Title
Identification of new signaling components in neutrophil chemotaxis

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Identification of new signaling components in neutrophil chemotaxis

by

Julie Wu

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Biochemistry and Molecular Biology

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO
Dedicated to my family
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My mom and dad, for their support and guidance throughout my life. Special thanks to my mom for instilling a desire to know how human health works. And to the rest of my family, especially my uncle and aunts and cousins living the Bay Area, for providing me a home away from home and helping me keep life in perspective.

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And to Leo, for helping me become a real person.
STATEMENT REGARDING AUTHOR CONTRIBUTIONS

Chapter 2 is my work except for the schematics in Fig. 2A and Fig. 4AB (borrowed from previously published papers) and measurement of phyB expression in HL-60 cell lines in figure 3, which was performed by Delquin Gong. The rapamycin system constructs were a kind gift from the Meyer lab. The phyB-mCherry and Pif-YFP- Rac(Q61L) constructs were a kind gift from Delquin Gong.

Chapter 3 was performed by me in collaboration with Michael Bassik and Martin Kampmann in the Weissman lab, who created the shRNA libraries used for the screen. The transwell chemotaxis assay protocol is a scaled up version of a protocol taught to me by Jason Park. The transwell assays were performed on the FlexStation machine in the Pathway to Medicine section of Wendell Lim’s lab.

Chapter 4 is a draft in preparation for submission. The mass spectrometry screen protocol was from Arjan Kortholt, and the mass spectrometry was performed by F. Fusetti and A. Keizer-Gunnink and bioinformatics analysis by W. Alkema. The pig’s blood preparation was done with contributions from members of the Weiner lab- Orion Weiner, Anna Goldberg, Miriam Genuth, Justin McLaurin, Alba Diz Munoz, and Sheel Dandekar. The GST protein purification protocol was taught to me by Daniel Süveges in the Jura lab. The PHAkt-Citrine, Hem1-YFP, and Pak-PBD-mCherry constructs were supplied by Delquin Gong. The initial analysis for the phalloidin stain was done with Liz Zhang and Shanshan Liu in the Atschuler and Wu lab.
ABSTRACT

Cell migration underlies many processes from development to cancer metastasis. Control of cell migration is highly orchestrated, involving many layers of regulation of key signaling nodes. However, despite knowing the identity of many of the core signaling components, the proteins that are responsible for their correct temporal activation and spatial organization are unknown. Here, we study the chemotactic signaling of neutrophils, a model system for eukaryotic chemotaxis.

In my first project, I studied the effects of exogenously activated Rac on neutrophil signaling. Rac GTPase is a core chemotaxis effector that is thought to drive a positive feedback loop that underlies cell polarization. However, this positive feedback had been inferred from epistasis experiments and had not been directly shown. We attempted to demonstrate Rac positive feedback by introducing exogenously activated Rac and, using readouts of endogenous Rac activity, determine whether exogenously activated Rac could activate endogenous Rac. Surprisingly, we found very little effect of exogenously activated Rac on neutrophil signaling, possibly indicating that Rac adapts to negate the effect of exogenous Rac.

In my second project, I optimized a large scale screening strategy for isolating chemotaxis-defective mutants from a pooled library of cells. Using my protocol, I was able to successfully enrich a known chemotaxis mutant from the background of wild type cells. This approach was combined with shRNA libraries provided by the Weissman lab to search for novel effectors of neutrophil
chemotaxis. It could be combined with other large scale, pooled genetic screens in the future for the same purpose.

In my third project, I identified Homer3, a novel Gai2 interacting protein that is also necessary for efficient neutrophil chemotaxis. Gai2 is specifically required for neutrophil chemotaxis, but whether it has its own effectors for chemotaxis or is just a binding partner for Gbetagamma is an open question. Using a biochemical mass spectrometry pulldown screen, we identified putative binding partners for Gai2 and identified one, Homer3, as having a role in neutrophil chemotaxis. We establish that Homer3 does not affect the initial burst of signaling in response to stimulation but rather affects the spatial organization of those signaling molecules at later timepoints.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Title page</td>
<td>i</td>
</tr>
<tr>
<td>Copyright page</td>
<td>ii</td>
</tr>
<tr>
<td>Dedication and Acknowledgments</td>
<td>iii</td>
</tr>
<tr>
<td>Statement regarding author contributions</td>
<td>vi</td>
</tr>
<tr>
<td>Abstract</td>
<td>vii</td>
</tr>
<tr>
<td>Table of contents</td>
<td>ix</td>
</tr>
<tr>
<td>List of Figures</td>
<td>x</td>
</tr>
<tr>
<td><strong>Chapter 1: Introduction</strong></td>
<td>1</td>
</tr>
<tr>
<td><strong>Chapter 2: Effects of constitutively active Rac on chemotactic signaling</strong></td>
<td>7</td>
</tr>
<tr>
<td><strong>Chapter 3: A genetic screen for chemotactic effectors</strong></td>
<td>34</td>
</tr>
<tr>
<td><strong>Chapter 4: Homer3 regulates the establishment of neutrophil polarity</strong></td>
<td>55</td>
</tr>
<tr>
<td><strong>Chapter 5: Summary</strong></td>
<td>101</td>
</tr>
<tr>
<td>Publishing agreement</td>
<td>104</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES

### Chapter 1
None

### Chapter 2

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Synthetic activation of exogenous Rac and readout of endogenous Rac activation.</td>
<td>27</td>
</tr>
<tr>
<td>2</td>
<td>Controlling protein localization and cell signaling with light.</td>
<td>28</td>
</tr>
<tr>
<td>3</td>
<td>HL-60s lose phyB expression over time.</td>
<td>29</td>
</tr>
<tr>
<td>4</td>
<td>Controlling protein localization and cell spreading with rapamycin.</td>
<td>30</td>
</tr>
<tr>
<td>5</td>
<td>Rac(Q61L) expressing cells show decreased tail retraction in comparison to wildtype.</td>
<td>31</td>
</tr>
<tr>
<td>6</td>
<td>Rac(Q61L) expressing cells show slightly increased basal Pak phosphorylation and unaltered filamentous actin content in comparison to wildtype.</td>
<td>32</td>
</tr>
<tr>
<td>7</td>
<td>Rac(Q61L) expressing cells show unaltered Rac activation kinetics</td>
<td>33</td>
</tr>
</tbody>
</table>

### Chapter 3

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Genome-wide shRNA screen for genes involved in neutrophil chemotaxis.</td>
<td>52</td>
</tr>
<tr>
<td>2</td>
<td>Results of the pilot shRNA neutrophil migration screen.</td>
<td>53</td>
</tr>
<tr>
<td>3</td>
<td>HUVEC cells dramatically decrease basal neutrophil migration in transwell assay.</td>
<td>54</td>
</tr>
</tbody>
</table>

### Chapter 4

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Homer3 can bind Gai2 in neutrophil lysates.</td>
<td>92</td>
</tr>
</tbody>
</table>
Figure 2: Homer3 knockdown impairs HL-60 chemotaxis.

Figure 3: Homer3 plays a role in initiation of HL-60 migration.

Figure 4: Homer3 depletion does not affect levels of Pak phosphorylation and Akt phosphorylation.

Figure 5: Spatial persistence of Rac and PI3K activity is affected in immotile Homer3 knockdown cells.

Figure 6: Homer3 depletion reduces persistence of actin polymerization.

Supplementary Figure 1: Screen schematic

Supplementary Figure 2: Calcium signaling is intact in Homer3 knockdown cells.
CHAPTER ONE

Introduction
BACKGROUND AND SIGNIFICANCE

Ischemia-reperfusion injury and neutrophil chemotaxis

Ischemia-reperfusion injury occurs in myocardium following loss and restoration of blood flow, such as in a myocardial infarction. Inflammatory signals generated by injured endothelial cells and cardiomyocytes recruit and activate neutrophils, which then release proteases and oxygen radicals that do further damage. To prevent neutrophil-induced inflammatory damage, one strategy is to prevent neutrophils from arriving at healthy tissues. To do so, we must understand the mechanisms by which neutrophils migrate to sites of injury. Understanding neutrophil chemotaxis also has implications for other conditions involving leukocyte migration, such as atherosclerosis and chronic inflammation.

Neutrophil polarity and positive feedback

To migrate, the neutrophil must restrict cytoskeletal signaling to two distinct regions, actin assembly at the leading edge and myosin-based contraction at the trailing edge. When presented with a gradient of chemoattractant, cells orient their axis of polarity up the external gradient. When in uniform chemoattractant, cells also polarize, but in random directions. Neutrophils and other cells are thought to polarize by combining short-range positive feedback and long-range inhibition (Fig. 1A). The short-range positive feedback is thought to be generated by Rac, a Rho family GTPase. Rho family GTPase positive feedback loops are thought to organize polarity in systems from neutrophils to Dictyostelium to yeast. However, these feedback loops have been
inferred based on indirect data (primarily components appearing to act upstream and downstream of one another) and thus are not conclusive for positive feedback.\textsuperscript{5,8} For example, if A is required for the activation of B and B is required for the activation of A, they could either be involved in a positive feedback loops or could simply be co-permissive (for example ATP is required for protein kinases, but the levels of ATP don’t set the level of phosphosubstrates).

To directly demonstrate Rac positive feedback, we need to show that an activated Rac GTPase can activate more Rac. My lab previously demonstrated PIP\textsubscript{3} positive feedback by demonstrating that exogenous PIP\textsubscript{3} could induce cells to generate more PIP\textsubscript{3} through endogenous enzymes.\textsuperscript{5} I wanted to do a similar experiment to investigate Rac positive feedback using an inducible Rac activation system. Elucidating the mechanism of positive feedback, which underlies all models of neutrophil polarity,\textsuperscript{4} will lead to a more fundamental understanding of neutrophil motility and could lead to new targets for anti-inflammatory drugs.

**Screening for new chemotaxis effectors**

Our lab focuses on understanding how neutrophils (professional migratory cells of the innate immune system) regulate cell polarity and motility in response to external gradients of chemoattractant. We are combining optogenetic activation of individual nodes of the signal transduction cascade with live cell activity readouts to interrogate the logic of information flow through the signaling network. This approach can only be applied to known components of the neutrophil signaling cascade, but there are significant gaps in our understanding of the full suite of components involved in chemotactic signaling. There have not been any systematic biochemical or forward-
genetic-based screens for proteins involved in signal relay in neutrophil chemotaxis. In this project, we propose to use biochemical pulldowns and genome-wide shRNA screens to identify new components of the neutrophil chemotaxis cascade. The components that are identified in these screens can be used to generate additional optogenetic inputs, activity readouts, and loss-of-function perturbations to better understand how the signaling cascade generates adaptation, signal amplification, and other key behaviors necessary for neutrophil chemotaxis.

**Forward genetic screen for genes involved in neutrophil chemotaxis.**

We lack a complete parts list of the proteins that regulate neutrophil migration. No genome-wide forward genetic screens have been conducted for neutrophil chemotaxis. shRNA-based screens represent a high-throughput platform for this approach, but previous screens have been plagued by false positives (not all shRNAs work to knock down the gene of interest) and false negatives (many shRNAs have off-target phenotypes; at a genome wide scale, for many screens it has been estimated that the bulk of the hits represent off-target effects). To overcome these difficulties, we are collaborating with Jonathan Weissman’s lab, who have developed an innovative shRNA screening approach that uses the statistical power of ultracomplex libraries (25 shRNAs per gene) to dramatically decrease the false positive and false negative rate. Because so many shRNAs are targeted per gene, there is a very low probability that the majority of the shRNAs fail to knock down the gene of interest. Furthermore, the pooled infection and deep sequencing used in the screen demands that a significant proportion of the shRNAs for any given gene yield a phenotype, dramatically decreasing the
likelihood of false positives. By adapting this screen to a transwell migration assay in which we can purify the chemotactic population of neutrophils away from the non-chemotactic population, we are conducting a genome-wide screen for the proteins involved in neutrophil chemotaxis.

