THE PENNSYLVANIA STATE UNIVERSITY
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DEPARTMENT OF BIOCHEMISTRY AND MOLECULAR BIOLOGY

THE CONSERVED CNK CRAC DOMAIN FUNCTIONS AS A CYTOHESIN-2 BINDING SITE

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ABSTRACT

Epithelial cells grow as sheets to form barriers that separate different compartments of the body. Though epithelia are usually stationary under normal circumstances, they may become migratory during embryonic development, wound healing, or tumor metastasis. The adoption of a motile phenotype requires regulated changes in the actin cytoskeleton and in the attachment of cells to the substratum. This protein cascade, started by HGF, is tightly regulated. The HGF ligand binds to and activates the c-Met receptor tyrosine kinase, which activates a signaling branch that contains the small GTPase Ras. The pathway downstream of Ras has not been well characterized in literature. CNKs (connector enhancer of KSR) are scaffold proteins that promote Ras to MEK signaling.

Mammalian CNKs have a special conserved region, the CRAC domain, in the C-terminal. The function of this region was previously unknown. Pip3-E is homologous to the C-terminal half of mammalian CNKs and was studied extensively in this paper. This research investigates the cytohesin 2 binding location to scaffolding protein Pip3-E/IPCEF. A series of truncation mutants of Pip3-E were created to more precisely localize its cytohesin-binding domain. From the results obtained, it was determined the CRAC domain functions as the cytohesin 2 binding region.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF GRAPHS

Introduction

Epithelial cells form barriers that separate different compartments of the body. Columnar epithelia grow as sheets in which neighboring cells are attached to each other by junctional complexes, including tight junctions and adherens junctions (Santy and Casanova 2001). This strongly regulated network requires that the epithelia are stationary under normal circumstances. However, they may become migratory during embryonic development, wound healing, or tumor metastasis (Gumbiner 1996). The adoption of a motile phenotype requires monitored changes in the actin cytoskeleton and in the attachment of cells to the substratum. Epithelial cells can migrate either individually or collectively. If migrating individually, the junctions on neighboring cells are broken and the motile cell separates from the epithelial monolayer. Or, if the entire monolayer moves as one unit, the intercellular junctions are maintained. Hepatocyte growth factor (HGF) enhances both individual and collective cell migration (Santy and Casanova 2001).

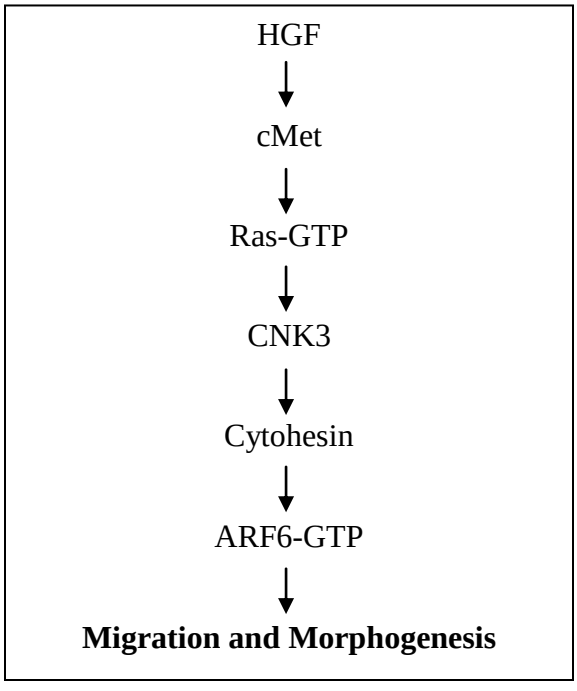


Figure 1: Schematic cascade for growth factor activation of ARF6-GTP.

Small GTPase regulation of Motility

Ras

The HGF ligand binds to and activates the c-Met receptor tyrosine kinase. Met activates a signaling branch that contains the small GTPase Ras as a key component to control cytoskeletal rearrangement and cell adhesion. Ras is an important part of both the extracellular signal-regulated kinase (ERK) pathway and the mitogen-activated protein kinase (MAPK) pathway, which is triggered in response to Met activation. (Birchmeier, Birchmeier et al. 2003). Inhibition of the Ras-pathway inhibits migration in response to HGF (Derman, Cunha et al. 1995). Because Ras is required for the migration of multiple carcinoma cell lines, this protein has been extensively studied as a cell growth regulator. However, the pathways by which it regulates migration have been less well characterized in scientific literature.

ARF6

The ADP-ribosylation factor (ARF) family of proteins belongs to the Ras superfamily of small GTPases. Though initially characterized as vesicular trafficking regulators, subsequent work demonstrated that ARF family members can also regulate assembly of the actin cytoskeleton (Radhakrishna, Klausner et al. 1996). The ARF proteins are ubiquitously expressed and the amino-acid sequences seem to be well maintained in all eukaryotes with strong fidelity. For example, six ARF family members were identified in *Giardia lamblia*, a protist of early diverging lineage that lacks representatives of the Ras and G-protein families (Murtagh, Mowatt et al. 1992). This

finding illustrates early eukaryotic origin for the ARF family members relative to other GTPases (D'Souza-Schorey and Chavrier 2006).

Based on amino-acid sequence identity, the six known mammalian ARFs can be categorized into three classes (Kahn, Cherfils et al. 2006). Class I ARF proteins (ARF1, ARF2 and ARF3) share more than 96% similarity, regulate the assembly of different coat complexes onto budding vesicles, and activate lipid-modifying enzymes (Bonifacino and Glick 2004). The functions of the class II ARFs (ARF4 and ARF5) still remain largely unclear. However, some studies have indicated that ARF5 participates in early Golgi transport (Claude, Zhao et al. 1999). ARF6, which is the sole member of class III, is believed to regulate endosomal membrane traffic and structural organization at the cell surface (D'Souza-Schorey, Li et al. 1995).

ARF6 is the least-conserved member of the ARF family and shares only 66% amino-acid identity with ARF1, but unlike ARF1, ARF6 does not influence Golgi membrane pathways. Rather, it localizes to the plasma membrane and endosomal compartments to regulate endocytic membrane trafficking. ARF6 has also been implicated in actin remodelling at the cellular periphery in addition to rapid cell-surface morphology changing events (D'Souza-Schorey and Chavrier 2006). ARF6-regulated actin remodelling is believed to be required for the formation of pseudopods (Radhakrishna, Klausner et al. 1996), endosome comet-tails (Schafer, D'Souza-Schorey et al. 2000), cell spreading (Albertinazzi, Za et al. 2003), cell migration (Santy and Casanova 2001), and phagocytosis (Zhang, Cox et al. 1998).

The effects of ARF6 on the actin cytoskeleton are thought to occur either through the activation of the Rac1 GTPase and/or lipid metabolism (see **Fig. 2**). In the first

scenario, cytohesin2/ARNO in migrating epithelia has been shown to promote the activation of Rac1 via a Rac1 GEF, the DOCK180–ELMO complex (Santy, Ravichandran et al. 2005). In the second scenario, ARF6 has been shown to activate PLD and PIP5K, leading to the accumulation of PI(4,5)P2, which regulates actin capping (Hilpela, Vartiainen et al. 2004). In fact, ARF6-induced PLD activity functions together with Rac1 activation to promote cell migration (Santy and Casanova 2001).

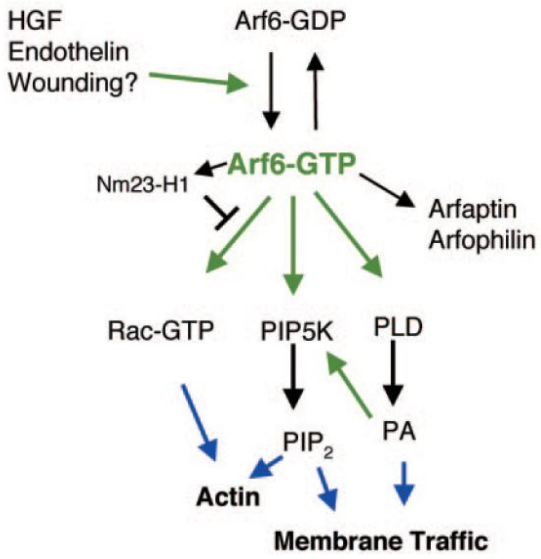


Figure 2: Proteins and pathways affected by Arf6 (Donaldson 2003)

Like all GTPases, ARF6 requires specific factors to cycle between its active-GTP-bound and inactive-GDP-bound conformations. Hydrolysis of bound GTP is mediated by GTPase-activating proteins (GAPs), and the exchange of GDP for GTP is mediated by guanine nucleotide exchange factors (GEFs). Though several ARF-specific GEFs and GAPs can interact with one or more ARF proteins *in vitro*, distinct GAPs and GEFs regulate individual ARF proteins *in vivo* (D'Souza-Schorey and Chavrier 2006). All ARF proteins are myristoylated at the second Gly residue of the N terminus, and this lipid

modification seems to be important to link the ARF family to membranes (D'Souza-Schorey and Chavrier 2006).

ARF GEFs are divided into two major groups: those of high molecular weight and those of low molecular weight (see **Fig. 3**). They can additionally be cataloged on the basis of sequence similarity, functional differences, and sensitivity to brefeldin A (a fungal metabolite that inhibits activation of only ARF1). The high molecular weight mammalian ARF GEFs (>100 kDa) consist of BIG1, BIG2, and GBF1 (Golgi-specific brefeldin A resistance factor 1). They localize to and regulate Golgi membrane trafficking (Jackson 2000). The low molecular weight mammalian ARF GEFs (<100 kDa) include the cytohesin family, the EFA6 (exchange factor for ARF6) family, and ARF-GEP100. These ARF GEFs seem to be involved in ARF6-mediated cytoskeletal reorganization (Jackson 2000).

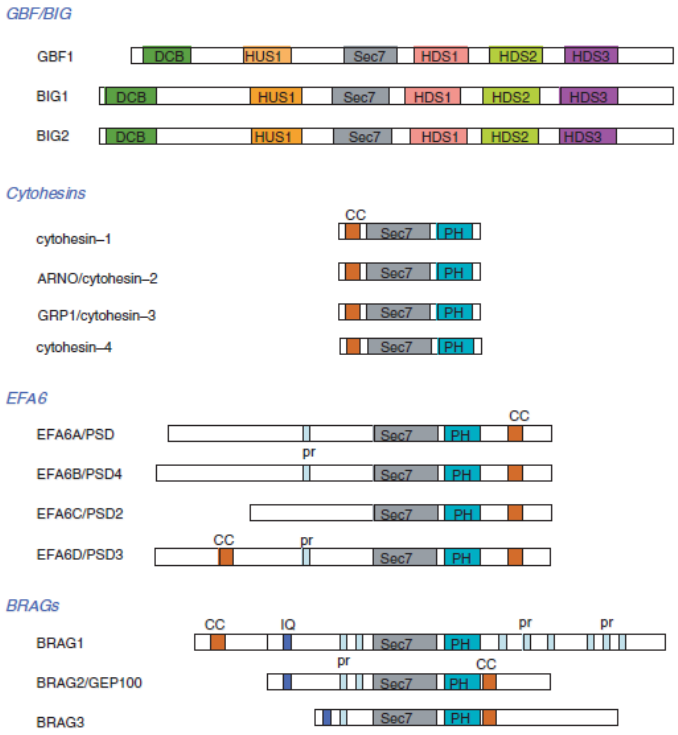


Figure 3: Domain organization of the Sec7 family Arf GEFs (Jackson 2000)

In particular, the cytohesin family of ARF GEFs has been implicated in the regulation of the actin cytoskeleton and cell shape. Cytohesins induce rearrangements in the cortical actin cytoskeleton (Klarlund, Guilherme et al. 1997), and are recruited to the plasma membrane in response to growth factors (Frank, Upender et al. 1998), (Venkateswarlu, Oatey et al. 1998). Upon expression of ARNO in MDCKs, cells quickly form large fan-shaped lamellipodia and pull away from the tight islands. This causes ARNO-expressing cells to exhibit increased migratory activity due to activation of Rac1 (Santy and Casanova 2001).

