The Rho-family GEF Asef2 activates Rac to modulate adhesion and actin dynamics and thereby regulate cell migration

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Summary

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 HT1080 cells, but only Rac1 is crucial 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 crucial 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.

Supplementary material available online at
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Key words: Rho family GTPases, Cell migration, Adhesion dynamics, Actin

Introduction

Cell migration is a highly coordinated, tightly regulated process that begins with the extension of a protrusion, which is probably driven by the polymerization of the actin cytoskeleton at the leading edge of cells (Borisy and Svitkina, 2000; Carson et al., 1986; Lauffenburger and Horwitz, 1996; Wang, 1985). 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 forward translocation of the cell body (Beningo et al., 2001; Miyamoto et al., 1995; Turner et al., 1990; Zaidel-Bar 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 et al., 2004), but is poorly understood on a molecular level. Emerging data indicate that the actin cytoskeleton has a crucial role in the formation, dynamics and turnover of leading-edge adhesions (Alexandrova et al., 2008; Choi et al., 2008; Gardel 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 (Nobes and Hall, 1995; Ridley and Hall, 1992; Rottner 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 (Chrzanowska-Wodnicka and Burridge, 1996; Ridley and Hall, 1992; Rottner et al., 1999). Cdc42 can regulate the formation of filopodia and promote the establishment and maintenance of cell polarity, which is essential for directed migration (Etienne-Manneville and Hall, 2003; Nobes and Hall, 1995). Although 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 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 and SH3 domains are associated with the C-terminus of the molecule (Hamann et al., 2007). Following activation, Asef2

significantly increases the level of active Rac1 and Cdc42 in epithelial cells, indicating that it has GEF activity toward these GTPases (Kawasaki et al., 2007). Asef2 is thought to regulate actin dynamics, possibly through its interaction with the F-actin-binding protein Neurabin2 (Sagara et al., 2009). It is also implicated in the modulation of cell migration (Sagara et al., 2009), but the molecular mechanisms by which it contributes to this process are currently unknown.

In this study, we show that 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 crucial 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.

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 et al., 2006). One of the molecules that we detected was a 652 amino acid protein, Asef2 (Fig. 1A), which is reported to have GEF activity for Rac1 and Cdc42 (Kawasaki 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 effect of Asef2 on Rho-family GTPases by assaying for active Rac1, Cdc42 and RhoA in HEK293 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 supplementary material Fig. S1, GFP-Asef2 expression increased the level of active Rac and Cdc42 twofold and threefold, respectively, which is consistent with Asef2 having GEF activity toward these GTPases (Kawasaki et al., 2007). Interestingly, RhoA activity was decreased by 50% in GFP-Asef2-expressing cells. These results indicate that Asef2 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 HT1080 cell lines stably expressing low levels of GFP-Asef2 or GFP as a control. In these cells, the level of GFP-Asef2 expression was less than three times that of endogenous Asef2, as determined by western blot analysis (Fig. 1B,C). The amount of active Rac and Cdc42 increased 1.5-fold, whereas in HT1080 cells stably expressing GFP-Asef2, the level of active RhoA was decreased by greater than 50% compared to that in control cells expressing GFP (Fig. 1D), which is consistent with the results obtained in HEK293 cells. Thus, these cells provided us with stable cell lines for examining the role of Asef2 in regulating 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 et al., 2009), which is consistent with it playing an important role in the regulation of migration. In our study, endogenous Asef2 localized with actin at the leading edge of wild-type HT1080 cells (Fig. 2D), suggesting that Asef2 indeed functions to regulate HT1080 cell migration via

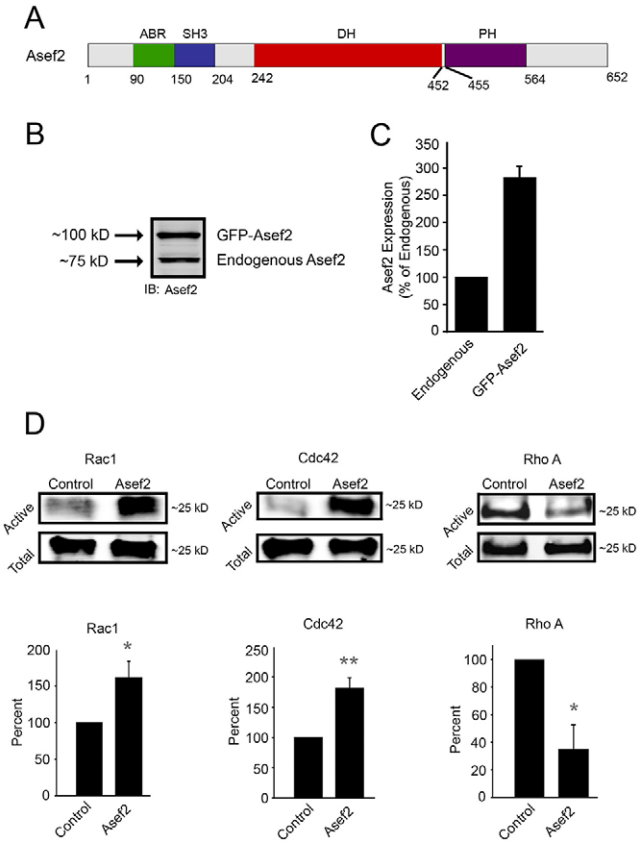

Fig. 1. Asef2 increases the amount of active Rac1 and Cdc42, but decreases Rho activity in HT1080 cells. (A) Schematic of the domain structure of Asef2. APC-binding region (ABR), Src homology-3 (SH3), Dbl homology (DH) and Pleckstrin homology (PH) domains are shown. (B) Immunoblot for Asef2 from HT1080 cells stably expressing GFP-Asef2. The ~75 kDa band represents endogenous Asef2 whereas the ~100 kDa band corresponds to exogenously expressed GFP-Asef2. (C) Quantification of the amount of GFP-Asef2 in stably expressing HT1080 cells relative to endogenous levels of the protein. Error bar represents s.e.m. from four separate experiments. (D) The GTP-bound (active) forms of Rac1, Cdc42 and RhoA were 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 (lower panels). Error bars represent s.e.m. (*P<0.04; **P<0.003).

