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Dictyostelium NF1-mediated Ras Signaling is Essential for Directional Sensing, Polarization and Cell Motility

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in

Biology

by

Sheng Zhang

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2008
The dissertation of Sheng Zhang is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

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Co-Chair

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Chair

University of California, San Diego

2008
DEDICATION

In recognition of my loving wife, Xihong Wang,
my sweet daughter, Samantha Zhihan Zhang,
my parents, Xilin Zhang and Lingli Wang,
my parents in-laws, Genbao Wang and Juan Li
and all who have supported me.
This thesis is dedicated to you.
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ACKNOWLEDGEMENTS

First and foremost, I would like to express my gratitude and appreciation to my dissertation advisor: Dr. Richard Firtel for his enthusiastic supervision, invaluable guidance, encouragement and patience during the past 5 years. I would like to acknowledge my committee members: Dr. Steve Briggs, Dr. Randy Hampton, Dr. Joan Heller Brown and Dr. Tony Hunter for their helpful advice and suggestions on my project and my career.

To my wife Xihong, I am deeply appreciative of her love and support. She and my daughter Samantha give me all the passion and motivation to complete my Ph.D. study. I also thank my parents for their generous love and encouragement all these years.

Last, but not least, I would like to thank all the previous and current research scholars in Firtel’s lab and collaborators in Jamora’s lab for their stimulating discussions, helpful suggestions and technical assistance.
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ABSTRACT OF THE DISSERTATION

*Dictyostelium* NF1-mediated Ras Signaling is Essential for Directional Sensing, Polarization and Cell Motility

by

Sheng Zhang

Doctor of Philosophy

University of California, San Diego, 2008

Professor Richard Firtel, Chair
Professor Steve Briggs, Co-Chair

In response to a chemoattractant signal, amoeboid cells such as neutrophils, macrophages, and *Dictyostelium* cells are able to polarize and move towards the chemoattractant source. During *Dictyostelium* chemotaxis, it is known that cells amplify and differentially localize specific signaling responses at the future anterior and posterior of the cell leading to outwardly directed F-actin polymerization and myosin II-mediated
contractility at the front and back, respectively. However, how cells initially detect and orient themselves in chemoattractant gradients remains largely unknown.

Ras activation is the earliest polarized response to chemoattractant gradients downstream from heterotrimeric G proteins in Dictyostelium and inhibition of Ras signaling results in directional migration defects. Activated Ras is enriched at the leading edge, promoting the localized activation of key chemotactic effectors, such as PI3K and TORC2. To investigate the role of Ras in directional sensing, I studied the effect of its misregulation using cells with disrupted RasGAP activity. I identified an orthologue of mammalian NF1, DdNF1, as a major regulator of Ras activity in Dictyostelium. nfaA- cells fail to spatially and temporally regulate Ras activity, which leads to misregulated downstream PI3K activity and F-actin polymerization. As a result, severe cytokinesis and chemotaxis defects in nfaA- cells are observed. Through both genetic and biochemical approaches, I identified RasG as the major target of DdNF1 GAP activity in chemotaxis. Using unpolarized, latrunculin-treated cells, I showed that tight regulation of Ras is required for directional sensing. It is speculated that the uniformly distributed DdNF1 functions as a global inactivator of Ras activity, coupled to putative local activators (e.g. RasGEFs), unidentified global inhibitors as well as positive feedback signaling, leads to persistence and amplification of the Ras signal at the front and promotes leading edge formation. Consequently, cells migrate up the gradients. Together, it is suggested that Ras is part of the cell’s compass, and that the RasGAP-mediated regulation of Ras activity affects directional sensing. Further, growing nfaA- cells exhibit elevated Ras activity and display enhanced random cellular movement, consistent with the model that a G protein-independent Ras/PI3K/F-actin circuit regulates basic cell motility.

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Chapter 1 Introduction to Chemotaxis and Ras Signaling

1.1 Chemotaxis

Chemotaxis is a fundamental cellular process in which cells sense gradients of extracellular chemical cues (chemoattractants) and respond by polarizing and directionally migrating towards the source. Single-celled organisms use chemotaxis as a principal mechanism to search for food and optimal environmental conditions as well as to avoid predators (Konijn et al., 1967). Chemotaxis is required for the cell aggregation in the life cycle of the social amoebae, Dictyostelium discoideum (Manahan et al., 2004). In higher organisms, it plays pivotal roles in a wide range of cellular responses, including morphogenesis, embryogenesis, angiogenesis, neuronal patterning, and tissue maintenance (Campbell and Butcher, 2000; Crone and Lee, 2002; Martin and Parkhurst, 2004; Moser and Loetscher, 2001; Rubel and Cramer, 2002). One representative chemotactic response is exemplified by neutrophils as they find the way to the sites of inflammation in the process of wound healing. In addition to the roles in normal physiology, chemotaxis is also involved in pathological events, such as cancer metastasis and various chronic inflammatory diseases (Condeelis et al., 2005; Kunkel and Godessart, 2002; Murphy, 2001; Tarrant and Patel, 2006).

Signaling mechanisms controlling chemotaxis are remarkably conserved between mammalian leukocytes and the simple eukaryotic amoeboid organism Dictyostelium discoideum, although both are separated by millions of years of evolution (Parent, 2004). Research on chemotaxis of eukaryotic cells has progressed substantially, mainly through
the study of *Dictyostelium* and neutrophils as model systems (Merlot and Firtel, 2003; Van Haastert and Veltman, 2007; Weiner, 2002). In order for cells to respond to and migrate up a chemoattractant gradient, a series of complex processes needs to be organized in succession. Chemotaxis is initiated when chemoattractants/ligands bind to trans-membrane G-protein coupled receptors (GPCRs), which leads to the dissociation of the coupled heterotrimeric G protein into $\alpha$ and $\beta\gamma$ subunits. The dissociated G protein subunits further activate a plethora of effectors and cognate downstream signaling cascades that synergistically convert shallow extracellular gradients of chemoattractants to highly polarized intracellular signaling responses. As a result, cells amplify and localize certain signaling proteins in an asymmetric manner. These polarized signaling molecules further induce coordinated local remodeling of the cytoskeleton as well as cell adhesion to the substratum, resulting in outwardly directed F-actin polymerization and myosin II-mediated contractility at the front and back of the chemotaxing cells, respectively.

The sensitivity of eukaryotic cells to chemoattractant gradients is extremely high. Both neutrophils and *Dictyostelium* are able to detect as low as $\sim$2% difference in concentration between the front and the back of cell, and recent experiments with growth cones have claimed to exhibit axonal guidance in concentration differences as little as 0.1% (Parent and Devreotes, 1999; Rosoff et al., 2004). In *Dictyostelium* cells, the GPCRs and heterotrimeric G proteins are evenly distributed throughout the plasma membrane while the receptor occupancy and G-protein activation directly mirrors the concentration of chemoattractant with little amplification upon the directional stimulation of chemoattractant (Jin et al., 2000; Ueda et al., 2001). In contrast, trans-membrane
chemoreceptors in bacteria are not uniformly distributed, localizing at one or both cell poles in multimeric assemblies, coupled with other cytosolic components (Baker et al., 2006).

The first amplified, asymmetric signaling response downstream of heterotrimeric G proteins is activated Ras, which is activated at the leading edge of chemotaxing cells in response to chemoattractant gradients, as shown by the localization of a GFP fused Ras binding domain of the mammalian protein Raf1 (GFP-RBD$_{Raf1}$) (Sasaki et al., 2004). GPCR/heterotrimeric G-protein signaling promotes Ras activation, presumably through the Ras guanine nucleotide exchange factors (RasGEFs) by a yet-to-be-identified mechanism. Abrogation of Ras signaling results in severe defects in directional migration in Dictyostelium, indicating that Ras may be an upstream component of the integrated cellular compass (Insall et al., 1996; Sasaki et al., 2004). Ras regulates Dictyostelium and metazoan class I phosphatidylinositol-3-kinase (PI3K) through its binding to the Ras binding domain (RBD) on the p110 catalytic subunit (Funamoto et al., 2002; Pacold et al., 2000). Ras also activates Dictyostelium Target of Rapamycin Complex 2 (TORC2) in a PIP3-independent manner, through its binding to one TORC2 component, Ras Interacting Protein 3 (RIP3/SIN1) (Kamimura et al., 2008; Lee et al., 2005).

Localized Ras-GTP binds and activates membrane-bound PI3Ks at the front of the cell, i.e. the region of the membrane closest to the chemoattractant source, to convert phosphatidylinositol-4,5-bisphosphate (PIP2) to phosphatidylinositol-3,4,5-trisphosphate (PIP3). A positive feedback loop between Ras/PI3K and F-actin has been proposed that aids in recruiting more cytosolic PI3K molecules to the leading edge to amplify PIP3 production. Consistent with this proposal, Sasaki and colleagues observed that
translocation of PI3K is abolished in latrunculin A (an inhibitor of F-actin polymerization) treated cells upon chemoattractant stimulation (Sasaki et al., 2004). On the other hand, the phosphatase and tensin homologue deleted on chromosome ten (PTEN) phosphatidylinositol 3’-phosphatase delocalizes from the front and becomes restricted to the back of the cell, i.e. the region of membrane further away from the chemoattractant source (Funamoto et al., 2002; Iijima and Devreotes, 2002). This “backness” localization of PTEN is probably further stabilized and strengthened by the cortical cytoskeleton and a positive feedback loop between PTEN and PIP2 (Iijima et al., 2004; Janetopoulos and Firtel, 2008). Moreover, a detailed mechanism has been revealed in neutrophils such that PTEN can be phosphorylated by a RhoA-GTP regulated Ser/Thr kinase (ROCK), which leads to its localization and activation at the back (Li et al., 2005; Meili et al., 2005). PTEN seems indispensable for spatiotemporally restricting both PI3K activity and F-actin production to the leading edge because depletion of PTEN leads to extended and non-spatially restricted PI3K activity, and elevated levels of PIP3 and F-actin. Chemotaxing pten− Dictyostelium cells are poorly polarized with an increased number of lateral pseudopodia, a phenotype similar to that of cells expressing membrane-targeted PI3K (Funamoto et al., 2002; Iijima and Devreotes, 2002). In addition, Nishio and colleagues found in neutrophils that one phosphatidylinositol 5’-phosphatase, Src homology 2 (SH2) domain-containing inositol-5-phosphatase1 (SHIP1) also regulates PIP3 levels and cells lacking SHIP1 have severe polarity and motility defects (Nishio et al., 2007). Together, the reciprocal regulation of PI3Ks and PTEN results in the localized synthesis and accumulation of PIP3 at the leading edge of the cell, which functions as the docking site for various downstream PH domain/basic domain containing effectors, such
as PKB, *Dictyostelium* PH-domain-containing protein A (PhdA) and cytosolic regulator of adenylyl cyclase (CRAC). In neutrophils, it has been shown that asymmetrical localization of PIP3 allows dominant pseudopodia formation at the leading edge of chemotaxing cells through the modulation of Rho-GTPases Rac and Cdc42, and the downstream Wiskott-Aldrich syndrome protein (WASP)/SCAR complex that activates the Arp2/3 complex to produce robust F-actin polymerization (Benard et al., 1999; Srinivasan et al., 2003; Stradal et al., 2004). In *Dictyostelium*, similar mechanisms also exist through the recruitment and/or activation of Dock180 family Rac guanine nucleotide exchange factors (RacGEFs), RacGEF1, as well as Rac effectors like WASP to promote F-actin polymerization and pseudopod formation (Janetopoulos and Firtel, 2008; Myers et al., 2005; Park et al., 2004).

*Dictyostelium* cells lacking PI3K1/2 or the PI3K inhibitor LY294002-treated cells fail to chemotax properly up linear/shallow chemoattractant gradients, suggesting that PI3K is an important mediator of chemotactic responses, especially when the concentration of chemoattractant is at an appropriate physiological level (Takeda et al., 2007). However, other studies indicate that in the absence of PI3K signaling, cells can still chemotax reasonably well up steep chemoattractant gradients generated by micropipette filled with chemoattractant, albeit more slowly, suggesting the existence of parallel directional sensing pathways (Hoeller and Kay, 2007). Chen and colleagues identified a gene encoding a phospholipase A2 (PLA2) homologue as a component of such a parallel pathway in a genetic screen, based on the assumption that the chemotaxis of the mutant cells with the parallel pathway disrupted in the presence of LY294002 should be severely inhibited (Chen et al., 2007). This finding is supported by the work of
van Haastert and colleagues using a PLA2 pharmacological inhibitor combined with mutant analysis (van Haastert et al., 2007). However, neither PLA2 nor its hydrolytic products arachidonic acid and lysophospholipid, exhibits an asymmetric localization as PI3K or PIP3 does. The mechanism of how PLA2 mediates chemotactic responses is yet to be determined. As for other lipid signaling pathways, van Haastert and colleagues have recently suggested that phospholipase C (PLC) is activated in response to chemoattractant stimulation, hydrolyzing PIP2 into inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). The degradation of PIP2 on the plasma membrane may disrupt PIP2-PTEN binding, and hence PTEN dissociates from the plasma membrane and shuttles back to the cytoplasm (van Haastert et al., 2007). Another parallel pathway probably involving soluble guanylyl cyclase (sGC) has been suggested as well, based on the observation that inhibition of both PI3K and PLA2 results in much less severe chemotaxis phenotype when Dictyostelium cells are highly polarized at their late stage of aggregation (Veltman et al., 2008; Veltman and Van Haastert, 2006).

In order for cells to chemotax efficiently, the signaling networks regulating the back of the cell are equally important as those at the front, which provide mechanisms to suppress the lateral/back pseudopodia, maintain the rigidity of lateral cell cortex, and to generate appropriate force for contraction and retraction in the posterior. The central mediator of above processes is myosin filaments, whose assembly depends on the phosphorylation state of the myosin heavy chain (MHC) regulated via myosin heavy chain kinase A (MHCKA) and protein phosphatase 2A (PP2A), respectively (De la Roche et al., 2002; Murphy and Egelhoff, 1999). On the other hand, myosin light chain kinase (MLCK) phosphorylates MLC to promote myosin motor activity, which provides
the contractile force for posterior retraction (Bosgraaf and van Haastert, 2006). It has been reported that MHCKA enriches at the leading edge during *Dictyostelium* chemotaxis to phosphorylate and dissemble the myosin filaments (Steimle et al., 2001). In this way, actomyosin meshworks become passively restricted to the rear of the cell. Recent studies by Mondal and colleagues have revealed a novel pathway that RasGEF Q activates RasB to regulate myosin II assembly through a possible regulation of MHCKA (Mondal et al., 2008). In addition, small GTPase Rap1 has been shown to regulate myosin II functions through a Rap1/Phg2/MHCKA/MyoII signaling pathway (Jeon et al., 2001c). Another component involved in the regulation of myosin II is *Dictyostelium* p21-activated kinase A (PAKa) (Chung and Firtel, 1999; Chung et al., 2001b). PAKa is colocalized with myosin II at the posterior during chemotaxis, and *pak1−* cells exhibit increased numbers of lateral pseudopodia, phenocopying cells lacking myosin II. Furthermore, PKB recruited by PIP3 at the leading edge phosphorylates and activates PAKa in the posterior via an unknown mechanism of dynamic cross-talk. Studies of guanylyl cyclases (GC), cGMP phosphodiesterases and cGMP-target/binding protein show that chemotactant-induced intracellular cGMP is another key regulator for myosin II filament formation in *Dictyostelium* (Bosgraaf et al., 2002; Bosgraaf and van Haastert, 2006; Goldberg et al., 2002). Elevated cGMP levels enhance the number of myosin II filament in the cortex, suppress the lateral pseudopodia and improve chemotaxis, while reduced cGMP levels cause the opposite effects. In neutrophils, the signaling pathways regulating “backness” are distinct from those in *Dictyostelium*. Myosin filament formation is mediated by the activation of RhoA and its downstream effector ROCK (Alblas et al., 2001; Xu et al., 2003). Interestingly, studies have
demonstrated that loss of microtubules enhances RhoA activity, leading to increased cell polarity and impaired chemotactic efficiency, which implicates a potential role for microtubule-mediated signaling in the back (Alblas et al., 2001; Xu et al., 2005).

1.2 Dictyostelium: a Powerful Model System

Chemotaxis is essential during the entire life cycle of social amoeba Dictyostelium discoideum (Bagorda et al., 2006). During the growth phase, Dictyostelium exist as single, free-living amoeboïd cells, which are able to hunt bacteria via chemotaxis towards folic acid, a by-product of bacterial metabolism, and engulf them by phagocytosis similar to macrophages (Konijn et al., 1967). When challenged with adverse conditions, such as starvation, Dictyostelium cells stop dividing and enter a unique differentiation/development program. Accordingly, a set of newly-expressed proteins is upregulated within several hours that shifts the chemotactic sensitivity of the cells from folic acid to cAMP. Cells form a multicellular organism (within ~8 hours) through the chemoattractant aggregation of up to $10^5$ cells. Cells secrete and relay cAMP through the periodic activation of adenylyl cyclase (AC). The differentiating prestalk cells then migrate to the top of the mound, eventually extending to a tip and forming the migrating slug (~15 hours). In ~24 hours, development culminates with the formation of terminally differentiated fruiting bodies of mature stalk and spore cells that are resistant to environmental stresses.