Biochemical interaction screen for neutrophil chemotaxis signaling network.

We know a number of the key nodes involved in neutrophil chemotaxis, but we don’t fully understand how these nodes are connected together in the full signaling network. For instance, we know that $\alpha_i$-based heterotrimeric G-protein signaling is required for neutrophil chemotaxis, but don’t know how $\alpha_i$ and its associated $\beta\gamma$ communicate with downstream effectors in the signaling cascade. Similarly, we know that the Rac GTPase is essential for actin polymerization and cell migration in neutrophils and that higher-order positive and negative feedback loops are used to sculpt the spatiotemporal dynamics of Rac activation, but we do not know which Rac activators and inhibitors are involved in these feedback loops. One straightforward means to identify the molecules that connect these signaling nodes is to perform biochemical-based pulldowns with these signaling proteins. We used primary neutrophil lysates (which can be isolated at large scale from pig blood and prepared into high-quality lysate by nitrogen cavitation) and bait presented in column format to identify even weakly interacting proteins; these weak binders can be physiologically relevant— for instance Rac acts through the WAVE complex to stimulate actin assembly, but Rac only binds the WAVE complex with a 7 micromolar affinity. By fishing with different nucleotide states of GTPases (Gai, Rac, Arf), we can identify the upstream and downstream modulators of these GTPases. By fishing with Gbg, we can identify the effectors of this key neutrophil signaling module.
For this project, we are collaborating with Arjan Kortholt who has successfully used a similar approach to identify novel proteins involved in *Dictyostelium* chemotaxis. For further investigation, we will prioritize proteins that are identified in both our genetic screen and biochemical screen.
CHAPTER TWO

Effects of constitutively active Rac GTPase on chemotactic signaling
SUMMARY

Directed cell migration is involved in many physiological and pathological processes including angiogenesis and cancer metastasis. To achieve the proper spatial regulation of actin assembly during directed migration, cells polarize intracellular signals such as the Rac GTPase. Rac activation is both necessary\(^1\) and sufficient\(^2\) to direct cell migration, but how Rac achieves its proper localization and dynamics is not known. Our lab studies the polarization of neutrophils, immune cells that contribute to inflammation, atherosclerosis and ischemia/reperfusion injury. My long-term goal was to understand how neutrophils generate and maintain polarity. All models of cell polarity for systems from budding yeast to Dictyostelium to neurons to leukocytes rely on GTPase positive feedback loops. For neutrophils, this positive feedback loop is thought to involve Rac induced Rac activation, but the mechanism for this feedback loop is unknown. Here, I generated cells with both inducible and constitutive exogenous Rac activation and measured their effect on Rac activation.
RESULTS

Specific Aim 1: Measuring Rac-induced Rac activation in neutrophils.

Rationale: Previous experiments have provided indirect evidence for Rac positive feedback. For example, components (like the WAVE complex) that act downstream of Rac for actin assembly are themselves required for Rac activation. Furthermore, the WAVE complex is recruited to active Rac and scaffolds several Rac GEFs that could participate in Rac autocatalysis. To directly demonstrate Rac positive feedback, I wanted to test the ability of exogenously activated Rac (through light-mediated plasma membrane recruitment of a constitutively active Rac allele) to trans-activate endogenous Rac in neutrophils (Fig. 1B). Failure to see evidence of Rac positive feedback would have strongly limited the potential role of Rac feedback and trigger important reconsideration of a widely held mechanism.

Experiment – verifying Rac input: Our lab has developed a light-controlled protein localization system that can be used for spatiotemporal control of protein activation in live mammalian cells. We have previously used this system to activate Rac globally in fibroblasts, where it is capable of inducing lamellipodia formation (See Methods & Fig. 2C). I tried this experiment in neutrophils because the evidence of Rac positive feedback is the most well-established in these cells. We successfully ported this light system into HL-60 cells (See Methods & Fig. 2D) and verified that the light-gated Rac input can couple to Rac effector recruitment. We first generated a HL-60 line stably expressing phyB-RasCAAX-mCherry and Pif-YFP-Rac1(Q61L). We then differentiated the cells into neutrophils and exposed one population to uniform 750nm (off) light, and
another to 650nm (on) light. We used phosphorylation of p21-activated kinase as a readout of Rac activation. Using western blot and phospho-specific antibodies against phospho-Pak, we saw an increase in phosphorylated Pak in the 650nm light-exposed population versus the 750nm light-exposed population.

However, the light system was unstable in HL-60 cell lines. Upon differentiation, 50 to 85% of the phyB expression was lost (Fig. 3A). In addition, phyB expression would decline over time (Fig. 3B). An alternative method for inducibly recruiting an activated Rac allele to the plasma relies on rapamycin, a chemical inducer of dimerization. In this method, rapamycin is used to translocate a tagged constitutively active Rac to a membrane-bound construct with the partner domain. This method has been shown to be effective in fibroblasts. In transient transfections, I could achieve drug-induced control of Rac activity and cell spreading in neutrophils (Fig. 4). Because rapamycin inhibits mTOR, which could effect chemotactic signaling, another option was to use analogs of the drug rapamycin (such as AP21967 and C20 methylallyl-rapamycin) that rapidly induce the dimerization of fusion proteins containing mutant FKBP and FRB domains, without influencing endogenous mTor signaling. At the doses used for my experiments (100nM), though, I have not seen any measurable effects of even unmodified rapamycin on neutrophil migration. However, this approach also proved unusable. Though one could use the rapamycin system to achieve Rac activation in a subset of successfully transfected cells, this ability to induce Rac activation was lost in lines stably expressing the rapamycin system components.

I moved toward a transcriptional control approach due to difficulty in activating Rac using light or rapamycin dimerization systems. I generated neutrophil cell lines that
will express a constitutively active Rac mutant (Q61L) upon induction from a doxycycline-inducible promoter. This constitutively active mutant was also engineered to have mutations so that it would also not bind GAPs (E91H/N92H) and not bind RhoGDI (R66E). By microscopy the constitutively active Rac-expressing cells were slightly more adherent than wildtype (Fig. 5). Their tails were elongated, possibly due to antagonism of RhoA- myosin-mediated retraction of the rear. Some cells also showed a flatter morphology, consistent with increased Rac activity. They retained their ability to respond to agonist by ruffling and further flattening, similar to wildtype. This is in contrast with what is seen with neutrophils transiently transfected with RacQ61L, which uniformly spread out and ruffle and lose their polarized morphology.

Neutrophils with increased Rac activity should also have elevated Pak signaling and elevated actin polymerization. We sought to quantify how much Rac activity was elevated in the RacQ61L-expressing neutrophils using the phospho-Pak assay to assess Pak signaling and phallodoin staining to assess actin polymerization. We measured the Pak signaling before fMLP addition and at the peak after stimulation. At both timepoints there was slightly elevated Pak and actin, but the increase was small compared to the increase seen with chemoattractant addition (Fig. 6).

I then used a Rac pulldown assay to quantify the level of exogenous constitutive Rac being expressed in the neutrophils. Since the exogenous Rac is CFP-tagged, it is significantly different in size than endogenous Rac and can be differentiated on a western blot. With the pulldown I could see that the levels of constitutively active exogenous Rac were in excess of the peak levels of activated Rac in response to
Therefore, low expression of exogenous Rac is not the reason for relatively low Rac activity. Using the pulldown assay I can detect a small but detectable contribution of exogenous activated Rac to endogenous Rac activation, demonstrating the presence of Rac positive feedback (Fig. 7). However, this small contribution cannot account for the large burst of Rac activity that follows stimulation, which was thought to be the result of positive feedback. Possible explanations for the seemingly small effect that I observed are adaptation prior to assay, possible limiting cofactors, or a genuinely small contribution of Rac positive feedback to the peak response of activated Rac in response to stimulation.

**Specific Aim 2: Investigate the role of Dock2, P-Rex1, and Arhgap15 in Rac adaptation.**

**Rationale:** In neutrophils PIP3 is known to engage a Rac-dependent positive feedback loop. Exogenous delivery of PIP3 stimulates endogenous production of more PIP3, demonstrating positive feedback. Inhibition of Rac abolishes feedback. We have recently discovered that Rac is activated and adapts directly downstream of PI3K activation. Using a light-controlled PI3K, we can activate PI3K independent of chemoattractant stimulation and therefore isolate the effects of PI3K from the parallel pathways activated by fMLP. In addition, with the light system we can keep PI3K activity at high levels throughout a timecourse. Under the normal timecourse following stimulation, PI3K is activated but then adapts to lower levels at later timepoints. Therefore we can look specifically at the effects of a single level of PI3K activity over
time. Using persistently elevated PI3K signaling as an input, we can see Rac is activated and then adapts back to baseline, similar to what is seen with chemoattractant stimulation. This implies that Rac adaptation lies downstream of PI3K.

To find a molecular mechanism of what is mediating Rac adaptation downstream of PI3K, we looked through the literature for proteins that are known to interact directly with both Rac and PIP3. Three such candidates were Dock2, P-Rex1, and Arhgap15. Deletion of these genes are known to affect chemotaxis. Dock2 and P-Rex1 are GEFs for Rac activation and Arhgap15 reported to be a Rac GAP. All also directly bind to PIP3. Dock2 and P-Rex1 localization is mediated by PIP3, and inhibition of PI3K leads to their mislocalization.

Experiment – knock down candidate GEFs: We used an RNAi approach to knockdown P-Rex1, Dock2, and Arhgap15 expression in neutrophils. We then confirmed the level of knockdown using RT-PCR. Lines with 80% or greater knockdown efficiency were selected for further analysis. Since all these genes have been previously shown to play roles in neutrophil chemotaxis, we next tested the knockdown lines for migration efficiency in a transwell migration assay. The transwell migration assay has the advantage of being fast, parallelizable, and quantitative. In addition, as a final functional output of Rac activation, it is a test of whether we were able to achieve physiologically relevant levels of gene knockdown. Dock2 knockdown neutrophils migrated normally relative to the control neutrophils carrying a nonsense shRNA. This could be because the levels of knockdown were not high enough to see an effect on migration. Previous
results showing a role for Dock2 in chemotaxis have relied on mouse knockout neutrophils, so Dock2 may need to be completely eliminated in order to see an effect.

Both P-Rex1 and Arhgap15 knockdown lines showed a chemotaxis defect by the transwell migration assay. For the P-Rex1 line, this is consistent with previous data. However, for Arhgap15, previous groups have seen a potentiation of migration, not inhibition. This contradictory result could be a result of off-target effects, since we only used one shRNA against Arhgap15. An alternative, possibly more exciting explanation is that Arhgap15 can have different effects depending on the level expressed. The study that found potentiation of migration used an Arhgap15 knockout mouse neutrophil. Therefore, the residual Arhgap15 in the shRNA knockdown line could have a different effect on migration than getting rid of it altogether.

METHODS

Rac activation. The light system relies on the heterodimerization of chromophore (PCB)-associated phytochrome (PhyB) to Phytochrome Interacting Factors (PIFs) in response to red (650 nm) light. The proteins dissociate in infrared (750 nm) light (Fig. 3A). For control of protein localization in mammalian cells, PhyB is tethered to the membrane with the prenylation sequence of K-Ras. The protein of interest is fused to PIF and can be directed to the membrane with 650nm light (Fig. 3D). Rac requires membrane association and GTP-loading to activate downstream effectors. Thus, a PIF-tagged Rac mutant (RacQ61L/E91H/N92H ΔCAAX, which is constitutively GTP-bound, cannot interact with GTPase activating proteins and lacking the prenyl tail normally used for
membrane association) basally localizes to the cytosol where it incapable of activating downstream effectors but can be recruited to and released from the membrane in a in a light-dependent manner to drive actin assembly and other signaling events (Fig. 3B,C). Sustained Rac activation can successfully stimulate actin assembly in fibroblasts (Fig. 3C).