modulation of the actin cytoskeleton. Similarly to endogenous Asef2, GFP-Asef2 localized with actin at the leading edge whereas GFP alone was found to distribute diffusely throughout HT1080 cells (Fig. 2A,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 cells and normalized it to the unit area. The normalized leading edge fluorescent intensity was significantly enhanced in GFP-Asef2 cells compared with control cells (Fig. 2B), indicating that GFP-Asef2 is enriched at the leading edge. Furthermore, these results show that 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 effect of Asef2 on migration using live-cell imaging. Fig. 2E shows individual migration tracks of GFP and GFP-Asef2 stable cells. Interestingly, the migration paths of GFP-Asef2 cells

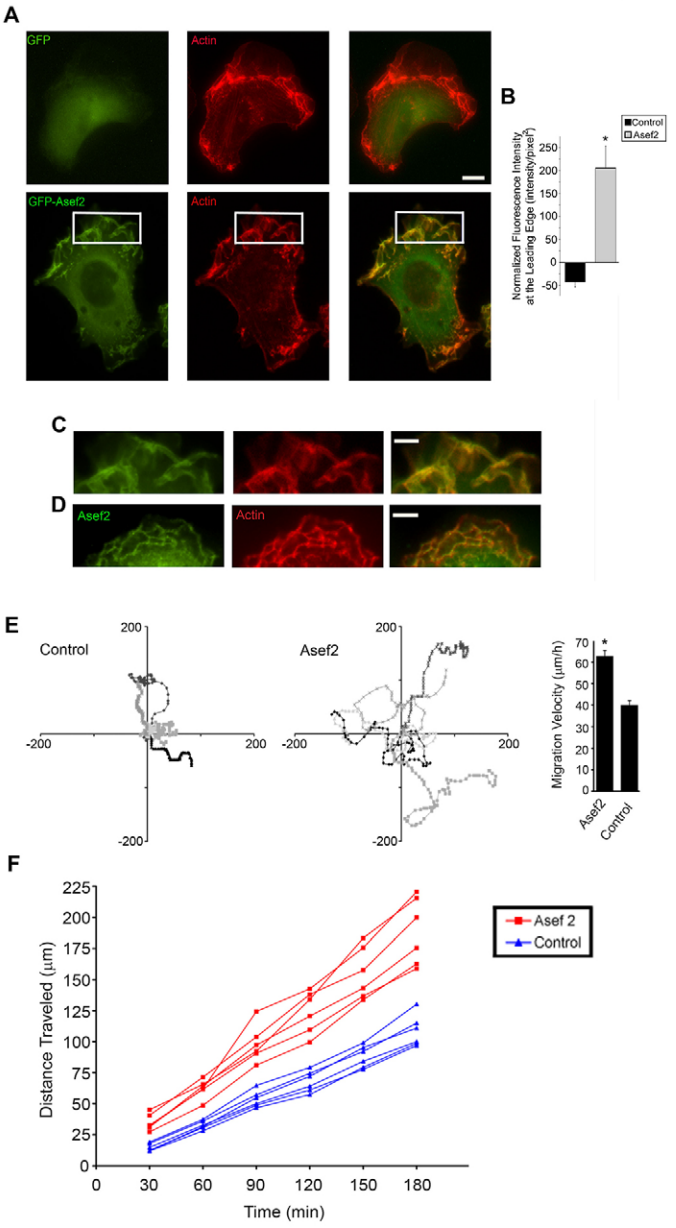

Fig. 2. Asef2 localizes with actin at the leading edge and promotes cell migration. (A) Cells stably expressing GFP (top panels) or GFP-Asef2 (bottom panels) were fixed and stained for actin using TRITC-phalloidin (red). Overlays are shown (right panels). Scale bar: 10 μm. (B) Quantification of the normalized 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 that 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 A. Scale bar: 5 μm. (D) Wild-type HT1080 cells were fixed and co-immunostained for endogenous Asef2 (green) and actin (red). The overlay is shown (far right panel). Scale bar: 5 μm. (E) GFP (control) and GFP-Asef2 stable cells were plated on fibronectin and images were collected every 5 minutes 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.

were significantly longer than those in control cells (Fig. 2E,F; supplementary material Movies 1 and 2). The migration velocity of GFP-Asef2 cells increased 1.6-fold compared with control GFP cells (Fig. 2E), suggesting that Asef2 regulates the migration of HT1080 cells.

Knockdown of endogenous Asef2 in HT1080 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 HT1080 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. 3A,B). By 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 HT1080 cells resulted in an approximately twofold decrease in the migration velocity compared with cells expressing scrambled siRNA or empty pSUPER vector (Fig. 3C,D; supplementary material Movies 3 and 4). To further show that 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 et al., 2007). Since *ASEF2* siRNA #2 is designed against nucleotides within this N-terminal region, it does not affect expression of Asef2Δ204 (Fig. 3A). 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. 3C,D). These results indicate that endogenous loss of Asef2 inhibits migration and point to a crucial role for Asef2 in regulating this process.

Since our results suggested that 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 50% decrease in the amount of active Rac and Cdc42 compared with that observed in cells expressing scrambled siRNA or empty pSUPER vector (Fig. 3E). In addition, RhoA activity was increased 1.7-fold in Asef2-knockdown cells compared with control cells expressing either scrambled siRNA or pSUPER vector (Fig. 3E). 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 (Nayal et al., 2006; Rottner et al., 1999; Webb et al., 2004), we hypothesized that Asef2 affects migration by regulating adhesion turnover. To test this hypothesis, GFP and GFP-Asef2 cells were immunostained for two adhesion markers, paxillin and vinculin, and observed with total internal reflection microscopy (TIRF). Adhesions in GFP-Asef2 cells were very small and located primarily around the cell perimeter usually within 1 μm of the leading edge (Fig. 4A). By contrast, in control cells, numerous large adhesions were found throughout the cell body (Fig. 4A). Since the smaller, peripherally located adhesions in GFP-Asef2 cells could