The cytohesin family [cytohesin 1, cytohesin 2/ARNO (ARF nucleotide binding site opener), cytohesin 3/GRP1 (general receptor for phosphoinositides 1), and cytohesin 4] all share a similar domain organization, as shown in **Figure 3** (Jackson and Casanova 2000). The NH_2-terminal 60 amino acids comprise a coiled coil (CC) domain that functions in homodimerization. A central catalytic region, the Sec7 domain, is conserved among all ARF-GEFs. Mutation of a key residue within this region (E156K in ARNO) completely halts the exchange activity of all Sec7 family members (Beraud-Dufour, Robineau et al. 1998). The COOH-terminal portion of the cytohesin family contains a pleckstrin homology (PH) domain to mediate the interaction of these proteins with membrane surfaces (Jackson and Casanova 2000).

Pip3-E/IPCEF

Phosphoinositides are membrane phospholipids that direct the localization and function of intracellular target proteins. These target proteins affect many important pathways in eukaryotic cells, including signaling, vesicle trafficking, and cytoskeletal

assembly. Pip3-E was first identified as a phosphoinositide binding protein that bound with high affinity to a PI(3,4,5)P3 matrix (Krugmann, Anderson et al. 2002). It was subsequently isolated in a yeast two-hybrid screening for proteins that bind cytohesin 2 and named "Interaction protein for cytohesin exchange factors 1" (IPCEF1/Pip3-E) (Venkateswarlu 2003). Cytohesin 2-IPCEF1 interact at the coiled-coil domain of cytohesin 2 and the C-terminal 121 amino acids of IPCEF1. IPCEF1 also interacts with the other members of the cytohesin family of ARF GEFs, which suggests that the interaction with IPCEF1 is highly conserved among the ARF GEF cytohesins. IPCEF1 associates with cytohesin 2 and localizes to the cytosol in unstimulated cells and relocates (along with with cytohesin 2) to the plasma membrane of cells stimulated with epidermal growth factor (EGF). There, it modulates the ARF6 GEF activity of cytohesin 2 (Venkateswarlu 2003).

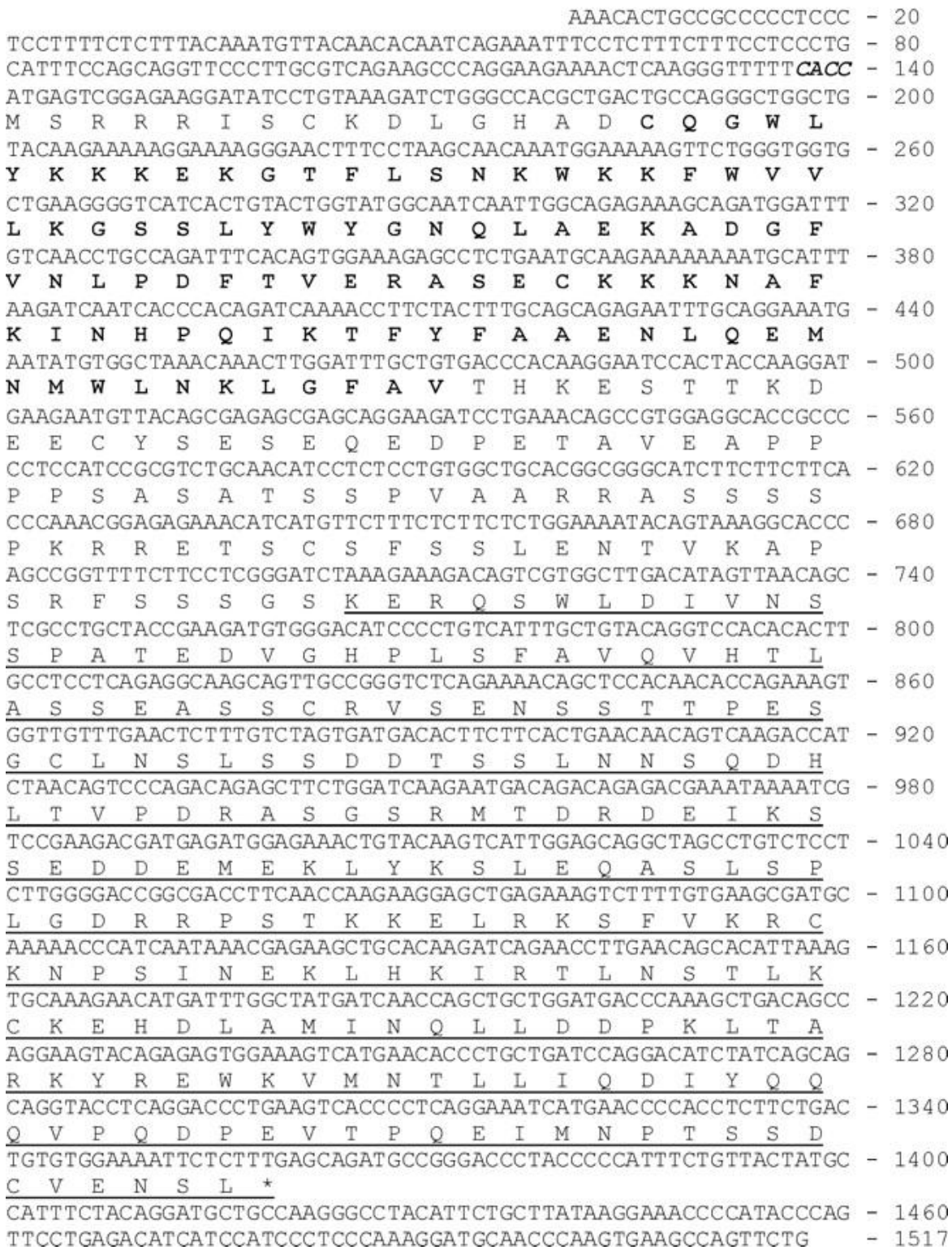


Figure 4: The nucleotide and amino acid sequence of IPCEF1. This figure shows the amino acid sequences of the PH domain (in bold) and the partial cDNA (underlined) (Venkateswarlu 2003).

CNKs

The scaffold protein CNK (connector enhancer of KSR) family was first isolated in Drosophila (Therrien, Wong et al. 1998) and was initially identified as a positive regulator of Ras pathway signaling (Anselmo, Bumeister et al. 2002). Its C-terminal portion directly binds to Raf while its N-terminal portion cooperates with Ras (Therrien, Wong et al. 1998). Drosophila CNK suppresses Raf to MEK signaling without active Ras and promotes Raf to MEK signaling with active Ras (see **Fig. 5**). Though CNK has not been strongly well characterized in literature, its functions have been shown to include RTK-mediated developmental events affecting cell proliferation, differentiation and migration (Clapéron, 2007).

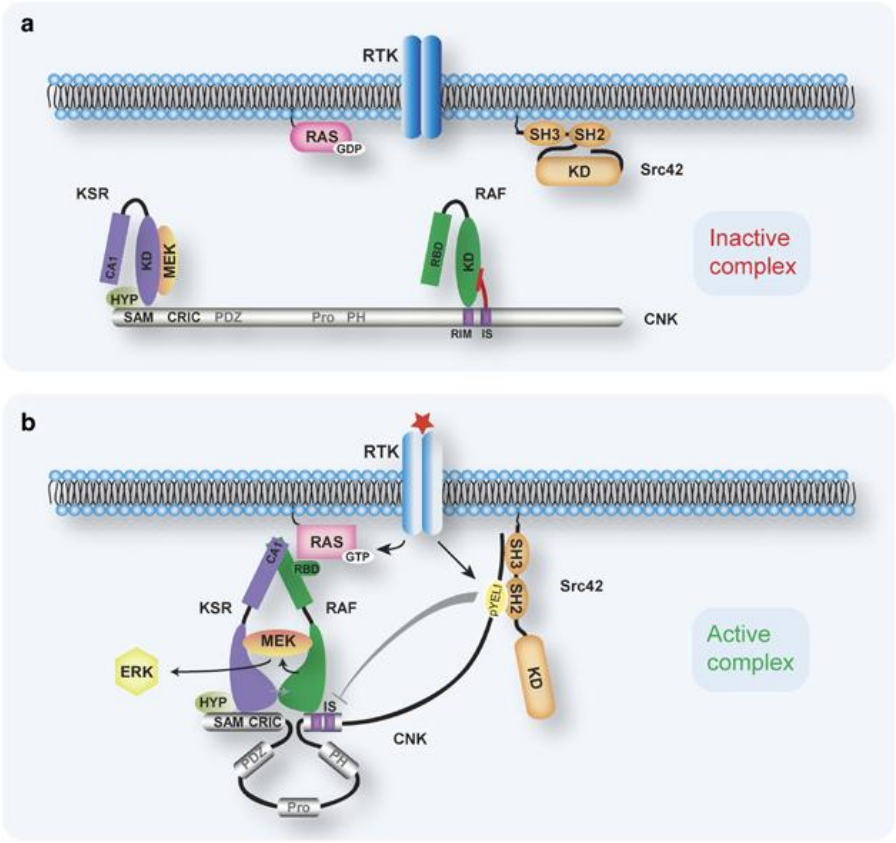


Figure 5: Summary of the effects of CNK on the RAS/MAPK module (Clapéron 2007).

CNK proteins are found in all multicellular organisms and three mammalian homologues have been identified (see **Figure 6: Schematic diagram of CNK domain structures**.) (Therrien, Wong et al. 1998). Like the fly CNK, mammalian CNKs function as protein scaffolds that can facilitate Ras-dependent ERK activation. They can also mediate crosstalk between various Ras effector cascades (Lanigan, Liu et al. 2003).

Though the N-terminal portion of mammalian CNKs is well conserved, the C-terminal portions of fly and mammalian CNKs are not conserved. This suggests that this region may have unique functions (Yao, Hata et al. 1999). Mammalian CNKs have a special conserved region, the CRAC domain, that is absent in fly CNK (Douziech, Roy et al. 2003). Pip3-E/IPCEF1 is homologous to the C-terminal half of mammalian CNK1 and CNK2 (Douziech, Roy et al. 2003). Ras is currently thought to act via CNK3 to activate ARF6 in migrating cells when exposed to growth factor.