**Phospho-Pak assay.** Cells were resuspended to a concentration of 2 million ml⁻¹ in RPMI+0.2%FBS. We stimulated cells with 10 nM fMLP and quenched the reaction by adding aliquots of the cell mixture to ice-cold 20% trichloroacetic acid plus 40 mM sodium fluoride plus 20 mM beta-glycerol phosphate (50020, Fluka) at the indicated time points. Pellets were washed with 0.5% trichloroacetic acid and resuspended in Laemmli protein sample buffer (161–0737, BioRad) containing 5% β-mercapto-ethanol. Protein bands were separated by SDS-PAGE gel electrophoresis, transferred to nitrocellulose, blocked with Odyssey block, and incubated at 4°C overnight with 1:1000 dilutions of anti-phosphoPAK (Cell Signaling 2605S) and anti-Pak2(Cell Signaling, 4825S)). The blot was developed with the fluorescent secondary antibodies and protein bands were imaged using ODYSSEY INFRARED IMAGING SYSTEM (Li-COR, Biosciences).

**Filamentous Actin staining.** Cells were resuspended in mHBSS with 0.2% BSA. Cells were stimulated with addition of 10nM fMLP, fixed with 3.7% paraformaldehyde in intracellular buffer (140mM KCl, 1mM MgCl2, 2mM EGTA, 320mM sucrose, 20mM Hepes pH7.5), and incubated on ice for 20 minutes. After centrifugation at 400g for 2 minutes, the cell pellet was resuspended in intracellular buffer plus 0.2% triton and
1:500 rhodamine phallodin and stained for 20 minutes. The cells were centrifuged and resuspended in mHBSS and analyzed on a FACS Aria.

**Cell culture.** HL-60 cells were cultured as described. Briefly, cells were grown at 37°C, 5% CO2, in RPMI 1640 media with L-glutamine and 25 mM HEPES (10–041-CM, Mediatech), with 10% heat-inactivated fetal bovine serum (FBS). Cell differentiation was initiated by adding 1.5% DMSO (endotoxin-free, hybridoma-tested; D2650, Sigma) to cells in media. Cells for all experiments were used at 2–3 days after differentiation.

**RNAi knockdown.** Knockdown of Dock2, P-Rex1, and ARHGAP15 in neutrophils was achieved with lentivirally derived cell lines expressing shRNA for the genes. Our lab has used this approach to knock down the WAVE complex in HL-60 cells. Levels of knockdown were confirmed by RT-PCR with primers for mRNA of the appropriate protein. Knockdown lines were compared to cells expressing control (scrambled or irrelevant) shRNAs.

**Microscopy.** All of the phytochrome system experiments will be performed on a light patterning system built around a Nikon Ti-E inverted Total Internal Reflection Fluorescence microscope.

**Transwell Chemotaxis Assay.** Transwell chemotaxis assays were performed using 24-well Fluoroblok transwell chambers (pore size, 3.0 µm; Corning) as previously described. Briefly, cells were stained with the membrane dye DiD and 300,000 cells were loaded to each top well. They were allowed to migrate towards the bottom well for two hours at 37°C. The migrated cells were measured by fluorescence from the bottom of the insert (the opaque filter prevented excitation of cells on top of the filter). The percentage of migrating neutrophils was calculated by dividing the fluorescence reading
from each well by the fluorescence reading of the total input cells. Analysis was performed with a FlexStation 3 Microplate Reader (Molecular Devices).
CONCLUSIONS

Through Aim 1, I attempted to develop an assay to directly demonstrate Rac positive feedback. This required the ability to activate Rac directly without also activating the upstream signaling cascade. However, I was unable to generate a cell line that could have high baseline Rac activity, despite having high levels of GTP-loaded Rac. Even more surprisingly, the kinetics of endogenous Rac activation were unaffected by the presence of exogenous GTP-Rac. One can imagine if there was increased Rac inhibition to compensate for the increased levels of GTP-Rac, one might not see higher Rac activity at baseline but would see reduced peak Rac activation or faster Rac turnoff or both. That we see neither means that endogenous Rac is almost completely blind to exogenous GTP-Rac. This suggests that there is a post-translational mechanism for turning off excessive Rac activity.

Through Aim 2, I attempted to uncover the mechanism of Rac adaptation downstream of PI3K by testing candidates that were known to interact with both PIP3 and Rac. Though PIP3 is known to engage in Rac-dependent positive feedback, how PIP3 engages Rac activation and deactivation is unknown. We generated Dock2, P-Rex1, and Arhgap15 knockdown strains and then assessed them for migration defects. Although P-Rex1 knockdown cells showed a chemotaxis defect as expected, the Arhgap15 line also showed a migration defect, contrary to prior published results.

One possibility for the contradictory results was that Arhgap15 depletion led to increased Rac activation globally in the cell, causing it to be unable to polarize and chemotax efficiently. However, further characterization of these lines showed that,
similar to the migration assay results, both the P-Rex1 and Arhgap15 knockdown lines showed a decrease in Rac activation in response to chemoattractant. Depletion of a Rac GEF like P-Rex1 should lead to decreased Rac activation, but the Arhgap15 result is unexpected.

What could explain the contradictory Arhgap15 result? One explanation may be the complex relationship between Rac activation and its own positive and negative feedback loops. In such a setting, the levels of each component need to be tuned such that the positive feedback or negative feedback arms don’t predominate. Arhgap15 may control the balance of positive and negative feedback by acting as a Rac GAP. In its complete absence, positive feedback dominates and Rac activity is increased and does not adapt. However, at low levels, Arhgap15 may inhibit the positive feedback arm by some yet unknown mechanism.
REFERENCES CITED


FIGURE LEGENDS.

Fig. 1. Synthetic activation of exogenous Rac and readout of endogenous Rac activation. A) The working model has a GEF-mediated Rac positive feedback loop that drives polarization and chemotaxis. B) Schematic representation of synthetic activation of Rac. Light or rapamycin recruits a GTPase deficient (Q61L) Rac allele lacking a prenylation sequence to the plasma membrane. If the positive feedback model is correct, recruitment of active Rac to the membrane should result in the activation of endogenous Rac.

Figure 2. Controlling protein localization and cell signaling with light. A) 650 nm light causes membrane-bound PhyB to associate with cytosolic PIF. 750 nm light causes the two to dissociate. PhyB needs to be bound to PCB to recruit PIF. B) HL-60 cells expressing membrane-bound PhyB and PIF-YFP-Rac(CA) were illuminated in suspension with the indicated wavelength of light for 40 minutes. Protein was precipitated with TCA and subjected to western analysis using antibodies specific for phosphorylated Pak1 (Rac activity readout) and actin (loading control).

Fig. 3. HL-60s lose phyB expression over time. A) FACS analysis of mCherry-phyB expression in undifferentiated and differentiated HL-60s. Sx labels different times the same line was analyzed, with x being the number of times that line has been sorted. B) FACS of mCherry-phyB expression in undifferentiated cells selected with drug selection or FACS sorting.

Figure 4. Controlling protein localization and cell spreading with rapamycin. A) Rapamycin brings a constitutively active Rac allele to the plasma membrane by chemically heterodimerizing the FKBP domain attached to RacQ61LDCAAX to a FRB
membrane anchor (Lyn N-terminal sequence). B) FKBP-RacQ61LDCAAX recruitment induces lamellipodia formation in NIH3T3 cells. Adapted from Inoue et al. 2005.  
C) Rapamycin-induced Rac activation induces morphological changes in HL-60 cells. TIRF images show recruitment of a YFP-tagged Rac GEF to the membrane at 30 seconds and consequent cell spreading at 2 minutes.

**Figure 5. Rac(Q61L) expressing cells show decreased tail retraction in comparison to wildtype.** Wildtype (A) or CFP-Rac1(Q61L) expressing differentiated HL-60 cells were plated in culture media on a coverslip coated with fibronectin. After incubation at 37°C for one hour, non-adhered cells were washed away and the culture media was replaced by mHBSS. The cells were then imaged on a 20X objective.

**Figure 6. Rac(Q61L) expressing cells show slightly increased basal Pak phosphorylation and unaltered filamentous actin content in comparison to wildtype.** A) Pak phosphorylation of wildtype or CFP-Rac1(Q61L) expressing cells was measured by western blot and normalized to beta-actin. The unstimulated and peak stimulated levels are shown. B) Actin polymerization of wildtype or CFP-Rac1(Q61L) expressing cells was measured by phalloidin staining and FACS analysis. The unstimulated and peak stimulated levels are shown.

**Figure 7. Rac(Q61L) expressing cells show unaltered Rac activation kinetics.** A) Pak pulldown of GTP-Rac in response to fMLP stimulation in wildtype or CFP-Rac1(Q61L) expressing cells was measured by western blot and normalized by cell number. The fMLP stimulation timecourse and levels of exogenous GTP-loaded Rac are shown. B) Quantification of the western blot shown in (A).
Figure 1-Synthetic activation of exogenous Rac and readout of endogenous Rac activation.
Figure 2- Controlling protein localization and cell signaling with light.
Figure 3-HL-60s lose phyB expression over time.

A

% Cells

PI3K

undiff
d5 diff

B

% positive cells

% Infected (initial)  Re-induction (days)  Re-assess (weeks)

FACS sort only  Drug select only
Figure 4-Controlling protein localization and cell spreading with rapamycin.
Figure 5-Rac(Q61L) expressing cells show decreased tail retraction in comparison to wildtype.
Figure 6-Rac(Q61L) expressing cells show slightly increased basal Pak phosphorylation and unaltered filamentous actin content in comparison to wildtype.
Figure 7—Rac(Q61L) expressing cells show unaltered Rac activation kinetics

A

Rac-GTP

100nM fMLP

1uM fMLP

no fMLP

15s 60s 15s 60s

no fMLP

15s

CA Rac

CFP-CA Rac

total Rac

B

Graph showing arbitrary units (arbitrary units) vs. time (no fMLP, 15 sec, 60 sec) for different conditions: CA Rac (100nM fMLP), CA Rac (1uM fMLP), wildtype (1uM fMLP)
CHAPTER THREE

A genetic screen for chemotactic effectors
SUMMARY.

Our lab focuses on understanding how neutrophils (professional migratory cells of the innate immune system) regulate cell polarity and motility in response to external gradients of chemoattractant. We are combining optogenetic activation of individual nodes of the signal transduction cascade with live cell activity readouts to interrogate the logic of information flow through the neutrophil signaling network. Our optogenetic approach can only be applied to known components of the neutrophil signaling cascade, but there are significant gaps in our understanding of the full suite of components involved in chemotactic signaling. There have not been any systematic biochemical or forward-genetic-based screens for proteins involved in signal relay in chemotaxis. In this work, we use genome-wide shRNA screens to identify new components of the neutrophil chemotaxis cascade. The more complete parts list of the signaling network that will be generated through these experiments will be essential to take full advantage of our optogenetic-based approach for understanding neutrophil chemotaxis.
RESULTS.

Rationale: Our lab and others have identified key regulators of neutrophil chemotaxis through reverse genetics. A number of investigators have performed shRNA screens to identify genes involved in cell migration. However, many of these screens investigate the phenotypic consequence of knockdown in a one knockdown per well format, which typically has limited the throughput to a maximum of one to two thousand genes (19056882, 19160483, 20473299). In addition, there is dishearteningly little overlap when different groups perform similar migration screens (19056882, 19160483, 21062900). These previous screens have been plagued by false positives (not all shRNAs work to knock down the gene of interest) and false negatives (many shRNAs have off-target phenotypes; at a genome wide scale, for many screens it has been estimated that the bulk of the hits represent off-target effects). To overcome these difficulties, we are collaborating with Jonathan Weissman’s lab, who have developed an innovative shRNA screening approach that uses the statistical power of ultracomplex libraries (25 shRNAs/ gene vs. 5/ gene for previous studies) to dramatically decrease the false positive and false negative rate (19448642). Because so many shRNAs are targeted per gene, there is a very low probability that the majority of the shRNAs fail to knock down the gene of interest. Furthermore, the pooled infection and deep sequencing used in the screen demands that a significant proportion of the shRNAs for any given gene yield a phenotype, dramatically decreasing the likelihood of false positives. This technology will enable us to screen through far more genes with more accuracy and sensitivity than previously possible. Here we apply this powerful next-generation shRNA approach to cell migration for the first time.
Experiment – screening methodology: This screening approach relies on infection of a cell population with an shRNA library, selection of the population (in previous cases cell survival following exposure to a toxin), then next-generation sequencing to identify shRNAs that either enhance or suppress cell sensitivity to the perturbation. In our case, we are using a transwell device that enables us to isolate cells that are either enhanced or suppressed in their capacity for chemotaxis. Our genetic screen for the proteins involved in neutrophil chemotaxis is based on an shRNA approach developed in the Weissman lab in which lentiviruses are used to generate a pooled library of cells expressing the shRNAs (25 shRNAs/gene; one shRNA/cell), cells are exposed to a phenotypic-based selection/purification, and deep sequencing is used to identify shRNAs that either enhance or suppress the phenotype. Because so many shRNAs are used per gene, consistency in phenotypic effect of the shRNAs across the population of the 25 shRNAs can be used to identify true hits from the background. The Weissman lab has used this approach to identify genes involved in ricin sensitivity, where they used simple cell survival as the selection. For our migration screen, we used a transwell assay to purify chemotactic versus non-chemotactic cells.