Dictyostelium discoideum has proven indispensable as a powerful model system to elucidate the signaling events that regulate chemotaxis (Franca-Koh et al., 2006). As a matter of fact, many GFP fused reporters for identifying in vivo spatial localization of
well-conserved signaling proteins were first developed in *Dictyostelium*. Most of the molecular genetics techniques used in other experimental systems are available in *Dictyostelium*, including homologous recombination for gene targeting, insertional mutagenesis for gene disruption, random mutagenesis for structure-function analysis, RNA interference, and multicopy suppression libraries (Landree and Devreotes, 2004). Gene disruption is simple, fast and straightforward and a variety of autotrophic and dominant drug selectable markers (G418, uracil, thymidine, blasticidin, and hygromycin) are available to construct multiple gene knockout strains and/or to express proteins and reporter constructs. *Dictyostelium* cells move rapidly (averaging ~10 μm/min), in a manner that closely resembles movement of mammalian neutrophils. They are flat and relatively big (~20 μm), making them accessible for *in vivo* cell imaging. Upon starvation, *Dictyostelium* cells develop to form multicellular structure, allowing morphogenetic cell movements to be studied. Its small (34 Mbp) haploid genome has been completely sequenced, aligned and annotated (available at [www.ddictybase.org](http://www.ddictybase.org)) and harbors many genes homologous to those found in higher eukaryotes (Eichinger et al., 2005). The online database greatly facilitates collaborations and resource sharing, with a web-based ordering system for vectors, strains, and reporters from a central stock (Kreppel et al., 2004). In addition, *Dictyostelium* grow and divide rapidly, so that up to \(10^{12}\) clonal cells can be obtained in just few days for various biochemical and protein purification assays. For all above reasons, *Dictyostelium* is perfectly poised for studies of fundamental cellular processes such as chemotaxis, as well as cytokinesis, basic cell motility and development.
1.3 Directional Sensing and Current Models

Chemotaxis is a highly coordinated and dynamic response involving three distinct processes: directional sensing, cell polarization and cell motility (Chung et al., 2001a; Devreotes and Janetopoulos, 2003). In addition, cellular adhesion may also participate in this type of amoeboid crawling, in part through the regulation of small GTPase Rap1 and its downstream effectors (Jeon et al., 2007a; Kinashi and Katagiri, 2005). For decades, we have known that cells have the ability to sense asymmetric extracellular cues and generate amplified responses, as visualized by GFP-fused signaling and cytoskeleton proteins as biosensors (Haugh et al., 2000; Jin et al., 2000; Meili et al., 1999; Parent et al., 1998; Sasaki et al., 2004; Servant et al., 2000). The directional sensing response does not require cellular polarization and also occurs in mobility-disrupted cell treated with F-actin polymerization inhibitors such as latrunculin. These studies suggested that eukaryotic cells possess a spatial sensing mechanism that is independent of either cell polarization or motility. This spatial sensing mechanism requires the cell to constantly measure the receptor occupancy across the entire perimeter of the cell, with measurements occurring continuously as a cell migrates up a chemoattractant gradient. In addition, the chemoattractant-induced response of gradient sensing is highly adaptive. For both Dictostelium and mammalian neutrophils, cells rapidly respond to changes in receptor occupancy while they quickly adapt when occupancy is held constant (Dinauer et al., 1980; Lauffenburger et al., 1983).

The signaling network regulating chemotaxis was first understood in bacterial cells, where only 6 proteins are involved (Baker et al., 2006). These prokaryotic cells use the temporal strategy alone to sense the right direction by comparing the concentration of
chemoattractant at one time with that of a moment before. Because of their cell sizes of 1-2 µm, it is likely that they are too small to process spatial information. Cells randomly walk, frequently tumble, and move in a new random direction when the surrounding concentration of chemoattractant keeps constant. Tumbling frequency is reduced and movement in a certain direction is reinforced once cells experience an increase in chemoattractant concentration, i.e. cells move up the gradient (Bourret and Stock, 2002). For eukaryotic cells, the detailed temporal sensing mechanism is still elusive. Some recent studies suggest that chemotaxis in shallow gradients might be achieved by temporal and not spatial mechanisms (Andrew and Insall, 2007; Miyanaga et al., 2007; Van Haastert and Postma, 2007). In this scenario, chemotaxis is mediated by biased choices between random pseudopod extension that is a stochastic process and is uncoupled from the chemoattractant gradient. As a result, one pseudopod experiencing increased concentration of attractant would be extended and reinforced while the other pseudopod experiencing decreasing concentration of attractant extinguished. Such a temporal sensing mechanism is also suggested in Dictyostelium chemotaxis towards folic acid (Janetopoulos and Firtel, 2008).

Eukaryotic chemotaxing cells are able to take advantage of the strategy of spatial sensing because these cells are about 10 times bigger than bacteria. These cells are able to compare chemoattractant concentrations across the cell surface, point themselves towards the highest concentration, and move directly up the gradient. A number of mathematical models have been proposed to explore different mechanisms for gradient sensing and spatial localization in eukaryotic chemotaxis, which in turn guide more experiments (Iglesias and Devreotes, 2008; Janetopoulos and Firtel, 2008). A common mechanism is
based on a local excitation, global inhibition (LEGI) principle (Kutscher et al., 2004; Parent and Devreotes, 1999). Briefly, receptor occupancy triggers a rapid, local excitatory signal as well as a slower, global inhibitory signal. The activator binds to the plasma membrane at a rate proportional to the local degree of receptor activation, leading to more activators bound at the front than at the rear of the cell in relative to the chemoattractant source. On the other hand, the global inhibitor is proportional to the average concentration of receptor occupancy across the length of the cell. The difference between these two processes controls the overall cellular response (Figure 1).

Qualitatively, the LEGI model can account for the observed responses of most signaling and cytoskeletal proteins that have been shown in neutrophils and Dictyostelium cells in response to a uniform stimulus or a chemoattractant gradient. However, by itself, it cannot explain the amplification from the external shallow gradient to the internal highly polarized response. A two-LEGI model, therefore, is proposed in which parallel local excitation, global inhibition mechanisms control the membrane binding of PI3K and PTEN at the front and rear, respectively, of a chemotaxing cell (Ma et al., 2004). The activation of these enzymes induces an amplified PIP3 response that agrees quantitatively with experimentally obtained plekstrin homology (PH)-GFP accumulation in latrunculin-treated cells. In addition, it is likely that LEGI based models may also rely on the robust internal positive feedback loops which could provide a full explanation of the responses observed in polarized cells (Sasaki et al., 2004; Weiner et al., 2002). Experimental observations that PI3K localization is severely inhibited in latrunculin treated cells and that posterior-localized PTEN in a highly polarized cell does not dissociate upon the uniform stimulation suggest that the cytoskeleton may stabilize and reinforce the
localization/activation of PI3K and PTEN, probably through a positive feed back loop from cytoskeletal proteins.

![Diagram of localization/activation](image)

**Figure 1.** Local excitation, global inhibition (LEGI) model of directional sensing (adapted from Iglesias and Devreotes, 2008)

All LEGI models proposed to date rely on the existence of one or more “global inhibitor”, which is assumed to be a rapid diffusing protein or a small molecule either on the membrane or in the cytoplasm (Kutscher et al., 2004; Postma et al., 2004). Despite
intense research during the past decade, the biochemical identification of such hypothetic inhibitor(s) is still lacking. Therefore, a number of alternative models for eukaryotic chemotaxis not involving global inhibitors have also been put forward (Meier-Schellersheim et al., 2006; Postma and Van Haastert, 2001; Schneider and Haugh, 2005). One model based on neutrophils gradient sensing and polarization proposed a coincidence circuit involving PI3K and Ras that obviates the need for the global inhibitors (Onsum and Rao, 2007). This model is able to give explanation on how “front” and “back” pathways are correctly localized in response to both uniform stimulus and gradients of chemoattractant, even when cells are treated with latrunculin. A “balanced inactivation” model also eliminates the need for the global inhibitors by proposing Gα and Gβγ subunits act as activator and inhibitor, respectively, although this scheme is contradicted by the current experimental findings (Levine et al., 2006). An additional appeal of this model is that a third component, a membrane-bound inactivator, is added that is mutually antagonistic to the response of activation. The addition of this component makes it possible to fully elucidate the switch-like behavior (i.e. the rapid reversal of the internal asymmetry) upon reversal of the external chemoattractant gradient direction, while other LEGI models are unable to address the same issue.

1.4 Introduction to Ras

*ras* was originally described as the cellular counterpart of the viral oncogene, *v-ras* (Shih et al., 1979). The Ras subfamily consists of small, monomeric GTPases that function as molecular switches in intracellular signal transduction and regulate a wide variety of cellular events including proliferation, differentiation, cell motility, cell
polarity and trafficking of vesicles and macromolecules (Campbell et al., 1998; Hancock, 2003; Shields et al., 2000). When bound to GDP, Ras proteins are in the inactive configuration. Extracellular signals stimulate Ras guanine nucleotide exchange factors (GEFs) that catalyze the exchange of GDP for GTP, resulting in a conformational change of Ras to the activated state (Boguski and McCormick, 1993; Mitin et al., 2005). Ras-GTP then interacts with one or more effectors, leading to activation of downstream signaling pathways, including mitogen-activated protein kinase (MAPK) cascades, PI3K-regulated pathways, and RalGDS/Ral pathway (Ehrhardt et al., 2002). In addition, Ras regulates the activity of small G proteins that belongs to the family of Rho, Rap, and Rab GTPase via their respective exchange factors, which have influences on cytoskeleton arrangement, cell adhesion, cell cycle, endocytosis and gene expression (Hall, 2005; Mitin et al., 2005). Ras proteins contain a C-terminal CaaX motif, where a cysteine is followed by two aliphatic residues and one random amino acid. This motif is targeted for enzymatic prenylation, which is required for Ras binding to the membrane. In order to achieve functional anchorage, H-Ras, N-Ras and K-Ras 4A require additional cysteine palmitoylation, whereas K-Ras 4B and M-Ras need to interact with the negatively charged head groups of membrane phospholipids through their polybasic motif (Hancock et al., 1989; Hancock et al., 1990).

As one of the most important Ras regulators, RasGEF was first characterized in yeast Saccharomyces cerevisiae, in which loss of cdc25 results in the growth arrest due to inhibition of Ras-induced adenylyl cyclase activity (Broek et al., 1987). Subsequently, Drosophila son of sevenless (Sos) and mammalian hSos1 were identified, each of which contains a catalytic domain related to that in cdc25 (Bonfini et al., 1992; Fath et al.,
Canonically, RasGEF forms complex with the adaptor protein Grb2, which is recruited by activated receptor tyrosine kinase (RTK) to the plasma membrane in presence of stimuli. This recruitment brings RasGEF to the proximity of Ras to activate it (Aronheim et al., 1994). Other RasGEFs, such as the Ras-GRF family containing EF hands, are able to activate Ras in response to calcium and DAG signaling (Farnsworth et al., 1995). It appears that the domains surrounding the RasGEF catalytic domain are critical for their potential roles in mediating membrane localization and activation.

Ras signaling in mammalian cells is extremely complicated, involving 36 Ras subfamily proteins that fall into five subgroups including p21 Ras, R-Ras, M-Ras, Rap, and Ral (Wennerberg et al., 2005). Although functional redundancy within the Ras subfamily has been speculated, it is becoming increasingly evident that different Ras proteins may have distinct functions that depend on not only differences in their affinities to regulators or effectors but also their precise subcellular localization (Ehrhardt et al., 2002). For example, K-Ras 4B is activated exclusively at the plasma membrane while H- and N-Ras are activated not only at the plasma membrane but also at internal membranes such as ER and Golgi (Choy et al., 1999). Studies have shown that Ras proteins interact dynamically with specific microdomains on the membrane, which may compartmentalize different Ras isoforms and provides signal specificity. It has been found that H-Ras resides in lipid rafts whereas K-Ras 4B is excluded from rafts and localizes to disordered plasma membrane regions, which might explain why H- and K-Ras attain different preference to activate downstream effectors in vivo, but not in vitro (Prior et al., 2001).

Ras regulates normal and cancer cell motility in fibroblasts and epithelial cells and overexpression of oncogenic Ras induces rearrangement of the actin cytoskeleton
(Oxford and Theodorescu, 2003). In PDGF-stimulated chemotaxis, fibroblasts overexpressing a dominant negative mutant of Ras or RasGAP display suppressed chemotaxis while cells overexpressing Ras guanine releasing factor (GRF) or constitutively active Ras also fail to chemotax towards PDGF (Kundra et al., 1995). Targeted deletion of oncogenic K-Ras or expression of dominant negative H-Ras^{S17N} reduces cellular motility in some carcinoma cells (Pollock et al., 2005; Ueoka et al., 2000). In breast epithelial cells, activated R-Ras and TC21 stimulate cell migration through PI3K- and PKC-dependent pathways (Keely et al., 1999). Ras inhibitor S-trans, 

$tans$-farnesylthiosalicylic acid (FTS) can prevent the transformation of human glioblastoma multiforme cells by inhibiting their migration as well as their anchorage-independent proliferation (Goldberg and Kloog, 2006). However, so far only a limited number of studies have investigated the role of Ras in regulating amoeboid-type chemotaxis, cell polarization, and directional sensing. Questions like where Ras is activated in a chemotaxing cell, and how Ras activates downstream effector to mediate directional movement, are yet to be answered. Fortunately, recent studies performed in Dictyostelium have shed light on these important issues.

1.5 RasGAPs

Ras GTPase activating proteins (RasGAPs) negatively regulate Ras signaling by accelerating the otherwise low intrinsic GTPase activity of Ras by 5 orders of magnitude, converting the conformation from the active GTP-bound form back to the inactive GDP-bound form. Some early studies also suggest that RasGAP may function as a Ras effector in addition to a Ras signal terminator (Duchesne et al., 1993; Martin et al., 1992; Yang et
Mammalian RasGAPs consist of p120GAP, NF1, the Syn GAP family, and the GAP1 family, all of which contain a common ~250 amino acid RasGAP catalytic domain. p120GAP, the first GAP identified, provided important biochemical explanations for why mis-sense mutations at residues G12 and Q61 result in constitutive Ras activation. Structural studies of Ras-p120GAP complex demonstrate that the invariant arginines Arg789 and Arg903 in p120GAP are critical for the interaction between RasGAP domain and GTP-bound Ras, and the acceleration of GTP hydrolysis. When Ras-p120GAP complex forms, Arg789 points into the active site and neutralizes the negative charges of the γ-phosphate group, and by means of the finger loop, anchors Gln61 of Ras. Arg789 also interacts with Gly12 of Ras through van der Waals forces. If Gly12 is mutated to any other amino acid, this would lead to steric clashes. Another invariant residue, Arg903 further stabilizes the finger loop of Arg789 (Scheffzek et al., 1997). It is of note that even the relatively conservative mutation of arginine at 789 to lysine would have a detrimental effect on the catalysis in Ras proteins.

In contrast to RasGEFs, there is considerably less information regarding the mechanisms that regulate RasGAP activity via extracellular signals, and it has been generally thought that RasGAPs are constitutively active. However, the presence of a variety of modular domains within different RasGAPs implies that these proteins are subject to a diverse array of cellular interactions and regulations. CAPRI, a RasGAP that contains a N-terminal C2 domain and a C-terminal PH domain in addition to the RasGAP domain, is normally cytosolic and inactive. It rapidly translocates to the plasma membrane in a C2-domain-dependent manner, when Ca^{2+} levels are elevated. This translocation subsequently activates the RasGAP activity of CAPRI and leads to
decreased Ras activity (Lockyer et al., 2001). In another example, annexin A6, a Ca\(^{2+}\)-dependent membrane binding protein, forms complex with p120GAP to promote p120GAP-Ras assembly on the plasma membrane, leading to the inactivation of Ras and downstream Raf-MAPK pathway (Grewal et al., 2005). Therefore, as compartmentalization of different Ras isoforms ultimately contributes to the specificity of Ras signaling, differential association of various RasGAPs with distinct signal transducing complexes at different subcellular locations may lead to specific inactivation of individual Ras isoforms (Bernards and Settleman, 2004).

1.6 Neurofibromin (NF1)

One intensively studied RasGAP protein is neurofibromin (NF1), the protein product of the tumor suppressor gene \(Nf1\), alterations of which are responsible for the pathogenesis of von Recklinghausen’s neurofibromatosis, a common genetic disorder with a predisposition to tumor formation (Lee and Stephenson, 2007; Trovo-Marqui and Tajara, 2006). Neurofibromatosis has a \textit{de novo} incidence of one in 3500 individuals without a special predilection for sex or race. NF1 is ubiquitously expressed but most abundant in neurons, Schwann cells, astrocytes, oligodendrocytes and leukocytes (Daston et al., 1992; Gutmann et al., 1991). It has been found in keratinocytes and melanocytes as well (Malhotra and Ratner, 1994). The subcellular localization of NF1 is still inconclusive. It has been mostly reported in the cytoplasm but also found in mitochondria, nucleus, and endoplasmic reticulum (DeClue et al., 1991; Nordlund et al., 1993; Roudebush et al., 1997; Vandenbroucke et al., 2004).
A central region of ~360 amino acids in NF1 is identified as a RasGAP catalytic domain, based on its significant homology to the GAP domain of mammalian p120GAP, yeast IRA1/2, and *Drosophila* GAP1 (Scheffzek et al., 1997). Structural and biochemical data have shown that Arg1276 and Arg1391 within the RasGAP domain of NF1, which correspond to Arg789 and Arg903 in p120GAP, are essential for the interaction with p21 Ras as well as the acceleration of GTPase activity (Scheffzek et al., 1998). The replacement of Arg1276 by alanine or proline causes a drastic reduction in GAP activity by 3-4 orders of magnitude with no substantial influence on the binding affinity of NF1 to Ras (Klose et al., 1998; Sermon et al., 1998). The lack or mutation of NF1 leads to elevated Ras activity, followed by upregulation of various Ras effectors. Disrupted RasGAP function has been characterized as the major cause for increased cell proliferation, cell survival, tumor formation and other clinical manifestations in NF1 patients.