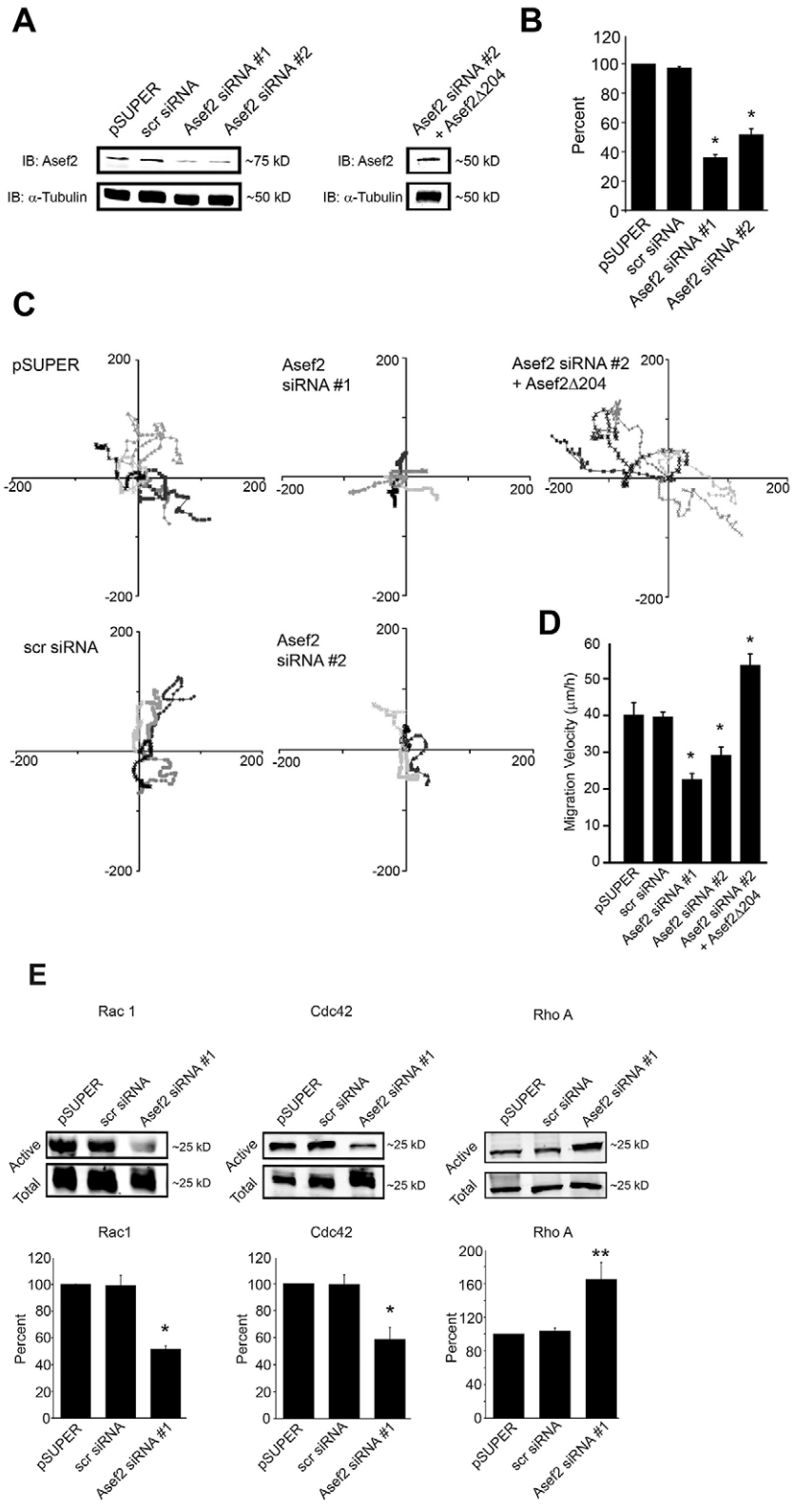

Fig. 3. Knockdown of endogenous Asef2 significantly impairs migration. (A) Wild-type HT1080 cells were transfected with empty pSUPER vector, scrambled siRNA (scr siRNA) or *ASEF2* siRNAs. In some experiments, wild-type HT1080 cells were co-transfected with *ASEF2* siRNA #2 and a truncated form of Asef2 (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 HT1080 cells were transfected with empty pSUPER vector, scrambled siRNA (scr siRNA) or *ASEF2* siRNAs and used in live-cell migration assays 3 days later. To show that the migration phenotype observed with cells expressing *ASEF2* siRNA 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 constructs from C. Error bars represent s.e.m. for 30-35 cells from four separate experiments (**P*<0.009). (E) HT1080 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 (lower panels). Error bars represent s.e.m. (**P*<0.003; ***P*<0.03). For B, D and E, asterisks denote a statistically significant difference compared with pSUPER-transfected cells.

result from enhanced adhesion turnover, we further explored this possibility. Adhesion turnover was quantitatively assessed by transfecting GFP-Asef2 cells with mCherry-paxillin and measuring the $t_{1/2}$s for the assembly and disassembly of adhesions, as previously described (Nayal et al., 2006; Webb et al., 2004). In GFP-Asef2 cells, the apparent $t_{1/2}$ for adhesion assembly was decreased by 50% compared with control cells, indicating that adhesions were forming significantly faster in the Asef2 cells (Fig. 4B,C; Table 1; supplementary material Movies 5 and 6). Asef2 had a similar effect on the disassembly of adhesions, because the $t_{1/2}$ for adhesion disassembly was decreased by 75% in GFP-Asef2 stable cells compared with control cells (Fig. 4B,C; Table 1; supplementary