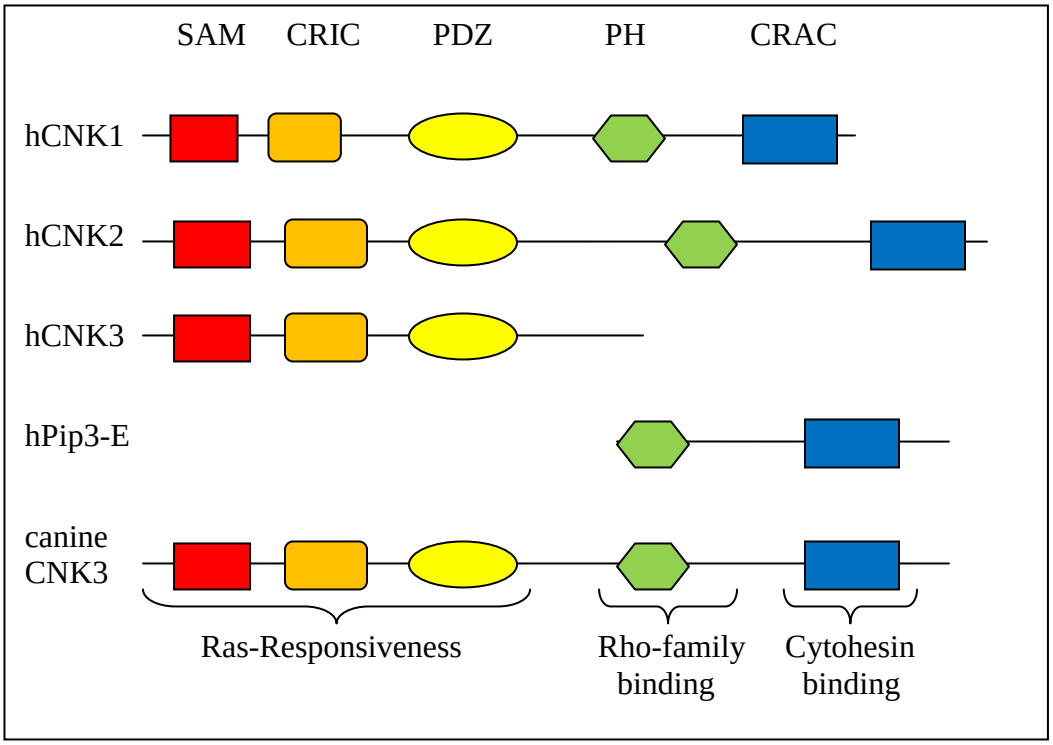


Figure 6: Schematic diagram of CNK domain structures.

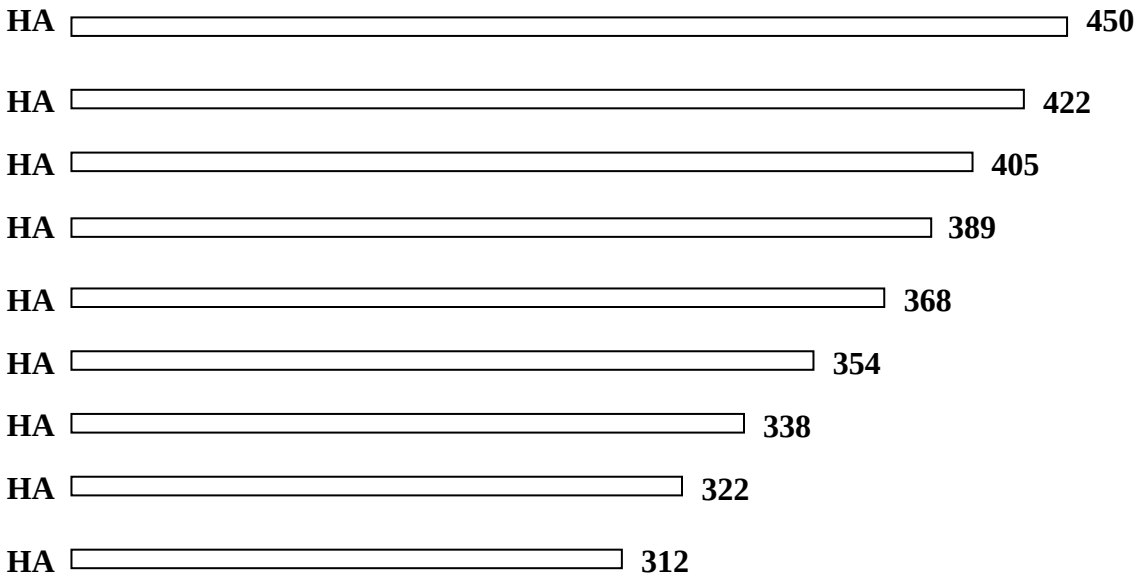
Experimental Design

This paper investigates the cytohesin 2 binding location to scaffold protein Pip3-E/IPCEF. The first study that isolated PIP3-E as a cytohesin-2 binding partner determined that the binding site is located within 120 amino acids of the C-terminus (Venkateswarlu, 2003). This region contains both the conserved CRAC and a series of non-conserved amino acids.

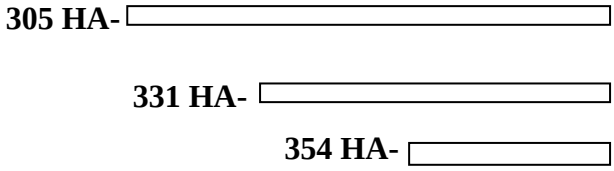
In this paper, a series of truncation mutants of Pip3-E was created to more precisely localize its cytohesin-binding domain. Stop codons were introduced throughout the C-terminus 120 amino acids every 10 to 20 residues. Similar truncations were added to the N-terminus of Pip3-E (see **Fig. 7**). Constructs encoding myc-cytohesin 2 and HA-Pip3-E mutants were co-transfected into HeLa cells. The cells were lysed and co-immunoprecipitated using rabbit α HA (Covance). Co-immunoprecipitation was checked by Western blotting the immunoprecipitates with α-myc antibody (Covance). From these results, the C-terminal boundary of the Pip3-E region required for cytohesin 2 binding was determined.

Once the N- and C-terminal boundaries were defined, the region was then confirmed to be sufficient for cytohesin-binding. PCR was used to create GFP-tagged constructs encoding only the predetermined cytohesin-binding domain (see **Fig. 7**). Co-immunoprecipitation was used as described above to confirm that this domain alone can bind cytohesin 2.

C-Terminal Truncations of Pip3-E:



N-Terminal Truncations of Pip3-E:



GFP Constructs of Pip3-E:

Figure 7: Schematic diagram of IPCEF1/Pip3-E constructs

Materials and Methods

Cell Cultures

Hela cells were cultured in Dulbecco's Modified Eagle's Medium (Cellgro) supplemented with penicillin, streptomycin, fungizone, 10% FBS, glutamine and NEAA. The T23 line of MDCK cells were maintained in DMEM supplemented with penicillin, streptomycin, fungizone and 10% FBS. All cell lines were cultured at 37ºC in a 5% CO_2 water-jacketed incubator.

Migration assays

Migration assays were performed using two different procedures. The first method was a wound-healing assay. In this method, the T23 line of MDCK cells was incubated at 37ºC overnight in a 35mm plate and allowed to form a monolayer. Next, the confluent cells were disrupted by gently wounding the culture with a cell scraper. The media in the plate was refreshed and a thin film of mineral oil was pipetted on top of the culture to avoid media evaporation. Time-lapse microscopy was performed using a Zeiss AxioPlan microscope equipped with a Spot RT3 camera (Diagnostic Instruments). Images were captured every ten minutes for two hours as the 35mm plate was incubated in a culture chamber at 37ºC. During each experiment, standardized microscope and camera settings were used and all images were recorded using the same exposure times. Images of the motile MDCK cells were taken, and cell migration was quantitiated by measuring the remaining cell-free surface area using ImageJ (Image Processing and Analysis in Java, NIH).

The second cell migration assay method used involved Oris migration chambers (Oris^{TM} Universal Cell Migration Assembly Kit: Platypus Technologies, Madison, WI).

The T23 line of MDCK cells was transfected with siRNAs targeting either ARNO or Arf6 for 2 days. Cells ($5x10^4$) were then seeded into each test well and incubated at 37ºC in a 5% CO_2 humidified chamber. After 24 hours, all well inserts were removed, and the media was refreshed. The cells were incubated for 24 hours to permit cellular migration and then fixed with 4% formaldehyde. The fixed cells were stained with 0.1% crystal violet. Images of each chamber were taken, and cell migration was quantitiated by measuring the remaining cell-free surface area using ImageJ.

siRNA and Plasmids

siRNA duplexes against the sequence GCAAUGGGCAGGAAGAAGU targeting ARNO, and siRNA duplexes against the sequence CAATCCTGTACAAGTTGAA targeting Arf6 were obtained from Dharmacon. The siRNAs were transfected with Lipofectamine 2000 (Invitrogen®) in MDCK cells using the manufacturer's suggested protocol for transfection (Invitrogen®). Experiments were carried out 48 hours after transfection with siRNAs. Myc-tagged ARNO was obtained from James Casanova.

Primer Design and PCR

Select sequences of human Pip3-E/IPCEF were amplified by PCR using 100µg/mL full length pcHA-IPCEF plasmid as a template, 10x ThermPol Buffer (New England Biolabs), 10µM forward or reverse primer, dNTPs, *Pfu* DNA polymerase (New England Biolabs) and the following sets of primers with indicated restriction sites:

C-terminal Truncation	Forward Primer Sequence	Reverse Primer Sequence	Digest Enzymes
C312	5’-GAGACAAAAGTGTCTG AAGACGATTAAATGGAG AAGC-3’	5’-GCTTCTCCATTTAATC GTCTTCAGACACTTTTGT CTC-3’	BbsI
C322	5’-GCTGTACAAATCACTCG AGTAAGCTAGTCTATCT CC-3’	5’-GGAGATAGACTAGCT TACTCGAGTGATTTGTAC AG-3’	XhoI
C338	5’-CGACCTTCGACTAAATA GGAGCTCAGAAAATCCTT TGTT-3’	5’-AACAAAGGATTTTCTG AGCTCCTATTTAGTCGAAG GTC-3’	SacI
C354	5’-CCATCTATAAACTAGA ACTTCACAAAATCCGAAC ATT-3’	5’-CCATCTATAAACTAGA AGCTTCACAAAATCCGAA CATT-3’	HindIII
C368	5’CATTGAATAGCACACTT AAGTGAAAAGAACATGAT CTGGC-3’	5’-GCCAGATCATGTTCTT TTCACTTAAGTGTGCTATT CAAT-3’	AfLII
C422	5’-CTGATGACACCCCCTA GGAGCTCAAGAAATCA CC-3’	5’-GATTTCTTGAGTTCCTG GGGGGTGTCATCAGTGTC ATC-3’	SacI

Table 1: Primer information for each HA-IPCEF/Pip3-E construct.

Problems were encountered when both forward and reverse primers were added to the same PCR reaction for each individual pcHA-IPCEF construct. Therefore, reactions were performed with only the forward or reverse primers in the first round of PCR. The PCR products were then combined, 0.5 µL Pfu enzyme added, and this final PCR reaction produced each of the six desired pcHA-IPCEF constructs (see **Table 2**).

	Reaction 1	Reaction 2	Reaction 3
Reaction Components	1µL 100µM pcHA-IPCEF 0.5 µL 10 µM forward primer 1 µL 2.5mM dNTP 2.5 µL 10X ThermoPol buffer 0.5 µL Pfu enzyme 19.5 µL RNAse-free H_2O	1µL 100µM pcHA-IPCEF 0.5 µL 10 µM reverse primer 1 µL 2.5mM dNTP 2.5 µL 10X ThermoPol buffer 0.5 µL Pfu enzyme 19.5 µL RNAse-free H_2O	Combine PCR products of Reactions 1 and 2 0.5 µL Pfu enzyme
Cycle Conditions	10 min., 95ºC 30 sec., 95ºC 1 min., 55ºC 12 min., 68ºC } 15 cycles 10 min., 72ºC	10 min., 95ºC 30 sec., 95ºC 1 min., 55ºC 12 min., 68ºC } 15 cycles 10 min., 72ºC	10 min., 95ºC 30 sec., 95ºC 1 min., 55ºC 12 min., 68ºC } 15 cycles 10 min., 72ºC

Table 2: PCR Reactions and Conditions each HA-IPCEF/Pip3-E construct

The resulting cDNAs were digested with DpnI for one hour at 37ºC and transformed into DH5α cells. Cells were plated on LB-Ampicillin plates at 37ºC overnight and isolated colonies inoculated in 3mL of sterile LB containing 3µL of 50mg/mL Ampicillin shaking overnight at 37ºC. The cultures were mini-prepped using Qiagen® Minipreps DNA Purification System to obtain the plasmid. The plasmid was eluted in 30µL elution buffer. To verify the accuracy of the insert, 2µL of the plasmid DNA was digested with 0.5µL of the indicated enzyme (New England Biolabs) and appropriate buffer (New England Biolabs) with or without 10X BSA (New England Biolabs) for one hour at 37ºC (see **Table 2**). The digests were run on a 1% agarose gel at 120 volts for one hour, and the band sizes confirmed. The authenticity of all constructs was then verified by using Sanger sequencing (The Genomics Core Facility, University Park).

