RESEARCH ARTICLE

A RhoG-mediated signaling pathway that modulates invadopodia dynamics in breast cancer cells

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ABSTRACT

One of the hallmarks of cancer is the ability of tumor cells to invade surrounding tissues and metastasize. During metastasis, cancer cells degrade the extracellular matrix, which acts as a physical barrier, by developing specialized actin-rich membrane protrusion structures called invadopodia. The formation of invadopodia is regulated by Rho GTPases, a family of proteins that regulates the actin cytoskeleton. Here, we describe a novel role for RhoG in the regulation of invadopodia disassembly in human breast cancer cells. Our results show that RhoG and Rac1 have independent and opposite roles in the regulation of invadopodia dynamics. We also show that SGEF (also known as ARHGDEF26) is the exchange factor responsible for the activation of RhoG during invadopodia disassembly. When the expression of either RhoG or SGEF is silenced, invadopodia are more stable and have a longer lifetime than in control cells. Our findings also demonstrate that RhoG and SGEF modulate the phosphorylation of paxillin, which plays a key role during invadopodia disassembly. In summary, we have identified a novel signaling pathway involving SGEF, RhoG and paxillin phosphorylation, which functions in the regulation of invadopodia disassembly in breast cancer cells.

KEY WORDS: RhoG, Invadopodia, SGEF, Guanine-nucleotide exchange factors, Src, Paxillin, Rac1

INTRODUCTION

Rho GTPases control many aspects of cell behavior ranging from the regulation of cytoskeletal organization, cell motility and cell polarity, to nuclear gene expression and control of cell growth (Hodge and Ridley, 2016). Rho proteins cycle between an active (GTP-bound) and an inactive (GDP-bound) state. The activation of Rho proteins involves the exchange of GDP for GTP, which is catalyzed by specific guanine-nucleotide-exchange factors (GEFs). Once activated, Rho GTPases interact with a wide variety of downstream effectors to modulate their activity and/or localization. The hydrolysis of GTP to GDP, a reaction that is stimulated by GTPase-activating proteins (GAPs), inactivates the GTPases and terminates the signal. With more than 80 Rho GEFs, 70 Rho GAPs and over 100 effectors, cells regulate the activity of Rho proteins through multiple pathways, thus acting as key nodes for signal integration and dissemination (Bustelo et al., 2007; Rossman et al., 2005; Tcherkezian and Lamarche-Vane, 2007).

RhoG, a Rho protein related to Rac, has been associated with cell migration, neurite outgrowth, microtubule dynamics, micropinocytosis, bacterial uptake, phagocytosis and leukocyte trans-endothelial migration (deBakker et al., 2004; Ellerbroek et al., 2004; Jackson et al., 2015; Katoh et al., 2006, 2000; van Buul et al., 2007). Recent studies have revealed that RhoG plays a role in tumor cell invasion and may contribute to the formation of invadopodia (Hiramoto-Yamaki et al., 2010; Kwiatkowska et al., 2012). Invadopodia are actin-rich adhesive structures that form in the ventral surface of cancer cells and allow them to degrade the extracellular matrix (ECM) (Gimona et al., 2008). Formation of invadopodia involves a series of steps that include the disassembly of focal adhesions and stress fibers, and the relocalization of several of their components into the newly formed invadopodia (Hoshino et al., 2012; Oikawa et al., 2008). Invadopodia assembly starts with the formation of actin- and cortactin-rich puncta, followed by the recruitment of adhesion proteins, such as vinculin and paxillin, and proteinases that allow ECM degradation (Hoshino et al., 2013). Even though many of the molecular components required for invadopodia formation have been identified, the signaling pathways that regulate these events are still poorly understood (Linder et al., 2011). Invadopodia formation is controlled by the integrated activity of several GTPases, including Cdc42, Rac1, RhoC and SGEF (Spuul et al., 2014). Cdc42 promotes invadopodia formation in almost every system tested (Ayala et al., 2009; Di Martino et al., 2014; Moreau et al., 2006, 2003; Nakahara et al., 2003; Tatin et al., 2006). However, generalized conclusions cannot be drawn from studies of other Rho GTPases, since their activities can both inhibit or promote invadopodia formation depending on the experimental conditions or cell type used (Spuul et al., 2014).

In this study, we describe a novel function for RhoG in the regulation of invadopodia dynamics in human breast cancer cells. Our findings describe a signaling pathway involving SGEF and RhoG that regulates invadopodia disassembly independently of Rac1 through regulation of paxillin phosphorylation.

RESULTS

**RhoG is a negative regulator of invadopodia formation**

We have previously shown that the breast cancer cell line SUM159 forms invadopodia when treated with phorbol esters such phorbol 12,13-dibutyrate (PDBu) and 12-O-tetradecanoylphorbol-13-acetate (PMA), and that the formation of these structures correlates with their metastatic potential (Goicoechea et al., 2009). To determine the requirement of RhoG in invadopodia formation, we generated stable SUM159 cell lines in which RhoG expression was silenced using lentivirally encoded shRNA. We used two different RhoG-specific shRNAs (shRNA#1 and shRNA#4) to rule out off-target effects. A stable cell line expressing a non-targeting shRNA was used as control (CTRL). Fig. 1A shows that RhoG silencing was efficient, especially for shRNA #4. SUM159 cells are able to form invadopodia spontaneously, so we first looked at the effect of RhoG depletion in untreated cells. To identify invadopodia structures, we stained the cells with the invadopodia markers...
cortactin and actin. We found that silencing RhoG induced a significant increase in the number of spontaneous invadopodia when compared to CTRL cells, from 7% in CTRL cells to 14% and 23% in RhoG shRNA#1 and shRNA#4 cells, respectively (Fig. 1B, untreated; Fig. S1A). We next looked at the effect of RhoG depletion in cells treated with phorbol esters. In PDBu-treated cells, the number of cells with invadopodia increased significantly in RhoG-knockdown (KD) cells, from 35% in CTRL cells to 64% and 81% in RhoG shRNA#1 and shRNA#4 cells respectively (Fig. 1B,C). Similarly, the percentage of cells with invadopodia...
Enhanced invadopodia formation after RhoG depletion requires Src activity

Phorbol esters mimic diacylglycerol (DAG), which activates protein kinase C (PKC) family members. The role and positioning of PKC in these pathways remains largely unknown, but it involves the function of Src family kinases (Hai et al., 2002; Tatin et al., 2006).