Transwell assay for purifying chemotactic versus non-chemotactic neutrophils. Our genetic screen was based on a scaled-up version of the transwell chemotaxis assay, which has been the platform for previous migration screens (21062900, 20473299). In transwell assays, cells are placed on top of a porous filter with chemoattractant below the filter. Chemotactic cells migrate through the filter to the bottom chamber, where they can be purified away from the non-migratory cells. For these experiments, we are using the human HL-60 cell line, which can be infected with
the lentiviral shRNA library (Fig. 1A), propagated indefinitely in culture, and
differentiated into neutrophil-like cells that we and others have demonstrated to be a
good model for neutrophil chemotaxis. We have verified that the lentiviral-based
shRNAs knock down mRNAs in HL60s to a similar extent as the K562 cells used in the
Weissman lab screen (Fig. 1B). By competing wildtype cells against those expressing
shRNA against the Hem-1 subunit of the WAVE complex (which we previously
demonstrated to cause a chemotaxis defect 16417406), we verified the ability of our
transwell approach to enrich for shRNAs that interfere with cell migration (Fig. 1C,D).

**Pooled shRNA migratory screen in neutrophils.** The Weissman lab recently
published the shRNA libraries that we are using for these experiments (23394947,
19448642, 23739767). The genome-wide library is divided into nine sublibraries of
55,000 shRNA each and target each gene with 25 independent shRNAs. Each
sublibrary also contains 500 or more negative control shRNAs, which have been
designed to match the base composition of the targeted shRNAs within the same
sublibrary without targeting any transcript in the human genome. The shRNAs are
expressed from a Pol II promoter in a miR-30 context.

**Pilot genetic screen.** To pilot our genetic migration screen, we infected 200
million HL-60 cells with one of the Weissman lab shRNA libraries at a multiplicity of
infection of 0.3 with the goal of having most cells expressing one or less shRNAs. Then
we selected the cells for three days with puromycin to kill cells that fail to express any
shRNAs. HL60 cells were then differentiated into neutrophils and subjected to large-
scale transwell assays to purify cells that do and do not chemotax towards the bacterial
chemoattractant fMLP. Under these conditions, the majority of the cells migrate with
similar efficiency to wildtype (Fig. 2A), demonstrating that, as expected, the shRNA library is not broadly perturbative to migration. We used PCR and deep sequencing to identify shRNAs that were differentially enriched in the migratory versus non-migratory cell populations. To ensure we had decent coverage of each shRNA, we scaled the assay such that both the top and bottom fractions had a representation of 400 cells per library element. From the pilot screen we identified genes that either enhance or suppress neutrophil chemotaxis in a statistically significant fashion (two example genes are shown in Fig. 2B). Following further optimization of the transwell migration assay and shRNA libraries (see below), we will repeat the screen for each sublibrary to achieve genome-wide coverage. For followup we will prioritize shRNA hits potentially linked to known nodes of the signaling cascade (for instance there are over 60 Rho family GEFs and a similar number of GAPs, but we do not know which are linked to chemotaxis) or those that overlap with hits in our biochemical screen (Aim 2).

Potential problems and alternate approaches for the migration screen.

Improving the transwell assay. While the pilot screen was able to identify some genes that are involved in neutrophil chemotaxis, the screen would greatly benefit by increased discrimination between chemotactic versus non-chemotactic cells. In the presence of chemoattractant, approximately 30% of wt cells migrate through the transwell assay. In the absence of chemoattractant, approximately 5% of wt cells migrate through the transwell. If we could further reduce the proportion of cells that migrate in the absence of chemoattractant, this would significantly improve our ability to discriminate shRNAs that alter cell migration. For this purpose, we plating epithelial
cells on top of the transwell filter, forcing the neutrophils to migrate both past the epithelial monolayer as well as the transwell filter. This modified assay only slightly decreases the number of neutrophils that migrate to the bottom chamber in the presence of chemoattractant but decreases the proportion of cells that migrate through in the absence of chemoattractant by more than an order of magnitude (Fig. 3). We anticipate that this will lower our false positive rate and enable improved identification of genes involved in migration.

**Improving the shRNA library.** Before we repeat this screen to achieve genome-wide coverage, we are not only working to improve the transwell assay but also aim to use better shRNA libraries. Because each gene for the Weissman lab’s ricin screen is covered by 25 shRNAs and because a phenotype can be measured for each shRNA, they can not only identify which gene products are involved in ricin sensitivity, but they can also determine which shRNAs most potently yield a phenotype (and thus knock down the target gene most effectively). Iterated on a genome wide scale, this has enabled the Weissman lab to improve the rules for picking which 25 shRNAs to use for each gene to maximize the likelihood that these are effective in knockdown. This will further improve the statistical power of the approach. The Weissman lab plans to have a next generation of shRNA libraries in the next 6 months, which we will use these improved libraries for our genome-wide screen.

**Secondary screens.** As for any primary genetic screen, secondary screens will be necessary to validate hits and prioritize them for further investigation. One possible set of false positives for the migration screen are shRNAs that interfere with cell differentiation, because only differentiated neutrophils express chemoattractant
receptors. To exclude this class of hits, we evaluate whether shRNAs that give chemotactic defects also affect expression of end-stage differentiation markers such as the formyl peptide receptor, which can be measured in FACS. Any shRNAs that alter differentiation will be excluded. Inhibition of some signaling pathways gives a chemotaxis defect for only a subset of chemokines (XXX)—these represent receptor-specific inputs to migration. Other signaling pathways are required for chemotaxis to a broad range of receptor inputs XX—these likely represent core components of the chemotaxis machinery. We will perform secondary screens with chemoattractants other than fMLP to distinguish between these classes of signaling proteins and will prioritize shRNAs that give effects for a range of ligands.
METHODS

**Cell culture.** HL-60 cells were cultured as described. Briefly, cells were grown at 37°C, 5% CO₂, in RPMI 1640 media with L-glutamine and 25 mM HEPES (10–041-CM, Mediatech), with 10% heat-inactivated fetal bovine serum (FBS). Cell differentiation was initiated by adding 1.5% DMSO (endotoxin-free, hybridoma-tested; D2650, Sigma) to cells in media. Cells for all experiments were used at 2–3 days after differentiation.

**RNAi knockdown.** Knockdown of Rab1A, Rps25, and Trappc8 in neutrophils was achieved with lentivirally derived cell lines expressing shRNA for the genes. Levels of knockdown were confirmed by RT-PCR with primers for mRNA of the appropriate protein. Knockdown lines were compared to cells expressing control (scrambled or irrelevant) shRNAs.

**Quantitative real-time PCR.** Total RNA was extracted from differentiated HL-60 cells using RNEasy (Qiagen) according to the manufacturer’s instruction. Next, 1 µg of total RNA was reverse-transcribed with the QuantiTect reverse transcription kit (Qiagen, Mississauga, ON, Canada). An equal amount of cDNA from each cell line was amplified by real-time PCR using SYBR Green QPCR Master Mix. We normalized gene expression to a housekeeping gene (GAPDH), and the relative expression value between the samples was calculated based on the threshold cycle (CT) value using the standard curve method.

**Transwell Chemotaxis Assay.** Transwell chemotaxis assays were performed using 24-well Fluoroblok transwell chambers (pore size, 3.0 µm; Corning) as previously described. Briefly, cells were stained with the membrane dye DiD and 300,000 cells were loaded to each top well. They were allowed to migrate towards the bottom well for
two hours at 37°C. The migrated cells were measured by fluorescence from the bottom of the insert (the opaque filter prevented excitation of cells on top of the filter). The percentage of migrating neutrophils was calculated by dividing the fluorescence reading from each well by the fluorescence reading of the total input cells. Analysis was performed with a FlexStation 3 Microplate Reader (Molecular Devices).

**Large scale transwell protocol (for >20 million cells)**

Materials: 75mm Transwell® with 3.0μm Pore Polycarbonate Membrane Insert, Sterile (Product #3420), Corning

- mHBSS+0.2%low endotoxin BSA
- RPMI
- fMLP

1. Infect HL-60 cells with library and select to >90% purity.
2. Differentiate >200 million HL-60s with 1.5% DMSO 4 days before the screen.
3. Count cells and measure viability day of screen. Ideal viability is at least 70%, discard cells if below 60% viability.
4. Calculate the number of transwell plates to run (36 million cells/plate). Make enough mHBSS for transwells (22mL/plate). Warm RPMI for starvation step.
5. Run four plates at a time until out of cells-
   a. Spin down four tubes of cells, 36 million cells per 50 mL Falcon tube, at 400g x 5min
b. Resuspend each tube in 18 mL RPMI. Loosen cap and let sit in 37
   incubator for 8-15 min.

c. Meanwhile, prepare four plates by adding 13 mL mHBSS with 10nM fMLP
to each plate to the bottom well while top insert is in place. This is to
prevent the weight of cells added to the top insert from tearing the insert
membrane when they’re added.

d. Spin down cells and resuspend each tube in 9 mL mHBSS.

e. Add the contents of each tube to the top of the insert.

f. Carefully transfer each plate to a 37 incubator.

g. Collect cells at the end of 2 hours.
   i. Collect top well first.
   ii. Collect bottom well and add 4-5 mL trypsin to the bottom well.
   iii. Incubate at 37 for 5-10 min.
   iv. Scrape cells from the bottom of the transwell insert and bottom well
       and add to the trypsin. Combine the trypsin and previously
       collected bottom fraction.
   v. Spin down bottom fractions and combine to concentrate cells.

   Expected yield is 1-3 million cells per plate.

6. Count total cells collected. Freeze cells from combined top fraction and combined
   bottom fraction in 50 million cell/tube in FBS+10%DMSO.
Bibliography and References Cited


FIGURE LEGENDS.

Figure 1. Genome-wide shRNA screen for genes involved in neutrophil chemotaxis. (A) Experimental strategy: A population of HL-60 cells was infected with a pooled high-coverage shRNA library and differentiated into neutrophils. The cells were placed in a transwell chamber with 100 nM fMLP in the bottom chamber. The frequency of shRNA-encoding constructs in each subpopulation was determined by deep sequencing. (B) Quantitative PCR analysis of mRNA abundance in K562 (the line used by the Weissman lab) and HL-60 cells infected with viruses targeting genes encoding TRAPPC8, RAB1A, and RPS25, presented relative to the abundance of GAPDH mRNA. (C) Transwell chemotaxis of differentiated HL-60 cells either infected with virus targeting Hem1 or uninfected. Migrated cells across an optically opaque transwell membrane were quantified by DiD labeled cells using a microplate reader. (D) Transwell chemotaxis in a large scale format with a mixed population of wildtype or CFP-tagged Hem1 knockdown cells. Cell samples were drawn from the top and bottom wells and the proportion of CFP expressing Hem1 knockdown cells was measured by FACS after two hours.

