (PH) domains are shown. (B) Immunoblot for Asef2 from HT-1080 cells stably expressing GFP-Asef2. The ~ 75 kDa band represents endogenous Asef2 while the ~100 kDa band corresponds to exogenously expressed GFP-Asef2. (C) Quantification of the amount of GFP-Asef2 in stably expressing HT-1080 cells relative to endogenous levels of the protein. Error bar represents S.E.M. from four separate experiments. (D) The GTP bound (active) form of Rac1, Cdc42, and RhoA was pulled down from lysates of GFP (Control) or GFP-Asef2 stable cells. The total amount of each of these GTPases in cells is included as a loading control. Quantification of the amount of active GTPases from blots from four separate experiments is shown in the lower panels. Error bars represent S.E.M. (*, $p < 0.04$; **, $p < 0.003$).


A
Asef2
ABR
SH3
DH
PH
1
90
150
204
242
452
455
564
652
B
~100 kD
~75 kD
GFP-Asef2
Endogenous Asef2
IB: Asef2
C
Asef2 Expression
(% of Endogenous)
350
300
250
200
150
100
50
0
Endogenous
GFP-Asef2
D
Rac1
Cdc42
Rho A
Control
Asef2
Active
Total
~25 kD
Rac1
Cdc42
Rho A
Percent
*
**
Control
Asef2

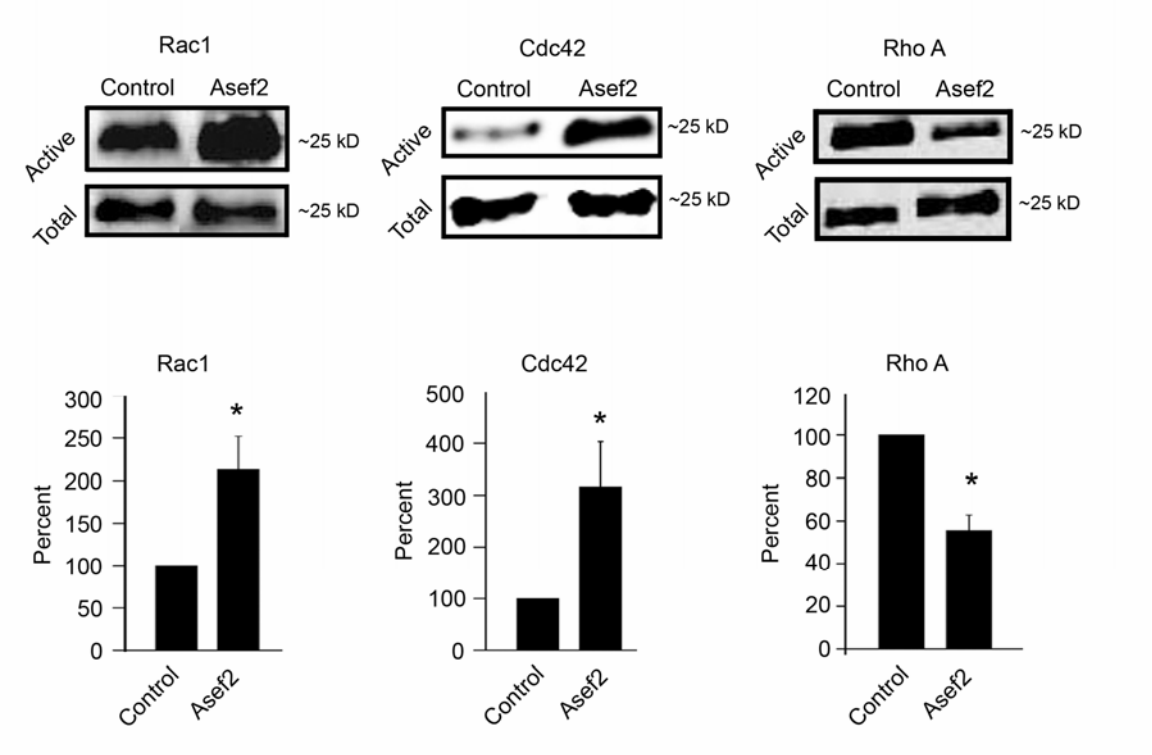


Figure 8. Asef2 increases Rac1 and Cdc42 activity and decreases the amount of active Rho in HEK-293 cells. The active form of Rac1, Cdc42, and RhoA was pulled down from lysates of HEK-293 cells expressing GFP (Control) or GFP-Asef2. The total amount of each of these GTPases in the cells is included as a loading control. Quantification of the amount of active Rac1, Cdc42, and Rho in HEK-293 cells expressing GFP (Control) or GFP-Asef2 is shown (lower panels). Error bars represent S.E.M. from at least three separate experiments. Asterisks denote statistically significant differences when compared with control GFP expressing cells ($p < 0.0001$).

< 3-fold over endogenous Asef2 as determined by Western blot analysis (Fig. 7B and C). The amount of active Rac and Cdc42 was increased 1.5-fold while active RhoA was decreased greater than two-fold in GFP-Asef2 stably expressing HT-1080 cells compared with control cells expressing GFP (Fig. 7D), which is consistent with the results obtained in the HEK-293 cells. Thus, these cells provided us with stable cell lines for examining the effects of Asef2 on migration and were used in subsequent experiments.

Asef2 localizes with actin at the leading edge of cells and regulates migration

Asef2 was previously reported to localize in membrane ruffles at the leading edge of HeLa cells (Sagara, Kawasaki et al. 2009), which is consistent with it playing an important role in regulating migration. In our study, endogenous Asef2 localized with actin at the leading edge of wild-type HT-1080 cells (Fig. 9D), suggesting that Asef2 may indeed function to regulate HT-1080 cell migration via modulation of the actin cytoskeleton. Like endogenous Asef2, GFP-Asef2 localized with actin at the leading edge while GFP alone was found to distribute diffusely throughout HT-1080 cells (Fig. 9A and C). To confirm the enrichment of GFP-Asef2 at the leading edge, we measured the background subtracted, integrated fluorescence intensity at the edge of GFP and GFP-Asef2 stable cells and normalized it to the unit area. Indeed, the normalized leading

Figure 9. Asef2 localizes with actin at the leading edge and promotes cell migration. (A) GFP (top panels) and GFP-Asef2 (bottom panels) stable cells were fixed and stained for actin using TRITC-phalloidin (red). The overlays are shown in the far right panels. Bar, 10 μm. (B) Quantification of the normalized leading edge fluorescent intensity for GFP (Control) and GFP-Asef2 stable cells is shown. The normalized fluorescent intensity was significantly greater at the leading edge of GFP-Asef2 stable cells compared with GFP cells, confirming an enrichment of GFP-Asef2 at this region. Unlike Asef2, the normalized leading edge fluorescent intensity in GFP stable cells was negative because the fluorescent intensity in the cytosol was higher than at the leading edge, indicating GFP is diffusely distributed in cells. Error bars represent S.E.M. for 20 cells from three separate experiments (*, $p < 0.0001$). (C) Enlargements of the boxed regions in panel A are shown. Bar, 5 μm. (D) Wild-type HT-1080 cells were fixed and co-immunostained for endogenous Asef2 (green) and actin (red). The overlay is shown in the far right panel. Bar, 5 μm. (E) GFP (Control) and GFP-Asef2 stable cells were plated on Fn and images were collected every 5 min using time-lapse microscopy. Rose plots showing individual migration tracks for these cells are shown. Quantification of the migration velocity of GFP (Control) and GFP-Asef2 stable cells is shown (far right). Error bars represent S.E.M. for 28-32 cells from at least three independent experiments (*, $p < 0.0001$). (F) Plot of the migratory distance traveled for individual GFP (Control) and GFP-Asef2 stable cells are shown.

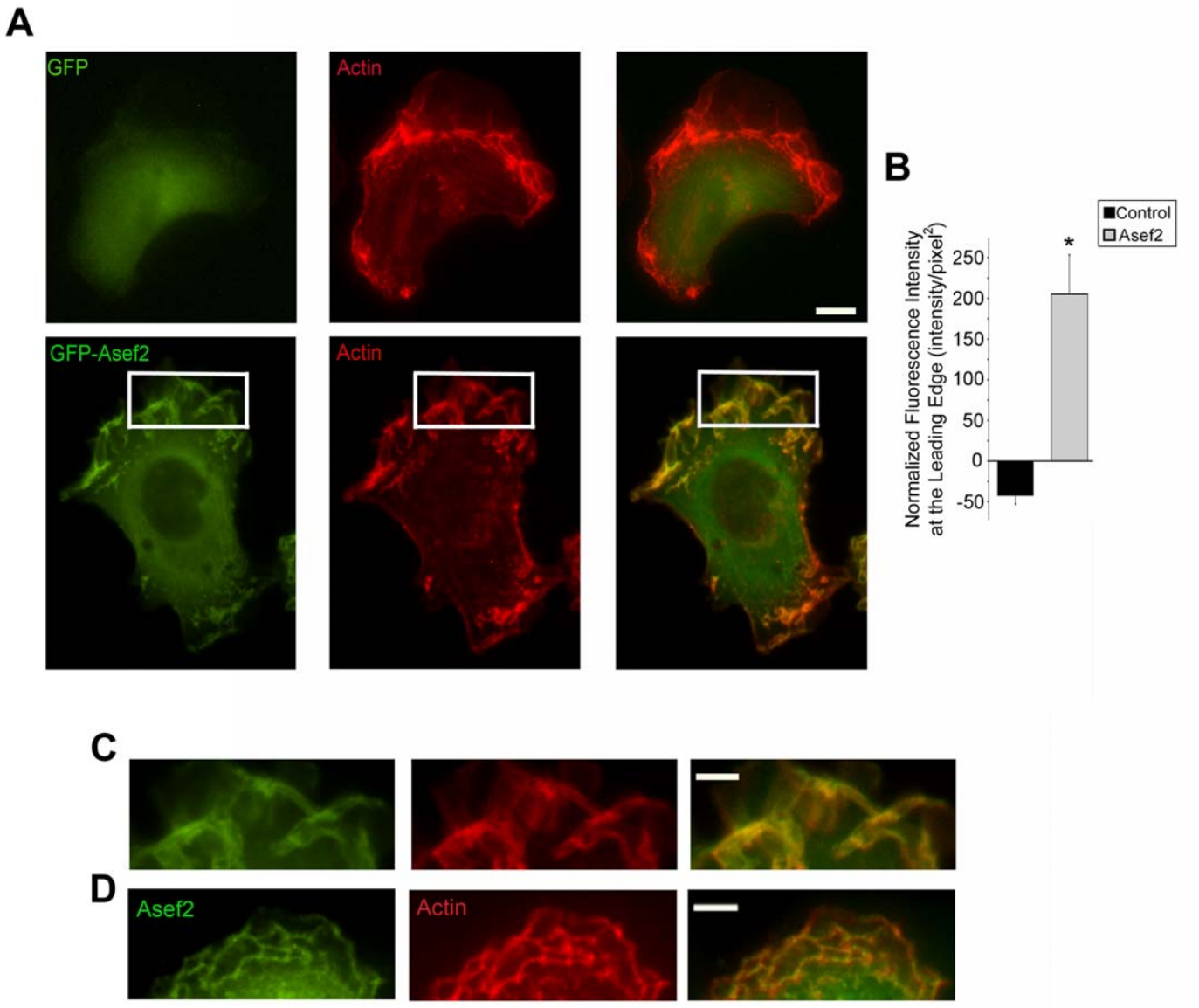
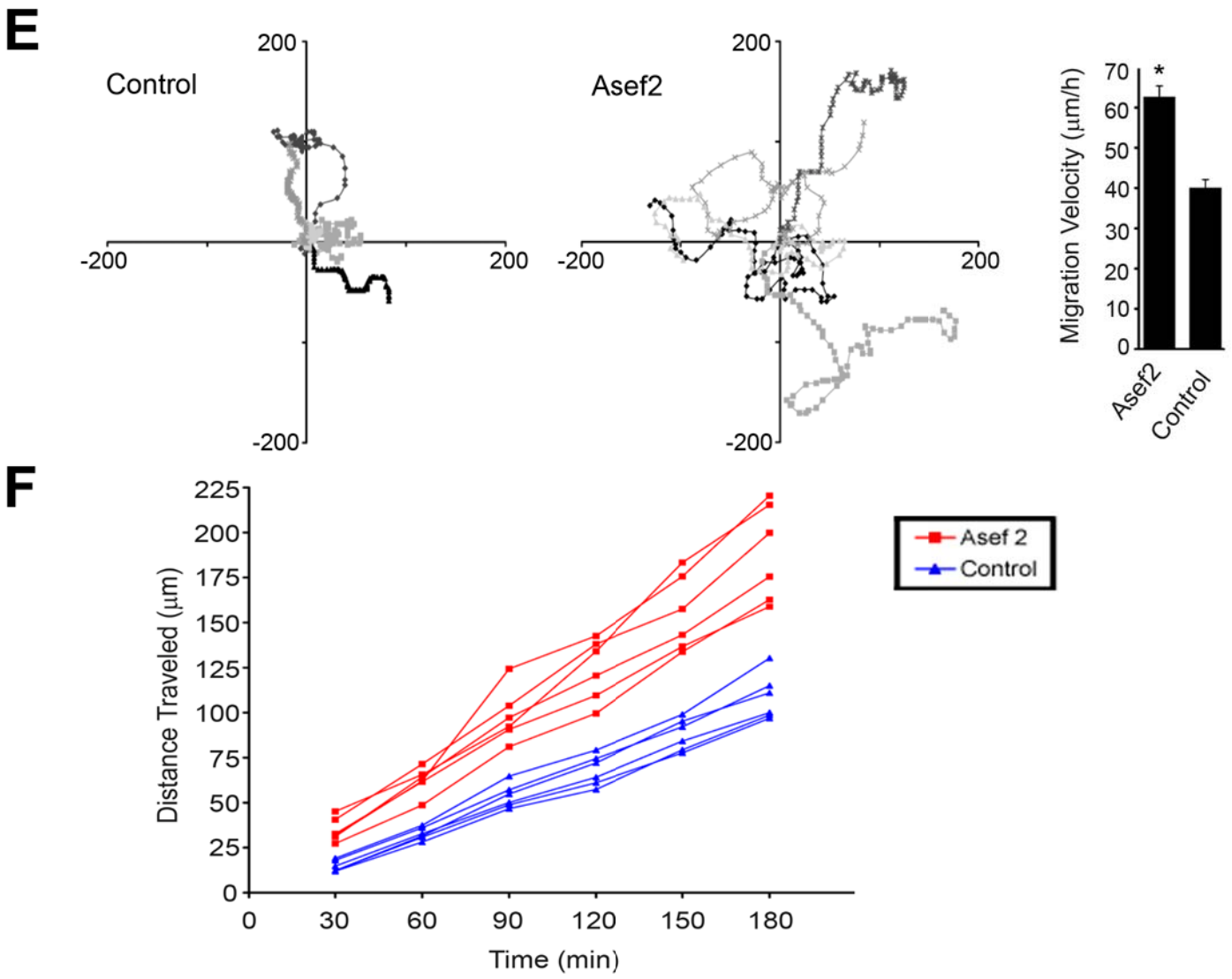

A
GFP
Actin
GFP-Asef2
Actin
B
Control
Asef2
*
Normalized Fluorescence Intensity at the Leading Edge (intensity/pixel²)
250
200
150
100
50
0
-50
C
D
Asef2
Actin

Figure 9—cont.



edge fluorescent intensity was significantly enhanced in GFP-Asef2 cells compared with control cells expressing GFP (Fig. 9B), indicating GFP-Asef2 is enriched at the leading edge. Furthermore, these results show GFP-Asef2 localizes similarly to the endogenous molecule and is a valid marker for examining Asef2 function.

The leading edge localization of Asef2 led us to hypothesize that it functions in the regulation of cell migration. To test this, we assayed the effects of Asef2 on migration using live-cell imaging. Fig. 9E shows individual migration tracks of GFP and GFP-Asef2 stable cells. Interestingly, the migration paths of GFP-Asef2 stable cells were significantly longer than those in control cells (Fig. 8E and F; Movies 1 and 2). The migration velocity of GFP-Asef2 stable cells was increased 1.6-fold compared with control GFP stable cells (Fig. 9E), suggesting Asef2 regulates the migration of HT-1080 cells.

Knockdown of endogenous Asef2 in HT-1080 cells decreases migration

To further explore the role of Asef2 in regulating migration, we generated two small interfering RNA (siRNA) constructs to knock down endogenous expression of the protein. Transfection of wild-type HT-1080 cells with the two siRNA constructs resulted in a significant decrease in the expression of endogenous Asef2. As determined by Western blot analysis, Asef2 siRNA #1 knocked down endogenous expression of the protein by almost 65% and Asef2 siRNA #2 decreased expression by approximately 50% compared with empty pSUPER vector (Fig. 10A and B). In contrast, transfection with scrambled siRNA

did not significantly affect expression of endogenous Asef2. Thus, the Asef2 siRNAs were effective in decreasing expression of endogenous Asef2 and were used to assess the effect of Asef2 on migration. Expression of the Asef2 siRNAs in wild-type HT-1080 cells resulted in an approximately two-fold decrease in the migration velocity compared with cells expressing scrambled siRNA or empty pSUPER vector (Fig. 10C and D; Movies 3 and 4). To further show the decrease in migration was due to knockdown of Asef2, we performed a "rescue" experiment with Asef2 lacking the N-terminal ABR/SH3 domains (Asef2Δ204), which is an active form of Asef2 in terms of its GEF activity (Kawasaki, Sagara et al. 2007). Since Asef2 siRNA #2 is designed against nucleotides within this N-terminal region, it does not affect expression of Asef2Δ204. Expression of Asef2Δ204 in cells transfected with Asef2 siRNA #2 led to a significant increase in the migration velocity, completely rescuing the defect in migration observed with Asef2 knockdown (Fig. 10C and D). These results indicate that endogenous loss of Asef2 inhibits migration and point to a critical role for Asef2 in regulating this process.

Since our results suggested Asef2 regulates the activity of Rho family GTPases, we assayed for active Rac, Cdc42, and Rho in cells in which endogenous Asef2 expression was knocked down. Expression of Asef2 siRNA #1 led to a two-fold decrease in the amount of active Rac and Cdc42 compared to that observed in cells expressing scrambled siRNA or empty pSUPER vector (Fig. 10E). In addition, RhoA activity was increased 1.7-fold in Asef2-knockdown cells compared with control cells expressing either scrambled siRNA or pSUPER

Figure 10. Knockdown of endogenous Asef2 significantly impairs migration. (A) Wild-type HT-1080 cells were transfected with empty pSUPER vector, scrambled siRNA (scr siRNA), or Asef2 siRNAs. In some experiments, wild-type HT-1080 cells were co-transfected with Asef2 siRNA #2 and a truncated form of Asef2 (Asef2Δ204) (right panels). Cell lysates were immunoblotted for Asef2 or α-tubulin (loading control). (B) Quantification of endogenous amounts of Asef2 from cells transfected with the indicated constructs is shown. Error bars represent S.E.M. from four independent experiments (*, $p < 0.0001$). (C) Wild-type HT-1080 cells were transfected with empty pSUPER vector, scrambled siRNA (scr siRNA), or Asef2 siRNAs and used in live-cell migration assays three days later. To show the migration phenotype observed with Asef2 siRNA expressing cells was due to endogenous loss of the protein, Asef2Δ204, which is a truncated, active form of Asef2, was co-expressed with Asef2 siRNA #2. Rose plots with individual migration tracks for cells expressing the indicated constructs are shown. (D) Quantification of the migration velocity of cells transfected with the constructs from panel C is shown. Error bars represent S.E.M. for 30-35 cells from four separate experiments (*, $p < 0.009$). (E) HT-1080 cells were transfected with empty pSUPER vector, scrambled siRNA (scr siRNA), or Asef2 siRNA #1 and cell lysates were assayed for active Rac1, Cdc42, and RhoA. Quantification of the amount of active GTPases from blots from four separate experiments is shown in the lower panels. Error bars represents S.E.M. (*, $p < 0.003$; **, $p < 0.03$). For panels B, D, and E, asterisks denote a statistically significant difference compared with pSUPER transfected cells.

