IMPACT OF SUBCELLULAR LOCALIZATION ON ONCOGENIC FUNCTIONS OF THE RHOGEF ECT2 AND ON ITS RHO GTPASE TARGETS

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ABSTRACT

Lauren Parker Huff: Impact of subcellular localization on oncogenic functions of the RhoGEF Ect2 and on its Rho GTPase targets
(Under the direction of Adrienne D. Cox)

Rho GTPases are molecular switches that canonically signal from the plasma membrane or endomembranes to control a wide variety of cellular processes. Their activation is tightly regulated spatiotemporally via regulatory proteins and post-translational modifications. Here, I summarize the recently appreciated consequences of GTPase C-terminal phosphorylation on their localization, effector utilization and biological functions. I also describe in detail the use of recruitment assays for monitoring the subcellular locations of GTPase activity. Misregulation of Rho GTPases is associated with many types of cancer. For example, Rho Guanine nucleotide exchange factors (GEFs), which stimulate exchange of GDP for GTP to turn GTPases "on," are frequently found overexpressed in tumors and often are necessary for cellular transformation. Here I report validation of a RhoGEF inhibitor with a dose-dependent selectivity for Rho GTPase signaling and anti-transformation activity. I have focused primarily on the RhoGEF Ect2 and its role in ovarian tumors, where it is chromosomally amplified and its mRNA is overexpressed. I observed that Ect2 protein is highly expressed and predominantly nuclear, and that nuclear but not cytoplasmic Ect2 increases with advanced ovarian disease. Knockdown of Ect2 decreased anchorage-independent (but not anchorage-dependent) growth of ovarian cancer cell lines. Restoration of Ect2 expression rescued the anchorage-independent growth defect, which required both the DH catalytic domain and the nuclear localization sequences (NLS) of Ect2. This

suggested a novel mechanism whereby Ect2 could drive transformation in ovarian cancer cells by acting as a RhoGEF within the nucleus. Interestingly, Ect2 had an intrinsically distinct GTPase specificity profile in the nucleus versus the cytoplasm. Nuclear Ect2 bound preferentially to Rac1, while cytoplasmic Ect2 bound to RhoA but not Rac. Consistent with nuclear activation of endogenous Rac, Ect2 overexpression recruited Rac effectors to the nucleus, a process that required a functional Ect2 catalytic domain. Further, expression of active nuclear Rac1 rescued the defect in transformed growth caused by Ect2 knockdown. My work suggests a novel mechanism of Ect2-driven transformation, identifies subcellular localization as a regulator of GEF specificity, and implicates activation of nuclear Rac1 in cellular transformation.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS AND SYMBOLS

a.a.	amino acid
Akt	protein kinase B
AP-1	activator protein 1
APC	adenomatous polyposis coli or anaphase promoting complex
Arf	ADP ribosylation factor
ARHGEF6	Rho guanine nucleotide exchange factor 6
ARP2/3	actin-related protein 2/3
ASEF	APC-stimulated guanine nucleotide exchange factor
BDK	bradykinin
BRCT	BRCA1 C-terminus
Brd-U	bromodeoxyuridine
C-terminus	carboxyl-terminus
CAAX	cysteine-aliphatic-aliphatic-unconserved amino acid
CaM	calmodulin
cAMP	cyclic AMP
CCM1	cerebral cavernous malformation protein 1
Cdc42	cell division cycle 42 small GTPase
Cdk-1, -5	cyclin-dependent kinase-1, -5
cDNA	complementary deoxyribonucleic acid
CENP-A	centromere protein A
CIP	calf intestinal alkaline phosphatase
CK1	casein kinase 1

COSMIC	catalogue of somatic mutations in cancer
CRIB	Cdc42- and Rac- interactive binding domain
CYT	cytoplasm
DAPI	4',6-diamidino-2-phenylindole
Dbl	diffuse B-cell lymphoma
Dbs	Dbl's big sister
ddH2O	double distilled water
DH	Dbl homology
DHR1/DHR2	Dock-homology region-1 and -2
DLC1	deleted in liver cancer 1
DMEM-H	high glucose Dulbecco's modified Eagle medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DOCK	dedicator of cytokinesis
DPBS	Dulbecco's phosphate-buffered saline
ECM	extracellular matrix
Ect2	epithelial cell transforming sequence #2
EGFR	epidermal growth factor receptor
ELM	the eukaryotic linear motif resource
ELMO	engulfment and cell motility
ERK	extracellular-signal-regulated kinase
ESCC	esophageal squamous cell carcinoma
FAK	focal adhesion kinase

FBS	fetal bovine serum
FIP3	Rab11 family-interacting protein
FRET	fluorescence resonance energy transfer
FTase	farnesyltransferase
FTI	farnesyltransferase inhibitor
Gal3	galectin-3
GalT	galactosyl transferase
GAP	GTPase-activating protein
GBD	GTPase-binding domain
GBM	glioblastoma multiforme
GCS	GIBCO calf serum
GDF	GDI displacement factor
GDI	guanine nucleotide-dissociation inhibitor
GDP	guanine diphosphate
GEF	guanine nucleotide exchange factor
GFP	green fluorescent protein
GGTase-I,-II	geranylgeranyltransferase-I, -II
GGTI	geranylgeranyltransferase inhibitor
GPCR	G protein-coupled receptor
Grb2	growth factor receptor-bound protein 2
GST	glutathione-*S*-transferase
GTP	guanine triphosphate
GTPγS	guanosine 5'-O-[gamma-thio]triphosphate

H-Ras	Harvey rat sarcoma virus oncogene homolog small GTPase
HA	hemagglutinin
HVR or HVD	hypervariable region or hypervariable domain
Icmt	isoprenylcysteine carboxyl methyltransferase
IHC	immunohistochemistry
IM	internal membrane
ITSN-1	intersectin-1
JNK	c-Jun N-terminal kinase
K-Ras	Kirsten rat sarcoma virus oncogene homolog small GTPase
LARG	leukemia-associated RhoGEF
LIMK	LIM domain kinase
LPA	lysophosphatidic acid
MAL	megakaryocytic acute leukemia
mDia	mammalian diaphanous-related formin-1
MEK	mitogen-activated protein kinase kinase
MgcRacGAP	Rac GTPase-activating protein 1 (RacGAP1)
min	minute
MKLP1	mitotic kinesin-like protein 1
MLCK	myosin light chain kinase
MLK3	mixed lineage kinase 3
MMP	matrix metalloproteinase
MRCK	myotonic dystrophy kinase-related CDC42-binding kinase
mRNA	messenger ribonucleic acid

MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
MYPT1	myosin-binding subunit of myosin phosphatase 1
N-Ras	neuroblastoma-Ras
N-terminus	amino-terminus
N-WASP	neural Wiskott-Aldrich syndrome protein
NADPH oxidase	nicotinamide adenine dinucleotide phosphate oxidase
NES	nuclear export signal
Net1	neuroepithelial cell transforming gene 1
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NLS	nuclear localization signal
NSCLC	non-small cell lung cancer
NT	non-targeted
NUC	nucleus
PAK	p21-activated kinase
PARP	poly (ADP-ribose) polymerase
PBD	p21-binding domain
PBR	polybasic region
PBS	phosphate-buffered saline
PDEδ	retinal rod rhodopsin-sensitive cGMP 3',5'-cyclic phosphodiesterase subunit delta
PDGF	platelet-derived growth factor
PDK1	phosphoinositide-dependent kinase 1
PFA	paraformaldehyde

PH	pleckstrin homology
PI3K	phosphatidylinositol 3-kinase
PIP3	phosphatidylinositol (3,4,5)-triphosphate
PKA	cAMP-dependent protein kinases
PKCi	protein kinase C iota
PKG	cGMP-dependent protein kinase
PLD	phospholipase D
Plk-1,-4	polo-like kinase-1, -4
PM	plasma membrane
PMA	phorbol-12-myristate-13-acetate
POSH	plenty of SH3 domains
PRA1	prenylated Rab acceptor 1
PTEN	phosphatase and tensin homolog
RA	Ras association domain
Rab	rat brain small GTPase
Rac-1, -2, -3	Ras-related C3 botulinum toxin substrate-1, -2, -3 small GTPase
RacGAP1	Rac GTPase-activating protein 1 (MgcRacGAP)
Raf	rapidly accelerated fibrosarcoma kinase
Ral	Ras-like small GTPase
Ral BP1	Ral-binding protein 1
RalGDS	Ral guanine nucleotide dissociation stimulator
Ran	Ras-related nuclear protein small GTPase
Rap	Ras-proximate small GTPase

RBD	Ras (or Rho) binding domain
Rce1	Ras and a-factor converting enzyme 1
RhoA,-B,-C,-D,-E,-F,-H	Ras homology small GTPase
RhoBTB-1,-2,-3	Rho-related bric-à-brac domain containing small GTPase
ROCK/ROK	Rho-associated protein kinase
RPMI	Roswell Park Memorial Institute cell culture medium
RT-PCR	reverse transcription polymerase chain reaction
SAAX	serine-aliphatic-aliphatic-unconserved amino acid
SEM	standard error of the mean
shRNA	short hairpin ribonucleic acid
siRNA	small interfering ribonucleic acid
SLAT	SWAP70-like adaptor of T cells
SLK	Ste20-related kinase
SMART	simple modular architecture research tool
SmgGDS	small GTPase GDP-dissociation stimulator 1
SNRNP200	U5 small nuclear ribonucleoprotein 200 kDa helicase
SOS	son of sevenless
SRE	serum response element
SRF	serum response factor
STAT-3,-5a	signal transducer and activator of transcription-3, -5a
SV40T	simian vacuolating virus 40 large T antigen
SWAP70	switch-associated protein 70
TC10	teratocarcinoma 10 small GTPase

TCF	T-cell factor
TCL	TC10-like small GTPase
Tiam1	T-cell invasion and metastasis gene 1
TMA	tissue microarray or tumor microarray
TPA	12-O-tetradecanoylphorbol-13-acetate
Trio	triple functional domain protein
TUNEL	terminal deoxynucleotidyl transferase dUTP nick-end labeling
Wrch-1, -2	Wnt-regulated Cdc42 homolog-1, -2 small GTPase
WT	wild type
YopT	Yersinia outer protein T
Zn2+	zinc
μm	micron
μM	micromolar

CHAPTER 1: INTRODUCTION

THE RAS SUPERFAMILY OF SMALL GTPASES

Definition/structure/function/regulation

The Ras superfamily is a diverse family of >150 small GTPases that can be further categorized into 5 subfamilies: Ras, Rho, Arf, Ran, and Rab (1). Each member contains a catalytic G domain, composed of 2 well-conserved loop regions termed switch I and switch II, and a C-terminal hypervariable region (2). By definition, a Ras superfamily GTPase is a monomeric protein that binds to and hydrolyzes guanine nucleotides. However, GTPases are poor hydrolases and can stably interact with either GDP or GTP (1). This allows GTPases to function as molecular switches within the cell (Fig.1.1). GTPases are considered "off" while GDP-bound, because they bind with low affinity to downstream effectors in this state. Exchange of GDP for GTP causes a conformational change in switch I and switch II, turning the GTPase "on" and allowing switch I to interact more strongly with downstream effectors. Guanine nucleotide exchange factors (GEFs) promote release of guanine nucleotides from GTPases. Since cellular GTP is 10x more abundant than GDP this enhances binding of GTP to activate the GTPase (2). In contrast, GTPase activating proteins (GAPs) enhance the intrinsic hydrolase activity of the GTPase, causing it to become GDP-bound and "off."

Summary of subfamilies

Members of the Ras superfamily are categorized into 5 subfamilies based on their structure and function (1). Rabs and Arfs are regulators of vesicular transport (3, 4). The Ran family is comprised of a single human GTPase, Ran (1), that shuttles cargo in and out of the nucleus (5). Ras and Rho family GTPases are the focus of this dissertation. GTPases within these families act as signaling nodes within the cell. A large array of signaling cascades converges on activation of these GTPases, which are then responsible for diverse consequences including regulation of gene expression, the cell cycle, cell polarity, and cell survival (1, 2). Thus it is not surprising that both Ras and Rho family GTPases play critical roles in cellular transformation and tumorigenesis (summarized below). Rho GTPases are also particularly well known for their regulation of the actin cytoskeleton to drive changes in cellular morphology and migration (6-8).

Subcellular localization and its regulation by prenylation + a second signal

In addition to being in the GTP-bound "on" state, small GTPases must be properly localized within the cell to promote signal transduction (2). The founding members of the Ras superfamily (H-Ras and K-Ras) are predominantly (>90% of total protein) localized to the plasma membrane (9, 10) and many Ras superfamily proteins are associated with membranes (plasma or internal) as well (1). For Ras and Rho family GTPases, this membrane localization requires both prenylation of the GTPase and a second signal in the C-terminal membrane targeting domain. Ras and Rho family GTPases typically terminate in a CAAX (C=cysteine, A=aliphatic amino acid, X=any amino acid) motif, which is recognized by either farnesyltransferase (FTase) or geranylgeranyltransferase-I (GGTase-I) (11). FTase

attaches a farnesyl isoprenoid group whereas GGTase-I attaches a geranylgeranyl isoprenoid group to the cysteine of the CAAX box. FTase generally acts on small GTPases terminating in a serine, threonine, alanine, or glutamine, whereas GGTase-I shows preference for GTPases terminating in a leucine (12). For most small GTPases, addition of either moiety is sufficient to drive downstream signaling (13-15), however the two isoprenoids have opposing downstream consequences when attached to RhoB (16).

Prenylation (isoprenoid modification) of the GTPase targets it for further processing, by Rce1 (Ras and a-factor converting enzyme-1) and Icmt (isoprenylcysteine carboxyl methyltransferase), to cleave the terminal AAX from the GTPase and carboxylmethylate the prenylated cysteine. The modified GTPase is capable of interacting with the plasma membrane, such that the attached lipid moiety is inserted into the hydrophobic core of the membrane. However, this plasma membrane association occurs only when a second membrane targeting signal is present (9). The signal is found in the C-terminal hypervariable region of the GTPase and consists either of a sequence of basic amino acids (polybasic region, PBR) that form electrostatic interactions with the plasma membrane, or of the addition of a palmitate fatty acid to a palmitoylatable cysteine residue (9). Phosphorylation of Ras and Rho family GTPases within their hypervariable region allows for regulation of GTPase subcellular localization and function with additional spatio-temporal precision (reviewed in Chapter 2).

Rab and Arf GTPases are also membrane-bound because of post-translational modifications. Geranylgeranyltransferase-II (GGTase-II) recognizes di-cysteine motifs in the C-terminus of Rab proteins and adds a geranylgeranyl group to each cysteine, likely through independent reactions (17). *N*-myristoyltransferase is responsible for addition of a

myristate fatty acid to the N-terminus of many Arf proteins (18). Ran is not lipidated nor membrane-bound (1).

Roles for GTPase prenylation beyond regulation of localization

The role of prenylation in GTPase signaling cascades is frequently studied using "SAAX mutants", referring to GTPases in which the prenylated cysteine of the CAAX box has been mutated to a serine and thus cannot be prenylated. Since prenylation is required for membrane association, SAAX mutants are not membrane-bound. As would be expected due to their mislocalization (9, 19), SAAX mutants are impaired in most GTPase functions (9, 13, 14, 16, 20) (although there are reports of Rho family SAAX mutants retaining some ability to regulate certain transcriptional events (14, 16, 20)). Yet the requirement for prenylation vs. the requirement for proper subcellular localization of a GTPase should be considered distinctly.

This is evident when one considers that prenylation of the GTPase is required for full Ras-driven cellular transformation, yet ablation of the second signal impairs but does not ablate its transforming capabilities (9). Since both prenylation and a second signal are required for Ras to localize to the plasma membrane, there must be some functions of Ras that are prenylation dependent, but not dependent on proper plasma membrane localization (second signal independent).

Prenylation promotes certain protein:protein interactions with lipid-binding partners that recognize and bind to the isoprenoid moiety. For example, PRA1 (21) and PDEδ (22) bind to many Ras superfamily GTPases in a prenylation-dependent manner; RhoGDIs (described below) bind optimally to prenylated versus unprenylated Rho GTPases (23); and

galectins interact with Ras proteins in a farnesyl-dependent manner (24, 25). Binding of a prenyl group to a Rho GTPase destabilizes the GTPase and targets it for degradation (26). This is likely true for other Ras family GTPases as well (27). Therefore unless the prenyl group is inserted within a membrane or another protein, then the GTPase will be unstable. Thus these lipid binding proteins have been proposed to act as chaperones (27).

RHO FAMILY GTPASES

Definition/family

The Rho family of small GTPases is defined by the presence of an extra alpha helix within the G domain, composed of approximately 15 amino acids and termed the "Rho insert" (28, 29). The family includes 20 small GTPases that can be further clustered based on structural and functional similarity: RhoA-related (RhoA, RhoB, RhoC); Rac1-related (Rac1/1b, Rac2, Rac3); Cdc42-related (Cdc42/G25K, TC10, TCL, Chp/Wrch-2, Wrch-1); Rnd subfamily (Rnd1, Rnd2, Rnd3/RhoE); RhoBTB subfamily (RhoBTB-1, RhoBTB-2, RhoBTB-3); and other (RhoD, Rif, TTF/RhoH) (28). Among these GTPases, the canonical members RhoA, Rac1, and Cdc42 are the best studied.

Function- cytoskeleton/morphology regulation

In the 1990s Alan Hall and colleagues discovered that microinjection of RhoA into fibroblasts induced stress fiber formation (30), while Rac1 expression caused formation of membrane ruffles (31). It soon followed that injection of Cdc42 induced filopodia formation

(32). Since then it has been determined that all Rho GTPases, with the exception of RhoH and RhoBTB1-3, regulate the actin cytoskeleton and cell morphology (28).

These effects on morphology were not only observed via microinjection of Rho proteins. Lysophosphatidic acid (LPA) activates RhoA (33) and bradykinin (BDK) activates Cdc42 (32) through G protein-coupled receptor (GPCR) signaling, whereas platelet-derived growth factor (PDGF) stimulates Rac1 (31) activity through activation of receptor tyrosine kinases (34) to drive the same phenotypes. RhoA, Rac1, and Cdc42 control the actin cytoskeleton through a variety of signaling cascades. In addition to regulating the above described structures, these GTPases also regulate focal adhesion formation/turnover; cell:cell contacts, cell contraction, microtubule assembly, and cell polarity (35). Some of the primary RhoA, Rac1, and Cdc42 signaling pathways controlling these functions are summarized in Figure 1.2. As shown, these three small GTPases converge on many of the same molecules to either amplify or attenuate the action of other GTPases.

Additionally, activation of a given GTPase can have opposing effects on cellular structure, depending on the downstream effector pathway that is activated. For example, RhoA signaling to mDia enhances adherens junction stability, whereas RhoA signaling to ROCK causes disassembly of adherens junctions (36). RhoA binds with higher affinity to mDia than ROCK (Kd 6nM and 130nM respectively), therefore when there are low levels of RhoA activity within the cell, then RhoA will predominantly activate mDia and enhance adherens junction stability. However when there are high levels of RhoA activity, the effects of ROCK will also be observed and adherens junctions will disassemble (37). Thus the cell must very precisely control the activation of Rho GTPases (see below). This characteristic of Rho GTPases makes them difficult to study, since inhibition and activation experiments

sometimes result in the same end phenotype (38). Therefore common experimental manipulations, e.g., rescue of knockdown, can be technically challenging and require precise levels of expression.

Function- transcription

The function of Rho GTPases is not limited to regulation of cell morphology. Rho GTPases signal to an assortment of effectors. Some of the best-studied pathways for RhoA, Rac1, and Cdc42 are diagrammed in Figure 1.2. As shown, many of these pathways control regulation of gene transcription. For example, most Rho GTPases regulate the transcription factor SRF (serum response factor). When Rho GTPases cause globular actin (G-actin) to polymerize into filamentous actin (F-actin) this releases MAL from its sequestration with G-actin. MAL is then free to translocate to the nucleus, where it acts as a co-activator of SRF and helps drive transcription (39). RhoA, Rac1, and Cdc42 also all regulate the transcription factor NF-κB (40). Overexpression of any of these three GTPases results in phosphorylation of the inhibitory subunit (IκBα), targeting this subunit for degradation and allowing translocation of active dimers to the nucleus where they can regulate transcription (40). Similarly, multiple Rho GTPases regulate the phosphorylation of cytoplasmic STAT3 and STAT5a at key residues, thereby allowing for their dimerization, nuclear entry, and transcriptional activity (41).

In addition to activating relevant signaling cascades, Rac1 directly binds these STATs, in a GTP-dependent manner (42, 43). Through this binding, Rac1 is thought to chaperone STATs from the cytoplasm into the nucleus (43). A similar chaperoning mechanism has

been proposed for Rac1 and β-catenin, in which Rac1 enables β-catenin to regulate transcription via translocation to the nucleus (44).

Because of these roles of Rho proteins in transcription, luciferase reporter assays are commonly used as markers for Rho GTPase activation. In these assays, a promoter regulated by the transcription factor of interest (e.g., the serum response element, SRE, from the promoter of a gene such as c-fos can be used for SRF) is engineered to control luciferase expression (45). Therefore, a quick spectroscopy readout can be used to determine transcription factor activity, which correlates with Rho GTPase activity. The transcription factors regulated by Rho GTPases control the expression of a variety of genes, but most of them regulate cyclin D1 expression (41). Therefore, the cyclin D1 promoter is also commonly engineered to promote luciferase expression, and luminescence then correlates with activation of the cyclin D1 promoter and subsequent cyclin D1 expression (46).

Requirement for unusually precise regulation

The described signaling pathways are essential for most cell functions. Combined actions of Rho GTPases regulate cell migration, cell cycle progression, cytokinesis, apoptosis, cell polarity, and cell adhesion (47). Since many Rho GTPase pathways converge, and activation of a given GTPase can result in opposing outcomes (see above), proper completion of these functions requires precise spatial and temporal control of Rho GTPases. For example, in migration a coordinated influx of RhoA followed by Cdc42 and finally Rac1 activation within 2 mm of the leading edge produces a protrusion (48). Further coordination of Rho GTPases central to the cell and at its tail are also required for forward progression during cell migration (7). Similarly, during cytokinesis RhoA must become activated in a

narrow region to initiate cleavage furrow formation, but too broad a region of RhoA activity prevents furrow formation (49). It is therefore not surprising that Rho GTPase activation is very precisely controlled. Even a strong stimulus results in only about 5% of a specific Rho GTPase becoming activated (50, 51). This indicates that even very minor populations of active Rho GTPases can be functionally important.

Even though Rho GTPases must be GTP-bound to interact with downstream effectors, it has been suggested that they are not on/off switches like Ras GTPases. While turning a Ras GTPase "on" is sufficient to drive downstream functions, Rho GTPases (such as Rac1) may require continuous cycling to perform their cellular duties (see section on mutants below). This idea has been described as the "GTPase flux model" (52).

RhoGDIs (an extra level of regulation)

In addition to GEFs and GAPs, Rho GTPases are regulated by Rho guanine nucleotide dissociation inhibitors (RhoGDIs, Fig. 1.3). There are 3 types of mammalian RhoGDIs (α,β,γ, each with distinct specificity and expression profiles), that bind to the C-terminal prenyl group of Rho family members and to the GTPase itself (53-55). There is some debate over the affinity of GDIs for GDP-bound vs. GTP-bound GTPases. It seems that GDIs can interact with GTPases bound to either nucleotide, but interact with higher affinity to inactive GTPases (23). Once the bound GDIs inhibit nucleotide dissociation and GTP hydrolysis (55). They can also strip Rho GTPases from membranes and sequester them in the cytosol, where the Rho GTPases are unable to interact with their downstream effectors. Thus until recently, GDIs were considered simply as negative regulators of GTPase

activation. However, emerging evidence suggests that RhoGDIs are also essential for protein stability of prenylated Rho family GTPases (56).

Prenylation of Rho GTPases targets them for degradation through interactions with Hsp90 and the proteasome (26). However, expression of RhoGDI1 inhibits this degradation, presumably by binding to the prenyl moiety, preventing misfolding, and shielding the GTPase from its proteasome fate (26). This interaction of RhoGDI with Rho GTPases is essential for Rho GTPases to function, since prenylation is both irreversible and a necessary step in targeting Rho GTPases to the plasma membrane. Phosphorylation of both Rho GTPases and RhoGDIs controls their interaction (see Chapter 2). Thus kinase/phosphatase-induced release of GTP-bound GTPases from GDIs may be used by the cell to create a bolus of Rho GTPase activity (11).