Transformation

After one hour of digestion with DpnI, each reaction was used for transformation of DH5α competent cells. Once the cells were thawed on ice and resuspended, 1 µL of each digestion was added to 50µL of competent cells and the tubes were subsequently mixed gently and incubated on ice for 20 minutes. The cells were heat shocked for 90 seconds in a 42ºC water bath and then incubated on ice for an additional two minutes. The mixtures were added to 800µL sterile SOC media tubes and agitated for one hour at 37ºC. Each of the SOC mixtures were plated on three LB-Ampicillin plates in quantities of 50, 100, and 150µL, and allowed to incubate overnight at 37ºC.

Transfection of HeLa Cells

In Eppendorf tubes, 4.5µg of each HA-tagged IPCEF construct and 4.5µg myc-tagged ARNO were added to 0.5mL Optimem (Gibco®). In separate Eppendorf tubes, 10µL Lipofectamine 2000 (Invitrogen®) was added to 0.5mL Optimem. After a five minute incubation at room temperature, the mixtures were combined and allowed to incubate at room temperature for 30 minutes. The mixtures were then added to $1x10^6$ HeLa cells in 6cm plates along with 2mL of serum-free, antibiotic free DMEM. After five hours incubating at 37ºC and 5% CO_2, media was changed to DMEM supplemented with 10% FBS, glutamine and NEAA.

Co-Immunoprecipitation

HeLa cells were co-transfected with myc-tagged ARNO and the appropriate HA-tagged IPCEF construct. After 14 hours, HeLa cells were washed with phosphate-

buffered saline and lysed in 650µL of lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, and 1% protease inhibitor). Cell lysates were added to 25µL Sepharose® CL-4B (Fluka Biochemika) beads (prewashed with lysis buffer) and centrifuged at 14K for 2 minutes at 4ºC. 25µL supernatant was kept and refrigerated at 4ºC, and the remaining supernantents were incubated with 1 µg of polyclonal α HA antibody (Covance®) for 1 hour at 4°C. The immunocomplexes were then incubated with 25 µl of Immobilized Protein A (G Biosciences) for 2 h at 4 °C and washed three times with lysis buffer. 25µL 2x sample buffer was added to the centrifuged Immobilized Protein A beads and 10µL 2x sample buffer was added to the previously saved supernatant. All samples were boiled for three minutes.

Western Blotting

Protein samples were run on a 10% SDS gel and then transferred to a nitrocellulose membrane (Whatman®). Blocking was performed using 5% dry milk in 1X Phosphate Buffered Saline Tween (PBS-T) solution for one hour at room temperature on a rocking platform. Primary antibody incubation was performed at room temperature for one hour with 1:1,000 monoclonal mouse α myc antibody (Covance®) diluted in PBS-T. Membranes were washed three times with PBS-T for 10 minutes each on a rocking platform. The secondary antibody α Mouse HPR (Southern Biotech) was diluted 1:20,000 in PBS-T and incubated for 30 minutes at room temperature with gentle mixing. Membranes were again washed three times for ten minutes each in PBS-T solution. Immunoreactive bands were visualized by Millipore Immobilon Western

Chemiluminescent HRP Substrate. Images were developed on HyBlot Cl Autoradiography film.

The nitrocellulose membrane was then stripped (see procedure below) and another Western performed using the same procedure. The primary antibody used was 1:1,000 mouse α HA (Covance®) diluted in PBS-T and 1:20,000 α Mouse HRP (Southern Biotech) was used as the secondary antibody. Immunoreactive bands were again visualized by Millipore Immobilon Western Chemiluminescent HRP Substrate and images were developed on HyBlot Cl Autoradiography film.

Membrane Stripping

Membranes were submerged in stripping buffer (62.5 mM Tris-HCl, 100mM β-mercaptoethanol, 2% SDS, pH 6.7) for 30 minutes at 50ºC with occasional agitation. The membranes were washed with PBS-T three times for ten minutes each and re-blocked with 5% dry milk in PBS-T solution.

Primers and PCR

From the results of the Western blots to determine the N and C-terminal boundaries of the ARNO-binding region on Pip3-E/IPCEF, various primers were designed (see **Fig. 8, Table 3**) to amplify this specific region to confirm its sufficiency for cytohesin-binding.

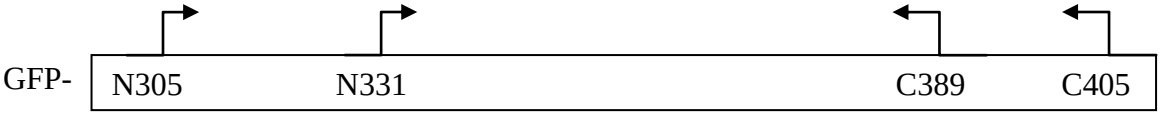


Figure 8: GFP-tagged IPCEF/Pip3-E construct primers.

Primer	Sequence	Digest Enzyme
N305 Forward	5'-CGAACTCGAGGATGATGAAATG GGAAGCTGTAC -3'	XhoI
N331 Forward	5'-CGAACTCGAGACTAAAAAGGAG TGAGAAAATCC -3'	XhoI
C389 Reverse	5'-CCTGAAGCTTTTACCTGGCTGTC AGCTTCGGGTC-3'	HindIII
C405 Reverse	5'-CCTGAAGCTTCTAGATGTCCTGG ATCAGCAGGG -3'	HindIII

Table 3: PCR Primers to create GFP-tagged construct encoding the putative cytohesin-binding region

Select sequences of human Pip3-E/IPCEF were amplified by PCR using 100µg/mL full length pcHAIPCEF plasmid as a template, 10X ThermPol Buffer, 10µM forward or reverse primer (from **Table 3**), dNTPs, *Pfu* DNA polymerase. The PCR reactions were as follows (**Table 4**):

	Reaction 1	Reaction 2	Reaction 3	Reaction 4
Reaction Reagents	1µL 100µM pcHA-IPCEF 1 µL N305 forward primer 1 µL C389 reverse primer 4 µL 2.5mM dNTP 5 µL 10x Pfu buffer 1 µL Pfu enzyme 37 µL RNAse-free H_2O	1µL 100µM pcHA-IPCEF 1 µL N305 forward primer 1 µL C405 reverse primer 4 µL 2.5mM dNTP 5 µL 10x Pfu buffer 1 µL Pfu enzyme 37 µL RNAse-free H_2O	1µL 100µM pcHA-IPCEF 1 µL N331 forward primer 1 µL C389 reverse primer 4 µL 2.5mM dNTP 5 µL 10x Pfu buffer 1 µL Pfu enzyme 37 µL RNAse-free H_2O	1µL 100µM pcHA-IPCEF 1 µL N331 forward primer 1 µL C405 reverse primer 4 µL 2.5mM dNTP 5 µL 10x Pfu buffer 1 µL Pfu enzyme 37 µL RNAse-free H_2O
Cycle	1 min., 94ºC 30 sec., 94ºC 1 min., 55ºC 2 min., 72ºC } 25 cycles 10 min., 72ºC	1 min., 94ºC 30 sec., 94ºC 1 min., 55ºC 2 min., 72ºC } 25 cycles 10 min., 72ºC	1 min., 94ºC 30 sec., 94ºC 1 min., 55ºC 2 min., 72ºC } 25 10 min., 72ºC	1 min., 94ºC 30 sec., 94ºC 1 min., 55ºC 2 min., 72ºC } 25 10 min., 72ºC

Table 4: PCR Reactions of GFP-tagged constructs encoding the putative cytohesin-binding region

Each PCR product was purified with Omega Bio-Tek CyclePure and eluted with 30µL elution buffer. Each reaction, along with the pEGFP-C3 vector (see **Fig. 9**), was prepared for ligation by double digestion with HindIII and XhoI at 37ºC overnight (see **Table 5**).

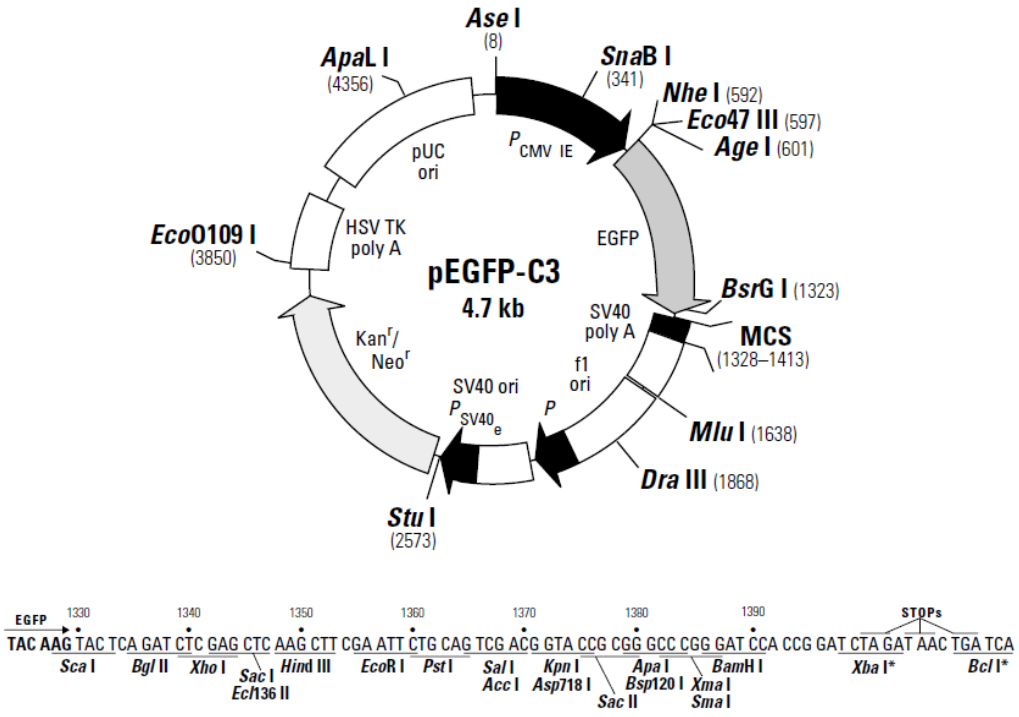


Figure 9: Restriction map and multiple cloning site of pEGFP-C3 (Clontech)

The digestion products were then run on a 2% agarose gel at 120 volts for one hour. Each band was cut out from the gel and purified with Omega Bio-Tek Gel Purification Kit. The purified insert and digested pEGFP-C3 vector were ligated using T4 DNA ligase at room temperature for 1 hour (see **Table 5**).