Although studies have shown that NF1 recognizes and binds to different Ras isoforms without any preference *in vitro, in vivo* targets of NF1 are likely specified and it is tempting to speculate that NF1 control the activity of different Ras isoform(s) in distinct tissues with certain extent of overlapping (Rey et al., 1994). As a matter of fact, it has been shown that NF1 preferentially inactivates K-Ras in mast cells and in astrocytes (Dasgupta et al., 2005a; Khalaf et al., 2007).

In addition to the RasGAP catalytic domain, NF1 contains a putative lipid-binding Sec14 domain and a PH-like domain (D'Angelo et al., 2006). The Sec14 domain is homologous to the yeast Sec14p, whose function is to regulate intracellular proteins and lipid trafficking (Mousley et al., 2006). Sec14 and PH-like domains presumably mediate
the membrane anchorage of NF1 but the biological evidence is significantly lacking. It has been reported that Sec14-PH binds to phosphorylated PI with the preference for the mono-phosphorylated head group through *in vitro* lipid binding assays while more recent studies suggest that Sec14-PH binds cellular glycerophospholipids (D'Angelo et al., 2006; Welti et al., 2007).

Two independent studies have showed that the mTOR pathway is tightly regulated by NF1 (Dasgupta et al., 2005b; Johannessen et al., 2005). mTOR is constitutively active in NF1-deficient cells in a Ras- and PI3K-dependent manner. This aberrant activation is mediated by the phosphorylation and inactivation of tuberin by PKB. A recent report from the same group further demonstrates that TORC1/mTOR activity is essential for NF1-associated tumorigenesis (Johannessen et al., 2008). Based on these studies, the potential application of rapamycin in treating NF1 tumors is suggested.

In addition to Ras-mediated function, NF1 plays an important role in regulation of the heterotrimeric G protein stimulated AC/cAMP/PKA pathway (Trovo-Marqui and Tajara, 2006). The small size defect associated with *Nf1*-deficient *Drosophila* can be rescued by over-expression of activated protein kinase A (PKA) but not by attenuating Ras1 activity (The et al., 1997). Furthermore, the expression of human NF1 can rescue the cAMP-related phenotypes of these mutant flies (Tong et al., 2002). Evidence is also presented that NF1 regulates the activity of adenylyl cyclase in mammalian cells (Dasgupta et al., 2003). Yeast IRA1 and IRA2 are homologues of NF1 and studies have shown that the kelch protein Gpb1/2 interacts with IRA1 and IRA2 through their
RasGAP domains. Loss of Gbp1/2 results in destabilization of IRA1/2, leading to enhanced Ras2 activity and unbridled PKA signaling (Harashima et al., 2006).

It has been shown clinically that malignant peripheral nerve sheath tumors (MPNSTs) transformed from plexiform neurofibroma are highly aggressive and frequently metastasize (Ferner et al., 2007). Some studies based on cell cultures start to reveal NF1’s function in cell motility. Nf1+/− astrocytes exhibit decreased cell attachment and increased cell motility (Gutmann et al., 2001). The same group later found that Nf1−/− astrocytes have fewer actin stress fibers and also exhibit increased cell motility in comparison with wild-type cells, in an mTOR-dependent manner. The phenotypes can be rescued by pharmacologic and genetic mTOR inhibition. Further studies implicate the involvement of a rapamycin-sensitive Rac1 and a critical mTOR effector, nucleophosmin, in the regulation of actin stress fiber formation and motility (Sandsmark et al., 2007). Huang and colleagues showed that Schwann cells from Nf1-deficient mice exhibit enhanced migration through an up-regulation of R-Ras2 and downstream PI3K (Huang et al., 2004). Mast cells in Nf1+/− mice exhibited increased motility through hyperactivation of the Ras-PI3K-Rac2 pathway in response to Kit ligand (kitL), which is secreted by homozygous Nf1 mutant Schwann cells (Yang et al., 2003). Ozawa and colleagues proposed that neurofibromin plays an important role in cell motility by regulating the dynamics and reorganization of actin filaments via the Rho-ROCK-LIMK2-cofilin pathway in a Ras-dependent manner (Ozawa et al., 2005). NF1 was also reported to interact with syndecan, a transmembrane coreceptor, although the biological relevance was unclear. Recent studies by Lin et al. show that NF1 mediates the syndecan signal to PKA, which subsequently phosphorylates vasodilator-stimulated
phosphoprotein (VASP), inducing F-actin polymerization and promoting filopodia formation in neurons (Lin et al., 2007).

In addition, it has been known for more than a decade that NF1 associates with cytoplasmic microtubules, with the region critical for the interaction being within the RasGAP domain (Gregory et al., 1993; Xu and Gutmann, 1997). Selected mutations of several highly conserved residues in the RasGAP domain impair microtubule association, which are probably responsible for the learning disabilities and cognitive problems. It was suggested that NF1 might interact with certain proteins that are also present on the microtubule to fulfill its function; however, the biological significance of the association has yet to be characterized.

Researchers have begun to unveil the mechanisms of how NF1 is regulated. Feng et al. showed that phosphorylation of a C-terminal region of NF1 followed by 14-3-3 interaction negatively regulates its RasGAP function (Feng et al., 2004). Mangoura and colleagues have shown that PKC phosphorylation of NF1 in neurons and astrocytes, prominently within the cysteine-serine-rich domain (CSRD) upstream of RasGAP domain in the N-terminus, leads to enhanced NF1 RasGAP activity and induces increased association with actin (Mangoura et al., 2006). It is proposed that in response to pleiotropic growth factors, PKC-dependent phosphorylation of NF1 may serve as a part of feedback mechanism to attenuate Ras signaling, resulting in a shift of cellular responses from cell proliferation to cell migration. NF1 is also regulated by posttranslational degradation. Cichowski and colleagues found that stimulation of cells with various growth factors results in a rapid degradation of neurofibromin via the ubiquitin-proteasome pathway. The authors reasoned that the efficient degradation of
NF1 under mitogenic conditions activates p21-Ras and induces proliferation (Cichowski et al., 2003).

Studies using mouse models of NF1 are beginning to unravel the mechanisms that underlie the various symptoms associated with the disease, including abnormalities in cellular differentiation, tumor formation and learning deficits. Homozygous Nf1-mutant mice display heart malformation and hyperplasia of sympathetic ganglia, and die at E13.5 (Brannan et al., 1994). Heterozygous Nf1-mutant mice are viable, but show an increasing incidence of tumor formation with age (Jacks et al., 1994). Moreover, Nf1+/- mice carrying a homozygous mutation in Schwann cells develop neurofibromatosis due to massive mast cell infiltration into nerve tissues, consistent with studies using tissue culture (Zhu et al., 2002). Heterozygous Nf1-mutant mice develop skin pigmentation abnormalities and papillomas, even in the resistant genetic background, when challenged with either a skin-cancer initiator or a tumor promoter (Atit et al., 2000). Several studies have shown that loss of RasGAP function underlies the learning deficits associated with NF1 (Costa et al., 2001; Silva et al., 1997). Genetic manipulations that reduce Ras activity or using a farnesyl-transferase inhibitor (FTI), which indirectly attenuates the active Ras, can reverse/rescue the learning disabilities (Costa et al., 2002). Furthermore, studies in Drosophila suggest that the malfunction of NF1 to regulate AC might also contribute to the cognitive dysfunction that requires a loss of heterozygosity (LOH) (Guo et al., 2000).

1.7 Dictyostelium Ras proteins
Surprisingly, *Dictyostelium* Ras GTPase subfamily is unexpectedly large, including 11 Ras, 3 Rap and one Rheb-related proteins (Kortholt and van Haastert, 2008). Evolutionarily, it is unlikely that most *Dictyostelium* Ras proteins are functionally redundant. One speculation for this unusually large number would be that a lack of certain types of signaling components in *Dictyostelium* might have necessitated an expansion and specialization of Ras GTPases to obtain the required signaling complexity. So far six ras subfamily genes: *rasB, rasC, rasD, rasG, rasS* and *rapA*, have been characterized (Lim et al., 2002). Table 1 summarizes their functions, mutant phenotypes, regulators, and effectors. RasB, RasD, and RasG are the closest relatives to human H-Ras, K-Ras and N-Ras and have conserved effector domains, while Rap1 is remarkably similar (76%) to human Rap1A. RasS and RasC are more divergent with differences in the effector-binding domain compared to H-Ras. Each member of Ras subfamily appears to have highly specific cellular functions, although some overlap (e.g. among RasG, RasD and RasB proteins) has been suggested (Khosla et al., 2000). All forms of *Dictyostelium* Ras identified lack a palmitoylation site; instead, they have polybasic domains at the C-terminus near the CaaX motif. It has been found that RasG uniformly distributes along the plasma membrane through prenylation and polybasic domain anchorage, in a manner similar to human K-Ras 4B localization (Sasaki et al., 2004).

The mechanism of Ras activation is largely unknown in *Dictyostelium*, but Ras proteins are apparently downstream of heterotrimeric G proteins, since Ras activation is insensitive to cAMP stimulation in mutants lacking Gβ or Gα2. In addition, Ras activation must be catalyzed by GEF proteins. There are at least 25 putative RasGEF genes encoded by the *Dictyostelium* genome, only 5 of which have been studied to some
extent. It has been suggested that each RasGEF has a precisely defined role. Such a large number of RasGEF proteins could maintain the complexity of Ras signaling in the absence of receptor tyrosine kinases (RTKs) in Dictyostelium, which are the major upstream controller for Ras signaling in metazoans. Mutant studies show that cells lacking RasGEFA fail to aggregate upon starvation and are unable to synthesize and respond to cAMP, phenocopying rasC cells, indicating a linear pathway from RasGEFA to RasC to regulate activation of adenylyl cyclase. In a recent report, both in vitro and in vivo evidence suggests that RasGEFA specifically activates RasC, while RasGEFR and other unknown RasGEF(s) are necessary for the maximal activation of RasG upon cAMP stimulation (Kae et al., 2007). Two cyclic guanosine monophosphate binding proteins GbpC and GbpD also contain RasGEF domains. GbpD appears to be required for maximal Rap1 activation, while the target of GbpC remains unknown.

As the first identified Ras protein, RasD was linked to the regulation of phototaxis and thermotaxis of multicellular slugs (Wilkins et al., 2000b). RasS may control the balance between feeding and movement by regulating macropinocytosis (Chubb et al., 2000). It is suggested that RasGEFB acts as an exchange factor for RasS as gefB− cells exhibit several phenotypes reminiscent of those of rasS− cells, including impaired growth, reduced macropinocytosis, and enhanced motility (Chubb et al., 2002; Wilkins et al., 2000a). It has been proposed that RasB plays a role in the regulation of mitosis. RasB was reported to localize in the nucleus except during mitosis/cytokinesis (Sutherland et al., 2001). Recent studies have suggested that RasB regulates processes requiring myosin II, including cell motility and cytokinesis, presumably mediated by MHCKA. Overexpressing RasGEFQ activates RasB, causes enhanced recruitment of MHCKA to
the cortex, and leads to cytokinesis defects in suspension, phenocopying cells expressing constitutively active RasB, and myoII mutants (Mondal et al., 2008). Multiple attempts to generate stable rasB’ cells have failed, indicating that rasB is an essential gene. Studies have underlined the important role of Dictyostelium Rap1 in cytoskeletal rearrangement, phagocytosis, adhesion and chemotaxis (Jeon et al., 2007a; Kang et al., 2002; Rebstein et al., 1997). Both biochemical and genetic evidences indicate that Rap1 executes its functions, at least partially by regulating myosin II assembly/disassembly through its downstream effector Phg2 and MHCKA (Jeon et al., 2007a). Recently, two upstream regulators of Rap1 have been identified, including cGMP-binding protein D (GbpD) as the exchange factor and RapGAP1 as the GTPase activating protein (Bosgraaf et al., 2005; Jeon et al., 2007b; Kortholt et al., 2006).

Other studies have revealed that two well-studied RasGTPases, RasC and RasG, are involved in different responses generated from the cAMP stimulus. RasC is predominantly involved in the activation of adenylyl cyclase and hence the cAMP relay, whereas RasG is important for PIP3 signaling and hence essential for cell polarity and actin polymerization (Kortholt and van Haastert, 2008). Recently, Kaminmura and colleagues identified RasC as the major but not exclusive activator of target of rapamycin complex 2 (TORC2) (Kamimura et al., 2008). Figure 2 illustrates the cAMP-induced Ras signaling that occurs at the leading edge of chemotaxing cells.

Sasaki and colleagues have shown that RasG, in part, is required for PI3K activation in Dictyostelium, while cells expressing a mutated form of PI3K carrying a point mutation in the RBD that disrupts Ras-GTP binding is unable to activate PI3K-mediated responses (Funamoto et al., 2002; Sasaki et al., 2004). Parallel studies also
show that mammalian PI3Ks are directly activated both *in vitro* and *in vivo* by Ras (Pacold et al., 2000; Suire et al., 2002). Recent studies have further disclosed that input from Ras signaling is required for maximal PI3K activity in neutrophils and *Drosophila* (Orme et al., 2006; Suire et al., 2006).

In response to cAMP, RasG activation is rapidly stimulated, peaking at ~3-5 sec., which is the most rapid signaling event reported to occur downstream of the heterotrimeric G proteins (Kae et al., 2004; Sasaki et al., 2004). This kinetics of activation and subsequent adaptation are consistent with those of cAMP-stimulated PI3K activity. Activated Ras (predominantly RasG) is restricted to the leading edge of the cell in chemoattractant gradients, which is known as the earliest polarized cellular response, suggesting its instructional role in transducing shallow extracellular gradients into sharp intracellular responses (Sasaki et al., 2004). Depletion of Ras signaling through overexpression of a dominant negative RasG<sup>517N</sup> mutant in a background lacking RasGEFA, results in severe defects in directionality and cell polarity, hinting that Ras may be involved in directional sensing (Sasaki et al., 2004). RasG is activated in presence of the PI3K inhibitor, LY294002, and in *pi3k1/2* null cells, consistent with Ras activation being initiated in the absence of PI3K signaling. A positive feedback loop, involving PIP3-mediated actin polymerization, recruitment of additional cytosolic PI3Ks, and Ras activation, has been suggested as an important mechanism for initial signal amplification and leading edge stabilization during chemotaxis (Sasaki et al., 2004). Such a positive feedback circuit is also employed by other fundamental cellular processes including random cell migration and cytokinesis in a heterotrimeric G protein independent manner, as suggested by Sasaki and colleagues (Sasaki et al., 2007).
Table 1. Six characterized Dictyostelium Ras proteins in regulating cytoskeleton-dependent processes

<table>
<thead>
<tr>
<th></th>
<th>RasB</th>
<th>RasC</th>
<th>RasD</th>
<th>RasG</th>
<th>RasS</th>
<th>Rap1</th>
</tr>
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<tbody>
<tr>
<td>Function</td>
<td>Myosin II assembly;</td>
<td>ACA activation; PKB activation; cAMP relay;</td>
<td>Phototaxis;</td>
<td>PIP3 signaling;</td>
<td>Endocytosis; Motility</td>
<td>Myosin II assembly;</td>
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<td></td>
<td>Cytokinesis; Motility</td>
<td>Chemotaxis</td>
<td>Thermotaxis</td>
<td>F-actin polymerization; Directional</td>
<td></td>
<td>Cell adhesion; Chemotaxis</td>
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<td>sensing; Chemotaxis</td>
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<td>Expressing constitutively</td>
<td>Cytokinesis</td>
<td>Cell flattened, Motility -</td>
<td>Aberrant</td>
<td>Cell flattened; many filopodia;</td>
<td>ND</td>
<td>Cell flattened and</td>
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<td>active mutant</td>
<td>defect</td>
<td></td>
<td>development</td>
<td>Chemotaxis</td>
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<td>chemotaxis-</td>
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<td>Knockout mutant</td>
<td>Cell line unstable</td>
<td>Motility - ACA activation - Developmental</td>
<td>Slug phototaxis and thermotaxis</td>
<td>Chemotaxis - Cell growth - Cytokinesis and developmental defects</td>
<td>Motility + Endocytosis - Cell growth -</td>
<td>ND</td>
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<td>defects</td>
<td>defects</td>
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<tr>
<td>Regulator</td>
<td>RasGEFQ, DdNFI?</td>
<td>RasGEFA (aimless)</td>
<td>ND</td>
<td>RasGEFR, DdNFI;</td>
<td>RasGEFB</td>
<td>GbpD, RapGAPA</td>
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<tr>
<td>Effector</td>
<td>MHCKA</td>
<td>TORC2(RIP3)</td>
<td>ND</td>
<td>PI3K, RIP3?</td>
<td>ND</td>
<td>Phg2</td>
</tr>
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ND: not determined; ?: speculated; +: increase; -: decrease
Figure 2. Ras signaling at the leading edge of a chemotaxing cell
TORC2 turns out to be another essential downstream effector under Ras signaling in *Dictyostelium*, mediated via Ras-interacting protein-3 (RIP3, a homolog of AVO1/hSIN1) (Kamimura et al., 2008; Lee et al., 2005; Lee et al., 1999). Expression of RIP3 carrying a mutation in RBD that abrogates binding to Ras-GTP is unable to complement the chemotactic and developmental defects of *rip3* cells, indicating that Ras regulates TORC2 activation (Lee et al., 2005; Lee et al., 1999). Knocking out TORC2 components, including RIP3, Pianissimo (a homolog of Rictor/mAVO3), or Lst8/Gbl, generates similar phenotypes of defective cell polarity and significantly reduced chemotactic speed. These studies suggested TORC2 as a distinct Ras-mediated chemotactic pathway in addition to PI3K pathway. It is assumed that RIP3 prefers to bind RasG-GTP over other Ras isoforms, based on the yeast two-hybrid data; however, the inability of *rip3* cells to activate adenylyl cyclase upon starvation indicates a possible linkage between RasC and TORC2. Studies in mammalian cells suggest that TORC2 is a *bona fide* complex that phosphorylates Ser473 in the C-terminal hydrophobic motif of PKB (Hresko and Mueckler, 2005; Sarbassov et al., 2005). Intriguingly, *Dictyostelium* PKB activity is severely reduced in the cells with either RIP3, Pianissimo, or Lst8 disrupted, indicating an evolutionarily conserved role of TORC2 in regulating PKB through phosphorylation at Thr435 (equivalent to Ser473 in mammalian PKB). Measurement of Thr435 phosphorylation provides a valuable readout to assess the Ras signaling input to TORC2 pathway. Recent work by Kamumira et al. demonstrated that cytosolic TORC2 is activated via binding to locally activated small G protein(s) at the leading edge of chemotaxing cell, and phosphorylates PKBA and PKBR1 in a PIP3-
independent manner, followed by the phosphorylation of a series of signaling and cytoskeletal proteins that participate in regulation of chemotaxis (Kamimura et al., 2008).