The sum of the molar quantities of RhoA, Rac1, and Cdc42 within a cell is approximately equal to the moles of RhoGDIα (57). The competition of Rho GTPases for limited binding to RhoGDIs determines which GTPases are protected from degradation (27) and again emphasizes the very precisely controlled functions of Rho GTPases. The phenotypic consequences of experimental overexpression of Rho GTPases within a cell are not only due to signaling from the exogenous GTPase but also due to degradation and loss of signaling from other Rho GTPases that are outcompeted for GDI binding.

There are GDIs specific to the Rab subfamily of GTPases as well, but Ras, Arf, and Ran subfamilies do not have classical GDIs to exhibit this extra level of regulation (58). This likely explains certain differences in subcellular localization between Ras and Rho family GTPases. Despite similar C-terminal targeting sequences, Ras GTPases are rarely observed away from membranes, whereas Rho GTPases are expressed more diffusely throughout the

cell ((19), see Table 3.1). This is likely due to GDI sequestration of prenylated Rho (but not Ras) GTPases in the cytoplasm. Still, it has been proposed that galectins and/or PDEδ may act similarly to GDIs for Ras family GTPases (22, 58, 59).

Nuclear GTPases, an emerging field

Consistent with their role in cytoskeletal rearrangement, Rho GTPases have been extensively studied for their functions within the cytoplasm and at membranes. Although the vast majority of Rho proteins are found at these locations (57), the existence of a minor pool of nuclear Rho GTPases is beginning to be appreciated as well. As described above, minor populations of Rho GTPases can have important functional consequences.

RhoA (60, 61), Rac1 (62-64), and Cdc42 (65) have all been observed within the cell nucleus, and other Rho GTPases (e.g. RhoC, TCL, Rnd1, etc…) contain nuclear localization signals (NLSs) in the polybasic region of their hypervariable domains (9, 64). NLSs for Rho GTPases might seem unnecessary, since these small GTPases are generally less than 40 kDa (29) and therefore are small enough to passively diffuse through nuclear pores (66). However, prenylation inhibits nuclear entry (62, 67). Thus, it has been proposed that prenylated Rho GTPases must conceal their prenyl groups within a protein complex to gain nuclear entry (62). Since the resulting protein complexes are larger than the size limit for passive diffusion, the NLSs are then required to interact with importin molecules for active nuclear transport of the GTPases (66). Indeed endogenous nuclear Rac1 (62) and RhoA (68) are prenylated, and a functional NLS is required for Rac1 to shuttle the scaffolding protein, SmgGDS, into the nucleus (63). Still, RhoA does not contain a known NLS (64), but a

portion of RhoA is observed in the nucleus (granted, at a lower fraction than Rac1) (62). It is currently unclear how RhoA transits to the nucleus.

Rac1 activity has been observed in the nucleus in response to bacterial infection by *Yersinia pseudotuberculosis* (67). The bacterial protein YopT acts as a cysteine protease and cleaves off the CAAX box of Rac1, de-prenylating Rac1 and allowing for its nuclear import, where it remains active (67). While the function of nuclear Rac1 activity was not explored experimentally, the authors suggested that it may regulate transcription. Indeed, experiments in the Bapat lab suggest that active nuclear (but not cytoplasmic) Rac1 positively regulates Wnt/β-catenin driven transcription in colon cancer cells (44). Active Rac1 enhances nuclear accumulation of β-catenin (44) and also interacts with Wnt-responsive promoters (69). In another system, a constitutively active (Q61L) SAAX mutant of Rac1 was artificially overexpressed in the nucleus of cells (62). This resulted in an increased mitotic index, but did not increase membrane ruffling (62). The mechanism through which active nuclear Rac1 enhances mitosis is unknown. However, the Rac1(Q61L/SAAX) mutant can activate NF-κB transcription to inhibit UV-induced apoptosis (70). It was proposed that Rac1 must be both nuclear and GTP-bound to drive NF-κB transcription, and that Rac1 activation of PAK1 causes nuclear accumulation of the NF-κB p65 protein (70). However these experiments focused on the function of Rac1 SAAX and prenylated nuclear Rac1 was not examined.

Less is known about the nuclear activity of other Rho GTPases. RhoA activation in the nucleus has been observed in response to irradiation (60) or to loss of CCM1 (61), yet the signaling and functional consequences of nuclear RhoA activity have not been explored. Cdc42 binds to and is essential for proper localization and function of CENP-A, a histone H3 variant that defines centromere loci and is key for kinectochore attachment (65). Knockdown

of either a GEF or a GAP that act on Cdc42 similarly impaired CENP-A function, thereby making the role of Cdc42 at this location hard to decipher (65).

While it is likely that unique signaling pathways exist within the nucleus, many downstream effectors of Rho GTPases have also been observed there. In the signaling cascades diagrammed in Figure 1.2, proteins that have been observed within the nucleus are highlighted in yellow. As shown, nuclear actin is thought to drive chromatin remodeling and to interact with RNA polymerase to regulate transcription (71). In fact, many signaling molecules downstream of Rho GTPases (such as PAK1, ROCKII, paxillin, and cofilin) have been described to regulate transcription when present in the nucleus (71-75). For example, the Rho effector ROCKII binds to and phosphorylates p300 acetyltransferase when in the nucleus (74). This enhances the ability of p300 to acetylate histones and drive transcription (74). Thus Rho GTPase activation in the nucleus, like its activation in the cytoplasm, may regulate transcription. Consistent with this, there are reports of Rho GTPase SAAX mutants (found to varying degrees in the nucleus) retaining some ability to activate certain transcriptional events (14, 16, 20).

Regulators of Rho GTPases have also been observed in the nucleus. The Rho GEFs Ect2 (76) and Net1 (77) are predominantly nuclear, and LARG (78), Vav3 (79), ARHGEF6/alpha-PIX (80), and Tiam1 (69) have also been detected there. Negative regulators MgcRacGAP (81), DLC1 (82, 83) and RacGAP1 (84) have been found in the nucleus as well. Because the stoichiometry of proteins within the nucleus and cytoplasm are distinct, even cascades involving the same signaling molecules may differ greatly between the two compartments.

RHOGEFS

Families of RhoGEFs

Within the Ras superfamily of GTPases, each subfamily has its own specific regulators (58). There are 3 varieties of RhoGEFs, able to catalyze nucleotide exchange on Rho GTPases: Dbl family RhoGEFs, DOCK-related RhoGEFs, and SWAP70/SLAT (85). The majority of RhoGEFs belong to the Dbl family and these will be the focus here. There are also 11 DOCK-related RhoGEFs, each of which activate either Rac1 or Cdc42. The DOCK family Rho GEFs contain 2 well-conserved regions, Dock-homology region-1 and -2 (DHR1 and DHR2), that are composed of a C2 (lipid-binding) domain and an armadillo array (protein-binding) domain (85). Some DOCK-related RhoGEFs require binding of the scaffolding protein ELMO to gain catalytic activity (86). SWAP70 and SLAT are outliers. They do not show homology with either Dbl or DOCK RhoGEFs, yet still catalyze nucleotide exchange on Rho GTPases (85, 87, 88).

Dbl family domains- DH

Dbl family RhoGEFs are characterized by the presence of a Dbl-homology (DH) catalytic domain that is responsible for enhancing nucleotide exchange (85). Although there is low sequence conservation among DH domains, their overall structure is well-conserved and is composed of helices that fold together to form a chaise longue shape (85). The DH domain interacts with switch I and switch II of the GTPase to disorganize the nucleotide binding pocket in the G domain of the GTPase. This releases both the nucleotide and its binding co-factor Mg^{2+}, and allows for GTP binding/activation of the GTPase (85).

Dbl family domains- PH

With the exception of Tuba, all Dbl-family RhoGEFs contain a pleckstrin homology (PH) domain located adjacent to and C-terminal of the DH domain (85). The PH domain is found in many signaling molecules, and interacts with lipid products of phosphatidylinositol-3-kinase (89). It is widely thought that PH domains aid in association of proteins with membranes via binding to phospholipids (89). However, many RhoGEF PH domains have low affinity for phospholipids and some RhoGEFs localize to plasma membranes despite mutations in their PH domains to prevent phospholipid binding (90, 91). Therefore the plasma membrane association of these GEFs/PH domains may not be due to phospholipid binding, but instead due to binding with other proteins. In fact, GTP-bound (but not GDP-bound) RhoA has recently been shown to bind to the PH domains of many RhoGEFs (such as Lbc, LARG, and GEF-H1) (92). When active RhoA is membrane-bound, it enhances the GEF activity of these RhoGEFs in a positive feedback loop, presumably through allowing the PH domain to bind to membranes such that the GEF can activate other inactive RhoA proteins (92). Alternatively, PH domain interactions with proteins can target them to other specific subcellular localizations where they activate Rho GTPases. For example, the PH domain of Trio is thought to direct Trio to actin structures via binding of filamin (93).

In vitro, GEFs containing functional DH and PH domains are frequently more efficient at catalyzing nucleotide exchange than those with an impaired PH domain (90, 94-96). Some PH domains directly interact with the GTPase to promote this exchange (95, 97), while for other PH domains the effect may be allosteric (as no interactions can be observed with crystallography (98)).

Whether there is a single global function of the RhoGEF PH domain is unclear. It likely varies among RhoGEFs, as the relative orientation of DH and PH domains differs greatly among them (85).

Dbl family domains- other

Beyond the DH/PH domains, RhoGEFs are an extremely variable class of proteins, containing many different protein:protein interaction and other domains (Fig. 1.4) (85). This diversity allows cells to respond to numerous different signaling inputs by activating a variety of Rho GTPases.

Specificity vs. promiscuity

There are 70 distinct Dbl-family RhoGEFs to regulate the 20 identified Rho family GTPases (99). Some of these Dbl-family GEFs act solely on a single GTPase: however, many have multiple substrates (Fig. 1.5) (85). Examination of crystal structures of GEF/GTPase interactions has allowed identification of key residues that regulate the specificity of the exchange reaction. For example, a single amino acid change in Rac1 to the corresponding Cdc42 residue (W56F) allows the Cdc42-specific GEF ITSN-1 to catalyze exchange on the mutated Rac1, and prevents binding of Rac1-specific GEF Tiam1 to the mutated Rac1 (100). Similarly, mutation of a couple of residues in the RhoA- and Cdc42-specific GEF Dbs allows for selective activation of each GTPase (101).

Regulation

For many RhoGEFs, the N-terminus is autoinhibitory, and therefore artificial truncation of the GEF activates it (85). *In cellulo* this autoinhibition is overcome either by post-translational modification or by protein binding. For example, Vav is N-terminally phosphorylated by Src family members. This phosphorylation causes the N-terminus to become unstructured, causing it to release the DH domain and activate Vav (102). Tiam1 is opened/activated by Ras binding to the RBD in its N-terminus (103). Similarly, APC binds the N-terminus of ASEF (104) and $G_{\alpha 13}$ binds the N-terminus of p115 Rho GEF (105) to activate these RhoGEFs.

Subcellular localization

In general, RhoGEFs are thought to localize similarly to RasGEFs (106). The prototype of GEF localization is the RasGEF Son of Sevenless (SOS). SOS is localized diffusely throughout the cytoplasm, until phosphorylation of receptor tyrosine kinases creates a docking site for the adaptor protein Grb2 (growth factor receptor-bound protein). Grb2 localization to the plasma membrane recruits SOS there, and SOS then activates Ras family GTPases (107). Indeed the RhoGEF Vav also translocates to the plasma membrane upon activation (108), and PDGF stimulation recruits the Rac1-specific GEF Tiam1 to membranes (109).

Yet, this model does not explain all the intricacies of RhoGEF subcellular localization. For example, the RhoA GEF, GEF-H1, is held in an inactive state while interacting with microtubules. Depolymerization of microtubules allows release of GEF-H1 and subsequent RhoA activation (110). Additionally, there are 2 RhoGEFs, Ect2 (76) and Net1 (77), that are

predominantly localized to the nucleus of interphase cells. It was originally thought that these RhoGEFs were simply sequestered from their Rho GTPase substrates when in the nucleus, (77, 111, 112). However, it has now been shown that Net1 can activate RhoA when in the nucleus (68). In Chapter 4 of this dissertation I discuss a nuclear role for Ect2.

ECT2

Domains

Ect2 is a Dbl family RhoGEF, containing both DH and PH domains. A diagram of the structure of Ect2 is shown in Fig. 1.6. While the DH domain of Ect2 alone is capable of inducing nucleotide exchange, this activity is enhanced by the presence of its PH domain both *in vitro* and *in cellulo* (94). Uniquely among RhoGEFs, Ect2 also contains two N-terminal BRCT domains. BRCT domains have been identified in 23 proteins, most of which are involved in DNA damage repair (113). BRCT domains are commonly found in pairs and bind to phospho-serines (114). The BRCT domains of Ect2 bind to its DH domain and autoinhibit its GEF catalytic activity (111, 115). Located between the BRCT domains and DH/PH domains of Ect2 are two nuclear localization signals (NLSs), which are unusual among RhoGEFs.

Although no other domains have been characterized in Ect2, the region C-terminal of the DH/PH domain is functionally significant. It is a required addition to the DH/PH domain, in order for Ect2 to activate Rac1 and Cdc42, and for optimal activation of RhoA (94). The C-terminus is also required for Ect2 to increase cyclin D1 transcription (46). Interestingly, 32% of the C-terminus is either a serine or threonine, suggesting the possibility of numerous phosphorylation sites. Also, a third functional NLS signal has been described in this region

(111, 116) as well as putative degrons, which are motifs that allow APC binding and polyubiquitination to target Ect2 for degradation (116).

Expression

Initial northern blot analysis revealed that Ect2 is most highly expressed in the testis and also in the kidney, liver, and spleen of adult mice. Ect2 transcript was not detected in lung tissue or in postmitotic (brain, heart, skeletal muscle) tissue of these same animals (117). However, in mouse embryos and tumor cells Ect2 mRNA was more ubiquitously expressed (45). It was therefore hypothesized that Ect2 expression is elevated during cell division. Indeed, after serum starving cells such that >90% were in GO/G1, Ect2 expression at both the mRNA and protein level was increased upon serum stimulation in conjunction with cells re-entering the cell cycle (45). And heightened Ect2 expression has been correlated with S/G2/M phase in a regenerating liver mouse model (118) and by double thymidine block (116). Yet others have not observed dramatic changes in Ect2 expression with the cell cycle and instead showed phosphorylation of Ect2 upon entry into G2/M (described further below) (76). Thus, the rules governing the expression patterns of Ect2 are somewhat unclear.

Subcellular localization

As beautifully captured by Tatsumoto and colleagues, the subcellular localization of Ect2 changes throughout the cell cycle (76). During interphase, Ect2 is one of two RhoGEFs that localizes predominantly to the nucleus, the other being Net1. Approximately 1-2 minutes before the nuclear envelope breaks down and mitosis is initiated, Ect2 spreads into the cytoplasm (119). During metaphase, Ect2 highlights the mitotic spindle (76). As

anaphase and teleophase progress, Ect2 is concentrated at the central spindle (120)/cleavage furrow (76), and in some cells it is also found at the cell cortex (121). Prior to abscission of the two cells, Ect2 is observed at the midbody (76, 122). It translocates to the nucleus as interphase begins again (76).

Within the nucleus, Ect2 has been described as nucleolar (119). I have observed this, but have also observed nucleolar exclusion in some circumstances (LPH, unpublished). Additionally Ect2 has been observed at cell:cell contacts (123), and specifically at the zonula adherens (124).

Specificity/GEF activity

The GTPase specificity of Ect2 has been context-dependent at best and controversial at worst. In the original Ect2 paper (117), the homology of Ect2 to other RhoGEFs was noted. However, Ect2 isolated from insect cells did not catalyze nucleotide exchange on purified RhoA, Rac1, or Cdc42 (117). Since then, it has been shown that full-length Ect2 immunoprecipitated from COS cells (monkey kidney fibroblasts) is capable of activating purified RhoA, Rac1, and Cdc42 (76). *In cellulo*, cell type and context-specific differences have led to a wide array of specificity profiles for Ect2 (111, 121, 123, 125-128). Table 1.1 summarizes the published specificity of Ect2 towards RhoA, Rac1, and Cdc42. Ect2 is known to bind other GTPases as well, with particularly high affinity for RhoC (117, 129). And expression of truncated Ect2 in COS cells causes activation of RhoB, RhoC, and Rac2 (111).

The GEF activity of Ect2 is thought to be autoinhibited by its BRCT domains because: 1) the BRCT domains bind the DH domain (111, 115); 2) expression of BRCT-

mutated or N-terminally truncated Ect2 drives more dramatic phenotypes than full-length Ect2 (111, 117, 118); and 3) the N-terminus of Ect2 alone acts as a dominant-negative (76, 118, 125). Therefore Ect2 must be activated before it can enhance nucleotide exchange, which may explain some of the conflicting reports about the GEF activity of Ect2.

The mechanism of Ect2 activation remains unclear, but may be achieved by phosphorylation ((76), see Chapter 6.1). As mentioned above, the C-terminus of Ect2 also regulates its GEF activity, through an unidentified mechanism. It is required for Ect2 to activate Rac1 and Cdc42 and to optimally activate RhoA (94). Thus, further work is required to understand the signals for Ect2 activation and what drives its specificity.

Role in cytokinesis/mitosis

The best-studied function of Ect2 is its role in cytokinesis of normal cells. Cytokinesis is the last step in cell division, during which a single cell that has replicated its genetic material physically divides into two daughter cells (130). Manipulation of Ect2 levels/activity (via knockout (131)/knockdown (120) or overexpression of constitutively active (118) or dominant-negative (76) Ect2) causes cells to become multinucleated, indicating an inability to complete this process. Thus, fully functional Ect2 is required for proper cytokinesis of many cells.

Cytokinesis can be divided into 4 stages: identification of the division site, cleavage furrow ingression, midbody formation, and abscission (49). It is initiated during anaphase. As chromosomes are pulled apart, interpolar microtubulues bundle together to form the central spindle and help define the site of cell division. A cleavage furrow/contractile ring is formed over this site, which is where the cell constricts. The contracted contractile ring,

which acts as a narrow bridge between the two newly distinct cells, is called the midbody. Abscission occurs when the plasma membrane is severed on either side of the midbody, allowing the two cells to separate (130). As detailed below, Ect2 regulates both cleavage furrow formation and abscission.

Centralspindlin is a complex of the proteins MgcRacGAP and MKLP1, that is localized to the central spindle. MgcRacGAP is phosphorylated on serine 157 by Plk1 (132, 133). This phosphorylation creates a docking site for the BRCT domains of Ect2 and recruits Ect2 to the central spindle (120, 132, 133). In addition to properly localizing Ect2, the binding of the BRCT domains of Ect2 to MgcRacGAP also prevents autoinhibition of Ect2, holding it in an active conformation (120). Recruitment of Ect2 to the central spindle allows for activation of RhoA in a narrow area of the cell cortex above the central spindle, which becomes the cleavage furrow (120). This activation of RhoA leads to activation of myosin-II and actin polymerization, causing cleavage furrow ingression and proper execution of cytokinesis (120).

The onset of cytokinesis is tightly regulated by phosphorylation. Ect2 is phosphorylated during metaphase at T341, likely by Cdk1, but is dephosphorylated during anaphase (120). This phosphorylation inhibits the ability of Ect2 to bind MgcRacGAP and therefore prevents cleavage furrow formation from occurring too early during metaphase (120). Additionally, during pro-metaphase, Ect2 is phosphorylated by Cdk1 at residue T412 (134). This phosphorylation event increases the affinity of Plk1 toward Ect2 (134). Taken with the above information, it is possible that, when the Ect2/Plk-1 complex comes in contact with centralspindlin, Plk-1 phosphorylates MgcRacGAP, creating the described binding site

for the BRCT domains of Ect2, and restricting Ect2 to the central spindle where it activates RhoA.

As described above, Ect2 localizes to the midbody in anaphase and early telophase (76, 122). However, for abscission of the two daughter cells to proceed, Ect2 must be released from the midbody (122). Ect2 competes with FIP_3 (Rab11 family interacting protein) for binding to MgcRacGAP. Since their binding is mutually exclusive and FIP_3 is required for abscission, only upon release of Ect2 from MgcRacGAP is abscission able to proceed (135). Therefore Ect2 localization to the midbody acts as checkpoint for the final step in cytokinesis. Plk-4 is required for proper localization of Ect2 to the midbody (136), thus it is likely that dephosphorylation of sites phosphorylated by Plk-4 allow release of Ect2 from the midbody.

Ect2 has also been implicated in the initiation of mitosis, independent of MgcRacGAP. One to two minutes prior to nuclear envelope breakdown, Ect2 is released from the nucleus into the cytoplasm. This release causes activation of RhoA at the cell cortex and results in cell rounding (one of the earliest steps in mitosis) (119).

In summary, phosphorylation and protein:protein interactions determine the localization and activation of Ect2 throughout the cell cycle. Ect2 then regulates the localization and activation of RhoA to drive both cytokinesis and mitosis. Ect2 also inhibits abscission of the two daughter cells, until the cell is ready to conclude cytokinesis. Some tumor cells have been described to undergo Ect2-independent cytokinesis (121, 137, 138). Despite this Ect2 has functional significance in the cellular transformation of these tumor cells (137, 138). Ect2 must have other functions in addition to its well-documented role in cytokinesis. This will be the focus of the rest of this section.

Oncogene- identification

Ect2 was originally identified in a screen for oncogenes performed by Dr. Toru Miki and colleagues (117). NIH 3T3 mouse fibroblast cells were transfected with a cDNA library isolated from mouse keratinocytes. The transfectants were then screened for focus formation, which represents a loss of contact-inhibition and therefore transformed growth (117). The cDNAs capable of creating foci were extracted from cells and identified using a technique they previously developed (139). In this way, Miki and colleagues identified genes from normal epithelial cells that were sufficient to drive transformation.

The original isolate of Ect2, Epithelial cell transforming sequence #2, coded for an N-terminally truncated version of the protein (117). Careful sequence analysis reveals that it lacked both BRCT domains and the first NLS sequence (Fig. 1.7). Therefore, it was not autoinhibited, and represented a constitutively active form of Ect2.

It is less clear whether the truncated active mutant was, like full-length Ect2, predominantly nuclear during interphase. Mutation of the NLSs individually in full-length Ect2 has been reported to drive Ect2 to the cytoplasm similar to mutation of both NLSs (111, 116), however the extent of nuclear exclusion/cytoplasmic enhancement of N-terminally truncated Ect2 lacking only the first NLS is unclear (111). Dr. Danielle R. Cook has observed differences in strength between the two NLSs. Mutation of NLS-1 caused a more dramatic shift to the cytoplasm than mutation of NLS-2 (Dr. Danielle R. Cook, personal communication). Regardless of its localization, the original isolate of Ect2 was extremely transforming. Cells expressing this N-terminally truncated form of Ect2 grew anchorage-independently, and 100% of mice injected subcutaneously with NIH 3T3 cells expressing it

formed tumors within 6 weeks, whereas cells not expressing Ect2 did not create tumors in mice (117). Thus, a constitutively active form Ect2 is capable of acting as potent oncogene.

Oncogene- domains required for fibroblast transformation

Numerous truncation mutants of the original Ect2 isolate were analyzed for their ability to transform NIH3T3 fibroblasts ((117), Fig. 1.7). As would be expected if the GEF activity of Ect2 were required for this phenotype, inclusion of the autoinhibitory BRCT domains or omission of the catalytic DH domain prevented the ability of Ect2 to transform these cells (117). This suggested that the GEF activity of Ect2 was indeed required, which was later confirmed with a point mutation in the DH domain that ablated its catalytic activity (111).

Consistent with its necessity for optimal nucleotide exchange (94), the PH domain is also required for Ect2 to transform fibroblasts (111). Surprisingly, tryptophan 752, a residue that is conserved across all RhoGEF PH domains and is usually necessary for optimal GEF function, is not required for Ect2-driven fibroblast transformation (94).

Multiple labs have determined that a DH/PH/C truncation mutant of Ect2 (activated by removal of the N-terminal BRCT domains, see domain structure in Fig. 1.6) is sufficient to drive cellular transformation in fibroblasts, but upon further removal of the C-terminus, the isolated DH/PH domains cannot recapitulate the phenotype (46, 94, 111). One may expect that this requirement for the C-terminus would provide clues regarding which GTPases Ect2 acts through to regulate transformation, because the C-terminus is required for activation of Rac1 and Cdc42 (94). However, the C-terminus also enhances Ect2-mediated

nucleotide exchange on RhoA. Therefore it is still unclear which GTPases Ect2 acts through to drive transformation.