	Double Digestion of PCR Products	Double Digestion of GFP vector	T4 Ligase Reaction
Reaction Components	30 µL PCR products 1.5 µL HindIII 1.5 µL XhoI 6 µL 10X BSA 6 µL Restriction Enzyme Buffer 2 15 µL RNAse free H_2O	30 µL GFP vector 1.5 µL HindIII 1.5 µL XhoI 6 µL 10X BSA 6 µL Restriction Enzyme Buffer 2 15 µL RNAse free H_2O	2 µL vector 5 µL insert 1 µL 10X ligase buffer 0.5 µL T4 DNA ligae 1.5 µL RNAse free H_2O
Reaction Conditions	Incubate at 37ºC overnight	Incubate at 37ºC overnight	Incubate at room temperature for 60 min.

Table 5: Reactions to construct GFP-tagged IPCEF/Pip3-E constructs

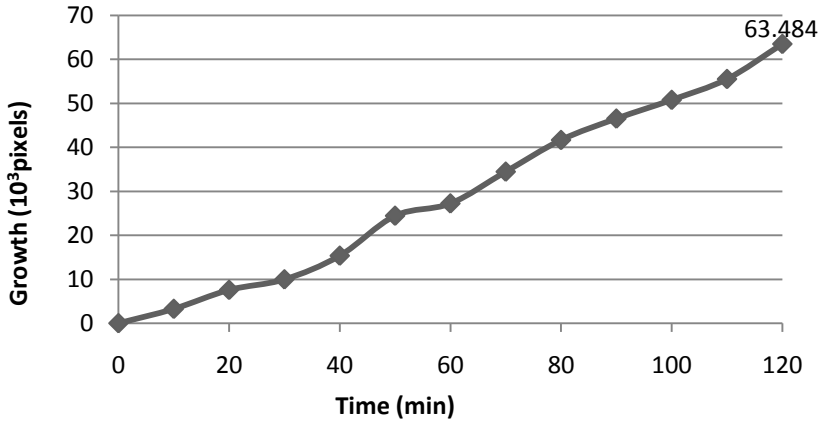
Each reaction was used for transformation of DH5α cells (as described above), plated on LB-Kanamycin at 37ºC overnight, cultured in 25mL of LB containing 25µL of 50mg/mL Kanamycin shaking overnight at 37ºC. The cultures were midiprepped using Qiagen® Midipreps DNA Purification System to obtain the plasmid, which was eluted in 500µL elution buffer. The authenticity of all constructs was then verified by using Sanger sequencing (The Genomics Core Facility, University Park).

The GFP-tagged IPCEF/Pip3-E constructs were transfected into HeLa cells, lysed, and co-immunoprecipitated with a rabbit polyclonal α GFP antibody. Westerns were performed as described above, using 1:1,000 monoclonal mouse α myc and 1:20,000 α Mouse HRP as the secondary antibody. After stripping, the membrane was re-probed with 1:5,000 mouse α GFP (FL) and 1:20,000 α mouse HRP as the secondary antibody. Immunoreactive bands were visualized as described above.

Results

Knockdowns of ARNO and Arf6 Reduce Cellular Migration in MDCK cells

To test the effects of cytohesin 2 and Arf6 knockdowns on the migration of MDCK cells, the migration of untreated control cells was first measured via time lapse microscopy. A monolayer of MDCK cells was wounded with a cell scraper, incubated at 37ºC (the media protected by a thin film of mineral oil), and a photograph of the collectively migrating epithelia was captured every ten minutes for two hours (see **Fig. 10**). Each image was analyzed and cell migration was quantitated using ImageJ software (see **Graph 1**).



Graph 1: Wound-healing assay of untreated MDCK cells. A monolayer of MDCK cells was disrupted by gently wounding the culture with a cell scraper. Time-lapse microscopy was performed as images were captured every ten minutes for two hours. Cellular migration was quantitiated in pixels by measuring the remaining cell-free surface area using the software ImageJ (NIH).

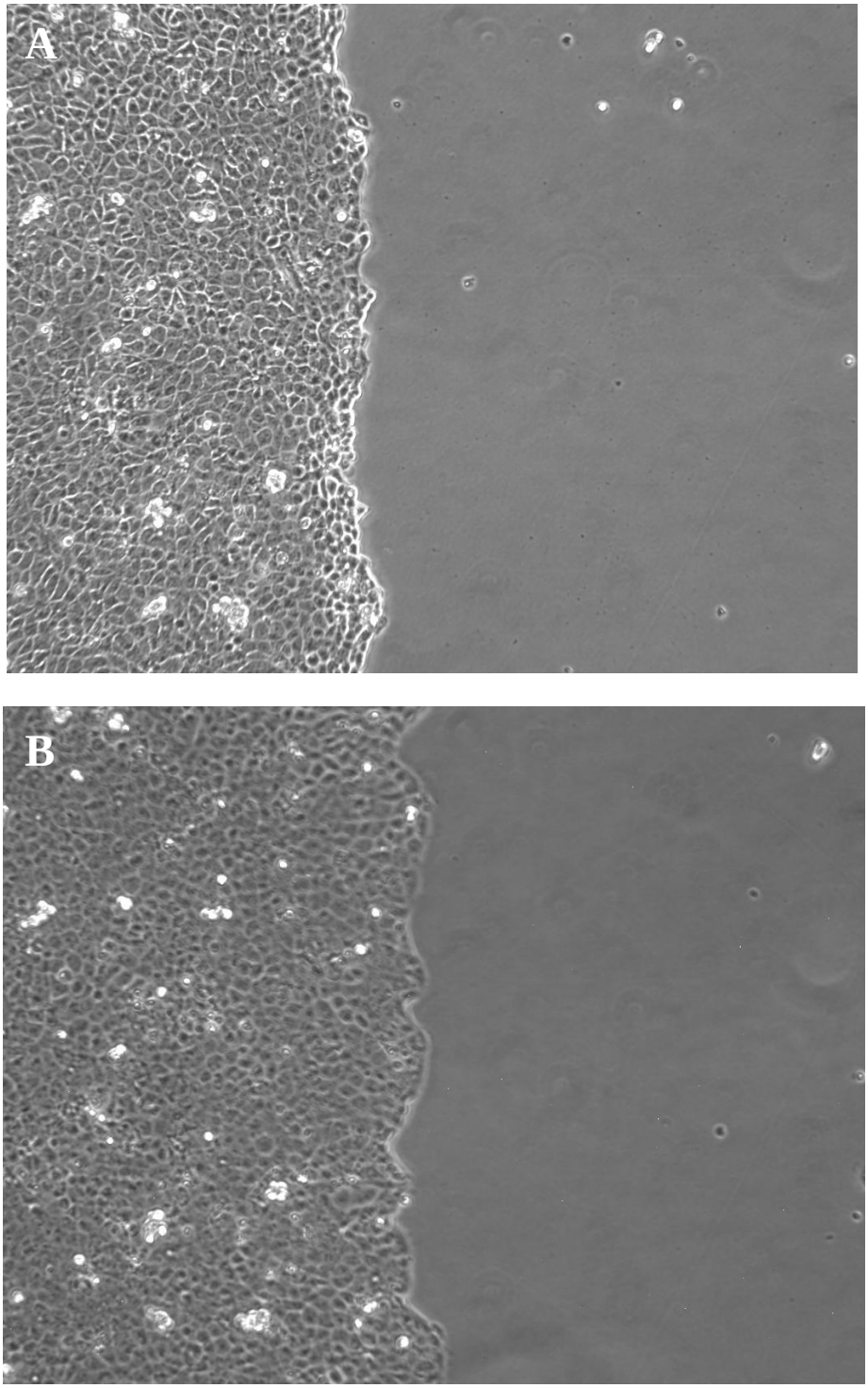
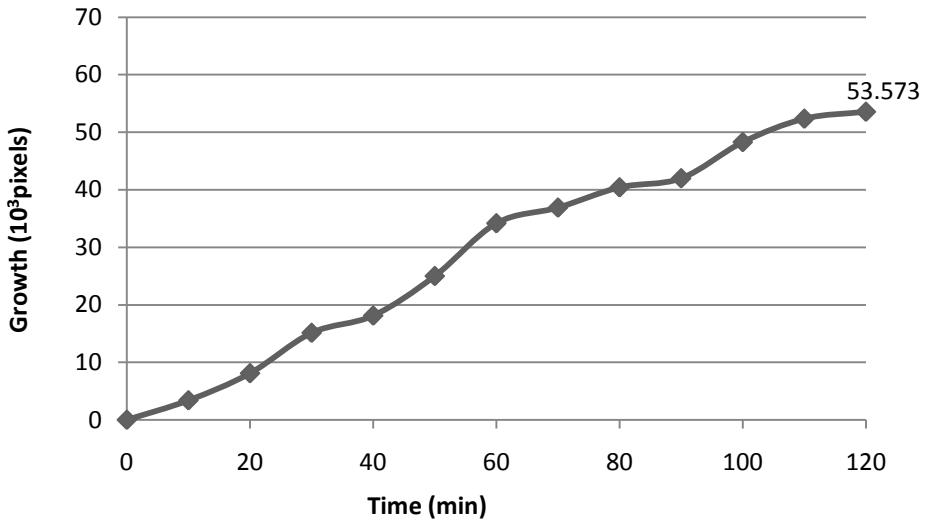


Figure 10: Wound-healing assay of untreated MDCK cells. Time-lapse microscopy was performed using a Zeiss AxioPlan microscope (50X objective) equipped with a Spot RT3 camera as images were captured every ten minutes for two hours. Migration images from **A)** minute 0 and **B)** minute 120.

To confirm that the GEF ARNO is involved in cellular migration, ARNO was inhibited both pharmacologically and by knockdown. The same migration assay was performed with MDCK cells pretreated with 15 µM SecinH3, a cytohesin inhibitor that binds to the Sec7 domains. After treatment, migration was inhibited (see **Fig. 11** and **Graph 2**).



Graph 2: Reduction of ARNO inhibits cell migration. Wound-healing assay of MDCK cells pretreated with 15 µM SecinH3 shows decreased migration. A monolayer of MDCK cells was disrupted by gently wounding the culture with a cell scraper. Time-lapse microscopy was performed as images were captured every ten minutes for two hours. Cellular migration was quantitiated in pixels by measuring the remaining cell-free surface area using the software ImageJ (NIH).