*rasG* cells exhibit reduced polarity and motility during chemotaxis in gradients of folate and cAMP (Tuxworth et al., 1997). Cells overexpressing constitutively active RasG, RasG<sup>G12T</sup>, exhibit reduced motility during random movement and chemotaxis (Khosla et al., 1996). It is interesting that the disruption of *rasG* and the expression of constitutively active RasG both inhibit motility and chemotaxis, indicating a precise dynamic regulation of RasG signaling is absolutely required for optimum control of chemotaxis.

RasC is essential for the cAMP relay, the process that allows cAMP-stimulated cells to transmit the signal to neighboring cells during *Dictyostelium* aggregation. *rasC* cells exhibit severely reduced ACA activity, and are unable to aggregate, a defect that can be recovered by exogenous application of a cAMP pulse (Lim et al., 2001). Vegetative *rasC* cells are less motile and chemotax poorly to folate, while cells overexpressing constitutively active RasC<sup>G13T</sup> are non-polarized and show severely reduced random motility (Lim et al., 2002). Early studies showed that the phosphorylation and activation of PKB upon cAMP stimulation in *rasC* cells is reduced, indicating that RasC has an input into PI3K (Lim et al., 2001). However, RasC seems not to be required for the generation of PH-domain recruitment sites, because GFP fusions of the PH-domains of PKB, CRAC and PhdA successfully translocate to the plasma membrane in cAMP-stimulated *rasC* cells. Recently, it has been found that the reduction of PKBA and PKBR phosphorylation and activation in *rasC* cells is PIP3-independent and results from decreased TORC2 activity in absence of RasC (Kamimura et al., 2008).
Interestingly, the phenotypes of both rasC and rasG cells are less severe than those of pi3k or pkbA null cells (Funamoto et al., 2001; Meili et al., 1999). It is reasoned that there may be some functional redundancy and that each Ras protein may compensate for the loss of the other. Consistent with this idea, rasG cells express elevated levels of RasC compared to those of wild-type cells (Kae et al., 2004). A recent study has shown that disruption of both rasC and rasG abolishes cAMP-mediated signaling, leading to much severer phenotypes so that cells are unable to aggregate, have no cAMP relay, and fail to chemotax towards cAMP (Bolourani et al., 2008).

1.8 Dictyostelium RasGAP

Thus far, there are no published reports that examine Dictyostelium RasGAPs. Two previously identified RasGAP-like proteins: DdGAP1 and DdGAPa, do not contain an arginine finger in their respective RasGAP domains, have no GAP activity, and turn out to be IQGAP-related proteins. These two IQGAPs modulate the F-actin cytoskeleton and control the cytokinesis and cell motility through interaction with activated Rac proteins.

Ras activation peaks at 3–5 sec followed by a rapid decline upon uniform chemoattractant stimulation. It is also observed that activated Ras at the leading edge of chemotaxing cell is rapidly deactivated when the chemoattractant is removed (Sasaki et al., 2004). Furthermore, cells expressing constitutively active RasG or RasC, show deficient cell motility and chemotaxis. These results indicate that the proper kinetics of Ras deactivation, presumably through RasGAP(s), is required for chemotaxis. To pinpoint the major Dictyostelium RasGAP and its substrate(s) will certainly broaden our
understanding on how integrated Ras signaling underlies such a sophisticated biological process as chemotaxis. Moreover, cells lacking RasGAP activity will provide a perfect experimental gain of function (GOF) system to appraise the potential role of Ras signaling in directional sensing as well as polarization and motility.

In this study, I demonstrate that the *Dictyostelium* RasGAP DdNF1, a homologue of human NF1, is required for the proper spatiotemporal regulation of chemoattractant-stimulated Ras activity, RasG in particular. Loss of DdNF1 leads to extended and non-spatially restricted Ras activity *in vivo*. Downstream PI3K activity and F-actin polymerization are upregulated and not temporally and spatially restricted to the side of the cell closest to the chemoattractant source, leading to severe directionality defects. Further, growing *nfaA* cells exhibit elevated Ras-GTP levels and display cytokinesis defects and enhanced random mobility, consistent with the model that a Gβ -independent Ras/PI3K/F-actin circuit regulates the cytokinesis and basic cell movement. These results lead to the conclusion that, spatial and temporal control of Ras activation through DdNF1 is an essential component of the regulatory circuit of the cell’s compass.
Chapter 2 Results

2.1 Identify Putative Dictyostelium RasGAPs Using a Bioinformatic Approach

13 genes encoding RasGAP consensus domain are found in the *Dictyostelium* genome using BLAST (Table 2). By constructing a phylogenetic tree using the conserved RasGAP domain of the 13 RasGAP candidates and that of human p120GAP and NF1, I have classified these into two subfamilies: 1) 7 putative *bona fide* RasGAPs (Figure 3A); and 2) 6 IQGAP-related proteins. Amino acid sequence alignment of the RasGAP domains shows that the 7 putative *bona fide* RasGAPs have both invariant arginine residues (i.e. Arg789 and Arg903 as in p120GAP) required for RasGAP activity while all IQGAP-related proteins are lacking the arginine finger (Figure 4). Therefore, the seven putative *bona fide* RasGAPs were chosen for the further studies.

I undertook a bioinformatic approach by examining the various motifs/domains that surround the RasGAP catalytic domain of the seven putative *bona fide* RasGAPs as these domains might provide important clues about potential regulatory mechanisms that control the activation and/or subcellular localization of the RasGAPs. Among the seven candidates, DdRasGAP_DDB0233756 and DdNGAP_DDB0220496 both contain a putative Ca$^{2+}$-dependent membrane-targeting C2 domain, while DdRasGAP_DDB0233497 has a RhoGEF catalytic domain, indicating the possible lipid binding or additional enzymatic function in these putative RasGAPs (Table 2). Single knockout strains of DdRasGAP_DDB0233756, DdNGAP_DDB0220496 and DdRasGAP_DDB0233762 were generated but no significant defects in cytokinesis,
development or chemotaxis were observed. Interestingly, GFP fused DdNGAP was located at the leading edge of chemotaxing cells, suggesting a possible role in directional sensing and/or leading edge formation. However, cells lacking DdNGAP chemotax normally, implying that other redundant RasGAP(s) may compensate the role of DdNGAP.

**Figure 3.** DdNF1 is a putative orthologue of the human RasGAP NF1. (A) Phylogenetic tree of 7 putative *bona fide* *Dictyostelium* RasGAPs (DdRasGAPs). DdNF1 is highlighted. (B) Comparison of protein domains between human NF1 (Hs_NF1) and DdNF1. RasGAP, Sec14 (Sec14 homology), and PH (pleckstrin homology-like) domains are shown.
Table 2. 13 Dictyostelium proteins containing RasGAP-related domain

<table>
<thead>
<tr>
<th>Subfamily</th>
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<th>SMART Diagram</th>
<th>Additional domain</th>
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<td>+</td>
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<td>in IQGAP-like proteins</td>
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Figure 4. Sequence alignment of the catalytic RasGAP domain of human p120GAP, NF1, and the 7 putative bona fide Dictyostelium RasGAPs. * in red and blue indicates the invariant arginines corresponding to Arg789 and Arg903 in p120GAP, respectively.
**Figure 5.** Disruption of the nfaA gene. (A) Schematic representation of the cloning strategy employed to disrupt the nfaA gene. (B) Southern blot of nfaA+ and wild-type parental genomic DNA. Genomic DNA from strains SZ15, SZ17 (nfaA+) and K-Ax3 (WT) were digested with EcoRI, separated on an 1% agarose gel, blotted onto NC membrane, and probed with the nfaA coding sequence (see panel A).
Of the most interest, DdNF1_DDB0233763 contains a Sec14 lipid-binding domain and a PH-like domain in addition to the RasGAP catalytic domain, which resembles the domain structure of neurofibromin and is hence suggested as a putative homolog of NF1 in *Dictyostelium*. DdNF1, however, lacks the long N-terminal region of neurofibromin, which lacks any known sequence motif but has been reported to be involved in the regulation of NF1 activity (Figure 3B).

2.2 Disruption of the *Dictyostelium nfaA* Gene

The *nfaA* gene was disrupted through the introduction of Blasticidin S-resistance gene (BSR) into the coding region of the RasGAP catalytic domain. Cells containing a disrupted *nfaA* gene were generated by homologous recombination by the strategy shown in Figure 5A. The knockout construct was transformed into wild type K-Ax3 cells, and clones were derived upon blasticidin selection. Southern blot results confirm that at least 2 independent clones (SZ15, SZ17) were positive as *nfaA* knockout strains and both were found to behave similarly (Figure 5B). Therefore, SZ17 was randomly chosen for all the work described here.

2.3 DdNF1 Regulates Cytokinesis

*nfaA* cells exhibit growth defects when cultured in suspension but not on a substratum. Mutant cells grow slowly in HL5 medium in suspension and cease growing at $5 \times 10^6$ cells/ml, a much lower final cell density than the parental wild type KAx-3 cells ($\sim 1 \times 10^7$ cells/ml) (Figure 6A). DAPI staining results show that more than 40% *nfaA* cells have >4 nuclei after 4 days of suspension growth, whereas *nfaA* cells grown on plates
maintain a wild type number of nuclei, which suggests normal karyokinesis but a defective cytokinesis in suspension culture (Figure 6B and 6C). This phenotype is similar to that of several Dictyostelium strains lacking structural proteins of the cytoskeleton or their regulators, including PKBR and Rac small GTPases (Robinson and Spudich, 2004; Rivero and Somesh, 2002). When these mutant cells are cultured on plates, they are able to divide by traction-mediated cytofission, a mechanism that allows Dictyostelium cytokinesis mutants to divide at nearly normal rates on a substratum (Fukui et al., 1990). Furthermore, cells expressing constitutively active RasB or RasG exhibit similar cytokinesis defects, implying that DdNF1 functions as an upstream inactivator of these Ras proteins to regulate cytokinesis (Lim et al., 2002; Mondal et al., 2008). Expression of myc-tagged DdNF1 under an actin15 promoter rescued the cytokinesis defects of nfaA− cells in suspension, suggesting that the phenotype mainly results from the lack of DdNF1.

2.4 Loss of DdNF1 Causes Delayed Aggregation

To investigate the effect of disrupting DdNF1 on multicellular development, nfaA− cells and wild type cells are plated on non-nutrient agar and their developmental programs are examined along the 24-hour time scale. A mild developmental delay of nfaA− cells during the aggregation stage between hour 4 and hour 10 is observed in comparison with wild type cells (Figure 7A). The mutant cells, however, are able to catch up in the developmental program, forming migration slugs and mature fruiting bodies promptly as do wild type cells. At a low cell density of $5 \times 10^5$ cells/cm$^2$, the delay seems much more significant as no aggregates are formed by ~ 6h for nfaA− cells (Figure 7B). It has been suggested that aggregation is mediated by both chemotaxis and cell-cell
adhesion. I reason that at high cell densities, cell-cell contacts may assist cells coalesce and form aggregates even when the mutant cells are unable to properly chemotax. In contrast, at low cell densities when the mutant cells distribute sparsely and are not touching each other, the aggregates can be formed only through inefficient chemotaxis and therefore cells exhibit stronger aggregation defects.

It is possible that the disruption of nfaA gene might affect the expression of early genes required for development and therefore indirectly result in the phenotype of delayed aggregation in nfaA− cells. In order to assess this possibility, I perform a RNA blot analysis examining the expression levels of the marker gene, cAMP receptor 1 (cAR1). cAR1 transcripts are induced normally in response to exogenous cAMP in nfaA− cells, indicating that the developmental defect of nfaA− cells arises from deficient chemotaxis due to the absence of DdNF1 (Figure 7C). Taken together, the delay at the aggregation stage of nfaA− cells implies a potential role of DdNF1 in regulating chemotaxis. nfaA− cells expressing myc-tagged DdNF1 exhibit no delay in aggregation stage and carry out a normal morphogenesis.
Figure 6. *nfaA* cells display cytokinesis defects. (A) Growth curves of suspension cell cultures of wild type, *nfaA*′, and *nfaA* cells expressing C-terminally myc-tagged DdNF1 (DdNF1-myc). Data represent mean ± SD of at least 3 independent experiments. (B) Number of nuclei per cell detected after 3, 4, and 5 days of growth in suspension, determined for at least 500 cells from 3 independent experiments. (C) Epifluorescent micrographs showing nuclei in cells grown in either suspension cultures for 4 days or on a Petri dish. Cells were stained with DAPI. Bar = 10 µm.
Figure 7. nfaA cells display delayed aggregation. Developmental phenotype of wild type and nfaA cells plated on non-nutrient agar at a cell density of (A) $2 \times 10^6$ cells/cm$^2$ (normal concentration) and (B) $5 \times 10^5$ cells/cm$^2$ (low concentration). Starvation time is indicated. (C) Northern blot analysis of cAR1 (cAMP receptor 1) gene expression in wild type and nfaA cells pulsed with cAMP for the indicated time, suggesting that the expression of developmental genes is unaffected by the disruption of nfaA.
2.5 DdNF1 Regulates Chemotaxis

To assess if DdNF1 plays a role in regulating *Dictyostelium* chemotaxis, presumably through its function as Ras inactivator, I studied the ability of *nfaA* cells to respond to and migrate in an exponential gradient of chemoattractant, as emitted by a micropipette filled with 150 μM cAMP, as well as in a linear gradient (0-1μM) produced in a Dunn chamber. Upon exposure to the cAMP released from a micropipette, wild-type cells respond by initially rounding up, a behavior associated with the reorganization of the actin cytoskeleton that disrupts the cell’s existing polarity (Van Haastert and Devreotes, 2004). This is rapidly followed by the extension of a pseudopod towards the micropipette. The cells then acquire an elongated morphology (polarization) and initiate directional migration up the gradient (Figure 8A). In contrast, *nfaA* cells assayed under the same condition display major polarity and chemotaxis defects, as indicated by reduced migration speed and directionality (Figures 8A and B). Although *nfaA* cells rapidly respond to the chemoattractant gradient by extending membrane protrusions/pseudopodia, in most cases these occur randomly relative to the axis of the gradient. It is calculated that the spatial gradients generated by a micropipette filled with cAMP in a pressurized system are proportional to 1/x², where x represents the distance from the pipette (Van Haastert and Postma, 2007). Therefore, it is deduced that the spatial gradient in the area close to the micropipette is steep and maintains an exponential shape, while the gradient in the area farther from the micropipette is shallow and close to linear. Correspondingly, I observe two types of cell behavior during the chemotaxis of *nfaA* cells: cells close to the needle can break their symmetry, but only after being exposed to the chemoattractant gradient for an extended time, and then move towards the
needle, albeit at a speed slower than wild-type cells and with reduced polarity (nfaA\(^{-}\) type 1); most cells farther from the micropipette do not polarize and move very little (nfaA\(^{-}\) type 2). Expression of myc-tagged DdNF1 in nfaA\(^{-}\) cells rescues these chemotaxis defects (Figure 8A).

To further dissect the aforementioned chemotaxis phenotypes, I count the number of pseudopodia extensions per 10 min during chemotaxis for wild-type, nfaA\(^{-}\) type 1 and type 2 cells, and categorize them in either forward or lateral/back pseudopodia based on the shape difference analysis (Figure 9A and C-E). More than 90\% of the pseudopodia generated in wild type cells extend towards the micropipette, with only occasional appearance of lateral/back pseudopod. In contrast, only \(~50\%\) and less than \(~30\%\) of the total pseudopodia extend up the gradient in nfaA\(^{-}\) type 1 and type 2 cells, respectively. The total number of pseudopodia extension in wild type cells is much less than that in nfaA\(^{-}\) cells (P<0.01), probably due to the frequent occurrence of random, lateral/back pseudopodia. I also measure the lifetime/persistence of these pseudopodia (Figure 9B). The majority of the pseudopodia (i.e. forward pseudopodia) in wild-type cells persist for more than 2 min whilst occasional lateral/back pseudopodia only last for \(~30\) sec. It is conceivable that forward pseudopodia are much more stabilized because they point in the right direction. Interestingly, for nfaA\(^{-}\) type 2 cells, the average lifetime of both forward pseudopodia and lateral/back pseudopodia is \(~40\) sec, indicating both kinds are composed of random pseudopodia regardless of the directions they extend in. Altogether, it appears that the deficiency of nfaA\(^{-}\) cells in chemotaxis is not because of the incapability of extending pseudopodia but due to the defects in deciphering the gradient of chemoattractant.
**Figure 8.** DdNF1 regulates chemotaxis. (A) Traces of representative chemotaxing cells in an exponential cAMP gradient delivered by a micropipette. Two types of chemotactic nfaA− cells are shown, type 1 and type 2, representative of cells in the steep and shallow parts of the gradient. (B) DIAS analysis of at least 10 traces from at least 3 independent experiments on cells migrating in an exponential gradient. Speed refers to the speed of the cell’s centroid movement along the total path; directionality indicates migration straightness; direction change refers to the number and frequency of turns; and roundness indicates the cell polarity. (C) Traces of cells chemotaxing in a linear gradient (Dunn chamber). The starting point of each migrating cell was apposed to the axis’ origin. A 6X close-up of the nfaA− cells’ traces near the origin is shown.
These chemotaxis defects become even clearer when analyzing the behavior of nfaA− cells placed in a linear chemoattractant gradient, as produced in a Dunn chamber. As depicted in Figure 8C, while wild-type cells become polarized and efficiently migrate up the chemoattractant gradient, the majority of nfaA− cells move completely randomly relative to the axis of the gradient, as if unable to sense its direction. One thing should be kept in mind is that using a Dunn chamber, it is impossible to examine the initial response of cells to the linear gradient because it takes ~10 min for the gradient to be established. These results suggest that DdNF1 regulates chemotaxis presumably via one or multiple Ras signaling pathways, and, therefore, nfaA− cells provide an ideal cellular context in which to assess the potential role of Ras signaling in directional sensing.