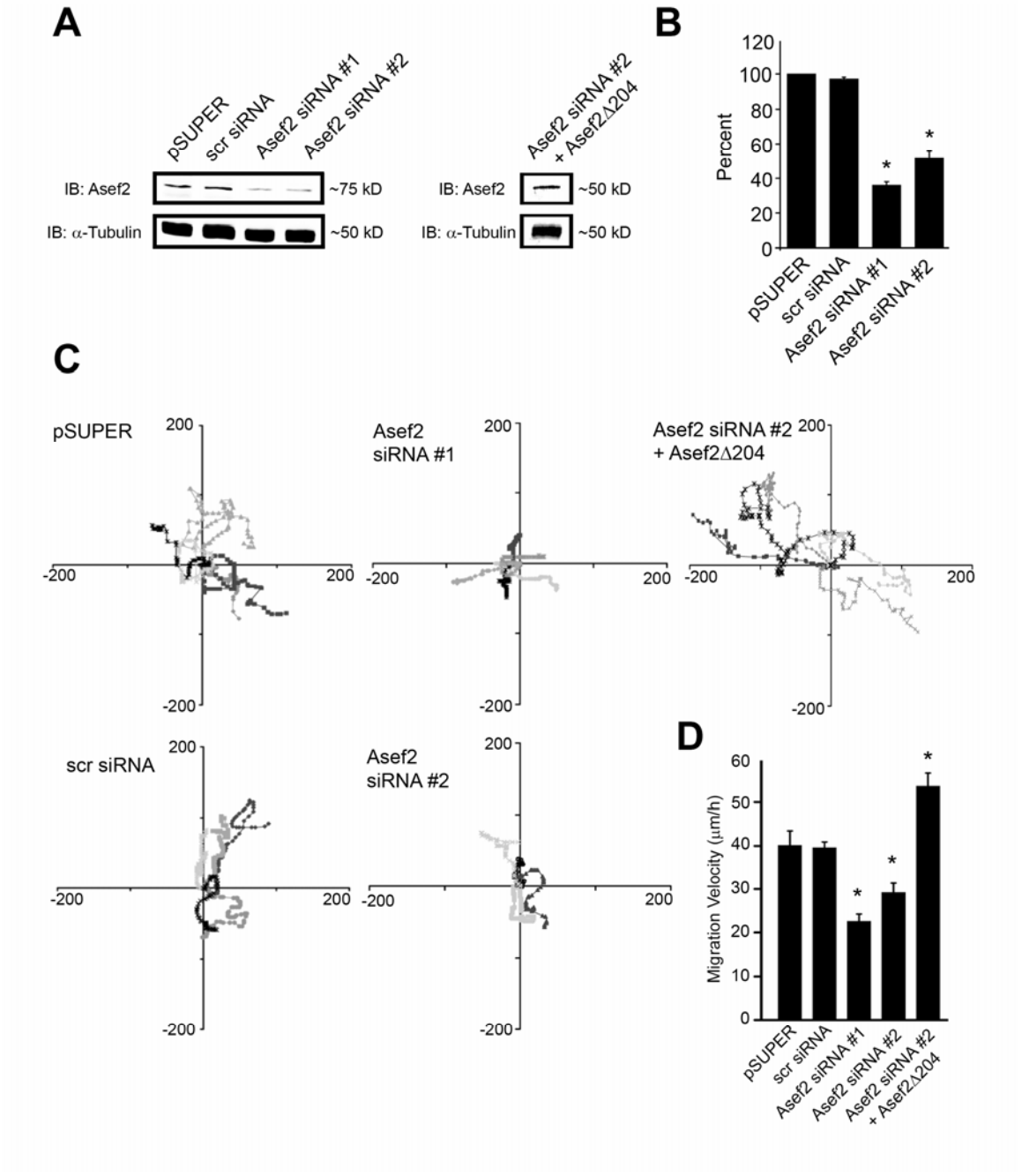
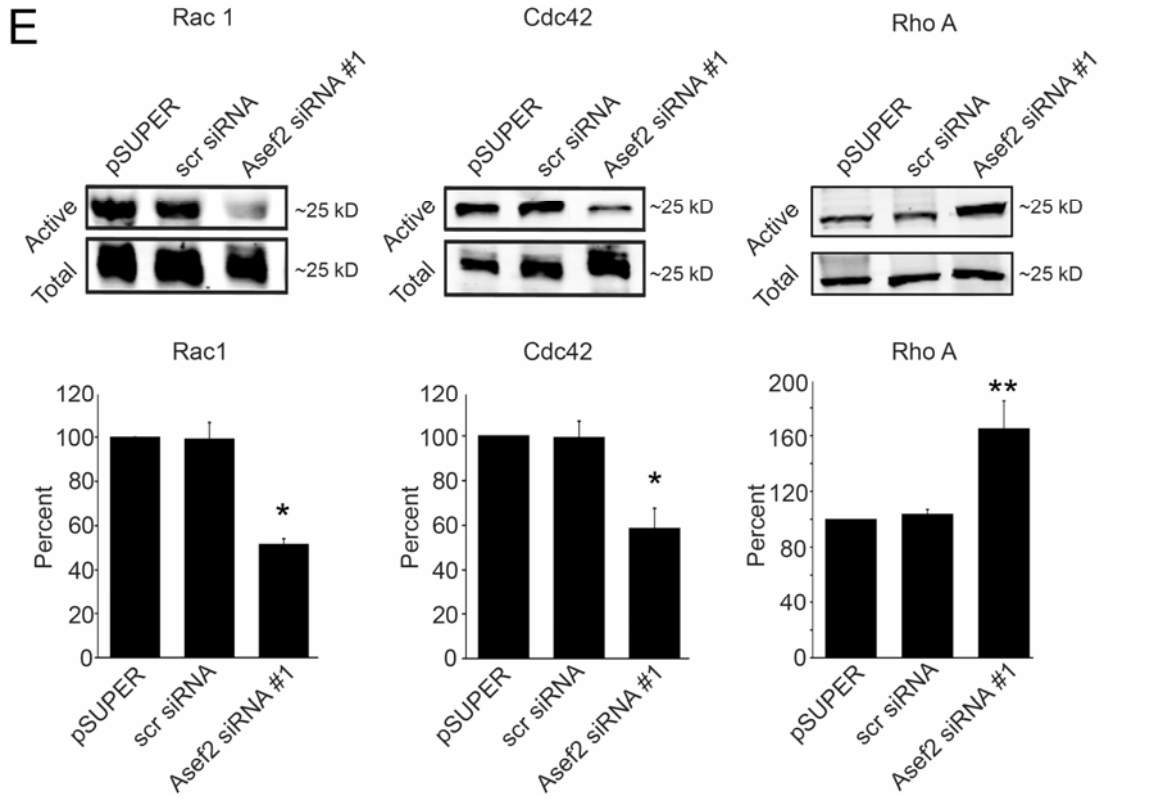

A
pSUPER
scr siRNA
Asef2 siRNA #1
Asef2 siRNA #2
IB: Asef2
~75 kD
IB: α-Tubulin
~50 kD
Asef2 siRNA #2
+ Asef2Δ204
IB: Asef2
~50 kD
IB: α-Tubulin
~50 kD
B
Percent
120
100
80
60
40
20
0
pSUPER
scr siRNA
Asef2 siRNA #1
Asef2 siRNA #2
C
pSUPER
Asef2
siRNA #1
Asef2 siRNA #2
+ Asef2Δ204
scr siRNA
Asef2
siRNA #2
200
-200
D
Migration Velocity (μm/h)
60
50
40
30
20
10
0
pSUPER
scr siRNA
Asef2 siRNA #1
Asef2 siRNA #2
Asef2 siRNA #2
+ Asef2Δ204

Figure 10-- cont.



vector (Fig. 10E). These results indicate Asef2 regulates the activity of Rho family GTPases.

Asef2 regulates adhesion turnover

Since assembly and disassembly of adhesions at the leading edge are essential for migration and Rho family GTPases are involved in this process (Rottner, Hall et al. 1999; Webb, Donais et al. 2004; Nayal, Webb et al. 2006), we hypothesized that Asef2 affects migration by regulating adhesion turnover. To test this hypothesis, GFP and GFP-Asef2 stable cells were immunostained for two adhesion markers, paxillin and vinculin, and observed with total internal reflection microscopy (TIRF). Adhesions in Asef2 stable cells were very small and located primarily around the cell perimeter usually within 1 μm of the leading edge (Fig. 11A). In contrast, in control cells, numerous large adhesions were found throughout the cell body (Fig. 11A). Since the smaller, peripherally located adhesions in Asef2 stable cells could be the result of enhanced adhesion turnover, we further explored this possibility. Adhesion turnover was quantitatively assessed by transfecting GFP and GFP-Asef2 stable cells with mCherry-paxillin and measuring the $t_{1/2}$s for the assembly and disassembly of adhesions as previously described (Webb, Donais et al. 2004; Nayal, Webb et al. 2006). In Asef2 stable cells, the apparent $t_{1/2}$ for adhesion assembly was decreased approximately two-fold compared with control cells, indicating adhesions were forming significantly faster in the Asef2 cells (Fig. 11B and C; Table 1; Movies 5 and 6). Asef2 had a similar effect on the disassembly of

Figure 11. Asef2 induces the formation of small, leading edge adhesions that turn over very rapidly. (A) GFP (Control) and GFP-Asef2 stable cells were immunostained for endogenous paxillin or vinculin and visualized with TIRF microscopy. Bar, 10 μm. (B) GFP and GFP-Asef2 stable cells were transfected with mCherry-paxillin, plated on Fn, and imaged in red fluorescence. Time-lapse images show adhesions at the leading edge assemble and disassemble on a much more rapid time scale in GFP-Asef2 stable cells compared with control GFP cells (arrowheads). Bar, 5 μm. (C) Quantification of the apparent $t_{1/2}$ for adhesion assembly and the $t_{1/2}$ for adhesion disassembly is shown (*, $p < 0.003$). Error bars represent S.E.M from 15-23 individual adhesions in 4-6 cells from at least three independent experiments.

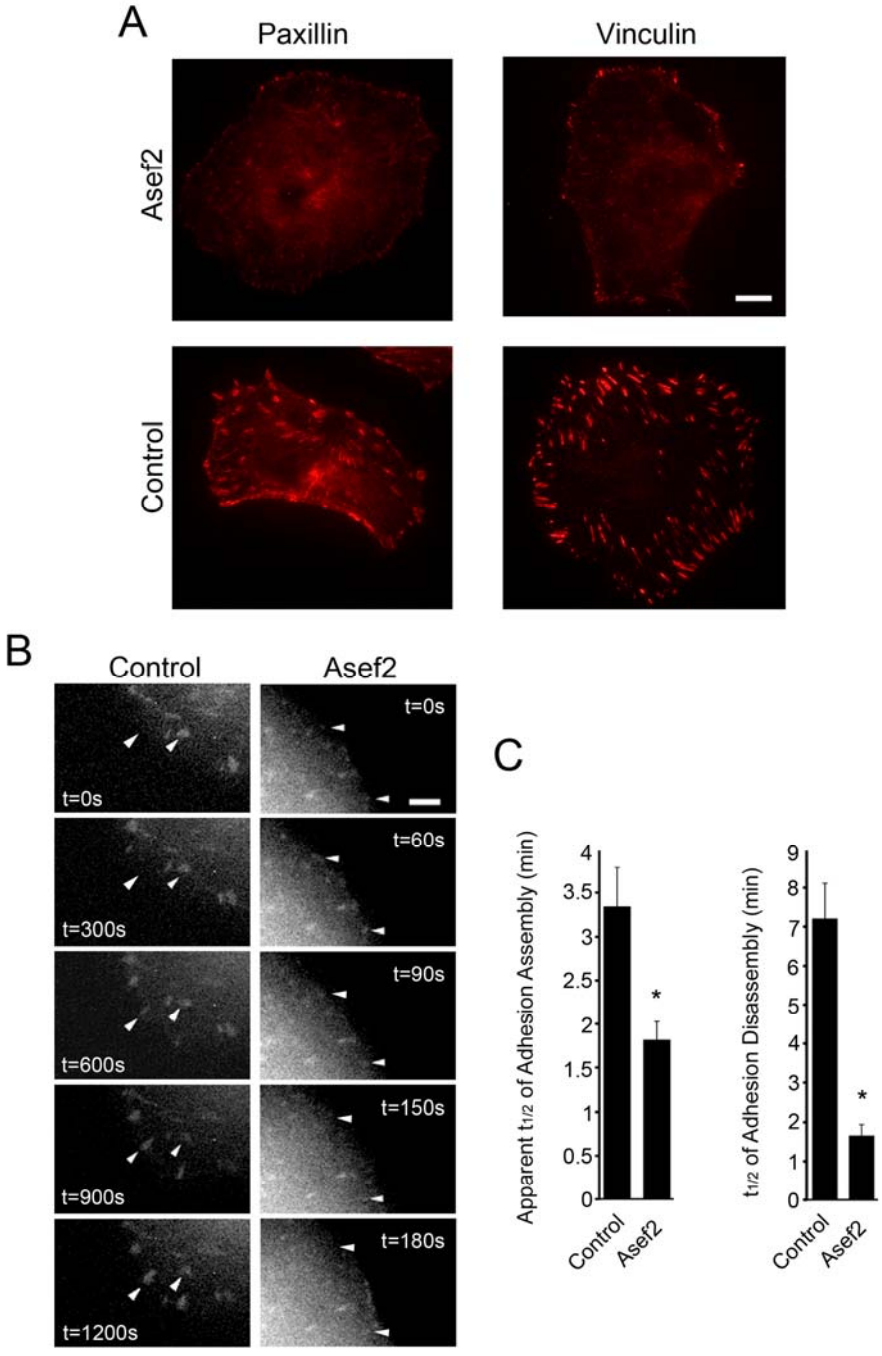

A
Paxillin
Vinculin
Asef2
Control
B
Control
Asef2
t=0s
t=0s
t=60s
t=300s
t=90s
t=600s
t=150s
t=900s
t=180s
t=1200s
C
Apparent t1/2 of Adhesion Assembly (min)
t1/2 of Adhesion Disassembly (min)
Control
Asef2
Control
Asef2

Table 1. Apparent $t_{1/2}$ for adhesion assembly and $t_{1/2}$ for adhesion disassembly in HT-1080 cells

The $t_{1/2}$s are reported as means ± S.E.M. For each $t_{1/2,}$ measurements were obtained from 15-23 individual adhesions in 4-6 cells from at least three independent experiments.

Construct expressed	**Apparent $t_{1/2}$ (min) (adhesion assembly)**	**$t_{1/2}$ (min) (adhesion disassembly)**
GFP	3.3 +/- 0.5	7.2 +/- 0.9
GFP-Asef2	1.8 +/- 0.2	1.7 +/- 0.3
pSUPER	3.0 +/- 0.3	6.9 +/- 0.6
scr siRNA	3.4 +/- 0.3	7.3 +/- 0.8
Asef2 siRNA#1	6.4 +/- 1.1	10.1 +/- 0.7

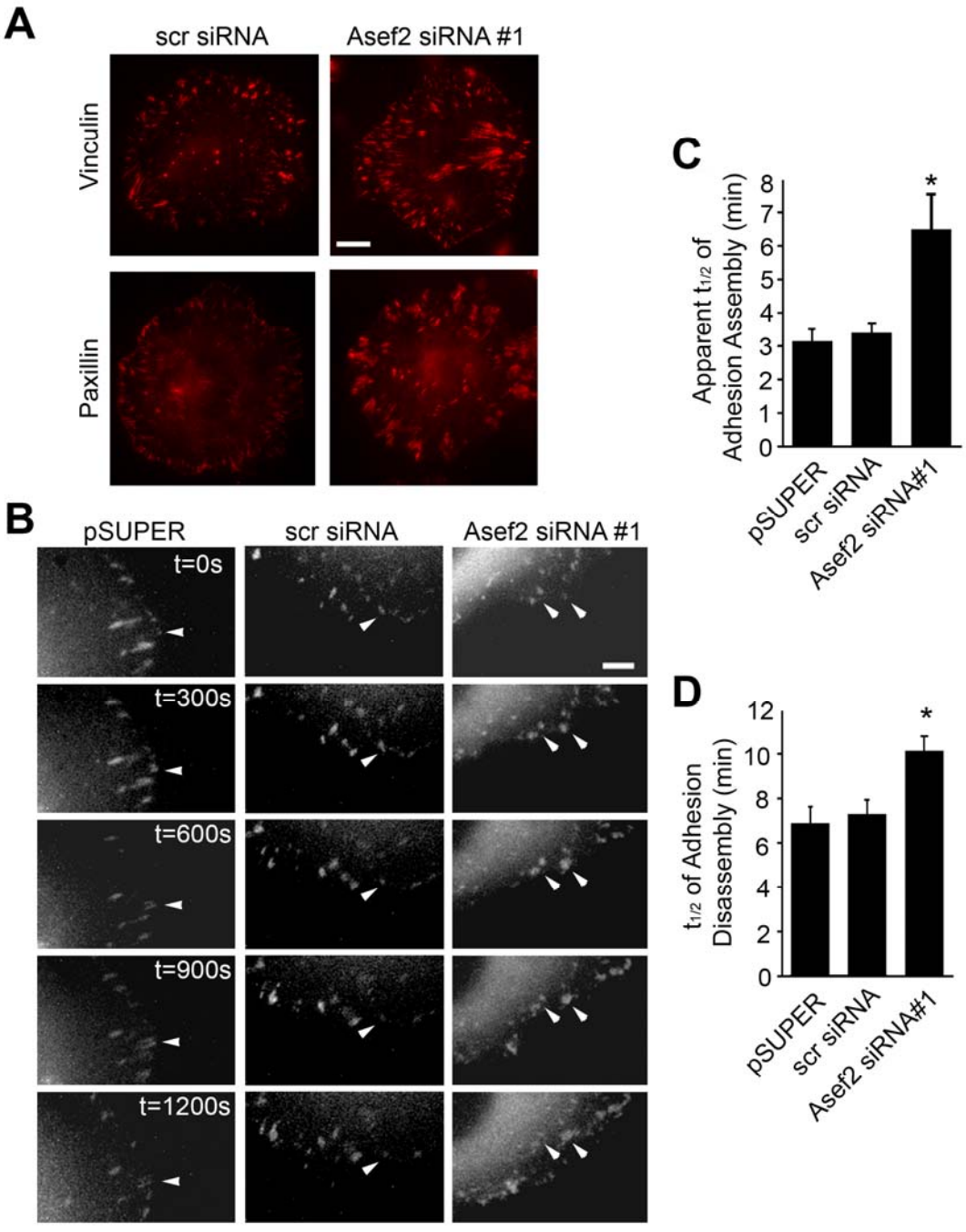
adhesions since the $t_{1/2}$ for adhesion disassembly was decreased greater than 4-fold in GFP-Asef2 stable cells compared with control cells (Fig. 11B and C; Table 1; Movies 5 and 6). These results suggest that Asef2 increases the rate of adhesion turnover, which could contribute to its effects on migration.

To confirm a role for Asef2 in adhesion turnover, we knocked down endogenous expression of the protein in wild-type HT-1080 cells with our siRNA construct. Many large adhesions were found throughout cells expressing Asef2 siRNA #1 (Fig. 12A). These adhesions appeared to be bigger than those observed in control cells expressing scrambled siRNA (Fig. 12A), which prompted us to quantify adhesion turnover in Asef2-knockdown and control cells. Expression of Asef2 siRNA #1 resulted in a two-fold increase in the apparent $t_{1/2}$ for adhesion assembly compared to that observed in control cells expressing empty pSUPER vector or scrambled siRNA (Fig. 12B and C; Table 1). Similarly, knockdown of endogenous Asef2 significantly increased the $t_{1/2}$ for adhesion disassembly when compared with that observed in cells expressing either empty pSUPER vector or scrambled siRNA (Fig. 12B and D; Table 1). Thus, knockdown of endogenous Asef2 significantly altered the rate of adhesion turnover, indicating it is an important regulator of this process.

Rac, but not Cdc42, is essential for Asef2-mediated migration and adhesion turnover

Since our results show GFP-Asef2 stable cells have increased levels of both Rac and Cdc42, this raised the question as to which of these GTPases is critical for the Asef2-mediated effects on migration and adhesion dynamics. To

Figure 12. Knockdown of endogenous Asef2 affects adhesion turnover. (A) Wild-type HT-1080 cells transfected with scrambled siRNA (scr siRNA) or Asef2 siRNA #1 were immunostained for endogenous paxillin or vinculin and visualized with TIRF microscopy. Bar, 10 μm. (B) Wild-type HT-1080 cells were co-transfected with mCherry-paxillin and empty pSUPER vector, scrambled siRNA (scr siRNA), or Asef2 siRNA #1 and adhesion assembly and disassembly were assessed. Time-lapse images show adhesions turn over more slowly in cells transfected with Asef2 siRNA #1 as compared with control cells transfected with either empty pSUPER vector or a scrambled siRNA (arrowheads). (C, D) Quantification of the apparent $t_{1/2}$ for adhesion assembly (panel C) and the $t_{1/2}$ for adhesion disassembly (panel D) is shown. Error bars represent S.E.M from 15-23 individual adhesions in 4-6 cells from at least three independent experiments (*, $p < 0.006$). Asterisks denote a statistically significant difference compared with pSUPER transfected cells.


A
scr siRNA
Asef2 siRNA #1
Vinculin
Paxillin
B
pSUPER
scr siRNA
Asef2 siRNA #1
t=0s
t=300s
t=600s
t=900s
t=1200s
C
Apparent t1/2 of Adhesion Assembly (min)
0
1
2
3
4
5
6
7
8
*
pSUPER
scr siRNA
Asef2 siRNA#1
D
t1/2 of Adhesion Disassembly (min)
0
2
4
6
8
10
12
*
pSUPER
scr siRNA
Asef2 siRNA#1

address this question, we generated Rac1 and Cdc42-specific siRNA constructs to knock down endogenous expression of the proteins and examined their effects on Asef2-mediated migration and adhesion turnover. Rac siRNA #1 and Rac siRNA #2 decreased endogenous expression of Rac by approximately 75% compared with empty pSUPER vector or scrambled siRNA (Fig. 13A). When the Rac siRNAs were transfected into GFP and GFP-Asef2 stable cells, an overall decrease in the migration velocity was observed (Fig. 13C and E), because Rac is an important regulator of cell migration. More importantly, expression of the Rac siRNAs completely abrogated the Asef2-mediated effects on migration (Fig. 13C and E). The migration velocities were almost identical in GFP and GFP-Asef2 stable cells transfected with the Rac siRNAs (Fig. 13E), indicating knockdown of endogenous Rac inhibited Asef2-mediated migration. In contrast, scrambled siRNA had no effect on Asef2-mediated migration.

Since Asef2 also increased Cdc42 activity in HT-1080 cells, we examined the effects of Cdc42 on Asef2-mediated migration using an siRNA approach. When Cdc42 siRNA #1 or Cdc42 siRNA #2 were transfected into HT-1080 cells, endogenous expression of Cdc42 was decreased by almost 75% compared with empty pSUPER vector or scrambled siRNA (Fig. 13B). Thus, the effectiveness of the Cdc42 siRNAs in decreasing endogenous expression of the protein was similar to that observed with the Rac siRNAs (Fig. 13A and B). As with the Rac siRNAs, transfection of GFP and GFP-Asef2 stable cells with the Cdc42 siRNAs resulted in an overall decrease in the migration velocity (Fig. 13D and F).