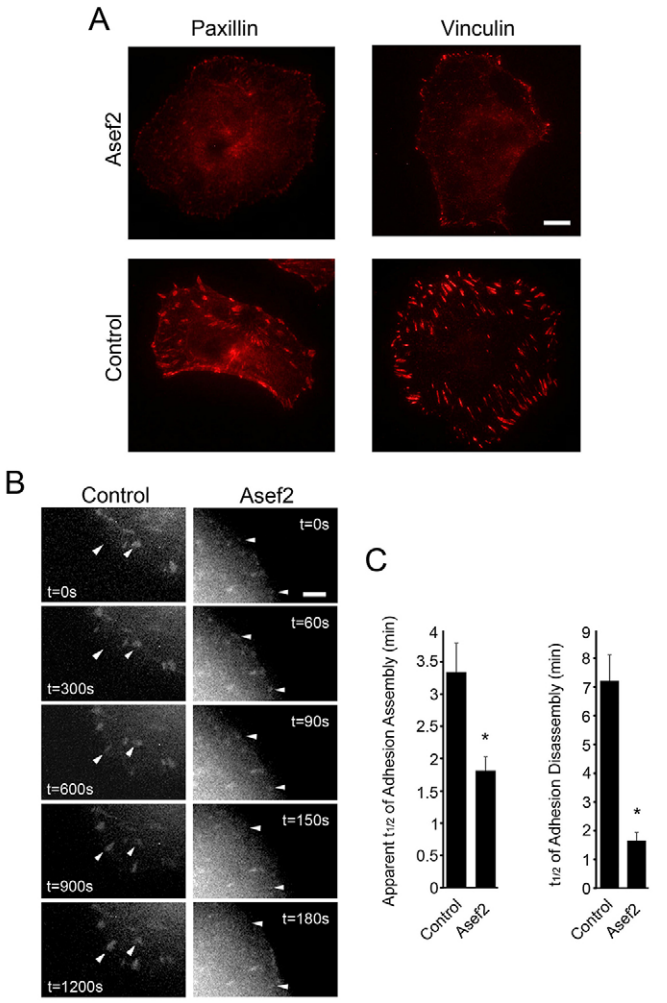

Fig. 4. Asef2 induces the formation of small, leading edge adhesions that turn over very rapidly. (A) GFP (control) and GFP-Asef2 stable cells were immunostained for endogenous paxillin or vinculin and visualized with TIRF microscopy. Scale bar: 10 μm. (B) Cells were transfected with mCherry-paxillin 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 cells compared with control cells (arrowheads). Scale 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.

material Movies 5 and 6). These results suggest that Asef2 increases the rate of adhesion turnover, which could contribute to its effect on migration.

To confirm a role for Asef2 in adhesion turnover, we knocked down endogenous expression of the protein in wild-type HT1080 cells with our siRNA construct. Many large adhesions were found throughout cells expressing *ASEF2* siRNA #1 (supplementary material Fig. S2A). These adhesions appeared to be larger than those observed in control cells expressing scrambled siRNA (supplementary material Fig. S2A), which prompted us to quantify adhesion turnover in Asef2-knockdown and control cells. Expression of *ASEF2* siRNA #1 resulted in a twofold increase in the apparent $t_{1/2}$ for adhesion assembly compared with that observed in control cells expressing empty pSUPER vector or scrambled siRNA (supplementary material Fig. S2B,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 (supplementary material Fig. S2B,D; Table 1). Thus, knockdown of endogenous Asef2 significantly altered the rate of adhesion turnover, indicating that it is an important regulator of this process.

Table 1. Apparent $t_{1/2}$ for adhesion assembly and $t_{1/2}$ for adhesion disassembly in HT1080 cells*

Construct	Adhesion assembly apparent $t_{1/2}$ (minutes)	Adhesion disassembly $t_{1/2}$ (minutes)
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
Scrambled siRNA	3.4±0.3	7.3±0.8
ASEF2 siRNA #1	6.4±1.1	10.1±0.7

*The $t_{1/2}$ values are reported as means ± s.e.m. For each $t_{1/2}$, measurements were obtained from 15-23 individual adhesions in four to six cells from at least three independent experiments.

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

Since our results showed that GFP-Asef2 cells have increased levels of both Rac and Cdc42, this raised the question as to which of these GTPases is crucial for the Asef2-mediated effects on migration and adhesion dynamics. To address this question, we generated *RAC* and *CDC42* 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. 5A). When the *RAC* siRNAs were transfected into GFP and GFP-Asef2 stable cells, an overall decrease in the migration velocity was observed (Fig. 5C,E), because Rac is an important regulator of cell migration. More importantly, expression of the *RAC* siRNAs completely abrogated the Asef2-mediated effect on migration (Fig. 5C,E). The migration velocities were almost identical in GFP and GFP-Asef2 stable cells transfected with the *RAC* siRNAs (Fig. 5E), indicating that knockdown of endogenous Rac inhibited Asef2-mediated migration. By contrast, scrambled siRNA had no effect on Asef2-mediated migration.

Since Asef2 also increased Cdc42 activity in HT1080 cells, we examined the effect of Cdc42 on Asef2-mediated migration using *CDC42* siRNA. When *CDC42* siRNA #1 or *CDC42* siRNA #2 was transfected into HT1080 cells, endogenous expression of Cdc42 decreased by almost 75% compared with empty pSUPER vector or scrambled siRNA (Fig. 5B). Thus, the effectiveness of the *CDC42* siRNAs in decreasing endogenous expression of the protein was similar to that observed with the *RAC* siRNAs (Fig. 5A,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. 5D,F). However, unlike the *RAC* siRNAs, expression of the *CDC42* siRNAs did not eradicate the Asef2-mediated effect on migration (Fig. 5D,F). The migration velocity of GFP-Asef2 cells was still increased 1.5-fold compared with control cells when both cell lines were transfected with *CDC42* siRNAs, indicating that knockdown of endogenous Cdc42 did not significantly affect Asef2-mediated migration.