It has been proposed that, in addition to activating its GEF activity, Ect2 must also be cytoplasmically mislocalized to drive transformation of fibroblasts (111). Indeed, Saito et al. showed that removal of NLS function (via point mutation or truncation) from Ect2 enhanced the ability of cells expressing it to grow free of contact inhibition (111). Yet, the original Ect2 paper, also from Miki and colleagues, shows that Ect2 with both NLSs, 1 NLS, or 0 NLSs are all transforming ((117), Fig. 1.7), These results suggest that Ect2 may be able to drive transformation from either the nucleus or the cytoplasm, or that all of these mutants shuttle between compartments.

A functional analysis of the domains of Ect2 utilized by tumor cells for transformed growth has not been performed. Since normal fibroblasts require Ect2 to regulate cytokinesis/mitosis (131) but some tumor cells may not (121, 137), Ect2 could cause transformation of fibroblasts via enhanced cytokinesis/mitosis, but this may not be relevant for tumor cells. Therefore, it is possible that the domains of Ect2 required for transformed growth in tumor cells is different from those sufficient to transform fibroblasts.

Oncogene- expression in tumor cells

No truncation/activation mutants of Ect2 have been described in human cancers. However, COSMIC (Catalogue of somatic mutations in cancer (140)) currently shows approximately 30 missense mutations in Ect2, one of which may attenuate its degradation (116). Interestingly, functional degrons and nuclear localization of Ect2 is required for ubiquitin-mediated Ect2 degradation (116). Additionally, Ect2 is overexpressed at the

mRNA level in a variety of tumor types, including lung (137), pancreatic (141, 142), recurrent liver (143), advanced kidney (144), oral (145), esophageal (146), brain (138, 147, 148), head & neck (149), cervical (149), and ovarian tumors (45, 150, 151). In some tumor types this is due to chromosomal amplification. *ECT2* is located on chromosome 3q26.1-3q26.2 (152). This region is frequently amplified in tumors of the: lung (153), pancreas (142), esophagus (154), head & neck (155), cervix (156), and ovaries (151, 157). In fact, 3q26 is the most common amplicon in ovarian tumors (158).

Although protein expression of Ect2 in ovarian tumors had not been investigated, Ect2 was known to be overexpressed at the protein level in lung (137, 146), oral (145), esophageal (146), and brain (138, 147, 148) tumors. In Chapter 4 I describe my analyses of Ect2 expression in ovarian tumors and cell lines.

Oncogene- multiple transforming functions

High expression of Ect2 in lung (146), esophageal (146), and brain (138, 148) tumors is correlated with poor patient outcome. Additionally, chromosomal amplification/mRNA expression of Ect2 in pancreatic ductal adenocarcinoma (PDAC) cell lines correlated with dependence on Ect2 for cell viability (142). Indeed, Ect2 has many functions related to cellular transformation that may explain these correlations (see below). Interestingly, many of these functions appear to be distinct from the role of Ect2 in cytokinesis (121, 137, 138).

Knockdown of Ect2 prior to subcutaneous injection of non-small cell lung cancer (NSCLC) cells (137) or intracranial injection of astrocytoma cells (138) attenuates growth of these tumors in mice. The tumors initiated from Ect2 knockdown cells in NSCLC showed lower levels of Brd-U staining, but no difference in TUNEL staining (137); and astrocytoma

tumors with Ect2 knocked down did not show increased multinucleation (138). These results suggest that the phenotype of decreased tumor growth is due to decreased proliferation but not increased apoptosis or cytokinesis defects. The decreased growth rate could also be seen in soft agar colony formation assays of NSCLC cells (137) but Ect2 knockdown did not affect proliferation on plastic of either NSCLC or astrocytoma cells (137, 138). Therefore the role of Ect2 in both of these cell types appears to be related to proliferation during anchorage-independent but not anchorage-dependent growth.

However, there have been conflicting reports about the role of Ect2 in tumor cell proliferation. Hirata and colleagues also found that Ect2 knockdown decreases soft agar growth of NSCLC cells and esophageal squamous cell carcinoma (ESCC) cells. Yet, in their experiments a general proliferation defect was observed, as NSCLC and ESCC cells with Ect2 knocked down became trapped in G1 phase of the cell cycle (146). Similarly knockdown of Ect2 in oral squamous cell carcinoma cells caused growth arrest in G1 (145), and there are reports in glioblastoma (GBM) cells of Ect2 knockdown causing decreased growth (147, 148) and increased multinucleation (147). Thus it is clear that Ect2 is required for anchorage-independent growth of tumor cells, however, it is less clear if this function is distinct from its role in mitosis/cytokinesis (described above) in all cell types.

A general proliferation defect was observed repeatedly when siRNA was utilized for Ect2 knockdown (146-148), whereas when shRNA was used the defect was specific to anchorage-independent growth (121, 137, 138). Thus, one may speculate that all cells require Ect2 for cytokinesis/mitosis, but upon stable knockdown a population of cells is able to evolve and compensate for loss of Ect2, whereas with transient knockdown the population has less selection pressure and time to evolve. Still, a comparison of multinucleation after

stable knockdown of Ect2 in tumor vs. normal cells (137) (and also in two types of tumor cells (121)) shows that cell types vary in their dependence on Ect2 for cytokinesis/mitosis. A much lower percentage of A549 and H1703 (NSCLC) cells showed multinucleation upon stable knockdown of Ect2 when compared directly with MDCK (canine kidney) cells (137).

As described above, overexpression of Ect2 in NIH 3T3 cells can induce soft agar colony formation (117). Together with the data from the previously described knockdown experiments, these results show that Ect2 can be both necessary and sufficient for anchorage-independent growth. Overexpression of activated Ect2 can also induce focus formation (46, 94, 111, 117), indicating that Ect2 is also sufficient to allow cells to bypass contact-inhibition and continue to grow. In summary, Ect2 is responsible for multiple aspects of transformed growth.

Beyond regulation of tumor cell growth, Ect2 also regulates migration and invasion. Intracranial injection of astrocytoma cells expressing endogenous Ect2 created tumors with more invasive borders, compared to the rare and discrete tumors formed when Ect2 knockdown cells were injected (138). And knockdown of Ect2 has been shown to decrease tumor cell invasion using both Matrigel invasion assays (137, 148) and organotypic brain slice invasion assays (147, 159). Similarly, overexpression of Ect2 is sufficient to increase invasion through Matrigel of non-transformed cells (146). With regard to migration the story is a little more complicated. Knockdown of Ect2 has been described to decrease the rate of migration (147). Conversely, overexpression of full length Ect2 can speed migration of some brain tumor and chicken fibroblast cells (138, 159), but also ectopic expression of truncated constitutively active Ect2 decreases the rate of migration of human fibrosarcoma cells (121). Given how tightly regulated Rho GTPases are and that different levels of activation of a

given GTPase can lead to opposite phenotypes (discussed above), perhaps these seemingly contradictory results should not be surprising.

There are two major types of single cell migration: amoeboid and mesenchymal, both of which require precise activation of Rho family GTPases (160). Mesenchymal migration consists of a leading edge ruffling, extending forward, and adhering to a matrix, followed by the cell contracting to bring the rest of the cell forward and finally releasing its adhesions to a previous location (160). Amoeboid migration requires much less adhesion and is powered by cycles of cellular expansion and contraction (160). During amoeboid migration through a matrix the cell rounds into a ball, squeezing and blebbing its way through a matrix (37). In contrast, during mesenchymal migration within a matrix, tumor cells typically degrade the matrix to create a tunnel through which they migrate (160). Tumor cells utilize different forms of motion based on cell-type and the properties of the surrounding extracellular matrix (37). Amoeboid migration is significantly faster than mesenchymal migration (160). It has been proposed that overexpression of Ect2 can speed cell migration by transforming the preferred cellular migration pattern from mesenchymal to amoeboid (138). Alternatively, for cells consistently moving in a mesenchymal fashion, Ect2 overexpression can slow migration by increasing focal adhesions and preventing forward motion (121). Still, some level of Ect2 activation appears to be required for proper membrane ruffling and optimal mesenchymal migration (159).

The cell-type differences described indicate that further examination of the role of Ect2 in tumor cells is required and may vary with tumor type.

Oncogene- focus on Rac-driven transformation

Although the importance of the DH domain/GEF activity of Ect2 in tumor cells has not been examined, the function of Ect2 in tumor cells has been correlated with its ability to activate Rac1. In NSCLC, decreased anchorage-independent growth that results from Ect2 knockdown can be rescued by expression of constitutively active Rac1 (137). While this experiment does not prove that Ect2 acts through Rac1 to drive transformation, it shows that activated Rac1 is sufficient to drive transformation in these cells. Further correlative data indicates that knockdown of Ect2 attenuates Rac1 activity (137, 138). And a T327 (T328 in reference, see Fig. 1.6 legend) phospho-deficient mutant of Ect2 that cannot rescue Rac1 activity also does not rescue anchorage-independent growth. In contrast, WT and T327 phospho-mimetic Ect2 rescue both Rac1 activity and anchorage-independent growth in NSCLC cells (161). Therefore it has been hypothesized that Ect2 drives NSCLC cellular transformation through activation of Rac1 (137).

Additional correlative data suggests that Rac1 activation may be a key function of Ect2 for driving cellular transformation. As described above, overexpression of Ect2 DH/PH/C transforms fibroblasts, while Ect2 DH/PH does not (46, 94, 111). While the C-terminus enhances nucleotide exchange on RhoA, Rac1, and Cdc42, fibroblasts expressing the DH/PH mutant have a morphology reminiscent of cells expressing active RhoA (94), whereas fibroblasts expressing DH/PH/C (46, 94) have a morphology suggestive of active Rac1. Therefore one could speculate that Rac1 is dominating the GTPase signaling in these cells and may be driving transformation as well.

Oncogene- cytoplasmic mislocalization hypothesis

Based on work in NIH 3T3 mouse fibroblasts (described above), the current model of Ect2 oncogenic function requires mislocalization of Ect2 from the nucleus to the cytoplasm in order to activate Rho GTPases and drive cellular transformation (111). Consistent with this idea Ect2 has been observed in the cytoplasm of a variety of tumor cells. Immunohistochemistry (IHC) has been used to stain normal lung and NSCLC tumors for Ect2. In normal lung epithelia Ect2 was observed in the nucleus, whereas in tumor epithelia it appeared to be nuclear excluded with high cytoplasmic expression (137). Although initially convincing, the primary tumor subcellular localization did not match that of NSCLC cell lines, where examination via immunofluorescence showed intense nuclear staining of Ect2, with low cytoplasmic signal (137). Additionally, when I tried to validate the specificity of the Santa Cruz antibody used, many non-specific bands appeared upon immunoblot and the immunofluorescence signal did not decrease upon Ect2 knockdown (Chapter 4 and data not shown). Therefore while the antibody (from the lot I tested) is sufficient for use by immunoblot where proteins can be separated by apparent molecular weight, it should not be used for immunofluorescence and likely not for immunohistochemistry. Examination of Ect2 expression in NSCLC by another group, utilizing a non-commercial antibody, indicated that both nuclear and cytoplasmic staining of Ect2 increased with tumor progression (146). This is consistent with other tissue types that have been examined (oral, esophageal, brain). Indeed, in normal tissue that has been stained, Ect2 is either expressed at low levels in the nucleus (137, 138, 146), low levels in the cytoplasm (147), or at undetectable levels (145, 146), and upon tumor progression Ect2 strongly stains the nucleus and also faintly highlights the cytoplasm (138, 145-147). Clearly Ect2 is not mislocalized in all tumor types. For

example, the initial characterization of Ect2 subcellular localization was performed in HeLa cells, which were derived from a cervical tumor (162), and in these cells Ect2 is predominantly nuclear during interphase (76).

Nuclear Ect2 has been assumed to be in an autoinhibited state, and sequestered from Rho GTPases (111). Consistent with this idea the majority of Rho GTPases are localized in the cytoplasm (47). However, pools of multiple Rho GTPases have also been observed in the nucleus (described above). Additionally, not all nuclear Ect2 is in an autoinhibited state, since, at least in HEK293T cells, a portion of nuclear Ect2 can interact with nucleotide-free RhoA (68). Therefore, the previous model requires revising (Fig. 1.8).

Also, the mechanism behind cytoplasmic mislocalization of Ect2 in tumor cells remains unclear. Exogenous Ect2 localizes to both the nucleus and cytoplasm of astrocyte progenitor cells, astrocytes, and astrocytomas, but only to the nucleus of some non-astrocytoma cell lines (138). Thus it has been hypothesized that overexpression alone is not sufficient to induce cytoplasmic mislocalization of Ect2, but instead that cytoplasmic mislocalization is regulated in some way (138). The Fields lab proposed that this regulation is conferred by PKCi phosphorylation of Ect2 on residue T327, and that the negatively charged phosphate opposes the adjacent basic NLSs to enhance cytoplasmic localization of Ect2 (137, 161). However, it is unclear if/how phosphorylation of Ect2 regulates its subcellular localization (See Chapter 6.1).

RAS AND RHO GTPASES IN CANCER

Identification

The study of Ras superfamily GTPases began in the 1960s when 2 viruses capable of inducing tumors in mice were identified (163, 164). These viruses contained genes from rodent cells that drove cellular transformation, which were later identified as the GDP/GTP binding proteins that we today call H-Ras (Harvey-Rat sarcoma) and K-Ras (Kirsten-Rat sarcoma) (2). Thus it is not surprising that misregulation of Ras and Rho GTPases drives tumor formation and many aspects of the transformed phenotype (2, 47).

Mutants- observed in tumors and used as tools

Ras family GTPases are frequently mutated in tumor cells (165). Alterations in residues G12, G13, and Q61 account for 97-99% of all observed Ras mutations in cancer (2). A variety of amino acids replace the WT residues in tumors (2), which is not surprising, because many amino acid substitutions result in the same phenotype. For example, mutation of G12 to any residue other than proline is sufficient to drive cellular transformation (166). Mutation of G12, G13, or Q61 creates a constitutively active GTPase by further impairing its intrinsically low ability to hydrolyze GTP and by conferring GAP insensitivity (167).

Frequency of mutation differs between isoforms and GTPases. K-Ras is mutated in 22% of tumors, while N- and H-Ras mutations are less common (8% and 3% respectively) (165). For a long time, Rho GTPases were considered distinct from their Ras counterparts by not being mutated in human tumors. Instead, Rho GTPases, RhoGEFs, and Rho downstream effectors were observed overexpressed in tumors, while GAPs were downregulated (47). It was suggested that this was due to the tighter regulation and GDP/GTP cycling required for

optimal Rho GTPase activity (52). However, recent deep sequencing studies have allowed the identification of Rac mutations in melanoma tumor cells and a variety of tumor cell lines (168-170). In fact, Rac1(P29S) is the third most common mutation in sun-exposed melanomas (168, 169). This mutation is located in the switch I region of GTPase and activates Rac (168-170). The mechanism by which P29S activates Rac1 is distinct from the activation of Ras by the mutations described above. P29S causes rapid nucleotide dissociation from Rac1 (170, 171) (hence, it is referred to as a "fast-cycling" mutant), which results in a GTPase that is frequently GTP-bound due to the higher prevalence of cellular GTP compared to GDP. Additionally, mutation of this residue may destabilize the GDP-bound conformation of Rac1 (169). The P29S mutation promotes proliferation (168); has an anti-apoptotic effect (170); is sufficient to drive anchorage-independent and tumor growth (170); enhances membrane ruffling (171); and increases speed of migration (168) of cells expressing mutant Rac1. Interestingly, Rac1(P29S) did not cause a dramatic increase in multinucleation whereas expression of an artificial Q61L Rac1 mutant did (171), suggesting that a fast cycling mutant allows progression through cytokinesis that is disrupted by a GTPase-deficient Rac1. N92I and C157Y mutations of Rac have also been observed in cancer cell lines, are fast cycling, and can drive anchorage-independent and tumor growth (170). These recent advances suggest that more Rho GTPase mutations may await discovery.

In addition to the mutations observed in tumor cells, other Ras and Rho GTPase mutations are frequently utilized to better understand cellular signaling. Rho family GTPases are mutated at residues homologous to Ras G12 and Q61 to create constitutively active GTPases. Additionally, fast-cycling mutants of Ras (172) and Rho family (173) GTPases are sometimes utilized as mimics of chronically on GTPase activity. For example, mutations

homologous to Ras(F28L) cause rapid nucleotide dissociation from GTPases without affecting GTP hydrolysis (172, 173). Although the engineered fast-cycling mutants result in the same effect that is now understood for the Rac1(P29S) mutation identified in melanomas, the structures of the two fast-cycling proteins are distinct (171).

In a screen for engineered Ras mutations that inhibit its activity, Ras(S17N) (174) and Ras(G15A) (175) were found. These mutants act as dominant-negatives. They have a low affinity for downstream effectors or nucleotides, but bind to GEFs with higher affinity than WT GTPases (176). Therefore expression of these mutants outcompetes endogenous Ras for GEFs and prevents activation of endogenous Ras (176). The G15A mutation creates the strongest interaction between Ras and GEFs, which has been described as irreversible (176). Yet, G15A is not stable in cells, so the less dramatic mutant, S17N, is commonly used as a dominant-negative *in cellulo* (176). Experiments utilizing these dominant-negative mutants must be interpreted with caution, especially for Rho GTPases which have very promiscuous GEFs (85). Since these mutants bind the GEF to prevent further activation of GTPases, expression of a dominant-negative GTPase (e.g. Rac(S17N)) will likely inhibit signaling to other GTPases as well (e.g. RhoA and Cdc42 GEF-dependent activation) (176).

Effector domain mutants have also been constructed, which show specificity to various Ras signaling cascades (2). For example, Ras Y40C signals to PI3K but not Raf or Ral-GDS (177), whereas Ras T35S signals to Raf and Ras E37G signals to Ral-GDS more specifically (177, 178). Yet as more signaling cascades are identified downstream of Ras the specificity of these mutants requires further testing.

Rho GTPases and GEFs are necessary and sufficient to drive many tumor phenotypes

The first associations of Rho GTPases/RhoGEFs with cellular transformation utilized many of the mutants described above. Expression of dominant-negative mutants of Rac1, Cdc42, or RhoA inhibit Ras-driven cellular transformation (179-182). These studies have since been confirmed with GEF knockout studies *in vivo*. For example, knockout of the Rac1 GEF Tiam1 dramatically reduced the number of tumors formed in a Ras-induced skin tumor model (183). Yet, the tumors that did form in Tiam1 knockout mice were more malignant than their Tiam1-expressing counterparts, suggesting that Tiam1 plays multiple roles throughout tumor initiation and progression (183). The role of RhoGEFs in tumors has since been expanded beyond Ras-driven oncogenesis. In some tumor-derived cell lines, knockdown of RhoGEFs such as Ect2 (137), Vav1 (184), and Trio (185) reduces tumor growth.

It was also observed early-on that overexpression of Rac1(G12V) is sufficient to induce focus formation (180) and RhoA(G14V) enhances Raf-driven focus formation (179). Now we know that fast-cycling mutants of Rho GTPases are more likely than these GTP-locked mutants to be observed in tumor cells (186), and that fast-cycling mutants of Rac1 drive cell proliferation, anchorage-independent growth, tumor growth, and inhibit apoptosis (168, 170). Similarly, activated RhoGEFs such as Ect2 (117), LARG (when overexpressed with active Raf) (187), Vav (188), Dbl (189), and a variety of others (190), are sufficient to induce these phenotypes in fibroblasts. In fact, expression of activated Rho GEFs is frequently more transforming than active Rho GTPases (46).

Because of their function in regulating the cytoskeleton and adhesion (discussed above), Rho GTPases are frequently studied for their role in tumor cell migration/invasion

and tumor metastasis. For example, RhoC expression is elevated in cells derived from pulmonary metastasis compared to initiating tumor cells (191) and knockout of RhoC dramatically reduces tumor metastasis (but not primary tumor formation) in a mouse model (192). To enhance migration/invasion/metastasis, Rho GTPases directly regulate cell movement (7), cell adhesion (193), and cell polarity (194). Rho GTPases can also activate expression/secretion of matrix metalloproteinases (MMPs) to degrade the ECM and allow cell movement through it (195). This is true not only for epithelial cells, but also for endothelial cells allowing for angiogenesis and vascularization of tumors (196, 197). It was recently shown that mice with the RhoGEF Asef knocked out have dramatically reduced ability to vascularize tumors (198).

Only a few of the many examples of Rho GTPase misregulation in cancer have been described above. As shown from this sampling, Rho GTPases regulate a variety of tumor cell functions, including tumor initiation, tumor growth/progression, tumor invasion/metastasis, and angiogenesis (47, 186).

How Rho GTPases drive cancer

Hanahan and Weinberg have famously summarized the "hallmarks" of cancer cells as sustaining proliferative signaling, evading growth suppressors, avoiding immune destruction, enabling replicative immortality, tumor-promoting inflammation, activating invasion and metastasis, inducing angiogenesis, genome instability and mutation, resisting cell death, and deregulating cellular energetics (199). Ras and Rho family GTPases are involved in many of these processes, through a variety of signaling cascades (37, 200-202). Figure 1.2 highlights

in orange some effectors of Rho family GTPases that are key regulators of one or more of these functions.

Prenylation of Rho GTPases is thought to be required for them to drive cellular transformation, yet unprenylated GTPases have still been reported to regulate transcription (14, 16, 20). Therefore, the role of GTPases in gene transcription has been uncoupled from its role in cellular transformation under at least some conditions. This was confirmed with multiple RhoA and Rac1 mutants capable of inducing SRF-transcription but not cellular transformation (203, 204). Still, Rho GTPases regulate the transcription of many genes, so some may be more relevant for cellular transformation. Westwick and colleagues observed that the transforming potential of RhoGEFs in NIH 3T3 mouse fibroblasts correlates with their ability to drive cyclin D1 expression, but does not correlate with their ability to regulate transcription factors SRF and c-Jun (46). In the same study, the ability of a panel of RhoGEFs to activate p38 and JNK also did not correlate with their ability to transform fibroblasts (46). This is not surprising, because it seems that there is not a single common mechanism through which all Rho GTPases drive cellular transformation, but instead cell-type and context differences decide the implications of a given signal. Some examples are given below.

Rac effector domain mutants have been used to determine that neither PAK nor JNK binding to Rac1 is required for Rac1 to induce cellular transformation in fibroblasts (204). This is surprising given the myriad of oncogenic pathways that PAKs regulate ((205) and Fig 1.2). A more recent study on skin tumors suggests that PAK activation of MEK (and the subsequent activation of ERK) mediates the ability of Rac1 to drive transformation (206).

Therefore, it is likely that in some cell types Rac acts through PAK for this phenotype, while in others it does not.

Many have suggested that Rac1 drives cell survival and cellular transformation via signaling to ERK, Akt, and NF-κB, whereas Rac1 activation of p38 and JNK inhibits transformation by promoting apoptosis (reviewed in (202)). ERK is a family of kinases that, upon phosphorylation, translocates into the nucleus to regulate a variety of mitogenic transcription factors (207). Rac1 has been described to activate ERK by converging in a variety of ways on the EGFR→SOS→Ras→RAF→MEK→ERK pathway (206). A direct interaction between Rac1 and ERK has been described (208). However, most mechanisms are through PAK. Rac1 activation of PAK has been shown to promote phosphorylation of both RAF (209) and MEK (206) to ultimately activate ERK. Similarly, PAK1 can bind to Akt and act as a scaffold for PDK1 to phosphorylate and activate Akt (210). Akt is a serine/threonine kinase that phosphorylates a variety of substrates to regulate cell survival, metabolism, angiogenesis, etc. (211). It is most commonly activated when PDK phosphorylates it after PI3K production of PIP_3 recruits both kinases to the plasma membrane (211). Rac1 can directly bind to PI3K to regulate Akt signaling as well (212).

Cdc42 also activates PAKs (205) and directly binds PI3K (212); therefore similar signaling pathways to those for Rac1 have been described to explain its role in cellular transformation (201). Additionally, activated Cdc42 binds the ubiquitin ligase Cbl and prevents EGFR degradation. Therefore, sustained Cdc42 activity leads to sustained EGFR signaling, ERK activation, and transformation (213). Yet, as with Rac1, Cdc42 can also initiate apoptosis via activation of JNK (214).