2.6 DdNF1 Temporally Regulates Chemoattractant-induced Ras Activity

To confirm whether DdNF1 functions as an authentic GAP to downregulate the chemoattractant-induced Ras activity, I assess the activation of Ras upon cAMP stimulation in nfaA− cells and compare it to that in wild type cells. In a pull-down assay using the Ras-binding domain (RBD) of human Raf1, I find that whereas the level of activated Ras (Ras-GTP) in wild type cells peaks at ~5 sec after cAMP stimulation and then rapidly declines, consistent with previous reports (Kae et al., 2004; Sasaki et al., 2004; Van Haastert and Postma, 2007), nfaA− cells display elevated basal levels as well as extended kinetics of cAMP-induced Ras-GTP (Figure 10A). The extended kinetics is confirmed by assessing the cAMP-induced translocation of a Ras-GTP reporter [RBD fused to GFP (GFP-RBD)] to the plasma membrane in live cells (Figure 10B). Uniform cAMP stimulation of wild type cells induces the translocation of the GFP-RBD reporter
from the cytoplasm to the plasma membrane within 3-5 sec, followed by its rapid re-localization to the cytoplasm at 10-15 sec after stimulation. In contrast, GFP-RBD remains localized to the plasma membrane of \( nfaA \) cells for more than 20-30 sec following cAMP stimulation. Both biochemical and live cell imaging data indicate that DdNF1 plays an important role in the rapid downregulation of chemoattractant-induced Ras activity. Note that RBD from human Raf1 shows high affinity for RasG and possibly other \textit{Dictyostelium} Ras isoforms but not RasC.

2.7 DdNF1 Spatially Controls Chemoattractant-induced Ras Activity

To determine whether the DdNF1-induced inactivation of Ras-GTP is also involved in the spatial regulation of Ras signal during chemotaxis, I examine and compare the localization of the GFP-RBD reporter in migrating wild-type and \( nfaA \) cells (Figure 10C). As described previously (Sasaki et al., 2004), upon exposure to chemoattractants released by a micropipette, wild-type cells initially produce a global response in which Ras is activated uniformly along the cortex (as observed by the uniform membrane localization of GFP-RBD), followed by global adaptation and the preferential activation of Ras at the site closest to the chemoattractant source, which subsequently becomes the leading edge. Then, Ras-GTP remains strictly restricted to the leading edge of wild type cells throughout their migration up the chemoattractant gradient. In contrast, \( nfaA \) cells, however, after the initial global activation, take a longer time to adapt than wild type cells. Subsequently, activated Ras does not display a similar restricted localization as seen in wild type cells, indicated by the constantly changing localization of GFP-RBD. Enrichment of Ras-GTP seems to occur at random locations.
along the plasma membrane of chemotaxing *nfaA* cells, resulting in pseudopodia/lamellipodia-like extensions at these sites. Moreover, the presence of activated Ras at a specific site on the membrane is transient, and sometimes multiple sites can be observed simultaneously. These observations indicate that DdNF1 assists to restrict Ras activity to the leading edge of chemotaxing cells by preventing Ras-GTP from accumulating at other sites along the plasma membrane, therefore forming a Ras-GTP gradient from the front of the cell to the back. Altogether, these unexpected findings imply that DdNF1 plays a crucial role in the spatial regulation of Ras activity during chemotaxis.

2.8 DdNF1 Regulates Chemotaxis through Downstream Target RasG

As mentioned above, *Dictyostelium* Ras proteins compose a large family. To identify which one is the potential substrate of DdNF1, I perform a small-scale yeast-two-hybrid assay between various constitutively active Ras mutants, including RasB, C, D, G and S, and the RasGAP domain of DdNF1, but no positive interaction is detected (Figure 11). One possible explanation for this is that when DdNF1 catalyzes the conversion from Ras-GTP to Ras-GDP, its interaction with Ras protein(s) is very transient and not sufficient to be identified in the two-hybrid assay.
**Figure 9.** *nfaA* cells produce more protrusions laterally or away from the chemoattractant source. (A) Number of pseudopodia extended per cell migrating for 10 min in an exponential cAMP gradient. (B) Lifetime of extended pseudopodia. The shades reflect the relative amount of pseudopodia that displayed a similar lifetime, with the darker color corresponding to the highest number. (C-E) Shape difference analysis between each frame of recordings of representative wild type or *nfaA* (Type 1 and 2) cells migrating in an exponential cAMP gradient. Protrusions and retractions are illustrated in green and red, respectively, as determined by DIAS analysis. Red and green star indicate the initiation of lateral/back and forward pseudopod, respectively. Arrows from the cells’ centroid indicate the direction of movement. *, direction of the gradient.
**Figure 10.** DdNF1 spatiotemporally regulates chemoattractant-induced Ras activity. (A) cAMP-induced Ras activation detected in a pull-down assay and (B) live cell imaging of GFP-RBD upon uniform cAMP stimulation. Ras-GTP or total Ras proteins were detected in a Western blot. Quantification of the pull-down data and the relative fluorescence intensity of membrane-localized GFP-RBD are shown on the right. Bar = 5 μm. (C) Imaging of GFP-RBD in wild type and nfuA<sup>+</sup> cells migrating in an exponential cAMP gradient. *, position of the micropipette. Bar = 10 μm.

**Figure 11.** No substrate of DdNF1 identified in the yeast two-hybrid assay. The RasGAP domain of DdNF1 shows no interaction with constitutively active (B) RasB (C) RasC (D) RasD (E) RasG and (F) RasS. (A) Positive control.
I, therefore, decide to take advantage of an *in vivo* approach by examining the comparing the cAMP-induced activation profile of individual Ras proteins in cells with or without DdNF1. Two Ras proteins, RasC and RasG, have been linked to the regulation of chemotaxis, including the activation of PI3K and TORC2 (Bolourani et al., 2006; Kae et al., 2004; Kamimura et al., 2008; Lim et al., 2001; Sasaki et al., 2004; Van Haastert and Postma, 2007; Wessels et al., 2004). To assess if DdNF1 regulates either RasC or RasG, I express FLAG-tagged RasC or RasG in wild type and *nfaA* cells and measure their profile of chemoattractant-induced activity in a pull-down assay using the RBD of the yeast protein Byr2, as this RBD binds RasC in addition to RasG, whereas that of Raf1 shows a preference for RasG and not for RasC (Kae et al., 2004; Van Haastert and Postma, 2007). Interestingly, the kinetics of RasG activation is both delayed and extended in *nfaA* cells compared to that of wild type cells, whereas the profile of cAMP-induced RasC activity is the same in both wild type and mutant strains (Figure 12A). I also investigate the cAMP-induced activity of other Ras proteins, including Rap1, RasB and RasD. The activation of RasD and Rap1 in *nfaA* cells upon cAMP stimulation is unaffected while the kinetics of RasB activation, which has recently been suggested to regulate myosin function, is extended (Figure 12A) (Mondal et al., 2008). Taking together, it appears that DdNF1 exhibits the substrate specificity for RasG and RasB *in vivo*; however, the possibility that DdNF1 regulates other uncharacterized Ras protein(s) cannot be ruled out.
Figure 12. The DdNF1-derived regulation of RasG mediates chemotaxis. (A). cAMP-induced activation of Rap1 and exogenously expressed FLAG-RasB, -RasC, -RasG, and myc-RasD was assessed in pull-down assays. The Ras proteins were detected by Western blot with anti-Ras (Ab-3), anti-FLAG (M2), anti-myc (9E10), or anti-Rap1 antibodies. Quantification of data is shown on the right. Quantified data represent mean ± SD of at least 3 independent experiments. (B) Traces of rasG^− and rasG/nfaA^− cells chemotaxing in an exponential cAMP gradient. *, position of the micropipette. The table shows the results of the DIAS analysis of 10 traces from at least 3 independent experiments. (C) Shape difference analysis between each frame of recordings of representative rasG^− or rasG/nfaA^− cells migrating in an exponential cAMP gradient. Protrusions and retractions are illustrated in green and red, respectively, as determined by DIAS analysis. Arrows from the cells’ centroid indicate the direction of movement. *, direction of the gradient.
Figure 13. PI3K is temporally misregulated in nfaA^- cells. (A) Imaging of GFP-PH upon uniform cAMP stimulation. Bar = 5 μm. The relative fluorescence intensity of membrane-localized GFP-PH is shown on the right. Quantified data represent mean ± SD of at least 3 independent experiments. (B) Activity of immunopurified PKB determined in a kinase assay using the H2B substrate. Quantification of the data is shown on the right. Quantified data represent mean ± SD of at least 3 independent experiments. (C) Imaging of GFP-PH upon uniform cAMP stimulation in FLAG-RasGQ61L/rasG^- compared to wild type cells. Bar = 5 μm. The relative fluorescence intensity of membrane-localized GFP-PH is shown on the right. (D) Basal and cAMP-induced activity of FLAG-RasG and FLAG-RasGQ61L expressed in rasG^- cells assessed in a pull-down assay. The Ras proteins were detected by Western blot with and anti-FLAG (M2) antibody. Quantified data represent mean ± SD of 3 independent experiments.
To further identify whether RasG is the major substrate of DdNF1 in vivo, I take advantage of a genetic approach. I generate a rasG/nfaA double null mutant and compare the chemotaxis phenotypes with those of nfaA and rasG single null strains. It has been reported that rasG single null cells exhibit fairly efficient chemotaxis, albeit a little slower in speed, presumably through a PIP3-independent parallel pathway(s) such as PLA2 or TORC2 (Kamimura et al., 2008; Sasaki et al., 2004; Van Haastert and Veltman, 2007). It is observed that rasG/nfaA double null cells chemotax with a relatively good directionality, polarity and speed, which are comparable to those of rasG single null cells but not to those of nfaA⁻ cells (Figure 12B-C). This finding reinforces the conclusion that DdNF1 regulates chemotaxis mainly through the downstream RasG.

2.9 PI3K Activity is Misregulated in nfaA⁻ Cells

I investigate the downstream PI3K pathway as the lack of proper Ras inactivation, RasG in particular, is expected to affect Ras-dependent PI3K signaling. I first examine the translocation kinetics of PIP3 in nfaA⁻ cells compared to that in wild type cells upon cAMP stimulation by means of a PIP3 reporter, consisting of the PH domain of CRAC fused to either GFP or RFP (GFP-PH and RFP-PH). I observe a rapid and transient translocation of GFP-PH from the cytoplasm to the plasma membrane, peaking at ~5-8 sec and back to the cytoplasm by 20 sec after cAMP stimulation, consistent with previous reports (Meili et al., 1999; Parent et al., 1998). In contrast, cAMP-induced translocation of GFP-PH in nfaA⁻ cells is delayed and greatly extended compared to wild type cells, reaching a maximum at ~15 sec and then slowly returning to the cytoplasm to reach complete dissociation ~70 sec following stimulation (Figure 13A). These observations
are supported by the finding that cAMP-induced activation of PKBA, a downstream effector of PI3K and regulator of the cytoskeleton (Sasaki and Firtel, 2006), is delayed and considerably prolonged in *nfaA*<sup>+</sup> compared to wild type cells (Figure 13B). Consistent with the idea that such an extended kinetics of PIP3 translocation in *nfaA*<sup>+</sup> cells results from the prolonged RasG activation, *rasG*<sup>+</sup> cells expressing “constitutively” active RasG<sup>Q61L</sup> exhibit a similar but slightly abbreviated translocation profile for the PIP3 reporter compared to that of *nfaA*<sup>+</sup> cells upon uniform stimulation with cAMP (Figure 13C). It is noted that although the RasG<sup>Q61L</sup> mutant has a higher basal activity and extended activation kinetics compared to the wild-type RasG, it is not an authentic “constitutively active RasG” and its activity can be elevated further upon cAMP stimulation. Figure 13D shows that, despite the fact that the activation peak of Flag-tagged RasG<sup>Q61L</sup> induced by cAMP is much broader than that of Flag-tagged wild-type RasG, its activity still wanes down to the basal level at ~40-60 sec following stimulation. Together, these findings indicate that misregulated RasG activity in *nfaA*<sup>+</sup> cells elicits upregulated and prolonged PI3K activity.

The next question is whether the spatial accumulation of PIP3 is affected as well in chemotaxing *nfaA*<sup>+</sup> cells. It is observed that while RFP-PH concentrates at the forming and established leading edge in chemotaxing wild type cells, the PIP3 reporter localizes to multiple and seemingly random sites along the plasma membrane of chemotaxing *nfaA*<sup>+</sup> cells (Figure 14A). These sites of PIP3 accumulation also correspond to where F-actin polymerizes as shown by the co-localization with the F-actin reporter GFP-LimEd<sup>Δ</sup>coil (Schneider et al., 2003). These abnormal patterns of PIP3 accumulation and F-actin polymerization in migrating *nfaA*<sup>+</sup> cells are reminiscent of those of activated Ras
(Figure 10C), strongly suggesting that the misregulated Ras activity in these cells leads to aberrant chemoattractant-induced PIP3 accumulation and F-actin polymerization. Consistent with these observations, the basal as well as the chemoattractant-induced F-actin polymerization levels are both elevated in nfaA- cells compared to those in wild type cells (Figures 14B).

The temporally and spatially unregulated PIP3 accumulation in nfaA- cells most likely results from misregulated PI3K activity. However, it has been shown that depletion of PTEN, the reciprocal regulator of PIP3 signaling, can cause extended and non-spatially restricted PIP3 production and F-actin polymerization (Funamoto et al., 2002; Iijima and Devreotes, 2002). To assess the possibility that loss of DdNF1 affects the PTEN signaling, I evaluate the chemoattractant-induced translocation of PTEN-GFP in wild type and nfaA- cells. I find that the two strains attain the same profile of chemoattractant-induced translocation of PTEN-GFP; upon uniform stimulation of cAMP, membrane-localized PTEN-GFP is initially delocalized from the plasma membrane followed by a gradual re-translocation to the cell cortex, starting at ~8 sec (Figure 15A). In addition, as illustrated in Figure 15B and as previously reported (Funamoto et al., 2002; Iijima and Devreotes, 2002), PTEN is excluded from PIP3 enriched regions of the plasma membrane in chemotaxing cells (the leading edge in wild-type cells and the multiple protrusions produced in nfaA- cells), suggesting that PTEN function is unaltered in nfaA- cells. Consequently, these findings confirm that the misregulation of PI3K signaling but not PTEN leads to uncontrolled F-actin polymerization and deficient chemotaxis.
Figure 14. PIP3 and F-actin polymerization are spatially misregulated. (A) Imaging of the RFP-PH and GFP-LimEΔcoil in wild type and nfaA<sup>−</sup> cells migrating in an exponential cAMP gradient. *, position of the micropipette. Bar = 10 μm. (B) cAMP-induced F-actin polymerization of wild type and nfaA<sup>−</sup> cells. Data represent mean ± SD of at least 3 independent experiments.
Figure 15. The function of PTEN is unaltered in nfaA\(^{-}\) cells. (A) Relative fluorescence intensity of membrane-localized PTEN-GFP upon uniform cAMP stimulation. Data represent mean ± SD of at least 3 independent experiments. (B) Localization of PTEN-GFP in migrating cells. The images show that PTEN-GFP is absent from the leading edge and from the pseudopodia of chemotaxing wild type and nfaA\(^{-}\) cells, respectively. *, position of the micropipette. Arrows indicate membrane protrusions. Bar = 5 µm.
2.10 LY294002 Treatment Partially Improve the Chemotaxis of nfaA\(^{-}\) Cells

Because it is Ras-dependent PI3K misregulation in nfaA\(^{-}\) cells that elicits defects in directionality, polarity and speed during chemotaxis, it is conceivable that modest inhibition of either Ras or PI3K would partially suppress those chemotaxis phenotypes in nfaA\(^{-}\) cells by improving the efficiency of the directional migration. Although some of Ras inhibitors, such as FTIs, have been exploited in various studies and tested in clinical trials to antagonize hyperactive Ras signaling, these inhibitors are likely to block the farnesylation of other non-Ras proteins containing a C-terminus CaaX motif in a non-specific manner and therefore complicate the interpretation (Barkan et al., 2006; Goldberg and Kloog, 2006). In comparison, LY294002 has been widely used as a rather specific PI3K inhibitor and is hence employed in this study. After a treatment with 10 \(\mu\)M final concentration of LY294002 for 30 min, nfaA\(^{-}\) cells exhibit significantly improved chemotaxis while the chemotaxis of wide-type cells is almost not affected (Figure 16). When wild-type cells are treated with 50 \(\mu\)M LY294002, which almost completely eliminates PI3K activity, cells lose the polarity and round up but are still able to chemotax in a relatively efficient way after being exposed to an exponential chemoattractant gradient for an extended time (Figure 16), as previously described (Takeda et al., 2007). Additional parallel chemotactic pathway(s) have been suggested to account for the remaining competence of chemotaxis. nfaA\(^{-}\) cells with the same treatment display identical directionality, cell polarity and speed as those treated wild-type cells (Figure 16). This is another piece of evidence that DdNF1 regulates chemotaxis mainly through Ras/PI3K pathway.
Figure 16. Inhibition of PI3K suppresses the chemotaxis phenotype of nfaA<sup>-</sup> cells. (A) Traces of nfaA<sup>-</sup> cells chemotaxing in an exponential cAMP gradient emitted by a micropipette, treated either with the carrier alone (control) or with the indicated concentrations of LY294002 for 30 min prior recording. nfaA<sup>-</sup> orange traces, type 1 cells; blue traces, type 2 cells. *, position of the micropipette. (B) DIAS analysis of at least 10 traces from at least 3 independent experiments of nfaA<sup>-</sup> cells, treated 10µM, 50µM or not with LY294002, chemotaxing in an exponential chemoattractant gradient.
2.11 nfaA− Cells Exhibit Impaired Directional Sensing

