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Regulation of ROCKII Membrane Localization through its C-terminus

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Abstract

RhoA activated kinases (ROCKs) are potent effectors of RhoA signaling for regulation of the cytoskeleton. ROCKs have been shown to be localized to several different subcellular locations, suggesting that its localization is context specific and regulated. However, the signaling mechanisms that control ROCK localization have not been clearly described. In this study we measured ROCKII localization following stimulation with the chemokine CXCL12 or adhesion to collagen 1. Strikingly, each of these extracellular signals targeted ROCKII to membrane protrusions. We further determined that both RhoA and PI3-kinase signaling are required for these stimuli to induce efficient membrane localization. Furthermore, we used a mutational approach to show that two separate domains predicted to respond to these localization signals, the Rho Binding Domain (RBD) and the Pleckstrin Homology domain (PH). Unexpectedly, we found that these two domains work synergistically to lead to membrane localization. This suggests a novel mechanism for controlling ROCKII localization at the membrane, in which the ROCKII C-terminus acts as a coincidence detector for spatial regulatory signals. In other words, efficient membrane targeting requires the ROCKII RBD to receive the RhoA signal and the PH domain to receive the phospholipid signal.

Keywords

ROCK; Rho; PI3-kinase; membrane targeting; coincidence detector

Introduction

The serine/threonine kinases, ROCKI and ROCKII, are primary effectors of the small GTPase, RhoA. ROCKs are multifunctional kinases, coordinating inputs from a wide variety of cell surface receptors and translating the signals into effects on the cytoskeleton, cell migration, and cell growth [1, 2]. While it is understood that spatiotemporal regulation of Rho GTPases is an essential mechanism for linking inputs to outputs[3], the equivalent regulation of Rho GTPase effectors has not been thoroughly examined. An outstanding question in the field is how extracellular stimuli can regulate ROCK activity in space and time to yield the appropriate physiological consequence.

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One possible mechanism for determining ROCK signaling specificity is through regulation of its subcellular localization. Numerous locations for ROCKs have been described, including the cytosol, membrane compartments, and the nucleus [4–8]. This suggests that dynamic regulation of ROCK localization is an important regulatory mechanism, which has not been rigorously investigated. One study suggested that ROCKII membrane localization was mediated by its pleckstrin homology (PH) domain [8]. While this study indicates a targeting role for the ROCKII PH domain, a quantitative study to determine the overall contribution of the PH domain to ROCKII subcellular localization has not been performed. In addition, it is likely that the specific subcellular localization of ROCKs depends upon stimuli received from extracellular cues. Thus, both intrinsic and extrinsic targeting signals are likely mechanisms for determining selective targeting of ROCKs in a particular cellular context.

Here we performed a rigorous analysis of ROCKII localization in cells using a GFP fusion protein. We found that stimulation with chemokine or integrin engagement transiently localizes GFP-ROCKII to the plasma membrane in membrane protrusions. Further investigation unexpectedly revealed that two independent domains in the ROCKII C-terminus were required for efficient membrane localization. Importantly, a mutant of ROCKII, which does not localize to the membrane, inhibits migration demonstrating the physiological significance of ROCKII membrane localization. Thus, our data support a novel mechanism in which the ROCKII C-terminus acts as a coincidence detector for regulating membrane targeting, such that distinct ROCKII domains integrate two independent signals emanating from extracellular stimuli.

Materials and Methods

Cell Culture

MCF7 cells stably overexpressing the CXCR4 receptor (MCF7-CX) cells [9] were cultured as previously described. Cells were routinely transiently transfected with Lipfectamine2000 (Invitrogen, Carlsbad, CA) or Lipofectamine LTX (Invitrogen, Carlsbad, CA) according to manufacturer's instructions, and assays were performed 24 hours post transfection. Transfected cells were serum starved for 2 hours prior to plating on coverslips coated with either 10 μ g/ml polylysine (Sigma, St. Louis, MO) or 10 μ g/ml collagen 1 (Invitrogen, Carlsbad, CA), followed by blocking with 1% fatty acid free BSA, for 1 hour prior to microscopic analysis. In some experiments, cells were serum starved for 2 hours, followed by the addition of 10ng/ml CXCL12 (R&D Systems) for 1 hour.