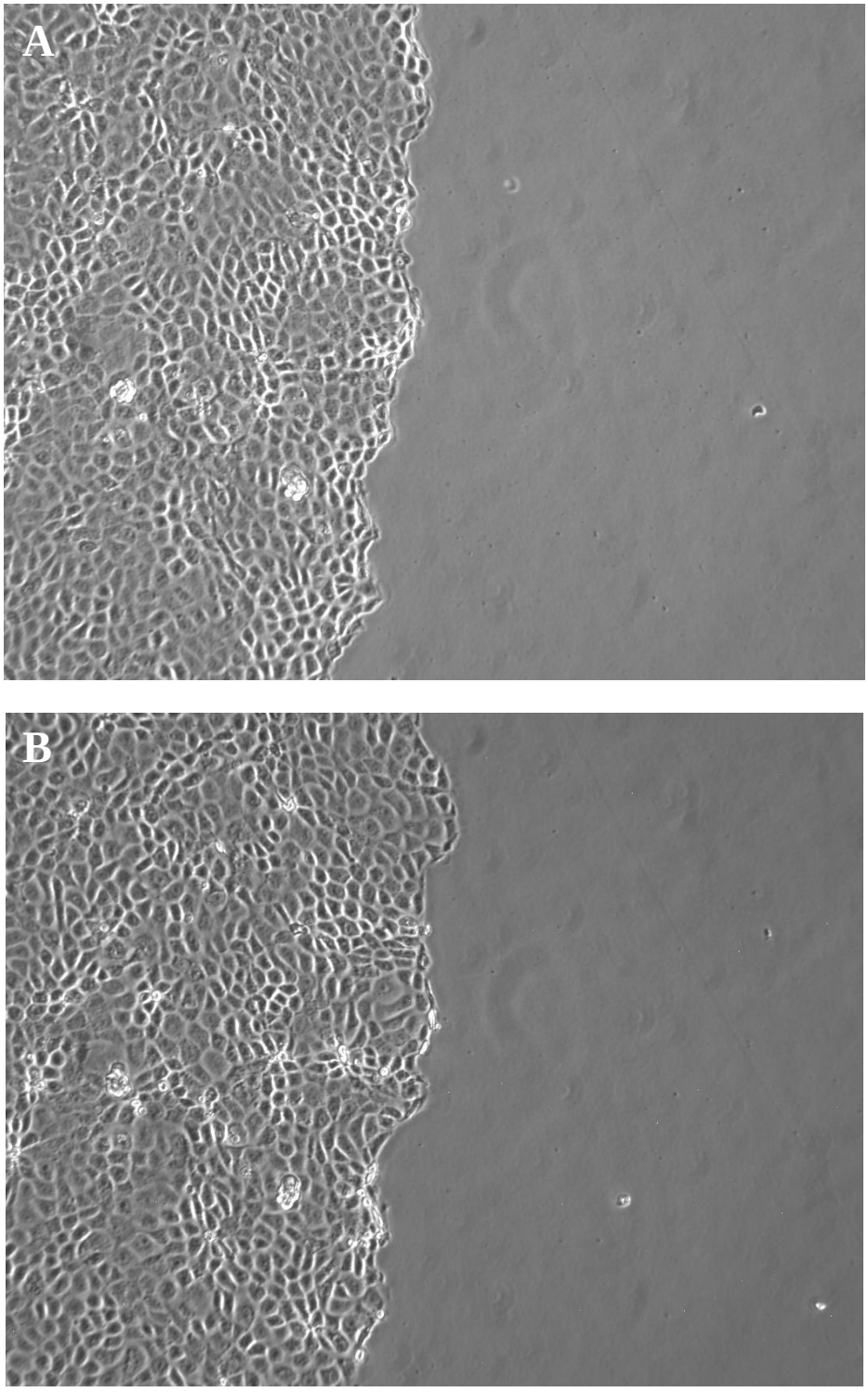
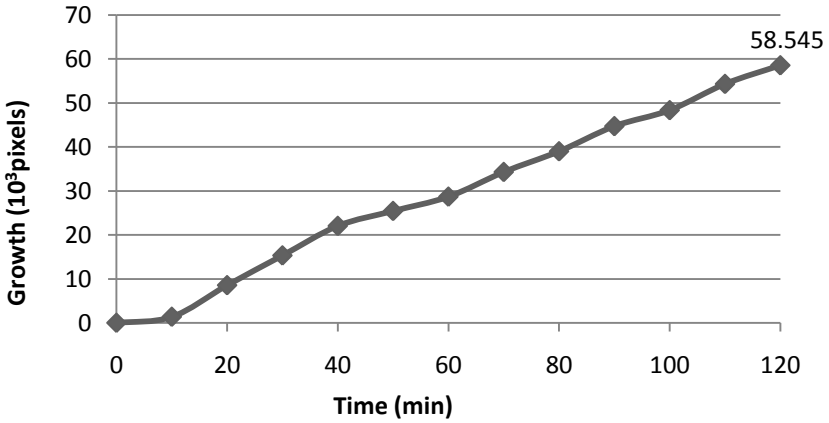


Figure 11: Wound-healing assay of MDCK cells pretreated with 15 µM SecinH3. Time-lapse microscopy was performed using a Zeiss AxioPlan microscope (50X objective) equipped with a Spot RT3 camera as images were captured every ten minutes for two hours. Migration images from **A)** minute 0 and **B)** minute 120.

ARNO was again inhibited by performing an ARNO knockdown on MDCK cells, as described in the Materials and Methods. The same wound-healing migration assay was completed on the transfected cells. ARNO-knockdown cells displayed decreased migration patterns (see **Fig. 12** and **Graph 3**).



Graph 3: Reduction of ARNO inhibits cell migration. ARNO levels in MDCK cells were reduced by transfection of siRNA against ARNO for 2 days before the wound healing assay was performed. A monolayer of MDCK cells was disrupted by gently wounding the culture with a cell scraper. Time-lapse microscopy was performed as images were captured every ten minutes for two hours. Cellular migration was quantitiated in pixels by measuring the remaining cell-free surface area using the software ImageJ (NIH).

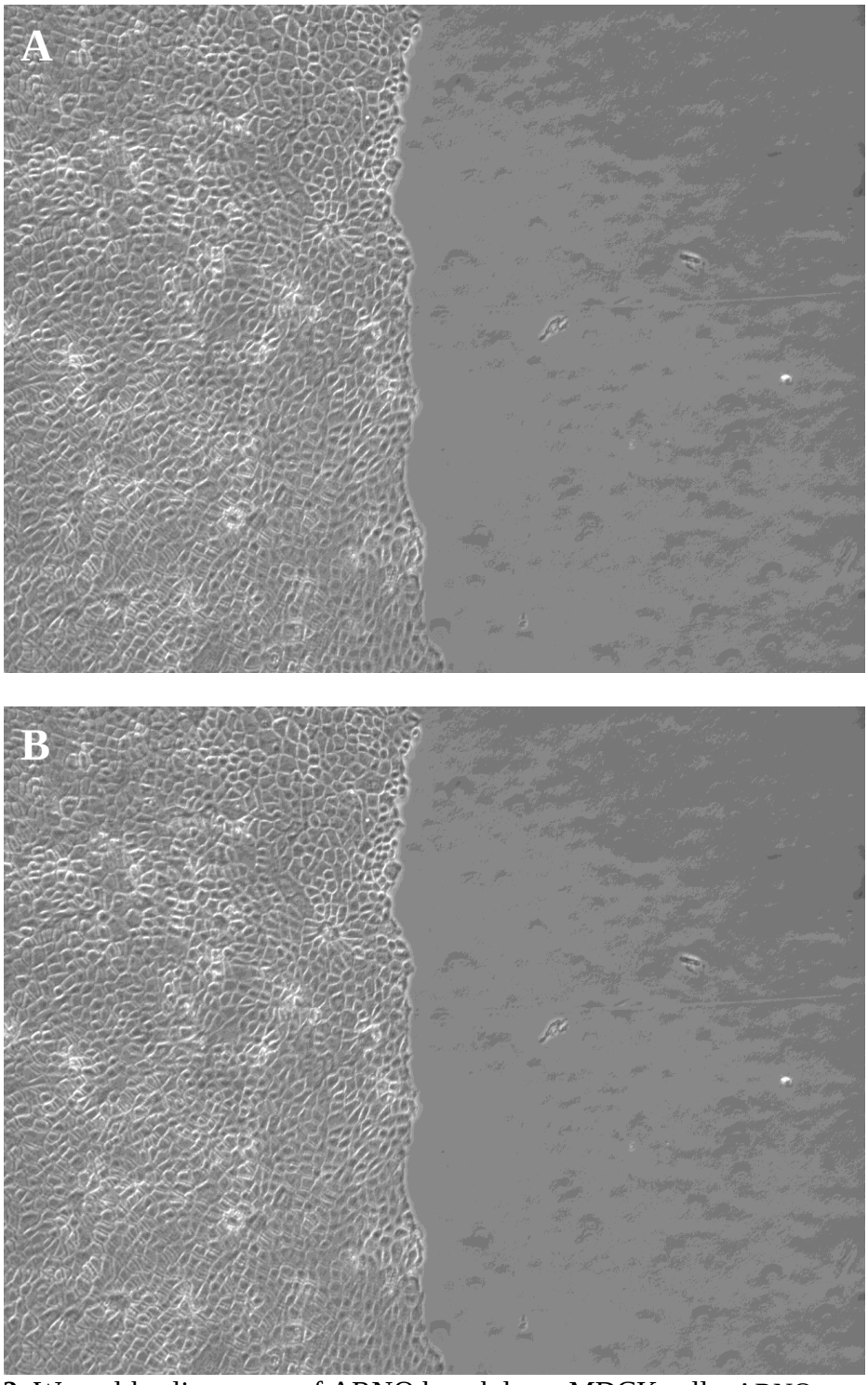
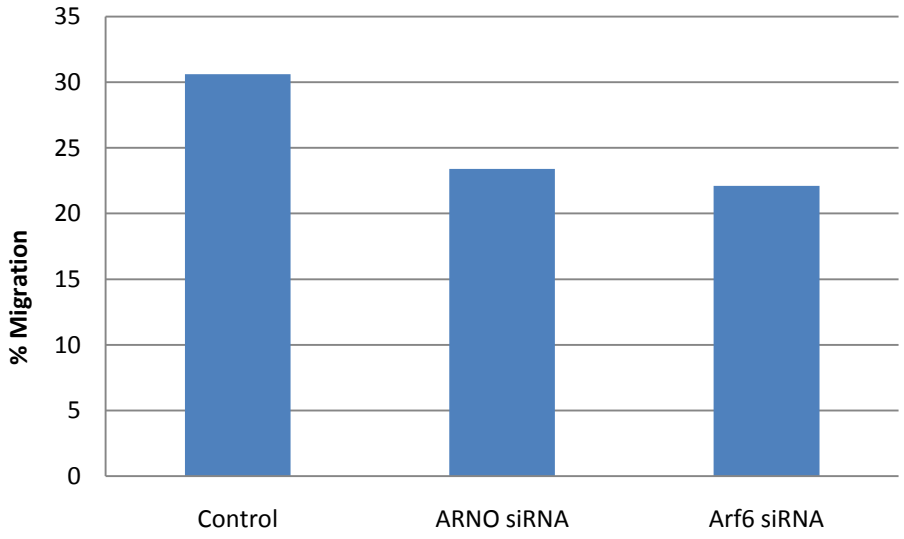


Figure 12: Wound-healing assay of ARNO knockdown MDCK cells. ARNO levels in MDCK cells were reduced by transfection of siRNA for 2 days before the wound healing assay was performed.. Time-lapse microscopy was performed using a Zeiss AxioPlan microscope (50X objective) equipped with a Spot RT3 camera as images were captured every ten minutes for two hours. Migration images from **A)** minute 0 and **B)** minute 120.

During the course of this research, a modification of this procedure became commercially available, the ORIS Migration Camber (Oris™ Universal Cell Migration Assembly Kit: Platypus Technologies, Madison, WI). In this assay, cells are grown in a 96-well plate around a silicon plug. The plug is then removed and the cells migrate into the open area. MDCK cells ($5x10^4$) were seeded in the chambers. After incubation at 37ºC overnight, the plug was removed to initiate the cellular migration into the vacated area. As expected, cellular migration was reduced in cells transfected with siRNA targeting ARNO, as was seen previously. Cell migration was also reduced in cells treated with siRNA targeting Arf6.



Graph 4: Reduction of ARNO and Arf6 both inhibit MDCK cell migration. ARNO or Arf6 levels in MDCK cells were reduced by transfection of siRNA for 2 days, and cell migration measured using the Oris migration chamber as described in Experimental Procedures. Images of each well were taken and cellular migration was quantitated with the software Image J by measuring the percent of the starting open area covered by the migrating cells. Results shown were an average of ten separate experiments.