Although evidence suggests that directional sensing involves mechanisms that do not require global cell polarity or an intact cytoskeleton (Devreotes and Janetopoulos, 2003), F-actin-dependent positive feedback loops play an important role in the amplification of the PIP3 signal, in part, through the up-regulation of Ras and PI3K signaling (Charest and Firtel, 2006; Sasaki et al., 2004). Therefore, to determine whether the regulation of Ras activity directly affects gradient sensing independent of its role in controlling cell motility and polarity, I assess the spatiotemporal activation of Ras in cells treated with the actin polymerization inhibitor Latrunculin (LatB), which generates motility-paralyzed, symmetrical, and spherical cells without pseudopodia (Parent et al., 1998). As previously reported (Sasaki et al., 2004), in wild-type cells, the kinetics and the spatial activation of Ras following exposure to a chemoattractant gradient are unaffected by LatB treatment, as revealed by the localization profile of GFP-RBD (Figure 17A). After the initial uniform activation and adaptation that follow placing the cAMP-filled micropipette in close proximity to the cell, GFP-RBD rapidly accumulates in a crescent shape along the plasma membrane closest to the chemoattractant source. Upon repositioning of the micropipette, GFP-RBD is rapidly delocalized from its previous site and rapidly accumulates at the cortex closest to the new position of the micropipette, reflecting the prompt deactivation and activation of Ras at each site respectively. Interestingly, in a similar experiment, LatB-treated nfaA− cells present a markedly different profile of spatiotemporal regulation of Ras activity upon changes in gradient orientation. After repositioning the micropipette, I observe a considerable delay of ~40 sec before GFP-RBD fully dissociates from its original site on the plasma membrane, as
might be expected from a loss of GAP activity (Figure 17A). Unexpectedly, however, the chemoattractant-induced Ras activity at the new site closest to the chemoattractant source is also noticeably delayed, as illustrated by the significantly slower rise in Ras-GTP levels, which took ~30 sec to reach their maximum in nfaA− cells compared to <10 sec in wild type cells (Figure 17B). As a result, two crescents of plasma membrane-localized GFP-RBD are observed simultaneously, which never occurs in wild type cells, demonstrating that the misregulation of Ras activity affects the ability of cells to sense changes in gradient orientation (Figure 17C).

2.12 Subcellular Localization of DdNF1

In order to understand the mechanism of how DdNF1 regulates Ras activity in vivo, I decide to look into the subcellular localization of DdNF1 first. To do this, I take advantage of in vivo cell imaging. I construct a DdNF1-GFP vector, which is proved as functional since it rescues all the chemotaxis and cytokinesis phenotypes when expressed in nfaA− cells. In growing nfaA− cells, I observe that DdNF1-GFP predominantly distributes throughout the cytoplasm while occasionally is enriched on the spontaneous membrane protrusions (Figure 18A). Since activated Ras is known to be restricted to the leading edge of chemotaxing cells, it is tempting to speculate that the negative regulator, DdNF1, is localized at the lateral sides and rear of the cell to focus Ras-GTP on the front, reminiscent of the localization of PTEN, which restricts PIP3 accumulation at the leading edge. However, DdNF1-GFP does not show any preferential enrichment and exists in the cytoplasm all the time during chemotaxis (Figure 18A). Immunofluorescence staining of epitope tagged DdNF1 in chemotaxing nfaA− cells exhibits the same localization in the
cytoplasm (Figure 18A). Upon uniform stimulation of cAMP, neither DdNF1-GFP translocation to the cell cortex nor its disassociation from the plasma membrane is observed (data not shown).

Using the confocal microscope, due to the excessive amount of green fluorescence (i.e. DdNF1-GFP) in the cytosol, it is not feasible to examine whether DdNF1 also localizes on the plasma membrane/cortex when cells are chemotaxing. A cortical DdNF1 pool, therefore, may be occluded by the large cytoplasmic pool. With the assistance from other colleagues, I exploit the total internal reflection fluorescence microscopy (TIRFM) to exclusively examine the basal plasma membrane of chemotaxing cells. Figure 18B shows a wild type cell expressing GFP-PH as a positive control. GFP-PH binds PIP3 on the plasma membrane, which is also enriched at the cortex towards the chemoattractant source. By contrast, DdNF1-GFP in a chemotaxing nfaA− cell is present on the plasma member as well but do not show any preferential enrichment. The membrane localization of DdNF1 is confirmed when cytoplasmic fraction of nfaA− cell is removed by mild Triton X-100 treatment (Figure 18B). Altogether, DdNF1 localizes uniformly both in the cytosol and on the plasma member and the localization is not altered upon chemoattractant stimulation. Such a uniform localization pattern suggests that DdNF1 functions as a global inhibitor/inactivator of Ras throughout the cell but does not necessarily rule out the possibility that DdNF1 interacts with and/or is regulated by other asymmetrical distributed components, for example, phospholipids (D'Angelo et al., 2006; Welti et al., 2007).
Figure 17. Depletion of RasGAP activity causes gradient sensing defects. (A) Imaging of GFP-RBD in LatB-treated cells responding to changes in cAMP gradient orientation. *, position of the micropipette. Data are representative of at least 3 independent experiments. Bar = 10 µm. (B) Quantification of relative fluorescence intensity of membrane-localized GFP-RBD in (A). (C) Illustration of GFP-RBD translocation kinetics upon changes in gradient orientation.
**Figure 18.** Subcellular localization of DdNF1 and its truncated forms in growing and chemotactic cells. (A) Subcellular localization of DdNF1 in (a) a growing *nfaA* cell (b) a chemotaxing *nfaA* cell; (c) immunofluorescence staining of myc-tagged DdNF1 in a migrating cell; F-actin stained with TRITC-phalloidin. *, direction of chemoattractant gradient. (B) TIRFM images of (a) GFP-PH in wild type cells and (b) DdNF1-GFP in *nfaA* cells chemotaxing under agar. (c) Fluorescent images of *nfaA* cells expressing DdNF1-GFP. Cells were treated with 0.01% Triton X-100 before fixation. (C) Illustration of a series of truncated DdNF1 forms fused with GFP and their subcellular localization respectively.
To further examine the roles of each individual domains, especially the putative lipid-binding Sec14 domain and PH-like domain, in DdNF1’s subcellular localization, a series of truncated forms of DdNF1 fused with GFP are examined for their localization in growing and chemotaxis-competent cells. Figure 18C demonstrates that Sec14-GFP preferentially localizes in the nucleus while other truncated GFP fusion proteins are evenly distributed throughout the growing *nfaA* cells. In addition, no changes of localization for these truncated fusion proteins are detected upon the uniform stimulation with cAMP (data not shown). Despite these interesting findings, the underlying mechanism of DdNF1 localization and regulation is still elusive and under investigation.

### 2.13 DdNF1 Regulates the Random Cellular Movement

Our lab recently demonstrated that the basal motility of growing *Dictyostelium* cells is controlled by a Gβ-independent, regulatory circuit involving Ras, PI3K, and F-actin (Sasaki et al., 2007). I therefore investigate whether DdNF1-regulated Ras activity also affects the motility of vegetative cells, in addition to cAMP-induced chemotaxis. I find that growing *nfaA* cells display a ~2-fold increase of Ras and PKB activities compared to wild-type cells (Figure 19A), demonstrating an important role of DdNF1 in regulating basal activity of Ras and downstream PI3K. Both wild type and *nfaA* cells are stained with FITC-phalloidin followed by quantification by means of FACS. When the cell size of *nfaA* population is approximately same as that of wild type, indicated by the identical level of DNA staining of propidium iodide (PI), the level of polymerized F-actin in *nfaA* cells is 30% higher than that in wild type cells (Figure 19B). When growing cells are incubated in 12 mM Na/K phosphate buffer and subsequently attach to the
substratum, I observe more spontaneous pseudopodia that are F-actin enriched in nfaA\(^-\) cells than in wild-type cells (Figure 19C). As a result, growing nfaA\(^-\) cells exhibit considerably enhanced random cell motility: 7.4 \(\mu\)m/min of nfaA\(^-\) cells versus 5.8 \(\mu\)m/min of wild type cells (Figures 19D and 20). These results are consistent with the role of DdNF1 as a negative regulator of Ras, which attenuates the activation level of the downstream PI3K-F-actin signaling in growing cells as well as in chemotaxis competent cells.

2.14 Expression of DdNF1\(^{R91/A}\) Elicits Dominant Negative Phenotypes

It has been shown that a substitution of the arginine finger (i.e. Arg1276 in mammalian NF1) with alanine or proline causes drastic reduction in GAP activity with no substantial influence on the binding affinity of NF1 to Ras-GTP (Klose et al., 1998; Sermon et al., 1998). In order to further understand if RasGAP activity of DdNF1 is required for proper Dictyostelium chemotaxis, I express the GAP-deficient form of DdNF1, DdNF1\(^{R91/A}\) (with the arginine finger mutated to alanine), in wild type cells and investigate the chemotaxis phenotypes. Interestingly, such cells display strong dominant negative phenotypes, including extended cAMP-stimulated Ras activity and PKB activity, failure to chemotaxis, similar but more severe than those of nfaA\(^-\) cells (Figure 21-22). It is assumed that overabundant DdNF1\(^{R91/A}\) competitively binds Ras-GTP and blocks all endogenous RasGAPs, including DdNF1, from interacting with Ras-GTP, preventing the hydrolysis of GTP to GDP. As a result, the downstream effectors such as PI3K are activated with extended kinetics, indicated by prolonged PKB activity. In comparison, wild-type cells expressing myc-tagged DdNF1 do not display any defects in
terms of Ras activation or chemotaxis (Figures 21 and 22). Ongoing experiments have been designed to express a membrane-bound form of DdNF1 in wild-type cells to localize DdNF1 in proximity to Ras, which could possibly lead to some dominant phenotypes. Expressing DdNF1\textsuperscript{R91/A} in \textit{nfaA}\textsuperscript{−} cells does not aggravate the phenotypes of wild type cells expressing DdNF1\textsuperscript{R91/A}, suggesting that defects due to expression of DdNF1\textsuperscript{R91/A} or disruption of DdNF1 both result from the same cause, depletion of RasGAP activity. Consistently, the expression of DdNF1\textsuperscript{R91/A} in both wild-type and \textit{nfaA}\textsuperscript{−} cells leads to the same developmental phenotypes (Figure 23). The development of both strains is slightly delayed around 10-12 h compared to that of wild-type cells; by 23 h, both strains are able to form mature multicellular structures but are much smaller than those of wild-type cells. Surprisingly, these developmental defects are different from those of \textit{nfaA}\textsuperscript{−} cells. This, together with the observation that cells expressing DdNF1\textsuperscript{R91/A} display more severe chemotaxis phenotypes than \textit{nfaA}\textsuperscript{−} cells, indicates other possible functions of DdNF1 in regulating chemotaxis in addition to its RasGAP activity. In the past, RasGAP proteins were suggested as either Ras effectors or scaffold proteins (Tocque et al., 1997), and therefore, it is worthwhile to first look for potential binding partners of DdNF1 via protein complex analysis.
Figure 19. nfaA− cells exhibit random cell motility defects. (A) Spontaneous Ras and PKB activities (B) F-actin levels determined by FACS. Fluorescence histograms are shown. (C) Epifluorescent micrographs of TRITC-phalloidin-labelled cells. Bar = 5 µm. (D) Traces of representative randomly moving vegetative cells. The speed (movement of cell’s centroid) was determined by analyzing 15 traces obtained from 3 independent experiments using DIAS software. Data represent mean ± SD of at least 3 independent experiments.
Figure 20. Shape difference analysis of randomly moving wild type and \textit{nfaA} cells. Images represent the shape difference analysis between each frame of recordings of representative growing cells undergoing random cell migration. Protrusions and retractions are illustrated in green and red, respectively, using DIAS analysis. Arrows from the cells’ centroid indicate the direction of movement.
Figure 21. cAMP-induced activations of Ras and PKBA are significantly extended in cells expressing DdNF1\textsuperscript{R91A}–myc. (A) cAMP-induced Ras activation detected in a pull-down assay upon uniform cAMP stimulation. Ras-GTP or total Ras proteins were detected in a Western blot. Wild type or \textit{nfaA}\textsuperscript{−} cells expressing DdNF1\textsuperscript{R91A}–myc exhibit prolonged kinetics of Ras activation while wild type cells expressing wild type DdNF1 display a similar kinetics to wild type cells. (B) Activity of immunopurified PKB determined in a kinase assay for wild type and wild type cells expressing DdNF1\textsuperscript{R91A}–myc using H2B as the substrate. Wild type cells expressing DdNF1\textsuperscript{R91A}–myc exhibit prolonged kinetics of PKB activation compared to wild type cells.
Figure 22. Expression of a RasGAP-deficient form of DdNF1, DdNF1<sup>R91A</sup>-myc elicits severe chemotactic defects. (A) Traces of representative chemotaxing cells in an exponential cAMP gradient emitted by a micropipette including wild type, nfa<sup>A</sup> type 1 and 2, DdNF1<sup>R91A</sup>-myc/WT, DdNF1<sup>R91A</sup>-myc/nfa<sup>A</sup>, DdNF1-myc/WT, and DdNF1-myc/nfa<sup>A</sup> cells. (B) DIAS analysis of at least 10 traces from at least 3 independent experiments of cells chemotaxing in an exponential chemoattractant gradient.
**Figure 23.** DdNF1<sup>R91A</sup>-myc/WT and DdNF1<sup>R91A</sup>-myc/nfaA<sup>−</sup> cells exhibit same developmental defects. Wild type, nfaA<sup>−</sup>, DdNF1<sup>R91A</sup>-myc/WT, and DdNF1<sup>R91A</sup>-myc/nfaA<sup>−</sup> cells are plated on non-nutrient agar. Starvation time is indicated.
Chapter 3 Discussion

3.1 Ras is an Upstream Component of the Cellular Compass

It was suggested that *Dictyostelium* chemotaxis is instructed by a PIP3-mediated cellular compass based on the finding that the lipid second messenger PIP3 accumulates at the leading edge of chemotaxing cells, where it induces cytoskeletal rearrangements leading to pseudopod protrusion towards the chemoattractant source (Funamoto et al., 2002). PIP3 production is spatiotemporally controlled by two reciprocally distributed regulators, PI3K and PTEN. PI3K is activated at the site of the plasma membrane closest to the chemoattractant source, whereas PTEN is delocalized from this site (Charest and Firtel, 2006; Franca-Koh et al., 2006; Funamoto et al., 2001; Meili et al., 1999; Parent et al., 1998; Van Haastert and Veltman, 2007). Two positive feedback loops of PI3K binding to F-actin in the front and PTEN binding to PIP2 in the back respectively, are suggested to cause symmetry breaking and rapid amplification of a shallow chemoattractant gradient to a steep gradient of PIP3. Despite the important functions of PIP3 signaling in chemotaxis, some studies have challenged the idea that PIP3 is the essential player in directional sensing. It was found that a N-terminal fragment of PI3K2 expressed in *pi3k1/pi3k2* cells, where PIP3 production is almost abolished, continues to be localized at the leading edge, suggesting a cellular compass upstream of PI3K signaling (Funamoto et al., 2002). Directed PIP3 accumulation occurs in LatA-treated *pten* cells, implicating that directional sensing event can be achieved without PI3K translocation and PTEN activity (Sasaki et al., 2004). In addition, *Dictyostelium* cells can
chemotax in a fairly efficient way up an exponential gradient of cAMP when all 5 genes encoding type I PI3Ks are disrupted (Hoeller and Kay, 2007). Altogether, these reports suggest that chemoattractant-mediated PI3K signaling is mostly involved in controlling the motility of chemotaxing cells through cytoskeleton rearrangement with little effect on directional sensing, particularly in a steep gradient.

In the absence of receptor tyrosine kinases (RTKs) in Dictyostelium, Ras signaling is thought to exclusively lie downstream of the GPCR and heterotrimeric G protein and immediately upstream of PI3K. Disruption of the RBD in PI3K prevents its binding to Ras-GTP and consequently eliminates PI3K-dependent responses to chemoattractant (Funamoto et al., 2002). Activated Ras localizes to the leading edge of chemotaxing cells in a PIP3- and F-actin-independent manner (Sasaki et al., 2004). Chemoattractant-stimulated Ras activation in Dictyostelium occurs rapidly and transiently, peaking at ~3 sec after stimulation, which is the most rapid signaling event reported to occur downstream of the heterotrimeric G proteins (Sasaki et al., 2004). Inhibition of Ras signaling, by the overexpression of a dominant negative RasG<sup>S17N</sup> mutant in a background lacking the RasGEFA, results in severe directional movement defects, hinting that Ras may be involved in directional sensing (Sasaki et al., 2004). Altogether, these previous findings place Ras in an ideal position within the chemotactic signaling cascade to be a key component of directional sensing mechanism, but substantial evidence has been lacking.