Because both FAK and Src activities have been shown to be upregulated to promote invadopodia formation, and both play critical roles in cell invasion (Murphy and Courtnidge, 2011), we wanted to determine whether RhoG modulates invadopodia formation through a FAK- and/or Src-dependent pathway (FAK is also known as PTK2). Western blot analysis showed that there was no difference in the expression level of endogenous FAK and Src between CTRL and RhoG KD cells (Fig. 2A,B). However, the activity of both increased by ~2-fold in the absence of RhoG (Fig. 2A,B). We next assayed the ability of CTRL and RhoG KD cells to form invadopodia when either Src or FAK were inhibited. Our results show that inhibition of Src with PP2 significantly inhibited invadopodia formation in both CTRL and RhoG KD cells (Fig. 2C,D), whereas inhibition of FAK with PF-573228 had no effect (Fig. 2E,F). These findings suggest that the increase in Src activity in RhoG KD cells may contribute to the increase observed in the number of cells with invadopodia, and that RhoG may function downstream of Src to regulate invadopodia formation in SUM159 cells.

Sgef functions upstream of RhoG to regulate invadopodia formation

Several GEFs have been described to stimulate nucleotide exchange on RhoG (Bellanger et al., 2000; Blangy et al., 2000; Bustelo et al., 2007; D’Angelo et al., 2007; Damoulakis et al., 2014; Ellerbroek et al., 2004; Krishna Subbaiah et al., 2012; May et al., 2002; Wennerberg et al., 2002). To identify the GEF that regulates RhoG during invadopodia formation, we performed a candidate-based shRNA screen. We stably silenced the best characterized RhoG-specific GEFs, including SGEF (also known as ARHGEF26), ephexin 4 (also known as ARHGEF16), PLEKHG6 and Trio in SUM159 cells, using specific shRNAs. We tested two independent shRNAs for each of the GEFs and compared them to cells expressing a non-targeting shRNA (CTRL). The KD efficiency was verified either by quantitative real-time PCR (qRT-PCR; Fig. 3A; Fig. S3A) or western blotting (Fig. S3B). Cells were treated with PDBu, and invadopodia structures were identified in cells stained for cortactin and actin (Fig. 3B; Fig. S3C). Our results show that the number of cells with invadopodia increased significantly when SGEF was silenced (from 42% in CTRL cells, to 70% and 85% upon treatment with SGEF shRNA#4 and shRNA#5, respectively, Fig. 3C), whereas silencing PLEKHG6, ephexin 4 or Trio had no significant effect on invadopodia numbers (Fig. S3D). The phenotype observed in SGEF KD cells was similar to that observed in RhoG KD cells, both in terms of the number of cells that formed invadopodia and their appearance (Figs 3B and 1C). Based on these results, we used shRNA#5 for the rest of these studies (referred to as SGEF KD). To determine whether SGEF function was mediated through RhoG we attempted to rescue the effects of RhoG KD on invadopodia by expressing exogenous Myc–SGEF in RhoG KD cells. Our results showed that Myc–SGEF expression could not rescue the RhoG KD phenotype (Fig. 3D,E), suggesting that the role of SGEF in invadopodia is mostly mediated by RhoG. Consistent with the results shown in Fig. 1H, overexpression of Myc–SEGF wt, significantly inhibited the formation of invadopodia (Fig. 3F,G). In contrast, overexpression of a catalytically inactive mutant of SGEF (Myc–SGEF 446/621, which has E446A and N621A mutations) (Ellerbroek et al., 2004) had no effect (Fig. 3F,G), which demonstrates that SGEF exchange activity, and thus RhoG activation, is required. Only 5% of the cells transfected with Myc–SGEF wt formed invadopodia, whereas cells transfected with the Myc–SGEF 446/621 show invadopodia levels comparable to those of non-transfected cells (~30%) (Fig. 3F). Interestingly, Myc–SGEF 446/621 localized to invadopodia (Fig. 3G). We also observed Myc–SGEF wt at invadopodia, although at a much lower frequency (~1.25% of Myc–SGEF wt transfected cells) (Fig. S3E). This suggests that SGEF could localize transiently to invadopodia and be released to the cytoplasm after the exchange reaction is completed. In the absence of catalytic activity, SGEF may not be efficiently released and would thus be found at invadopodia.

Our results suggest that RhoG activity needs tight regulation during invadopodia formation, so we next investigated the kinetics of RhoG activation at different time points following PDBu exposure. We measured RhoG activity by using GST–ELMO to pulldown GTP-RhoG (van Buul et al., 2007). Our results show a rapid and transient decrease of RhoG activity in the first 5 min of PDBu treatment followed by a peak of activation at 15 min (Fig. 3H,J). We obtained similar kinetics of RhoG activation after treatment of cells with PMA (Fig. S4A,B). To test whether SGEF plays a role in the regulation of RhoG activation during PDBu stimulation, we measured RhoG activity in SGEF KD cells at different times following PDBu addition. Interestingly, the peak of RhoG activation observed at 15 min was completely abrogated by SGEF KD (Fig. 3I,J). In contrast, the activity of RhoG in the absence of PDBu was not significantly affected in the absence of SGEF. Therefore, our results show that SGEF specifically mediates the PDBu-induced activation of RhoG that occurs at 15 min.

Enhanced invadopodia formation in RhoG- and SGEF-depleted cells is not sufficient for invasion

In order to determine whether the invadopodia that formed following RhoG depletion retained their capacity to degrade the...
ECM, we plated CTRL and RhoG KD cells on Oregon Green 488-conjugated gelatin-coated coverslips and assessed invadopodia activity by quantifying the area of degradation (visible as dark on the green background) (Artym et al., 2009; Chen et al., 1985; Martin et al., 2012). Our results show that the number of cells that form invadopodia on gelatin matrix is also increased when RhoG is knocked down (CTRL=35%; RhoG KD=71%, Fig. 4A). RhoG-depleted cells also exhibited larger areas of matrix degradation compared to CTRL cells, which showed discrete areas of degradative puncta (Fig. 4B). Quantification showed a ∼9-fold increase in degradation area in RhoG KD cells relative to CTRL cells (Fig. 4C). These results demonstrate that invadopodia are still functional in the absence of RhoG. We obtained similar results, when we silenced RhoG in MDA-MB-231 cells (Fig. S2E,F). We then assayed the ability of serum-starved CTRL, RhoG KD and RhoG KD cells expressing a Myc-tagged shRNA-resistant version of RhoG (Rescue) to invade through Matrigel-coated membranes (Fig. 4D). Our results show that, even though there are more cells with invadopodia in RhoG KD cells than in CTRL cells, their ability to invade through Matrigel was significantly impaired. The ability to invade was partially restored by re-expressing Myc–RhoG (Fig. 4D). As observed in RhoG KD cells, SGEF KD cells also displayed a lower invasive capacity compared to CTRL cells (Fig. 4E). These results suggest that enhanced formation of invadopodia and increased degradation capacity in RhoG- and SGEF-deficient cells are not sufficient to enhance invasiveness. It is possible that the invasion defect observed results from the inability of cells to migrate in the absence of RhoG, since RhoG has been previously shown to play a role during cell migration (Hiramoto-Yamaki et al., 2010; Katoh et al., 2006). Supporting this, our results show that RhoG KD also impairs cell migration in SUM159 cells (Fig. S2G). In summary, our results suggest that proper coordination of matrix degradation and migration is required for efficient invasion.