Figure 13. Rac, but not Cdc42, is necessary for the Asef2-mediated effect on migration. (A) Wild-type HT-1080 cells were transfected with empty pSUPER vector, scrambled siRNA (scr siRNA), or Rac siRNAs. Three days later cells were lysed and immunoblotted for Rac or actin (loading control). Quantification of the amount of endogenous Rac from blots of cells transfected with the indicated constructs is shown (lower panels). Error bars represent S.E.M. from four independent experiments (*, $p < 0.0001$). (B) HT-1080 cells were transfected with empty pSUPER vector, scrambled siRNA (scr siRNA), or Cdc42 siRNAs. After three days, cell lysates were immunoblotted for Cdc42 or α-tubulin (loading control). Quantification of endogenous levels of Cdc42 from blots of cells transfected with the indicated constructs is shown (lower panels). Error bars represent S.E.M. from four independent experiments (*, $p < 0.0001$). For panels A and B, asterisks denote a statistically significant difference compared with pSUPER transfected cells. (C, D) GFP and GFP-Asef2 stable cells were transfected with empty pSUPER vector, scrambled siRNA (scr siRNA), Rac or Cdc42 siRNAs and used in migration assays. Rose plots with individual migration tracks are shown for cells expressing Rac siRNAs (panel C) or Cdc42 siRNAs (panel D). (E, F) Quantification of the migration velocity of cells transfected with the indicated constructs is shown. Error bars represent S.E.M. for 30-35 cells from four separate experiments (panel E *, $p < 0.0001$; panel F *, $p < 0.0002$). For panels E and F, asterisks denote statistically significant differences compared with GFP (Control) stable cells.

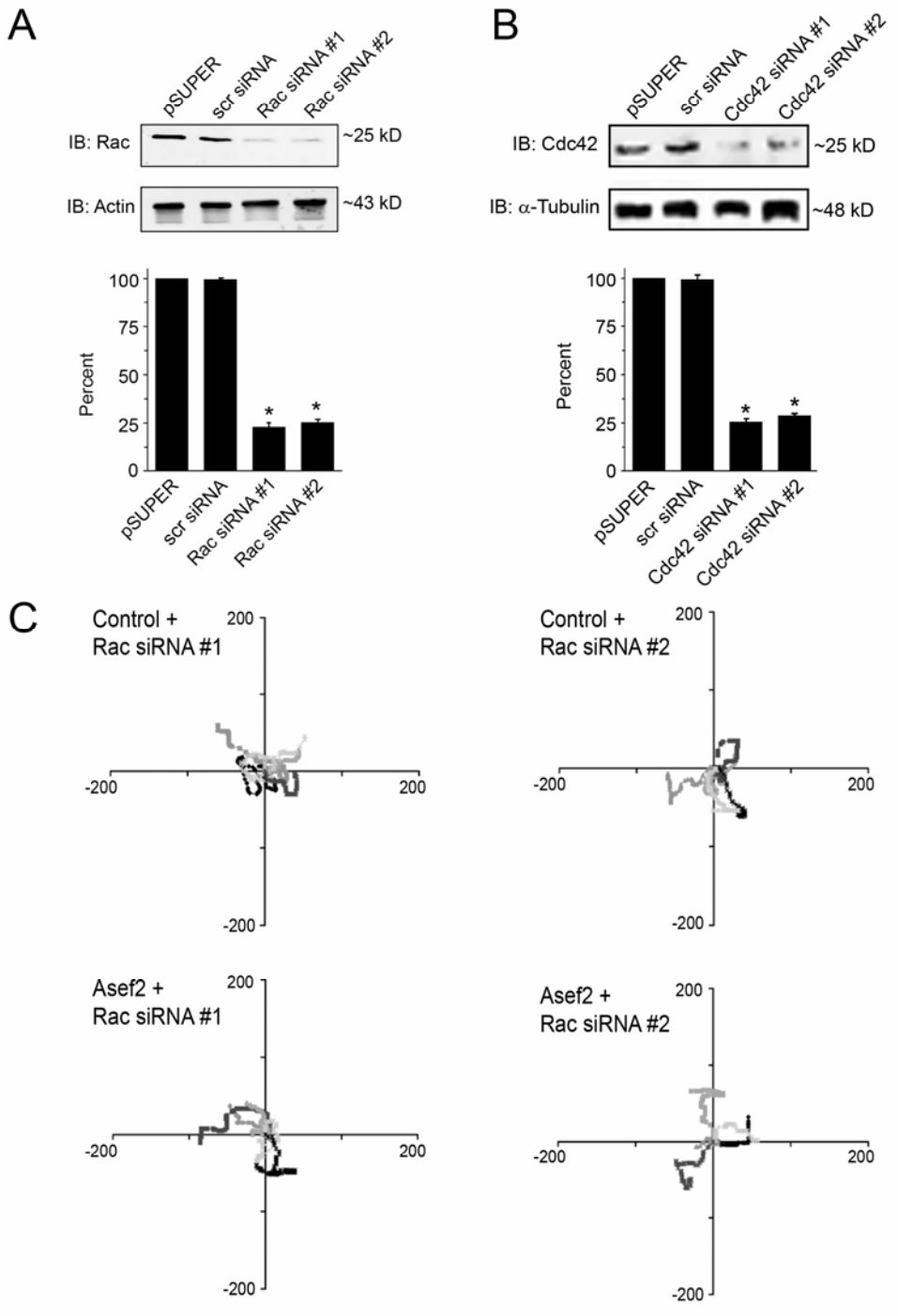
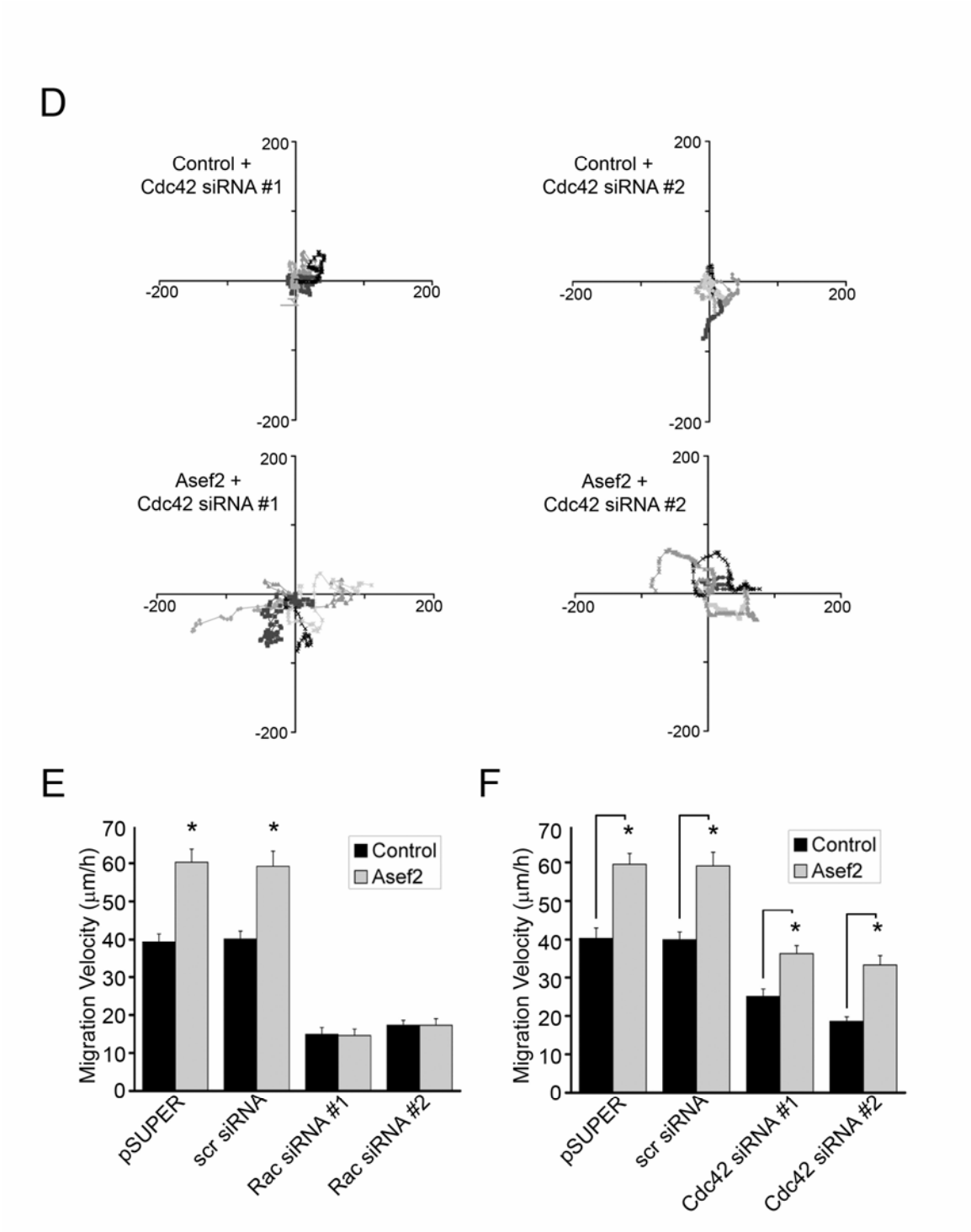

A
pSUPER
scr siRNA
Rac siRNA #1
Rac siRNA #2
IB: Rac
~25 kD
IB: Actin
~43 kD
Percent
100
75
50
25
0
B
pSUPER
scr siRNA
Cdc42 siRNA #1
Cdc42 siRNA #2
IB: Cdc42
~25 kD
IB: α-Tubulin
~48 kD
C
Control +
Rac siRNA #1
Control +
Rac siRNA #2
Asef2 +
Rac siRNA #1
Asef2 +
Rac siRNA #2
200
-200

Figure 13—cont.



However, unlike the Rac siRNAs, expression of the Cdc42 siRNAs did not eradicate the Asef2-mediated effects on migration (Fig. 13D and F). The migration velocity of GFP-Asef2 stable cells was still increased 1.5-fold compared with control cells when both cell lines were transfected with Cdc42 siRNAs, indicating knockdown of endogenous Cdc42 did not significantly affect Asef2-mediated migration.

To further confirm that Cdc42 is not involved in Asef2-mediated migration, we treated GFP and GFP-Asef2 stable cells with the Cdc42-specific inhibitor secramine A (2.5 μM) (Pelish, Peterson et al. 2006; Xu B 2006). Secramine A treatment resulted in an overall decrease in the migration velocity, but no effect was observed on Asef2-mediated migration. The migration velocity of GFP and GFP-Asef2 stable cells treated with secramine A was 22.1 +/- 2.2 μm/h and 36.8 +/- 2.9 μm/h (n= 12 from 4 separate experiments; p=0.0005), respectively. These results further establish that Rac, but not Cdc42, is necessary for Asef2-promoted migration.

Since our results indicated that Asef2 regulated migration through Rac, we next determined whether the Asef2-mediated effects on adhesion turnover were also Rac-dependent. When GFP and GFP-Asef2 stable cells were transfected with empty pSUPER vector or scrambled siRNA, the apparent $t_{1/2}$ for adhesion assembly was decreased two-fold in GFP-Asef2 cells compared with control GFP cells (Table 2). However, transfection of these cells with Rac siRNA #1

Table 2. Effect of endogenous Rac1 knockdown on Asef2-mediated adhesion assembly and disassembly.

The $t_{1/2}$s are reported as means ± S.E.M. For each $t_{1/2,}$ measurements were obtained from 15-18 individual adhesions in 4-6 cells from at least three independent experiments.

Constructs Expressed	Apparent $t_{1/2}$ (min) (adhesion assembly)	$t_{1/2}$ (min) (adhesion disassembly)
GFP + pSUPER	3.6 +/- 0.3	7.0 +/- 0.9
GFP + scr siRNA	3.5 +/- 0.4	7.1 +/- 0.9
GFP + Rac1 siRNA#1	6.9 +/- 0.4	8.4 +/- 0.9
GFP-Asef2 + pSUPER	1.7 +/- 0.2	1.8 +/- 0.2
GFP-Asef2 + scr siRNA	1.7 +/- 0.3	1.7 +/- 0.2
GFP-Asef2 + Rac1 siRNA#1	7.2 +/- 0.8	7.4 +/- 0.9

completely abrogated the Asef2-mediated effect on adhesion assembly and no significant difference in the apparent $t_{1/2}$ for the assembly of adhesions was observed between GFP and GFP-Asef2 stable cells (Table 2). As with migration, the knockdown of endogenous Rac affected the overall rate of adhesion assembly. Similarly, the Asef2-mediated effect on adhesion disassembly was eradicated by knockdown of endogenous Rac. A 4-fold decrease in the $t_{1/2}$ for adhesion disassembly was observed in GFP-Asef2 stable cells compared with controls cells transfected with either empty pSUPER vector or scrambled siRNA (Table 2). In contrast, when these cells were transfected with Rac siRNA #1, the $t_{1/2}$ for adhesion disassembly was similar in GFP and GFP-Asef2 stable cells (Table 2). Taken together, these results indicate Rac is necessary for the Asef2-mediated effects on adhesion turnover and support our hypothesis that Asef2 regulates migration and adhesion dynamics through a Rac-dependent mechanism.

PI3K plays an important role in Asef2-mediated migration and adhesion turnover

Since PI3K is implicated in the modulation of cell migration and Rho family signaling (for review see (Cain and Ridley 2009)), we hypothesized it is a component of the Asef2 pathway that regulates migration and adhesion turnover. To examine the effects of PI3K on Asef2-mediated migration, GFP and GFP-Asef2 stable cells were treated with various concentrations of the PI3K inhibitor wortmannin (Wymann 1996) for 30 min prior to imaging and then, migration was

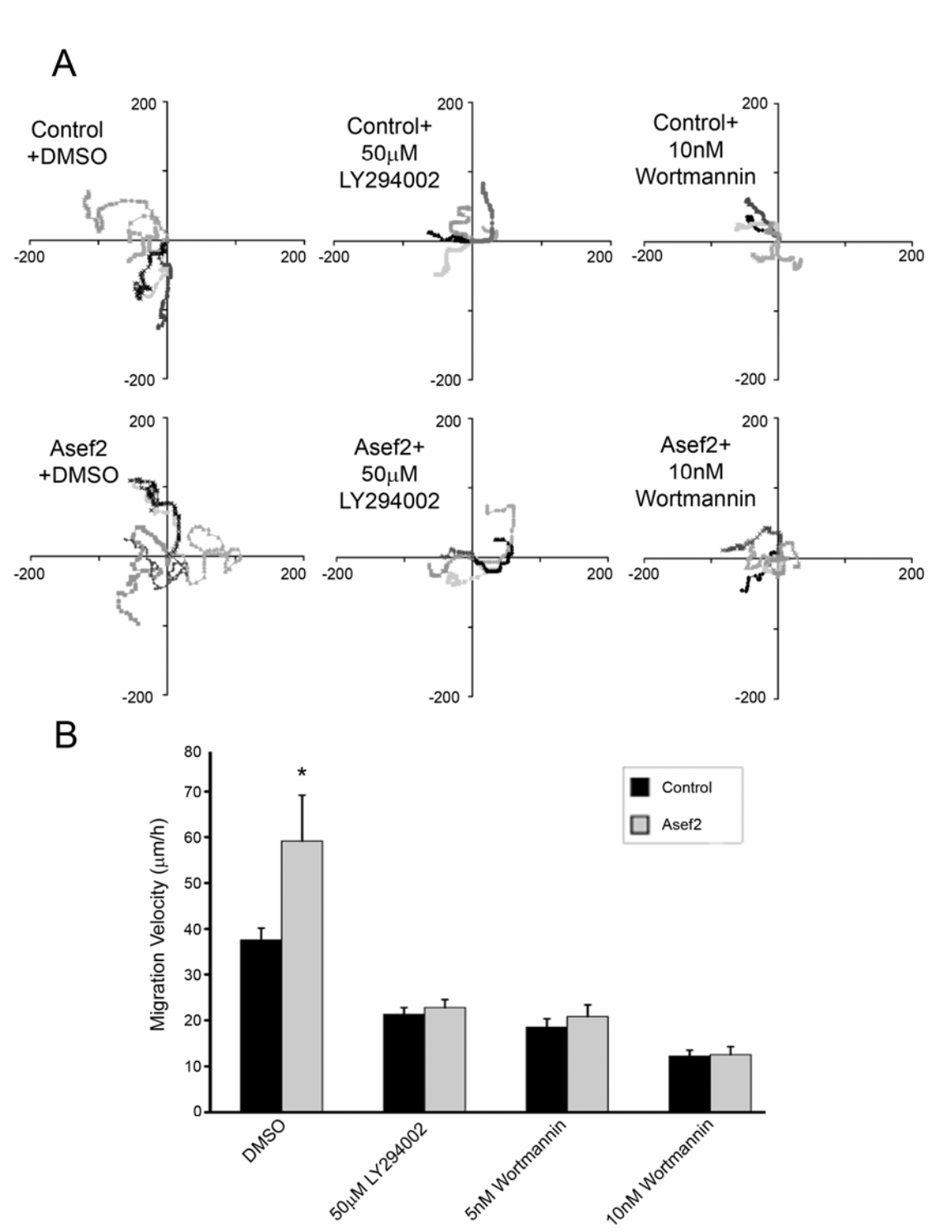

A
Control
+DMSO
Control+
50μM
LY294002
Control+
10nM
Wortmannin
Asef2
+DMSO
Asef2+
50μM
LY294002
Asef2+
10nM
Wortmannin
200
-200
B
Migration Velocity (μm/h)
80
70
60
50
40
30
20
10
0
*
Control
Asef2
DMSO
50μM LY294002
5nM Wortmannin
10nM Wortmannin

Figure 14-- cont.

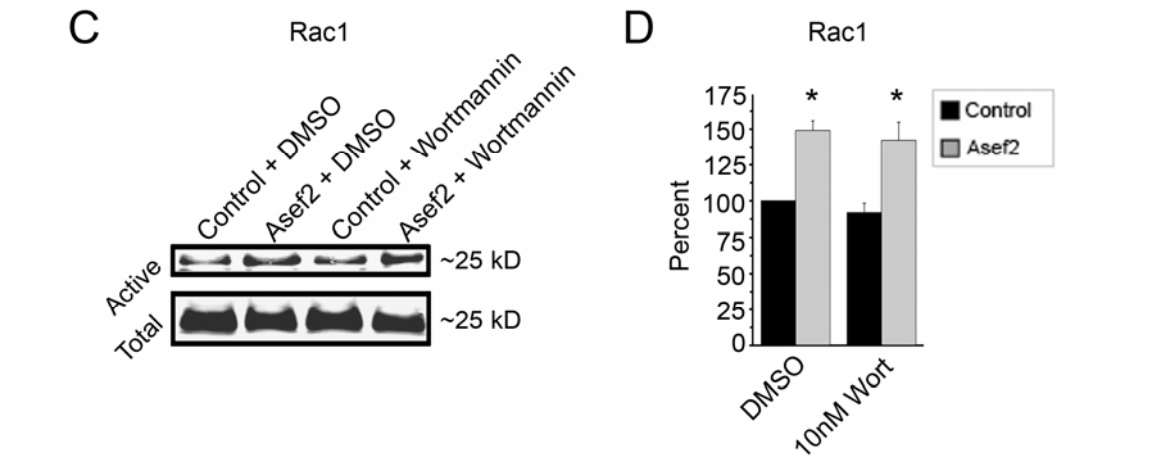


Figure 14 PI3K is necessary for Asef2-mediated migration. (A) GFP (Control) and GFP-Asef2 stable cells were incubated with vehicle (DMSO), wortmannin, or LY294002 (50 □M) and then used in live-cell migration assays. Rose plots with individual migration tracks are shown. (B) Quantification of the migration velocity of GFP (Control) and GFP-Asef2 stable cells treated with DMSO, wortmannin, or LY294002 is shown. Error bars represent S.E.M. for 30-35 cells from four separate experiments (*, $p < 0.0001$). (C) GFP (Control) and GFP-Asef2 stable cells were treated with vehicle (DMSO) or 10 nM wortmannin for 2 h and then cell lysates were assayed for active Rac. (D) Quantification of blots from five separate experiments is shown. Error bar represents S.E.M. (*, $p < 0.0001$). For panels B and D, asterisks denote statistically significant differences compared with GFP (Control) stable cells.

assessed. Since PI3K contributes to the regulation of cell migration, treatment with wortmannin resulted in a dose-dependent decrease in the migration velocity (Fig. 14A and B). Significantly, wortmannin treatment had a profound effect on the Asef2-promoted increase in migration. The lower dose of wortmannin (5 nM) almost entirely abrogated the Asef2-mediated effect on migration since the migration velocities were almost identical in GFP and GFP-Asef2 stable cells (Fig. 14B). Treatment of GFP-Asef2 stable cells with a higher concentration of wortmannin (10 nM) completely negated the Asef2-promoted increase in migration. To confirm that PI3K is necessary for the Asef2-mediated effect on migration, GFP and GFP-Asef2 stable cells were treated with another PI3K inhibitor, LY294002 (50 μM) for 1 h and then migration was assessed. Like wortmannin, LY294002 treatment led to a decrease in the migration velocity of both GFP and GFP-Asef2 stable cells, and importantly, treatment with LY294002 completely abrogated the Asef2-promoted increase in migration (Fig. 14A and B). These results indicate that PI3K is required for Asef2-promoted migration.