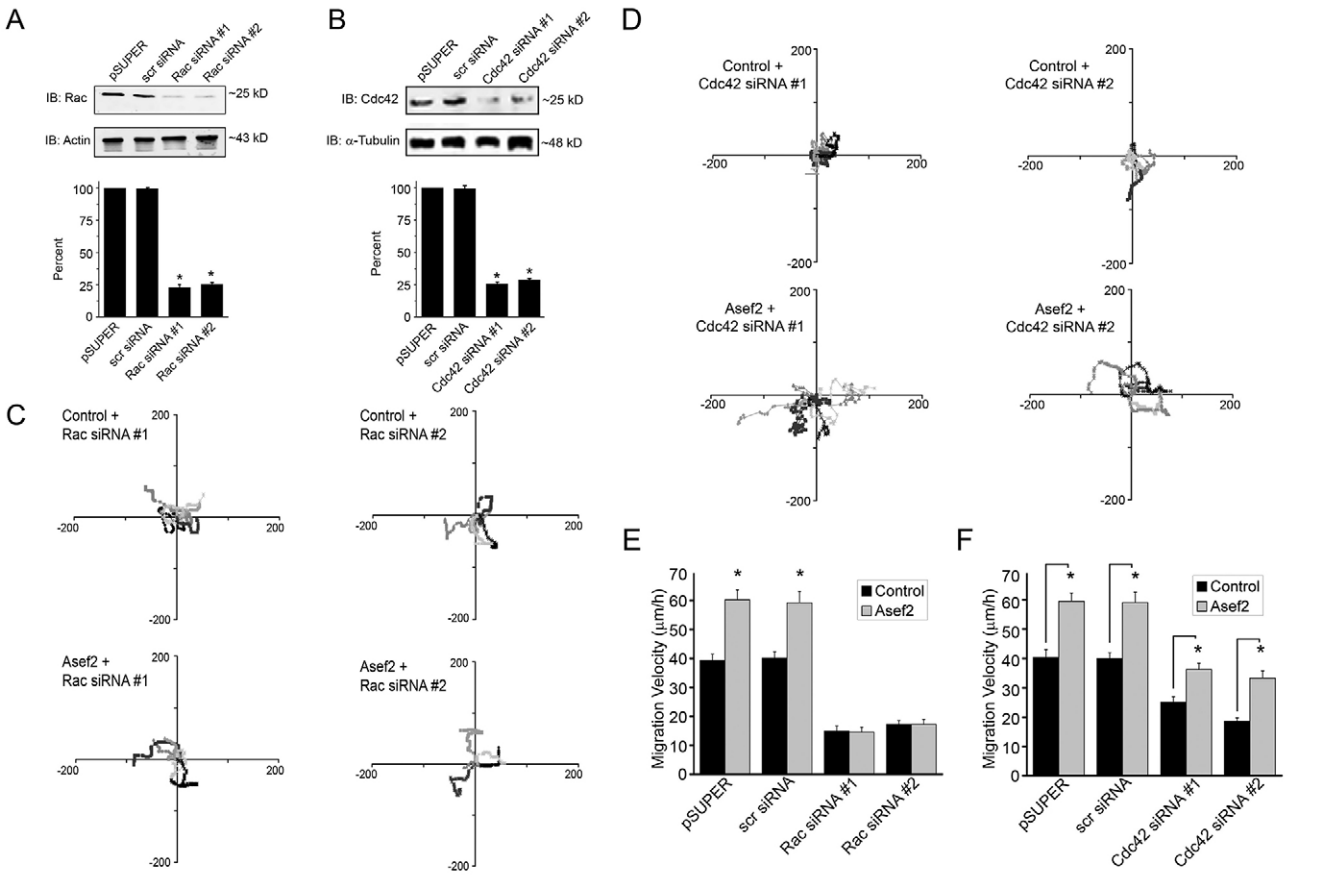

Fig. 5. Rac, but not Cdc42, is necessary for the Asef2-mediated effect on migration. (A) Wild-type HT1080 cells were transfected with empty pSUPER vector, scrambled siRNA (scr siRNA) or *RAC* siRNAs. Three days later, cells were lysed and immunoblotted for Rac or actin (loading control). Quantification 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) HT1080 cells were transfected with empty pSUPER vector, scrambled siRNA (scr siRNA) or *CDC42* siRNAs. After 3 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 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 (C) or *CDC42* siRNAs (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 (E, **P*<0.0001; F, **P*<0.0002). For E and F, asterisks denote statistically significant differences compared with GFP (control) cells.

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 et al., 2006; Xu et al., 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/hour and 36.8±2.9 μm/hour (n=12 from four 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 effect on adhesion turnover was 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 by 50% in GFP-Asef2 cells compared with control GFP cells (Table 2). However, transfection of these cells with *RAC* siRNA #1 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 75% decrease in the $t_{1/2}$ for adhesion disassembly was observed in GFP-Asef2 cells compared with control cells transfected with either empty pSUPER vector or scrambled siRNA (Table 2). By 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 that Rac is necessary for the Asef2-mediated effect on adhesion turnover and support our hypothesis that Asef2 regulates migration and adhesion dynamics through a Rac-dependent mechanism.

PI3K has 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 a review, see Cain and Ridley, 2009), we hypothesized that it is a component of the Asef2 pathway that

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

Construct	Adhesion assembly apparent $t_{1/2}$ (minutes)	Adhesion disassembly $t_{1/2}$ (minutes)
GFP + pSUPER	3.6±0.3	7.0±0.9
GFP + scrambled siRNA	3.5±0.4	7.1±0.9
GFP + *RAC* siRNA #1	6.9±0.4	8.4±0.9
GFP-Asef2 + pSUPER	1.7±0.2	1.8±0.2
GFP-Asef2 + scrambled siRNA	1.7±0.3	1.7±0.2
GFP-Asef2 + *RAC* siRNA #1	7.2±0.8	7.4±0.9

*The $t_{1/2}$ values are reported as means ± s.e.m. For each $t_{1/2}$, measurements were obtained from 15-18 individual adhesions in four to six cells from at least three independent experiments.