Plasmids

GFP-ROCKII (KIAA0619), GFP ROCKII Δ PH (delta PH, amino acids 1–1149), GFP-PH (amino acids 1150–1349), GFP-RBD (amino acids 979–1047), and GFP-RBD-PH (amino acids 979–1349), GFP-ROCKII Δ RBD-PH (amino acids 1 – 978), and GFP-ROCKII RBDmut (amino acid mutation I1041A) [10] were generated by PCR from the KIAA0619 template, and subcloned into nGFP-DEST [11] using Gateway cloning (Invitrogen, Carlsbad, CA). All constructs were verified by sequencing.

qPCR

Transcript levels for ROCKI and ROCKII were determined by qPCR using validated primer sets from SA Biosystems (Carlsbad, CA). RNA was isolated from MCF7-CX cell using the SA Biosystems kit, and qPCR reactions run according to manufacturers protocol. Ct values from control GAPDH primers were subtracted from Ct values from ROCKI or ROCKII primers, performed in simultaneous runs, to calculate Δ Ct ROCKII and Δ Ct ROCKI. The

fold difference between ROCKII and ROCKI gene expression as calculated as $2^{(\Delta\text{Ct ROCKII} - \Delta\text{Ct ROCKI})}$.

Inhibitor Treatments

To inhibit RhoA, cells were treated with 2mg/ml tat-C3 (Cytoskeleton, Denver, CO) in serum free medium for 2 hours prior to plating. To inhibit PI3kinase, cells were treated with 5 μ M LY294002 (Calbiochem, San Diego, CA) for the last 15 minutes of the two hour serum starvation prior to plating.

Microscopy

Cells were fixed with 4% paraformaldehyde in PBS for 15 minutes, permeabilized with 0.2% TritonX-100 for 5 minutes, then stained with Alexafluor594-Phalloidin (Invitrogen, Carlsbad, CA) for 1 hour. Imaging was performed with an Olympus IX81 equipped with a disc spinning unit (DSU), and images were captured with a Hamamatsu EM camera. Alternatively, laser confocal images were acquired with an Olympus Fluoview1000 system. Images were processed with Slidebook software, and Adobe Photoshop using only linear adjustments.

Quantitating membrane localization

Cells selected for analysis had to meet 2 criteria, 1) moderate expression level of GFP-fusion protein; 2) be isolated or in small clusters with ample space for the cell membrane to protrude (ie not in dense clusters with tight cell-cell contacts). A minimum of 20 cells were counted per experiment, and a minimum of 3 independent experiments were performed per condition. Cells exhibiting membrane localization in either one broad or multiple small protrusions were scored as positive, and the percentage of cells with membrane localization was calculated. We noted no detectable differences in the efficiency of cell adhesion following replating on collagen 1, indicating that the transfected constructs did not overtly alter normal cell behavior. In addition, the gross cellular morphology was not significantly altered with the cells expressing moderate levels of the GFP-fusion proteins.

Migration assay

Cells were grown as a monolayer and a wound was introduced with a plastic pipet tip to introduce an area for migration. Cells were then cultured in serum free medium $\pm$ 10 μ M CXCL12 for 24 hours and placed in an environmental control chamber on top of a microscope stage for time lapse recording of migration. Migration was determined by measuring the wound area at 0 and 24 hours in 5 different sites along the wound, in three separate experiments.

Results

ROCKII localizes to membrane protrusions

ROCKs have been found in multiple cellular locations. There are two different ROCKs, ROCKI and ROCKII in mammalian cells; however, we have found that in MCF7-CX cells the gene expression was 76 fold greater ROCKII than ROCKI by qPCR, so we have focused on ROCKII (see materials and methods). We have previously used subcellular fractionation and found ROCKII to be present in the cytosol, nucleus, endosomal vesicles, and plasma membrane [7]. These findings suggest that ROCKII localization may depend upon the specific cell type and environmental conditions. Because we have previously examined the role of ROCKII in the migration of MCF7 tumor cells that stably express the CXCR4 receptor (MCF7-CX) [9], we chose to perform our study of ROCKII localization in the same cell system. We expressed GFP-ROCKII in MCF7-CX cells and used fluorescent

microscopy to analyze its subcellular localization in a migrating cell. Figure 1A shows significant distribution of GFP-ROCKII in membrane protrusions. In keeping with the multiple locations previously reported for ROCKII, we found that ROCKII localization was not restricted to the membrane; however, the fraction of cells that expressed prominent membrane localization was striking.