Since Asef2-mediated migration was dependent on PI3K, we next determined whether PI3K was part of the Asef2 pathway regulating adhesion turnover. GFP and GFP-Asef2 stable cells were treated with 10 nM wortmannin for 30 min prior to imaging and then, adhesion assembly and disassembly were assessed. As with migration, wortmannin treatment significantly altered the $t_{1/2}$s for adhesion assembly and disassembly in both cell types, which is consistent with PI3K playing a role in regulating adhesion turnover. Notably, after wortmannin treatment, the $t_{1/2}$s for adhesion assembly and disassembly in GFP-

Table 3. Effect of inhibition of PI3K on Asef2-mediated adhesion assembly and disassembly

The $t_{1/2}$s are reported as means ± S.E.M. For each $t_{1/2,}$ measurements were obtained from 15-18 individual adhesions in 4-6 cells from at least three independent experiments.

Treatment	Apparent $t_{1/2}$ (min) (adhesion assembly)	$t_{1/2}$ (min) (adhesion disassembly)
GFP + DMSO	3.6 +/- 0.5	7.1 +/- 1.4
GFP + wortmannin	9.1 +/- 1.1	13.5 +/- 1.7
GFP + LY294002	6.8 +/- 0.6	8.9 +/- 0.9
GFP-Asef2 + DMSO	1.9 +/- 0.2	1.6 +/- 0.4
GFP-Asef2 + wortmannin	10.6 +/- 2.0	11.2 +/- 2.1
GFP-Asef2 + LY294002	6.0 +/- 0.7	7.7 +/- 1.1

Asef2 stable cells were comparable to those observed in control GFP cells (Table 3). Similar results were obtained when GFP and GFP-Asef2 stable cells were treated with LY294002 (Table 3), indicating inhibition of PI3K nullified the Asef2-promoted effect on adhesion turnover. Taken together, these results suggest PI3K is essential for the Asef2-mediated regulation of cell migration and adhesion turnover.

Our results indicate PI3K and Rac are critical components by which Asef2 regulates migration and adhesion dynamics; however, since PI3K can function upstream or downstream of Rac (Welch, Coadwell et al. 2003), it was unclear where PI3K acted. To address this question, we inhibited PI3K activity in GFP and GFP-Asef2 stable cells by incubation with 10 nM wortmannin for 2 hours prior to lysis and then assessed the level of active Rac. As expected, in the absence of wortmannin, the amount of active Rac was increased 1.5-fold in GFP-Asef2 stable cells compared with control cells (Fig. 14C and D). Even after wortmannin treatment, a 1.5-fold increase in the amount of active Rac was still observed in GFP-Asef2 stable cells compared with control cells. Thus, inhibition of PI3K activity by wortmannin did not significantly affect the Asef2-promoted increase in the amount of active Rac, suggesting PI3K is not upstream of Rac in the Asef2 pathway regulating migration and adhesion turnover.

Since a Rac-PI3K-dependent mechanism is reported to be critical for the recruitment of some GEFs to the leading edge (Lin, Yang et al. 2006; Sjoblom, Jones et al. 2006), we inhibited PI3K activity and examined the effect on Asef2 localization. GFP-Asef2 stable cells were treated with LY294002 and the amount

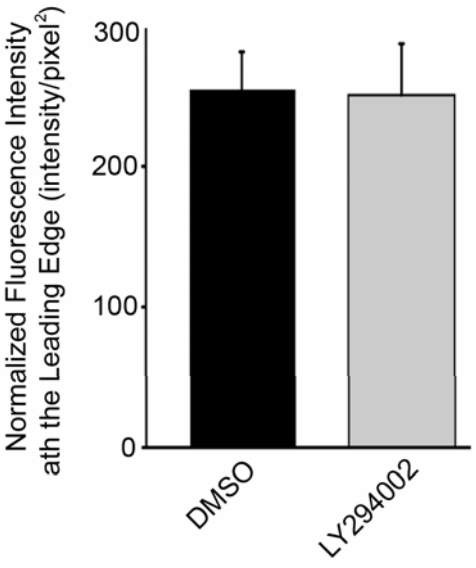


Figure 15. Inhibition of PI3K does not affect the localization of GFP-Asef2 to the leading edge. GFP-Asef2 stable cells were treated with vehicle (DMSO) or LY294002 (50 μM) for 1 h and then fixed. Quantification of the normalized leading edge fluorescent intensity is shown. Error bars represent S.E.M. for 20 cells from three separate experiments.

of Asef2 at the leading edge was quantified. Treatment with LY294002 did not significantly affect the leading edge localization of Asef2 (Fig. 15). Similar results were obtained when GFP-Asef2 stable cells were treated with wortmannin, indicating PI3K is not necessary for the recruitment of Asef2 to the leading edge.

Akt is a component of the Asef2 pathway that regulates migration and adhesion turnover

Since the serine/threonine kinase Akt is activated downstream of PI3K (Franke, Yang et al. 1995), we hypothesized that it contributes to the Asef2-mediated regulation of cell migration and adhesion turnover. To test this, we immunostained for active Akt in GFP and GFP-Asef2 stable cells using a phospho-specific antibody against Thr308 as Akt is activated by phosphorylation of Thr308 and Ser473 (Bokoch 2003). The level of active Akt co-localizing with actin at the leading edge of Asef2 stable cells was increased significantly compared with control cells (Fig. 16A). When we quantified the normalized fluorescent intensity, we found greater than a 5-fold increase in the amount of active Akt at the leading edge of GFP-Asef2 stable cells compared with control cells (Fig. 16B). Interestingly, the total amount of Akt at the leading edge was also increased in Asef2 stable cells compared with control cells (Fig.17) To further examine the role of Akt in Asef2-mediated migration, we transiently transfected GFP and GFP-Asef2 stable cells with a dominant negative (DN-Akt) or a kinase dead Akt mutant (KD-Akt) and analyzed their migration velocities. Both Akt mutants decreased the overall migration velocity; however, more

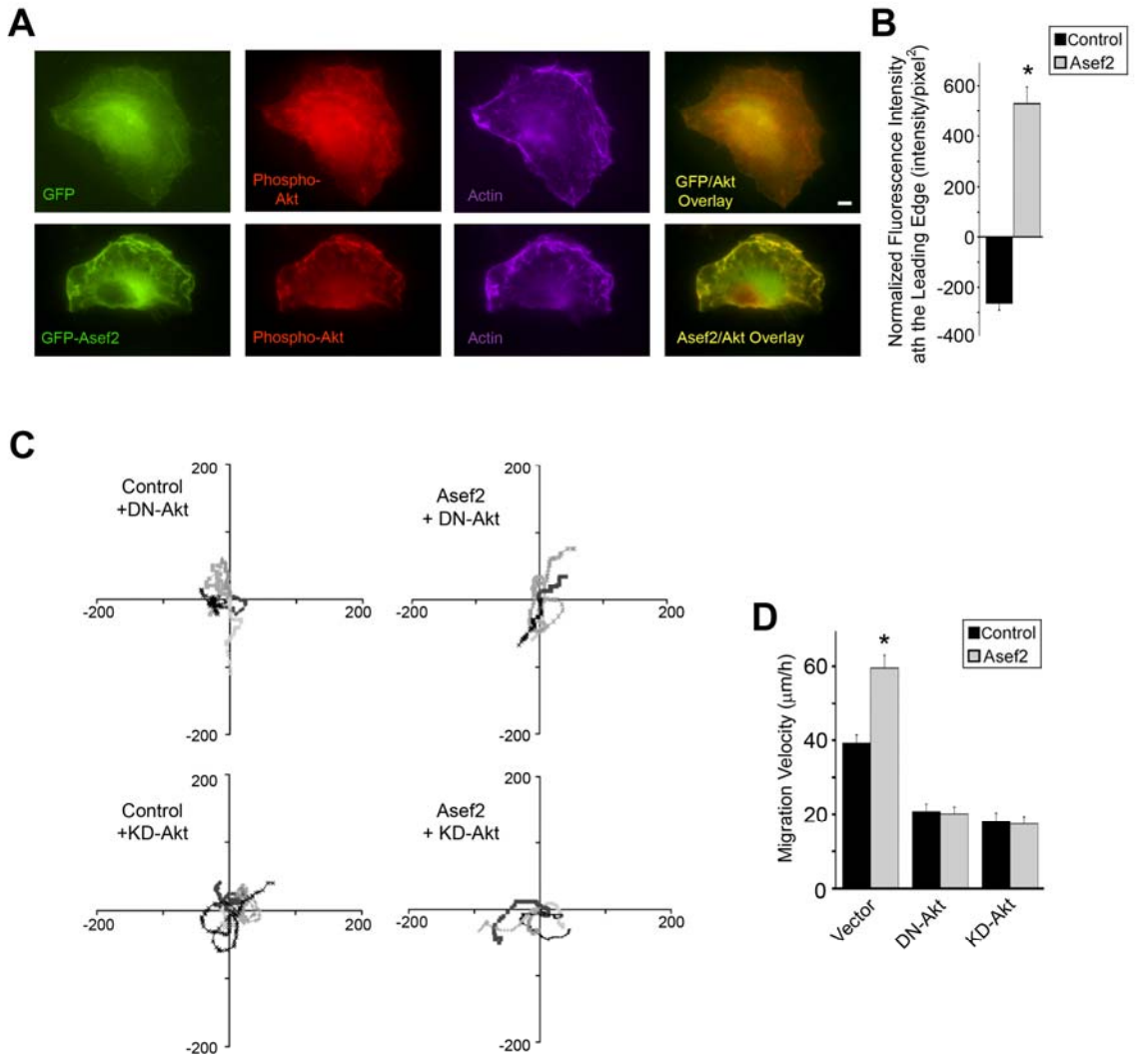


Figure 16. Akt plays a role in Asef2-promoted migration. (A) GFP and GFP-Asef2 stable cells were fixed and co-immunostained for active Akt using a phospho-specific antibody against Thr308 and actin with Alexa Fluor® 647-phalloidin (false colored purple). (B) Quantification of the normalized fluorescent intensity of active Akt at the leading edge of GFP (Control) and GFP-Asef2 stable cells is shown. Error bars represent S.E.M. for 20 cells from three separate experiments (*, $p < 0.0001$). (C) GFP (Control) and GFP-Asef2 stable cells were transfected with DN-Akt, KD-Akt, or empty vector and used in live-cell migration assays. Rose plots with individual migration tracks are shown. (D) Quantification of the migration velocity of GFP (Control) and GFP-Asef2 stable cells are shown. Error bars represent S.E.M. for 30-35 cells from three separate experiments (*, $p < 0.0001$). Asterisk denotes a statistically significant difference compared with GFP (Control) stable cells.

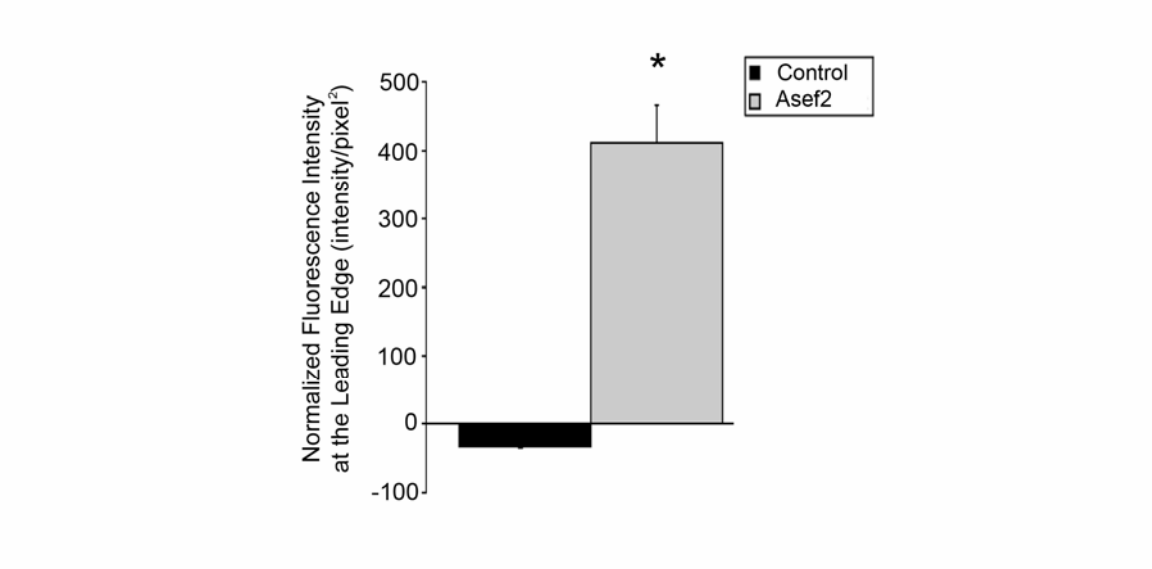


Figure 17. Asef2 increases the total amount of Akt at the leading edge. GFP and GFP-Asef2 stable cells were fixed and co-immunostained for total Akt using a pan-Akt antibody. Quantification of the normalized fluorescent intensity of total Akt at the leading edge of GFP (Control) and GFP-Asef2 stable cells is shown. Error bars represent S.E.M. for 15 cells from three separate experiments (*, $p<0.0001$).

importantly, expression of DN-Akt or KD-Akt completely abolished the Asef2-promoted increase in migration (Fig. 16C and D), suggesting the Asef2-mediated effect on migration is dependent on Akt. We next generated two siRNA constructs to knock down endogenous expression of Akt and determined their effect on Asef2-mediated migration. Akt siRNA #1 and Akt siRNA #2 decreased endogenous expression of the protein by 53% and 46%, respectively, compared with empty pSUPER vector or scrambled siRNA (Fig. 18A and B). As with DN- and KD-Akt, expression of the Akt siRNAs in GFP and GFP-Asef2 stable cells completely abrogated the Asef2-mediated effect on migration (Fig. 18C and D). These results indicate Akt is essential for the Asef2-mediated regulation of cell migration.

Similarly, inhibition of Akt activity had a significant effect on adhesion turnover, suggesting a new role for Akt in regulating this process. When GFP and GFP-Asef2 stable cells were transfected with KD-Akt or Akt siRNA #1, the Asef2-mediated effect on adhesion assembly and disassembly was eliminated (Table 4), indicating Akt is also critical for Asef2-promoted adhesion turnover. Taken together, our results suggest that Akt is necessary for the Asef2-mediated regulation of cell migration and adhesion turnover.

Asef2-mediated regulation of Rho activity is important for cell migration

Our results showed a two-fold decrease in the level of active Rho in GFP-Asef2 stable cells compared with control cells (Fig. 7D), which led us to

Figure 18. Knockdown of endogenous Akt significantly inhibits Asef2-promoted migration. (A) Wild-type HT-1080 cells were transfected with empty pSUPER vector, scrambled siRNA (scr siRNA), or Akt siRNAs. Cell lysates were immunoblotted for Akt or α-tubulin (loading control). (B) Quantification of the amount of endogenous Akt in cells transfected with the indicated constructs. Error bars represent S.E.M. from four independent experiments (*, $p < 0.0003$). Asterisks denote a statistically significant difference compared with pSUPER transfected cells. (C) GFP (Control) and GFP-Asef2 stable cells were transfected with Akt siRNAs and used in live-cell migration assays. Rose plots with individual migration tracks are shown. (D) Quantification of the migration velocity of GFP (Control) and GFP-Asef2 stable cells transfected with the indicated constructs is shown. Error bars represent S.E.M. for 30-35 cells from three separate experiments (*, $p < 0.0001$). Asterisks denote statistically significant differences compared with GFP (Control) stable cells.

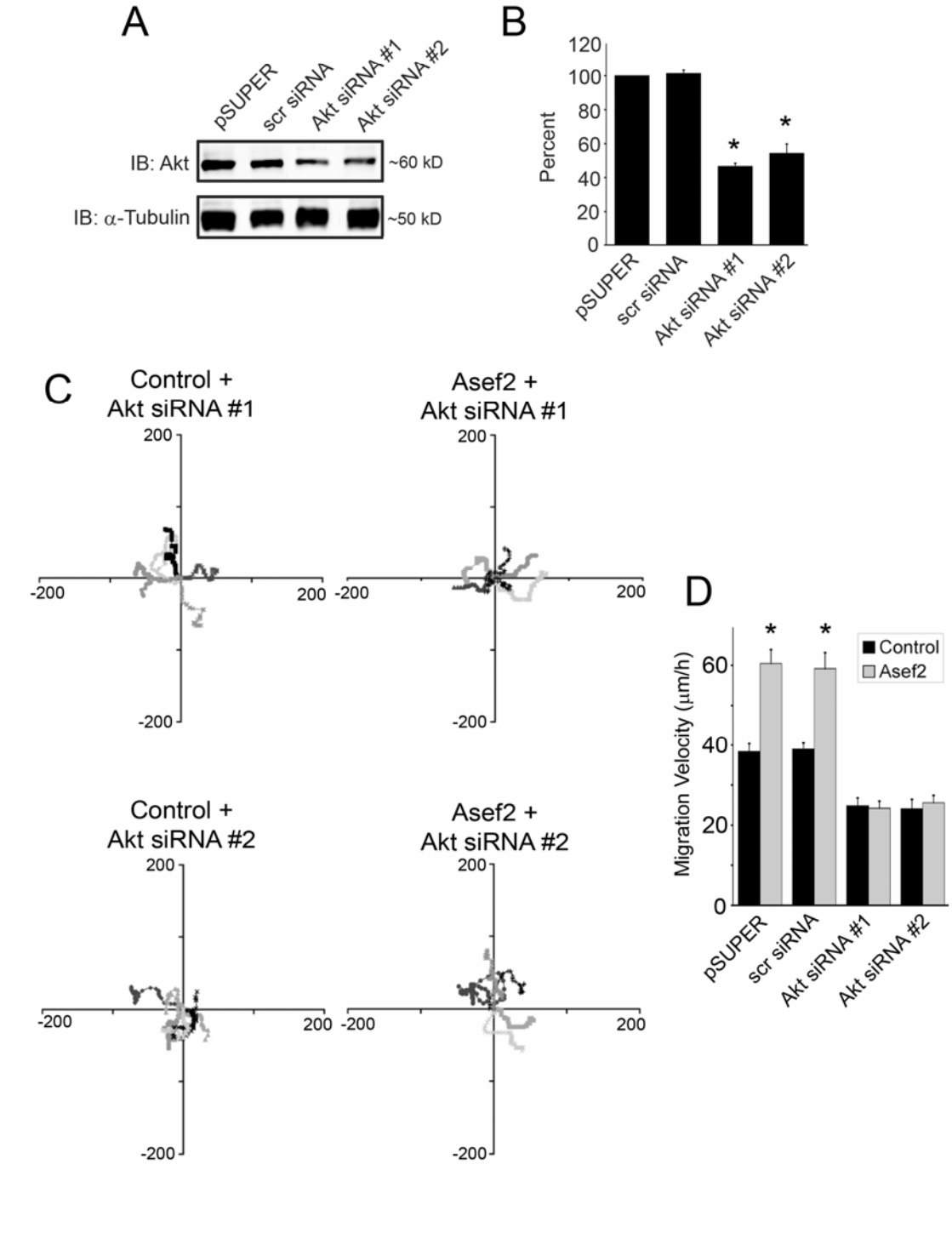

A
pSUPER
scr siRNA
Akt siRNA #1
Akt siRNA #2
IB: Akt
~60 kD
IB: α-Tubulin
~50 kD
B
Percent
120
100
80
60
40
20
0
*
*
pSUPER
scr siRNA
Akt siRNA #1
Akt siRNA #2
C
Control +
Akt siRNA #1
Asef2 +
Akt siRNA #1
Control +
Akt siRNA #2
Asef2 +
Akt siRNA #2
200
-200
200
-200
D
Migration Velocity (μm/h)
60
40
20
0
Control
Asef2
*
*
pSUPER
scr siRNA
Akt siRNA #1
Akt siRNA #2

Table 4. Effect of kinase-dead Akt and Akt knockdown on Asef2-mediated adhesion assembly and disassembly

The $t_{1/2}$s are reported as means ± S.E.M. For each $t_{1/2,}$ measurements were obtained from 15-18 individual adhesions in 4-6 cells from at least three independent experiments.

Constructs Expressed	Apparent $t_{1/2}$ (min) (Adhesion Assembly)	$t_{1/2}$ (min) (Adhesion Disassembly)
GFP + empty vector	3.1 +/- 0.5	6.7 +/- 1.0
GFP + KD-Akt	7.6 +/- 0.9	7.2 +/- 1.2
GFP + Akt siRNA #1	6.6 +/- 0.7	7.1 +/- 0.9
GFP-Asef2 + empty vector	1.8 +/- 0.2	1.8 +/- 0.3
GFP-Asef2 + KD-Akt	7.8 +/- 1.1	7.5 +/- 0.6
GFP-Asef2 + Akt siRNA #1	5.7 +/- 0.7	7.1 +/- 0.8

hypothesize that Rho is an effector of the Asef2 pathway. To test this hypothesis, we expressed constitutively active Rho (CA-Rho) in GFP and GFP-Asef2 stable cells and examined the effect on migration. If the Asef2-promoted increase in migration is due to a decrease in the amount of active Rho, then expression of CA-Rho should abrogate this migration phenotype. Indeed, expression of CA-Rho in GFP-Asef2 stable cells eliminated the increased migration seen with these cells and their migration velocity was identical to that observed in control cells. The migration velocities of GFP and GFP-Asef2 stable cells expressing CA-Rho were 10.8 +/- 1.4 μm/h and 10.8 +/- 1.3 μm/h (n= 20 cells from three separate experiments), respectively, indicating expression of CA-Rho eradicated the Asef2-promoted effect on migration.