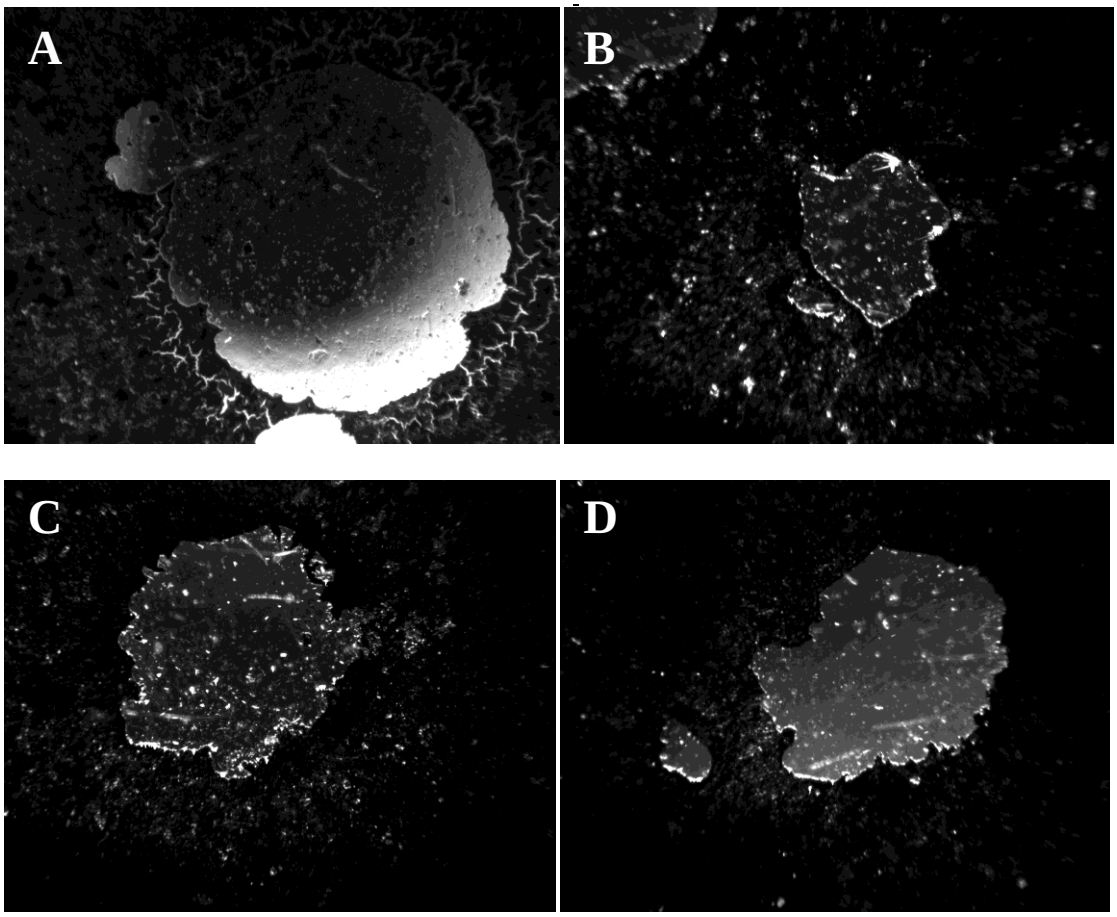


Figure 13: Reduction of ARNO and Arf6 both inhibit MDCK cell migration. ARNO or Arf6 levels in MDCK cells were reduced by transfection of siRNA for 2 days, and cell migration measured using the Oris migration chamber as described in Experimental Procedures. Images of each well were taken and cellular migration using a Zeiss AxioPlan microscope (10X objective) equipped with a Spot RT3 camera. Images shown are **A)** no migration, **B)** untreated MDCK control, **C)** MDCK cells transfected with ARNO siRNA, **D)** MDCK cells transfected with Arf6 siRNA.

Determining the ARNO-binding Site to the CRAC Domain

The Materials and Methods section described the construction of six HA-IPCEF/Pip3-E C-terminal truncations (C312, C322, C338, C354, C368, and C422). Two additional C-terminal truncations were constructed previously (C389 and C405) and three N-terminal truncations (N305, N331, N354) were made beforehand as well. In total, eleven different HA-tagged IPCEF truncations constructs were used in this experiment. The eleven HA-IPCEF/Pip3-E and myc-tagged ARNO were co-transfected into HeLa cells, lysed, and co-immunorecipitated with rabbit polyclonal α HA. Western blotting was then performed, using mouse monoclonal α HA as the primary antibody to visualize the HA-tagged IPCEF constructs from the co-immunoprecipiates (see **Fig. 14**).

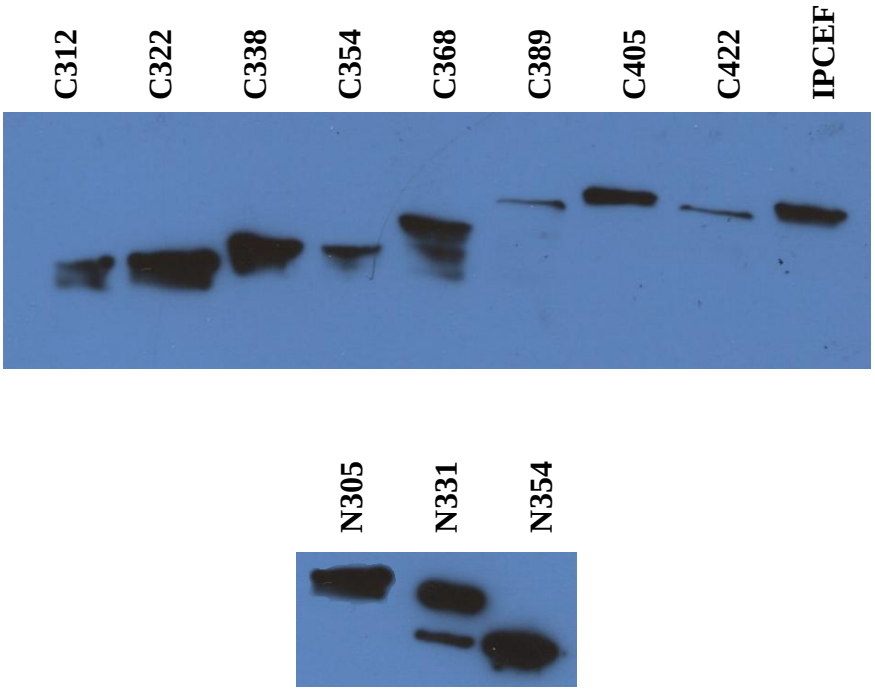


Figure 14: HA-IPCEF/Pip3-E appears in all co-immunoprecipitations. Western Blot analysis was performed on co-immunoprecipitated HeLa cells previously co-transfected with myc-tagged ARNO and the appropriate HA-tagged IPCEF construct. HeLa cells were lysed, co-immunoprecipitated with polyclonal α HA antibody, and Western blotted for monoclonal mouse α HA antibody.

The same nitrocellulose membrane was stripped and probed with mouse monoclonal α myc to visualize the myc-tagged ARNO that co-immunoprecipiated with HA-tagged IPCEF (see **Fig. 15**).

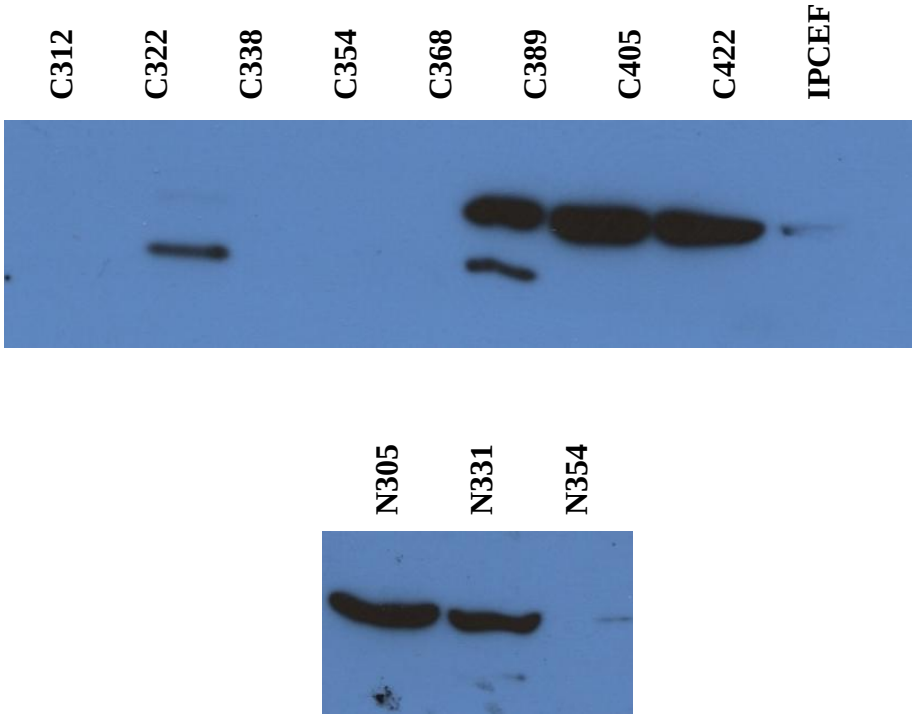


Figure 15: myc-ARNO appears in HA-IPCEF/Pip3-E constructs C389-C422 and N305-N331, along with full length HA-IPCEF (used as a control). Western Blot analysis was performed on co-immunoprecipitated HeLa cells previously co-transfected with myc-tagged ARNO and the appropriate HA-tagged IPCEF construct. HeLa cells were lysed, co-immunoprecipitated with polyclonal α HA antibody, and Western blotted for monoclonal mouse α myc antibody.

To confirm that myc-ARNO was indeed present in the lysed HeLa cells before the co-immunoprecipitation was performed, the supernatants were also Western blotted with mouse α myc (see **Fig. 16**).

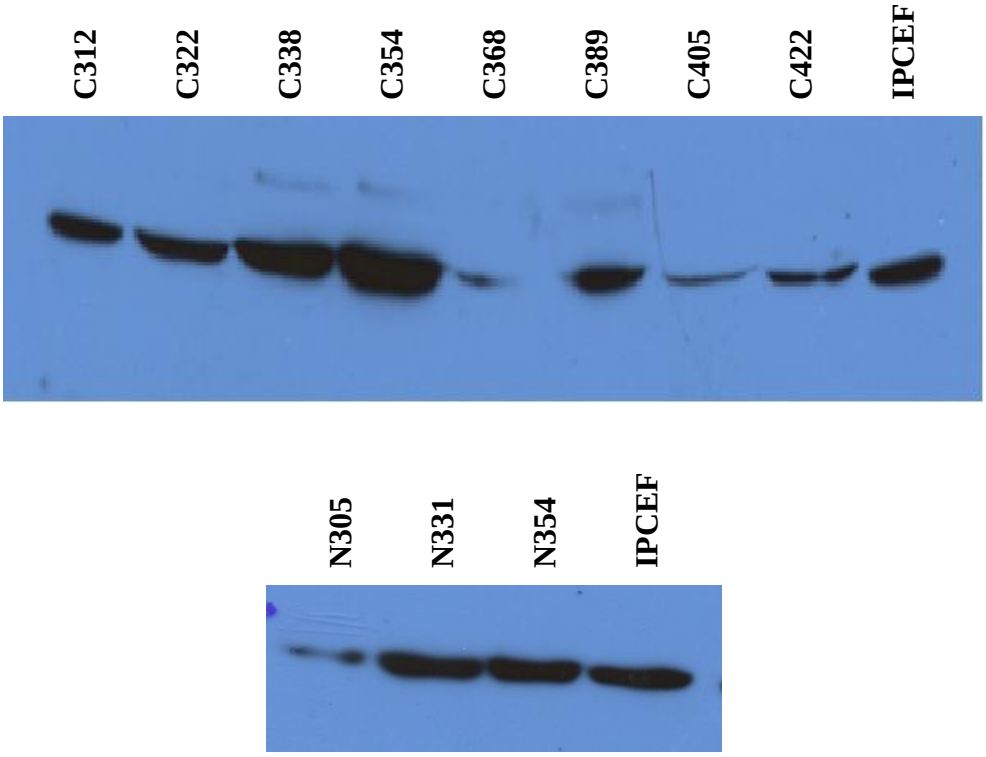


Figure 16: myc-ARNO appears in all supernatants of lysed HeLa cells before co-immunoprecipitation with polyclonal α HA antibody. Supernatants were Western blotted for monoclonal mouse α myc antibody.