**Rac1 stimulates invadopodia formation**

RhoG is a key upstream regulator of Rac1 in migrating cells (Elfenbein et al., 2009; Hiramoto et al., 2006; Katoh et al., 2006; Katoh and Negishi, 2003). However, RhoG has also been shown to function independently of Rac1 (Samson et al., 2010; Wennerberg et al., 2002). To determine whether the role of RhoG in invadopodia depends on Rac1, we first generated stable cell lines in which Rac1 expression was silenced using lentivirally encoded shRNA. Since knockdown of Rac1 was not efficient for any of the four shRNAs tested (not shown), we generated Rac1-knockout cells (Rac1 KO) using the double nicking RNA-guided Cas9 nucleases from the microbial CRISPR/Cas system. After isolation of single cell colonies, gene knockout efficiency was assayed by western...
Fig. 3. See next page for legend.
Fig. 3. SGEF regulates invadopodia formation. (A) Cell lysates from SUM159 cells stably expressing non-targeting (CTRL) or SGEF-specific shRNAs (shRNA#4 and shRNA #5) were analyzed by qRT-PCR for expression of SGEF. (B) CTRL and SGEF KD SUM159 cells were treated with PDBu for 30 min and stained with anti-cortactin antibody (red), Alexa-Flour-488–phalloidin (green) and Hoechst 33342 (blue). Arrowheads indicate representative invadopodia. (C) Quantification of cortactin- and actin-containing invadopodia in CTRL and SGEF KD cells expressed as the percentage of cells with invadopodia. Data are mean±s.e.m. of four independent experiments. (D) Cell lysates from CTRL, RhOG KD and RhOG KD cells expressing Myc–SGEF (rescue Myc–SGEF) were immunoblotted with anti-RhoG and -Myc antibodies. Tubulin was used as a loading control. (E) Quantification of cortactin- and actin-containing invadopodia in CTRL, RhOG KD and rescue Myc–SGEF cells. Data are mean±s.e.m. of three independent experiments in which at least 200 cells per experiment were counted. (F) Quantification of cortactin- and actin-containing invadopodia in SUM159 cells transiently transfected with either Myc-tagged wild-type SGEF (SGEF wt) or catalytically inactive SGEF (SGEF 446/621). Data are mean±s.e.m. of at least three independent experiments in which at least 200 cells per experiment were counted. (G) Representative images of cells transfected with either Myc-tagged wild-type SGEF (SGEF wt) or catalytically inactive SGEF (SGEF 446/621). Cells were stained with anti-cortactin antibody (red), anti-Myc antibody (green), Alexa-Flour-488–phalloidin (magenta) and Hoechst 33342 (blue). Arrows indicate transfected cells and arrowheads indicate representative invadopodia. (H–J) CTRL (H) and SGEF KD (I) cells were treated with PDBu for the indicated times. Active RhoG was precipitated from total lysates using GST–ELMO and immunoblotted with RhoG antibody. (J) For quantification, active RhoG levels were normalized to total RhoG levels. Data are mean±s.e.m. of at least three independent experiments. Scale bars: 10 µm. *P<0.05; **P<0.01; ***P<0.001.

blotting (Fig. 5A). Remarkably, invadopodia structures were completely absent in Rac1 KO cells (Fig. 5B). PDBu-treated Rac1 KO cells showed a very consistent morphology, with actin and cortactin concentrated at defined regions of the leading edge in small lamellipodia-like structures (Fig. 5B). Quantitative analysis in the three single cell colonies analyzed showed that Rac1 KO completely blocked the ability of cells to form invadopodia as compared to CTRL cells (Fig. 5C, Movies 1 and 2). Re-expression of Myc-tagged Rac1 in KO cells (rescue) restored the ability of the cells to form invadopodia to CTRL levels (Fig. 5D, Movie 3). To explore the role of Rac1 in more detail, we overexpressed Myc–Rac1 in SUM159 cells (Rac1 OE) and tested the cells for their ability to form invadopodia. As expected from the Rac1 KO results, cells overexpressing Rac1 formed more invadopodia than CTRL cells (Fig. 5E). We also attempted to rescue RhoG KD cells with Myc-tagged Rac1 (RhoG KD+Myc–Rac1). The rationale for this experiment was that if Rac1 is being activated downstream of RhoG, an excess of Rac1 would be able to rescue RhoG deficiency. Fig. 5F shows that ectopic expression of Rac1 could not rescue the RhoG KD invadopodia phenotype. In contrast, RhoG KD Myc–Rac1 rescue cells formed even more invadopodia than RhoG KD cells suggesting an additive effect. Taken together, these results demonstrated that Rac1 and RhoG function independently and play opposing roles in the regulation of invadopodia formation in breast cancer cells.

RhoG is involved in invadopodia dynamics

To further characterize the differences in invadopodia formation between CTRL and RhoG KD cells, we analyzed invadopodia formation over time by fixing and staining SUM159 cells at different times after PDBu addition. Surprisingly, at early time points, the number of cells with invadopodia was almost identical between CTRL and RhoG KD cells, peaking at ~70–80% (Fig. 6A). In CTRL cells, the number of cells with invadopodia decreased rapidly to ~40% at 30 min, and remained stable even after 3 h. In contrast, in the absence of RhoG, the number of cells with invadopodia did not decrease as much, and remained stable over time at ~70% (Fig. 6A). These results suggest that, in CTRL cells, following a rapid burst of initial invadopodia assembly in response to PDBu, invadopodia start to disassemble and the percentage of cells showing invadopodia stabilizes, probably reflecting an equilibrium between assembly and disassembly. In contrast, the percentage of cells with invadopodia remains significantly higher in RhoG KD cells, which may suggest that invadopodia are more stable or longer lived in the absence of RhoG.