We observed a 1.7-fold increase in RhoA activity and impaired migration in Asef2-knockdown cells, supporting our hypothesis that Asef2 regulates migration though Rho. To demonstrate that the migration defect seen in Asef2-knockdown cells was due to an increased amount of active Rho, we inhibited the activity of a Rho effector, Rho-associated kinase (ROCK) and examined the effect on migration. As expected, expression of Asef2 siRNA #1 resulted in a two-fold decrease in the migration velocity compared with cells expressing scrambled siRNA or empty pSUPER vector (Fig. 19). Treatment with the ROCK specific-inhibitor Y-27632 (10 μM) (Uehata, Ishizaki et al. 1997) led to an overall increase in the migration velocity of cells. More significantly, Y-27632 treatment alleviated the migration defect observed with Asef2-knockdown cells and the migration velocity was increased almost 5-fold in these cells (Fig. 19). Taken

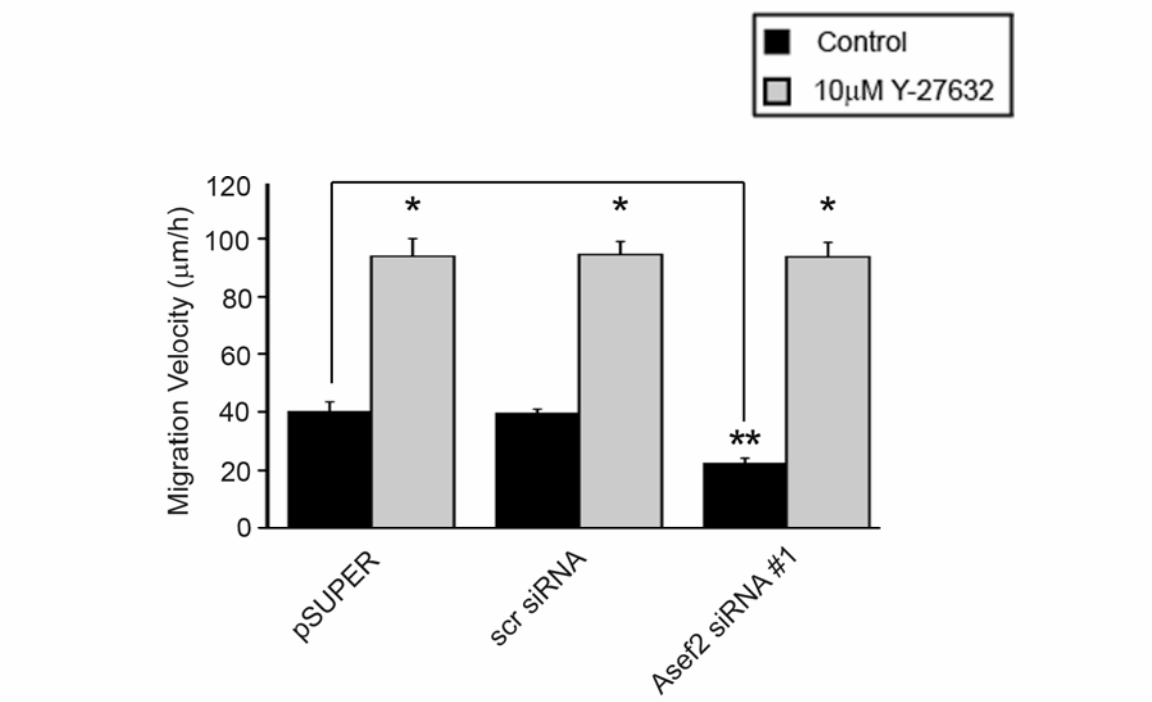


Figure 19. Inhibition of ROCK activity eliminates the migration defect seen in Asef2-knockdown cells. Wild-type HT-1080 cells were transfected with empty pSUPER vector, scrambled siRNA (scr siRNA), or Asef2 siRNA #1. Three days later, cells were treated with vehicle (water), labeled “Control”, or Y-27632 (10 μM) for 30 min and then used in live-cell migration assays. Error bars represent S.E.M. for 20 cells from three separate experiments (*, $p < 0.0001$; **, $p < 0.0001$). The “*” denote statistically significant differences compared with cells treated with vehicle.

together, these results suggest that Rho is an important effector by which Asef2 regulates migration.

Discussion

While it is well understood that Rho GTPase activity is regulated by GEFs, very little is known about the specific role GEFs play in regulating cellular processes, such as migration. Our results show Asef2 coordinately regulates the activities of Rho family members to promote cell migration by stimulating the rapid turnover of adhesions. Asef2 signaling leads to an overall decrease in the amount of active RhoA, which is critical for the effects of Asef2 on migration. RhoA induces the formation of stress fibers and can promote the maturation of nascent cell-matrix contacts into large, focal adhesions (Ridley and Hall 1992; Chrzanowska-Wodnicka and Burridge 1996; Rottner, Hall et al. 1999). Thus, the loss of RhoA activity in Asef2 stable cells could inhibit the maturation of nascent adhesions and contribute to the more rapid turnover of these structures. These described activities of Rho would be expected to inhibit migration. Indeed, in some cell types, high levels of Rho have been shown to impede migration (Cox, Sastry et al. 2001).

Asef2 increases the activity of Rac1 and Cdc42, which is consistent with previous studies showing this molecule has GEF activity for these GTPases (Hamann, Lubking et al. 2007; Kawasaki, Sagara et al. 2007). However, these studies did not detect an effect of Asef2 on RhoA. This is most likely because Asef2 does not directly affect RhoA activity. Our preliminary data indicate the

Asef2-mediated decrease in the amount of active RhoA is dependent upon Rac, PI3K, and Akt (data not shown). The exact mechanism by which these molecules contribute to the inhibition of RhoA activity is currently unknown, but probably involves the regulation of Rho GEFs and/or GAPs, and represents an interesting avenue for future study.

Although Asef2 increases both Rac and Cdc42 activity, it regulates cell migration in a Cdc42-independent manner. The role of Cdc42 in regulating migration is currently not fully understood and seems to be somewhat dependent on cell type. In macrophages, Cdc42 is necessary for the response of these cells to a chemotactic gradient, but not for their migration (Allen, Zicha et al. 1998). Cdc42 is apparently not essential for directed migration of some cells since loss of this GTPase did not significantly affect the migration velocity of fibroblastoid cells in a wound closure assay (Czuchra, Wu et al. 2005). In contrast, Cdc42-deficient mouse embryonic fibroblasts exhibited a defect in their ability to migrate directionally and to respond to a chemotactic gradient (Yang, Wang et al. 2006). In our study, knockdown of Cdc42 in HT-1080 cells significantly decreased their migration velocity, suggesting Cdc42 does contribute to this process. However, Cdc42 does not appear to play a significant role in Asef2-mediated random migration, but we cannot eliminate the possibility that Cdc42 is important for Asef2-promoted chemotaxis or directed migration. Indeed, another Asef family member, Asef1, was recently shown to promote chemotaxis of HeLa cells toward hepatocyte growth factor in a trans-well assay (Kawasaki, Tsuji et al. 2009). While it is not clear whether Cdc42 plays a role in this process, PI3K appears to

be necessary for the Asef1-promoted effect on HeLa cell chemotaxis. Future studies will be needed to understand whether the Asef2-mediated increase in Cdc42 activity that we observed contributes to chemotaxis and directed migration.

The establishment of a Rac-PI3K feedback loop at the leading edge of cells is reported to be a mechanism for recruitment and activation of some GEFs (Lin, Yang et al. 2006; Sjoblom, Jones et al. 2006). This indicates the regulation of GEF activity can be downstream of GTPase signaling. While we cannot completely discount the possibility that this feedback loop occurs with Asef2, inhibition of PI3K activity by treatment with LY294002 or wortmannin did not significantly affect the membrane localization of Asef2, suggesting translocation of Asef2 to the plasma membrane is not PI3K-dependent.

Although Rac can be activated downstream of PI3K signaling (Hawkins, Eguinoa et al. 1995; Hooshmand-Rad, Claesson-Welsh et al. 1997; Cain and Ridley 2009), our results suggest that PI3K is not upstream of Rac in the mechanism by which Asef2 regulates migration. One possibility is PI3K and Akt are downstream effectors of Rac in the Asef2 pathway that regulates cell migration. Consistent with this, Rac has been shown to act upstream of PI3K signaling (Welch, Coadwell et al. 2002; Srinivasan, Wang et al. 2003; Cain and Ridley 2009). Thus, the most likely scenario is that Asef2-promoted activation of Rac leads to an increase in PI3K and Akt activity.

Akt is well known for its role in modulating cell growth and survival, but more recently, there has been a growing interest in the function of Akt in

regulating cell migration. In most cases, Akt stimulates the migration of epithelial cells, fibroblasts, and fibrosarcomas, including HT-1080 cells, but this function of Akt seems to be partially dependent on the isoform present (Kim, Kim et al. 2001; Irie, Pearline et al. 2005; Zhou, Tucker et al. 2006). In our study, expression of Akt siRNAs, DN-Akt, or KD-Akt resulted in a decrease in the migration velocity, which is consistent with Akt promoting migration in HT-1080 cells (Kim, Kim et al. 2001). Interestingly, inhibition of Akt function led to a significant decrease in the turnover of nascent adhesions, which suggests a prominent, new role for Akt in regulating adhesion turnover. Akt has several downstream effectors, including p21-activated kinase (PAK) and glycogen synthase kinase 3 β (GSK-3 β), which are known to regulate cell migration and actin dynamics. The phosphorylation of PAK1 by Akt promotes the activation of this protein, which in turn, modulates reorganization of the actin cytoskeleton and thereby regulates migration (Bokoch 2003; Yang, Tschopp et al. 2003). Akt phosphorylation of GSK-3β on serine 9 results in inactivation of this kinase, which is important for the maintenance of a polarized migratory phenotype (Cross 1995; Etienne-Manneville and Hall 2003).

Our results point to a working model in which Asef2 stimulates cell migration through an increase in Rac activity. A local increase in Rac activity can promote the formation of nascent adhesions at the leading edge. At least one model for migration suggests it is important to keep Rho activity low at the leading edge of cells (Ridley, Schwartz et al. 2003). The Asef2-induced decrease in Rho activity could impair the maturation of nascent adhesions into large, focal adhesions. As a result, these adhesions do not mature into focal

adhesions, but instead turn over. Highly motile cells, such as keratocytes and neutrophils, do not have large, well organized adhesions (Yuruker and Niggli 1992; Lee and Jacobson 1997) while slower moving cells form larger, more mature adhesions (Couchman and Rees 1979). It has been proposed that the turnover of small, dynamic, leading edge adhesions drives the rapid migration of cells (Nayal, Webb et al. 2006). In this way, Asef2 could significantly enhance the migration velocity of cells by stimulating the rapid turnover of adhesions through the coordinated regulation of the Rho GTPases. The Asef2-mediated regulation of migration and adhesion turnover are dependent on PI3K and Akt, which could contribute to these processes by modulating the activity of Rho GTPases.

CHAPTER IV

ASEF2 INDIRECTLY INHIBITS RHO ACTIVITY

Asef2 decreases Rho activity in a Rac-dependent manner

As discussed earlier, we found by active GTPase pull-down that Asef2 increases Rac and Cdc42 activation by approximately two-fold, each. These data are supported by the findings of other groups that Asef2 binds to and activates both Cdc42 and Rac. These groups have also reported that Asef2 does not bind or activate Rho (Hamann, Lubking et al. 2007; Kawasaki, Sagara et al. 2007). However, our data show that Asef2 decreases the amount of active Rho in cells by two-fold. This led us to hypothesize that Rho is an effector of the Asef2/Rac pathway, and that Asef2's activation of Rac indirectly inhibits Rho. In order to test this hypothesis, we used siRNA to knock down endogenous expression of Rac in both GFP and GFP-Asef2-expressing cells, and assessed the relative amounts of active Rho in these cells compared to controls. As a control, GFP and GFP-Asef2-expressing cells were co-transfected with empty pSUPER vector and myc-tagged Rho. When the amount of active Rho was assayed, we found a two-fold decrease in the Asef2-expressing cells compared to that in GFP cells. In contrast, co-transfection of these cells with myc-Rho and Rac siRNA abolished the Asef2-mediated decrease in active Rho (Fig. 20A and B). Following siRNA-mediated knockdown of endogenous Rac, GFP and GFP-Asef2 cells contained the same relative amount of active Rho. This strongly suggests that Rho is

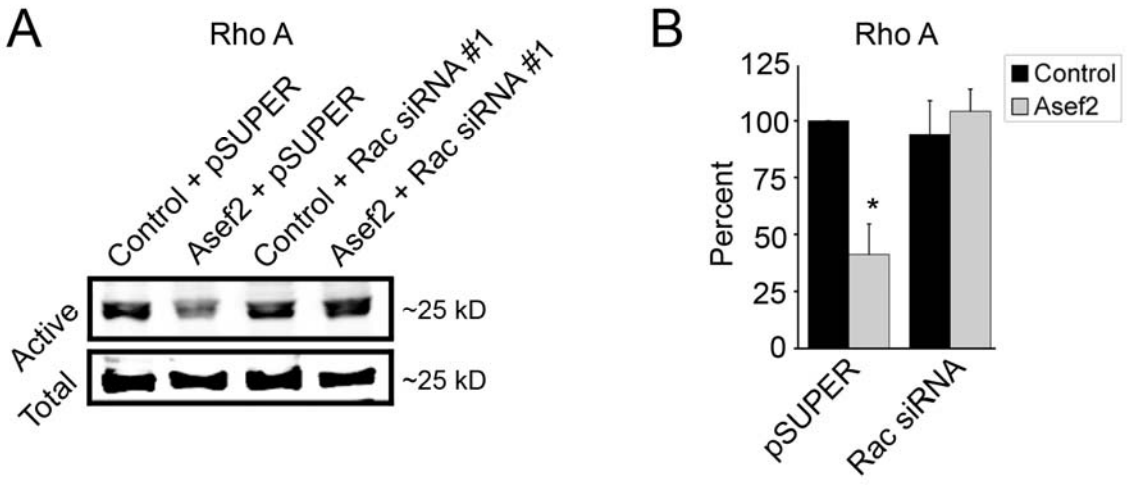


Figure 20. The Asef2-mediated decrease in Rho activity is dependent on active Rac. (A) GFP (Control) and GFP-Asef2 expressing cells were transfected with Rac siRNA #1 or empty pSUPER vector and cell lysates were assayed for active Rho. (B) Quantification of blots as described in panel A is shown. Error bars represents S.E.M. from four separate experiments (*, $p < 0.003$). For panel B, asterisks denote statistically significant difference when compared with GFP (Control) expressing cells.

an effector of the Asef2/Rac pathway where it is inhibited by Asef2 in a Rac-dependent manner.

Asef2 activates Rac in a PI3K-independent manner

Our data indicate that Asef2 promotes migration and rapid adhesion turnover in a PI3K-dependent, Rac-dependent manner. We next examined whether PI3K activation was necessary for Asef2 to increase Rac activation. To assess the effect of PI3K activation on Asef2-promoted Rac activation, we transfected GFP control cells and GFP-Asef2-expressing HT-1080 cells with flag-tagged Rac, then treated the cells for 2 h with the chemical PI3K inhibitor, wortmannin (10 nM), prior to lysis. In the absence of wortmannin (treated with DMSO alone), the amount of active Rac in Asef2 cells increased approximately two-fold over that in control cells. Interestingly, we found the same results in Asef2-expressing cells compared to controls following treatment with wortmannin (Fig. 21). These data suggest PI3K activation is not necessary for Asef2-promoted Rac activation, but rather suggest that it may function elsewhere in the Asef2 pathway.

Asef2 suppresses Rho activation through PI3K

To determine whether PI3K played a role in the observed Asef2-mediated decrease in Rho activation, we transfected GFP and GFP-Asef2-expressing cells with myc-Rho, then treated the cells with 10 nM wortmannin prior to lysis. The amount of active Rho was decreased significantly in GFP-Asef2-expressing cells

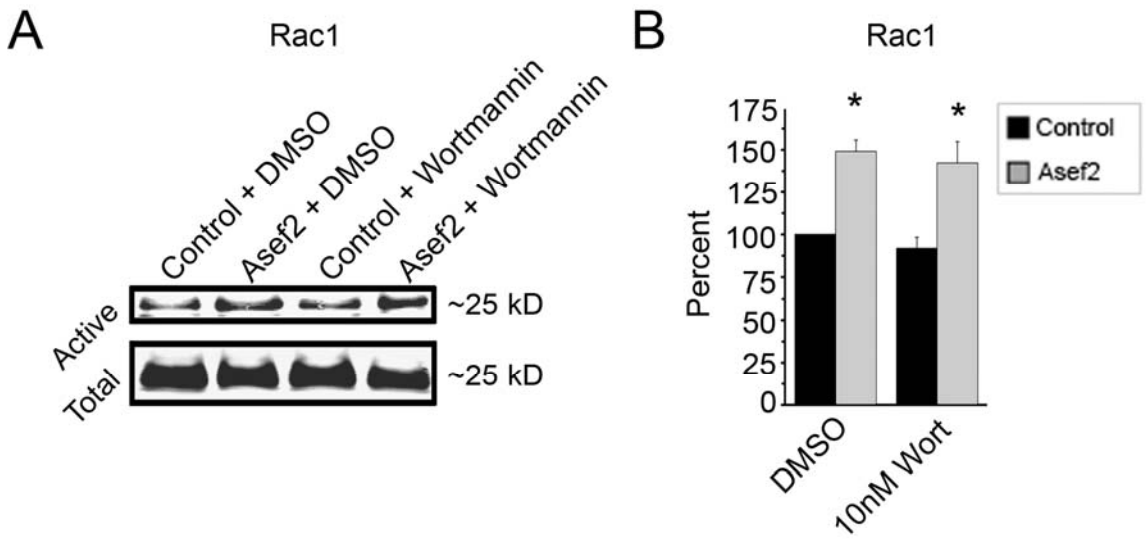


Figure 21. The Asef2-mediated increase in Rac activity is independent of PI3K. (A) GFP (Control) and GFP-Asef2 expressing cells were treated with vehicle (DMSO) or 10 nM wortmannin and cell lysates were assayed for active Rac. (B) Quantification of blots as described in panel A is shown. Error bar represents S.E.M. from five separate experiments (*, $p < 0.0001$). For panel B, asterisks denote statistically significant difference when compared with GFP (Control) expressing cells.

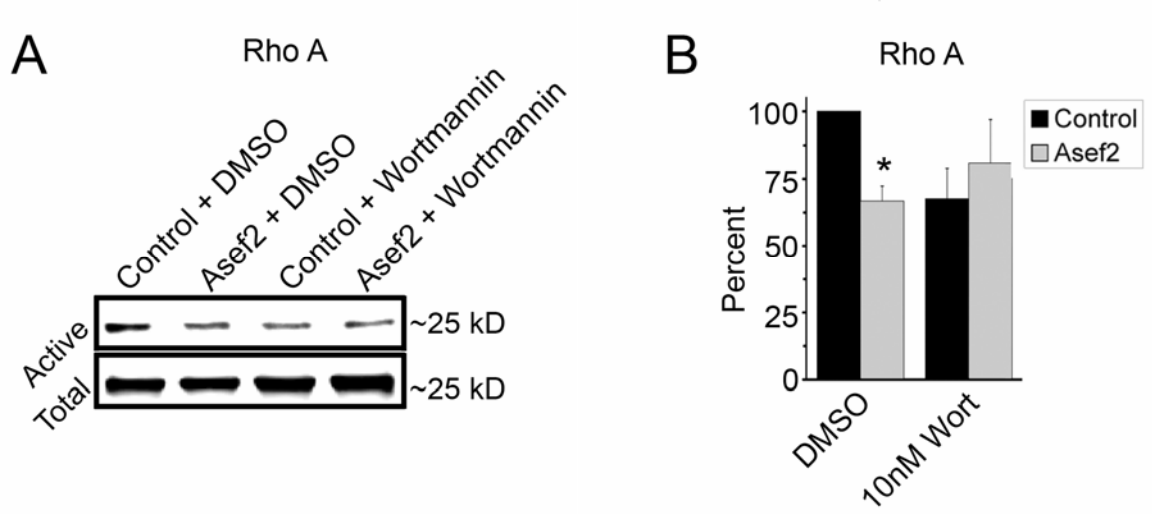


Figure 22. The Asef2-mediated decrease in Rho activity is dependent on PI3K. (A) GFP (Control) and GFP-Asef2 expressing cells were treated with vehicle (DMSO) or 10 nM wortmannin and cell lysates were assayed for active Rho. (B) Quantification of blots as described in panel A is shown. Error bar represents S.E.M. from five separate experiments (*, $p < 0.0001$). For panel B, asterisks denote statistically significant difference when compared with GFP (Control) expressing cells.

compared with GFP control cells, when treated with DMSO alone (Fig. 22). However, treatment of GFP and GFP-Asef2-expressing cells with 10 nM wortmannin prior to lysis completely abolished the Asef2-promoted decrease in Rho activity. The amount of active Rho measured in control and Asef2-expressing cells was similar when PI3K was inhibited, suggesting PI3K activity is necessary for Asef2-stimulated Rho inhibition.

Asef2-promoted Rho suppression is Akt-dependent

Migration and adhesion turnover data also indicated Akt as an important member of the Asef2 pathway. Akt is commonly a downstream effector of PI3K, so having determined that Asef2's regulation of Rho activity is PI3K-dependent, we next examined Akt's role in this pathway. We used a kinase-dead mutant of Akt to determine whether Akt activation is necessary for Asef2-mediated Rho inhibition. The expression of KD-Akt had a similar effect to that of Rac knockdown and PI3K inhibition on the Asef2-promoted decrease of Rho activation. Consistent with our previous findings, GFP-Asef2 cells co-transfected with myc-Rho and empty vector (control) had significantly less active Rho than GFP cells under the same conditions. In contrast, GFP and GFP-Asef2-expressing cells co-transfected with myc-Rho and KD-Akt had comparable amounts of active Rho. This indicates that the Asef2-mediated decrease of Rho activity is abrogated by Akt inhibition (Fig. 23), suggesting that Akt is a downstream effector of Asef2 in a pathway that leads to Rho inhibition.