regulates migration and adhesion turnover. To examine the effect 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 minutes before imaging and migration was then assessed. Since PI3K contributes to the regulation of cell migration, treatment with wortmannin resulted in a dose-dependent decrease in the migration velocity (Fig. 6A,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 because the migration velocities were almost identical in GFP and GFP-Asef2 stable cells (Fig. 6B). 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, cells were treated with another PI3K inhibitor, LY294002 (50 μM) for 1 hour and then migration was assessed. Similarly to 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. 6A,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 minutes before imaging and then adhesion assembly and disassembly were assessed. As with migration, wortmannin treatment significantly altered the $t_{1/2}$ values for adhesion assembly and disassembly in both cell types, which is consistent with a role for PI3K in regulating adhesion turnover. Notably, after wortmannin treatment, the $t_{1/2}$ values for adhesion assembly and disassembly in GFP-Asef2 stable cells were comparable with those observed in control GFP cells (Table 3). Similar results were obtained when cells were treated with LY294002 (Table 3), indicating that 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 that PI3K and Rac are crucial components by which Asef2 regulates migration and adhesion dynamics; however, since PI3K can function upstream or downstream of Rac (Welch et al., 2003), it was unclear where PI3K acted. To address this question, we inhibited PI3K activity by incubation of cells with 10 nM wortmannin for 2 hours before lysis and then assessed the

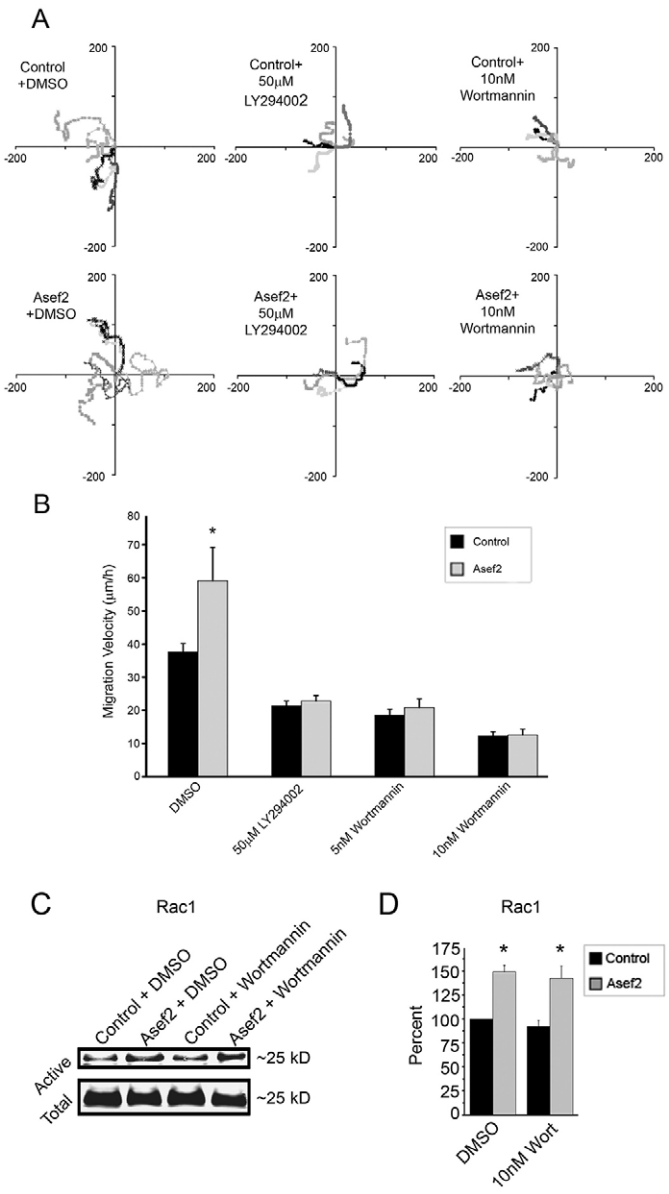

Fig. 6. PI3K is necessary for Asef2-mediated migration. (A) GFP (control) and GFP-Asef2 stable cells were incubated with vehicle (DMSO), wortmannin or LY294002 (50 μM) and then used in live-cell migration assays. Rose plots with individual migration tracks are shown. (B) Quantification of the migration 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 hours 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 B and D, asterisks denote statistically significant differences compared with control cells.

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. 6C,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

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

Treatment	Adhesion assembly apparent $t_{1/2}$ (minutes)	Adhesion disassembly $t_{1/2}$ (minutes)
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

*The $t_{1/2}$ values are reported as means ± s.e.m. For each $t_{1/2}$, measurements were obtained from 15-18 individual adhesions in four to six cells from at least three independent experiments.

of active Rac, suggesting that 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 crucial for the recruitment of some GEFs to the leading edge (Lin et al., 2006; Park et al., 2004), we inhibited PI3K activity and examined the effect on Asef2 localization. GFP-Asef2 stable cells were treated with LY294002, and the amount of Asef2 at the leading edge was quantified. Treatment with LY294002 did not significantly affect the leading-edge localization of Asef2 (supplementary material Fig. S3). Similar results were obtained when GFP-Asef2 stable cells were treated with wortmannin, indicating that 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 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, because Akt is activated by phosphorylation of Thr308 and Ser473 (Bokoch, 2003). The level of active Akt that co-localized with actin at the leading edge of GFP-Asef2 stable cells was increased significantly compared with control cells (Fig. 7A). When we quantified the normalized fluorescent intensity, we found that the amount of active Akt at the leading edge of GFP-Asef2 stable cells was increased more than fivefold compared with control cells (Fig. 7B). Interestingly, the total amount of Akt at the leading edge was also increased in GFP-Asef2 stable cells compared with control cells (supplementary material Fig. S4). Collectively, these results indicate a dramatic enhancement of Akt activity in GFP-Asef2 stable cells.

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 importantly, expression of DN-Akt or KD-Akt completely abolished the Asef2-promoted increase in migration (Fig. 7C and D), suggesting the Asef2-mediated effect on migration is dependent on Akt. We next generated two siRNA constructs against *AKT* 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. 8A and B). As with DN-Akt and KD-Akt, expression of the *AKT* siRNAs in GFP and GFP-Asef2 stable cells completely abrogated the Asef2-mediated effect on migration (Fig. 8C and D). These results indicate that Akt is essential for the Asef2-mediated regulation of cell migration.