As shown in Figure 1.2, RhoA signals to a variety of downstream effectors, including ROCK, mDia, and rhotekin (215). Effector domain mutants revealed a correlation between ROCK binding and the ability of RhoA mutants to transform cells (in conjunction with Raf) (203). Also, inhibition of ROCK minimizes Dbl-, Net1-, and RhoA-induced anchorage-independent growth (216). This indicates that signaling to ROCK is a key pathway by which RhoA drives cellular transformation. This is not surprising, because ROCK phosphorylates important downstream substrates that are involved in cellular transformation signaling (as shown in Fig. 1.2). Still, activated ROCK is not as transforming as active RhoA, suggesting that RhoA works through other pathways as well (216). It has been proposed that the actin polymerization functions of RhoA can help drive transformation (37). For example, data suggest that RhoA activation of mDia to enhance stress fiber formation could allow for proper localization of the oncogene Src kinase to drive transformation. (37).

Ovarian cancer overview

Projected to kill over 14 thousand women in the United States in 2013, ovarian cancer is the deadliest of gynecological cancers and the fifth leading cause of cancer-related deaths of American women (217). When diagnosed while the tumor is confined to the ovaries, ovarian cancer has a 5-year survival rate of 92% (217). However, this drops to 27% if the disease is diagnosed after it has spread to distant lymph nodes or organs (217). Unfortunately, most ovarian tumors (61%) are discovered after distant metastasis has occurred (217). Therefore either better biomarkers of ovarian cancer that allow for earlier detection or better treatment for late stage tumors are essential to fight this disease.

A comparison of somatic copy number alterations between serous ovarian tumors and GBM tumors (218), highlights the fact that ovarian tumors are extremely heterogeneous. Therefore specific advances in treatment will likely be effective only in a subset of ovarian malignancies. Epithelial tumors account for 90% of ovarian cancers (219), and are further divided into four common subtypes, based on the gynecological tissue each tumor resembles pathologically: serous (fallopian tubes); endometrioid (endometrium); mucinous (endocervix); and clear cell (vagina) (219). Molecularly, ovarian cancer can be stratified into two types. Type I ovarian cancer is characterized by mutations in K-Ras, B-Raf, PI3K, or PTEN. Type II ovarian cancers generally have inactivating mutations in the tumor suppressor p53 (219). In addition to these mutations, ovarian tumors also show chromosomal instability. One of the most frequent genomic amplifications in ovarian cancer is 3q26, which includes the genetic locus for *ECT2* (151, 218).

Even with these known genetic errors, no targeted chemotherapeutics are used in the clinic. The standard of care for advanced stage ovarian cancer is debulking surgery followed by a combination of conventional platinum- and taxane- based chemotherapeutics (220, 221). Many ovarian tumors are initially responsive to this treatment; however, recurrence usually leads to fatality. Targeted chemotherapeutics that show specificity to tumor cells and allow a higher concentration of drug to be tolerated may be the key to killing more resistant tumor cells and preventing recurrence.

Rho GTPases in ovarian cancer

As with many tumor types, Rho GTPases have been implicated in ovarian tumor signaling. RhoA and RhoC mRNAs are more highly expressed in advanced ovarian tumors

compared to benign cysts and early stage tumors, and also in metastases compared to primary tumors (222). Both GTPases appear to function in ovarian tumor cell motility. Knockdown of RhoC decreases tumor cell invasion and migration (223). Similarly, inhibition of RhoA decreases Matrigel invasion *in cellulo* (222) and peritoneal dissemination in a mouse model (224). Overexpression of RhoA increased peritoneal dissemination, but did not affect proliferation of ovarian tumor cells (224). The role of RhoA in ovarian cancer cell invasion and metastasis may be through activation of p160ROCK. This downstream effector of RhoA (225) is necessary for optimal ovarian cancer cell migration and invasion *in cellulo*, and its expression is sufficient to enhance both phenotypes (226).

Although neither Rac1 nor Cdc42 has been reported to be upregulated in ovarian cancer cells, upstream and downstream effectors of these GTPases are important in ovarian tumor cells. For example, PAKs are major downstream effectors of Rac1 and Cdc42 (205). PAK1 (227) and PAK4 (228) mRNA and protein are expressed more highly in ovarian tumor cells compared to cysts. Further, activated phospho-PAK1 (T212), phospho-PAK2 (S20), and phospho-PAK4 (S474) are found significantly more often in ovarian tumor cells when compared to benign lesions (227, 228). These expression data match patient outcome. Higher levels of either total or phosphorylated PAK1 (227) or PAK4 (228) correlate with poorer patient survival. Interestingly, the phosphorylated form of both of these kinases is seen predominantly in the nucleus, whereas total protein is mostly cytoplasmic (227, 228). Knockdown of PAK1, PAK2, or PAK4 in ovarian cancer cell lines decreases migration and invasion whereas overexpression of constitutively active PAKs has the opposite effect (227, 228). PAK4 also enhances ovarian cancer cell proliferation (via increasing expression of cyclin D1), but PAK1 and PAK2 do not regulate proliferation of these cells (227, 228).

Regulators of Rho GTPases that have also been implicated in ovarian tumor signaling include the RhoGEF DOCK180 (229) and the RhoGAPs IQGAP (230) and DLC-1-3 (231, 232). As mentioned above, Ect2 has been shown to be both amplified and upregulated at the mRNA level in ovarian cancer cells (45, 150, 151). Yet the protein level expression and the role of Ect2 in this disease had not been previously investigated (see Chapter 4).

Table 1.1. Summary of the controversy surrounding the GTPase specificity of Ect2. A list of references is given for papers determining that Ect2 activates or does not activate RhoA, Rac1, and Cdc42. The domains of Ect2 and the type of assay used to draw these conclusions are indicated.

	Activates		**Does NOT activate**	
RhoA	(76)	full length, *in vitro*	(117)	"transforming Ect2", *in vitro*
	(46)	DH/PH, morphology	(112)	full length, *in vitro*
	(125)	dominant-negative, function & pulldown	(137)	knockdown, pulldown
	(111)	DH/PH/C, pulldown	(138)	knockdown, pulldown
	(94)	DH/PH, *in vitro* & morphology & pulldown; DH/PH/C morphology & pulldown		
	(112)	DH/PH/C & BRCT mutant, *in vitro*		
	(121)	DH/PH/C, pulldown		
	(138)	"WT" (overexpression), morphology		
Rac1	(76)	full length, *in vitro*	(117)	"transforming Ect2", *in vitro*
	(46)	DH/PH/C, morphology	(111)	DH/PH/C, pulldown
	(94)	DH/PH/C, morphology & pulldown	(94)	DH/PH, *in vitro*
	(123)	DH/PH/C & knockdown, pulldown	(121)	DH/PH/C, pulldown
	(137)	knockdown, pulldown	(159)	suggests role in Rac activation is indirect via Trio
	(138)	knockdown, pulldown		
Cdc42	(76)	full length, *in vitro*	(117)	"transforming Ect2", *in vitro*
	(233)	Xenopus Ect2, functional correlation	(111)	DH/PH/C, pulldown
	(94)	DH/PH/C morphology & pulldown	(94)	DH/PH, *in vitro*
	(123)	DH/PH/C & knockdown, pulldown (MDCK)	(123)	DH/PH/C, pulldown (HeLa)
	(234)	knockdown & dominant-negative, pulldown	(137)	knockdown, pulldown
	(65)	functional/location correlation		
	(159)	knockdown, pulldown post stimulation		
	(138)	knockdown, pulldown		

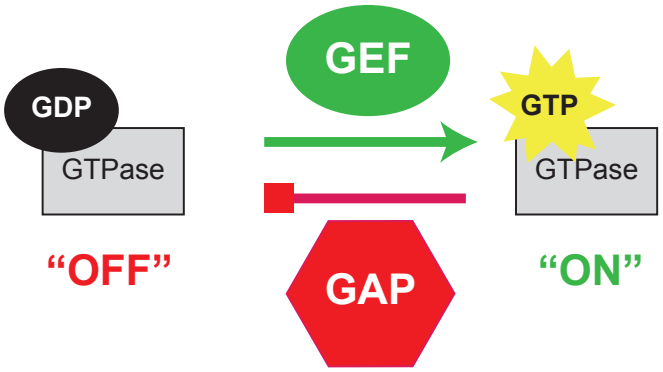

Figure 1.1. GTPases activity is regulated by GEFs and GAPs. GTPases act as molecular switches within the cell. They are "on" and able to interact with downstream effectors while GTP bound and "off" while GDP bound. Guanine nucleotide exchange factors (GEFs) catalyze the release of bound nucleotide enabling GTP to bind to and activate the GTPase. GTPase activating proteins (GAPs) enhance the intrinsic ability of the GTPase to hydrolyze GTP and turn the GTPase "off."

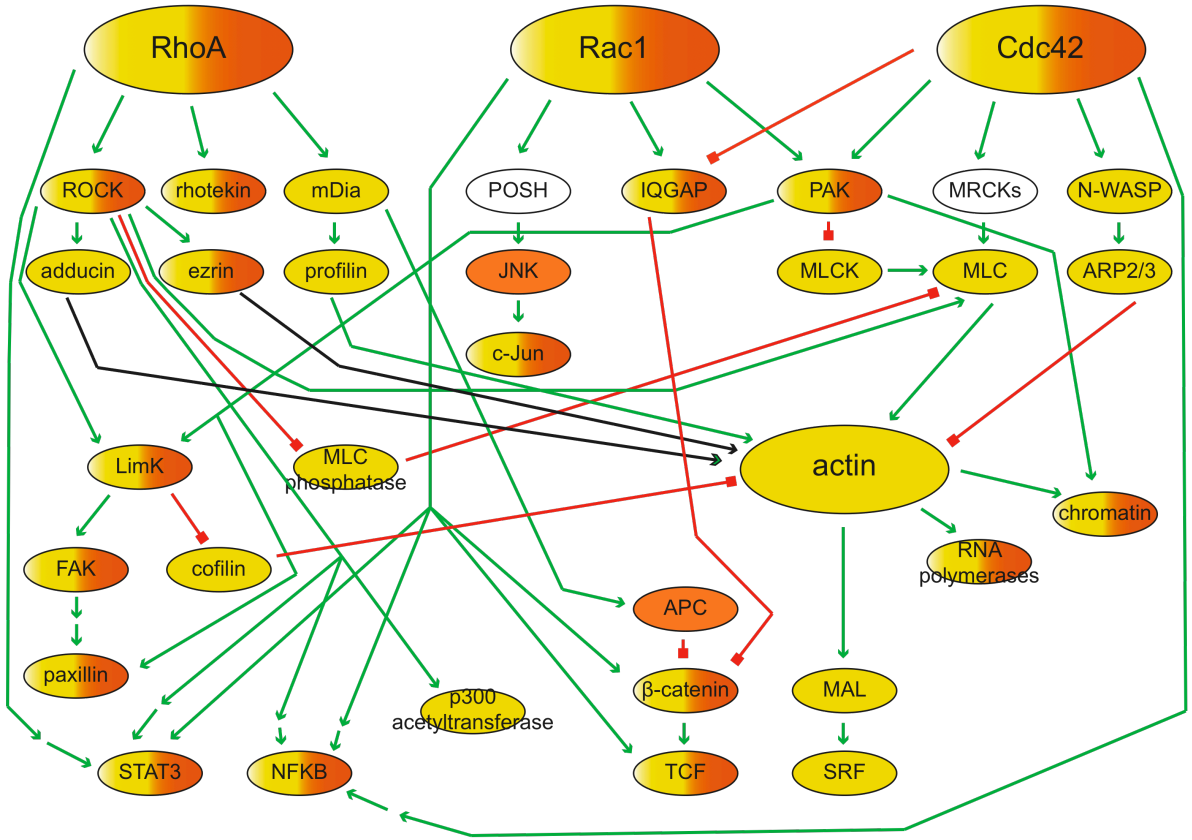

Figure 1.2. Examples of RhoA, Rac1, and Cdc42 signaling cascades, highlighting signaling molecules described in the nucleus and/or involved in cellular transformation. Some (of the many) well-established RhoA, Rac1, and Cdc42 signaling cascades are shown. Activating interactions are shown with green arrows, while inhibitory interactions are shown with red lines. With respect to actin, green arrows initiate at proteins that enhance actin polymerization, red lines initiate at proteins that enhance actin severing or branching, and black lines indicate other actin binding proteins. Proteins that have been documented in the nucleus are highlighted in yellow. Proteins that have important roles in cellular transformation are highlighted in orange. See list of abbreviations and symbols for additional information.

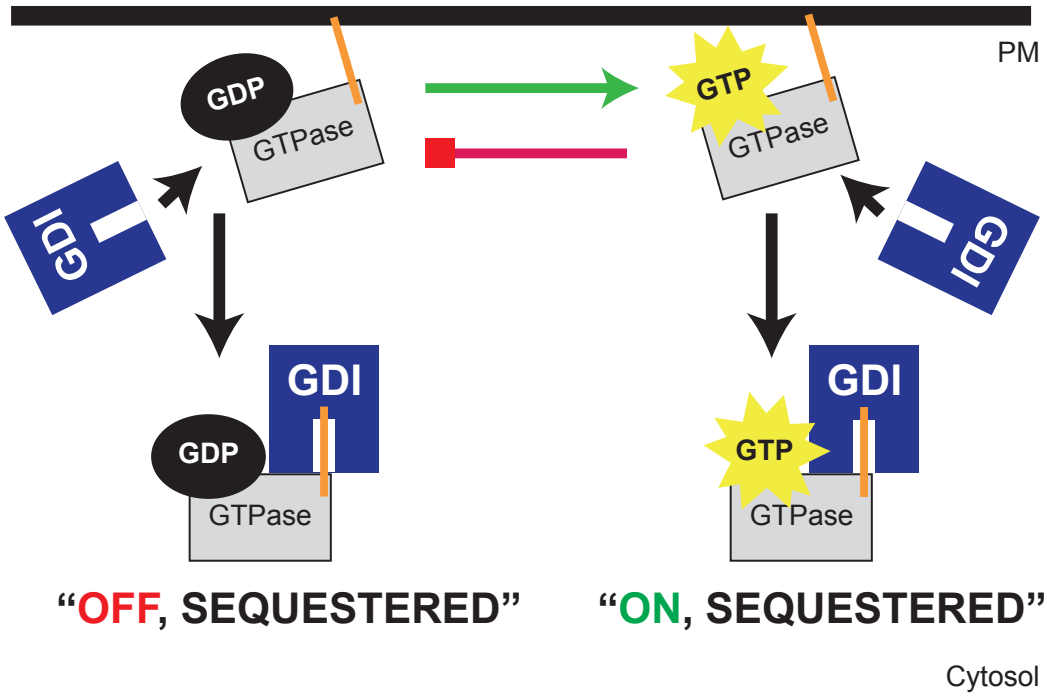

Figure 1.3. RhoGDI strip GTPases from the plasma membrane and sequester GTPases from effectors. In addition to GEFs and GAPs, Rho GTPases are regulated by Rho guanine nucleotide dissociation inhibitors (RhoGDI). RhoGDIs bind to the C-terminal prenyl group of Rho family members and to the GTPase itself. GDIs strip Rho GTPases from membranes and sequester them in the cytosol, where the Rho GTPases are unable to interact with their downstream effectors. Additionally, GDIs inhibit nucleotide dissociation and GTP hydrolysis.

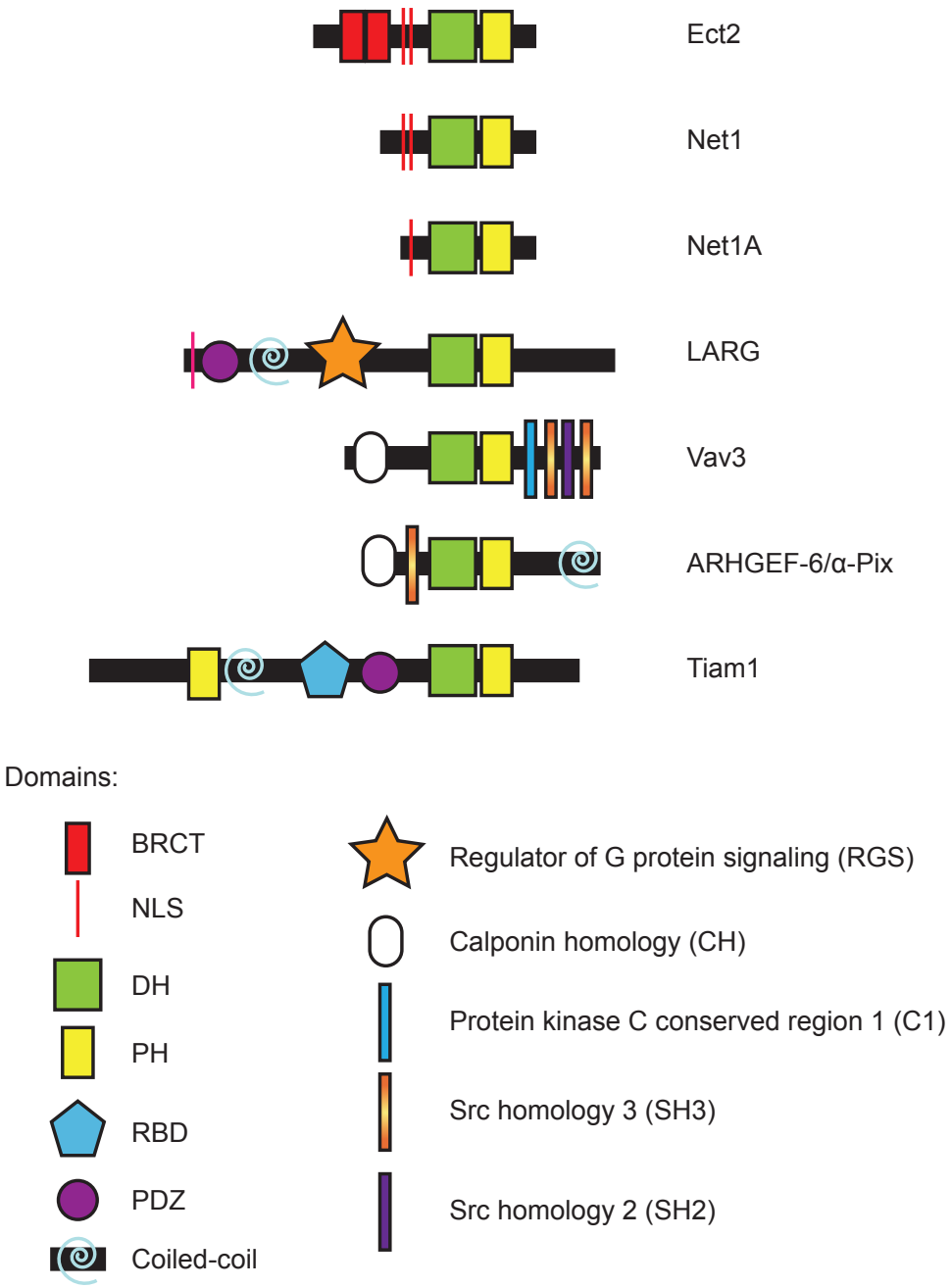

Figure 1.4. Dbl family RhoGEFs contain a Dbl homology (DH), a pleckstrin homology (PH), and a variety of other domains. The variability in additional domains allows cells to respond to a variety of signaling inputs by activating Rho GTPases. Ribbon diagrams of some example Dbl family RhoGEFs are shown. Here I have focused on Rho GEFs that have been observed in the nucleus. See the list of abbreviations and symbols for further details.

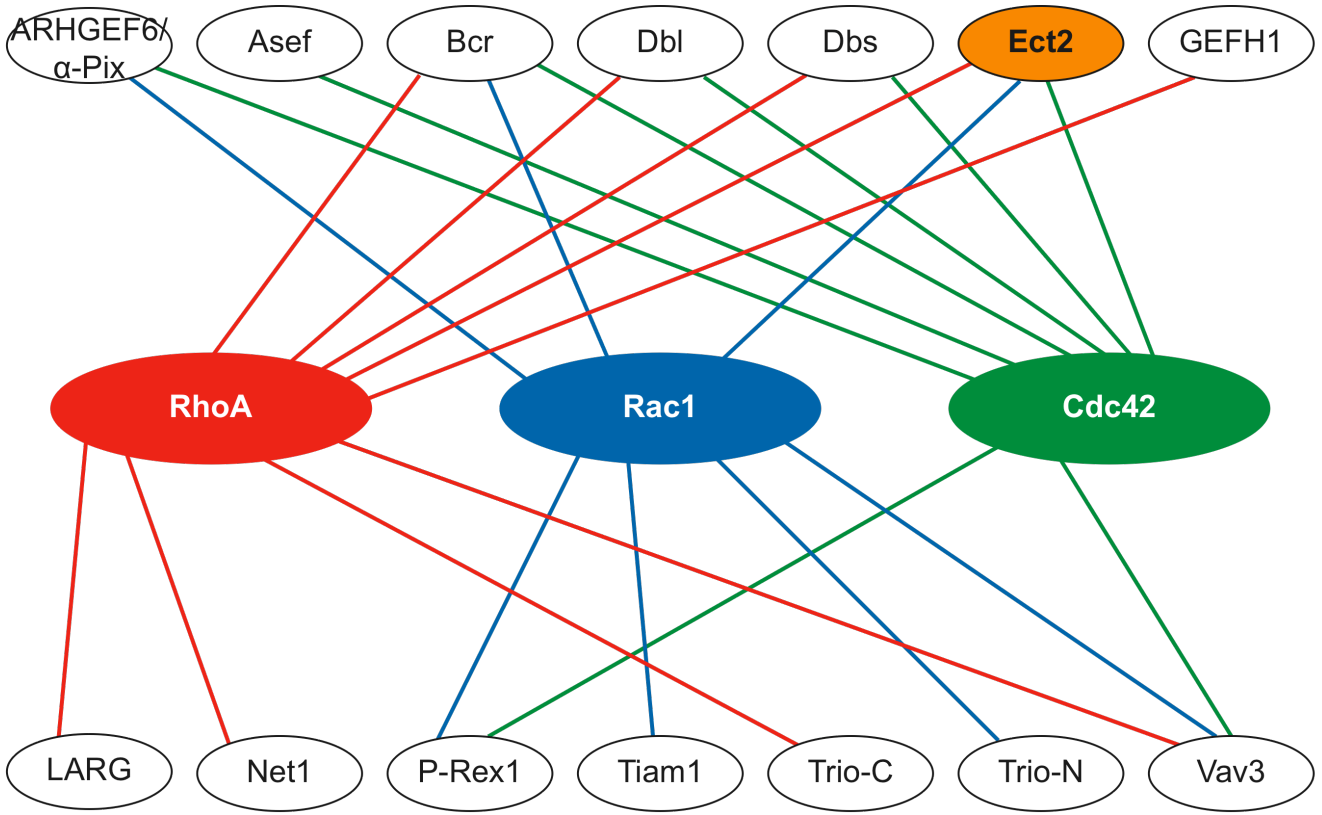

Figure 1.5. Dbl family RhoGEFs vary in specificity. Some Dbl family GEFs act solely on a single GTPase, however, many have multiple effectors. The specificity of example Dbl RhoGEFs towards RhoA, Rac1, and Cdc42 is shown here. Lines connect GEFs to GTPases they activate.

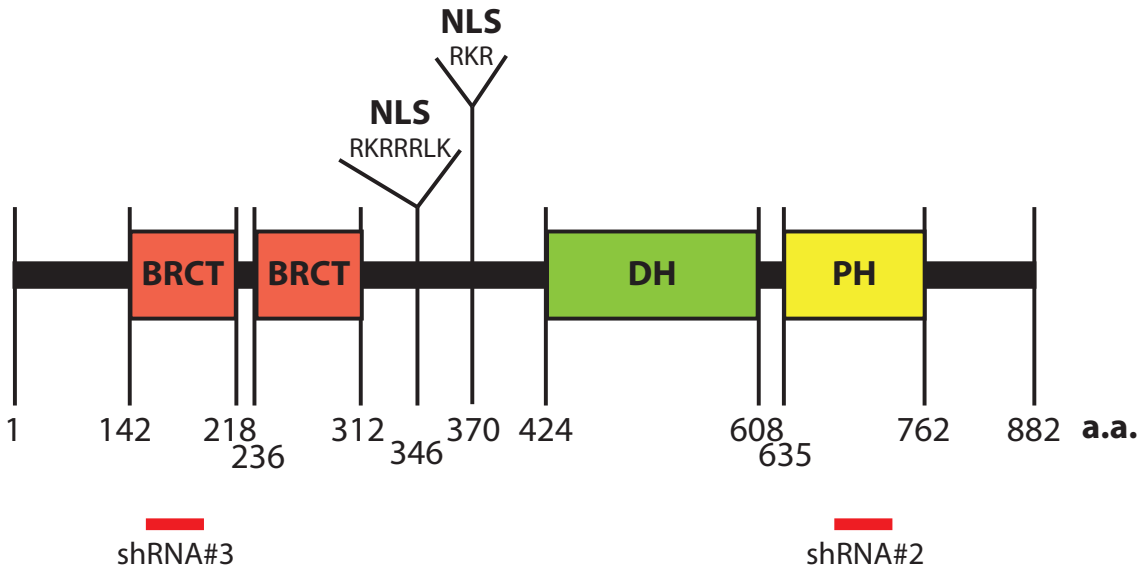

Figure 1.6. Domain structure of Ect2. SMART (simple modular architecture research tool) was used to define the domains of the *Homo sapiens* Ect2 sequence (GenBank accession number AY376439). See list of abbreviations and symbols for full domain names. Amino acid (a.a.) numbers and nuclear localization signals (NLS) are shown. Regions of homology with each Ect2 shRNA are indicated with red lines. Some Ect2 papers utilize an alternative sequence for Ect2, with an additional glutamic acid at residue 45. Thus occasionally, the numbering described in referenced papers will be one a.a. higher than the numbering used within this dissertation.