Figure 2. Results of the pilot shRNA neutrophil migration screen. (A) Transwell chemotaxis of wildtype differentiated HL-60 cells and HL-60 cells infected with the pooled shRNA library, performed in the same way as Fig. 3C. (B) Based on the frequency in the top and bottom well subpopulations of the transwell assay, a quantitative chemotaxis enhancement/suppression phenotype $\rho$ was calculated for each shRNA. Comparing the distribution of $\rho$s for shRNAs targeting a gene of interest to the
p distribution for negative control shRNAs using the Mann-Whitney U test yielded a p value for the gene. \textit{RhoU} knockdown enhances cell migration, whereas knockdown of \textit{SEP12} inhibits cell migration.

\textbf{Figure 3.} HUVEC cells dramatically decrease basal neutrophil migration in transwell assay.
Figure 1. Genome-wide shRNA screen for genes involved in neutrophil chemotaxis.

A

HL-60 cells

High coverage shRNA library

lentiviral infection and differentiation

Infected cells in a transwell chamber

cells allowed to migrate towards chemoattractant for 2 hours

top well (non-migratory cells)

bottom well (migratory cells)

separate cells in different wells

Extract genomic DNA and PCR amplify shRNAs

Count shRNAs by deep sequencing

B

fold change (over nonsense shRNA control)

percentage migrated (%)

TRAPPc8, RAB1a, RPS25

gene targeted by shRNA

K562

HL-60

C

percentage migrated (%)

buffer fMLP

wildtype Hem1 KD

D

Hem1 knockdown cells as a percentage of the total population (%)

pre-transwell post-transwell

original top well bottom well
Figure 2. Results of the pilot shRNA neutrophil migration screen.
Figure 3. HUVEC cells dramatically decrease basal neutrophil migration in transwell assay.
CHAPTER FOUR

Homer3 regulates the establishment of neutrophil polarity
Summary

Most chemoattractants rely on activation of Gαi to regulate directional cell migration, but few links from Gαi to chemotactic effectors are known. Through affinity chromatography using primary neutrophil lysate, we identify Homer3 as a novel Gαi2-binding protein. Homer3 is an EVH1 domain-containing scaffold protein that binds active Rac and regulates actin dynamics in T cells and neurons. RNAi-mediated knockdown of Homer3 in neutrophil-like HL-60 cells impairs chemotaxis and the establishment of actin polarity. Most proteins that are required for cell polarity are needed for actin assembly or activation of core chemotactic effectors such as PI3K and Rac. In contrast, Homer3 knockdown cells show normal kinetics and magnitude of chemoattractant induced activation of PI3K and Rac effectors as well as other Gαi effectors such as calcium mobilization. Although chemoattractant-stimulated Homer3 knockdown cells exhibit normal initial actin assembly, they fail to sustain and spatially organize actin assembly into cell polarity and motility. Our data suggests that Homer3 acts as a scaffold that spatially organizes actin assembly and cell polarity downstream of GPCR activation.
Introduction

Directed cell migration plays a central role in many physiological and pathological processes. Neutrophils are innate immune cells that use chemotaxis to arrive at sites of infection. Most chemoattractant receptors mediate activation of motility effectors through Gi-family heterotrimeric G-proteins (Neptune and Bourne, 1997; Rickert et al., 2000). Knockout or knockdown of Gαi2 and pharmacological inhibition of Gαi signaling blocks neutrophil chemotaxis towards most agonists (Spangrude et al., 1985; Wiege et al., 2012).

Ligand binding to GPCRs triggers GDP to be exchanged for GTP on Gαi2, which leads to dissociation of the Gαi from Gβγ heterodimers. Both Gαi2-GTP and Gβγ can interact with downstream signaling partners (Oldham and Hamm, 2008). Many potential links from Gβγ to downstream chemotactic effectors are known, including PI3K-gamma (which stimulates PIP₃ production) (Stephens et al., 2008), P-Rex1 and Dock2 (activators of Rac1 and Rac2 GTPases) (Dong et al., 2005; Welch et al., 2005; Kunisaki et al., 2006a, 2006b), phospholipase beta (which hydrolyzes PIP2 into DAG and IP₃), and several others (Tang et al., 2011). Whether Gαi2 has its own suite of distinct chemotactic effectors or whether it is simply a handle to release Gβγ is only beginning to be understood. Recent studies have begun to identify Gαi2-specific effectors in chemotaxis. An example of a recently discovered potential Gαi2 effector is mInsc, which binds Gαi2-GDP at the leading edge to recruit polarity complexes to the leading edge and control neutrophil directionality (Kamakura et al., 2013). Dock180, a Rac
activator highly homologous to Dock2, has also been shown to be a potential Gαi2 effector (Li et al., 2013).

The Gαβγ signaling pathway controls signaling molecules that need to be spatially organized to lead to cell polarity. In response to extracellular stimuli, neutrophils activate intracellular signaling cascades that result in the formation of a F-actin-rich leading edge and actomyosin filaments in the rear; these asymmetries are dependent on the polarization of intracellular signals (Wang, 2009; Berzat and Hall, 2010). Efficient movement requires the establishment and maintenance of these signaling asymmetries. Rac, a Rho GTPase, is localized to the leading edge and is essential for actin assembly and migration (Gardiner et al., 2002; Sun et al., 2004; Zhang et al., 2009). It activates the WAVE2 complex, which promotes actin polymerization and itself is necessary for Rac activation (Eden et al., 2002; Weiner et al., 2006). Though we know many of the signaling molecules necessary for migration, less is known about how they are spatially organized.

Here we identify Homer3 as a novel Gαi2 interacting protein that regulates actin organization in neutrophils. Homer3, part of a larger family of Homer scaffolding proteins, has been shown to play a role in actin dynamics following stimulation in neutrons and T cells (Ishiguro and Xavier, 2004; Shiraishi-Yamaguchi and Furuichi, 2007; Shiraishi-Yamaguchi et al., 2009). Its role and regulation in chemotaxis has not yet been characterized. Homer3 depletion dramatically reduces cell motility but does not affect global activation of Rac and PIP₃ effectors downstream of chemoattractant. These data suggest that Homer is
essential for proper spatial organization of the actin cytoskeleton downstream of chemotactic stimulation.
Materials and Methods

Cell lines and culture
HL-60 cells were cultured as described. Briefly, cells were grown at 37°C, 5% CO₂, in RPMI 1640 media with L-glutamine and 25 mM HEPES (10–041-CM, Mediatech), with 10% heat-inactivated fetal bovine serum (FBS). Cell differentiation was initiated by adding 1.5% DMSO (endotoxin-free, hybridoma-tested; D2650, Sigma) to cells in media. Cells for all experiments were used at 2–3 days after differentiation. Differentiation was confirmed with PE-conjugated anti-CDllb antibody (BD).

Imaging and analysis
TIRF images were acquired on a Nikon Ti-E inverted microscope with a 60× Apo TIRF 1.49 NA objective and an electron-multiplying charge-coupled device (EM-CCD) camera (Evolve; Photometrics, Tucson, AZ) controlled by NIS-Elements (Nikon, Melville, NY). Sample drift was minimized using an autofocus system (Perfect Focus; Nikon). Laser lines (514, 561 nm; all 200 mW) were supplied from a Spectral Applied Research LMM5 Laser Merge Module (Richmond Hill, Ontario, Canada). This laser launch uses acousto-optic tunable filters (AOTFs) to control laser output to a TIRF fiber for imaging or a Mosaic Digital Micromirror device (Andor, Belfast, UK) for spatially controlled photobleaching. TIRF or oblique imaging was performed with 50 mW or less (through AOTF and neutral density–based laser attenuation).
Confocal images were acquired in a custom built environmental chamber with temperature and CO2 control (In Vivo Scientific) on a Nikon Eclipse Ti inverted microscope equipped with a Yokogawa CSU-X1 spinning disk confocal, a 60× Plan Apo TIRF 1.49 NA objective, and a Clara interline CCD (Andor). 405 nm, 488 nm, and 561 nm laser wavelengths (MLC400B, Agilent) were used for excitation.

Calcium assays and time-lapse migration assays were imaged using a CCD camera (Cool Snap HQ; Photometrics) and a Nikon TE-2000 inverted microscope with a 20× PlanFluor 0.5 NA objective in an In Vivo Scientific microscope incubator (St. Louis, MO) to create a 37°C climate.

NIS-Elements was used for image acquisition, and NIS-Elements, ImageJ, and Microsoft Excel (Redmond, WA) were used for data analysis. Graphing and statistical analyses were performed using Prism 6 (GraphPad). All p values were calculated using t-test (populations were of equal variance) or, where indicated, paired t-test.

Knockdown line generation
Lentiviral Homer3 and nonsense shRNA control in pLKO.1 were purchased from Sigma. Sequences used in this study were Homer3 shRNA 1, 5′-CGGCTAAAGAAGATGTTGTCT-3′; Homer3 shRNA 2, 5′-GAACAGCATCTGACACAGTTT-3′; and control shRNA, 5′-GCGCGATAGCGCTAATAATTT-3′. HEK293T cells were grown to 70% confluency in a 6 well plate for each lentiviral target and transfected using 0.5 µg
Homer3 or nonsense shRNA, 50 ng VSV-G, and 0.5 µg CMV 8.9 with TransIT-293T (Mirus Bio) according to manufacturer’s instructions. Media was changed at 18 hours after transfection, and viral supernatant was collected at 42 hours and 66 hours post-transfection. 4 mLs of combined viral supernatant was used to infect 10^6 undifferentiated HL-60 cells by spinfection in the presence of 8 µg/ml polybrene. Stable cell lines were generated with 1 µg/ml puromycin selection.

Quantitative real-time PCR
Total RNA was extracted from differentiated HL-60 cells using RNEasy (Qiagen) according to the manufacturer’s instruction. Next, 1 µg of total RNA was reverse-transcribed with the QuantiTect reverse transcription kit (Qiagen, Mississauga, ON, Canada). An equal amount of cDNA from each cell line was amplified by real-time PCR using SYBR Green QPCR Master Mix. We normalized gene expression to a housekeeping gene (GAPDH), and the relative expression value between the samples was calculated based on the threshold cycle (CT) value using the standard curve method.

Preparation of high-speed cytosol
Pig leukocyte cytosol was prepared essentially as described. Pig blood was obtained from Rancho Veal (Petaluma, California, United States). 40L of blood was collected into 5 polypropylene jugs containing a total of 9L of 1× sterile ACD anticoagulant (80 mM sodium citrate, 15 mM NaH₂PO₄, 160 mM glucose, 17 mM citric acid, and 2 mM adenine). Blood was transported to the laboratory at room
temperature. At the laboratory, 250mL of 154 mM NaCl/3% polyvinylpyrrolidone (MW 360,000) was added per liter of blood plus anticoagulant, mixed thoroughly, poured into 2-l polypropylene containers, and allowed to settle into two phases for 30 to 45 min. The upper phase (containing leukocytes and contaminating red blood cells) was decanted and pelleted at 1,500g for 15 min at room temperature in an IEC swingout rotor. The supernatant was poured off, and the pellets were resuspended in calcium-free mHBSS (buffer A) containing 0.2% BSA. Cells were pelleted tightly at 1,500g for 15 min and resuspended in buffer A.