To further characterize the membrane localization, we performed the experiment by analyzing the localization of myc-tagged ROCKII instead of GFP, and in an alternative cell line, MDA-MB-231. Figure 1B shows that both GFP-ROCKII and myc-tagged ROCKII show similar patterns of subcellular localization. In addition, to more clearly visualize the localization, regions of active membrane activity were magnified for clarity. Figure 1C shows that ROCKII localizes to membrane ruffles in MDA-MB-231 cells, demonstrating that this phenomenon is not restricted to a single cell type.

Regulation of ROCKII localization by extracellular stimuli

The chemokine CXCL12 induces membrane protrusions and cell migration in our MCF7-CX cells, so we tested if CXCL12 stimulation affected ROCKII localization [12]. Cells were serum starved, then treated $-/+$ 10nM CXCL12 for 1 hour to induce membrane activity prior to fixation. Figure 2A shows the typical distribution pattern of GFP-ROCKII in control vs. CXCL12 stimulated cells. The efficiency of localization was quantitated (see materials and methods) and the data is presented in figure 2B, which shows that cells stimulated with CXCL12 had twice the membrane localization as compared to controls (33% to 65%).

Another important upstream signal that stimulates membrane protrusions is integrin engagement. To test if integrin engagement could regulate ROCKII membrane localization, we plated cells on either collagen 1, fibronectin, or the non-specifically charged polylysine coated surface, and analyzed the localization of GFP-ROCKII. Figure 3A shows the distribution of GFP-ROCKII following 1 hour of adhesion to collagen 1 or fibronectin, as compared to polylysine. While cells plated on collagen 1 or fibronectin showed membrane protrusions with robust GFP-ROCKII localization, the protrusions that formed on polylysine were devoid of GFP-ROCKII. The quantitation of this data is shown in figure 3B, which shows that cells adhering to collagen 1 or fibronectin had a 2–3 fold increase (22% to 62% or 48%) in ROCKII membrane localization as compared cells adhering to polylysine. These results show that extracellular stimuli can induce ROCKII targeting to membrane protrusions.

Signaling pathways that regulate ROCKII localization

We next investigated the signaling pathways downstream from CXCL12 or integrin engagement that controlled the membrane targeting of GFP-ROCKII. The C-terminus of Rho and other related Ras-family GTPases are lipid modified, which is thought to target the GTPases and their effectors to membrane compartments [13]. Both CXCL12 and integrin binding lead to activation of RhoA [12, 14], so we hypothesized that induction of ROCKII membrane localization was dependent upon RhoA signaling. To test if RhoA activation was required to induce ROCKII membrane localization, we treated the cells with C3 toxin prior to stimulation with CXCL12 or collagen 1. C3 toxin ADP-ribosylates RhoA, potentially inhibiting RhoA signaling [15]. To confirm that the C3 treatment did block Rho activity, we measured the levels of p-MYPT by western blot, as a readout (figure 4B). GFP-ROCKII was typically absent from the membrane protrusions formed in C3 treated cells, and the quantitation of the localization data (Figure 4A) shows that the membrane protrusions formed in C3-treated cells had significantly reduced numbers of cells displaying membrane targeted GFP-ROCKII (reduced by ~50% for both CXCL12 and collagen 1 stimulated cells).

Membrane targeting was not completely abolished, demonstrating that RhoA activity is required for efficient induction of ROCKII to membrane protrusions.

Another signaling pathway stimulated by CXCL12 or integrin engagement to collagen 1 is PI3-kinase [16–20]. Membrane bound phosphoinositides have been known to target proteins to the membrane [21, 22]. Thus, we predicted that PH domain mediated ROCKII membrane localization in response to upstream signals from PI-3K. To test this hypothesis, we treated cells with the PI3-kinase inhibitor LY294002 prior to stimulation with CXCL12 or collagen 1. To confirm that LY294002 was indeed blocking PI3-kinase activity, we performed a western blot for P-AKT as a readout (figure 4B). Quantitation of cells displaying prominent ROCKII membrane localization showed that LY294002 significantly blocked ROCKII membrane localization in response to both stimuli (reduced by ~50%), figure 4A. Again, inhibition of PI3-kinase signaling did not completely abolish ROCKII membrane localization, but reduced the efficiency. Because each of these inhibitors partially blocked ROCKII targeting, we tried treating the cells with both C3 and LY294002 in combination. This double inhibitor treatment resulted in nearly complete abrogation of ROCKII membrane localization (13% with CXCL12, 10% with collagen 1). Together, these experiments demonstrate that both RhoA and PI3-kinase signaling pathways are required for efficient ROCKII membrane targeting in response to extracellular cues.