Truncation	Transforming?	NLS present
1	No	NLS1, NLS2
2	Yes	NLS1, NLS2
3	Yes	NLS2
4	Yes	NLS2
5	Yes	NLS2
6	Yes	none
7	No	none

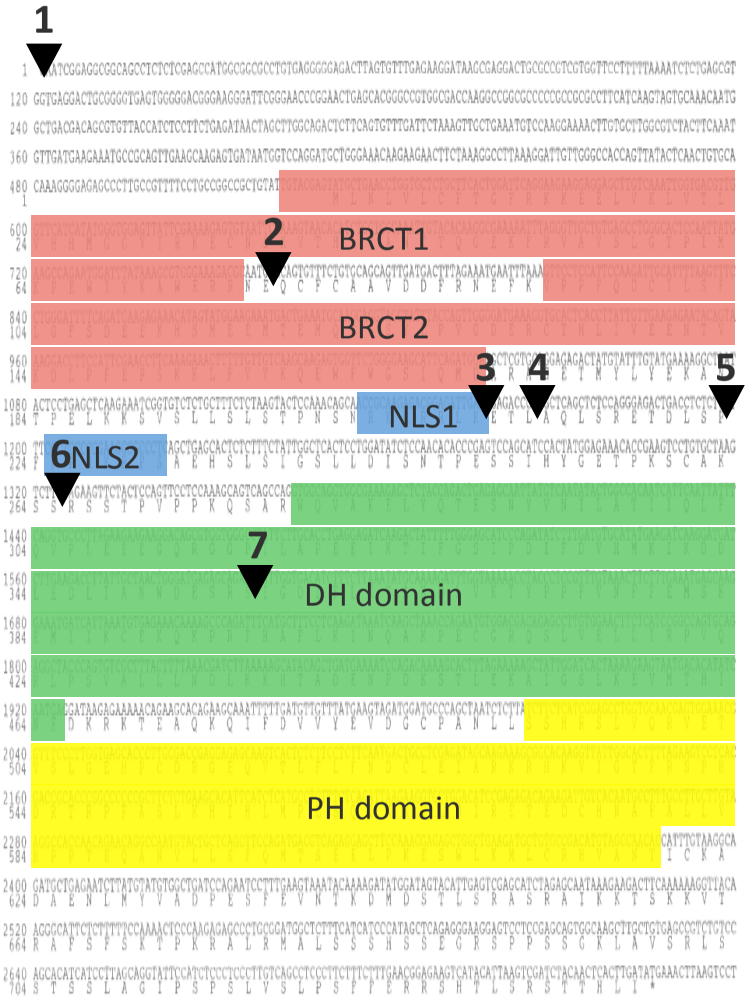

Figure 1.7. The domains and transforming capabilities of the originally isolated Ect2 truncation mutant cDNAs. The original Ect2 cDNA sequence (117) is shown and domains are highlighted. Black triangles represent the start site for each truncation mutant. Truncation #4 was the first cDNA isolated and was described in detail by Miki et al. (117).

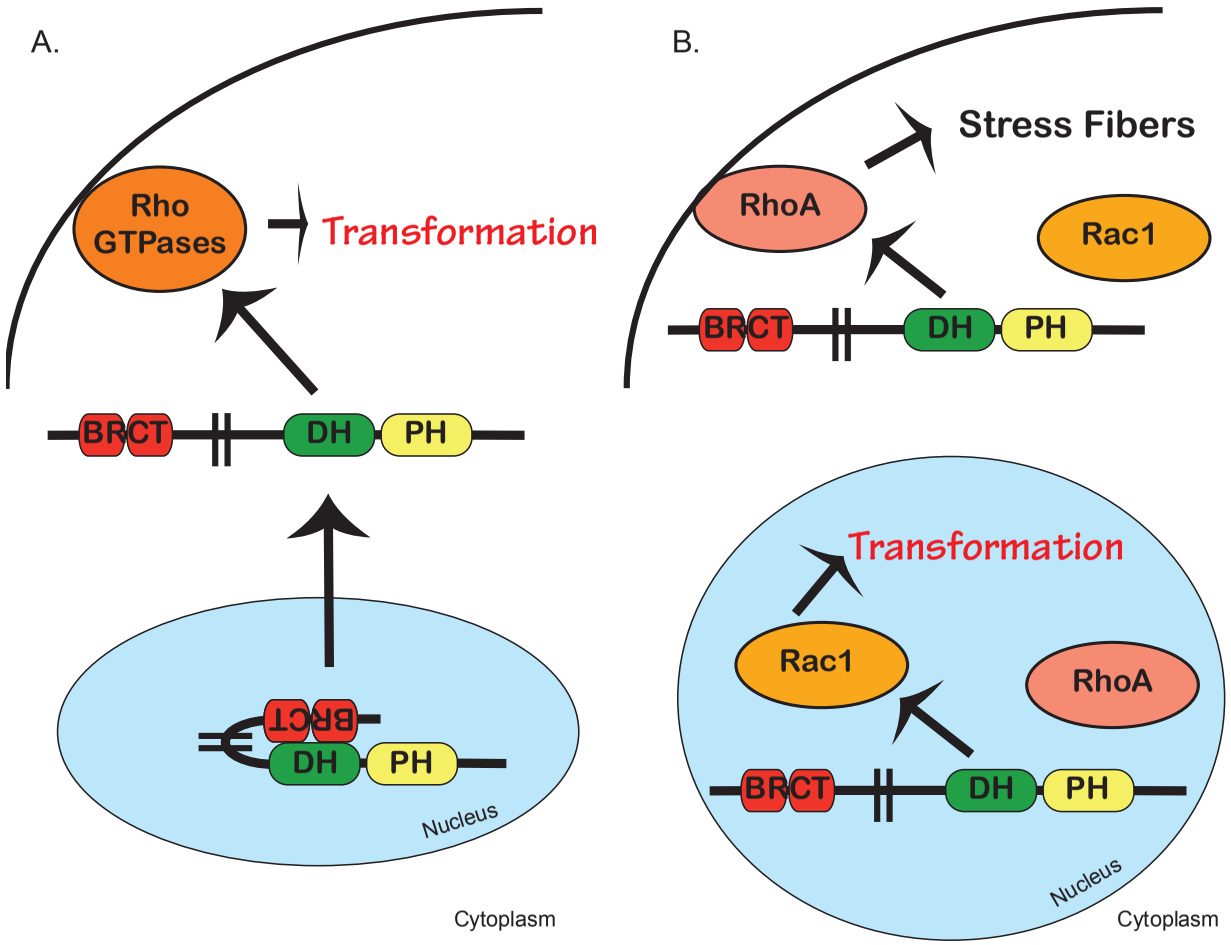

Figure 1.8. Comparison of models of Ect2-driven cellular transformation. *A.* The previous model of Ect2-driven cellular transformation (111) proposed that Ect2 is auto-inhibited and sequestered from Rho GTPases while in the nucleus. Therefore, it was thought that Ect2 must move to the cytoplasm, release its autoinhibition, and activate extra-nuclear Rho family GTPases to drive transformation. *B.* We determined that there is an active pool of Ect2 in both the nucleus and the cytoplasm and that Rho GTPases can be observed in both subcellular compartments. Ect2 preferentially activates Rac1 when in the nucleus and RhoA when in the cytoplasm. We propose nuclear Ect2 activation of nuclear Rac1 as a novel mechanism of cellular transformation.

CHAPTER 2: PRENYLATION AND PHOSPHORYLATION OF RHO FAMILY GTPASES[1]

OVERVIEW

Canonical small GTPases of the Ras, Rho and Rab families are modified in their C-terminal membrane targeting domains by farnesyl or geranylgeranyl isoprenoid groups, and these prenyl lipid modifications are strictly required for their correct subcellular localization and biological functions. Some of these proteins are also modified by nearby acylation with palmitate(s) that dynamically regulate their membrane binding and activities and permit more precise spatiotemporal control of their functions. In recent years it has become appreciated that additional posttranslational modifications such as phosphorylation may be a common mechanism for further dynamic modulation of these permanently prenylated proteins. In this chapter, we discuss the current state of knowledge regarding the C-terminal sites of phosphorylation of prenylated small GTPases, the kinases involved, and the consequences of these phosphorylation events for GTPase activation, subcellular localization, effector utilization and biological functions.

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[1]This chapter is reprinted from The Enzymes, Vol 30, Protein Prenylation part B (11), with permission from Elsevier. I wrote the section on Rho family GTPases. Heather B. Hodges-Loaiza wrote the section on Ras family GTPases and the introductory sections. We worked together on the conclusions and figures. Adrienne D. Cox edited all sections of this chapter.

INTRODUCTION

The Ras superfamily of small GTPases encompasses the Ras, Rho, Rab, Arf and Ran subfamilies, whose diverse cellular functions include regulation of cellular metabolism, proliferation, differentiation and survival, cytoskeletal organization and motility, and vesicular trafficking (1, 2). Not surprisingly, their precise and correct regulation is critical to maintain cellular homeostasis and proper functioning, whereas perturbation can lead to pathophysiological states. These proteins function as GDP-/GTP-regulated molecular switches to transduce cellular signals through multiple signaling cascades. Each subfamily includes highly related isoforms in which the primary regions of difference occur not in their ordered GTP-binding regions but in their highly flexible C-terminal membrane targeting regions, which are thus designated as the hypervariable regions (HVRs) or hypervariable domains (HVDs). HVD sequence differences between isoforms are highly conserved at the protein level throughout evolution, supporting the importance of these regions for protein function.

Spatiotemporal control of small GTPase signaling is conferred by a variety of posttranslational modifications of the HVD sequences that dictate targeting to specific subcellular compartments (e.g., plasma membrane [PM] versus Golgi), or to regions within those compartments (e.g., PM lipid rafts or non-raft PM regions). These posttranslational modifications are currently known to include prenylation, acylation, carboxymethylation, ubiquitination, SUMOylation and phosphorylation. Here, we focus on phosphorylation of the C-terminal HVDs of prenylated small GTPases. The most common consequence of such phosphorylation is translocation from the plasma membrane to the cytosol and endomembranes, where the translocated GTPases then interact with different pools of

regulators and effectors, resulting in distinct biological outcomes for the phosphorylated and unphosphorylated forms.

SMALL GTPASE PRENYLATION

Prenylation modifies eukaryotic proteins from a diverse spectrum of families, employing branched, unsaturated lipid groups that are biosynthesized from the mevalonate metabolic pathway, which provides the minimal five-carbon isoprene subunit of prenyl groups. Among the Ras superfamily, farnesyl or geranylgeranyl moieties are attached to the C-terminal "CAAX" motifs of most members of the Ras and Rho branches, where C is invariantly a cysteine, A is generally an aliphatic amino acid, and X = a variable amino acid, usually M, Q, S, T or A for farnesyl and L or I for geranylgeranyl (12). The farnesyl moiety is a 15-carbon isoprenyl chain that exists as the precursor lipid substrate farnesyl diphosphate (FPP) prior to its transfer to the target protein substrate by the enzyme farnesyl transferase (FTase). FTase transfers FPP to the sulfhydryl side chain of the cysteine within the C-terminal CAAX motif, forming a stable thioether bond. Modification of small GTPases by a geranylgeranyl moiety, from the 20-carbon geranylgeranyl diphosphate, requires the enzymatic activity of either geranylgeranyl transferase I (GGTase I) or geranylgeranyl transferase II (GGTase II). The latter enzyme specifically modifies members of the Rab subfamily of small GTPases, which terminate in CC, CXC, CCXX or CCXXX motifs and which all bind to a critical accessory protein, REP (Rab escort protein). The Rab:REP complex is then recognized by GGTase II for prenylation. In contrast, Arf family GTPases are not prenylated, but instead are stably acylated by the fatty acid myristate on N-terminal glycine residues, whereas the Ran GTPase is not modified by any lipid.

Newly prenylated C-terminal CAAX motifs then signal for further modifications by post-prenylation processing enzymes; these modifications do not take place in the absence of prenylation. Ras converting enzyme 1 (Rce1) performs the first of two post-prenylation modifications of the CAAX motif itself. Rce1 is an endoplasmic reticulum (ER)-integral membrane metalloprotease that recognizes C-terminal farnesylated or geranylgeranylated CAAX motifs and subsequently hydrolyzes the three -AAX amino acids from the prenylated CAAX motif. The newly exposed C-terminal prenylcysteine is then recognized as a substrate for esterification on its α-carboxyl group by the enzyme isoprenylcysteine carboxyl methyltransferase (Icmt), which transfers a methoxy functional group from the methyl donor *S*-adenosyl-L-methionine (SAM) to afford water and *S*-adenosyl-L-homocysteine as byproducts. The protein product of the three CAAX-signaled modifications of prenylation, proteolysis and carboxylmethylation includes a C-terminus with increased hydrophobicity and the appropriate biological attributes to be recognized by additional cellular machinery necessary for proper subcellular localization and functionality.

Prenylation has long been described as an obligate modification for membrane binding of the small GTPases so modified, as either structural mutation of the prenylated cysteines or pharmacological inhibition of the prenylation enzymes results in cytosolic proteins. However, prenylation is insufficient to support full biological activity. Because the diversity of small GTPase functionality requires very precise spatiotemporal control, multiple levels of regulation are required to confer such precision. CAAX-signaled processing alone is sufficient to promote weak membrane binding but is insufficient to promote either strong binding or binding to specific membranes. To accomplish this, both a "second signal" for membrane binding - which can be either a stretch of polybasic residues (PBR; lysines and/or

arginines) or acylation by one or more palmitates (9, 235) - and additional contextual sequence information (236) are necessary. Related small GTPase isoforms frequently contain either a PBR or a palmitoylation site, suggesting that it is important to maintain the diversity of functional consequences conferred by each type of second signal. Finally, since both the prenyl lipid and the PBR are permanent, there is strong rationale for additional posttranslational modifications that can confer more dynamic modulation.

C-TERMINAL PHOSPHORYLATION OF PRENYLATED RAS FAMILY SMALL GTPASES

Phosphorylation is becoming more widely recognized as another major posttranslational modification that influences the localization and function of prenylated small GTPases, by modulating the affinity of their C-terminal HVDs for specific biological membranes and for specific protein:protein interactions. These phosphorylation events are performed by an assortment of kinases and can be both cell type- and cell context-dependent. The contributions of C-terminal phosphorylation to the Ras branch of small GTPases include direct effects on protein-protein interactions, as is the case for K-Ras4B phosphorylation inhibiting calmodulin or galectin-3 binding, as well as indirect effects such as the allosteric changes caused by phosphorylation that perturb distal effector binding to Rap1.

K-Ras4b phosphorylation by PKC-alpha alters its protein:protein interactions with calmodulin and galectin-3, and translocates it from the PM, converting it to a death-inducing protein

Ras proteins normally play critical roles in transduction of extracellular signals to intracellular signaling to regulate gene expression and cellular proliferation and survival, and the genes that encode them are particularly known as the most frequently mutated oncogenes in human cancers (2, 237). Therefore there is particular interest in understanding the mechanisms that regulate Ras activity, which can so easily go awry with dire consequences. Although there are four Ras isoforms (H-Ras, N-Ras, and the alternatively spliced K-Ras4A and K-Ras4B), all of which are farnesylated, only K-Ras4B is also C-terminally phosphorylated. K-Ras4B was the first small GTPase in which this type of phosphorylation was reported, although it was not until many years later that the cellular consequences of this modification would be discovered. In 1987, Ballester, Furth and Rosen demonstrated (238), through metabolic labeling and immunoprecipitation studies of NIH 3T3 mouse fibroblasts, as well as *in vitro* phosphorylation assays, that K-Ras4B became phosphorylated in response to short-term stimulation of protein kinase C (PKC) with phorbol-12-myristate-13-acetate (PMA), also known as 12-O-tetradecanoylphorbol-13-acetate (TPA). They concluded that K-Ras4B is a direct substrate of PKC and that the likely site of phosphorylation was serine 181. This residue is just upstream of the farnesylated CAAX motif in the HVD, but the existence of neither farnesylation nor CAAX motifs in small GTPases was known at the time. In 2006, Philips and colleagues confirmed not only that transient stimulation of PKCalpha activity resulted in rapid phosphorylation of a subset of K-Ras4B on serine 181, but also that this phosphorylation induced a rapid translocation of phosphorylated K-Ras4B from the

plasma membrane (PM) to internal membranes and increased interaction with Bcl-XL (239). Translocation of K-Ras4B also resulted in cell death, a process that our laboratory then observed to be mimicked by the K-Ras4B phosphomimetic mutant S181E, and that tumor xenograft growth could be modulated by bryostatin-1, an activator of PKC, only when S181 was intact (239). These results collectively indicated the critical role played by phosphorylation of the HVD for both localization and biological activity of this important Ras small GTPase, and led to hopes, as yet unrealized, that this process could one day be exploited as a tractable avenue for managing the activity of oncogenic K-Ras4B.

Interestingly, members of the Faller laboratory demonstrated that chronic PMA treatment of mouse fibroblasts that depletes PKC activation caused a K-Ras-dependent apoptotic mechanism (240) that was not well suppressed by the apoptotic inhibitor Z-VADfmk (241). Thus, any deregulation of PKC activity, whether stimulation or suppression, may alter K-Ras control of cell survival in a context-dependent manner, either directly or indirectly, further highlighting the complexity of interfering in Ras function in a controllable way.

The Agell laboratory has explored the relationship between PKC-mediated phosphorylation of K-Ras at S181 and binding of the second messenger calmodulin (CaM) to this residue, and the consequences of these interactions for K-Ras localization and activity. They demonstrated that CaM interacts with GTP-bound K-Ras, but not with H-Ras or N-Ras (242), and, consistent with this selectivity, that the farnesyl group, polybasic residues and S181 of K-Ras are all required for this interaction (243). Phosphorylation of S181 and CaM binding are mutually exclusive, as a phosphomimetic mutant S181D cannot bind to CaM (244). PMA-mediated transient stimulation of PKC in NIH 3T3 cells resulted in increased

levels of K-Ras-GTP, but only if CaM was inhibited (244). Finally, an S181D phosphomimetic mutation but not a phosphodeficient S181A mutation in wild-type K-Ras was able to support proliferation when expressed in otherwise "Ras-less" fibroblasts (245). These findings, together with their previous studies that showed PM colocalization of CaM with K-Ras, as well as a PM localization of S181 phosphorylated K-Ras (243), led them to propose that this CaM / K-Ras S181 phosphorylation interplay provides a mechanism for regulating K-Ras localization into discrete PM microdomains in concert with the known mechanisms of K-Ras localization to specific nanoclusters, as described below.

In 2008, Plowman, Hancock and colleagues utilized the S181E phosphomimetic mutant of K-Ras4B to determine the effects of phosphorylation at this residue on K-Ras nanocluster assembly and function (246). Activated K-Ras-GTP organizes into functional nanoclusters on the inner leaflet of the plasma membrane, forming PM “hot spots” that support the transduction of high fidelity signals through intracellular signaling cascades (247, 248). K-Ras nanocluster organization, which is distinct from that of H-Ras or N-Ras, is dependent on both cellular context, including PM lipid content and actin cytoskeleton organization, and on K-Ras sequences (25, 247-250). Galectin-3 (Gal3), a predominantly cytosolic protein, is recruited to the PM upon K-Ras activation to facilitate K-Ras-GTP nanocluster formation through interactions with the G-domain and with the farnesyl group of K-Ras (25). The Gal3 hydrophobic binding pocket is also a Ras prenyl-binding pocket, and the recognition of K-Ras-GTP by Gal3 is structurally similar to that of RhoGDIs with Rho GTPases, in which the GTP-dependent conformational change of the GTPase within the switch I and switch II regions allows for direct interaction of the binding partner within the G-domain. Phosphorylation of K-Ras(G12V) at S181 inhibited nanocluster formation, but

surprisingly had no effect on the scaffolding ability of Gal3 in organizing the nanoclusters, indicating that Gal3 and the polybasic domain make distinct contributions to the formation of K-Ras-GTP nanoclusters.

Rap1 phosphorylation by PKA translocates it from membranes and may induce allosteric conformational changes to alter interactions with downstream effectors

The Rap (Ras proximate) proteins, Rap1a and Rap1b, and Rap2a/b/c, belong to the Ras subfamily of small GTPases, sharing ~50% sequence similarity with Ras (251). Rap proteins function primarily in cell adhesion and migration, and polarity (252, 253). Rap1a (CAAX = CLLL) is 95% identical to Rap1b (CAAX = CQLL), with the differences found largely in the C-terminal HVD and in a few N-terminal residues (254). These geranylgeranylated proteins are phosphorylated by cyclic AMP (cAMP)-dependent protein kinase (PKA) at serine 180 in Rap1a (254, 255) and serine 179 in Rap1b (255-258). None of the 3 isoforms of Rap2 have been shown to be phosphorylated, although Rap2a and Rap2b both carry potentially phosphorylatable serine residues proximal to the CAAX motif. Early *in vitro* kinetic studies found that Rap1 C-terminal phosphorylation is neither dependent on nor affects its nucleotide binding status, rate of hydrolysis, or interaction with a negative regulatory RapGAP (255, 259). Soon after, however, it was reported that Rap1b C-terminal phosphorylation enhances its ability to bind to and be stimulated by a noncanonical GEF, SmgGDS (260, 261), which supports a possible role for phosphorylation in modulating Rap1 activation. Therefore, stimulation of Rap1 by SmgGDS involves both prenylation (262) and phosphorylation, in which the latter modification decreases the affinity of the Rap1 polybasic domain for anionic membrane lipids, thereby allowing for release from the membrane and

subsequent interaction with the cytosolic GDS as a complex (263, 264). Here, phosphorylation and prenylation may work together to enhance GTPase activation.

Rap1 proteins are found predominantly at perinuclear compartments including the Golgi apparatus and late endosomes (264), whereas the primary activation site is at the plasma membrane (265). In fractionation studies of human platelets, phosphorylation has been reported to induce Rap1b translocation from membrane to cytosolic compartments (257), consistent with its cytosolic association with SmgGDS and may suggest a potential influence of C-terminal phosphorylation on Rap1 effector engagement. Indeed, phosphorylation by PKA has been reported to modulate Rap1 association with binding partners such as cytochrome b_{558} (266) and Raf-1 (267). An additional domain in Rap1a, spanning residues 85-89, has been shown to be necessary for its perinuclear localization, and may therefore regulate Rap1 localization and function in concert with C-terminal modifications (268).

In 2002, it was found that Rap1b activation and C-terminal phosphorylation are synergistic in promoting cAMP-mediated entry into G_1/S phase (269). Based on these findings, the authors proposed that C-terminal phosphorylation acts as a molecular switch to mediate Rap1 effector interactions. Recent biophysical experiments and computational modeling may now mechanistically explain the interplay between phosphorylation and activation status of Rap1b (270). Specifically, hydrogen/deuterium mass spectrometry experiments revealed that, upon phosphorylation of Ser-179, a domain around the phosphorylation site and also the regions containing the two switch loops have an increased surface exposure, supporting allosteric effects of Rap1b C-terminal phosphorylation on its effector domain. According to this model, the Rap1b polybasic domain dynamically

interacts with the negative environment of Glu-45 and Asp-47 in a β2-β3 turn, such that phosphorylation of Ser-179 within the polybasic domain presents a repulsive charge that perturbs this dynamic interaction and allows for an increase in β2-β3 turn flexibility, which can be transduced to the switch I and switch II regions. The authors proposed that C-terminal Rap1 phosphorylation behaves as an additional regulatory switch in parallel with its regulation by nucleotide binding, and furthermore that this newly proposed regulatory switch acts by allosterically discriminating between different conformational states of Rap1. This proposed mechanism by which C-terminal phosphorylation influences conformational dynamics and protein interactions within distal effector domains of GTPases supports the idea that this type of modification may have both indirect and direct effects on regulation of effector interaction. First, it may alter subcellular localization, thereby indirectly modulating regulator and effector availability, and second, it may have direct consequences to effector domain interactions with selected effector targets that were otherwise thought to be either "on" or "off" depending on whether the protein was GTP- or GDP-bound, respectively.