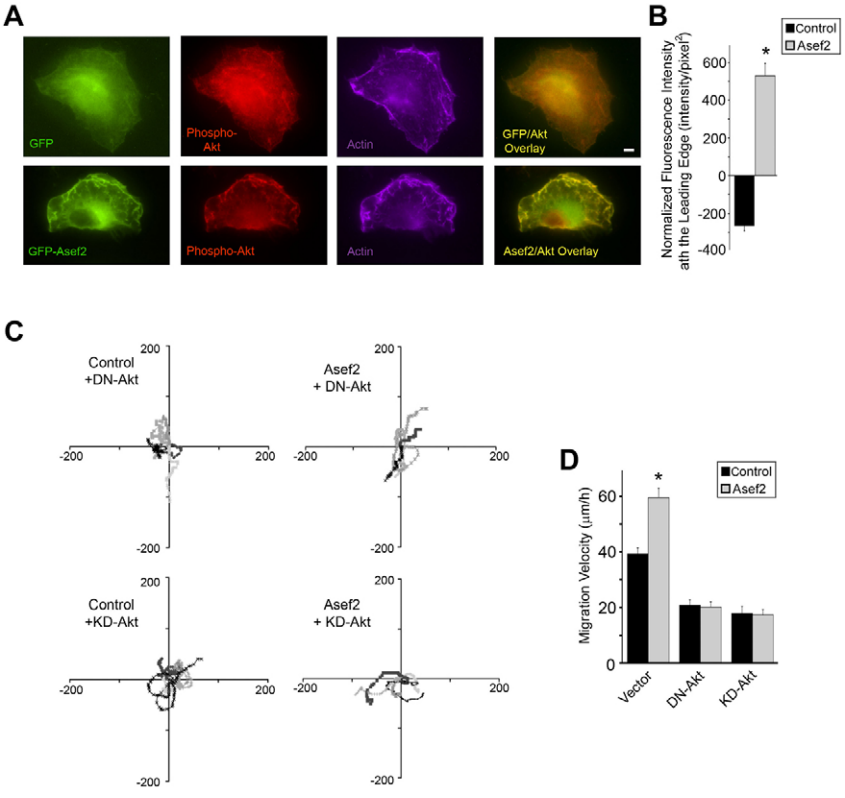

Fig. 7. Akt has a role in Asef2-promoted migration. (A) GFP (control) and GFP-Asef2 stable cells were fixed and co-immunostained for active Akt using a phospho-specific antibody against Thr308, and for actin with Alexa Fluor 647-phalloidin (false-colored purple). (B) Quantification of the normalized 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.

Similarly, inhibition of Akt activity had a significant effect on adhesion turnover, suggesting a new role for Akt in regulating this process. When cells were transfected with KD-Akt or *AKT* siRNA #1, the Asef2-mediated effect on adhesion assembly and disassembly was eliminated (Table 4), indicating that Akt is also crucial 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 50% decrease in the level of active Rho in GFP-Asef2 stable cells compared with control cells (Fig. 1D), which led us to 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/hour and 10.8±1.3 μm/hour (n=20 cells from three separate experiments), respectively, indicating that 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 through 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 50% decrease in the migration velocity compared with cells expressing scrambled siRNA or empty pSUPER vector (supplementary material Fig. S5). Treatment with the ROCK specific-inhibitor Y-27632 (10 μM) (Uehata 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 fivefold in these cells (supplementary material Fig. S5). Taken together, these results suggest that Rho is an important effector by which Asef2 regulates migration.

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

Construct	Adhesion assembly apparent $t_{1/2}$ (minutes)	Adhesion disassembly $t_{1/2}$ (minutes)
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

*The $t_{1/2}$ values are reported as means ± s.e.m. For each $t_{1/2}$, measurements were obtained from 15-18 individual adhesions in four to six cells from at least three independent experiments.

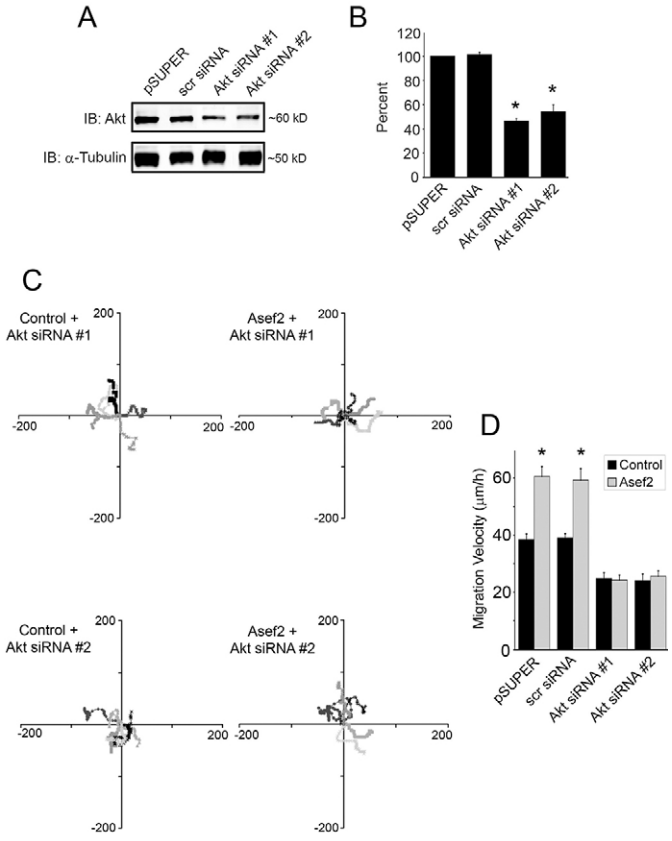

Fig. 8. Knockdown of endogenous Akt significantly inhibits Asef2-promoted migration. (A) Wild-type HT1080 cells were transfected with empty pSUPER vector, scrambled siRNA (scr siRNA) or *AKT* siRNAs. Cell lysates were immunoblotted for Akt or α-tubulin (loading control). (B) Quantification of the amount 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.

Discussion

Although 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 that 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 crucial for the effect 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 (Chrzanowska-Wodnicka and Burridge, 1996; Ridley and Hall, 1992; Rottner et al., 1999). Thus, the loss of RhoA activity in GFP-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 et al., 2001).