Spatial regulation of ROCKII by intrinsic domains

To investigate the spatial regulation of ROCKII, our first strategy was to determine which sequences within ROCKII were responsible for membrane targeting. We tested if ROCKII kinase activity was required by treating cells with the inhibitor H1152 (0.5 μ m), but found that the kinase activity was not required for efficient membrane targeting (Figure 5). Truncation mutants were then used to determine which ROCKII domain(s) conferred membrane localization of ROCKII. We focused on two candidate domains: the Rho Binding Domain (RBD) and the Pleckstrin Homology (PH) domain. The RBD could target ROCKII to the membrane through binding of prenylated RhoA; alternatively, the ROCKII PH domain has been shown to bind preferentially to lipid vesicles enriched in 3' phosphorylated phosphoinositides [8, 23].

GFP fusion proteins of different ROCKII truncation mutants were transfected into cells, and the efficiency of localization determined. Figure 5 shows the micrographs from representative cells (panel 5A), for the different constructs (panel 5B), and the quantitation of GFP-ROCKII localization (panel 5C). The data shows that deletion of the PH domain (ROCKII- Δ PH) reduces membrane localization more than 3 fold (53% to 18%), indicating an important role in ROCKII association with the membrane. Interestingly, the PH domain on its own was not efficiently localized to the membrane (22%), figure 5. Likewise, mutation of the Rho binding domain (ROCKII^{RBDmut}) reduced membrane localization (31%), while the isolated RBD was also unable to efficiently localize to membrane protrusions (11%), figure 5. Note that the fact that the deletion mutants (ie GFP-RBD) do not efficiently localize to the membrane also demonstrates that the membrane targeting of GFP-ROCKII is dependent upon sequences within ROCKII and not a feature of the GFP protein, alone. To further verify this, we expressed GFP alone as a control and measured membrane localization in only 11% of cells, figure 5. Thus, our data indicate that both the PH domain and the RBD are required for normal ROCKII membrane localization.

While PH domains are often associated with regulating membrane targeting, it has been found that the PH domain is often not a sufficient localization signal, and that a second domain cooperates for spatial regulation of the cognate protein [24]. Because the RBD and PH domains are located next to one another in the C-terminus of ROCKII, we hypothesized that they could work in concert to regulate membrane targeting of ROCKII. Figure 5 shows

that a construct containing both the RBD and PH domain (RBD-PH) showed synergistic membrane targeting activity, as compared to the isolated RBD or PH domains alone. Interestingly, the membrane localization was more efficient in the RBD-PH construct as compared to the full length protein. This is possibly due to the fact that the full length molecule is autoinhibited and requires activation to open the protein and allow the C-terminal domains to target the protein effectively. To further test that the RBD and PH domains are both required for efficient ROCKII membrane localization we measured the localization of a GFP-ROCKII mutant where both the RBD and PH domains were deleted (ROCKII^{ΔRBD-PH}). This mutant only localized to the membrane in 8% of cells (Figure 5). This data supports a novel mechanism for spatial regulation of ROCKII, in which two domains in the C-terminus act in concert to efficiently target ROCKII to the membrane.

ROCKII C-terminus is required for migration

We have previously shown that MCF7-CX cells migrate in response to the chemokine CXCL12 [12]. To determine if the C-terminus, which is responsible for membrane targeting, was required for migration we performed a wound healing assay to measure migration. Figure 6 shows that while control cells showed nearly a 3 fold increase in migration when treated with CXCL12, cells transfected with ROCKII^{ΔRBD-PH} had no migration response to the chemokine. Thus, abrogation of ROCKII membrane localization by deletion of the RBD and PH domains was associated with a block in migration, suggesting that membrane localization is required for motility in response to CXCL12.