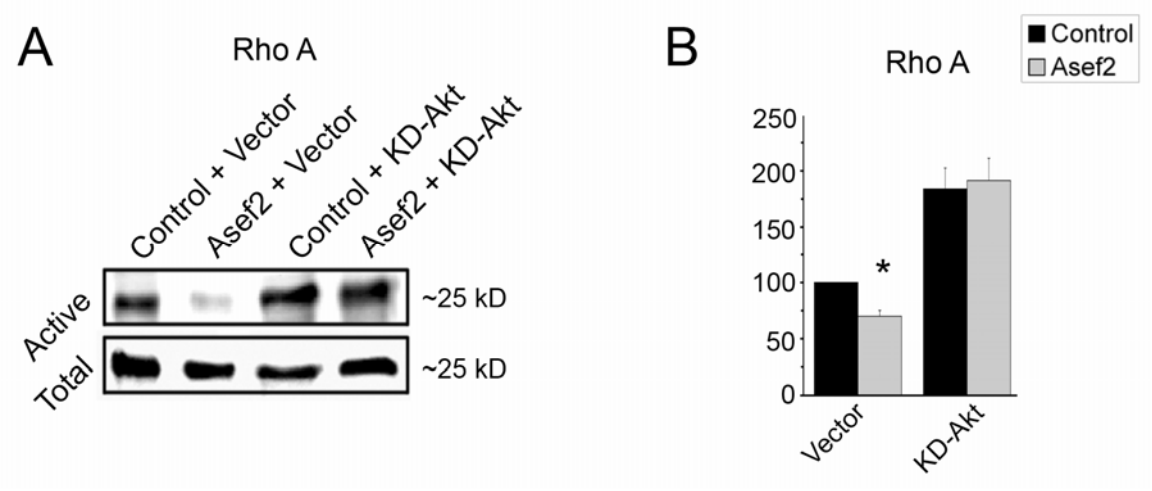


Figure 23. The Asef2-mediated decrease in Rho activity is dependent on Akt. (A) GFP (Control) and GFP-Asef2 expressing cells were transfected with KD-Akt or empty vector and cell lysates were assayed for active Rho. (B) Quantification of blots as described in panel A is shown. Error bar represents S.E.M. from three separate experiments (*, $p < 0.005$). For panel B, asterisks denote statistically significant difference when compared with GFP (Control) expressing cells.

Summary

Here we offer evidence that Rho is a downstream effector of Asef2. Active GTPase pull-down assays were used to determine that Asef2 inhibits Rho activation in a Rac-dependent manner. Further, PI3K activation, which was earlier shown to play an important role in Asef2-mediated promotion of migration and rapid adhesion turnover, is not required for Asef2-promoted activation of Rac, but is necessary for Asef2-mediated Rho inhibition. Finally, Akt activation is also necessary for suppression of Rho activity by Asef2. As Akt frequently acts as a downstream effector of PI3K, it is likely to function similarly here.

CHAPTER V

SUMMARY AND CONCLUSIONS

Significance

The research presented in this dissertation demonstrates that the Rho Family GEF, Asef2, functions as an important regulator of cell migration, and specifically, that it regulates the assembly and disassembly, or turnover, of cell-matrix adhesions. This is of note, because it is thought that an optimal adherence of the cell to its substrate must be maintained for migration to proceed ideally, and tighter or looser adherence to the substrate typically leads to a defect in migration (Palecek, Huttenlocher et al. 1998). In this case, the decreased adherence to the substrate improves the cell's ability to migrate. Here, we show that a rapid turnover of adhesions, promoted by Asef2, contributes significantly to increase random migration velocity.

Asef2 increases the activation of Rac and Cdc42, but decreases Rho activation. Prior to this dissertation, Asef2 was shown to bind to and activate both Cdc42 and Rac, but appeared to have no effect on Rho activation. Further, the activation of Rac or Cdc42 seemed to occur separately in a cell type-specific manner. We did not find any evidence of cell-type specificity in the course of this body of work. In HEK 293 or HT-1080 cells, Asef2 was found to increase both Rac and Cdc42 while decreasing Rho activity, as demonstrated by our active GTPase pull-down assays, described previously. This suggests the possibility of

complex differential regulation of Asef2 in various cell types, but more importantly, the data presented here indicate an important role for Asef2 as an activator of Rac and Cdc42 as well as a negative regulator of Rho that had previously not been described.

Moreover, the research presented in this dissertation suggests a mechanism by which the activation of Rac leads to the inhibition of Rho, thereby promoting rapid adhesion turnover. Although many pathways have been examined in which the antagonistic regulation of Rho by Rac and vice versa is well documented, this is the first evidence that Rac activation regulates adhesion turnover in particular by inhibiting Rho.

Although PI3K and Akt are well-established as regulators of cell migration, specific mechanisms through which they function as such are not well described. Here, we offer evidence that PI3K and Akt function downstream of Asef2 to promote migration through a novel mechanism that involves the modulation of adhesions leading to their rapid assembly and disassembly. Furthermore, in this mechanism, PI3K and Akt function to negatively regulate Rho activation. Recent studies have implicated PI3K and Akt as important negative regulators of Rho activation (Kakinuma N 2008). However, these studies have revealed a mechanism by which these molecules decrease Rho activation to promote migration by disrupting stress fiber formation, but have not detected an effect on adhesion turnover. The data presented in this thesis suggest a novel role for Akt as an important regulator of adhesion dynamics, as well as providing a novel mechanism by which PI3K and Akt modulate Rho activity.

Asef2 is an important regulator of cell migration and, in particular, adhesion assembly and disassembly. It modulates these processes by increasing Rac activity and decreasing Rho activity. The role of Asef2-mediated Cdc42 activation is still not clear, but may be an important mechanism for regulating cell polarity and directional migration. Asef2 appears to indirectly inhibit Rho activation in a novel mechanism that requires increased Rac, PI3K, and Akt activity. This previously-undescribed mechanism functions to promote cell migration and rapid adhesion turnover, and suggests a possible role for Asef2 in pathological conditions such as cancer, where dysregulation of Asef2 could potentially promote cancer cell metastasis.

Summary

The work detailed in this dissertation began with a proteomics search for migration-related molecules by Donna Webb in which an unknown protein was pulled down from cell lysates in separate experiments by PAK and paxillin, and identified by mass spec. Analysis indicated the presence of PH, DH, and SH3 domains within the protein, suggesting a probable role as a Rho Family GEF. At that time, there was nothing in the literature describing this molecule, and early work in the Webb lab determined it increased Rac and Cdc42 activity. Thus, it was termed CRAG for Cdc42 and Rac Activating GEF. After the identification of CRAG by the Webb lab and before the publication of this dissertation or any peer-reviewed research articles related to CRAG, two groups nearly simultaneously identified a Rho Family GEF called Asef2 in a database search

for molecules with homology to Asef (now Asef1). The first data identifying Asef2 was published in 2007 (Hamann, Lubking et al. 2007; Kawasaki, Sagara et al. 2007). It has since been determined by sequence analysis that CRAG and Asef2 are one and the same. Fortunately, the data published by others on Asef2 does not overlap significantly with that presented in this dissertation.

As a regulator of Rho GTPases, Asef2 was thought to be a potential regulator of cell migration, and the data in this dissertation strongly supports this hypothesis. Like GFP-Asef2, endogenous Asef2 co-localizes with actin at the leading edge of migrating cells. This localization not only suggests Asef2 may be interacting with actin, even if only indirectly, but is also similar to localization of known actin modulators and other molecules known to regulate migration.

Using an active GTPase pull-down assay, we have shown that Asef2 increases the amount of active Cdc42 and Rac significantly in both HEK 293 and HT-1080 cell lines. This is supported by data recently published by Kawasaki *et al.* (2007) and Hamann *et al.* (2007) showing that Asef2 binds to and activates Cdc42 and Rac *in vitro* and in cell lines. However, these studies found Asef2-mediated activation of Rac and Cdc42 occurred in a cell type-specific manner. Our data indicate Asef2 is capable of increasing activation of both GTPases in HEK 293 and HT-1080 cell lines to approximately the same degree. However, none of the previous studies included either of these cell lines. Also of note, data presented in this study indicate that Asef2 is capable of decreasing the amount of active Rho in cells. This was not previously discovered, probably because other groups who have investigated Asef2 tested direct activation of GTPases by

Asef2 in biochemical *in vitro* assays. An indirect inhibition of Rho by Asef2 via a downstream pathway would not be detected in such a manner. As an activator of Cdc42 and Rac and an inhibitor of Rho, Asef2 has great potential to play an important role as a regulator of cell migration and its underlying processes.

Previous studies have shown that Asef2 expression increases migration of MDCK and HeLa cells in a trans-well assay, but little else is known about Asef2-mediated effects on migration (Kawasaki, Sagara et al. 2007). Data presented in this thesis support previous findings, as MCF-7 human carcinoma cells transiently transfected with GFP-Asef2 exhibited increased migration in a trans-well assay compared to control cells expressing GFP alone (Figure 24). Furthermore, Asef2 promotes undirected migration, and this is evident from data collected in random migration assays. HT-1080 cells expressing low levels of GFP-Asef2 migrate 1.5-fold faster on average than GFP control cells on a Fn substrate. The specificity of Asef2's role in this increased migration effect is confirmed by siRNA-mediated knockdown of endogenous Asef2 in HT-1080 cells. Asef2 knockdown significantly decreases the migration velocity, compared to HT-1080 cells expressing empty vector or scrambled siRNA, which lack any migration defects. This migration defect is completely rescued with the expression of constitutively active Asef2, supporting the hypothesis that Asef2 is an important regulator of cell migration.

Asef2 also increases migration of cells into a scratch-wound, which is an indicator that Asef2 may play an important role in directional migration, since the cell monolayer is migrating generally in one direction—into the wound (Fig. 25).

The ability of Asef2-expressing cells to fill a scratch-wound faster than control cells are able to fill a similarly sized wound is the result of Asef2-mediated enhanced migration, and not due to differences in cell proliferation. Since Cdc42 is attributed with regulating cell polarity and directional migration, it would be interesting to determine whether these data are the result of an Asef2-mediated increase of Cdc42 activation. Clearly, Asef2 is an important regulator of migration, and these data suggest it may affect both random and directional migration. However, the act of migration is a multi-step process, and these random migration and scratch-wound assays have not offered adequate insight into Asef2's role in regulating the individual steps involved in migration, such as adhesion assembly and disassembly.

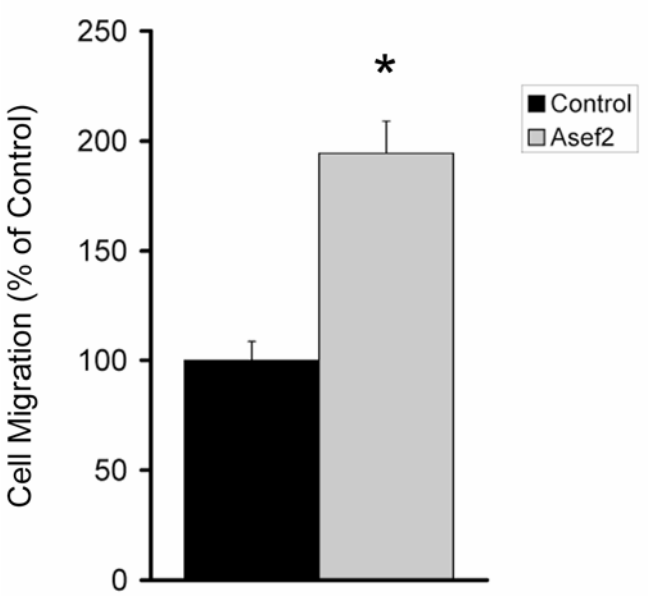


Figure 24. Asef2 increases MCF-7 cell migration through a trans-well. Transiently transfected MCF-7 cells expressing GFP-Asef2 traversed a trans-well membrane more effectively than GFP-expressing controls. The number of migrating cells was normalized according to the transfection efficiency. The average number of control cells able to pass through the trans-well was set to 100%. (Control cells, 100% +/- 8.6; GFP-Asef2 cells, 194.4% +/- 14.7; n=11-12 wells from 3 separate experiments; * $p<0.0001$)

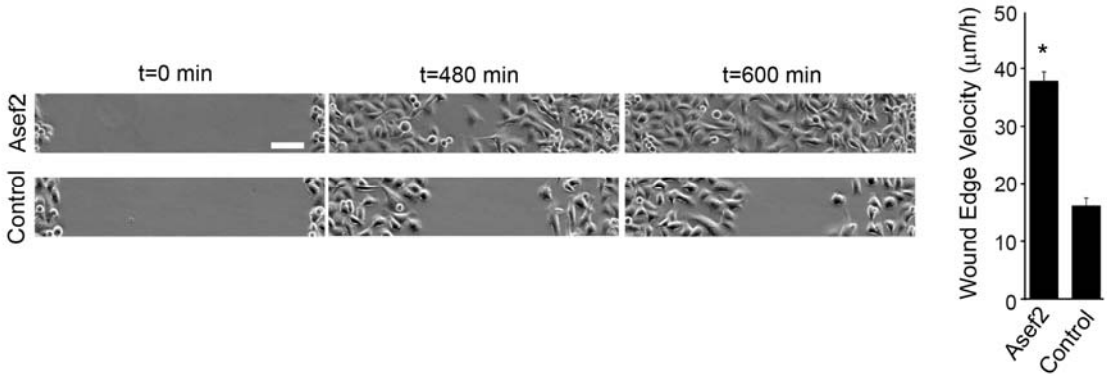


Figure 25. Asef2 promotes scratch-wound closure. A confluent monolayer of GFP (control) and GFP-Asef2 stably expressing HT-1080 cells was "wounded" to generate an area denuded of cells. Time-lapse images were collected as the cells migrated to close the wound and images from the time-lapse movies are shown. Bar, 100 μm. Quantification of the migration velocity at the wound edge is shown (far right). Error bars represent S.E.M. for 42-56 data points from three separate experiments. * $p<0.0001$.

The antagonistic relationship between Rac activation and Rho inhibition has been well established over the years. As discussed earlier, Asef2 increases Rac activation and decreases Rho activity. This suggests an intriguing possible role for Asef2 as a player in the push-pull relationship between these molecules. Asef2 increases migration, and it has a curious effect on Rac and Rho activity. Both of these GTPases have been associated with the formation of cell-matrix adhesions, and are important regulators of adhesion turnover. This led us to ask whether Asef2 regulates adhesion dynamics, so we compared TIRF images of GFP-Asef2 cells stained for paxillin or vinculin to visualize adhesions with control cells under the same conditions. Adhesions in GFP-Asef2 cells were localized primarily at the perimeter of the cell, and were considerably smaller than those in control cells, which had several larger adhesions spread throughout the cell body. Quantification of this phenomenon was achieved with use of an adhesion turnover assay in which individual adhesions in a time-lapse movie were observed as they assembled and disassembled over time. A striking difference in the apparent $t_{1/2}$ of adhesion assembly and the $t_{1/2}$ of adhesion disassembly was observed in cells expressing GFP-Asef2 compared to control cells. Adhesions assembled in Asef2 cells 1.8-fold faster and disassembled 4.4-fold faster than those in control cells. These data are strongly supported by the significant increase in apparent $t_{1/2}$ of adhesion assembly and $t_{1/2}$ of adhesion disassembly in wild-type HT-1080 cells that occurs when endogenous Asef2 is knocked down by siRNA. Not surprisingly, adhesions in Asef2-knockdown cells appear larger and more abundant than those in control cells, as seen by TIRF microscopy.

Taken together, these data implicate Asef2 as an important regulator of adhesion turnover.

Others have shown that Asef2 binds to and activates Rac, as discussed previously, and data presented in this study support this—Rac activation is increased by Asef2 expression in HEK 293 and HT-1080 cells. Since cells expressing Asef2 have enhanced migration and increased levels of active Rac, we hypothesized Asef2 may promote migration in a Rac-dependent mechanism. Two Rac-specific siRNA constructs were expressed in Asef2 and GFP cells individually, and indeed, Rac knockdown not only decreased the migration velocity of both cell lines compared to empty vector and scrambled siRNA controls, but it abolished the Asef2-mediated migration advantage. Cells treated with Rac-siRNA all migrated at the same velocity, regardless of Asef2 expression. These data support a mechanism by which Asef2 increases Rac activity to promote cell migration.

In contrast, Asef2-mediated activation of Cdc42 does not function as a random migration-promoting mechanism. Expression of Cdc42-specific siRNA in Asef2-expressing and GFP control cells failed to eliminate the Asef2-mediated increase in migration velocity. Although there was an overall decrease in the absolute velocities of both cell lines, the Asef2-expressers were still significantly faster than control cells under the same conditions. Treatment with the Cdc42-specific chemical inhibitor, secramine A, confirmed these findings. Taken together, these data strongly suggest Asef2-promoted random migration is Rac-dependent, but Cdc42-independent. This is not surprising, as Cdc42 is typically

associated with filopodial formation, establishment and maintenance of cell polarity, and directional migration, rather than undirected migration.

As Cdc42 does not appear to regulate Asef2-mediated migration, we chose to look elsewhere for potential downstream targets of Asef2 that may regulate rapid adhesion turnover. Rac is well established as a regulator of adhesion dynamics. As an activator of Rac and an important regulator of adhesion dynamics, Asef2 is likely to regulate adhesions in a mechanism that involves Rac activation. Analysis of adhesion turnover in Asef2-expressing cells treated with Rac-specific siRNA, compared to control cells under the same conditions revealed that Asef2 promotes adhesion turnover in a Rac-dependent manner.

Molecular regulators of cell migration other than Rho GTPases were examined as potentially integral players in the Asef2 pathway. Inhibition of PI3K with wortmannin or LY294002 in control and GFP-Asef2 cells decreased migration overall, but importantly, cells expressing Asef2 no longer migrated any more rapidly than control cells. Like Rac, PI3K activation is required for Asef2-mediated migration. In addition, adhesion turnover in both Asef2 and control cells is drastically slowed to the same apparent $t_{1/2}$ of assembly and $t_{1/2}$ of disassembly in both cell lines when PI3K is inhibited, demonstrating a dependence of Asef2-promoted adhesion turnover on PI3K activation.

As a well-established downstream effector of PI3K with a known ability to affect cell migration, Akt is another molecule we deemed likely to interact with members of the Asef2 pathway in a manner that could affect migration and

adhesion dynamics. We found that GFP-Asef2-expressing cells, unlike GFP-expressing controls, have an enrichment of active Akt at the leading edge that co-localizes with Asef2 and actin, as indicated by immunofluorescence. Not surprisingly, expression of kinase-dead or dominant negative Akt mutants or Akt siRNA in control and Asef2-expressing cells caused a global decrease in migration velocity in both cell lines and abolished the Asef2-mediated increase in migration velocity seen previously. Therefore, Akt is also an important regulator of Asef2-mediated adhesion turnover, apparent by significantly slowed turnover--both assembly and disassembly-- seen in Asef2 and control cells expressing KD-Akt or Akt siRNA. As was demonstrated previously with Rac and PI3K, disrupting Akt function abrogates Asef2-mediated effects on adhesion dynamics.

Previously, we found that Asef2 decreases Rho activity in cells, and hypothesized that this Rho inhibition may be integral to the observed Asef2-mediated enhanced migration. We confirmed this by circumventing Asef2's effects on Rho with the expression of a constitutively-active form of Rho (CA-Rho), which decreased the observed migration velocities of GFP control cells and Asef2 cells equally. Further, we treated Asef2-knockdown cells with the Rho-associated kinase (ROCK)-specific inhibitor, Y-276362, and saw a complete alleviation of the migration defects previously seen in cells treated with Asef2 siRNA. Indeed, Rho inhibition appears to be an important aspect of Asef2-mediated migration.

Data presented in this dissertation offer some insight to the molecular mechanism by which Asef2 promotes cell migration and adhesion turnover.

Having identified PI3K and Akt as important molecular regulators in the Asef2 pathway, in addition to Rac, we sought to elucidate the relationship of these molecules to one another, and to clarify the mechanism by which Asef2 inhibits Rho. Interestingly, we found that Asef2 continues to localize normally to the plasma membrane when PI3K is inhibited with wortmannin or LY294002. Using an active GTPase pull-down, we showed that PI3K activation, while necessary for Asef2-mediated migration and adhesion regulation, is not necessary upstream of Rac in this pathway. Treating cells with wortmannin prior to active-Rac pull-down had no effect on the relative amount of active Rac in Asef2 cells compared to controls. A similar experiment determined PI3K activation is required for Asef2-mediated Rho inhibition. Inhibition of PI3K by wortmannin alleviated the Asef2-mediated suppression of Rho activity seen in untreated cells. It is interesting to note that although the Asef2-mediated suppression of Rho activity is relieved by PI3K inhibition, overall Rho activity is decreased by wortmannin in control cells. This is likely a side-effect of global PI3K suppression in the cell, indicating multiple PI3K-mediated pathways may be responsible for Rho regulation in cells, some of which may lead to Rho activation.

As suggested earlier, it is possible Asef2-mediated activation of Rac could potentially lead to a downstream inhibition of Rho activity. Data presented here indicate this is indeed the case. By knocking down endogenous Rac with siRNA prior to pulling down active Rho, we have shown that Asef2-mediated Rho suppression is Rac-dependent. Cells expressing Rac siRNA have the same relative amount of active Rho, regardless of Asef2 expression. When taken

together with the data indicating PI3K acts in this pathway upstream of Rho, but not upstream of Rac, these data suggest a pathway in which Asef2 increases Rac activation upstream of PI3K activation, which leads to downstream Rho inhibition.