Cells were resuspended in a minimum volume of buffer A, and then 10× volume of ddH2O was added for 20 s to lyse contaminating red blood cells. Then 1.1× volume 10× buffer A was added to regain an isotonic solution. Cells were pelleted, washed, and then resuspended in freshly prepared 3 mM diisopropylfluorophosphate in buffer A to inactivate serine proteases, then allowed to sit for 20 min on ice. Cells were pelleted, washed, and then resuspended in cavitation buffer (50 mM NaCl, 50 mM Tris [pH 7.5] at 4 °C, 5 mM MgCl₂, 5 mM DTT, 1× EDTA-free protease inhibitor tablets [Roche, Basel, Switzerland] per 50 ml of solution). Cells were cavitated in a nitrogen parr bomb (350 psi, 20 min) into a collection vessel containing EGTA for a final concentration of 2 mM EGTA. Disrupted cells were spun at 1500g for 15 min to remove nuclei and unbroken cells and then 96,000g for 60 min to remove membranes. High-speed supernatant was carefully removed without disturbing the pellet. For purifications, the lysate was typically used fresh, but it was also stable when snap frozen in liquid nitrogen and stored at −80 °C for at least a year.
Pull-Down Assay

Rat G\(\alpha\)i2, bacterially expressed as a GST-fusion protein, was purified with glutathione-sepharose FF (GE Healthcare) as previously described (Ghosh et al., 2008). GST-G\(\alpha\)i2 was loaded with GDP or the non-hydrolyzable GTP analogue Gpp(NH)p using the alkaline phosphatase protocol. Nucleotide loading was assessed with HPLC. For GDP-AlF\(_4\) loading, 50 \(\mu\)M AlCl\(_3\) and 30mM NaF was added to GST-G\(\alpha\)i2 and incubated at 30°C for 30 minutes. For the mass spectrometry screen, 3 to 5mg GST or GST-G\(\alpha\)i2 bound to glutathione-sepharose FF was incubated with 50mL of 3mg/mL leukocyte lysate overnight with recirculation. The column was washed with three column volumes of wash buffer and bait and bound proteins were eluted with glutathione. Peak protein fractions were pooled and concentrated prior to running an SDS-PAGE gel and cutting bands for mass spec.

For preparation of FLAG–Homer3, 293T cells were transfected with the expression construct and lysed with 0.4% NP-40 48 hours post transfection. The lysate clarified by centrifugation and was combined with leukocyte lysate prepared as described. GST–G\(\alpha\)i2 bound to glutathione-sepharose FF was incubated with FLAG–Homer3 and leukocyte lysate overnight. Proteins were eluted with glutathione and subjected to SDS-PAGE, followed by staining with CBB or processing for Western blot with an anti-FLAG antibody.
Protein identification by mass spectrometry

Protein samples were concentrated and separated by 1D-SDS-PAGE. After Coomassie staining each lane was cut into 24 slices and subjected to in-gel digestion with 100 ng trypsin (Trypsin Gold, Promega), prior reduction with 10 mM DTT and alkylation with 55 mM iodoacetamide. Peptide mixtures were trapped on C18 reversed phase EASY-Column and separated on a 100 mm C18 reversed phase column (75 µm x 100 mm, 3-µm particle size, Thermo Scientific) using a linear gradient from 0% to 35% (v/v) acetonitrile in 0.1% formic acid, over 70 min at a constant flow rate of 300 nL/min. Nanoflow LC-MS/MS was performed on an EASYII LC system (Thermo Scientific) coupled to an LTQ-Orbitrap XL mass spectrometer (Thermo Scientific) operating in positive mode. MS scans were acquired in the Orbitrap in the range from 350 to 1800 m/z, with a resolution of 60,000 (FWHM). The 7 most intense ions per scan were submitted to MS/MS fragmentation (35% Normalized Collision Energy™) and detected in the linear ion trap. Peak lists were obtained from raw data files using the Proteome Discoverer v1.3 software. Mascot (version 2.1, MatrixScience) was used for searching aGαinst a sequence database obtained by combining the E. coli with the Dictyostelium proteome sequences. The peptide tolerance was set to 40 ppm and the fragment ion tolerance to 2.0 Da, using semi-trypsin as protease specificity and allowing for up to 2 missed cleavages. Oxidation of methionine residues, deamidation of asparagine and glutamine, and carboamidomethylation of cysteines were specified as variable modifications. Peptide and protein identifications were further validated with the program
Scaffold (Version 3.2, Proteome Software Inc., Portland, OR). Protein identifications based on at least 2 unique peptides identified by MS/MS, each with a confidence of identification probability higher than 95%, were accepted.

Phospho-Pak and phospho-Akt assay
Cells were resuspended to a concentration of 2 million ml\(^{-1}\) in RPMI+0.2%FBS. We stimulated cells with 10 nM fMLP and quenched the reaction by adding aliquots of the cell mixture to ice-cold 20% trichloroacetic acid plus 40 mM sodium fluoride plus 20 mM beta-glycerol phosphate (50020, Fluka) at the indicated time points. Pellets were washed with 0.5% trichloroacetic acid and resuspended in Laemmli protein sample buffer (161–0737, BioRad) containing 5% β-mercapto-ethanol. Protein bands were separated by SDS-PAGE gel electrophoresis, transferred to nitrocellulose, blocked with Odyssey block, and incubated at 4°C overnight with 1:1000 dilutions of anti-phosphoPAK (Cell Signaling 2605S) and anti-Pak2(Cell Signaling, 4825S) or anti-phosphoAkt (Cell Signaling, 4060S) and anti-Akt (Cell Signaling, 40D4). The blot was developed with the fluorescent secondary antibodies and protein bands were imaged using ODYSSEY INFRARED IMAGING SYSTEM (Li-COR, Biosciences).

Filamentous Actin staining
Cells were resuspended in mHBSS with 0.2% BSA. Cells were stimulated with addition of 10nM fMLP, fixed with 3.7% paraformaldehyde in intracellular buffer (140mM KCl, 1mM MgCl2, 2mM EGTA, 320mM sucrose, 20mM Hepes pH7.5),
and incubated on ice for 20 minutes. After centrifugation at 400g for 2 minutes, the cell pellet was resuspended in intracellular buffer plus 0.2% triton and 1:500 rhodamine phallodin and stained for 20 minutes. The cells were centrifuged and resuspended in mHBSS and analyzed on a FACSaria. Size correction for fluorescence intensity was derived from the ratio of background fluorescence from unstained control and Homer3 knockdown cells. Data gating and Gaussian fit analysis was performed on FlowJo (TreeStar, Ashland, OR).

Time-lapse migration assays
Cells were resuspended in 12.5 µLmHBSS with 2% BSA and 10nM fMLP and plated on 5 µg/ml (FN) coated coverslips previously blocked with BSA. A coverslip was placed over the cell suspension, and the edges were sealed with VALAP, forming a squeeze chamber 15µm in height. Starting 10 minutes after plating cells are imaged by phase contrast every 10 seconds for 1 hour on a Nikon TE-2000 with a 20× (.5 NA) objective. The cells were kept at 37°C using a thermostatic chamber.

Cell tracking was performed manually using the MTrackJ plugin of ImageJ. Error associated with manual rendering of images can result in low speeds in otherwise stationary cells as well as fluctuation in instantaneous velocity from any given frame. Thus cells were scored as “motile” if the maximum displacement from the origin was ≥5 µm (~ one half a cell radius) over a given time frame. To determine pauses, a moving average of the instantaneous velocity from the current frame and three previous frames was calculated for
each frame of each track. If the moving average fell one standard deviation below the mean velocity, the cell was scored as paused.

Transwell Chemotaxis Assay

Transwell chemotaxis assays were performed using 24-well Fluoroblok transwell chambers (pore size, 3.0 µm; Corning) as previously described (Park et al., 2014). Briefly, cells were stained with the membrane dye DiD and 300,000 cells were loaded to each top well. They were allowed to migrate towards the bottom well for two hours at 37°C. The migrated cells were measured by fluorescence from the bottom of the insert (the opaque filter prevented excitation of cells on top of the filter). The percentage of migrating neutrophils was calculated by dividing the fluorescence reading from each well by the fluorescence reading of the total input cells. Analysis was performed with a FlexStation 3 Microplate Reader (Molecular Devices).

Results

We have previously used affinity chromatography to identify novel G-alpha effectors in Dictyostelium and sought to use a similar approach to identify Gαi effectors in neutrophils (Kataria et al., 2013). GST-tagged Gαi2 was purified and incubated with neutrophil lysate harvested from cavitated pig leukocytes. The GST-Gαi2 and associated proteins were then isolated and separated by SDS-PAGE. The protein bands were analyzed by mass spectrometry. Two independent screenings identified several known Gαi interacting proteins, such as RASA3, TNFAIP8, Gβγ, RGS3 and RIC8A (Fig. 1A) (Neptune and Bourne,
1997; Anger et al., 2007; Nafisi et al., 2008; Laliberté et al., 2010; Kataria et al., 2013). From this screen, we were most interested in proteins that have functional domains suggesting that they could be activators or inhibitors of our bait proteins or represent potential links to other central nodes of the neutrophil chemotaxis signaling pathway. From this prioritized list of $G_{\alpha i}$2 interactors, we tested each individually for a role in chemotaxis with a follow-up genetic screen (Fig. S1).

Homer3, a novel $G_{\alpha i}$ interactor, was identified in both screens. Homer3 bound to both GDP-loaded GST-$G_{\alpha i}$2 and GST-$G_{\alpha i}$2 loaded with GTP analogues GDP-$\text{AlF}_4$ and Gpp(NH)p with relatively similar peptide counts. The interaction between Homer3 and $G_{\alpha i}$2 was confirmed with a tagged Homer3 construct (Fig. 1B). Since we were searching for a novel $G_{\alpha i}$2 interactor relevant to chemotactic signaling, we confirmed whether Homer3 was necessary for neutrophil chemotaxis. We used the neutrophil-like differentiated HL-60 cell line to knockdown expression of Homer3 (Hauert et al., 2002). HL-60s infected with control- or Homer3-shRNA were assessed with a transwell chemotaxis assay, in which cells migrated through a microporous filter towards a source of chemoattractant. When presented with a gradient of the $G_{\alpha i}$-coupled GPCR ligand fMLP, Homer3 knockdown cells migrated less efficiently than control cells (Fig. 2A). A similar defect was seen when using C5a, another chemoattractant that activates the $G_{\alpha i}$-coupled C5a receptor (data not shown). The chemotaxis defect was observed in two independent lines each expressing a different shRNA against Homer3. The magnitude of the defect scaled with the degree of Homer3
knockdown measured by RT-PCR (Fig. 2B), suggesting that the defect is likely to be specific to Homer3. We chose the line with the higher knockdown efficiency for follow-up characterization.

Homer3 is part of a family of scaffolds that bind a variety of proteins relevant to chemotaxis signaling, including actin and Rho family GTPase Rac1 (Shiraishi et al., 1999; Shiraishi-Yamaguchi and Furuichi, 2007; Shiraishi-Yamaguchi et al., 2009). There are three members of the Homer family, which share a highly conserved amino-terminal Enabled/vasodilator-stimulated phosphoprotein homology 1 (EVH1) domain and long Homer-specific carboxy-terminal coiled-coil domain (Shiraishi-Yamaguchi and Furuichi, 2007). Homer proteins have been primarily studied in neurons, where they localize to the synapse and participate in calcium signaling, axon guidance, and dendritic spine morphology (Foa et al., 2001; Sala et al., 2001; Fagni et al., 2002; Hwang et al., 2003; Moutin et al., 2012). In neurons, Homer2 interacts directly with the actin cytoskeleton and Drebrin, a dendritic F-actin-binding protein, via the conserved EVH1 domain (Shiraishi-Yamaguchi et al., 2009).

The transwell assay can show defects in chemotaxis but cannot assess the source of the defect. One cannot determine whether the defect is in speed, directionality, or persistence of migrating neutrophils. To do so we needed to observe control and Homer3 knockdown cells during migration with microscopy. To assess the effect of Homer3 knockdown on random cell migration, we used
time-lapse migration assays to compare control cells infected with a nonsense shRNA and Homer3 knockdown cells. As described previously (Weiner et al., 2006), we used a “chimney assay,” in which cells are resuspended into a small volume sandwiched between two coverslips. In this context, migration is not dependent on cellular adhesion, enabling us to screen for cells whose lack of movement is not due to a lack of adhesion. A substantial fraction of the Homer3 knockdown cells fail to move in this context (Fig. 3A). These non-motile cells either fail to polarize or extend short protrusions that are quickly retracted. Homer3 knockdown cells exhibit a significant increase in the percentage of non-motile cells compared to the control line.