Determining the Sufficiency of the CRAC Domain for Cytohesin Binding

From the results of the Western blots to determine the N and C-terminal boundaries of the ARNO-binding region on Pip3-E/IPCEF, various primers were designed (see **Fig. 8, Table 3** of Materials and Methods) to amplify this specific region to confirm its sufficiency for cytohesin-binding. Three regions were constructed: N305F-C389R, N331F-C389R, and N331F-C405R (the fourth construct, N305F-C405R, could not be transformed into DH5α cells).

Again, the three different GFP-tagged IPCEF/Pip3-E constructs and myc-ARNO were each co-transfected into HeLa cells, lysed, and co-immunorecipitated with rabbit polyclonal α GFP. Western blotting was then performed, using mouse monoclonal α GFP

as the primary antibody to visualize the GFP-tagged IPCEF constructs from the co-immunoprecipiates (see **Fig. 14**).

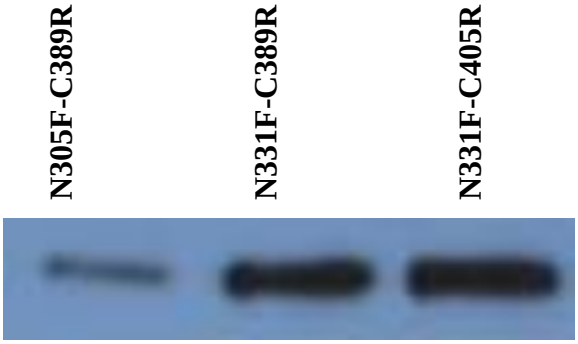


Figure 17: GFP-IPCEF/Pip3-E appears in all co-immunoprecipitations. Western Blot analysis was performed on co-immunoprecipitated HeLa cells previously co-transfected with myc-tagged ARNO and the appropriate GFP-tagged IPCEF construct. HeLa cells were lysed, co-immunoprecipitated with polyclonal α GFP antibody, and Western blotted for monoclonal mouse α GFP antibody.

The same nitrocellulose membrane was stripped and probed with mouse monoclonal α myc to visualize the myc-tagged ARNO that co-immunoprecipiated with GFP-tagged IPCEF (see **Fig. 18**).

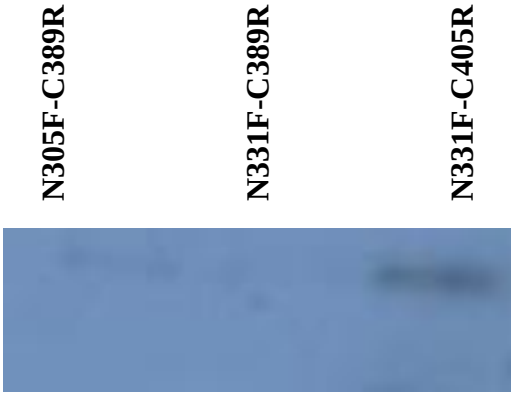


Figure 18: myc-ARNO appears only slightly in GFP-IPCEF/Pip3-E construct N331F-C405R. Western Blot analysis was performed on co-immunoprecipitated HeLa cells previously co-transfected with myc-tagged ARNO and the appropriate GFP-tagged IPCEF construct. HeLa cells were lysed, co-immunoprecipitated with polyclonal α GFP antibody, and Western blotted for monoclonal mouse α myc antibody.

To confirm that myc-ARNO was indeed present in the lysed HeLa cells before the co-immunoprecipitation was performed, the supernatants were also Western blotted with mouse α myc (see **Fig. 19**).

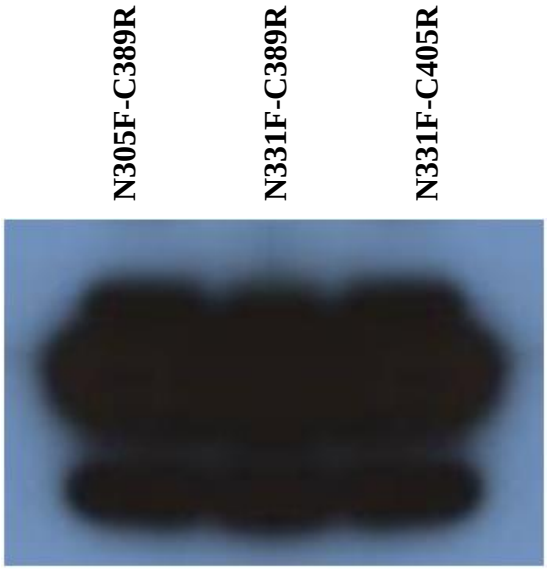


Figure 19: myc-ARNO appears in all supernatants of lysed HeLa cells before co-immunoprecipitation with polyclonal α GFP antibody. Supernatants were Western blotted for monoclonal mouse α myc antibody.

Discussion

Many common cancers, including those of the breast, colon, lung, and prostate, arise from epithelial tissues. Though the cells making up the epithelia are normally non-motile, they can become migratory (such as during tumor metastasis). Understanding the pathways that control migration in these cells is critical to developing strategies to halt metastasis of epithelial cancers.

Though activating Ras mutations are found in one fifth of human tumors (Bos 1989), the pathways acting downstream of Ras to alter cell shape and initiate migration remain unclear. Likewise, while Arf6 acts downstream of growth factor receptors to alter cell shape and regulate migration, the pathway that activates this GTPase is not well understood. Until these pathways are elucidated, our knowledge of growth factor regulated migration is strongly limited. The research for this thesis was a small part in defining a Ras-dependent pathway leading from growth factor receptors to the activation of small GTPases that can alter cell shape and regulate epithelial migration.

The overall objective of this study was to investigate epithelial cell motility. In order to accomplish this, first cell migration assays were conducted on the T23 line of MDCK cells. Knockdowns of both ARNO and ARF6 inhibited migration, implicating their respective functions in promoting cellular migration. The inhibition of ARNO via SecinH3 also decreased cellular migration.

Secondly, this paper investigated the binding of cytohesins to the CNK protein family. This was accomplished by a series of truncation mutants of Pip3-E/IPCEF, which is homologous to the C-terminal half of mammalian CNK1 and CNK2. Stop codons were introduced throughout the C-terminus 120 amino acids every 10 to 20 residues. Similar

truncations were added to the N-terminus of Pip3-E/IPCEF. From the results obtained, cytohesin 2/ARNO was determined to bind to C-terminal truncations C389, C405, C422, and full-length IPCEF/Pip3-E. ARNO also bound to N-terminal truncations N305 and N331.

The cytohesin-binding region, located in the CRAC domain, was then confirmed for its sufficiency to bind ARNO. Primers were designed to amplify this specific region of the CRAC domain to confirm its ability to interact with ARNO. Of the three regions constructed (N305-C389, N331-C389, and N331-C405), only the N331-C405 domain was able to bind cytohesin 2. The interaction, though, was not strong as evidenced by the weak band corresponding to myc-ARNO on the Western blot (**Fig. 18, Lane 3**). Though the C389 region bound cytohesin 2 strongly in the C-terminal truncation mutant, only including the Pip3-E/IPCEF region up to the C389 primer location was not sufficient to bind ARNO (neither the N305-C389 nor the N331-C389 mutant bound cytohesin 2). ARNO clearly needs additional C-terminal amino acids (up to those included by the C405 primer) in order to bind sufficiently.

These results confirm that cytohesin 2 binds to the CRAC region of the C-terminal. Since the CRAC domain is conserved, it is therefore predicted that CNK1 and CNK2 will also bind cytohesins. This research found that the previously undefined conserved CRAC domain of the CNK family most likely functions as a cytohesin binding region.

Others projects currently being conducted at the time of this research include definitively linking Ras to CNK activation via cytohesin binding. This would further elucidate the Ras-dependent pathway of cellular migration. Additional experiments to

further study the CRAC domain of CNKs include performing co-immunoprecipitations as described above, transfecting ARNO along with either CNK1 or CNK2. The results from these experiments can finally confirm that all members of the CNK family bind cytohesins in the conserved CRAC domain. After this is proven, knockdowns can be performed on CNKs 1 and 2 to show impairment of ARF6 functions. This would link the CNK family to ARF6 activation, further elucidating the Ras-dependent pathway of cellular migration.

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Academic Vita

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EDUCATION

The Pennsylvania State University, University Park, PA
B.S. in Biochemistry and Molecular Biology, May 2010
Minors, History and Biology
Schreyer Honors College

RESEARCH

Lorraine Santy Lab: Penn State University, University Park, PA (May 2007 – May 2010)

- Engage in epithelial protein-protein interaction research
- Study small GTPase regulation of epithelial motility
- Perform PCR, agarose gels, DNA purifications, mini- and midi-preps, transductions, cell culture, transfections, immunoprecipiatations, SDS-PAGE gels, Western blots, and more
- Received 2008 Summer Discovery Grant

Dr. Sheetij Dutta Lab at Walter Reed (January 2009-August 2009)

- Conducted site-directed mutagenesis on malaria antigen "X"
- Constructed protein chimera from DNA through to protein purification
- Skills include PCR, DNA extraction, cloning, minipreps, DNA digestion, ligation, transformation, induction, microfluidizing, HPLC columns, Westerns, Coumassie gels, ELISAs, GIAs, native gels, parasites, mouse injections/bleeds, and more

WORK EXPERIENCE

Women in Science Camp, University Park, PA (May - August 2008)

- Worked as science mentor to high school-aged girls
- Served as mentor for girls interested in science-related careers
- Created science-related activities for participants

Program Assistant in Pennypacker Hall, University Park, PA (August 2008 – May 2008)

- Serve as academic mentor to residents of Pennypacker
- Hold weekly office hours to tutor science subjects
- Provide bi-weekly programs on academic subjects
- Foster sense of community among residents

ACTIVITIES

Oboe (August 2006 – May 2010)
Double Reed Ensemble, Campus Band, Symphonic Band, Sinfonietta Orchestra, Chamber Orchestra, Small Woodwind Trio Ensemble

Student Red Cross Club (January 2007 – May 2010)
On-Site Coordinator
Mt. Nittany Medical Center Volunteer (January 2007 – May 2010)
Volunteer in Oncology Unit, Sterile Processing, Pharmacy, Emergency Departments

Science LionPride (January 2008 – May 2010)
Founding member
Participate in Open House Days, Group Tours, Alumni events, and more
Serve on Tour Guide Committee

Biochemistry Society (August 2008 – December 2008)
President and Founder
Provide educational seminars on biochemistry topics, invite lecturers to speak, foster sense of community in BMB department

ACCOMPLISHMENTS AND AWARDS

- Dean's List
- Member of the Schreyer Honors College (August 2006-present)
- Academic Excellence Scholarship (every semester from August 2006-present)
- Discovery Research Grant (Summer 2008)
- Biochemistry Departmental Award for Academic Excellence (May 2008)
- Awarded the Ruth Ott Memorial Scholarship for Academic Excellence
- Member of Phi Beta Kappa Honor Society