Discussion

Our study was designed to investigate the spatial regulation of the RhoA activated kinase, ROCKII. In this study we examined the localization of ROCKII in response to stimulation with the chemokine CXCL12 or adhesion to collagen 1 and fibronectin, and found strong, transient localization in membrane protrusions (Figures 1–3). Thus, we hypothesized that the localization of ROCKII to membrane protrusions was regulated. Interestingly, signaling from both RhoA and PI3-kinase were required for its membrane localization in response to CXCL12 or collagen 1 (figure 4). This further suggested that multiple domains within ROCKII might be used to integrate these two signals for efficient membrane targeting. Mutational analysis showed that both the pleckstrin homology (PH) domain and the RhoA binding domain (RBD) work synergistically for ROCKII membrane localization (Figure 5). Taken together, we find a novel mechanism for regulation of ROCKII membrane localization through the combinatorial action of the RBD and PH domains in the C-terminus.

ROCKs are well established regulators of cell migration; however their function has traditionally thought to be restricted to the back end of migrating cells. In recent years, several investigators have made use of sophisticated FRET-probes to show that RhoA is active in both the front and back ends of the cell [25, 26]. However it is not clear how downstream effectors link to RhoA's function at the leading edge. One study found that the actin regulatory protein, mDia, is the critical RhoA target at the leading edge [27]. Our findings demonstrate that ROCKII localizes to membrane protrusions, especially when stimulated with CXCL12 or collagen 1 and fibronectin; thus, RhoA likely signals to more than one downstream effector at the membrane, which may depend on the extracellular stimuli.

ROCKs are known to be multifunctional kinases, regulating cellular functions ranging from cytokinesis to phagocytosis [2]. They also respond to a wide range of extracellular signals, including soluble cytokines and the extracellular matrix. This implies regulation of ROCK signaling by mechanisms that respond to the specific cellular context, in order to achieve the

appropriate outcome. A previous study showed that in 3-dimensional amoeboid migration of tumor cells, PDK1 and RhoE regulate the membrane localization of ROCKI [28]; however, the mechanisms governing the spatial regulation of ROCKII in response to specific upstream stimuli have not been thoroughly investigated. Here we show that ROCKII localizes to membrane protrusions, and that this is promoted by chemokine or integrin signaling (figure 2 & 3). In addition, we find that two independent signals are required for efficient ROCKII membrane localization: RhoA and PI3-kinase (figure 4). Our data suggests that these signals are received by two distinct domains in the C-terminus of ROCKII, the RBD and PH domains, respectively. Thus, our study suggests an integrated mechanism for spatial regulation of ROCKII in which intrinsic domains within ROCKII are coordinated with upstream signaling pathways.

Regulation of protein localization by multiple domains is a mechanism employed by other molecules. A previous study showed that the ROCKII PH domain localized to the plasma membrane, consistent with our findings [8]. However, we have performed a quantitative analysis, which reveals that the PH domain alone does not efficiently target to the membrane. While PH domains are strongly associated with regulating the localization of its cognate protein, they often are not capable of strong membrane localization activity in isolation, and collaborate with another domain for spatial regulation [24]. In fact, there are other instances of small GTPases working in conjunction with PH domains to regulate the localization of a protein [29–31]. The purpose of signal integration in spatial regulation of proteins is unknown, but it has been proposed that integration of two independent localization signals enables the possibility of fine-tuning, perhaps using GTP hydrolysis to control the duration of time that a protein is associated with the membrane [24]. Our study demonstrates that the combination of the RBD with the PH domain results in synergistic membrane targeting (Figure 5), and suggests that the ROCKII C-terminus acts as a coincidence detector for RhoA and PI3-kinase signals.

Highlights

- ROCKII localizes to membrane protrusions.
- Localization is stimulated by extracellular stimuli like chemokine stimulation or integrin engagement.
- Localization is dependent on two distinct intracellular signals; RhoA and PI-3 kinase.
- ROCKII localization is driven by integration of extracellular stimuli by both the RBD and PH domains at the C terminus.

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Figure 1. ROCKII localizes to membrane protrusions

A. Cells transfected with GFP-ROCKII then plated onto collagen 1 coated coverslips, to determine the localization pattern of ROCKII. Cells were fixed and stained for actin, then analyzed with fluorescence microscopy. The first panel shows a phase contrast image of a migrating cell with a broad protrusive front. We observed distinct localization of ROCKII in membrane protrusions, which colocalized with f-actin. **B.** Cells transfected with either GFP-ROCKII or myc-ROCKII showing membrane targeting, and magnification of prominent area of membrane activity. **C.** GFP-ROCKII localization in MDA-MB-231 cells. Scale bar = 10 microns.