Asef2 increases the activity of Rac1 and Cdc42, which is consistent with previous studies showing that this molecule has GEF

activity for these GTPases (Hamann et al., 2007; Kawasaki 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 that 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 et al., 1998). Cdc42 is apparently not essential for directed migration of some cells because loss of this GTPase did not significantly affect the migration velocity of fibroblastoid cells in a wound-closure assay (Czuchra et al., 2005). By contrast, Cdc42-deficient mouse embryonic fibroblasts exhibit a defect in their ability to migrate directionally and to respond to a chemotactic gradient (Yang et al., 2006). In our study, knockdown of Cdc42 in HT1080 cells significantly decreased their migration velocity, suggesting that Cdc42 does contribute to this process. However, Cdc42 does not appear to have a significant role in Asef2-mediated random migration; however, 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 Transwell assay (Kawasaki et al., 2009). Although it is not clear whether Cdc42 has 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 et al., 2006; Park et al., 2004). This indicates the regulation of GEF activity can be downstream of GTPase signaling. Although 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 that translocation of Asef2 to the plasma membrane is not PI3K dependent.

Although Rac can be activated downstream of PI3K signaling (Cain and Ridley, 2009; Hawkins et al., 1995; Hooshmand-Rad et al., 1997), our results suggest that PI3K is not upstream of Rac in the mechanism by which Asef2 regulates migration. One possibility is that 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 (Cain and Ridley, 2009; Srinivasan et al., 2003; Welch et al., 2002). 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 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 HT1080 cells, but this function of Akt seems to be partially dependent on the isoform present (Irie et al., 2005; Kim et al., 2001; Zhou et al., 2006). In our study, expression of *AKT1* siRNAs, DN-Akt or KD-Akt resulted in a decrease in the migration velocity, which is consistent with Akt promoting migration in HT1080 cells (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 3β (GSK-3β), 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; Zhou et al., 2003). Akt phosphorylation of GSK-3β on Ser9 results in inactivation of this kinase, which is important for the maintenance of a polarized migratory phenotype (Cross et al., 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 that it is important to keep Rho activity low at the leading edge of cells (Ridley 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 (Lee and Jacobson, 1997; Yuruker and Niggli, 1992) whereas 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 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.

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 and pan-Akt C67E7 antibody were from Cell Signaling (Beverly, MA). Glutathione-Sepharose beads came from Amersham. CCM1 was purchased from Hyclone. Secramine A synthesized by Bo Xu and Gerald Hammond was generously provided by the laboratories of Tomas Kirchhausen (Harvard Medical School, Cambridge, MA) and Gerald Hammond (University of Louisville, Louisville, KY).

Plasmids

Full-length *ASEF2* cDNA was generated by reverse transcription of HEK293 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*, *RAC*, *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* siRNA #1, 5′-TTGCGCAGCTAGCCACTAT-3′; *ASEF2* siRNA #2, 5′-TTCGTCAGATTGCGAGTGA-3′; and *CDC42* siRNA #2, 5′-AAAGTGGGTGCCTGAGATA-3′. Both *RAC1* and *AKT1* target sequences and *CDC42* target sequence siRNA #1 have been previously described (Chan et al., 2005; Degtyarev et al., 2008; Katome et al., 2003; Wegner 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, TX).

Cell culture and transfection

HEK293 and HT1080 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, Palo Alto, CA) were cultured in DMEM with 10% heat-inactivated FBS and penicillin-streptomycin and selected as previously described (Bryce et al., 2005). Stable HT1080 cells expressing GFP or GFP-Asef2 were prepared by retroviral transduction as previously described (Bryce 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). HT1080 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 ×10 objective (NA 0.3) or a PlanApo ×60 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, Middleton, WI).

To quantify the enrichment of GFP-Asef2, phospho-Akt Thr308, and total Akt at the leading edge of HT1080 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 hour at 37°C and fixed with 4% paraformaldehyde and 4% glucose in phosphate buffered saline (PBS) for 5 minutes. Before cell plating, coverslips were pre-incubated with 10 μg/ml fibronectin (Fn). Following fixation, cells were permeabilized with 0.2% (v/v) Triton X-100 for 5 minutes at room temperature. Cells were then blocked with 20% goat serum in PBS for 1 hour and incubated with primary antibody for 1 hour followed by fluorescently conjugated secondary antibody for 1 hour. 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, 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 minutes at 37°C in the dark before fixation.

Migration assay

For random migration, cells were plated at low density on culture dishes, pre-incubated with 10 μg/ml Fn, and allowed to adhere for 1 hour at 37°C. Cells were then imaged in phase with a ×10 objective every 5 minutes for 12 hours. During microscopy, cells were maintained at 37°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 *X-Y* 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 HT1080 cells were transfected with 0.5 μg mCherry-paxillin cDNA and incubated at 37°C for 24 hours. In some experiments, cells were co-transfected with 0.5 μg mCherry-paxillin cDNA and 2.5 μg *ASEF2* siRNA #1, *RAC* siRNA #1, *AKT* siRNA #1, pSUPER vector, scrambled siRNA or KD-Akt cDNA. Transfected cells were then seeded at low density on glass-bottom Fn-coated microscopy dishes and incubated at 37°C for 1 hour. Fluorescent time-lapse images were obtained at 15- to 30-second intervals and the $t_{1/2}$ values for adhesion assembly and disassembly were determined as previously described (Webb et al., 2004) using Metamorph software.

Rho-family GTPase activity assays

HT1080 stable cell lines were transfected with 3 μg Myc-RhoA, FLAG-Cdc42 or FLAG-Rac1 cDNA and incubated at 37°C for 24 hours. In some experiments, wild-type HT1080 cells were co-transfected with 2 μg *RHOA*, *RAC* or *CDC42* cDNA and 4.5 μg *ASEF2* siRNA #1, pSUPER vector or scrambled siRNA. Cell lysates were collected and assayed as previously described (Ren et al., 1999). Active GTPase assays were performed in HEK293 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.

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