To investigate the impact of RhoG and SGEF on invadopodia lifetime, we analyzed the dynamics of invadopodia by time-lapse confocal microscopy in SUM159 cells expressing mCherry–cortactin after PDBu treatment (Fig. 6B). In CTRL cells, invadopodia were short-lived and motile with an average lifetime of ~10 min (Fig. 6B, C; Movie 1). However, when either RhoG or SGEF were silenced, the lifetime of invadopodia increased significantly to over 30 min, and they often persisted in a single location for time periods >1 h (Fig. 6B,C, Movies 4 and 5). When RhoG or SGEF were re-expressed in RhoG KD or SGEF KD cells, respectively, invadopodia lifetime returned to control levels (Fig. 6C; Movies 6 and 7). These results demonstrate the importance of RhoG and SGEF in the regulation of invadopodia dynamics, most likely during disassembly.

Paxillin has previously been found to be a component of invadopodia in other systems, and its tyrosine phosphorylation has been shown to play a role in invadopodia disassembly (Badowski et al., 2008). To test whether RhoG and SGEF regulate paxillin tyrosine phosphorylation, we first examined the levels of phosphorylated (phospho)-paxillin (PXN p118) and total paxillin (PXN) in CTRL, RhoG KD and SGEF KD cells by western blotting using both total and phospho-specific paxillin antibodies. Quantification of immunoblots showed that paxillin tyrosine-phosphorylation decreased ~2.5- and ~1.8-fold in RhoG KD and SGEF KD cells respectively compared to CTRL cells (Fig. 7A). Similar effects were also observed in MDA-MB-231 and MCF7 cells (Fig. 5A, Movies 4 and 5). When RhoG or SGEF were re-expressed in RhoG KD or SGEF KD cells, respectively, invadopodia lifetime returned to control levels (Fig. 7C; Movies 6 and 7). These results demonstrate the importance of RhoG and SGEF in the regulation of invadopodia dynamics, most likely during disassembly.

Both Src and FAK have been associated with paxillin phosphorylation (Bellis et al., 1995; Schaller and Parsons, 1995). However, our results show that even though the phosphorylation of both kinases increased when RhoG is silenced (Fig. 7A,B), paxillin phosphorylation levels are significantly decreased (Fig. 2A), which suggests that the effect of RhoG on paxillin phosphorylation may be independent of Src and FAK. To determine whether Src and/or FAK are involved in RhoG-mediated regulation of paxillin phosphorylation, we analyzed the levels of phospho-paxillin in CTRL or RhoG KD cells in the presence or absence of Src or FAK inhibitors. Our results show that treating CTRL or RhoG KD with a
FAK inhibitor did not have a significant effect in phospho-paxillin levels when compared to non-treated cells. In contrast, inhibition of Src family kinases promoted a decrease in phospho-paxillin levels in both CTRL and RhoG KD cells (Fig. 7E,F). These results suggest there are two pools of phospho-paxillin in the cells, one that is regulated by RhoG independently of Src, and the other is dependent on Src and independent of RhoG.

**DISCUSSION**

Cells assemble invadopodia during cell invasion, which involves a dramatic rearrangement of the actin cytoskeleton, a process that is regulated by the integrated activity of several GTPases (Spuul et al., 2014). While RhoG is known to promote migration and invasion and has been recently linked to invadopodia, the regulation and role of RhoG during invadopodia formation are still unknown. The data reported here describe a novel function for RhoG as a regulator of invadopodia disassembly in human breast cancer cells. We showed that when RhoG expression is silenced, invadopodia are more stable and live longer, whereas its overexpression has the opposite effect. We also identified SGEF as the exchange factor that regulates RhoG activation during invadopodia disassembly. Silencing SGEF, but not other RhoG-specific GEFs, phenocopied the results obtained in RhoG KD cells with a significant increase in the number of cells that form invadopodia. SGEF has been shown to direct actin cytoskeleton remodeling mainly at the dorsal surface of the cells, where it promotes the formation of dorsal ruffles (Ellerbroek et al., 2004; Patel and Galán, 2006; van Buul et al., 2007). These actin-rich dorsal structures share several of its molecular components with invadopodia and podosomes, as well as with focal adhesions, suggesting that some of the signaling pathways controlling their assembly may also be conserved (Buccione et al., 2004). In addition, recent studies have shown that SGEF may play a role during invasion in human papillomavirus-mediated cervical cancer, prostate cancer and glioblastoma (Fortin Ensign et al., 2013; Krishna Subbaiah et al., 2012; Wang et al., 2013). The molecular mechanisms by which SGEF contributes to these processes are unclear. Our studies suggest that SGEF activity is tightly regulated during the lifetime of invadopodia and that it is recruited to invadopodia where it activates RhoG during invadopodia disassembly.

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**Fig. 4. Silencing RhoG increases matrix degradation but not invasion.** (A) CTRL and RhoG KD cells were cultured on Oregon Green 488-conjugated gelatin and stained with anti-cortactin antibody (red). Invadopodia quantification in CTRL and RhoG KD cells is expressed as the percentage of cells with invadopodia. Data are mean±s.e.m. of three independent experiments. (B) Representative images showing matrix degradation in CTRL and RhoG KD cells. Scale bars: 20 µm. (C) Area of matrix degraded per cell area. Data are mean±s.e.m. of three independent experiments. (D) Invasion assay in CTRL, RhoG KD and RhoG KD/Myc-RhoG rescue cells. Representative images showing cells that have invaded across a Matrigel-coated transwell membrane are presented. Results are representative of at least four individual experiments. (E) Invasion assay of CTRL and SGEF KD cells. Representative images are showing on the left. Results are representative of at least four individual experiments. *P<0.05; **P<0.01.
RhoG can directly influence the activity of Rac1 by forming a ternary complex with its effector protein ELMO and the Rac1 exchange factor DOCK180 (also known as DOCK1) (Brugnera et al., 2002; Katoh and Negishi, 2003). However, RhoG can also signal independently of Rac1 (Gauthier-Rouviere et al., 1998; Samson et al., 2010; Wennerberg et al., 2002). Our results demonstrate that Rac1 and RhoG play opposing roles in the regulation of invadopodia dynamics, with Rac1 being essential for invadopodia formation whereas RhoG is involved in their disassembly. Rac1 has been recently shown, together with Trio and Pak1, to function in invadopodia disassembly in rat mammary adenocarcinoma cells (Moshfegh et al., 2014). In that study, silencing the expression of Rac1 or Trio was also shown to promote an increase in invadopodia lifetime, without affecting invadopodia numbers (Moshfegh et al., 2014). Even though our