To further elucidate this pathway, the effect of Akt activity on Asef2-mediated Rho inhibition was assessed. As a downstream effector of PI3K with a known role in regulation of cell migration, it was not surprising that Akt activation was required for Asef2-mediated Rho inhibition. KD-Akt relieved Asef2-mediated suppression of Rho activity. While this identifies Akt as an important regulator of Asef2-mediated Rho inhibition, it does not identify Akt's place in this pathway. It is, however, very appealing to suggest it lies just downstream of PI3K, where it is activated by a lipid product of PI3K.

In summary, this dissertation describes the role of a recently identified Rho family GEF in regulating cell migration and begins to elucidate the molecular mechanism by which this GEF acts. Asef2 increases Rac and Cdc42 activation, but decreases Rho activity. Asef2 co-localizes with actin at the leading edge of cells and promotes random and directional migration and rapid adhesion turnover. Asef2's effects on random migration are independent of Cdc42 activation, but are dependent on Rac, PI3K, and Akt activation. Adhesion turnover is also promoted by Asef2 in a manner that depends upon activation of Rac, PI3K, and Akt (Fig. 26). Asef2-mediated activation of Rac is not PI3K-dependent, but its inhibition of Rho is PI3K-dependent. Asef2-mediated inhibition of Rho is also dependent on Rac and Akt activation. These data suggest a novel

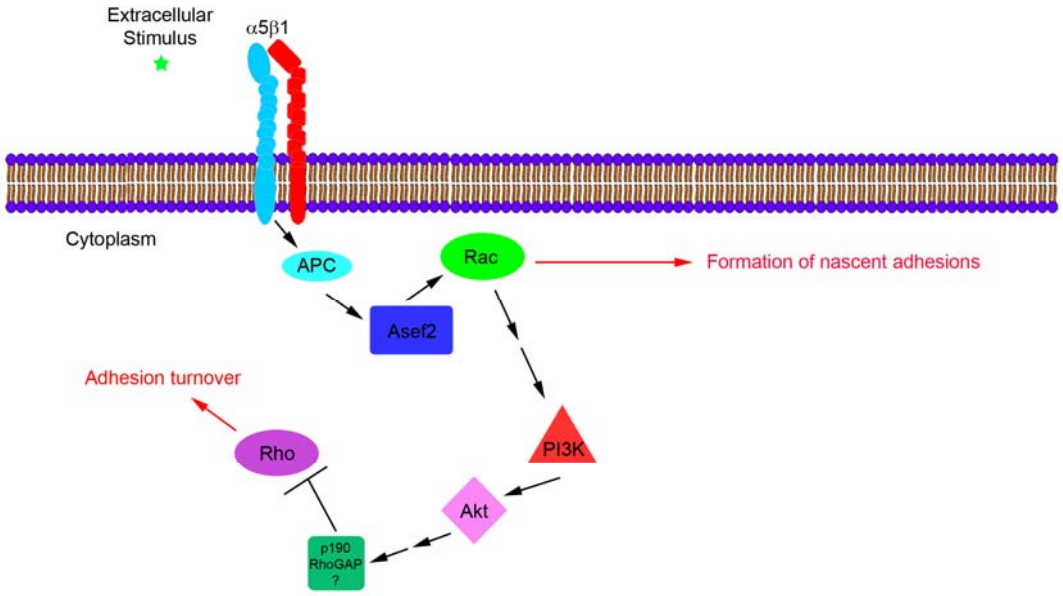


Figure 26. Predicted Asef2 model. An external stimulus activates integrin heterodimers, causing a signaling cascade that leads to Asef2 activation at the cell's leading edge, when APC binds it and relieves Asef2 from its autoinhibitory conformation. Now active, Asef2 can bind GDP-bound Rac, causing a conformational change that allows Rac to release GDP and bind GTP, becoming active. Active Rac then stimulates the formation of nascent adhesions at the leading edge that stabilize extending membrane protrusions. Active Rac leads to the activation of PI3K via a currently unknown mechanism, which leads to Akt activation. It is predicted that p190 RhoGAP becomes activated downstream of PI3K and Akt, which then causes the observed inhibition of Rho activity. The inhibition of Rho prevents adhesion maturation, facilitating rapid adhesion turnover.

mechanism of adhesion turnover regulation, and implicate Asef2 as an important regulator of cell migration.

Conclusions and Future Directions

Data presented in this dissertation and by others indicate Asef2 increases Rac and Cdc42 activation. While Kawasaki *et. al.* (2007) and Hamann *et. al* .(2007) used *in vitro* biochemical analysis to determine binding and activation specificity of Asef2 for Cdc42 and Rac with no interaction or influence on Rho activity, we have shown that Asef2 increases Rac and Cdc42 activation and decreases Rho activity in cells. Others have concluded that Asef2 activates Cdc42 or Rac in a cell-type specific manner when examining HeLa or MDCK cells—something we did not experience in our work with HEK 293 or HT-1080 cell lines. It would be interesting to determine Asef2-mediated GTPase activation in HEK and HT-1080 cells following stimulation with growth factors, such as PDGF or EGF or following monolayer wounding that may stimulate directional migration cues. Cells used for active GTPase pull-down assays were plated without a Fn substrate, and so adhered to ECM components secreted from the cells, themselves. It is possible that perhaps the GTPase specificity of Asef2 is determined by the external stimulus, as the cell interprets individual signals into differing pathways to elicit the appropriate response to fit the cue.

Cells expressing Asef2 migrate more rapidly than controls, whether in a trans-well, migrating randomly, or migrating to fill a scratch-wound. This supports data presented by others who examined the effect of Asef2 expression on the

ability of cells to migrate in a trans-well assay. The data presented in this thesis have been derived from experiments in which full-length Asef2 tagged with GFP has been stably transduced into cells or endogenous Asef2 was knocked down by siRNA. Previous studies by Kawasaki *et. al.* found similar results in a trans-well assay when they expressed full-length, un-tagged Asef2 in MDCK cells. Interestingly, they reported further enhanced migration when they expressed a truncated mutant of Asef2 that lacks the ABR and SH3 domain, which leads to constitutive activation of Asef2. Additionally, Kawasaki *et. al*. found that Asef2 is an important regulator of cell migration in SW480 colorectal cancer cells that express a mutated APC associated with tumorigenesis. Migration data from this group in addition to that presented in this thesis implicate Asef2 as an important regulator of cell migration and suggest it may be important in pathological conditions by promoting tumorigenesis and cancer cell metastasis. Future experiments should include studies to determine the effect of APC-mediated Asef2 activation in HEK 293 and HT-1080 cells, as expression of full-length Asef2 alone significantly increased migration of these cell lines without regard to APC. Whether APC-mediated activation of Asef2 is strictly necessary for Asef2 to elicit a biological response in these cells remains unclear. It would be interesting to screen a variety of cell types and cancer cell lines to determine Asef2's prevalence and correlate pathology with Asef2 or APC mutations that may lead to Asef2 dysregulation.

Asef2 is an important regulator of cell-matrix adhesion turnover. Its expression leads to rapid adhesion assembly and disassembly in cells, as

visualized by the incorporation of mCherry-paxillin into adhesions at the leading edge of lamellipodial protrusions and its elimination from adhesions as they disassemble. Asef2 promotes rapid turnover via a mechanism that requires Rac activation, and active GTPase pull-down data indicate Asef2 inhibits Rho activation in a Rac-dependent mechanism. It is tempting to extrapolate from this that Asef2 regulates adhesion dynamics through a mechanism that requires Rho inhibition via Rac activation. Others have shown that Tiam1-mediated Rac activation leads to Rho down-regulation, and that some basal level of Rho activity is required for normal Rac activation and migration (Sander, ten Klooster et al. 1999). This relationship between Rac and Rho typically describes a need for Rac to not only stimulate protrusion of the leading edge, but to also inhibit Rho from causing the formation of stress fibers, which are associated with migration inhibition. Previous reports state that Rac is active at the front of the cell while Rho is active at the back (Ridley, Schwartz et al. 2003), but this appears not to be entirely true. FRET biosensors have shown localized Rac activity leads to a decrease in Rho activation at the leading edge of migrating cells (Pertz, Hodgson et al. 2006). As a positive regulator of Rac activity and a negative regulator of Rho activity that localizes to the leading edge of cells, Asef2 appears to be an important regulator of a previously-undescribed mechanism through which Rac activation promotes adhesion turnover by inhibiting Rho activity.

The formation and maturation of cell-matrix adhesions is controlled by Rac and Rho, and there has been much debate over the classification of types of adhesions. Small nascent adhesions that form within 1 μm of the leading edge

help to stabilize new protrusions, and are considered the primary force generators in migrating cells (Beningo, Dembo et al. 2001; Nayal, Webb et al. 2006). They are typically referred to as focal complexes and their formation is stimulated by Rac. A second group of larger focal adhesions that are further from the leading edge may be important for traction as the cell propels itself forward across the ECM, but are associated with slower migration (Petit and Thiery 2000; Zaidel-Bar, Ballestrem et al. 2003). It is unclear whether formation of these adhesions occurs separately from focal complexes in a Rho-dependent manner, or if they are the result of Rho-mediated maturation of focal complexes that occurs during the migration process. TIRF images of GFP-Asef2 HT-1080 cells show relatively small adhesions that are located primarily at the perimeter of the cell and lack larger adhesions further from the cell edge, and fluorescent adhesion time-lapse movies indicate adhesions in these cells turn over rapidly near the edge. An attractive explanation of Asef2's role in adhesion turnover is that either Asef2 blocks the formation of large, Rho-dependent focal adhesions altogether, and/or it prevents the Rho-stimulated maturation of these adhesions from small nascent adhesions.

PI3K and Akt are integral members of the Asef2 pathway regulating adhesion dynamics, and this is a novel role for Akt. There are examples in the literature of pathways in which PI3K and Akt mediate a down-regulation of Rho, but such down-regulation has been associated with stress fiber regulation, not adhesion dynamics. For example, the adapter protein, Kank, is a phosphorylation target of Akt that is activated downstream of PI3K. Kank phosphorylation leads to

its association with 14-3-3, which inhibits Rho in a mechanism that still remains unclear; it has been suggested that p190 RhoGAP may mediate Rho inhibition in this pathway, as it is a binding partner of 14-3-3. This inhibition decreases stress fiber formation in cells and leads to increased cell motility (Kakinuma N 2008). The importance of p190A RhoGAP in the regulation of adhesions has recently been elucidated in a study that implicated it as an important point of convergence for Rho regulation by α5β1 integrins, via tyrosine kinases, and the fibronectin receptor, syndecan-4, via PKC. In this mechanism, the integrin pathway activates p190A RhoGAP, and the syndecan-4 pathway aids in localization of p190A RhoGAP through PKC activation, leading to a localized p190A RhoGAP-mediated decrease in Rho activation. Integration of these signals regulates fibronectin-stimulated formation of vinculin-containing adhesions at the leading edge (Bass, Morgan et al. 2008). All together, this makes p190 RhoGAP an attractive potential downstream effector of Asef2 that could function as an important modulator of adhesion dynamics via Rho inactivation.

In addition to p190 RhoGAP, there are several migration and adhesion-regulating molecules that could be important players in an Asef2 pathway. Future studies should include an investigation of kinases such as PAK, FAK, and Src that are of known importance for the assembly and disassembly of adhesions as well as the process of migration as a whole. The serine/threonine kinase, PAK, is a well-established downstream effector of both Cdc42 and Rac that serves many roles within the cell. It functions in the lamellipodium to promote protrusion by indirectly stimulating cofilin, and it is an important cell-matrix adhesion regulator,

where it is a part of a multimolecular complex containing PIX, GIT, and paxillin (Zhao, Manser et al. 2000; Delorme, Machacek et al. 2007). Interestingly, PAK seems particularly important for adhesion disassembly, although its mechanism of action is still unclear (Manser, Huang et al. 1997; Webb, Parsons et al. 2002). PAK is targeted to the focal adhesion sites by active Rac or Cdc42, and elevated levels of active PAK are associated with diminished focal adhesions, leaving only few, small adhesions at the cell periphery as seen by fluorescence microscopy (Manser, Huang et al. 1997). This affect on adhesions is very similar to that attributed to Asef2 activity as presented in this dissertation. As an activator of Cdc42 and Rac that causes a PAK-like adhesion phenotype, it is likely that Asef2 functions upstream of PAK to regulate adhesion turnover, and this is a promising avenue for future research.

Like Asef2-knockdown HT-1080 cells, FAK-null mouse keratinocytes have atypically large adhesions. Not surprisingly, these adhesions assemble at a decreased rate and, also like Asef2-knockdown HT-1080 cells, disassembly of adhesions in FAK-null keratinocytes occurs at a further decreased rate (Schober, Raghavan et al. 2007). Interestingly, FAK activity has been correlated to a localized decrease in Rho activation and a resulting increase in cell migration, spreading, and adhesion turnover (Ren, Kiosses et al. 2000). Additionally, FAK is thought to phosphorylate, and therefore activate, p190 RhoGAP at integrin-enriched focal adhesion sites, where FAK is also known to aid in recruitment of other important regulatory molecules, such as Src (Schaller, Hildebrand et al. 1994; Schober, Raghavan et al. 2007). For all these reasons, the role of FAK in

migration and adhesion dynamics regulation is likely to be closely linked to that of Asef2, and presents an interesting avenue of future investigation.

Following FAK autophosphorylation at sites of cell-matrix adhesion, Src binding sites are revealed, allowing Src to further activate FAK kinase activity while exposing additional protein-binding sites. This FAK-Src complex is able to recruit and activate scaffolding molecules that modulate Rac localization and activity, leading to protrusion. This complex can also phosphorylate paxillin, leading to the recruitment of GIT and PIX, which in turn can be phosphorylated by Src (Huveneers and Danen 2009). Like FAK-null cells and Asef2-knockdown cells, Src-null fibroblasts exhibit migration and adhesion turnover defects (Klinghoffer, Sachsenmaier et al. 1999; Huveneers and Danen 2009). FAK-Src signaling promotes adhesion disassembly, and is thought to prevent adhesion maturation (Webb, Donais et al. 2004). Interestingly, the FAK-Src complex promotes migration by mediating Rho inhibition through p190 RhoGAP in response to integrin ligation (Arthur, Petch et al. 2000; Ren, Kiosses et al. 2000; Huveneers and Danen 2009).

Their effects on cell migration, adhesion turnover, and the modulation of Rac and Rho activation make PAK, Src, FAK, and p190 RhoGAP likely downstream effectors of Asef2. Additionally, the function of Asef2's regulation by APC and the relationship of these two molecules to tumorigenesis and metastasis require further study. Further, the impact of Asef2 expression and regulation on directed or chemotactic cell migration and the function of Asef2-mediated Cdc42 activation still remain to be clarified.

Concluding Remarks

In conclusion, the data presented in this dissertation highlight the molecular complexity of cell migration regulation and the importance of its control by Rho family GEFs. Asef2 is a recently described GEF that regulates the activity of Rac, Rho, and Cdc42 to modulate cell migration. We have employed various microscopy techniques to determine Asef2's function as a promoter of migration and specifically as an agent of rapid adhesion turnover. These were complemented by biochemical analyses that determined Asef2 functions in a Rac-dependent pathway requiring PI3K and Akt to inhibit Rho. Our work has elucidated the role of Asef2 in cell migration and uncovered important new roles for PI3K and Akt as regulators of adhesion dynamics in a previously-undescribed pathway. Dysregulation of Rho family GEFs, such as Asef2, has been linked to several human cancers and as a result, our understanding of their function and regulation may prove to be the key to development of effective therapeutics in the future.

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REFERENCES

Abraham, V. C., V. Krishnamurthi, et al. (1999). "The Actin-Based Nanomachine at the Leading Edge of Migrating Cells." Biophys. J. **77**(3): 1721-1732.

Aghazadeh, B., W. E. Lowry, et al. (2000). "Structural Basis for Relief of Autoinhibition of the Dbl Homology Domain of Proto-Oncogene Vav by Tyrosine Phosphorylation." Cell **102**(5): 625-633.

Alexandrova, A. Y., K. Arnold, et al. (2008). "Comparative Dynamics of Retrograde Actin Flow and Focal Adhesions: Formation of Nascent Adhesions Triggers Transition from Fast to Slow Flow." PLoS ONE **3**(9): e3234.

Allen, W. E., D. Zicha, et al. (1998). "A Role for Cdc42 in Macrophage Chemotaxis." J. Cell Biol. **141**(5): 1147-1157.

Amann, K. J. and T. D. Pollard (2001). "Direct real-time observation of actin filament branching mediated by Arp2/3 complex using total internal reflection fluorescence microscopy." Proceedings of the National Academy of Sciences of the United States of America **98**(26): 15009-15013.

Arthur, W. T., L. A. Petch, et al. (2000). "Integrin engagement suppresses RhoA activity via a c-Src-dependent mechanism." Current Biology **10**(12): 719-722.

Aspenström, P. (1999). "The Rho GTPases Have Multiple Effects on the Actin Cytoskeleton." Experimental Cell Research **246**(1): 20-25.

Bass, M. D., M. R. Morgan, et al. (2008). "p190RhoGAP is the convergence point of adhesion signals from {alpha}5{beta}1 integrin and syndecan-4." J. Cell Biol. **181**(6): 1013-1026.

Bellacosa, A., C. C. Kumar, et al. (2005). Activation of AKT Kinases in Cancer: Implications for Therapeutic Targeting. Advances in Cancer Research, Academic Press. **Volume 94:** 29-86.

Beningo, K. A., M. Dembo, et al. (2001). "Nascent focal adhesions are responsible for the generation of strong propulsive forces in migrating fibroblasts." J. Cell Biol. **153**(4): 881-888.

Bi, F., B. Debreceni, et al. (2001). "Autoinhibition Mechanism of Proto-Dbl." Mol. Cell. Biol. **21**(5): 1463-1474.

Bishop, A. L. a. A. H. (2000). "Rho GTPases and their effector proteins." Biochem. J. **348**(2): 241-255.

Bokoch, G. M. (2003). "BIOLOGY OF THE P21-ACTIVATED KINASES." Annual Review of Biochemistry **72**(1): 743-781.

Borisy, G. G. and T. M. Svitkina (2000). "Actin machinery: pushing the envelope." Current Opinion in Cell Biology **12**(1): 104-112.

Borm, B., R. P. Requardt, et al. (2005). "Membrane ruffles in cell migration: indicators of inefficient lamellipodia adhesion and compartments of actin filament reorganization." Experimental Cell Research **302**(1): 83-95.

Broussard, J. A., D. J. Webb, et al. (2008). "Asymmetric focal adhesion disassembly in motile cells " Current Opinion in Cell Biology **20**(1): 85-90.

Bryce, N. S., E. S. Clark, et al. (2005). "Cortactin Promotes Cell Motility by Enhancing Lamellipodial Persistence." Current Biology **15**(14): 1276-1285.

Burridge, K. and K. Wennerberg (2004). "Rho and Rac Take Center Stage." Cell **116**(2): 167-179.

Bustos, R. I., M.-A. Forget, et al. (2008). "Coordination of Rho and Rac GTPase Function via p190B RhoGAP." Current Biology **18**(20): 1606-1611.

Cain, R. J. and A. J. Ridley (2009). "Phosphoinositide 3-kinases in cell migration." Biology of the Cell **101**(1): 13-29.

Carpenter, C. L. and L. C. Cantley (1996). "Phosphoinositide kinases." Current Opinion in Cell Biology **8**(2): 153-158.

Carson, M., A. Weber, et al. (1986). "An actin-nucleating activity in polymorphonuclear leukocytes is modulated by chemotactic peptides." J. Cell Biol. **103**(6): 2707-2714.

Cary, L. A., J. F. Chang, et al. (1996). "Stimulation of cell migration by overexpression of focal adhesion kinase and its association with Src and Fyn." J Cell Sci **109**(7): 1787-1794.

Cerione, R. A. and Y. Zheng (1996). "The Dbl family of oncogenes." Current Opinion in Cell Biology **8**(2): 216-222.

Chan, A. Y., S. J. Coniglio, et al. (2005). "Roles of the Rac1 and Rac3 GTPases in human tumor cell invasion." Oncogene **24**(53): 7821-7829.

Chhabra, E. S. and H. N. Higgs (2007). "The many faces of actin: matching assembly factors with cellular structures." Nat Cell Biol **9**(10): 1110-1121.

Choi, C. K., M. Vicente-Manzanares, et al. (2008). "Actin and [alpha]-actinin orchestrate the assembly and maturation of nascent adhesions in a myosin II motor-independent manner." Nat Cell Biol **10**(9): 1039-1050.

Chrzanowska-Wodnicka, M. and K. Burridge (1996). "Rho-stimulated contractility drives the formation of stress fibers and focal adhesions." J. Cell Biol. **133**(6): 1403-15.

Chrzanowska-Wodnicka, M. and K. Burridge (1996). "Rho-stimulated contractility drives the formation of stress fibers and focal adhesions." J. Cell Biol. **133**(6): 1403-1415.

Condeelis, J. (1993). "Life at the Leading Edge: The Formation of Cell Protrusions." Annual Review of Cell Biology **9**(1): 411-444.

Couchman, J. R. and D. A. Rees (1979). "The behaviour of fibroblasts migrating from chick heart explants: changes in adhesion, locomotion and growth, and in the distribution of actomyosin and fibronectin." J. Cell Sci. **39**: 149-65.

Cox, E. A., S. K. Sastry, et al. (2001). "Integrin-mediated Adhesion Regulates Cell Polarity and Membrane Protrusion through the Rho Family of GTPases." Mol. Biol. Cell **12**(2): 265-277.

Cross, D. A., D.R. Alessi, P. Cohen, M. Andjelkovich, and B.A. Hemmings (1995). "Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B
" Nature **378**(6559): 785 - 789

Czuchra, A., X. Wu, et al. (2005). "Cdc42 Is Not Essential for Filopodium Formation, Directed Migration, Cell Polarization, and Mitosis in Fibroblastoid Cells." Mol. Biol. Cell **16**(10): 4473-4484.