RalA and RalB are differentially phosphorylated

The Ras like proteins RalA and RalB share 55% sequence similarity with Ras (271). They are both geranylgeranylated, although artificially farnesylated forms can substitute functionally (272, 273). RalA (CAAX = CCIL) and RalB (CAAX = CCLL) are approximately 85% identical, with their peptide sequences varying mostly within the C-terminal HVD (271). Activation of Ral GTPases by RalGEFs, key effectors of Ras function, link them to Ras signaling (274). RalA and RalB contribute to and/or regulate a diversity of cellular functions including vesicular trafficking and exocytosis (275, 276), cellular

morphology and motility (277-280), cell survival and oncogenesis (281-286). Ral proteins are localized to both the plasma membrane and endomembranes, though the two isoforms have both overlapping and distinct subcellular localizations depending on their phosphorylation, activation status, and cellular context (285, 287, 288).

In 2005, Huang and colleagues reported that an expression screening search strategy for Aurora-A kinase-selective substrates identified RalA but not RalB as a target of Aurora-A-mediated phosphorylation (289). The basis for this isoform selectivity of the closely related Ral proteins was determined to be that a phosphorylatable serine, S194, was present within an RKSL motif found in the C-terminus of RalA but not RalB. These authors further demonstrated that phosphorylation of RalA at S194 promoted collagen I-induced cell motility and anchorage-independent growth of Aurora-A-expressing stable clones of MDCK cells, and that phosphorylation of S194 promoted RalA activation. Recently, a collaborative effort among three laboratories including the groups of Counter, Der and Cox demonstrated that phosphorylation of RalA at S194 induces both activation and translocation of RalA from the PM to endomembranes, with subsequent translocation and activation of the RhoGAP activity of the Ral effector RalBP1 (Ral binding protein-1/RLIP-76) (285). That the S194 residue is required for full transforming activity mediated by either Aurora-A or RalGEFs and in pancreatic cancer cell lines (285) is again consistent with C-terminal phosphorylation providing a strong functional complement to the nearby permanent prenyl modification of geranylgeranylation.

Interestingly, the RalA S194 residue also exists within a protein kinase A (PKA) consensus sequence and can be phosphorylated by PKA *in vitro* (33, 288), consistent with

links between PKA and RalA activation (290, 291). Thus, Aurora-A and PKA may compete to phosphorylate this site, depending on cellular context.

A study by Hahn and colleagues showed that S183 shares with S194 a common phosphatase, the tumor suppressor protein phosphatase 2A (PP2A Abeta). Phosphorylation of RalA at S183 was also shown to regulate RalA activation and function in anchorage-independent growth of HEK cells (292); however, the kinase responsible was not identified.

Not surprisingly, given their distinct HVDs, the RalA phosphorylation sites are not conserved in the RalB isoform. Instead, Theodorescu and colleagues found that, in contrast to RalA, RalB is C-terminally phosphorylated by protein kinase C (PKC), on S198 (288). Like RalA, phosphorylation of RalB induced its translocation from the PM to perinuclear regions. Furthermore, PKC-mediated phosphorylation of S198 supported anchorage-independent growth and cell motility of bladder cancer cell lines *in vitro*, and tumor growth and lung cancer cell line metastasis *in vivo*. Given the distinct isoform- and cell context-dependent roles of Ral proteins in disease, it will be of great interest to determine what rules dictate whether S198 phosphorylation is an activating or an inactivating event.

C-TERMINAL PHOSPHORYLATION OF PRENYLATED RHO FAMILY SMALL GTPASES

RhoA phosphorylation is complex and regulates its protein:protein interactions, activation, and effector binding

The role of phosphorylation in concert with prenylation to regulate Rho GTPase activity has been best characterized for the RhoA isoform, a geranylgeranylated small GTPase (CAAX = CLVL) best known for induction of stress fibers (33). *In vitro* kinase

assays and *in vivo* pharmacologic studies have shown that cAMP- and cGMP-dependent protein kinases (PKA and PKG, respectively), and Ste20-related kinase (SLK) are all capable of phosphorylating RhoA (293-298) at serine 188 in the C-terminal HVD (293, 294, 296, 297, 299). Although this phosphorylation site is only two amino acids upstream of cysteine 190 where RhoA is geranylgeranylated, the prenylation status of RhoA does not affect the ability of PKA to phosphorylate RhoA *in vitro* (293, 297). However, if RhoGDI is already bound to the prenyl group, then phosphorylation of RhoA is inhibited, presumably because of steric hindrance (297). Many studies have shown that phosphorylation of RhoA at S188 increases the binding affinity between RhoA and RhoGDI (294, 295, 297, 300, 301). This phosphorylation event has also been correlated with a variety of cellular phenotypes, all of which are consistent with increased RhoGDI binding.

For example, Rolli-Derkinderen, Pacaud and colleagues observed increased interaction between RhoGDI and phosphorylated RhoA in co-immunoprecipitation experiments (300). Further, PKG-phosphorylated and phosphomimetic RhoA S188E proteins were ubiquitinated to a lesser extent and were more stable than nonphosphorylated or phosphodeficient RhoA (300). They hypothesized that RhoGDI binding inhibits GTPase degradation, and that phosphorylation of RhoA controlled this interaction.

Consistent with the earlier findings of Hohl and colleagues (302), who determined that depletion of isoprenoid synthesis by depletion of the obligate precursor mevalonate decreased degradation of existing RhoA proteins, Pacaud and colleagues also found (300) that a non-prenylated mutant (C190A) of RhoA had a much longer half-life than prenylated RhoA.

Boulter, Burridge and colleagues found additional evidence to support the former notion while investigating the role of RhoGDI1. They noticed that knockdown of RhoGDI1 caused a significant decrease in protein but not mRNA levels of the Rho family members RhoA, Rac1, and Cdc42 (26). Further investigation revealed that the prenylated but not nonprenylated GTPases interact with Hsp90 and proteasome members. Using prenylation-free mutants (SAAX) and prenylation inhibitors (GGTIs) they showed that inhibition of Rho family GTPase (RhoA, Rac1, and Cdc42) geranylgeranylation increases protein levels of these Rho family GTPases (26), and concluded that prenylation of Rho GTPases targeted them for degradation. However, expression of RhoGDI1 could inhibit this degradation, presumably by binding to the prenyl moiety, preventing misfolding, and shielding the GTPase from its proteasome fate.

Phosphorylation of RhoA at S188 increases the amount of cytosolic or "free" RhoA and decreases membrane bound RhoA (295, 297, 300, 301, 303). Bertoglio and colleagues showed that this membrane extraction occurred only in the presence of RhoGDI (297). The effect of nucleotide (GDP vs. GTP) on GDI/GTPase interactions may depend on ionic conditions within a cell or *in vitro* system (295). However, it is generally thought that GDP-RhoA binds RhoGDI with higher affinity than GTP-RhoA (297, 304). PKA does not show preference for GTP- or GDP-bound RhoA and phosphorylates each equally (295). However, upon phosphorylation of RhoA, the binding affinity of GTP-RhoA for GDI is more strikingly increased than that of GDP-RhoA for GDI (297). This led Lang et al. to hypothesize that when active GTP-RhoA bound to the plasma membrane is phosphorylated by PKA, RhoGDI can then interact with RhoA as it couldn't previously, and extract it from the plasma membrane into the cytosol. Forget et al. have since shown that under certain ionic conditions

phosphorylation of GDP-RhoA can greatly enhance binding of GDP-RhoA to GDI as well (295).

As would be expected of a stimulus that extracts active RhoA from the plasma membrane, phosphorylation of RhoA also attenuates its downstream signaling. For example, activation of PKA and PKG impairs the ability of RhoA to induce stress fiber formation, a key biological activity of this canonical small GTPase (293, 297, 298, 305). Forskolin, which activates adenylate cyclase to increase production of cAMP and activate PKA, causes a marked decrease in the stress fibers induced by overexpression of constitutively active RhoA (G14V), but not if RhoA is nonphosphorylatable (G14V, S188A) (293, 294). These results suggest that phosphorylation of S188 is essential for these kinases to inhibit RhoA signaling to stress fiber formation, and that blocking the phosphorylation step impairs the ability of Rho to regulate this key downstream function. Expression of S188 phosphodeficient (S188A) and phosphomimetic (S188E) mutants in a variety of cell types confirms that this is the case in several RhoA-driven biological endpoints. For example, phosphodeficient RhoA S188A is more effective at preventing neurite outgrowth (299), slowing cell spreading (294) and inducing cell rounding (306) than RhoA that can be phosphorylated. Similarly, in mouse aorta rings, inhibition of RhoA causes smooth muscle relaxation, resulting in vasodilatation (296). This smooth muscle relaxation is mediated by SLK, presumably through its phosphorylation and inhibition of RhoA (296). Interestingly, PKG-mediated inhibition of SRF expression acts downstream of Rho phosphorylation (307), suggesting that PKG regulates RhoA function at multiple levels.

The interaction between RhoA and its key effector Rho-associated kinase (ROK or ROCK) is decreased upon RhoA phosphorylation, as shown by pulldown assays (299), and

RhoA phosphorylation is also inversely correlated with signaling to its downstream effector phospholipase D (PLD) (303, 308). However, not all effects of RhoA phosphorylation can be attributed to increased binding to RhoGDI leading to decreased signaling. RhoA expressed in bacteria (i.e. nonprenylated) and phosphorylated *in vitro* bound less efficiently to its downstream effector ROK *in vitro* than did nonphosphorylated RhoA (293). Since this was done without any RhoGDI present, there must be something intrinsic about RhoA phosphorylation that affects RhoA interactions with at least some of its downstream effectors. Also, RhoA phosphorylation does not affect its interaction with other effectors such as Rhotekin, mDia-1 or PKN, as examined by pulldown assays in neuronal PC12 cells (299). It is currently unclear where this specificity arises. It is clear, however, that RhoA phosphorylation does NOT affect the ability of RhoA to bind nucleotides or its intrinsic GAP activity (297). Nor does phosphorylation of RhoA affect the ability of positive regulatory GEFs, negative regulatory GAPs, or GGTase I prenyltransferase to act on RhoA *in vitro* (294).

In summary, PKA and PKG agonists stimulate these kinases to phosphorylate RhoA on S188. This phosphorylation event triggers increased RhoGDI binding to RhoA, extracting RhoA from the plasma membrane and relocating it to the cytosol where it is sequestered from downstream effectors. Yet, phosphorylated RhoA is also protected from degradation, and is likely in a GTP-bound state. Thus phosphorylated RhoA constitutes a pool of RhoA primed and ready for signaling upon release by RhoGDI. This release would presumably be triggered by a phosphatase or GDI displacement factor (GDF) that has yet to be identified for Rho family GDIs. Alternatively, phosphorylation of Rho GDI itself has been shown to

trigger release of Rho family GTPases (55). Additionally, phosphorylation of RhoA seems to intrinsically regulate downstream effector binding, by a currently unknown mechanism.

RhoB phosphorylation inhibits its function

Although RhoA and RhoB utilize a partially overlapping set of effectors, including Rhotekin and mDia1/2, and are both capable of inducing stress fibers, many of their functions differ greatly (28, 309). This is most apparent from the fact that RhoA is an oncogene and RhoB is generally thought of as a tumor suppressor (28). RhoB is expressed much more transiently than RhoA and is found at membranes (PM and endosomes) even at resting state, while RhoA is generally cytoplasmic and GDI-bound in resting cells and translocates to the plasma membrane upon stimulation (310). The amino acid sequence of RhoB is approximately 85% identical to that of RhoA, but their strikingly different localization and function can be attributed to their distinct C-terminal hypervariable domains. RhoA is targeted to membranes by geranylgeranylation and a series of basic residues in its C-terminus, whereas RhoB (CAAX = CCKVL) can be farnesylated or geranylated, and is palmitoylated (28).

Like RhoA, RhoB is also phosphorylated within its hypervariable domain (311). In 2008, Pradines and colleagues showed that CK1 is capable of phosphorylating RhoB *in vitro* and *in cellulo* at serine 185 (311). This phosphorylation was independent of the nucleotide bound to RhoB, because both constitutively active (G14V) and wild type RhoB were phosphorylated to similar extents (311). It is currently unknown how this phosphorylation relates to RhoB prenylation and/or palmitoylation, localization, and stability. However, prevention of RhoB phosphorylation with CK1 inhibitors or a phosphomimetic mutant

(S185A) caused greater stress fiber formation, enhanced EGFR internalization, and increased binding to Rhotekin-RBD (311). All of these phenotypes indicate that, as in RhoA, the hypervariable domain phosphorylation of RhoB inhibits RhoB function. The mechanism of this inhibition remains to be explored. While it is tempting to speculate that phosphorylation of RhoB increases its affinity for GDI in a mechanism similar to RhoA, there are other variables to take into account with RhoB. First, palmitoylation has been described to inhibit RhoGDI binding (57). Thus, depalmitoylation in addition to phosphorylation may be required to observe enhanced GDI binding. Reports on interactions between RhoB and GDI are conflicting (55), and it may be that the differing results were attained based on conditions suitable for palmitoylation/phosphorylation or not.

RhoG – phosphorylated in vitro only?

RhoG (CAAX = CILL) has the highest sequence similarity to Rac1 (72%) and Cdc42 (62%) and also shares activators, effectors, and biological functions with these proteins (312). Unlike Rac1, RhoG may be phosphorylated in its hypervariable domain. PKA was capable of phosphorylating RhoG, but not RhoG S187A, *in vitro* (294). However, it is unknown if this phosphorylation event occurs in cells, or, if so, what are the functional consequences.

RhoE/Rnd3 – phosphorylated everywhere?

Rnd3 (also known as RhoE or Rho6) terminates in a CTVM motif and is therefore farnesylated rather than geranylgeranylated like canonical Rho GTPases (313). It is also atypical in that it does not have the conventional residues at positions 12, 59 and 61 that regulate GTP/GDP cycling, and as such it lacks intrinsic GTPase activity and is

constitutively active (313). Therefore other mechanisms of regulating its activity/signaling must be employed. It has been suggested that this control occurs through phosphorylation. Rnd3/RhoE can be phosphorylated by both ROCKI and PKCα (314, 315). Mass spectrometry analysis of ^{32}P labeled RhoE, revealed that Rnd3/RhoE is phosphorylated in both its extreme N- and C- termini (315). Mutation of all seven serine/threonine residues in these regions (S7, 11, 210, 218, 222, 240; T214) prevented *in vitro* phosphorylation by ROCK1, but if any one was left intact it was still phosphorylated (315), suggesting that any of these residues can be phosphorylated by ROCK1. Of particular interest is S240, which has very similar positioning to S188 in RhoA, in that it is immediately adjacent to the cysteine (C241) on which the GTPase is prenylated. Ridley and colleagues hypothesized that phosphorylation at this site inhibits insertion of the farnesyl group into membranes (316), as has been hypothesized for RhoA phosphorylation (317). However, further investigation by our laboratory revealed that many of the functional roles of this phosphorylation are independent of S240 (314). Interestingly, we also noted that a Cys to Ser (SAAX) mutant, that cannot be prenylated, does not show a mobility shift upon activation of PKCα (314). One possible explanation for this is that phosphorylation requires that Rnd3/RhoE be prenylated, perhaps to direct it to the plasma membrane where it can interact with PKCα.

Phosphorylation of Rnd3/RhoE induces its internalization from the plasma membrane to internal membranes and the cytosol. Phosphorylation of RhoE also increased protein stability of the GTPase (314, 315). Thus, one may think that, like RhoA, Rnd3/RhoE phosphorylation increases its binding to GDI. RhoGDI binding normally attenuates GTPase downstream signaling, however, there are currently conflicting reports as to how RhoE phosphorylation affects downstream signaling. Rnd3/RhoE inhibits RhoA-ROCK signaling,

stress fiber formation and Ras-induced transformation (318). Despite the report that phosphorylation of RhoE does not alter downstream effector binding with ROCKI or p190-RhoGAP in pulldown assays (315), *in cellulo* S11 phosphorylation was necessary for increased stress fiber disassembly and inhibition of Ras-induced transformation, which are mediated by RhoE (315). Therefore the requirement for phosphorylation for RhoE activity in this study was presumably based on altered localization and not a change in intrinsic ability to bind effectors. Results in our laboratory suggest that phosphorylated RhoE is actually inactive, and that RhoE phosphorylation promotes greater stress fiber formation and phosphorylation of MYPT1 (which are normally inhibited by Rnd3/RhoE) (314). Some of the biological assays were carried out with an "all A" mutant in which S-7, 11, 220, 222, 240 and T-214 were all mutated to alanines, while others focused only on S11. It is therefore conceivable that phosphorylation of S11 alone activates Rnd3/RhoE, while phosphorylation of multiple residues has a combined inhibitory effect, or may be a cell context-dependent issue (HeLa versus NIH 3T3 cells). This question will require further experimentation to resolve.

Wrch-1/RhoU phosphorylation – an unusual palmitate / tyrosine pair

Wrch-1/RhoU is an atypical Rho family GTPase of the Cdc42 branch (57% sequence identity to Cdc42) but with additional N- and C-terminal extensions (319). Wrch-1 terminates in a CCFV motif, which upon first inspection appears to be a permanently prenylated CAAX motif but is not; rather, it specifies reversible palmitoylation of the second cysteine (CXX motif) (320). This palmitoylation step is necessary for membrane localization of Wrch-1, thereby serving a function similar to prenylation of other small GTPases. Since

Wrch-1 is not prenylated, it does not interact with RhoGDI. However, we have shown that phosphorylation of Wrch-1 within its HVD near the lipidated cysteine does drive changes in both protein localization and function (321, 322). This indicates that hypervariable domain phosphorylation has both prenylation-dependent and -independent effects on Rho family activity.

Unlike other phosphorylated Ras and Rho small GTPases identified to date, Wrch-1 is phosphorylated not on a serine or threonine but on a tyrosine residue, at position 254, two amino acids upstream of the palmitoylation site (321). The tyrosine kinase Src is essential for this modification. Few other small GTPases possess tyrosines in their HVDs at all, and, of those that do (K-Ras4A, N-Ras, Rap2A/B/C, Rheb2, RhoB, RhoBTB3) none is in such proximity to a lipidation site. Whether any of these tyrosines is phosphorylated has not been determined. Thus, Wrch-1 may be regulated uniquely.

As in these other GTPases, phosphorylation induces internalization of Wrch-1 from the plasma membrane to internal membranes (321), which negatively regulates its activity. Consistent with this, stimulation of Wrch-1 phosphorylation decreases its binding to and activation of its effectors PAK and Pyk-2, and phosphomimetic Wrch-1 (Y254E) displays decreased binding to GTP as shown by pulldown assays compared with wild type and phosphodeficient Wrch-1 (Y254F). Phosphodeficient Wrch-1 (Y254F) can recruit its downstream effector PAK to the plasma membrane, whereas phosphomimetic Wrch-1 (Y254E) cannot do the same to endosomes (321). However, if Wrch-1 is mutated to be GAP-insensitive (i.e., Q107L), then even phosphomimetic Wrch-1 (Y254E, Q107L) can recruit PAK to endosomes (321). This implies that there is an unidentified GAP that turns off Wrch-1 at endosomes.

TC10/RhoQ – a target for phosphorylation by CDK-5

TC10/RhoQ is also a member of the Cdc42 branch of the Rho family. The two proteins share many downstream effectors and both induce filopodia formation, yet their posttranslational modifications are distinct (28). Cdc42 is directed to endomembranes by a combination of geranygeranylation of the cysteine in its CAAX motif (CVLL) and an upstream polybasic sequence. TC10 is more complicated: it can be farnesylated or geranylgeranylated (CAAX = CCLIT), and both contains a polybasic domain and also becomes palmitoylated (C209 and/or C206). TC10 is best known for regulating GLUT4 transport in response to insulin stimulation. Under these conditions, TC10 can be phosphorylated by CDK-5 (323) on threonine 197, which is just upstream of the polybasic region of TC10 but is farther from the CAAX motif cysteine (C210) than other known phosphorylation sites described in this chapter. Still, this phosphorylation event has a dramatic effect on the localization and function of TC10.

TC10 normally congregates specifically at lipid rafts within the plasma membrane, whereas phosphodeficient TC10 (T197A) does not (323). This suggests that phosphorylation of TC10 positively regulates its association with lipid rafts. Using pulldown assays to measure TC10 activation, Mori and colleagues observed a lower basal level of activation with the phosphodeficient mutant (T197A) and a higher level with the phosphomimetic mutant (T197D) than wild type TC10. Insulin could stimulate activation of both the wild type and phosphodeficient mutant TC10, but the phosphomimetic mutant appeared to already be maximally activated. Also, the phosphodeficient mutant always showed lower levels of GTP binding than the corresponding wild type TC10 (323). These data all suggest that phosphorylation of TC10 is necessary for proper localization and activation, and biological

assays confirmed this (323). It is still unknown how phosphorylation triggers association of TC10 with lipid rafts and its subsequent activation.

Cdc42 – phosphorylated elsewhere, to regulate GDI binding

Cdc42, canonically known for its induction of filopodia, is a substrate for GGTase I, with the major Cdc42 isoform terminating in CVLL (1). While there is a putative PKA phosphorylation site three amino acids upstream of this geranygeranylated CAAX motif, whether this site is phosphorylated *in vivo* is less clear (317). Béliveau and colleagues showed phosphorylation of Cdc42 by PKA *in vitro* using autoradiography (295). Further, *in vitro* stimulation with PKA enhanced Cdc42 binding to GDI and removal of Cdc42 from isolated rat membranes (295). These observations implied that Cdc42 and RhoA might be regulated similarly by C-terminal PKA phosphorylation of the small GTPase. However, they did not test whether PKA directly phosphorylated Cdc42 in cells.

Arguing against this possibility, phosphorylation of Cdc42 by PKA was not observed in NIH 3T3 or HeLa cells, respectively (294, 305) under conditions where RhoA was robustly phosphorylated. And while PKA could phosphorylate Cdc42 *in vitro* (324), it was minimal compared to RhoA phosphorylation. Further, although stimulation of PKA, for example with 8-Br-cAMP, or transient expression of the catalytic subunit of PKA caused Cdc42 activation in both CHO-K1 and COS-7 cells (324), phosphorylation of Cdc42 was not observed under these conditions. Together, these results suggest that PKA phosphorylation does not occur directly on Cdc42 itself, but rather on its upstream regulator(s), either to activate a GEF or to impair interactions with RhoGDI.

Cdc42 is less controversially phosphorylated on tyrosine 64 by Src (325). This internal phosphorylation event enhances binding of Cdc42 with GDI (325). Since GDIs bind Rho GTPases both at the GTPase switch regions and the C-terminus/attached prenyl group (53), it is logical that phosphorylation on both of these residues would affect GDI binding. Interestingly, Y64 is in the switch II region and is conserved in most Rho family members, including all those discussed in this review. It would be interesting to determine if phosphorylation of this conserved residue affects GDI binding of other GTPases as well.

Rac1 – also phosphorylated elsewhere

Rac1, best known for its ability to induce lamellipodia formation and as an activator of NADPH oxidase, terminates in CLLL and, like all canonical Rho GTPases, is geranylgeranylated. It also possesses a polybasic region in its HVD, but lacks any serine, threonine, or tyrosine residues in that region and thus cannot be phosphorylated there. Rac1 is phosphorylated by Akt on serine 71, a modification that inhibits GTP binding *in vitro* and *in vivo* (326). Unlike the typical C-terminal phosphorylation, S71 phosphorylation status has not been correlated with changes in localization, and appears instead to regulate intrinsic capabilities of the GTPase.