Homer3 knockdown cells not only exhibited a significant increase in the proportion of non-motile cells but they also exhibited subtle defects in the motile population of cells as well. The speed of the motile cells of the Homer3 knockdown line was slightly lower compared to the control line (Fig. 3B). The Homer3 knockdown cells paused for significantly longer periods than the control cells (Fig. 3D), consistent with a general defect in initiation of migration. However, Homer3 knockdown cells do not pause more frequently than control (Fig. 3E). Consistent with this observation, the overall persistence of cell movement in motile Homer3 knockdown cells is not affected (Fig. 3B), since cells usually change direction following pauses. Therefore, Homer3 may play a role in initiation of migration but does not seem to affect the maintenance of migration.
Does the motility defect for Homer3 knockdown cells represent a general lack of activation of heterotrimeric G-protein effectors, as observed for the Ric8 protein in Dictyostelium? To investigate whether there is a general defect in signaling, we assayed calcium release from Homer3 knockdown cells following stimulation. Calcium release was assessed by loading cells with the cell-permeable calcium indicator dye Fluo-4AM and measuring fluorescence intensity in individual cells before and after stimulation. A similar proportion of cells responded in both the control (407 of 451 cells, 90%) and Homer3 knockdown (465 of 546 cells, 85%) cells (Fig. S2). Thus, Homer3 knockdown does not prevent cell signaling in response to chemoattractant. In addition, this means that Homer3 depletion does not affect differentiation of HL-60s into neutrophils, since differentiation is required for the expression of formyl-peptide receptor, which would be necessary to respond to fMLP (Kanayasu-Toyoda et al., 1999).

Given that one effector of G protein signaling (calcium release) was intact for Homer3 depleted cells, we next assayed the downstream heterotrimeric G-protein effectors Rac and PI3K, both of which contribute to regulation of chemotaxis (Wang, 2009; Berzat and Hall, 2010). We used phosphorylation of p21-activated kinase (Pak) as a downstream readout Rac activation (Knaus et al., 1995; Weiss-Haljiti et al., 2004). We used phosphorylation of Akt to read out signaling through the PI3K cascade (Burgering and Coffer, 1995; Franke et al., 1995; Stokoe et al., 1997). We observed the same stimulation kinetics of both the Homer3 knockdown and control cells (Fig. 4). This is in contrast to the transwell migration assay, where we saw a substantial decrease in chemotaxis for Homer3
knockdown cells. There is a small increase in the amount of phosphorylated Akt and Pak normalized to total Akt and Pak in the Homer3 knockdown cells versus the control at all time points. However, this difference is not significant if we control for the slight size decrease in Homer3 knockdown cells (Supplement). Overall, our results indicate that Ga signaling is intact in Homer3 knockdown cells and that Homer3 depletion does not significantly affect the kinetics or magnitude of Rac and PI3K activation.

If the overall magnitude of signaling is unchanged, we speculated that the localization of signaling molecules may have become affected in Homer3 knockdown cells. We assessed the localization of Rac activity in live cells using total internal reflection fluorescence (TIRF) imaging of fluorescently tagged Rac-GTP-binding domain, Pak-PBD-mCherry (Weiner et al., 2007). We assessed control and Homer3 knockdown cells in uniform chemoattractant in squeeze chambers. In control cells and motile Homer3 knockdown cells, Pak-PBD-mCherry persistently accumulates at the leading edge (Fig. 5A). However, in immotile Homer3 knockdown cells, Rac activity wanders throughout the cell. Thus, Homer3 depletion reduces persistence of Rac activity localization in immotile cells.

What happens to a signal’s localization before, during, and after stimulation? To assess the dynamic localization of a signaling molecule before and after fMLP addition, we used confocal microscopy to observe localization of PH-Akt-Citrine, which binds PIP₃ (Servant et al., 2000). Because confocal
microscopy does not require cells to be directly on the coverslip, unlike TIRF, we can image cells outside a squeeze chamber. Using a squeeze chamber precludes chemoattractant addition after the chamber has been sealed. Consistent with the Western blot results we saw a uniform translocation of PH-Akt-Citrine to the plasma membrane in both the control and Homer3 knockdown cells (Fig. 5B). However, while PH-Akt-Citrine preferentially accumulated at the leading edge of control cells, we saw no preferential localization in immotile Homer3 knockdown cells.

Since Homer proteins have been associated with actin regulation (Ishiguro and Xavier, 2004; Shiraishi-Yamaguchi et al., 2009), we investigated whether Homer3 plays a similar role in neutrophils. Control and Homer3 knockdown cells were stimulated with the chemotacttractant fMLP and stained with phalloidin, which binds F-actin. Exposure to fMLP induces a rapid and transient global F-actin accumulation, which peaks around 1 minute and later organizes into a polarized lamellipod at later timepoints. The mean fluorescence intensity of phallodin staining in control and Homer3 knockdown cells show similar kinetics, with a peak at 1 min and a gradual decrease afterwards (Fig. 6E). There is a slight overall decrease in actin stain intensity in Homer3 depleted cells at all time points. However, using the mean intensity averages out notable changes in the distribution of actin staining intensity of individual cells (Fig. 6C). For control cells, the distribution of staining intensity of individual cells remains unimodal throughout- before stimulus, during peak response, and after peak response.
Homer3 knockdown cells also have a unimodal distribution before stimulus and during peak response (Fig. 6C,D). However, Homer3 knockdown cells separate into a bimodal distribution at later timepoints, one dim and one bright. The dim population corresponds to cells without a bright, organized lamellipod and represents roughly half the total Homer3 knockdown cells 7 minutes post stimulation (Fig. 6A,B). In contrast, only one fifth of the control cells lack an organized lamellipod at that time point. This suggests that while Homer3 knockdown cells retain their ability to polymerize actin in response to stimulus, they lack the ability to maintain or organize the actin at later timepoints.

To document polarity in live cells, we added Hem1-YFP to nonsense or Homer3 shRNA expressing cells. Hem1- YFP shows strong leading edge localization in neutrophils and serves as a dynamic, discrete marker of leading edge polarity (Weiner et al., 2006, 2007). We imaged control and Homer3 knockdown cells in uniform chemoattractant in squeeze chambers using total internal reflection fluorescence microscopy. Hem1-YFP localized to the leading edge of control and motile Homer3 knockdown cells, as it does in wildtype (Fig. 6F). In immotile Homer3 knockdown cells, Hem1-YFP showed a non-persistent localization that wanders throughout the cell, similar to what we observed for Rac activity. This demonstrates that immotile Homer3 knockdown cells have no persistent axis of polarity when in uniform chemoattractant.
Discussion

Most chemoattractants act through Gαi proteins to mediate directional movement, but only a few chemotaxis-relevant effectors of Gαi are known. Recent work has indicated that mInsc, which binds Gαi2-GDP, helps maintain directionality in neutrophils and the Dock180/Elmo1 complex, a GEF for Rac activation, associates with Gαi2 upon stimulation of breast cancer cells (Kamakura et al., 2013; Li et al., 2013). Our work extends this suite of effectors with the identification of Homer3 as a Gαi2-biding protein that is essential for cell polarity and motility in neutrophils. It is not clear how Gαi2 regulates Homer3. Homer3 does not affect Gαi2 function since Rac activity and PIP₃ production, both which are dependent on Gαi2 function, are unaffected (Wang, 2009; Berzat and Hall, 2010). This distinguishes Homer3 from another class of Gαi2-interacting proteins that have been found- proteins that regulate Gαi activity. For example, the Gαi2 GEF Ric8A is responsible for amplifying the intracellular signaling gradient in Dictystelium (Kataria et al., 2013). Homer3 may serve as a scaffold to link Gαi2 with effector proteins or the actin cytoskeleton itself.

Our results show Homer3 is required for proper spatial dynamics of Rac and PI3K activity and persistent polarization of Hem1. We see that the magnitude and kinetics of activation of key signaling molecules such as Rac and PI3K in response to chemoattractant are largely unaffected by Homer3 depletion. However, though we can see a burst of PI3K activity in individual Homer3 knockdown cells, these cells fail to polarize at later timepoints. Live cell readouts of Rac activity and Hem1 localization show that the signal wanders throughout
the cell in immotile Homer3 knockdown cells. In sum, our results with Homer3 suggest its role may be to organize intracellular cell polarity.

Our work shows that Homer3 is necessary for the organization of polarity and not for the initial burst of activity in response to chemoattractant. Given that chemoattractant-stimulated neutrophils can polarize in suspension but Homer3 knockdowns exhibit a significant defect in this context, our data suggest that this defect is not due to mis-regulation of adhesion. Importantly, this is different from previous genetic manipulations of neutrophils, which interfere with organization of polarity but also response to chemoattractant. For example, Hem1 knockdown cells fail to polarize but also fail to increase Rac activity in response to fMLP (Weiner et al., 2006). Deletion of another actin regulatory protein, filamin, also leads to perturbations in Rac activity (Valle-Pérez et al., 2010). Deletion of Rac or PI3K will clearly have effects on Rac and PI3K activity, respectively. Deletion of downstream effectors of Gβγ, such as Rac GEFs P-Rex1 and Dock2, also inhibit Rac activation and cell migration (Kunisaki et al., 2006a; Lawson et al., 2011). This suggests that Gαi2 and Gβγ may coordinate the chemotactic response by organization and amplification of chemotactic signals such as active Rac, respectively.

How might Homer3 organize cell polarity? Previous studies have shown that Homer3 binds directly to active Rac1-GTP and not inactive Rac1-GDP via a C-terminal domain (amino acids 204 to 296) (Shiraishi et al., 1999). We also find that Rac2 preferentially interacts with GTP-loaded Gαi2 in our pulldown screen, possibly through association with Homer3 and GTP loading of the Rac2 from
GTP in the lysate. In addition, Homer3 is likely to bind directly to actin via its EVH1 domain (amino acids 1-120), which is highly homologous—82% identical—to the actin-binding EVH1 domain of Homer2 (Shiraishi et al., 1999). It has been shown in neurons that Homer colocalizes with actin and treatment with latrunculin A, an actin inhibitor, causes Homer mislocalization (Kuriu et al., 2006; Shiraishi-Yamaguchi and Furuichi, 2007). Homer3 could integrate signals from actin, Rac-GTP, and Gαi2 to help a neutrophil organize a persistent leading edge.

The lack of persistent polarity with Homer3 depletion could help explain the immotile phenotype we see in Homer3 knockdown cells. A large fraction of Homer3 knockdown cells do not form protrusions or only transient ones in uniform chemoattractant. In addition, the mobile Homer3 knockdown cells pause longer, supporting a role for initiation of protrusions. In contrast, most control and wildtype cells form persistent lamellipodia in uniform chemoattractant. If activated Rac does not localize in one spot long enough in Homer3 knockdown cells to polymerize enough actin to form a lamellipod, we could see transient or absent protrusions. The wandering Rac localization is similar to previous results from our lab using a drug cocktail to immobilize the actin cytoskeleton. In actin-arrested cells, Rac activity also wanders across the cell and does not persist at the leading edge (Peng et al., 2011). Further work could determine whether immobilization is secondary to improper localization of signals such as activated Rac or vice versa.
In our study we’ve found that Homer3 binds Gai2-GTP and Gai2-GDP with relatively equal affinity. However, this *in vitro* association with both forms may be signaling-dependent *in vivo* in cells. Gai2-GTP is produced in response to fMLP, and free Gai2-GDP is produced from Gai2 GTPase activity at the leading edge of neutrophils. Gβγ sequesters non-signaling Gai2-GDP within cells, interrupting the interaction of Gai2-GDP with binding partners. Notably, mInsC, a recently discovered Gai2 effector, does not show a preference for Gai2-GTP (Kamakura *et al.*, 2013). It plays a role in organizing polarity complexes at the leading edge and colocalizes with Gai2-GDP.