I identified DdNF1, a putative Dictyostelium orthologue of the human RasGAP NF1, which is a major negative regulator of basal as well as chemoattractant-induced Ras activity, RasG in particular. Analyzing cells with deficient RasGAP activity, i.e. nfaA
cells, allows me to further uncover the role of Ras in chemotaxis, especially its function in directional sensing. Cells lacking DdNFI exhibit major chemotaxis defects that include considerably reduced polarity, migration speed, and directionality. Correspondingly, \( nfaA^- \) cells display delayed aggregation upon starvation. Interestingly, these defects are not linked to reduced F-actin polymerization as previously shown in some mutant cell lines such as \( racB^- \). Instead, the basal and chemoattractant-induced F-actin polymerization is upregulated in \( nfaA^- \) cells and the total pseudopod extensions of a chemotaxing \( nfaA^- \) cell are more abundant than those of wild type. As might be expected from the loss of RasGAP activity, \( nfaA^- \) cells undergo an up-regulation of both basal and cAMP-stimulated Ras activity, leading to extended and elevated chemoattractant-mediated responses, including downstream PI3K activation and F-actin polymerization. Surprisingly, \( nfaA^- \) cells fail to spatially control the chemotactic response of activated Ras, which probably underlies the severely impaired ability of \( nfaA^- \) cells to properly sense chemoattractant gradients.

Upon directional sensing, a cell must identify the side of the cell that produces the strongest response to the gradient. This is most likely achieved through differential and sequential activation and inactivation of key responses along the cortex until the cell determines the side with the strongest response, which is closest to the chemoattractant source. My data indicate that cells depleted in RasGAP activity are unable to do this. The inability to rapidly down-regulate Ras responses during the initial stages of gradient sensing impairs the ability of cells to efficiently identify the side of the cell closest to the chemoattractant source. As a result, Ras and PI3K activation as well as F-actin polymerization do not exclusively localize to the side of the cell exposed to the highest
chemoattractant concentration, which would become the leading edge in wild-type cells. Instead, these chemotactic responses occur along the entire cell cortex, forming multiple, random “patches”. I propose that this process of gradient sensing continues to play a role as the cells migrate up the gradient, allowing the cells to acquire constant directional cues. Thus, although RasGAP-deficient cells are still able to respond to chemoattractant stimulation, the failure to spatially control the chemotactic responses prevents the cells from deciphering the direction, polarizing, and efficiently performing directional migration. The severity of the chemotactic phenotype observed when comparing RasGAP-depleted cells migrating within shallow and steep gradients is most likely due to the relative difference in chemoattractant concentration between the cell’s anterior and posterior, resulting in a greater difference in relative activation of the signaling responses between the side closest to and that farthest away from the source in a steep opposed to a shallow gradient. Consequently, this increase in the ratio of activation between the presumptive front and back may help the cell decipher the axis of the gradient in the absence of RasGAP function and may explain why some cells perform chemotaxis, albeit inefficiently, in exponential gradients but not in linear gradients.

3.2 DdNF1 Regulates RasG/PI3K/F-actin Pathway

I identified that RasB and RasG are downstream targets of DdNF1 in a pull down assay, demonstrating a substrate specificity of DdNF1 to some extent. Further experiments reveal that the disruption of rasG suppresses the nfaA− chemotaxis phenotypes, and that expression of a “constitutively active” RasGQ61L mutant in rasG− cells results in an increased and prolonged cAMP-induced PIP3 production, similar to
what was observed in nfaA− cells. These findings strongly suggest that the chemotaxis defects in nfaA− cells result from the misregulation of RasG and not RasB. In addition, the levels and kinetics of assembled myosin II upon uniform stimulation of chemoattractant show no difference between nfaA− and wild type cells (data not shown), which implies that the regulator of myosin II assembly, RasB, may not be the mastermind of the chemotaxis defects.

Interestingly, although the RasGQ61L mutant has a higher basal activity and an extended activation kinetics upon the uniform stimulation compared to wild-type RasG, it is not constitutively active. This might explain why the kinetics of PIP3 production in RasGQ61L/rasG− cells are not as extended as in nfaA− cells. However, I cannot rule out the possibility that DdNF1 downregulates other uncharacterized Ras protein(s) in addition to RasG, which is assumingly involved in the activation of PIP3 signalling.

As previously reported, rasG− cells do not display severe chemotaxis defects. It has been known that the functions of RasB, RasC and RasD overlap with those of RasG and their expression levels are elevated in rasG− cells. In the absence of RasG, it is likely that one or more of these Ras proteins compensates the functions of RasG including the regulation of PI3K (Bolourani et al., 2006; Khosla et al., 2000; Sasaki et al., 2004). Taking into consideration of this compensation mechanism between Ras subfamily proteins, loss of function study of a single ras gene by disrupting it may not properly elucidate its function. Rather, manipulating Ras regulators including GAP and GEF, expressing constitutively active or dominant negative form of Ras, or generating multiple ras knockout strain would provide more information on sophisticated Ras signaling in Dictyostelium.
The phenotype that chemotaxing nfaA− cells exhibit spatiotemporally unregulated PIP3 accumulation on the plasma membrane is highly similar to those of cells overexpressing a membrane-targeted PI3K and cells lacking the PI3P phosphatase PTEN (Funamoto et al., 2002; Iijima and Devreotes, 2002). Studies from our and other labs suggest that RasG is an important regulator of PI3K. It is most likely that RasG cannot be downregulated properly in nfaA− cells, eliciting the misregulation of PI3K, and consequently aberrant localization of PIP3. However, it is also possible that the disruption of nfaA would directly impair PTEN’s function rather than upregulate RasG/PI3K signaling to cause the similar phenotype. My observations suggest that the function of PTEN in nfaA− cells is unaltered, as the kinetics and profile of chemoattractant-induced translocation of PTEN-GFP upon uniform stimulation, as well as its localization in chemotaxing nfaA− cells, are identical to those in wild type cells. Together, these data suggest that DdNF1 downregulates PI3K signaling via its regulation of RasG. Such an inhibitory mechanism in Dictyostelium validates the proposition in several mathematic models that other inhibitory components may exist in addition to PTEN.

Since the directional sensing and chemotaxis defects in nfaA− cells result from aberrant upregulation of PI3K, pharmacologic or genetic inhibition of PI3K is expected to suppress the chemotactic phenotype of nfaA− cells. It has been shown that disruption of PI3K signaling by itself, as in Dictyostelium, only marginally affects chemotactic speed, especially in a steep gradient of chemoattractant. In addition, LY treated pten− cells exhibit an improved chemotaxis (Chen et al., 2003). The reason why Dictyostelium cells are able to chemotaxis relatively well when PIP3 signaling is completely blocked has
been ascribed to the presence of the parallel chemotactic pathways such as PLA2 (Chen et al., 2007; van Haastert et al., 2007). As a matter of fact, treatment of \textit{nfaA} \textsuperscript{−} cells with LY partially restores the chemotaxis defect, in terms of both directionality and speed, producing cells that can polarize and migrate as efficiently as LY-treated wild type cells, in a steep gradient of chemoattractant. This observation further strengthens the idea that RasG-induced misregulation of PI3K is mostly responsible for the chemotactic phenotype in \textit{nfaA} \textsuperscript{−} cells. In this sense, I would predict that the cells lacking both DdNF1 and PI3Ks would exhibit more efficient chemotaxis than \textit{nfaA} single null cells in a steep gradient.

There is a possibility that other putative RasG effectors, such as TORC2, which has been shown to interact with constitutively activated RasG in a yeast two-hybrid assay, are involved in RasG-dependent chemotactic signaling cascades (Lee et al., 2005). Mammalian TORC2 phosphorylates the C-terminus hydrophobic motif of PKB and further activates it (Hresko and Mueckler, 2005; Sarbassov et al., 2005). It is possible that the elevated and extended PKB activity detected in \textit{nfaA} \textsuperscript{−} cells upon cAMP stimulation partially results from upregulated TORC2 activity in addition to the aberrant PI3K signaling. However, Kamimura and colleagues recently found that TORC2-dependent phosphorylation of PKBR1 in \textit{rasG} cells is identical to that in wild type cells while the same phosphorylation event is significantly impaired in \textit{rasC/rasG} cells or cells lacking RasGEFA, a GEF specific for RasC, suggesting that RasC but not RasG is required for the activation of TORC2 (Kamimura et al., 2008). Therefore, the chemotactic phenotype of \textit{nfaA} \textsuperscript{−} cells is mainly due to the misregulated PI3K but not TORC2 activity. In addition, disruption of one component of TORC2, RIP3, leads to the cell’s inability to activate adenylyl cyclase upon starvation, similar to the defect in \textit{rasC} \textsuperscript{−} cells (Lee et al., 2005;
Lim et al., 2001). Knocking out RIP3, Pianissimo, or Lst8/Gbl generates cell lines that are unable to aggregate and fail to complete the developmental program while nfaA− cells have no problem to differentiate into the multiple cellular structure in 24 hours, albeit being delayed at the aggregation stage. The distinct developmental phenotypes between nfaA− cells and cells with disrupted TORC2 component further support that TORC2 is not directly regulated through DdNF1/RasG pathway.

3.3 DdNF1 is an Important Component of Ras Signaling to Decipher the Direction

The observation that chemotaxing nfaA− cells lose directionality, together with the finding that Ras cannot be activated and inactivated promptly in nfaA− cells upon the reversal of chemoattractant gradients, suggests that the downregulation of Ras by RasGAPs, including RasG and DdNF1, is a potential inhibitory mechanism implicated in directional sensing in Dictyostelium. I speculate that DdNF1 inhibits Ras activity throughout the cell, which is consistent with the data that DdNF1 is uniformly distributed in chemotaxing cells, thereby lowering the overall level of active Ras (Ras-GTP) both in the resting state and after stimulation (Figure 24A). This globally inhibitory mechanism is distinct from the locally controlled inhibitory mechanism recently proposed by Xu and colleagues in GPCR-mediated directional sensing (Xu et al., 2007). Whether DdNF1 could be the hypothetic “global inhibitor” in LEGI model remains to be answered and no evidence has been found that the activity of DdNF1 is stimulated in response to chemoattractant.

Based on the current data, we put forward a model of how RasGAP functions as an indispensable component of Ras signaling in direction sensing, with the assumption
that RasGAP is “constitutively active” (i.e. same activity before and after stimulation). In the resting state, \( nfaA^+ \) cells display higher levels of Ras-GTP compared to wild type cells due to a lack of RasGAP activity and the slow intrinsic GTPase activity of Ras. In wild type cells, upon directional chemoattractant stimulation, there is a global activation of Ras along the cell’s plasma membrane to reset any pre-existing intrinsic activity, which then rapidly adapts. I speculate that following adaptation, only the remaining activated Ras at the front is sufficient to trigger feedback signaling through the Ras-PI3K-F-actin positive feedback loop (Sasaki et al., 2004). This further leads to the localized persistence and amplification of the Ras signal, thereby creating a steep intracellular gradient of Ras and PI3K activity and promoting leading edge formation (Charest and Firtel, 2006). In the absence of RasGAP activity, the high levels of Ras-GTP throughout the plasma membrane persist, even after the global adaptation. This triggers the positive feedback signaling to amplify the chemotactic responses all around the cell, causing the “non-localized” extension of multiple pseudopodia (Figure 24A).

Taking a look from a different angle, the inhibitory effect of RasGAP can be alternatively considered as a hypothetic “threshold” for Ras activity throughout the plasma membrane. Figure 24B summarizes such a “threshold” based model for cells in a steady state following the initial global activation and subsequent adaptation, in response to a shallow chemoattractant gradient. In wild-type cells, Ras activity is polarized from the front to the back of the cell, reflecting the extracellular chemical gradient. Only Ras activity near the front is above the “threshold”, thereby leading to a localized and amplified response towards the chemoattractant source. In the absence of RasGAP, although the initial gradient of Ras activity is the same as that in wild type cells, the
“threshold” is significantly reduced and therefore Ras activity throughout the membrane is higher than the “threshold”. As a result, the chemotactic responses are stimulated and amplified all over the plasma membrane and consequently cells lose directionality.

Although cAMP-induced Ras activation is expected to extend in the absence of RasGAP activity, it is certainly a novel finding that Ras activity is spatially misregulated in \( nfaA^- \) cells. As illustrated in the above models, positive feedback loop of Ras/PI3K/F-actin could be responsible for the spatially unregulated Ras activation in RasGAP-depleted cells. Such a speculation is consistent with the finding that LatB treated \( nfaA^- \) cells do not display multiple accumulations of activated Ras along the plasma membrane (Figure 17A). I believe that the inhibitory mechanism of DdNF1, coupled with putative local activators (e.g. RasGEFs, Figure24C), global inhibitors as well as positive feedback signaling, leads to persistence and amplification of the Ras signal at the front and promotes leading edge formation, and consequently determines the right direction.

The finding that \( nfaA^- \) cells fail to promptly inactivate the localized Ras response when chemoattractant gradient is removed and shifted to the opposite site provides experimental evidence for Onsum and Rao’s prediction in their mathematical model of gradient sensing that cells with impaired RasGAP activity would respond sluggishly to changes in the direction of the gradients (Onsum and Rao, 2007). Moreover, Levine and colleagues proposed a membrane bound inactivator in addition to the “global inhibitor” that is mutually antagonistic to the response of activation (Levine et al., 2006). This hypothesized membrane-bound inactivator certainly shares some characteristics with DdNF1. The addition of this third component to LEGI model helps fully explicate the switch-like response upon reversal of the external chemoattractant gradient, which classic
LEGI models fail to explain. Last, examination of switch-like response upon changes of chemoattractant gradient direction is the key approach to reveal the role of RasGAP in directional sensing in this study. Despite the success, I was unable to quantitatively control the steepness and the background level of the chemoattractant gradient. Future studies in a well-controlled gradient environment using a microfluidic chamber and pressurized system will improve our understanding of directional sensing in a more quantitative way.
Figure 24. Models demonstrating that RasGAP-mediated Ras regulation assists the cells to determine the right direction. (A) In the resting state, *nfaA* cells display elevated levels of Ras-GTP compared to wild type cells due to the slow intrinsic GTPase activity of Ras. In wild type cells, upon directional chemoattractant stimulation, there is a global activation of Ras along the cell’s plasma membrane, which rapidly adapts. Low levels of polarized Ras activity at the plasma membrane that persist in the direction of the gradient lead to the local polymerization of F-actin and leading edge formation through signal amplification. We speculate that in *nfaA* cells, the high levels of Ras-GTP that persist, even after the global adaptation that follows the initial stimulation, trigger feedback signaling and amplification of the signal all around the cell and cause the extension of pseudopodia in every angle relative to the chemoattractant gradient. (B) An alternative “threshold” based model. The initial Ras activation (green line) mirrors the shallow gradient of directional chemoattractant stimulation. DdNFI1/RasGAPs are evenly distributed throughout the cell and function as an inactivator of Ras-GTP, setting up an inhibitory threshold (red line). As a result, in wild type cells, only at the front, the Ras activity is above the threshold, leading to a persistent, amplified, F-actin dependent response (yellow line). In absence of RasGAPs, we speculate that the threshold is significantly reduced and therefore Ras activity throughout the membrane is above the threshold, leading to multiple, random protrusions along the cell cortex. (C) RasGAPs, coupled to local activators (RasGEFs), positive feedback loops from the cytoskeleton, creates a steep intracellular gradient of Ras activity in response to a chemoattractant gradient.
3.4 RasGAP Involved in Ras Activation?

Interestingly, I observed that the loss of RasGAP activity also impairs the ability of cells to rapidly activate Ras when cells are exposed to changes in chemoattractant gradient orientation. This is consistent with the findings that chemoattractant-induced RasG activation, PIP3 production and PKB activation of nfaA cells upon uniform stimulation of chemoattractant are considerably delayed, in addition to being prolonged, in comparison with the wild-type cells. It is not clear whether RasGAP directly or indirectly has such an influence on Ras activation. One possibility would be that RasGAP-depleted cells fail to provide sufficient substrate, i.e. Ras-GDP for de novo Ras activation, because the conversion from Ras-GTP to Ras-GDP is decelerated in mutant cells. It was reported that regulators of G protein signaling (RGS), GAP for Gα subunit, can speed up the activation of signaling. A “kinetics scaffolding” model was proposed to explain how RGS enhances receptor-mediated G protein activation when GTP hydrolysis becomes the rate-limiting step (Zerangue and Jan, 1998; Zhong et al., 2003). Such a model may be extended to the regulation of Ras proteins through their respective GAPs. A similar speculation has been made in the study of Rac GTPase. Overexpression of constitutively active Rac1 unexpectedly reduces chemokinesis of HeLa cervical carcinoma cells toward serum and inhibits migration and chemotaxis/invasion of clearCa-28 renal carcinoma cells (Oxford and Theodorescu, 2003). It was proposed that the proper cycling between Rac GTP-bound and GDP-bound forms is required for the continuous activation of signaling and cell motility, which is disturbed when cells express Rac1G12V. For future studies, an active and cycling form of Ras mutant, RasF28L can be exploited to characterize the role of DdNF1 in Ras activation, because RasF28L does not
attenuate Ras-GTP/GDP cycling while still accumulating in the active GTP-bound form in vivo.