Fig. 5. Rac1 is necessary for invadopodia formation in SUM159 cells. (A) Cells lysates from CTRL and Rac1 KO SUM159 cells were analyzed by western blotting and probed for Rac1 and tubulin, as a loading control. (B) CTRL and Rac1 KO cells were treated with PDBu for 30 min and stained with anti-cortactin antibody (red), Alexa-Fluor-488–phalloidin (green) and Hoechst 33342 (blue). Arrowheads indicate representative invadopodia. Scale bars: 10 µm. (C) Quantification of cortactin- and actin-containing invadopodia in CTRL and Rac1 KO cell lines expressed as percentage of number of cells with invadopodia. Data are mean±s.e.m. of at least three independent experiments in which at least 200 cells per experiment were counted. (D) Cell lysates from CTRL, Rac1 KO and Rac1 KO cells expressing Myc–Rac1 (rescue) were immunblotted with anti-Rac1 and -Myc antibodies. Tubulin was used as a loading control (left panel). Quantification of cortactin- and actin-containing invadopodia in CTRL, Rac1 KO and rescue cells (right panel). Data are mean±s.e.m. of at least three independent experiments in which at least 200 cells per experiment were counted. (E) Cell lysates from CTRL and cells expressing Myc–Rac1 (Rac1 OE) were immunblotted with anti-Rac1 and -Myc antibodies. Tubulin was used as a loading control (left panel). Quantification of cortactin- and actin-containing invadopodia in CTRL cells and cells expressing Myc–Rac1 (right panel). Data are mean±s.e.m. of at least three independent experiments in which at least 200 cells per experiment were counted. (F) Cell lysates from CTRL, RhoG KD and RhoG KD cells expressing Myc–Rac1 (RhoG KD+Myc–Rac1) were immunblotted with anti-RhoG and -Myc antibodies. Tubulin was used as a loading control (left panel). Quantification of cortactin- and actin-containing invadopodia in CTRL, RhoG KD and RhoG KD +Myc–Rac1 cells (right panel). Data are mean±s.e.m. of at least three independent experiments in which at least 200 cells per experiment were counted. *P<0.05; **P<0.01; ***P<0.001.
results contradict those of Moshfegh and colleagues (2014), they are in agreement with several reports which show Rac1 is required for invadopodia and podosome formation in different cell lines (Furmaniak-Kazmierczak et al., 2007; Harper et al., 2010; Lin et al., 2014; Nascimento et al., 2011; Pignatelli et al., 2012; Wheeler et al., 2006). Moreover, our results show that silencing SGEF or RhoG affects both invadopodia numbers and lifetime, whereas silencing Trio has no significant effect on the number of cells that form invadopodia. Our results also differ with recent studies that analyzed the role for RhoG in invadopodia. In glioblastoma cells, depletion of RhoG inhibits invadopodia formation (Kwiatkowska et al., 2012), whereas in rat breast cancer cells it appears to have no significant effect (Moshfegh et al., 2014). The differences between these studies and the results reported here may not be necessarily contradictory. In the studies by Kwiatkowska et al., invadopodia formation was determined by measuring matrix degradation area (Kwiatkowska et al., 2012), whereas in the studies by Moshfegh et al. invadopodia were followed directly in live rat breast cancer cells following EGF stimulation (Moshfegh et al., 2014). Since matrix degradation does not necessarily correlate with invadopodia number, it is difficult to compare these studies. Alternatively, these distinct effects might reflect differences specific to the cell types used or the experimental conditions, as already reported for other GTPases (Spuu et al., 2014).

Little is known regarding the extracellular signals that regulate RhoG activity. In this study, we show that there is a rapid and transient decrease of RhoG activity in the first 5 min after PDBu and PMA treatment followed by a peak of activation at 15 min (invadopodia formation peaks at 10 min after PDBu treatment). Phorbol esters stimulate the formation of podosomes and invadopodia by activating PKCα, which lies upstream of Src (Gatesman et al., 2004; Hai et al., 2002; Tatin et al., 2006) but the mechanisms that connect them with SGEF and RhoG regulation are not known and are the focus of our future studies.

Efficient turnover of invadopodia is critical for effective cell invasion (Chan et al., 2009). It has been reported that RhoG and SGEF promote cell migration and invasion in several cell lines including glioblastoma cells, MDA-MB-231 breast cancer cells, PC-3 prostate cancer cells and HeLa cells (Chatterjee et al., 2011; Fortin Ensign et al., 2013; Hiramoto-Yamaki et al., 2010; Katoh et al., 2006; Krishna Subbajah et al., 2012; Kwiatkowska et al., 2012). Here, we also show that RhoG- and SGEF-deficient SUM159 cells show impaired invasion, despite showing enhanced invadopodia formation and ECM degradation. These results are consistent with the notion that the ability of cancer cells to form invadopodia alone is not sufficient for invasion to occur (Chan et al., 2009). This uncoupling between invadopodia formation and invasion has been described before, and may originate from the fact that several of the proteins involved in invadopodia formation also play a role during adhesion and migration. Silencing the expression of other proteins, including FAK, laminin-332 and ezrin, has also been shown to induce an increase in invadopodia while simultaneously decreasing their invasive capacity (Chan et al., 2009; Hoskin et al., 2015; Liu Fig. 6. Silencing RhoG or SGEF increases invadopodia lifetime. (A) CTRL or RhoG KD SUM159 cells were treated with PDBu for the indicated times and stained for cortactin and actin as invadopodia markers. Invadopodia were quantified and expressed as the percentage of cells with invadopodia. Data are mean ± s.e.m. of three independent experiments in which at least 200 cells were analyzed per condition. (B) Representative time series of invadopodia formation in CTRL and RhoG KD cells. SUM159 cells expressing mCherry–cortactin were imaged for 120 min at 15 s intervals following the addition of PDBu. White arrows point to an invadopodia cluster forming in the nuclear region. Scale bars: 20 µm. (C) Invadopodia lifetime increases when RhoG and SGEF expression are silenced. Re-expression of RhoG and SGEF in RhoG KD and SGEF KD cells respectively restores the lifetime to the levels of CTRL cells. Results are representative of three independent experiments in which at least five cells were analyzed per condition. The box represents the 25–75th percentiles, and the median is indicated. The whiskers show the 5th to 95th percentiles. *P<0.05; **P<0.01.
et al., 2010). It is possible that the invasion defect observed is related to impaired migration. Migration is also inhibited in RhoG KD cells, as has been shown previously by others (Hiramoto-Yamaki et al., 2010; Katoh et al., 2006). Our results and those of others suggest that RhoG may be working in parallel to regulate migration through Rac1 and invadopodia disassembly independently of Rac1 (Hiramoto et al., 2006; Katoh et al., 2006).