Das, B., X. Shu, et al. (2000). "Control of Intramolecular Interactions between the Pleckstrin Homology and Dbl Homology Domains of Vav and Sos1 Regulates Rac Binding." J. Biol. Chem. **275**(20): 15074-15081.

Deakin, N. O. and C. E. Turner (2008). "Paxillin comes of age." J Cell Sci **121**(15): 2435-2444.

Degtyarev, M., A. De Maziere, et al. (2008). "Akt inhibition promotes autophagy and sensitizes PTEN-null tumors to lysosomotropic agents." J. Cell Biol. **183**(1): 101-16.

Delorme, V., M. Machacek, et al. (2007). "Cofilin Activity Downstream of Pak1 Regulates Cell Protrusion Efficiency by Organizing Lamellipodium and Lamella Actin Networks." Developmental Cell **13**(5): 646-662.

Estrach, S., S. Schmidt, et al. (2002). "The Human Rho-GEF Trio and Its Target GTPase RhoG Are Involved in the NGF Pathway, Leading to Neurite Outgrowth." Current Biology **12**(4): 307-312.

Etienne-Manneville, S. (2004). "Cdc42 - the centre of polarity." J Cell Sci **117**(8): 1291-1300.

Etienne-Manneville, S. and A. Hall (2003). "Cdc42 regulates GSK-3beta and adenomatous polyposis coli to control cell polarity." Nature **421**(6924): 753-6.

Faix, J. and K. Rottner (2006). "The making of filopodia." Current Opinion in Cell Biology **18**(1): 18-25.

Fenteany, G. a. G., M. (2003). "Cytoskeletal remodeling in leukocyte function." Curr Opin Hematol **11**: 15-24.

Franco, S. J., M. A. Rodgers, et al. (2004). "Calpain-mediated proteolysis of talin regulates adhesion dynamics." Nat Cell Biol **6**(10): 977-983.

Franke, T. F., S.-I. Yang, et al. (1995). "The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase." Cell **81**(5): 727-736.

Hall, A. (1994). "Small GTP-Binding Proteins and the Regulation of the Actin Cytoskeleton." Annual Review of Cell Biology **10**(1): 31-54.

Hall, A. (1998). "Rho GTPases and the Actin Cytoskeleton." Science **279**(5350): 509-514.

Hamann, M. J., C. M. Lubking, et al. (2007). "Asef2 Functions as a Cdc42 Exchange Factor and Is Stimulated by the Release of an Autoinhibitory Module from a Concealed C-Terminal Activation Element." Mol. Cell. Biol. **27**(4): 1380-1393.

Harvey, K., I. C. Duguid, et al. (2004). "The GDP-GTP Exchange Factor Collybistin: An Essential Determinant of Neuronal Gephyrin Clustering." J. Neurosci. **24**(25): 5816-5826.

Hawkins, P. T., A. Eguinoa, et al. (1995). "PDGF stimulates an increase in GTP-Rac via activation of phosphoinositide 3-kinase." Current Biology **5**(4): 393-403.

Hooshmand-Rad, R., L. Claesson-Welsh, et al. (1997). "Involvement of Phosphatidylinositide 3'-Kinase and Rac in Platelet-Derived Growth Factor-Induced Actin Reorganization and Chemotaxis." Experimental Cell Research **234**(2): 434-441.

Horwitz, A. R. and J. T. Parsons (1999). "CELL BIOLOGY:Cell Migration--Movin' On." Science **286**(5442): 1102-1103.

Hotchin, N. A. and A. Hall (1995). "The assembly of integrin adhesion complexes requires both extracellular matrix and intracellular rho/rac GTPases." J. Cell Biol. **131**(6): 1857-1865.

Huveneers, S. and E. H. J. Danen (2009). "Adhesion signaling - crosstalk between integrins, Src and Rho." J Cell Sci **122**(8): 1059-1069.

Irie, H. Y., R. V. Pearline, et al. (2005). "Distinct roles of Akt1 and Akt2 in regulating cell migration and epithelial-mesenchymal transition." J. Cell Biol. **171**(6): 1023-1034.

Itoh, R. E., E. Kiyokawa, et al. (2008). "Phosphorylation and activation of the Rac1 and Cdc42 GEF Asef in A431 cells stimulated by EGF." J Cell Sci **121**(16): 2635-2642.

Jaffe, A. B., A. Hall, et al. (2005). "Association of CNK1 with Rho Guanine Nucleotide Exchange Factors Controls Signaling Specificity Downstream of Rho." Current Biology **15**(5): 405-412.

Kaibuchi, K., S. Kuroda, et al. (1999). "REGULATION OF THE CYTOSKELETON AND CELL ADHESION BY THE RHO FAMILY GTPASES IN MAMMALIAN CELLS." Annual Review of Biochemistry **68**(1): 459-486.

Kakinuma N, R. B., Zhu Y, Wang Y, Kiyama R. (2008). "Kank regulates RhoA-dependent formation of actin stress fibers and cell migration via 14-3-3 in PI3K-Akt signaling." J Cell Biol. **181**(3): 537-49.

Karnoub AE, W. D., Rossman KL, Pruitt WM, Campbell SL, Sondek J, Der CJ. (2001). "Molecular basis for Rac1 recognition by guanine nucleotide exchange factors." Nat Struct Biol. **8**(12): 1037-1041.

Katome, T., T. Obata, et al. (2003). "Use of RNA interference-mediated gene silencing and adenoviral overexpression to elucidate the roles of AKT/protein kinase B isoforms in insulin actions." J. Biol. Chem. **278**(30): 28312-23.

Kawasaki, Y., M. Sagara, et al. (2007). "Identification and characterization of Asef2, a guanine-nucleotide exchange factor specific for Rac1 and Cdc42." Oncogene **26**(55): 7620-267.

Kawasaki, Y., M. Sagara, et al. (2007). "Identification and characterization of Asef2, a guanine-nucleotide exchange factor specific for Rac1 and Cdc42." Oncogene **26**(55): 7620-7267.

Kawasaki, Y., T. Senda, et al. (2000). "Asef, a Link Between the Tumor Suppressor APC and G-Protein Signaling." Science **289**(5482): 1194-1197.

Kawasaki, Y., S. Tsuji, et al. (2009). "Adenomatous Polyposis Coli and Asef Function Downstream of Hepatocyte Growth Factor and Phosphatidylinositol 3-Kinase." J. Biol. Chem. **284**(33): 22436-22443.

Kim, D., S. Kim, et al. (2001). "Akt/PKB promotes cancer cell invasion via increased motility and metalloproteinase production." FASEB J. **15**(11): 1953-1962.

Kins, S., H. Betz, et al. (2000). "Collybistin, a newly identified brain-specific GEF, induces submembrane clustering of gephyrin." Nat Neurosci **3**(1): 22-29.

Klinghoffer, R. A., C. Sachsenmaier, et al. (1999). "Src family kinases are required for integrin but not PDGFR signal transduction." EMBO J **18**(9): 2459-2471.

Kozma, R., S. Ahmed, et al. (1995). "The Ras-related protein Cdc42Hs and bradykinin promote formation of peripheral actin microspikes and filopodia in Swiss 3T3 fibroblasts." Mol. Cell. Biol. **15**(4): 1942-1952.

Kraynov, V. S., C. Chamberlain, et al. (2000). "Localized Rac Activation Dynamics Visualized in Living Cells." Science **290**(5490): 333-337.

Kubiseski, T. J., J. Culotti, et al. (2003). "Functional Analysis of the Caenorhabditis elegans UNC-73B PH Domain Demonstrates a Role in Activation of the Rac GTPase In Vitro and Axon Guidance In Vivo." Mol. Cell. Biol. **23**(19): 6823-6835.

Larue, L. and A. Bellacosa (2005). "Epithelial-mesenchymal transition in development and cancer: role of phosphatidylinositol 3[prime] kinase//AKT pathways." Oncogene **24**(50): 7443-7454.

Lauffenburger, D. A. and A. F. Horwitz (1996). "Cell Migration: A Physically Integrated Molecular Process." Cell **84**(3): 359-369.

Laukaitis, C. M., D. J. Webb, et al. (2001). "Differential Dynamics of {alpha}5 Integrin, Paxillin, and {alpha}-Actinin during Formation and Disassembly of Adhesions in Migrating Cells." J. Cell Biol. **153**(7): 1427-1440.

Lee, J. and K. Jacobson (1997). "The composition and dynamics of cell-substratum adhesions in locomoting fish keratocytes." J Cell Sci **110**(22): 2833-2844.

Lim, Y., S.-T. Lim, et al. (2008). "PyK2 and FAK connections to p190Rho guanine nucleotide exchange factor regulate RhoA activity, focal adhesion formation, and cell motility." J. Cell Biol. **180**(1): 187-203.

Lin, Q., W. Yang, et al. (2006). "Identification of a DOCK180-related guanine nucleotide exchange factor that is capable of mediating a positive feedback activation of Cdc42." J. Biol. Chem. **281**(46): 35253-62.

Ilic, D. k., Y. Furuta, et al. (1995). "Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK-deficient mice." Nature **377**(6549): 539-544.

Lodish, H., A. Berk, et al. (2000). "Molecular Cell Biology, 4e." WHFreeman and Company.

Lorenzi MV, C. P., Chen Q, Hori Y, Miki T. (1999). "Distinct expression patterns and transforming properties of multiple isoforms of Ost, an exchange factor for RhoA and Cdc42." Oncogene **18**(33): 4742-55.

Manser, E., H. Y. Huang, et al. (1997). "Expression of constitutively active alpha-PAK reveals effects of the kinase on actin and focal complexes." Mol. Cell. Biol. **17**(3): 1129-1143.

Manser, E., T.-H. Loo, et al. (1998). "PAK Kinases Are Directly Coupled to the PIX Family of Nucleotide Exchange Factors." Molecular Cell **1**(2): 183-192.

Mayhew, M. W., D. J. Webb, et al. (2006). "Identification of Protein Networks Associated with the PAK1−βPIX−GIT1−Paxillin Signaling Complex by Mass Spectrometry." Journal of Proteome Research **5**(9): 2417-2423.

Mitin, N., L. Betts, et al. (2007). "Release of autoinhibition of ASEF by APC leads to CDC42 activation and tumor suppression." Nat Struct Mol Biol **14**(9): 814-823.

Miyamoto, S., H. Teramoto, et al. (1995). "Integrin function: molecular hierarchies of cytoskeletal and signaling molecules." J. Cell Biol. **131**(3): 791-805.

Murayama, K., M. Shirouzu, et al. (2007). "Crystal Structure of the Rac Activator, Asef, Reveals Its Autoinhibitory Mechanism." J. Biol. Chem. **282**(7): 4238-4242.

Nayal, A., D. J. Webb, et al. (2006). "Paxillin phosphorylation at Ser273 localizes a GIT1-PIX-PAK complex and regulates adhesion and protrusion dynamics." J. Cell Biol. **173**(4): 587-589.

Nimnual, A. S., L. J. Taylor, et al. (2003). "Redox-dependent downregulation of Rho by Rac." Nat Cell Biol **5**(3): 236-241.

Nobes, C. D. and A. Hall (1995). "Rho, Rac, and Cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia." Cell **81**(1): 53-62.

Oikawa, T., H. Yamaguchi, et al. (2004). "PtdIns(3,4,5)P3 binding is necessary for WAVE2-induced formation of lamellipodia." Nat Cell Biol **6**(5): 420-426.

Palecek, S. P., A. Huttenlocher, et al. (1998). "Physical and biochemical regulation of integrin release during rear detachment of migrating cells." J Cell Sci **111**(7): 929-940.

Papadopoulos, T., V. Eulenburg, et al. (2008). "Collybistin is required for both the formation and maintenance of GABAergic postsynapses in the hippocampus." Molecular and Cellular Neuroscience **39**(2): 161-169.

Pelish, H. E., J. R. Peterson, et al. (2006). "Secramine inhibits Cdc42-dependent functions in cells and Cdc42 activation in vitro." Nat. Chem. Biol. **2**(1): 39-46.

Pertz, O., L. Hodgson, et al. (2006). "Spatiotemporal dynamics of RhoA activity in migrating cells." Nature **440**(7087): 1069-1072.

Petit, V. and J.-P. Thiery (2000). "Focal adhesions: structure and dynamics." Biology of the Cell **92**(7): 477-494.

Pollard, T. D., L. Blanchoin, et al. (2000). "MOLECULAR MECHANISMS CONTROLLING ACTIN FILAMENT DYNAMICS IN NONMUSCLE CELLS." Annual Review of Biophysics and Biomolecular Structure **29**(1): 545.

Pollard, T. D. and G. G. Borisy (2003). "Cellular Motility Driven by Assembly and Disassembly of Actin Filaments." Cell **112**(4): 453-465.

Ponti, A., M. Machacek, et al. (2004). "Two distinct actin networks drive the protrusion of migrating cells." Science **305**(5691): 1782-6.

Rameh, L. E., A.-k. Arvidsson, et al. (1997). "A Comparative Analysis of the Phosphoinositide Binding Specificity of Pleckstrin Homology Domains." J. Biol. Chem. **272**(35): 22059-22066.

Ren, X. D., W. B. Kiosses, et al. (2000). "Focal adhesion kinase suppresses Rho activity to promote focal adhesion turnover." J Cell Sci **113**(20): 3673-3678.

Ren, X. D., W.B. Kiosses, and M.A. Schwartz. (1999). "Regulation of the small GTP-binding protein Rho by cell adhesion and the cytoskeleton." EMBO J.(18): 578-85.

Ridley, A. J. (2001). "Rho GTPases and cell migration." J Cell Sci **114**(15): 2713-2722.

Ridley, A. J. and A. Hall (1992). "The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors." Cell **70**(3): 389-399.

Ridley, A. J., M. A. Schwartz, et al. (2003). "Cell Migration: Integrating Signals from Front to Back." Science **302**(5651): 1704-1709.

Rossman, K. L., C. J. Der, et al. (2005). "GEF means go: turning on RHO GTPases with guanine nucleotide-exchange factors." Nat Rev Mol Cell Biol **6**(2): 167-180.

Rottner, K., A. Hall, et al. (1999). "Interplay between Rac and Rho in the control of substrate contact dynamics." Current Biology **9**(12): 640-649.

Russo, C., Y. Gao, et al. (2001). "Modulation of Oncogenic DBL Activity by Phosphoinositol Phosphate Binding to Pleckstrin Homology Domain." J. Biol. Chem. **276**(22): 19524-19531.

Sagara, M., Y. Kawasaki, et al. (2009). "Asef2 and Neurabin2 cooperatively regulate actin cytoskeletal organization and are involved in HGF-induced cell migration." Oncogene.

Sagara, M., Y. Kawasaki, et al. (2009). "Asef2 and Neurabin2 cooperatively regulate actin cytoskeletal organization and are involved in HGF-induced cell migration." Oncogene **28**(10): 1357-1365.

Sander, E. E., J. P. ten Klooster, et al. (1999). "Rac Downregulates Rho Activity: Reciprocal Balance between Both GTPases Determines Cellular Morphology and Migratory Behavior." J. Cell Biol. **147**(5): 1009-1022.

Schaller, M. D., J. D. Hildebrand, et al. (1994). "Autophosphorylation of the focal adhesion kinase, pp125FAK, directs SH2-dependent binding of pp60src." Mol. Cell. Biol. **14**(3): 1680-1688.

Schiller, M. R., K. Chakrabarti, et al. (2006). "Regulation of RhoGEF Activity by Intramolecular and Intermolecular SH3 Domain Interactions." J. Biol. Chem. **281**(27): 18774-18786.

Schmidt, A. and A. Hall (2002). "Guanine nucleotide exchange factors for Rho GTPases: turning on the switch." Genes & Development **16**(13): 1587-1609.

Schober, M., S. Raghavan, et al. (2007). "Focal adhesion kinase modulates tension signaling to control actin and focal adhesion dynamics." J. Cell Biol. **176**(5): 667-680.

Sjoblom, T., S. Jones, et al. (2006). "The Consensus Coding Sequences of Human Breast and Colorectal Cancers." Science **314**(5797): 268-274.

Srinivasan, S., F. Wang, et al. (2003). "Rac and Cdc42 play distinct roles in regulating PI(3,4,5)P3 and polarity during neutrophil chemotaxis." J. Cell Biol. **160**(3): 375-385.

Steffen, A., J. Faix, et al. (2006). "Filopodia Formation in the Absence of Functional WAVE- and Arp2/3-Complexes." Mol. Biol. Cell **17**(6): 2581-2591.

Tilghman, R. W., J. K. Slack-Davis, et al. (2005). "Focal adhesion kinase is required for the spatial organization of the leading edge in migrating cells." J Cell Sci **118**(12): 2613-2623.

Tolias, K. F., L. C. Cantley, et al. (1995). "Rho Family GTPases Bind to Phosphoinositide Kinases." J. Biol. Chem. **270**(30): 17656-17659.

Turner, C. E., M. C. Brown, et al. (1999). "Paxillin LD4 Motif Binds PAK and PIX through a Novel 95-kD Ankyrin Repeat, ARF-GAP Protein: A Role in Cytoskeletal Remodeling." J. Cell Biol. **145**(4): 851-863.

Turner, C. E., J. R. Glenney, Jr., et al. (1990). "Paxillin: a new vinculin-binding protein present in focal adhesions." J. Cell Biol. **111**(3): 1059-1068.

Uehata, M., T. Ishizaki, et al. (1997). "Calcium sensitization of smooth muscle mediated by a Rho-associated protein kinase in hypertension." Nature **389**(6654): 990-4.

Wang, Y. L. (1985). "Exchange of actin subunits at the leading edge of living fibroblasts: possible role of treadmilling." J. Cell Biol. **101**(2): 597-602.

Weaver, A. M., M. E. Young, et al. (2003). "Integration of signals to the Arp2/3 complex." Current Opinion in Cell Biology **15**(1): 23-30.

Webb, D. J., K. Donais, et al. (2004). "FAK-Src signalling through paxillin, ERK and MLCK regulates adhesion disassembly." Nat Cell Biol **6**(2): 154-161.

Webb, D. J., J. T. Parsons, et al. (2002). "Adhesion assembly, disassembly and turnover in migrating cells - over and over and over again." Nat Cell Biol **4**(4): E97-E100.

Wegner, A. M., C. A. Nebhan, et al. (2008). "N-WASP and the Arp2/3 Complex Are Critical Regulators of Actin in the Development of Dendritic Spines and Synapses." J. Biol. Chem. **283**(23): 15912-15920.

Welch, H. C. E., W. J. Coadwell, et al. (2002). "P-Rex1, a PtdIns(3,4,5)P3- and G[beta][gamma]-Regulated Guanine-Nucleotide Exchange Factor for Rac." Cell **108**(6): 809-821.

Welch, H. C. E., W. J. Coadwell, et al. (2003). "Phosphoinositide 3-kinase-dependent activation of Rac." FEBS Letters **546**(1): 93-97.

Wymann, M., Bulgarelli-Leva, G, Zvelebil, MJ, Pirola, L, Vanhaesebroeck, B, Waterfield, MD, Panayotou, G (1996). "Wortmannin inactivates phosphoinositide 3-kinase by covalent modification of Lys-802, a residue involved in the phosphate transfer reaction." Mol. Cell. Biol. **16**: 1722-1733

Xiang, S., E. Y. Kim, et al. (2006). "The Crystal Structure of Cdc42 in Complex with Collybistin II, a Gephyrin-interacting Guanine Nucleotide Exchange Factor." Journal of Molecular Biology **359**(1): 35-46.

Xu B, P. H., Kirchhausen T, Hammond GB. (2006). "Large scale synthesis of the Cdc42 inhibitor secramine A and its inhibition of cell spreading." Org Biomol Chem. **4**(22): 4149-57.

Yang, L., L. Wang, et al. (2006). "Gene Targeting of Cdc42 and Cdc42GAP Affirms the Critical Involvement of Cdc42 in Filopodia Induction, Directed Migration, and Proliferation in Primary Mouse Embryonic Fibroblasts." Mol. Biol. Cell **17**(11): 4675-4685.

Yang, Z.-Z., O. Tschopp, et al. (2003). "Protein Kinase B{alpha}/Akt1 Regulates Placental Development and Fetal Growth." J. Biol. Chem. **278**(34): 32124-32131.

Yin, H. L. and P. A. Janmey (2003). "PHOSPHOINOSITIDE REGULATION OF THE ACTIN CYTOSKELETON." Annual Review of Physiology **65**(1): 761-789.

Yuruker, B. and V. Niggli (1992). "Alpha-actinin and vinculin in human neutrophils: reorganization during adhesion and relation to the actin network." J Cell Sci **101**(2): 403-414.

Zaidel-Bar, R., C. Ballestrem, et al. (2003). "Early molecular events in the assembly of matrix adhesions at the leading edge of migrating cells." J Cell Sci **116**(22): 4605-4613.

Zaidel-Bar, R., R. Milo, et al. (2007). "A paxillin tyrosine phosphorylation switch regulates the assembly and form of cell-matrix adhesions." J Cell Sci **120**(1): 137-148.

Zhang, H. and I. G. Macara (2008). "The PAR-6 Polarity Protein Regulates Dendritic Spine Morphogenesis through p190 RhoGAP and the Rho GTPase." Developmental Cell **14**(2): 216-226.

Zhao, Z.-s., E. Manser, et al. (2000). "Coupling of PAK-Interacting Exchange Factor PIX to GIT1 Promotes Focal Complex Disassembly." Mol. Cell. Biol. **20**(17): 6354-6363.

Zhou, G. L., D. F. Tucker, et al. (2006). "Opposing roles for Akt1 and Akt2 in Rac/Pak signaling and cell migration." J. Biol. Chem. **281**(47): 36443-53.

Zigmond, S. H. (1996). "Signal transduction and actin filament organization." Current Opinion in Cell Biology **8**(1): 66-73.