3.5 DdNF1/RasG/PI3K/F-actin Circuit Regulates Cytokinesis and Basic Cell Movement

Cells lacking DdNF1 exhibit severe cytokinesis defects when grown in suspension but are mostly mono- or binucleated when grown on a plastic surface. Such a phenotype is reminiscent of those defects in mutant cells including $mhcA^*$, $gefQ^*$, $rasG$ and $racE^*$ (De Lozanne and Spudich, 1987; Lim et al., 2002; Mondal et al., 2008; Rivero and Somesh, 2002; Tuxworth et al., 1997). The karyokinesis in the mutant cells is normal but the following cytokinesis is impaired, which can be rescued through an alternative mechanism called traction-mediated cytofission when cells adhere to the substratum (Fukui et al., 1990). In cytokinesis, a series of cytoskeletal proteins and their regulators, including myosin II, cortexillin, coronin, RacE and scar, localize at the furrow or poles of the dividing cell (Robinson and Spudich, 2004). Moreover, signaling events at the plasma membrane in response to internal cues also play an instructive role in cytokinesis as PI3K and PTEN associate with and function at the poles and furrow of a dividing cell respectively to modulate the cell polarity (Janetopoulos et al., 2005).

Ras is found co-localized with PI3K at the polar ruffles of a dividing cell and a Gβ-independent Ras/PI3K/PTEN/F-actin feedback loop has been suggested to play a fundamental role in cytokinesis (Sasaki et al., 2007). Previous studies have demonstrated that RasG is involved in regulation of cytokinesis and PIP3 signaling. The finding that RasG is the substrate of DdNF1 suggests that DdNF1 may regulate cytokinesis through a
RasG/PI3K/F-actin circuit. Since DdNF1 also inactivates RasB \textit{in vivo}, it is possible that the cytokinesis defects of \textit{nfaA} \textsuperscript{-} cells partially result from the abnormal myosin II assembly due to misregulated RasB activation.

Growing \textit{nfaA} \textsuperscript{-} cells display elevated Ras activity, PKB activity, F-actin polymerization and random mobility. The same G\textbeta\text{-}independent Ras/PI3K/PTEN/F-actin circuit has been implicated in random cell movement (Sasaki et al., 2007). I speculate that, in such a stochastic process, the inhibitory component, RasGAP DdNF1, controls the level and time of Ras activation and sets up a hypothetical “threshold”. Only when Ras activity at a random site on the plasma membrane is above the threshold, can Ras-derived response be amplified through the positive feedback loop from PI3K and F-actin. As a result, a random pseudopod starts to form. In contrast, if Ras activation is inhibited or Ras-derived chemotactic responses are attenuated, cells would attain less random pseudopodia and be less motile. Such a speculation is in agreement with previous findings that cells lacking upstream Ras activator, RasGEFA, or cells lacking downstream effector, PI3K, move less than wild-type cells (Sasaki et al., 2007). Since the RasGAP activity is deficient in \textit{nfaA} \textsuperscript{-} cells, I reason that the hypothetical inhibitory “threshold” falls to a lower level than that of wild type cells. As a result, more Ras activation events along the plasma membrane, albeit stochastic and fluctuating, are above the threshold and can be distinguished and amplified instead of being suppressed, leading to more random pseudopodia and elevated motility. Altogether, identifying the role of DdNF1 in basic cell movement not only broadens our knowledge on how \textit{Dictyostelium} cells wander around but also sheds light on why tumor cells carrying abnormally high Ras activity tend to be more motile and sometimes highly metastatic.
3.6 Is RasS-derived Macropinocytosis Regulated by DdNF1 as well?

Interestingly, chemotaxing *nfaA* cells sometimes undergo a seemingly enhanced macropinocytosis, indicated by occurrence of F-actin or PIP3 enriched macropinosomes (Figure 14 and data not shown), which is much less pronounced in chemotaxing wild-type cells. It has been thought that macropinocytosis is an indication of cells that are in a growing state and/or not fully chemotaxis/aggregation-competent. However, based on the data of several independent indicators of chemotactic competency, it is clear that the occurrence of macropinosomes in *nfaA* cells may results from signaling defects rather than lack of developmental competency. First, the expression level of the developmental marker cAR1 of *nfaA* cells is indistinguishable from that of wild type cells after cells being pulsed for 5, 6, and 7 hours. Second, mutant cells are fully responsive to uniform stimulation of cAMP, and exhibit the similar biphasic profile of F-actin polymerization to that in wild type cells. Furthermore, RasC, RasD, and Rap1 in *nfaA* cells can be activated in response to cAMP and their level and kinetics of activation are indistinguishable from those in wild type cells. Therefore, it is likely that elevated and prolonged Ras-GTP in *nfaA* cells elicits enhanced macropinocytosis, even in chemotaxis-competent cells.

Loss of function analysis through the disruption of *rasS* gene has indicated its function in controlling the balance between two different actin-dependent processes, macropinocytosis and cell movement (Chubb et al., 2000). *rasS* cells migrate considerably faster than wild-type cells, while display impaired endocytosis. It is tempting to hypothesize that DdNF1 also regulates RasS and the upregulation of RasS due to a lack of RasGAP in *nfaA* cells leads to enhanced macropinocytosis and reduced
cell motility. Generating a cell line with both \textit{rasS} and \textit{nfaA} genes disrupted and examining if such a strain phenocopies either \textit{rasS} or \textit{nfaA} cells in terms of macropinocytosis and chemotaxis will be the first step to elucidate the hypothetical role of DdNF1 in inactivating RasS. Unfortunately, the effector region of RasS that interacts with RasGAP domain is not as conserved as those of RasB, C, D and G so that neither Raf1-RBD nor Byr2-RBD is able to pull down activated RasS. Such a technical obstacle, as well as the lack of a specific RasS antibody, hinders us to biochemically identify the linkage between RasS and DdNF1.

3.7 NF1’s Role in Directional Sensing and Chemotaxis Evolutionally Conserved

Given that Ras modulates PI3K function in migrating neutrophils (Suire et al., 2006), and that the signaling pathways regulating chemotaxis in \textit{Dictyostelium} and leukocytes are surprisingly well conserved (Parent, 2004), I believe that Ras and its regulation by RasGAPs are likely to play an important role in regulating directional sensing and migration in mammalian cells too. Furthermore, NF1 is remarkably conserved through evolution and in its domain architecture (D’Angelo et al., 2006; Trovo-Marqui and Tajara, 2006). Interestingly, mammalian NF1 is predominantly expressed in neurons, Schwann cells, oligodendrocytes, astrocytes, and leukocytes (Daston et al., 1992; DeClue et al., 1991; Gutmann et al., 1991). Since the function of these cells either depends on or includes their ability to undergo directed migration, it is suggested that NF1 might regulate directional sensing and migration in these cells. Studies using NF1-deficient cell cultures have demonstrated that mammalian NF1 certainly plays a role in cell movement. Different signaling cascades downstream of NF1
depending on the cell types, including PI3K and mTOR, are suggested to be involved in the regulation of cell motility (Huang et al., 2004; Johannessen et al., 2005). NF1-deficient cells, albeit from different tissues, all exhibit elevated F-actin levels, consistent with my finding in Dictyostelium. Most NF1-deficient cells possess enhanced mobility, which agrees with the observation that nfaA− cells acquire elevated random cell motility but seems to contradict the finding in Dictyostelium chemotaxis. However, the motility of mammalian NF1-deficient cells is typically assessed using endpoint assays such as in vitro transwell assay or artificial wound healing assay. These approaches, although useful in some circumstances like chemokinesis, may not provide reliable information on the ability of cells to sense directional cues. In addition, other potential parallel pathways of directional sensing in mammalian cells, such as Cdc42 (Benard et al., 1999; Srinivasan et al., 2003), which has not been identified in Dictyostelium, may compensate the loss of directionality in NF1-deficient cells. Therefore, these cells could still chemotax towards the right direction while acquiring higher motility. Our pioneering study of Dictyostelium DdNF1 has shed light on the potential functions of mammalian NF1 on directional sensing. In recognition of the importance of directional sensing in various physiological processes, such as wound healing, neuronal development and tumor metastasis, we are shifting from Dictyostelium to mammalian system to investigate the chemotaxis of neutrophils purified from the NF1 conditional knockout mouse, in collaboration with Kevin Shannon’s lab.
Appendix Experimental Procedures

A1. Materials, DNA Constructs, and Gene Disruption

I obtained adenosine 3’5’-cyclic monophosphate sodium salt monohydrate (cAMP), Latrunculin B, LY294002, FITC- or TRITC-phalloidin conjugate, anti-mouse IgG (Fc-specific)-FITC, aprotinin, leupeptin, propidium iodide (PI) and anti-FLAG (M2) antibody from Sigma-Aldrich, monoclonal anti-Ras (Ab-3) antibody from Oncogene Research Products, glutathione-Sepharose beads from GE Healthcare, monoclonal anti-myc (9E10) from Santa Cruz, and γ-[32P]ATP from MP Biomedicals. Polyclonal anti-Rap1 antibody is a gift from G. Weeks (University of British Columbia, Vancouver, Canada) (Kang et al., 2002). Polyclonal anti-PKB antibody was made as described previously (Meili et al., 1999)

GST-RBD(Raf1), GFP-RBD(Raf1), GFP-PH(CRAC), PTEN-GFP, Flag-RasG, Flag-RasG(Q61L) have been described previously (Funamoto et al., 2002; Iijima and Devreotes, 2002; Kae et al., 2004; Sasaki et al., 2004). RFP-PH(CRAC) and GFP-LimE-Acoil expression vectors were obtained from P. Devreotes (Johns Hopkins School of Medicine, Baltimore, MD) and G. Gerisch (Max-Planck-Institut für Biochemie, Germany) respectively. Myc-RasD, Flag-RasB and Flag-RasC were provided by P. Charest (University of California, San Diego, CA). GST-RalGDS(RBD) was a gift from J.L. Bos (University Medical Center Utrecht, Utrecht, Netherlands). GST-RBD(Byr2) was generated as a GST fusion by cloning nucleotides 1-360 of the cDNA sequence of *S. pombe* Byr2 (provided by S. Subramani, University of California, San Diego, CA), corresponding to the RBD, into pGEX-KG vector (Guan and Dixon, 1991).
DdNF1-myc was generated by adding the sequence of the myc epitope to the C-terminus of the full-length nfaA genomic DNA and subcloning into the expression vector Exp4- (+). DdNF1-GFP was generated by ligating DdNF1-myc to a EGFP fragment at its C-terminus through a linker sequence: 5’-TCAGGGTCCAGGCAGGTCAAGGT-3’ and subcloning into the expression vector Exp4- (+). DdNF1R91A-myc was generated using the QuikChange Site-Directed Mutagenesis kit (Stratagene). DdNF1-GAP construct used for yeast two-hybrid was generated by cloning a cDNA sequence of nfaA from nucleotide 1 to 1083 into pJG4-5 vector.

A nfaA knockout construct was made by inserting the blasticidin resistance cassette into a BamHI site created at nucleotide 1365 of nfaA genomic DNA and was used for a gene replacement in the KAx-3 parental strains. Randomly selected clones were screened for a gene disruption by PCR, which was then confirmed by Southern blot analysis. The rasG gene was disrupted as described previously (Tuxworth et al., 1997). The rasG/nfaA double null mutant was made by transforming the same rasG knockout construct containing hygromycin resistance cassette instead of blasticidin resistance cassette to nfaAΔ cells.

The dictyBase (dictybase.org) was screened for putative RasGAPs using the RasGAP domain of human p120GAP or NF1 (Genbank™ accession numbers P20936 and P21359, respectively). Sequence analyses were done using the Blast, Smart, and SCAN programs and the alignments were performed with ClustalW.

A2. Cell Culture and Biochemical Assays
All *Dictyostelium* cell lines were cultured in axenic HL5 medium at 22°C. I obtained all transformants through electroporation and maintained them in 40 µg/ml G418, 70 µg/ml hygromycin, or both as required.

4×10^6 wild type, *nfaA*−, and *nfaA*− cells expressing myc-tagged DdNF1 harvested from Petri plates were seeded into 20 ml axenic medium in shaken culture and the cells were counted at 24-hour intervals thereafter using a hematocytometer for 5 days. Cells were also sampled at same time and stained with DAPI to examine the number of nuclei per cell.

PKB kinase and Ras-GTP pull-down assays have been described previously (Jeon et al., 2007a; Kae et al., 2004; Meili et al., 1999; Sasaki et al., 2004). Briefly, PKB activity was measured in an immunocomplex kinase assay following immunoprecipitation with the anti-PKB antibody using H2B as the substrate. The GST-RBD (Raf1) was used to pull down active FLAG-RasB, myc-RasD, and FLAG-RasG, whereas GST-RBD (Bry2) was used to pull down activated FLAG-RasC and GST-RBD (RalGDS) was used to pull down activated Rap1. The cell extract was incubated with 10 µg of GST-RBD on glutathione-agarose beads at 4°C for 30 min in the presence of 5 mg/ml BSA. The beads were then washed three times and Ras proteins were separated on a 15% SDS-PAGE gel and immunoblotted with the appropriate antibody. Quantification of Ras and PKB activities were determined by densitometry and expressed as the ratio of Ras-GTP over total Ras and ^32^P-H2B over total PKB relative to the maximal activity determined for wild type cells. The F-actin-enriched, Triton X-100-insoluble cytoskeleton fraction was isolated and measured as described previously (Steimle et al., 2001). Briefly,
caffeine-treated, aggregation-competent cells were stimulated with 10 μM cAMP and at the indicated times, aliquots of cells were lysed by addition of an equal volume of 100 mM MES (pH 6.8) buffer containing 1% Triton X-100, 5 mM EGTA, and 10 mM MgCl₂. The cytoskeletal pellet was collected by centrifugation, suspended in 2 × sample buffer, and subjected to SDS–PAGE. Actin and myosin levels were determined by densitometric analysis of scanned Coomassie-stained gels.

Developmental assay and indirect immunofluorescence staining have been described before (Chung and Firtel, 1999). Gene expression level of cAR1 was measured by Northern blot for wild type and nfaA⁻ cells pulsed with cAMP at 0, 5, 6 and 7 h. Yeast two-hybrid assays were performed as described previously (Lee et al., 1999).

For the FACS assay, cells were harvested from dishes and labeled with FITC-phalloidin, as described previously (Chung and Firtel, 1999), and then resuspended in 1 ml 50 μg/ml propidium iodide (PI) solution containing 100 μg/ml RNase and incubated 30 min at 37°C prior to FACS. I performed analytic flow cytometric measurements using a FACSARia flow cytometer with a 488 nm coherent sapphire solid-state laser (Becton Dickinson, San Jose, CA). I detected green fluorescence (FITC-phalloidin) and red fluorescence (PI-DNA) through a 515-545 nm bandpass filter and a 600-620 nm bandpass filter, respectively. Data analysis was performed using FlowJo software (Tree Star, Inc.).

A3. Chemotactic and Random Cell Movement Assays

Chemotactic and random cell movement assays were performed and analyzed using DIAS as previously described (Chung and Firtel, 1999; Funamoto et al., 2002;
Sasaki et al., 2007; Takeda et al., 2007; Wessels et al., 1998). Briefly, cells were pulsed with 30 nM cAMP at 6-min intervals for 5.5 h, and cells were washed and resuspended in 12mM Na/KPO$_4$ buffer. A small volume of cells was placed on glass-bottomed microwell plates (MatTek Inc.) and allowed to adhere to the surface for 10 min. A micropipette filled with 150 µM cAMP solution was positioned to generate an exponential gradient to stimulate cells with Eppendorf micromanipulator, and the cell movement was recorded with a time-lapse video recorder. I added DMSO (control) or LY294002 to a final concentration of either 10 or 50 µM 20 min before real-time recording of chemotaxis toward the micropipette. Alternatively, I used a Dunn chamber to generate a linear gradient of 0-1 µM cAMP for chemotactic analysis. The Dunn chamber has two wells and a connecting bridge. 1 µM cAMP solution and buffer are placed in the respective well, and a linear gradient rapidly forms across the bridge. _Dictyostelium_ cells were placed on the bridge and the movement of the cells was readily viewed and recorded. Random cell movement was measured as described by Sasaki et al. (Sasaki et al., 2007). Briefly, vegetative cells were harvested, rinsed three times with an excess amount of Na/KPO$_4$ buffer, and incubated for 10 min before 1-hour recording.

For assaying directional sensing in LatB-treated cells, cells were pre-treated with 5 µM LatB for 30 min. A micropipette containing chemoattractant was positioned in the dish and gradually moved to within ~10 µm of a cell. Once GFP-RBD accumulated at the membrane closed to the micropipette and formed a stable fluorescent crescent, the micropipette was rapidly repositioned to the opposite side of the cell. The procedure was repeated several times and the kinetics of GFP-RBD membrane translocation and dissociation at both sides of the cell were measured as the relative fluorescence intensity
of membrane-localized GFP-RBD determined as the intensity difference between the plasma membrane and the cytosol pool.

A4. Imaging Acquisition

DIC images of cells were taken at intervals of 6 sec for 30 min using a confocal microscope (DM IRE2; Leica) equipped with an ORCA-285 camera (Hamamatsu Photonics) and a HCX plan Apo 1.25-0.75 40X objective lens (oil CS; Leica). Images were captured using the SimplePCI software (Compix Inc., Imaging Systems) and the data analyzed using the DIAS software (Wessels et al., 1998).

Fluorescence images were obtained using the above-mentioned microscope with HCX plan Apo NA 1.40 63X objective lens (oil CS; Leica) and ORCA-ER or dual view OI-11-EM-equipped EM-CCD cameras for simultaneous imaging (Hamamatsu Photonics). The images were analyzed using the Metamorph software (Molecular Devices). Quantification of membrane or cortical localization of GFP fusion proteins was performed as previously described (Sasaki et al., 2004).

TIRFM (total internal reflection fluorescence microscopy) images were taken using a Nikon TE300 microscope equipped with a Nikon Laser TIRF Unit Prototype and a Cascade II:512 EM-CCD camera (Photometrics), and captured with Image-Pro Plus software (Media Cybernetics).
References