Despite the significant advances in the characterization of the very early stages of invadopodia assembly and the role of adhesion proteins in promoting invadopodia maturation (Beaty and Condeelis, 2010).
activity needs to be downregulated for invadopodia to assemble, whereas Rac1 activity is absolutely essential. We propose that a yet-to-be-identified RhoG-specific GAP downregulates RhoG during invadopodia assembly. During invadopodia disassembly, SGEF is targeted to invadopodia, where it activates RhoG. Active RhoG then promotes the phosphorylation of the adaptor protein paxillin, which stimulates the disassembly of invadopodia. Taken together, our observations suggest that RhoG promotes invadopodia turnover as the cell protrudes and prepares for tissue invasion. Our findings provide novel insights into the mechanisms of RhoG signaling in breast cancer invasion.

**MATERIALS AND METHODS**

**Cell lines**

Three breast cancer cell lines were used: MCF7, MDA-MB-231 and SUM159. The cell lines were a gift from Carol Otey (UNC-Chapel Hill, NC). MCF7 and MDA-MB-231 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (GIBCO, Grand Island, NY) containing 10% fetal bovine serum (FBS) and antibiotics (penicillin-streptomycin). SUM159 cells were cultured in Ham’s F12 with 5% calf serum, 5 µg/ml insulin, 1 µg/ml hydrocortisone and antibiotics. All cell lines were grown at 37°C and 5% CO2. All experiments were conducted with early passage cells that were passaged no more than 15 times. Mycoplasma was tested regularly by staining with Hoechst 33342 (83218, AnaSpec Inc., San Jose, CA).

**Reagents**

Antibodies against the following proteins were used: RhoG (sc-26484), Trio (sc-6060), cortactin (sc-11408) and Myc (9E10, sc-131) (Santa Cruz, Santa Cruz, CA); tubulin (T9028, Sigma, St. Louis, MO); pY118-paxillin (2541), total FAK (13009), pY397 FAK (8556), total Src (2108) and pY418 Src (2101) (Cell Signaling, Danvers, MA); total paxillin (610051) and Rac1 (610650; BD Biosciences, San Jose, CA). The antibody dilutions used are listed in Table S1. Secondary antibodies were: Alexa Fluor 488 and Alexa Fluor 594-conjugated anti-mouse-IgG and anti-rabbit-IgG secondary antibodies (A12379 and Alexa Fluor-647 (A22287) conjugated to phalloidin (Life Technologies, Carlsbad, CA), and horseradish peroxidase (HRP)-conjugated anti-mouse-IgG, anti-rabbit-IgG and anti-goat-IgG secondary antibodies (715-035-151, 711-035-152 and 705-035-147; Jackson Immunoresearch, West Grove, PA). PP2 (529573, Calbiochem, San Diego, CA). Phorbol-12,13-dibutyrate (PDBu) (P1269), Phorbol 12-myristate 13-acetate (PMA) (P8139) and PF-573228 (PZ0117) were from Sigma, St Louis, MO.

**Transfections, immunofluorescence and treatments**

Transfection of SUM159 cells was performed using Lipofectamine 2000 (Life Technologies, Carlsbad, CA). For immunofluorescence, MCF7, MDA-MB-231 and SUM159 cells grown on coverslips were fixed in 3% paraformaldehyde, permeabilized with 0.1% Triton X-100 in PBS and then incubated with primary antibody for 1 h at room temperature. Primary antibodies were detected with Alexa Fluor 488- and Alexa Fluor 568-conjugated anti-mouse-IgG or anti-rabbit-IgG antibodies. Images were acquired on an Olympus IX81 inverted microscope using a PlanApo N 60×1.42 NA oil objective lens and a XM10 camera (Olympus, Tokyo, Japan). Image processing and quantitative analysis was performed using ImageJ. Invadopodia formation was induced by the addition of 1 µM PDBu or 7.5 µM PMA. To inhibit Src, cells were treated with PP2 (5 µM) or DMSO diluents for 30 min before treatment with PDBu. For FAK inhibition, we incubated cells with 5 µM PF-573228 for 30 min before PDBu treatment. Live imaging was performed with a Leica SP8 confocal microscope using a PlanApo CS2 63×1.4 NA objective (Leica, Wetzlar, Germany), and equipped with an environmental chamber that controls temperature, CO2 and humidity (Tokai Hit, Fujinomiya, Japan).

**Cell lysis and immunoblotting**

Cells cultured on 100 mm tissue culture dishes were rinsed with PBS and then scraped into a lysis buffer containing 50 mM Tris-HCl pH 7.4, 10 mM...
MgCl₂, 150 mM NaCl, 1% Triton X-100 and EZBlock protease inhibitor cocktail (BioVision, Mipitas, CA). The supernatant was collected after centrifugation at 16,800 g for 10 min. For immunoblotting, lysates were boiled in 2× Laemmli buffer, and 20 µg of protein were resolved by SDS-PAGE. The proteins were transferred onto PVDF and immunoblotted with the indicated antibodies. Immunocomplexes were visualized using the Immobillon Western Chemiluminescence HRP substrate (Millipore, Billerica, MA).

RhoG activity assay
Active RhoG pulldown experiments were performed as described previously (van Buul et al., 2007). Briefly, SUM159 cells were lysed in 50 mM Tris-HCl pH 7.4, 10 mM MgCl₂, 150 mM NaCl, 1% Triton X-100, and EZBlock protease inhibitor cocktail. After clearing the lysates by centrifugation at 14,000 g for 5 min, the protein concentrations of the supernatants were determined, and equal amounts of total protein were incubated with 50 µg of glutathione transferase (GST)-ELMO (GST fusion protein containing the full-length RhoG effector ELMO) bound to glutathione–Sepharose beads (GE Healthcare, Pittsburgh, PA), and rotated for 30 min at 4°C. Subsequently the beads were washed four times in lysis buffer. Pull-downs and lysates were then immunoblotted for RhoG.