C-TERMNAL PHOSPHORYLATION OF PRENYLATED RAB FAMILY SMALL GTPASES

Rab4 is a monomeric small GTPase of the Rab family that is associated during interphase with early endosomes and that plays a key role in endocytosis of such physiologically important molecules as GLUT4, integrins and angiotensin (327). Like other

Rab proteins, Rab4 is geranylgeranylated (CXC = CGC) by GGTase II (328). Unlike other Rabs, Rab4 is the only Rab protein to our knowledge that has been reported to be phosphorylated near the prenyl group, the latter of which is absolutely required for its function. Rab4 is a target of the mitotic kinase Cdc2, which phosphorylates it on S196 during mitosis (329), causing it to bind to the peptidyl-prolyl isomerase Pin-1 (330), become disassociated from endosomes and to accumulate in the cytosol (331). In that location, the phosphorylated Rab4 can no longer continue to promote endocytotic trafficking as it does during interphase, thereby allowing proper apportioning of vesicular structures and their contents to the daughter cells that form as cells divide. Which properties of Rab4 that make it apparently unique with regard to this particular combination of posttranslational modifications on Rab GTPases remain to be determined.

CONCLUSIONS

Prenylation of the membrane targeting sequences of small GTPases is necessary to anchor them to specific subcellular compartments and subdomains, where they can then interact with specific subsets of their upstream regulators and downstream effectors to elicit their distinct biological functions in response to particular environmental and contextual cues. However, prenylation is both irreversible and insufficient to promote correct membrane binding, and at least some prenylated small GTPases are unstable and targeted for degradation unless additionally modified. Thus, other more dynamic modifications are necessary for precise spatiotemporal control of GTPase localization and function. In this chapter, we have focused on the continually emerging role of C-terminal phosphorylation in GTPase regulation.

Phosphorylation of prenylated proteins has multiple consequences, the most prominent of which is to translocate them from the plasma membrane to a variety of internal membranes, where they are typically downregulated and/or altered in their biological functions. Downregulation of activity can occur by enhanced protein:protein interactions with cytosolic chaperones, such as RhoA with RhoGDI, to both increase cytosolic localization and decrease GTP-binding, or by enhanced protein:protein interactions with membrane-bound negative regulators restricted to specific compartments, such as Wrch-1/RhoU with an endosomally localized GAP. C-terminal phosphorylation can also fine-tune small GTPase localization, such as the phosphorylation of TC10/RhoQ that does not extract TC10 from the plasma membrane but rather directs it specifically to lipid rafts within that membrane compartment. Alterations in function can be as simple (but necessary) as an on/off switch, such as that seen with phosphorylated Rab4 no longer driving endocytosis during mitosis, or as dramatic as the conversion of K-Ras4B from a growth-promoting to a death-inducing protein. Finally, given that phosphomimetic and/or phosphodeficient mutants interfere with their function, it is likely that at least some of these C-terminally phosphorylated and prenylated small GTPases will turn out to require cycling of their phosphorylation / dephosphorylation states, just as they do cycling of their GDP-/GTP-bound states. Thus, hypervariable domain phosphorylation of the Ras superfamily of small GTPases, in conjunction with nearby prenylation, provides additional and critical regulatory elements to their subcellular localization and functions, whose precise mechanisms are still being unraveled. It will be interesting to explore how phosphorylation of specific residues is spatiotemporally coordinated with other dynamic posttranslational modifications at the same or adjacent sites.

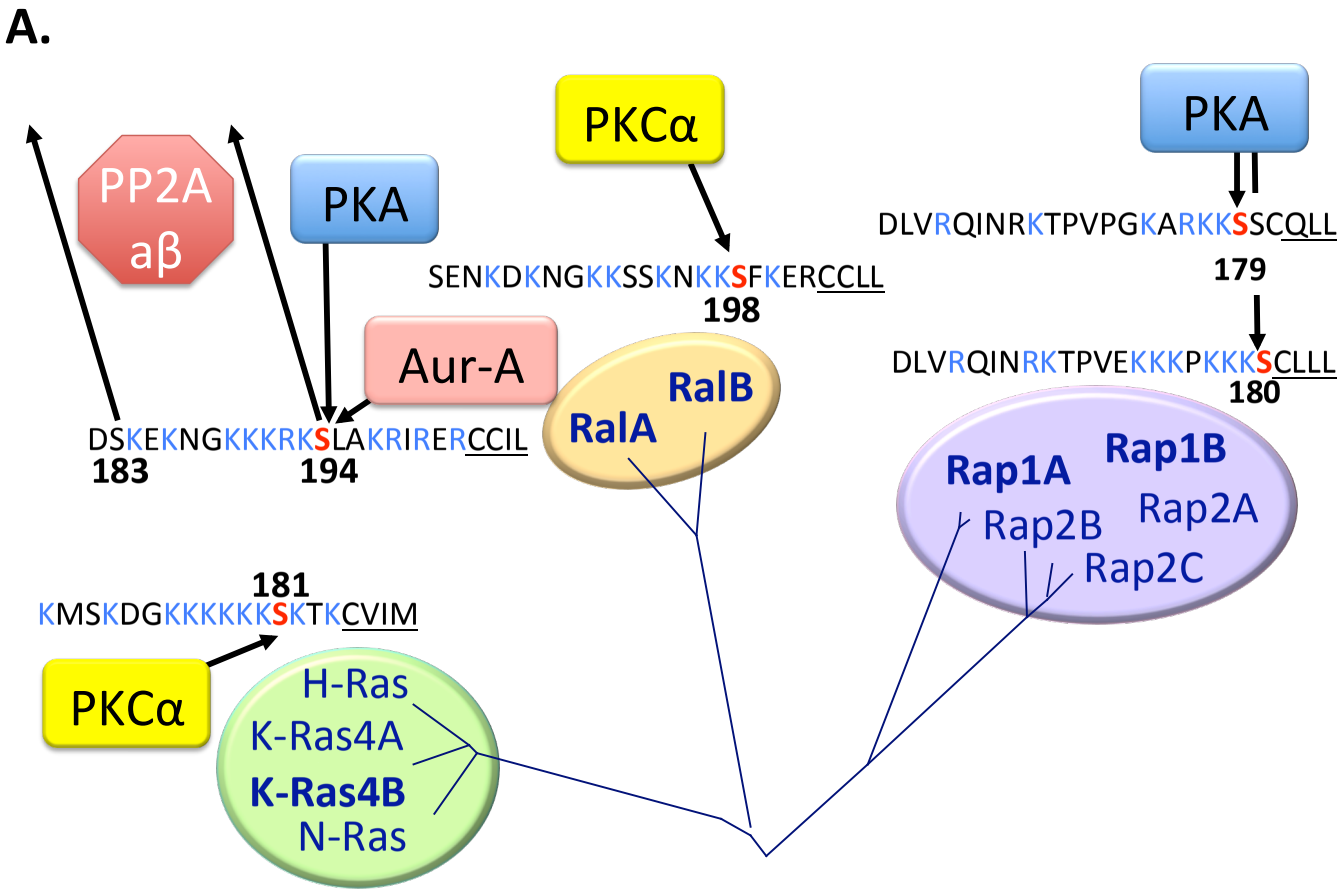
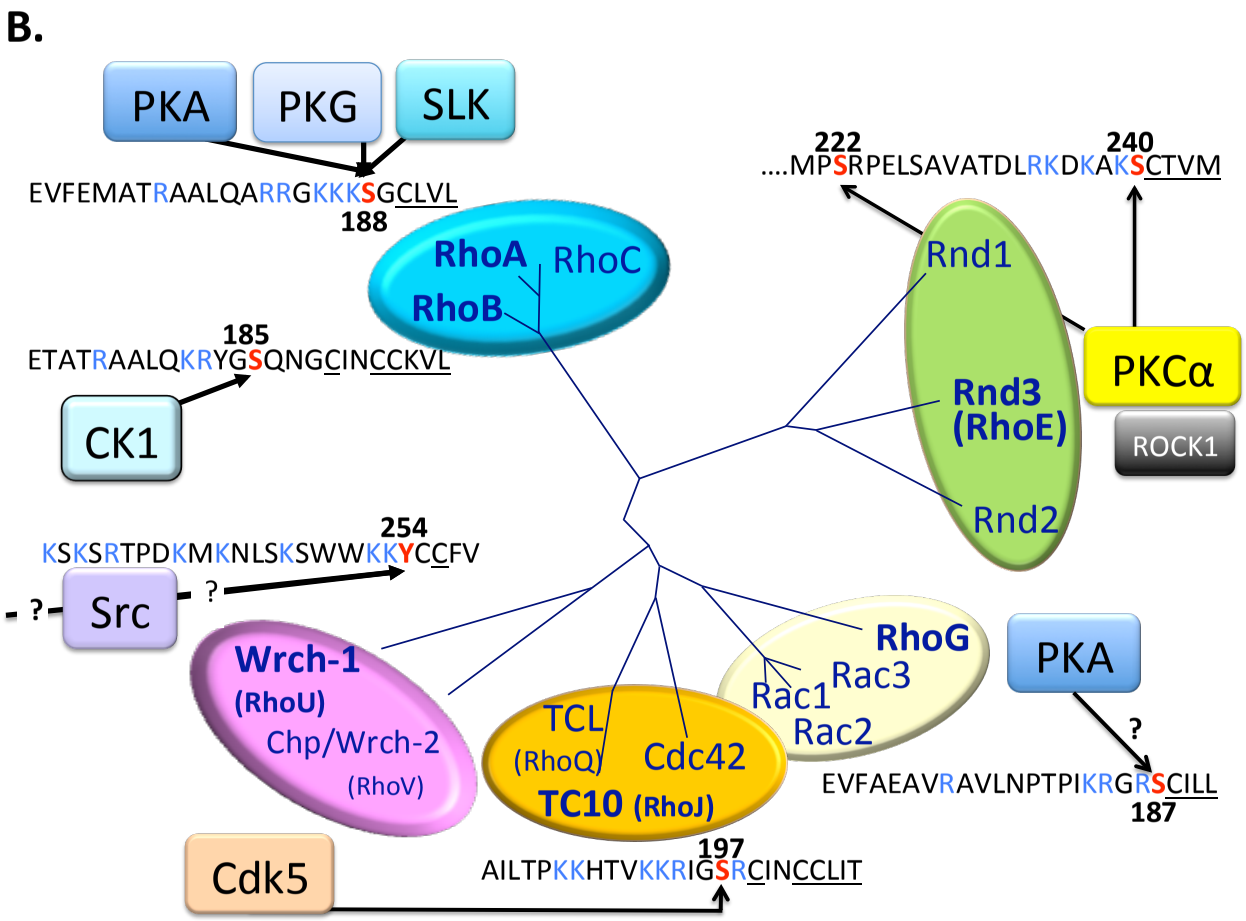


Figure. 2.1. Posttranslational modifications of Ras superfamily small GTPases in which both prenylation and C-terminal phosphorylation have been demonstrated. The sequences of the hypervariable (membrane-targeting) domain (HVD) of each such *(A)* Ras and (*B*) Rho GTPase are shown. Known phosphorylation sites are bolded and shown in red; numbers are amino acid residues. Basic residues (with a positive charge) are shown in blue. Responsible kinases are indicated. S = serine, T = threonine, Y = tyrosine.



The sequences of the hypervariable (membrane-targeting) domain (HVD) of each such *(A)* Ras and (*B*) Rho GTPase are shown. Known phosphorylation sites are bolded and shown in red; numbers are amino acid residues. Basic residues (with a positive charge) are shown in blue. Responsible kinases are indicated. S = serine, T = threonine, Y = tyrosine.

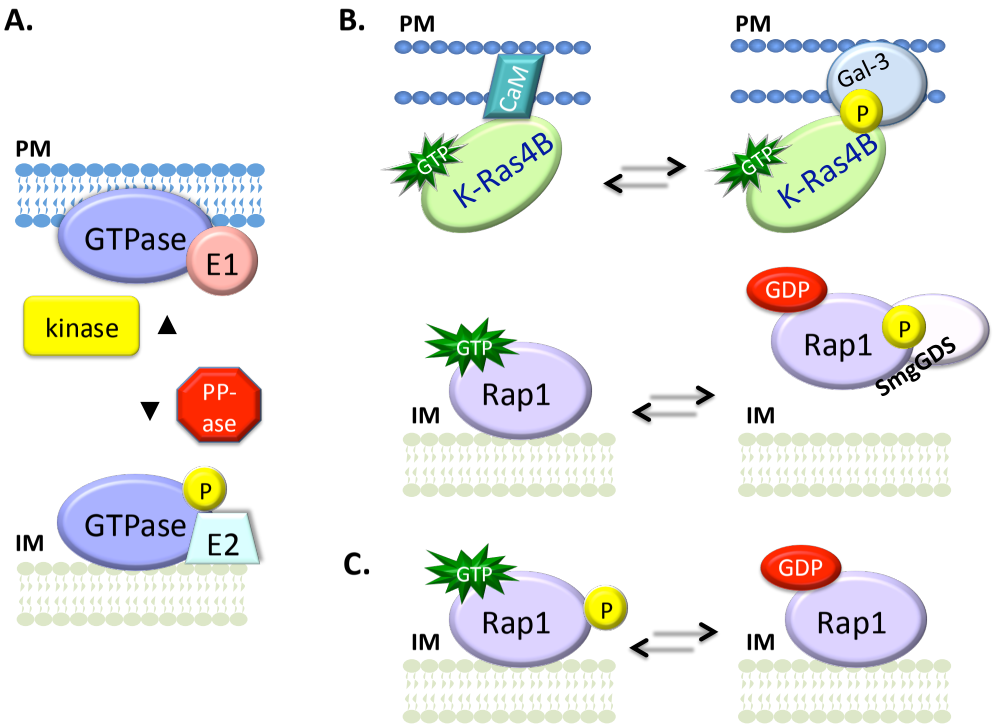


Figure. 2.2. C-terminal phosphorylation of prenylated small GTPases alters subcellular localization and protein:protein interactions, with consequences for activation state, effector interactions and signaling activities. *A.* Changes in subcellular localization can alter protein:protein interactions by exposing the small GTPase to distinct pools of available interacting partners, including both regulators (GEFs, GAPs) and effectors ("E1", "E2"). *B.* Altered interaction with chaperones (e.g., RhoGDI, galectin-3, calmodulin) or with regulatory molecules (RhoGDI, SmgGDS), whether due to physical characteristics of phosphorylated versus nonphosphorylated proteins, or due to competition for binding at the site of phosphorylation, can drive changes in GTP-binding activation state. *C.* Allosteric effects of phosphorylation on the switch regions that change conformation when bound to GDP or GTP can also promote changes in activation state or effector interactions. PM = plasma membrane; IM = internal membrane.

CHAPTER 3: SUBCELLULAR LOCALIZATION OF RAS AND RHO GTPASE ACTIVITIES AS DETERMINED BY EFFECTOR RECRUITMENT ASSAYS[2]

OVERVIEW

Ras and Rho family GTPases control a wide variety of cellular processes, and the signaling downstream of these GTPases is influenced by their subcellular localization when activated. Since only a minority of total cellular GTPases is active, observation of the total subcellular distribution of GTPases does not reveal where active GTPases are localized. In this chapter, we describe the use of effector recruitment assays to monitor the subcellular localization of active Ras and Rho family GTPases. The recruitment assay relies on preferential binding of downstream effectors to active GTPases versus inactive GTPases. Tagging the GTPase binding domain of a downstream effector with a fluorescent protein produces a probe that is recruited to compartments where GTPases are active. We describe an example of a recruitment assay using the GBD of PAK1 to monitor Rac1 activity and explain how the assay can be expanded to determine the subcellular localization of activation of other GTPases.

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[2]Reproduced with permission, from a chapter of the same title, in Methods in Molecular Biology, Ras Signaling, edited by Lorenza Trabalzini and Saverio Francesco Retta, and published by Springer. The author list is as follows: Huff LP, DeCristo MJ, and Cox AD. I wrote the chapter, collected the data for the tables, and generated the figure. Molly J. DeCristo helped with data collection for the tables and edited the chapter. Adrienne D. Cox edited the chapter.

INTRODUCTION

The Ras and Rho families of small GTPases are comprised of 55 family members that act as cellular signaling nodes (2). Numerous extracellular signals converge on these GTPases, which then regulate diverse downstream signaling cascades. The subcellular localization of GTPase activation strongly influences its specific outcomes. For example, when K-Ras4B is active at the plasma membrane, it signals for cell survival, but when it is active at internal membranes such as endoplasmic reticulum and mitochondria, it signals for cell death (239). However, the predominant subcellular localization of a GTPase does not necessarily correlate with where that GTPase is active (332) (see Table 3.1). A given stimulus may lead to activation of less than 10% or as much as 40% of the available cellular Ras (333) or Rho (50, 51). Therefore, typical observation of GTPase distribution (via immunofluorescence or fractionation) primarily detects the inactive pools of that GTPase, and alternative techniques must be utilized to determine where a GTPase is active. In this chapter we describe the use of effector recruitment assays to monitor the subcellular localization of active Ras and Rho family small GTPases.

Ras and Rho family GTPases are active when GTP-bound and inactive when GDP-bound. Guanine nucleotide exchange factors (GEFs) catalyze exchange of GDP for GTP to activate GTPases, and GTPase activating proteins (GAPs) catalyze GTP hydrolysis to inactivate GTPases. Effector molecules that transmit GTPase signals downstream preferentially bind to active, GTP-bound GTPases. For example, Raf-1, a key downstream effector of Ras, has a 1,000-10,000 fold higher affinity for Ras-GTP than for Ras-GDP (334, 335). Each GTPase signals to an array of disparate downstream effectors, each of which contains a GTPase binding domain (GBD) that is responsible for this preferential interaction

with the active GTPase. For instance, Ras downstream effectors contain Ras binding domains (RBD) or Ras association domains (RA), and downstream effectors of Rac, a Rho family GTPase, contain either p21 binding domains (PBD) or Cdc42 and Rac interactive binding (CRIB) domains. Many of these domains show little sequence identity, but they have a common structure and can be identified via databases such as SMART (the online Simple Modular Architecture Research Tool [http://smart.embl-heidelberg.de/]). Other downstream effectors (and GBDs) have only been identified empirically. For example, residues 292-362 of POSH have been shown to preferentially bind to Rac-GTP, so this is considered the PBD even though it shows little similarity to that of other downstream effectors of Rac (336). Notably, each downstream effector shows varying degrees of specificity among GTPases (Table 3.2). POSH is thought to be specific to Rac1 and does not bind other Rho GTPases tested (336), whereas mDia2 is a downstream effector for many Rho GTPases (RhoA, RhoB, RhoC, RhoD, RhoF, Rac1, Cdc42) (337-339).

In an effector recruitment assay, the GBD of a downstream effector is tagged with a fluorescent molecule such as GFP. This fluorescent probe is recruited to locations where there are active GTPases that the GBD binds. Thus, one can monitor changes in the subcellular localization of active GTPases due to different stimuli and also determine if specific subcellular pools of a GTPase are active or inactive. This method is similar to the commonly used biochemical pulldowns for active GTPases, in which GBD sequences are expressed in bacteria, bound to solid support such as Sepharose or agarose beads, and used to retrieve active GTPases from cellular lysates (340, 341). Both assays rely on the preferential binding of GBD to active GTPases over inactive GTPases. Table 3.2 compares the use of probes in each assay. In pulldown assays, GBD specificity for GTPases is less important,

because the pulldown is followed by immunoblotting with an antibody specific for the desired GTPase. However, in localization recruitment assays, identification of the GTPase which is responsible for recruitment of the GBD is much more heavily dependent on probe choice and experimental design (see Notes 2, 4, 5).

Other assays have been created to observe changes in the subcellular localization of active GTPases, including FRET-based biosensors (332, 342) and fractionation followed by GBD pulldowns (343). Each assay has advantages and disadvantages. Although FRET-based biosensors can be highly sensitive and are GTPase-specific, their use requires amenable cell conditions, sophisticated microscopy and time-intensive imaging and processing. These considerations limit the number of cells that can be observed in a single assay, and thereby limit throughput of an overall population. In addition, FRET-based biosensors generally require exogenous expression of the GTPase, whereas recruitment assays can often be used to monitor changes in endogenous GTPase activation (265, 344, 345). Like recruitment assays, fractionation-pulldown assays can measure changes in endogenous GTPase activation and do not require as specific a probe as recruitment assays, but they are also technically challenging. Additionally, hydrolysis of GTP into GDP during the hours it takes to fractionate the cells can lower the signal-to-noise ratio. Finally, recruitment assays yield better spatiotemporal resolution than fractionation-pulldowns, as recruitment assays can be used to observe more transient GTPase activation and to observe active pools of GTPases in microdomains within the cell that may be difficult to isolate by a biochemical fractionation protocol. Recruitment assays also have disadvantages, however. Negative results can be related to a variety of factors (see Notes 5 & 6) and therefore can be hard to interpret, and certain GTPases are easier to monitor with available probes than others

(see Note 5 & Table 3.2). Additionally, quantification of this assay can be challenging (see Note 17). However, many of these issues can be attenuated with proper experimental setup, as described in the Notes section.

In the Methods section of this chapter, we describe a method whereby a fluorescent GBD probe is used to reveal the subcellular localization of an active Ras or Rho small GTPase. We illustrate this method with an example in which a GFP-PAK1-PBD probe shows the location of active Rac1 (Fig. 3.1). In this example, an exogenously expressed Rac1 mutant contains two mutations, one to make it constitutively active (Q61L), and an additional mutation (C189S) at a residue that has been previously described to cause nuclear accumulation of Rac1 (62). PAK1 is a key effector of Rac1 (346). In resting cells, GFP-PAK1-PBD is not localized to specific subcellular compartments but is distributed diffusely throughout the nucleus and cytoplasm (Fig. 3.1). In contrast, in cells expressing the constitutively active, nuclearly localized Rac1 mutant, GFP-PAK1-PBD highlights the nucleus. Images of these two groups of cells are shown as a striking example of GBD recruitment by the presence of an active GTPase, in this case, to the cell nucleus. In the Notes section and Table 3.2 we describe variations on this technique that enable it to be used to observe the location of active forms of other Ras and Rho family small GTPases, both exogenous and endogenous. We have previously utilized this method to examine the subcellular localization of active Wrch-1 (321) and Ras (347-349), and recently used it to determine spatially regulated activation of Rho family GTPases by the RhoGEF Ect2 (see Chapter 4).

MATERIALS

(Listed in order of use. Indicated manufacturers are examples and can be substituted unless otherwise stated.)

Plating and transfecting cells

1. Coverslips (Fisherbrand, microscope cover glass 12 mm circles, 0.13-0.17 mm thick)
2. 70% ethanol
3. Tissue culture hood, equipped with a vacuum flask and attached aspirator
4. Pasteur pipets, autoclaved for sterility
5. Dulbecco's phosphate buffered saline (DPBS 1x with calcium chloride and magnesium chloride, GIBCO/Invitrogen)
6. 10 cm tissue culture plates and 12-well plates (Corning, sterile polystyrene dishes)
7. Fine-tip tweezers
8. Fibronectin (Fisher): reconstitute in DPBS to a concentration of 2.5 μg/ml, and filter-sterilize using a 0.2 micron filter. This solution can be stored at 4°C for many months.
9. RPMI-1640 cell culture medium (GIBCO, RPMI-1640 1x with L-glutamine) or other culture medium appropriate for cells of interest
10. Fetal bovine serum (FBS, Sigma): mix 1 volume of FBS with 9 volumes of RPMI-1640 to produce complete RPMI growth media
11. Tissue culture incubator: set to 37°C and 5% CO_2, or appropriate conditions for cells of interest
12. Trypsin (0.05% trypsin with EDTA, GIBCO)

13. Expression plasmids for proteins of interest, including the fluorescent GBD probe (here we used pEGFP-C1-PAK1-PBD, a.a. 75-118 [see Table 3.2]; pCGN-HA empty vector control and pCGN-HA-Rac1-Q61L-C189S)
14. Polystyrene 5 ml round bottom tubes (BD-Falcon)
15. Transfection reagent (TransIT-LTI, Mirus)

Fixing and staining cells

1. 10x phosphate buffered saline solution (PBS: 1370 mM NaCl, 100 mM Na_2HPO_4, 18 mM KH_2PO_4, 27 mM KCl in ddH_2O, pH to 7.4); mix 9 volumes of distilled water with 1 volume of 10x PBS for 1x working solution
2. Paraformaldehyde (PFA, Electron Microscopy Services, 20% EM grade PFA solution): dilute to working solution of 4% PFA using 1 volume of 10x PBS, 2 volumes 20% PFA, 7 volumes ddH_2O
3. Triton-X-100 (Triton-X-100 for molecular biology, Sigma)
4. Parafilm M or similar wrap (Sigma)
5. Anti-HA antibody (Covance, HA.11 monoclonal antibody clone 16B12 mouse; this is the best validated antibody available for this purpose, and we highly encourage its use); other secondary antibodies may be appropriate depending on the epitope-tag or primary antibody used to detect the GTPase of interest
6. Alexa Fluor® 594 or similar fluorescent anti-mouse antibody that works well in contrast to green GFP color (Alexa Fluor® 594 donkey anti-mouse IgG H+L, Invitrogen)
7. Double distilled H2O (ddH20)

8. Lab wipes (Kimwipes, Kimtech Science)
9. Mounting medium with DNA stain such as 4',*6*-diamidino-2-phenylindole, DAPI (Vectashield mounting medium for fluorescence with DAPI, Vector Laboratories)

Imaging and data analysis

1. Fluorescent confocal microscope, equipped with appropriate filters for the secondary antibodies used, and with a camera (we use a Zeiss LSM710 Spectral Confocal Laser Scanning Microscope, fitted with Plan-Apo 40x, 1.3 NA and 63x, 1.4 NA oil objectives, using Zen software, see Note 19).
2. Push-button manual cell counter (Veeder-Root)

METHODS

Plating and transfecting cells

Perform in a tissue culture hood, using sterile technique.