Figure 2. CXCL12 induces membrane localization of GFP-ROCKII

Cells were serum starved for 2 hours, followed by treatment with CXCL12 for 1 hour. Cells were then fixed and processed for actin staining and immunofluorescence analysis. The percentage of cells showing clear membrane localization of GFP-ROCKII (see materials and methods) is plotted as the average and standard error from at least 20 individual cells from 5 independent experiments (total of 107 cells). These data demonstrate that CXCL12 promotes membrane targeting of GFP-ROCKII.

Figure 3. Adhesion to Collagen 1 induces ROCKII membrane localization

To determine if integrin engagement regulated ROCKII localization, cells were transfected with GFP-ROCKII then plated onto either polylysine (PLL) or collagen 1 coated coverslips, and ROCKII localization was determined by fluorescence microscopy. **A.** Cells were fixed and stained for actin, then analyzed with fluorescence microscopy. Cells adhering to polylysine formed small membrane protrusions which did not contain ROCKII, whereas adhesion to collagen 1 or fibronectin promoted larger membrane protrusions which often contained ROCKII. **B.** The percentage of cells showing distinct membrane localization of ROCKII was quantitated as in figure 2. The bars represent the average and standard error from 60–80 cells from 3 independent experiments. We find that adhesion to Collagen 1 or fibronectin stimulates ROCKII membrane localization by 2 – 3 fold.

Figure 4. RhoA and PI3 Kinase activity are required for efficient membrane localization

To determine if RhoA activity was required for localization, cells were incubated with C3 for 2 hours (see experimental procedures) prior to adhesion on collagen. To determine if PI3 kinase signaling was required for localization, cells were incubated with LY249002 for 15 minutes prior to adhesion to collagen. Cells were fixed and stained for actin, then analyzed with fluorescence microscopy, and analyzed as in figure 2. **A.** Inhibition of RhoA or PI3-kinase, each partially inhibited the membrane localization of GFP-ROCKII when cells were stimulated with CXCL12. Treatment with both inhibitors together resulted in more significant reduction in membrane targeting. The bars represent the average and standard error from 60–80 cells from 3 independent experiments; * = $p < 0.05$, ** = $p < 0.01$. ROCKII membrane targeting was partially reduced by inhibition of either RhoA or PI3-kinase, suggesting that CXCL12 stimulation and collagen 1 engagement stimulate ROCKII membrane localization through both of these signaling pathways. **B.** Western blots for P-AKT or P-MYPT as indicators of the effectiveness of LY249002 or C3 treatments, as a control.

Figure 5. Both the PH and RBD domains are required for efficient membrane localization

To map the domain(s) in ROCKII that could mediate membrane targeting, cells were transfected with GFP-ROCKII truncations (see schematic diagram, panel B) and localization was measured as in figure 2. Two putative targeting domains in the C-terminus were tested, the Pleckstrin Homology (PH) domain and the Rho Binding Domain (RBD). **A.** Visualization of GFP-ROCKII constructs (left) and the overlay with actin staining (right) by fluorescence microscopy. **B.** Schematic diagram of ROCKII constructs. **C.** To quantitate ROCKII membrane localization we counted the percentage of cells that showed distinct membrane localization of ROCKII. $n=60-80$ cells per construct in a minimum of 3 independent experiments. Bars represent the average $\pm$ standard error; * = p values < 0.05 ,

**= $p < 0.01$ for each mutant as compared to the full length ROCKII. Together these data show that both the RBD and PH domains contribute to ROCKII localization, but neither one is sufficient for efficient localization. Furthermore, the combination of the two resulted in synergized localization activity.

Figure 6. Cell migration is blocked by localization deficient ROCKII mutant

To assess if membrane targeting of ROCKII was necessary for cell migration, we performed a scratch wound assay to measure migration $\pm$ CXCL12. **A.** Cell migration was monitored by live cell imaging, and representative sections of the wound were imaged at 0 and 24 hours. **B.** Migration was quantitated by measuring the area of the wound in multiple sites from 3 independent experiments. Data shown represents the average $\pm$ std error, and p values of the pairs with significant differences are shown. CXCL12 increases migration of MCF7-CX cells, which is blocked by the expression of ROCKII Δ RBD-PH.