Gelatin degradation assay
Oregon Green 488-conjugated gelatin-coated coverslips were prepared as described previously (Martin et al., 2012). Coverslips were coated with 50 µg/ml poly-D-lysine for 15 min, washed with PBS and cross-linked with 0.5% glutaraldehyde for 15 min. Coverslips were then inverted on a 60 µl drop of 1 mg/ml Oregon Green 488-conjugated gelatin (Molecular Probes, ThermoFisher) for 20 min. After washing with PBS, coverslips were quenched with 5 mg/ml sodium borohydride for 5 min followed by washes with PBS. Finally, they were transferred into complete growth medium for 1 h before use. Cells were seeded and cultured on cross-linked gelatin for 16 h and then fixed for immunofluorescence studies. For each experimental condition, 25 images were taken in a random fashion.

Migration and invasion assays
Migration assays were carried out using 24-well non-coated Transwell plates (Corning, Lowell, MA) and invasion was analyzed using BD BioCoat growth-factor-reduced Matrigel Invasion Chambers (BD Biosciences, Bedford, MA). After 2 h of serum starvation, cells (1.5×10⁴) were added to the upper chamber. The bottom chamber was filled with medium containing 10% FBS. Cells were allowed to migrate or invade for 16 h. Cells to the upper chamber. The bottom chamber was filled with medium containing 10% FBS. Cells were allowed to migrate or invade for 16 h. Cells at the upper side of the membrane were removed using a Q-tip. Cells on the bottom surface were fixed and stained using Diff-Quick (JHC World, LLC, Woodstock, MD). Cells were counted from at least four independent experiments performed in triplicates.

DNA constructs
Generation of eukaryotic expression vectors pCMV-myc-RhoG-wt, wild type (WT) and mutant (E446A-N621A) Myc-tagged SGEF has been previously described (Ellerbroek et al., 2004). The RhoG Q61L was generated by site-directe mutagenesis using the Quickchange Site-Directed Mutagenesis kit (Strategene, Santa Clara, CA). mCherry–Cortactin cDNA was a gift from James Bear, UNC-Chapel Hill, NC.

Overexpression using adenoviral system
shRNA-resistant versions of human Myc–RhoG, Myc–Rac1, Myc–SGEF and mCherry–Cortactin were subcloned into pAdCMV/VS-DEST using Gateway recombination technology (Life Technologies Carlsbad, CA). shRNA resistance in RhoG and SGEF was achieved by introducing silence mutations in at least three bases within the corresponding shRNA targeting region. Virus particles were produced using the Virapower Adenoviral Expression System (Life Technologies, Carlsbad, CA).

CRISPR/Cas9-mediated KO
The Rac1 gene was knocked out using CRISPR/Cas9 double nickase plasmids (Santa Cruz Biotechnology, Santa Cruz, CA). Briefly, cells were transfected with the plasmid mixture (2 gRNA plasmids; strand A, 5'-AGACACGATCGAGAAACTGA-3'; strand B, 5'-TTATGTTCCCTAGGATGA-3') and selected with puromycin 24 h after transfection. After selection, single cell colonies were isolated by serial dilution. The efficiency of the knockout was confirmed by western blotting.

Lentiviral constructs and transduction
pLKO lentiviral non-targeting shRNA control was from Sigma (SHC016-1EA). pLKO.1 shRNAs for human RhoG (#1 TRCN000048018, #2 TRCN000048021), SGEF (#4 TRCN000048291, #5 TRCN000048292), ephexin 4 (#1 TRCN0000047503, #3 TRCN0000047507), PLEKHG6 (#1 TRCN0000128030, #4 TRCN0000148892) and Trio (#1 TRCN000000871, #5 TRCN0000010561) were from Open Biosystems (Huntsville, AL). Lentiviruses were prepared at the Lenti-shRNA Core Facility (UNC-Chapel Hill, NC). Cells were infected with lentivirus particles overnight. The following day, the infection medium was removed and replaced with complete medium containing puromycin (2.5 µg/ml) to select for shRNA-expressing cells. Total cell lysates were subjected to western blot analysis for protein expression as described above. For some shRNAs, single cell colonies were isolated by serial dilution.

qRT-PCR
Total RNA was purified from SGEF KD, PLEKHG6 KD and Ephexin4 KD SUM159 cells using Trizol (Life Technologies, CA) and was treated with DNase I (NEB, Ipswich, MA). Reverse transcription was carried out using the iScript cDNA Synthesis kit (BioRad, Hercules, CA) on 1 µg of total RNA. qRT-PCR was performed with equal amounts of cDNA using the Taq PCR Master Mix kit (Qiagen, Valencia, CA); primer sequences will be made available upon request.

Statistical analysis
Values calculated from at least three independent experiments were compared by a Student’s t-test using GraphPad Prism (La Jolla, CA), and *P<0.05 was considered statistically significant. Error bars represent the s.e.m.

Acknowledgements
We would like to thank Carol Otey (UNC-Chapel Hill, NC) for sharing the cell lines utilized in these studies and James Bear (UNC-Chapel Hill, NC) for the mCherry–cortactin cDNA.

Competing interests
The authors declare no competing or financial interests.

Author contributions

Funding
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Data availability
Supplementary movies are also available from the Dryad Digital Repository (http://dx.doi.org/10.5061/dryad.q1j50).

Supplementary information
Supplementary information available online at http://jcs.biologists.org/lookup/doi/10.1242/jcs.195552.supplemental

References


**Supplementary Figure 1. A)** Spontaneous invadopodia formation in RhoG KD cells. Representative images of CTRL or RhoG KD SUM159 cells stained with anti-cortactin antibody (red), Alexa-fluor-488-phalloidin (green) and Hoechst (blue). Arrowhead indicates representative invadopodia. Scale bars, 10 µm. **B)** RhoG knockdown induces invadopodia after PMA treatment. Representative images of CTRL or RhoG KD SUM159 cells treated with PMA for 60 min and stained with anti-cortactin antibody (red), Alexa-fluor-488-phalloidin (green) and Hoechst (blue). Arrowheads indicate representative invadopodia. Scale bars, 10 µm.
A) MDA-MB-231 cells

B) Cells with invadopodia (%)

C) MCF7 cells

D) Cells with invadopodia (%)

E) Cortactin 488-gelatin Merge

F) Degradation area/cell area

G) 

H) 