In summary, we have found that Homer3 associates with Gai2 and is necessary for the initiation of cell migration. While nonmotile Homer3 knockdown cells can form transient protrusions, they cannot sustain a leading edge and morphological polarity. Homer3 may enhance the initiation of migration by scaffolding signaling proteins such as active Rac and actin that regulate early steps in leading edge organization. From our study we have found that the overall activation of Rac and PI3K activity is not affected by Homer3 depletion. However, persistent polarization of Rac activity and actin is significantly reduced with Homer3 depletion. We postulate that Homer3 aids in polarization by linking active Rac to actin and Gai2 at the leading edge. If so, Homer3 depletion represents an unusual opportunity to study the role of the signaling molecules that organize polarity independent of their effects on the initial burst of activity in response to chemoattractant. Further work could elaborate on the role of Homer3
in organizing cell polarity, whether it be through its association with actin and active Rac or through other molecular mechanisms.
FIGURE LEGENDS

Figure 1. Identification of Homer3 as a neutrophil protein that binds Gαi2. A) B) GST-Gαi2 or GST alone was pulled down with FLAG-Homer3 in neutrophil lysate. The wash before elution or eluted GST-tagged bait + associated proteins were subjected to SDS-PAGE and analyzed by immunoblot with anti-FLAG antibody.

Figure 2. Homer3 knockdown impairs HL-60 chemotaxis. A) RNA was isolated from control cells (nonsense shRNA) and two separate HL-60 cell lines containing different Homer3 shRNAs (shRNA 1 and shRNA 2). Relative expression of Homer3 was quantified by qRT-PCR using GAPDH as a reference gene. Results represent the mean with standard deviation of three replicates. B) Migration of control or Homer3 knockdown HL-60 differentiated cells in response to 10nM fMLP was measured in transwell chemotaxis chambers after two hours and ten minutes. Results are a representative example of five independent experiments.

Figure 3. Homer3 plays a role in the initiation of migration. A) Percentage of non-motile cells in time-lapse migration assays in uniform 10nM fMLP expressed as mean with standard error. Results are from three independent experiments with two replicates each. B) Speed of control (nonsense shRNA) and motile Homer3 knockdown cells were compared in the time-lapse migration assays. Dot plot shows the overall population distribution; box and whiskers plots show quartiles.
C) Persistence index (defined as final distance from start/total distance traveled) comparison between control and Homer3 knockdown. D) Length of pauses in tracks from time-lapse migration assays of control and Homer3 knockdown cells as defined in Methods. E) Frequency of pauses in control and Homer3 knockdown cell tracks.

Figure 4. Homer3 depletion does not affect levels of Pak phosphorylation or Akt phosphorylation. A, C) Timecourse following uniform 10nM fMLP stimulation of control (nonsense shRNA) or Homer3 knockdown cells of Akt (A) or Pak (C) phosphorylation. Total Akt (A) or total Pak (C) used as a loading control. B, D) Quantification of phosphorylated Akt (B) or Pak (D) for three independent runs of the experiment shown in (A,C). Phosphorylated Akt (B) or Pak (D) was normalized to total Akt (B) or Pak (D), and each subsequent value was normalized to the peak value of that timecourse.

Figure 5. Spatial persistence of Rac and PI3K activity is affected in immotile Homer3 knockdown cells. A) Rac activity was assessed by TIRF imaging of a fluorescent Rac-GTP-binding domain (Pak-PBD-mCherry). Control or Homer3 knockdown cells are in uniform 100nM fMLP in a squeeze chamber. B) Representative time-lapse confocal images of PHAkt-Citrine before and after addition of 100nM fMLP in control and Homer3 knockdown cells. White is highest fluorescence intensity. Images are representative of at least three cells.
Figure 6. Homer3 depletion reduces persistence of actin polymerization. A,B,C,D,E) Differentiated control or Homer3 knockdown HL-60 cells in suspension were stimulated with 10nM fMLP. At the timepoints indicated, cells were fixed and stained with rhodamine phalloidin to visualize filamentous actin. A) Representative epifluorescence images at timepoints representing before stimulation (0 min), at peak response (1 min), and after polarization (7 min). B) Quantification of polarization at the 7 min timepoint of control (n=577) and Homer3 knockdown (n=754) cells in epifluorescence images. Results are the mean and standard error of three independent experiments. C) Actin staining intensity was quantified by FACS analysis. The overlays of the intensity distributions of control and Homer3 knockdown cells at the indicated timepoints are representative of three independent experiments. D) Two Gaussian distributions were fit to the FACS data from (C). The percentage of the total cell population that was described by the largest distribution is shown. Results are the mean and standard error of three independent experiments. E) Average fluorescence intensity of the whole cell population, as quantified by FACS, was measured and normalized to the unstimulated control population to correct for FACS and staining variation between experiments. Results are the mean and standard error of three independent experiments. F) Polarization was assessed by TIRF imaging of a fluorescent component of the WAVE complex (Hem1-YFP). Control or Homer3 knockdown cells are in uniform 100nM fMLP in a squeeze chamber. White is highest fluorescence intensity. Images are representative of at least three cells.
Supplemental material

Figure S1. Screen schematic.

Figure S2. Calcium signaling is intact in Homer3 knockdown cells. Both control (n=451) and Homer3 knockdown (n=546) cells release calcium in response to stimulation with 100nM fMLP, as assayed by the calcium indicator fluo-4AM. Representative epifluorescence images of control and Homer3 knockdown cells before and after fMLP stimulation are shown.

Explanation of phospho-Pak and phospho-Akt levels

The discrepancy in phosphorylated Pak and Akt normalized to total protein in control and Homer3 knockdown cells can be explained by their difference in cell size. The difference lies between the different size dependence of surface area and volume. Total Pak and Akt are primarily cytosolic proteins and hence their levels are proportional to volume. Phosphorylated Pak and phosphorylated Akt are both activated at the plasma membrane by membrane-bound Rac-GTP and membrane lipid PIP3, respectively. Hence their levels are proportional to cell surface area. Assuming that a cell is roughly spherical, surface area is proportional to $r^2$, where $r$ is the radius of the cell, and volume is proportional to $r^3$. Therefore the surface area to volume ratio is proportional to $r^2/r^3 = 1/r$, and so the phosphorylated protein/total protein ratio is also proportional to $1/r$. 
References


Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase. Cell 81, 727–736.


Figure 1 - Homer3 can bind Gai2 in neutrophil lysates

A

B

Figure 2 - Homer3 knockdown impairs HL-60 chemotaxis

A

B
Figure 3 - Homer3 plays a role in initiation of HL-60 migration

A) Proportion of nonmotile cells

B) Speed in 10nM uniform fMLP

C) Persistence index

D) Pause length

E) Pause frequency

Legend:
- green: 3 min
- blue: 2 min
- red: 1 min
- black: 0 min

nonsense Homer3 kd

nonsense Homer3 (motile)

nonsense Homer3 kd

nonsense Homer3 (motile)

nonsense Homer3 kd

nonsense Homer3 (motile)

nonsense Homer3 kd

nonsense Homer3 (motile)

nonsense Homer3 kd

nonsense Homer3 (motile)
Figure 4- Homer3 depletion does not affect levels of Pak phosphorylation and Akt phosphorylation

A
phospho-Akt

[Image of Western blot for phospho-Akt]

[Image of Western blot for total Akt]

B
phospho-Akt/total Akt

[Graph showing time (s) vs. phospho-Akt/total Akt levels for nonsense and Homer3 kd]

C
phospho-Pak

[Image of Western blot for phospho-Pak]

[Image of Western blot for total Pak]

D
phospho-Pak/total Pak

[Graph showing time (s) vs. phospho-Pak/total Pak levels for nonsense and Homer3 kd]
Figure 5: Spatial persistence of Rac and PI3K activity is affected in immotile Homer3 knockdown cells.
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Figure 6- Homer3 depletion reduces persistence of actin polymerization

A

B

C

D

E

F

Hem1-YFP

Homer3 kd
Primary mass spectrometry screen

- Bait protein bound to agarose column
- Neutrophil lysate incubated over column overnight
- Wash away unbound protein and elute bait protein with interacting proteins
- Identify proteins by SDS-PAGE and mass spectrometry

Secondary migration screen

- HL-60 cells
- Experimental shRNA
- Infected cells in a transwell chamber
- Cells allowed to migrate towards chemoattractant for 2 hours
- Lentiviral infection and differentiation
- Control shRNA
- HL-60 cells

Supplementary Figure 1- Screen schematic
Supplementary figure 2 - Calcium signaling is intact in Homer3 knockdown cells.
CHAPTER FIVE

Summary
SUMMARY AND FUTURE DIRECTIONS

The main findings of this work are that

1) Constitutively active Rac (Q61L) has a surprisingly mild phenotype when stably expressed in HL-60 cell lines, compared to HL-60 cells transiently expressing the same construct.

2) Large scale transwell assays can efficiently discriminate wildtype HL-60 neutrophil cells from migration-deficient HL-60 cells. This can serve as a discovery platform for pooled genetic screens for chemotaxis genes in the future.

3) Homer3 is a Gai2 interacting protein that is necessary for efficient neutrophil chemotaxis and polarization of intracellular signaling molecules.

The future directions for the first two were explained in the discussion of those chapters.

Future directions for the last chapter include investigating the other Gai2 interacting proteins that came up in the screen, characterizing the interaction between Gai2 and Homer3, and investigating the other phenotypes of Homer3 knockdown.

The Gai2 mass spectrometry pulldown screen came up with more potential binding partners than could be reasonably followed with single gene knockouts. There were twenty genes that I tested before settling on Homer3, four of which showed a defect in chemotaxis (Ric8, GPSM3, Rsu1, and Homer3).
However, genes that were demonstrated to have a role in chemotaxis in other studies (ex, IQGAP1) failed to show a defect in our study. This shows that my follow-up was not only limited, but it may have missed chemotaxis effectors due to insufficient knockdown. One of the previous ideas for the project was to combine the mass spectrometry hits with a large-scale genetic screen for chemotaxis effectors (project 2). The overlap between the two screens could yield potential Gai2 effectors in chemotaxis more efficiently than testing genes individually. In addition, the mass spectrometry pulldown screen could be applied to any other proteins known to be involved in chemotaxis that can be efficiently purified, such as Gbetagamma or Rac GTPase.

Although Homer3 bound to Gai2 in neutrophil lysates, we did not characterize its interaction with Gai2 further. Importantly, Homer3 binds to both Gai2-GDP and Gai2-GTP with relatively equal affinity. It is unclear whether Homer3 is a scaffold that associates with Gai2 at all times or if its association is stimulation-dependent. Preliminary studies with a fluorescently tagged Homer3 did not show appreciable changes in localization upon stimulation. Our collaborators also did not see a stimulus-dependent change in localization of Ric8, a direct Gai2 interactor necessary for amplification of Gai2 activation downstream of chemoattractant receptor activation. Thus, low affinity binders like Ric8 or Homer3 may not have a dramatic enough change in localization to be visible by microscopy. Further work could clarify how Gai2 regulates Homer3 function or vice versa.
Homer3 depleted cells have a consistently smaller size than wildtype or nonsense shRNA control cells. On average, their diameter is about 80% of wildtype. This size difference was often vexing for interpreting results, since most of our protocols rely on counting cells to normalize across samples. However, the size difference itself may be interesting for further study. Few determinants of mammalian cell size are currently known. One hypothesis is that the size difference is due to misregulation of Akt signaling. Akt is known to play a role in cell size homeostasis. We noticed that in unstimulated Homer3 depleted cells, there is an elevated phospho-Akt level by Western blot compared to nonsense shRNA control cells. Although the stimulation kinetics following fMLP addition are the same, we cannot explain why the initial levels are so high. Another reason may be due to defects in actin polymerization in Homer3 knockdown cells. Although actin does not yet have a defined role in cell size, one recent study showed that nuclear actin may play a role in counteracting the effects of gravity on nuclear proteins in larger cells like oocytes. Thus, actin may serve as a measuring rod for cells to know how large they are. If actin polymerization or maintenance of actin organization is defective, as it seems with Homer3 knockdown cells, cells may not be accurately sensing their own cell size.
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