CTRL RhoG KD

CTRL RhoG KD

CTRL RhoG KD

CTRL RhoG KD

**Supplementary Figure 2.** RhoG knockdown induces invadopodia formation in MDA-MB-231 and MCF7 cells. Cell lysates from MDA-MB-231 (A) and MCF7 (C) cells stably expressing a non-targeting shRNA (CTRL) or RhoG-specific shRNA (RhoG KD) were analyzed by Western blotting and probed for RhoG and tubulin as a loading control. CTRL and RhoG KD MDA-MB-231 and MCF7 cells were plated in coverslips, treated with PDBu for 30 min and stained with anti-cortactin antibody, and Alexa-fluor-488-phalloidin. Quantification of cortactin- and actin-containing invadopodia in CTRL and RhoG KD cells in MDA-MB-231 (B) and MCF7 (D). All results are representative of three independent experiments in which at least 200 cells/experiment were counted. Data are mean ± SEM (error bars). *p<0.05, **p<0.01. E) CTRL and RhoG KD MDA-MB-231 cells were cultured on Oregon Green-488-conjugated gelatin and stained with anti-cortactin antibody (red). Scale bars, 10 µm. F) Quantification of the area of matrix degraded per cell area. Data are mean ± SEM of three independent experiments. **p<0.01. G) Transwell migration assay of CTRL and RhoG KD SUM159 cells. RhoG KD significantly reduces the number of cells migrating through a transwell filter. H) Results are representative of three individual experiments performed in triplicates. **p<0.001. Cells on the lower surface of the filter were fixed and counted 20 hours after plating (Representative images of migrating cells are included).
**A)**

Relative mRNA levels (A.U.)

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**B)**

shRNA:

- CTRL
- #1
- #3
- #4
- #5

Trio

Tubulin

**C)**

Actin

Cortactin

Merge

**D)**

 Cells with invadopodia (%)

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**E)**

myc-SGEF wt

Cortactin

Merge

3.75%

1.25%
Supplementary Figure 3. Ephexin, PLEKHG6 and Trio don’t affect invadopodia formation. Cell lysates from SUM159 cells stably expressing non-coding shRNA (CTRL), or shRNAs targeting the indicated RhoG-specific GEFs were analyzed by qRT-PCR (A) or Western blotting (B). C) Ephexin4, PLEKHG6 and Trio KD cells were treated with PDBu for 30 min and stained with anti-cortactin antibody (red), Alexa-fluor-488-phalloidin (green) and Hoechst (blue) to detect invadopodia. Arrowheads indicate representative invadopodia. Scale bars, 10 µm. D) Quantification of cortactin- and actin-containing invadopodia in CTRL and GEFs KD cell lines. Results are representative of four independent experiments in which at least 200 cells/experiment were counted. Data are means ± SEM (error bars). E) Representative images of cells showing myc-SGEF wt expressing cells that make invadopodia. Approximately 5% of the cells overexpressing SGEF form invadopodia. The majority of the 5% cells that make invadopodia do not have myc-SGEF wt localized at invadopodia (3.75% of transfected cells, top panel). The remaining 1.25% of the transfected cells showed myc-SGEF localized at invadopodia (bottom panel). Cells were stained with anti-cortactin antibody (red), anti-myc antibody (green), and Hoechst (blue). Arrowheads points to myc-SGEF positive invadopodia. Scale bars, 10 µm.
Supplementary Figure 4. 

**A)** SUM159 cells were treated with PMA for the indicated times. Active RhoG was precipitated from total lysates using GST-ELMO and immunoblotted with RhoG antibody. **B)** For quantification, active RhoG levels were normalized to total RhoG levels. Data are mean ± SEM of at least three independent experiments. Cell lysates from CTRL and RhoG KD MDA-MB-231 (C) and MCF7 (D) cells were analyzed by Western blotting and probed for Y118 phosho-paxillin (p-PXN), total paxillin (t-PXN) and tubulin as a loading control. Quantification represents the average of at least three independent experiments. *, p≤0.05. ***, p≤0.01.
**SUPPLEMENTARY MOVIE LEGENDS**

Movies were deposited at Dryad: doi:10.5061/dryad.q1j50

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**Supplementary Movie 1.** Invadopodia formation in CTRL SUM159 cells in response to PDBu. SUM159 cells expressing a CTRL shRNA were infected with mCherry-cortactin adenovirus and plated on glass-bottom coverslips 24 h after infection. Cells were imaged at 37°C in a CO₂ and humidity controlled environment. At indicated times cells were stimulated to form invadopodia with PDBu. Images were captured at 15 sec intervals for the duration of the movie.
Supplementary Movie 2. Invadopodia formation in Rac1 KO cells. Rac1 KO SUM159 cells were infected with adenovirus encoding mCherry-cortactin as in Supp Movie 1. At indicated times cells were stimulated to form invadopodia with PDBu. Images were captured at 15 sec intervals for the duration of the movie.
Supplementary Movie 3. Rac1 KO/rescue cells. Rac1 KO SUM159 cells were co-infected with adenovirus encoding mCherry-cortactin and mycRac1. At indicated times cells were stimulated to form invadopodia with PDBu. Images were captured at 15 sec intervals for the duration of the movie.
Supplementary Movie 4. Invadopodia in RhoG KD SUM159 cells. SUM159 cells stably expressing a RhoG targeting shRNA were infected with mCherry-cortactin adenovirus as in Supp Movie 1. At indicated times cells were stimulated to form invadopodia with PDBu. Images were captured at 15 sec intervals for the duration of the movie.
Supplementary Movie 5. Invadopodia formation in SGEF KD cells. SUM159 cells stably expressing SGEF targeting shRNA were infected with mCherry-cortactin adenovirus as in Supp Movie 1. At indicated times cells were stimulated to form invadopodia with PDBu. Images were captured at 15 sec intervals for the duration of the movie.
**Supplementary Movie 6.** Invadopodia formation in RhoG KD/rescue cells. RhoG KD SUM159 cells were co-infected with adenoviruses encoding mCherry-cortactin and shRNA resistant mycRhoG. At indicated times cells were stimulated to form invadopodia with PDBu. Images were captured at 15 sec intervals for the duration of the movie.
**Supplementary Movie 7.** Invadopodia formation in SGEF KD/rescue cells. SGEF KD SUM159 cells were co-infected with adenovirus encoding mCherry-cortactin and shRNA resistant mycSGEF. At indicated times cells were stimulated to form invadopodia with PDBu. Images were captured at 15 sec intervals for the duration of the movie.
Supplementary Table S1.

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