1. Place coverslips in a 10 cm plate.
2. Sterilize the coverslips by covering them completely with 70% ethanol.
3. Aspirate the ethanol off the coverslips and rinse them twice in sterile DPBS, leaving any excess DPBS after the second rinse for ease of isolating single coverslips.
4. Place a single coverslip in each well of a 12-well plate.
5. Cover each coverslip with 1 ml of 2.5 μg/ml sterile fibronectin (see Note 1) and incubate at room temperature for 20 minutes.

6. Remove the fibronectin from each well in a sterile manner and store it at 4°C for future use.
7. Aspirate residual fibronectin from the well and from under the coverslip by prodding the edges of the coverslip with a pasteur pipet attached to an aspirator, until the coverslip sticks to the bottom of the well and does not move when prodded further with the aspirator.
8. Add 1 ml RPMI complete growth medium to each well and place the 12-well plate in the incubator to equilibrate.
9. Remove a 75% confluent 10 cm plate of cells (those used in the example, Fig. 3.1, are OVCAR8 ovarian cancer cells, but numerous cell types are appropriate; see Note 2) from the incubator.
10. Aspirate the growth medium and rinse the cells 1x in DPBS to remove excess medium.
11. Add 1 ml trypsin and return the plate to the incubator to speed cell detachment.
12. Once the cells have detached, resuspend them in 7 ml of RPMI complete growth medium and add 250 µl of the cell suspension to each well of the prepared 12-well plate containing fibronectin-coated coverslips (see Note 3).
13. Allow cells to adhere overnight.
14. Transfect 1 well of cells with both 1 µg pCGN (see Note 4) and 1 µg pEGFP-C1-PAK1-PBD (see Note 5), and transfect another well of cells with 1 µg pCGN Rac1-Q61L-C189S and 1 µg pEGFP-C1-PAK1-PBD (see Note 6).
    a. Mix the DNA for each well with 100 µl serum-free RPMI in a polystyrene tube.

b. In a separate tube, combine 12 µl of TransIT-LTI and 200 µl serum-free RPMI.

c. Incubate both mixtures for 15 minutes at room temperature.

d. Add 100 µl of TransIt mixture to each tube of DNA.

e. Incubate for 45 minutes at room temperature.

f. Add each DNA/TransIt mixture to the appropriate well of cells, dropwise.

Fixing and staining cells

15. 24 hours after transfection, remove the growth medium from the cells by aspiration, and rinse twice with PBS (see Note 7). *After this step, minimize exposure of cells to light, to prevent photobleaching (see Note 8).*
16. Remove the PBS by aspiration, then fix the cells by adding 4% PFA to each well and incubating for 30 minutes at room temperature (see Note 9). *[Pause point: cells can remain in PFA overnight at 4°C, shielded from light.]*
17. Rinse the cells 5x with PBS.
18. Permeabilize the cells by incubating them in 0.2% Triton X-100 (diluted in PBS) for 5 minutes (see Note 10).
19. Rinse the cells 5x with PBS to remove Triton X-100 (see Note 11).
20. Incubate the coverslips in anti-HA antibody at a dilution of 1:500 for 1 hour at room temperature (see Note 12).
21. Rinse the cells 5x in PBS to remove excess primary antibody.
22. Incubate the coverslips in Alexa Fluor 594 anti-mouse antibody at a dilution of 1:500 for 2 hours at room temperature (see Note 13).

23. Rinse the cells 5x in PBS to remove excess secondary antibody.
24. Soak the coverslips in ddH20 for 5 minutes (see Note 14).
25. Remove excess water from the coverslip by holding it perpendicular to a lab wipe, touching it gently to the wipe, and dabbing off excess water. *Do not touch the cell-containing surface.*
26. Mount each coverslip on a slide by applying 10 µl of Vectashield with DAPI to a slide and placing the coverslip cell-side down on the Vectashield (see Notes 15 & 16). *[Pause point: mounted coverslips can be stored for weeks to months at 4°C, shielded from light.]*

Imaging and data analysis

27. Quantify localization of GFP-PAK1-PBD under each condition (see Note 17):
    a. On an appropriately equipped fluorescent microscope, use a red filter to visualize the Alexa Fluor® 594 (excitation maximum, 594 nm / emission maximum, 618 nm) and thereby identify cells expressing the HA-tagged Rac1 protein.
    b. Switch to the green filter to visualize GFP (excitation maximum, 488 nm / emission maximum, 509 nm) and thereby observe where GFP-PAK1-PBD is localized.
    c. Expression patterns can be divided into categories, such as: diffuse nuclear and cytoplasmic localization (probe is evenly distributed between the two compartments), nuclear-highlighted (probe is greater than 2x brighter in the nucleus than the cytoplasm), nuclear-excluded (probe in the nucleus is greater

than 2x dimmer than probe in cytoplasm), and other (plasma membrane highlighted/endomembrane highlighted/etc…). Classify each red (HA-Rac1-expressing) cell into the appropriate category based on localization of GFP-PAK1-PBD, and count it towards the given category using a push-button cell counter.

d. Repeat steps a-c for 100 cells per condition. Since vector-only cells do not visibly express the HA tag, skip step a for this condition.

e. The percentage of cells with the GFP-PAK1-PBD probe in each category can be compared between conditions.

28. Repeat the assay a minimum of 3 times (see Note 18).

29. Take images of representative cells (this may require use of a different, camera-equipped microscope; see Note 19). Recruitment of the probe can be shown using overlaid images of the probe and GTPase (see Note 20). Overlay with DAPI can confirm where the recruitment is relative to the nucleus.

NOTES

1. It is easier to visualize subcellular compartments in cells that are well-spread. Rho family GTPases regulate the actin cytoskleleton, cell adhesion, and cell spreading (350). Therefore, overexpression of GBDs downstream of Rho GTPases can result in undesired cell rounding and decreased cell adhesion. This can be minimized by coating the coverslips with fibronectin and also by thorough assay optimization (see Notes 6 & 7). If fibronectin causes unintended signaling consequences, alternatives

to increase cell adhesion include coating coverslips with 0.1% fish gelatin or treating them with poly-L-lysine.

2. There are many factors to consider when choosing the appropriate cells for an effector recruitment assay.
    a. Spread, adherent cells with easily visible organelles will allow for better identification of subcellular compartments than rounded or suspended cells.
    b. Cell choice can be used to help maximize probe specificity while monitoring endogenous GTPase activation (see Note 5). For example, POSH-PBD is a good probe for endogenous Rac1 activation because it does not bind to other Rho GTPases such as RhoA and Cdc42. While it has not been determined if POSH is a downstream effector for Rac2 in addition to Rac1, it likely is, given that the effector binding loops of Rac1 and Rac2 are identical (351). Therefore, changes in POSH-PBD localization could be due to activation of either Rac1 or Rac2. However, Rac2 is expressed largely in hematopoietic cells and some tumor cells. If it is desirable to maximize probe specificity for Rac1, POSH-PBD can be used in cell types that do not express Rac2.
    c. To increase the signal-to-noise ratio, it is best to use cells with low basal levels of GTPase activation when observing the effect of overexpressed GTPases.
    d. There are numerous examples of cell-type differences in GTPase activation (352); thus, cells of relevance to the topic of study must be used.

Effector recruitment assays have also been successfully utilized to monitor GTPase activation in model organisms, such as *Xenopus* (345, 353).

3. Given an approximately 24-hour doubling time, it is optimal that cells are ~40% confluent on the day after plating so they will still be in logarithmic growth phase when they are fixed. However, this can vary with cell lines and doubling times. Additionally, low density on the day of transfection can lead to low transfection efficiency. If this occurs, multiple coverslips may be needed for each condition in order to acquire enough cells to count.
4. In the example illustrated here, vector-only was used as a negative control and the Rac1 mutant served as a proof-of-principle that PAK1-PBD could be recruited to the nucleus by expression of active, nuclearly localized Rac1. Other comparisons could include constitutively active Rac1(Q61L) that recruits PAK1-PBD or POSH-PBD to the plasma membrane; dominant-negative mutants of Rac1 that fail to bind any effectors; effector domain mutants of Rac1 or non-Rac HA-tagged small GTPases, both of which fail to bind PAK or POSH. Assays for endogenous GTPase activation require more rigorous controls. For example, to confirm that recruitment in response to a given stimulus is due to activation of a specific GTPase, knockdown of the GTPase should impair recruitment. To examine whether expression of a GEF causes probe recruitment due to GTPase activation, use of a GEF mutant with impaired GEF activity should be used to determine if recruitment is then reduced. Additionally, controls for potential contributions of other structural or functional elements of the regulator, such as scaffolding functions, should also be included.

5. Probe selection greatly impacts the performance of recruitment assays and the conclusions regarding specificity that can be drawn from them. As mentioned previously, some GBDs are very specific while others are more promiscuous among GTPases. The specificity of the GBD probe aids in the determination of which GTPases are activated by a given stimulus in the observed location. A less specific probe may be desirable at first to explore the activation of multiple GTPases at once. However, this can also be complicated and lead to false negative results if two GTPases have opposing effects on the same probe, and eventually a more specific probe will likely be the most useful. Table 3.2 lists previously described probes and their GTPase specificity; however, none of the probes have been tested for specificity among all GTPases. Therefore, other methods (see Notes 2 and 4) to confirm specificity should also be used. Additionally, as new discoveries about which residues define the GTPase specificity of a GBD are made, designer probes can be made with a desired specificity. For example, based on current knowledge, PAK1-PBD can be mutated to interact specifically with Cdc42 instead of Rac (354).

   Additionally, the ability of a probe to localize to a given location must be considered. For example, if a probe is too large (GFP adds an additional 27 kDa) then it may not be able to enter the nucleus, even if there are active GTPases present. Ideally, under basal conditions, effective probes will be expressed diffusely throughout the cytoplasm and nucleus prior to GTPase manipulation. If this is not the case, then performing proof-of-principle experiments with constitutively activated GTPases targeted to specific subcellular regions (such as the one described here with

Rac in the nucleus) can be used to determine if a given probe is capable of interacting with active GTPases in a specific location.

6. The amounts of GBD probe and GTPase ectopically expressed will likely require optimization based on the probe and based on the cell type. As mentioned above, Rho GTPases regulate the cytoskeleton, and overexpression of GBD probes derived from their downstream effectors (or the GTPases themselves) can have undesired consequences (350, 355). Therefore, using the lowest amount of probe that can be easily observed is advantageous. We recommend testing multiple concentrations of each probe and GTPase to determine the best concentration for cell health, transfection efficiency, and low probe expression.

   Stoichiometry is very important in these assays. We caution that a lack of observed recruitment may be due to stoichiometry problems causing a false negative result. If an expected effect is not seen with endogenous GTPases, one should also attempt the experiment with overexpression of the GTPase of interest. This caution is exemplified with Raf1-RBD, which can detect changes in exogenous but not endogenous Ras localization, presumably due to relatively low levels of endogenous Ras (335, 349).

7. As with amount of probe added, timing must also be optimized. Upon expression of the GBD for long periods of time, some cells will change in morphology and may require fixation prior to 24 hours to preserve cell health. Alternatively, some cells are slower to transcribe the probe and will require more time before optimal probe expression is observed. Since the probes are fluorescently labeled and suitable for live cell imaging (e.g., GFP), the preliminary assay used to optimize DNA

concentrations for transfection (see Note 6) can be observed at multiple time points (12, 24, 36, 48, 60, 72 hours) to determine optimal timing.

8. To minimize photobleaching, cells expressing the fluorescent probe should be kept in the dark. A cardboard box can be wrapped with aluminum foil and then used to cover the coverslips between steps during the fixation and staining process. After mounting the coverslips on slides, the slides should be kept in a dark box at 4°C prior to counting and imaging.
9. The assay can also be performed with a fluorescently labeled GTPase (or regulator) or in response to stimulation. In this case, one can perform the assay with live cells and fixation is not necessary. However, fixation is useful to avoid phototoxicity and to establish more clearly defined time points.
10. All other time points related to fixing and staining can be estimated. However, it is important that incubation with Triton-X-100 is monitored more closely. Leaving detergent on the cells for too long can cause cellular degradation.
11. Some primary antibodies require blocking at this step to prevent non-specific binding. Consult the immunofluorescence staining instructions provided by the manufacturer.
12. To conserve antibody, tape a piece of Parafilm to a lab bench so that it is taut and free of wrinkles. Use a pipet to dispense 40 μl of diluted antibody onto the parafilm for each coverslip. Place the coverslips with the cell-side down onto the antibody droplets for incubation. For later rinsing steps, return coverslips to the 12-well plate with the cell-side of the coverslip facing up.
13. In the described assay, the PAK1-PBD probe was tagged with EGFP (excitation maximum, 488 nm/emission maximum, 509 nm), which pairs well with Alexa

Fluor® 594 (excitation maximum, 594 nm/emission maximum, 618 nm) because their excitation and emission spectra do not overlap. Other fluorescent probes can be paired, but it is very important that their spectra do not overlap. Otherwise, the observed "recruitment" might be an artifact of bleed-through of the fluorescence of one probe into the wavelength of the other probe. To rule out bleed-through, it is important to use both channels to image cells expressing only the probe and cells staining only for the GTPase, and confirm that the other channel is blank in each scenario.

14. Rinsing with water helps remove residual salt (in the PBS) from the coverslip. Areas on the coverslip with salt crystals cannot be viewed under the microscope.
15. In this assay, mounting medium containing DAPI was used. DAPI stains nucleic acids and acts as a nuclear marker. The assay can be expanded to look at recruitment to various other subcellular locations by utilizing different markers and stains. For example: galactosyl transferase (GalT) marks the Golgi (335); transferrin marks endosomes (321); and MitoTracker® Red stains mitochondria (239). Many antibody suppliers provide useful lists of their antibodies that are specific to distinct subcellular compartments.
16. It is important to avoid bubbles in the mounting medium, since this will obstruct viewing of the cells. Be sure that bubbles are not released while pipetting the mounting medium onto the slide. Bubbles can also be generated from mounting the coverslip too quickly. Holding the coverslip at a 45 degree angle to the slide, place an edge of the coverslip onto the mounting medium and slowly lower the coverslip towards the slide.

17. In the described assay, it is impossible to count the cells blindly (i.e., vector-only cells are not red as are Rac1-expressing cells); however, whenever possible, images should be counted blind.

Quantitation can also be done by analysis using image-processing applications such as ImageJ (available free as public domain software from the US National Institutes of Health [http://rsbweb.nih.gov/ij/] and other download sites). Each cell that expresses both Rac and the probe can be imaged, and ImageJ can then be used to quantify the pixel intensity in the area of interest (e.g., the nucleus) and another area in the cell (e.g., the cytoplasm). An arbitrary threshold can be set (e.g., for nuclear recruitment, a ratio of greater than 2:1 for nuclear:cytoplasmic intensity) and cells can be assigned to categories using this quantitative approach. If using this method, it is very important that the images are taken in the linear range of the microscope so that none of the pixel intensities are saturated (this can be controlled by exposure time and gain on the microscope). Also, it is important that it is the ratios that are compared and not the raw pixel intensities in a given area. The raw pixel intensity is a measure of how much GBD is being expressed, while the ratio signifies the distribution of the GBD among different locations. A change in the distribution (ratio) of the GBD among conditions is what is of interest in recruitment assays. This quantitative method of analysis may limit the number of cells that can be analyzed, in which case it will not give as accurate a representation of the population as a whole. The more cells counted (using any method) the better, because typically cell populations are heterogeneous, with a range of distributions of the GBD probe due to differences in the activation state of a variety of pathways within a given cell. The key task of data

analysis is to examine a heterogeneous population of cells and determine if, in a given condition, certain subpopulations become more highly represented.

18. Recruitment assay results are regulated by a variety of factors that cannot always be adequately controlled, including serum freshness, cell density, cell passage number, etc. This can cause results to vary significantly upon repetition. Therefore, it is not uncommon to require repetition of the assay 5-8 times before obtaining statistically significant results with a low standard deviation.
19. Imaging cells on a confocal microscope allows imaging of a narrow slice in the z-plane. Overlap observed with a confocal microscope therefore represents co-localization in -x, -y, & -z planes within a cell, whereas overlap observed with an epi-fluorescent microscope represents co-localization only in the -x and -y planes. If a confocal microscope is unavailable, an epi-fluorescent microscope with the capability of visualizing the chosen fluorophores can still be used for less precise observations of recruitment.
20. Recruited GBD probes should overlay well with activated GTPases. But, using recruitment assays to visualize endogenous activation of a GTPase by a regulator will likely result in an imperfect overlay between the probe and the regulator, since the regulator may activate the GTPase in this location and then be released from its close proximity.

TABLES AND FIGURES

Table 3.1. The subcellular locations of active, GTP-bound GTPases, are frequently distinct from the locations of total GTPase pools. All currently known Ras and Rho family members are listed. Column 2 lists subcellular locations where the GTPase has been detected in an active conformation, whereas column 3 lists locations where the GTPase has been described in significant abundance. PM=plasma membrane. * denotes predicted location, based on information in the associated reference. ** denotes subcellular localization determined in the associated reference by ectopic expression of a constitutively active GTPase.

	Subcellular localization	
GTPase	**Active GTPase pools**	**Total GTPase pools**
RAS FAMILY		
Di-Ras1	PM*(356), vacuolar membranes*(356)	PM(356), vacuolar membranes(356)
Di-Ras2	PM**(357), *(356), vacuolar membranes*(356)	PM(356), vacuolar membranes(356)
Di-Ras3 (ARH1, NOEY2)	PM**(357)	
E-Ras	PM*(358)	PM(358)
Gem (kir)	PM**(357)	cytosol(359), nucleus(359)
H-Ras	PM(349), **(357, 360) (non-lipid rafts(250, 361)), Golgi(250, 349), endoplasmic reticulum (ER)(349)	PM(10, 57) (lipid rafts(250, 361)), Golgi(57)
K-Ras(4A)	PM**(357)	PM*(9)
K-Ras(4B)	PM(349), **(357) (non-lipid rafts(361)), mitochondria*(239)	PM(9, 57) (non-lipid rafts(361))
N-Ras	PM(349), **(357) (lipid rafts(250)), Golgi(250, 349)	PM(9) (non-lipid rafts(250))
NKIRas1	cytosol**(357), nucleus**(357)	
NKIRas2	cytosol**(357), nucleus**(357)	
R-Ras	PM**(357), focal adhesions(362), endosomes(363)	perinuclear region(362), Golgi(362), early and recycling endosomes(363) >

		PM(362, 364)
R-Ras2 (TC21)	PM**(357) (lipid rafts and non-lipid rafts(365)), ER(365)	PM(364) (multiple microdomains(365)) > focal adhesions(365), perinuclear region (ER and cis-Golgi(365)), recycling endosomes(365)
R-Ras3 (M-Ras)	PM**(357) (non-lipid rafts(366))	PM(364) (non-lipid rafts(366))
Rad	PM**(357)	cytosol(367), nucleus(367)
RalA	PM**(357), (287, 368), perinuclear region (recycling endosomes(287)), mitochondria(369)	punctate cytoplasmic vesicles(287)
RalB	PM**(357), (287)	punctate cytoplasmic vesicles(287), endomembranes(370)
Rap1A	PM(265), perinuclear region(360)	perinuclear endosomes(335), late endosomes/lysosomes(371)
Rap1B	PM*(11), cytosol*(11)	late endosomes/lysosomes(371)
Rap2A	PM**(357), vesicles**(357)	Golgi(371)
Rap2B	PM**(357), vesicles**(357)	
Rap2C		PM(372), cytosol(373), recycling endosomes(374)
RasD1	cytosol**(357), nucleus**(357)	
RasD2	PM**(357), vesicles**(357)	
RasL 10A (RRP22)	nucleolus(375)	nucleus(375)
RasL 10B (RRP17)		perinuclear region(376)
RasL 11A		nucleolus(377)
RasL 11B	cytosol**(357), nucleus**(357)	cytosol(378)
RasL 12	nucleus**(357) > cytosol**(357)	
Rem1 (Ges)	PM**(357)	cytosol(367), nucleus(367)
Rem2		cytosol(379), nucleus(379)
Rerg	nucleus**(357) > cytosol**(357)	cytosol(380) > nucleus(380)
Rheb1	endomembranes*(358)	endomembranes(358)
Rheb2	endomembranes*(358)	endomembranes(358)

Rit1	PM**(357)	PM(381)
Rit2 (Rin)	nucleus**(357) > PM**(357)	PM(381)
RHO FAMILY		
Cdc42	trans-Golgi(382) > PM(57, 344, 345), cell periphery(382)	PM(57, 338), Golgi(57, 338), ER(57), nuclear envelope(57)
Chp (RhoV)	PM(383), focal adhesions**(384)	PM(338), endomembranes(338), Golgi(383)
Rac1	PM**(57, 357), lamellipodia, lamellae(57, 332, 385)	cytosol(355) > PM(57), nuclear envelope(332)
Rac2	membranes(386), PM**(357) (vs. not PM*(57))	Golgi(57), ER(57), cytosol(338), endomembranes(28), nuclear envelope(57) > PM(57)
Rac3	membranes(355), PM*(355), **(357)	membranes(355), PM*(355), endomembranes(338)
RhoA	PM(57, 345), **(384), cell periphery(342), nucleus(68)	cytosol(19, 57) > perinuclear region(342)
RhoB	PM**(357), endosomes**(384), *(387)	PM(57), Golgi(57), early endosomes(19), pre-lysosomes(19)
RhoBTB1	vesicles (not lysosomes or Golgi)*(384)	vesicles (not lysosomes or Golgi)(384)
RhoBTB2	vesicles (not lysosomes or Golgi)*(384)	vesicles (not lysosomes or Golgi)(384)
RhoC	PM**(357), **(384), perinuclear region**(387)	PM(388), cytosol(19)
RhoD	PM**(357), vesicles**(357), **(384)	PM(389), endosomes(389)
RhoF (Rif)	PM**(357), perinuclear region**(384)	PM(338)
RhoG	PM**(357), *(390), vesicles**(357), mitochondria**(384)	PM(338), endosomes(338), mitochondria(384)
RhoH (TTF)	PM**(357), cytosol*(391)	cytosol(391)
Rnd1	PM**(357), *(392), adherens junctions*(392)	PM(392), adherens junctions(392)
Rnd2 (Rho7)	endosomes*(314) > cytosol*(314)	endosomes(338) > cytosol(338)

Rnd3 (RhoE)	PM**(357), *(313), internal membranes*(313), Golgi*(313), cytosol*(313)	PM(314), internal membranes(314), Golgi(338), cytosol(314)
TC10	perinuclear region(393), vesicles**(357), (393) > PM**(357) (lipid rafts(323)), focal adhesions**(384)	PM(57, 393) (lipid rafts)(394), actin filaments(338), perinuclear endsosomes(57, 393), recycling endosomes(393), exocytosing vesicles(393)
TCL	PM**(357), vesicles**(357), **(384)	PM(338), endosomes(338)
Wrch-1 (RhoU)	PM(321)	PM(321), endomembranes(321)

Table 3.2: GBD probes have varying degrees of GTPase specificity. GBD probes were first used to determine the levels of active GTPases (pulldown assays) and since then have been adapted to determine the localization of GTPase activity (recruitment assays). The demonstrated specificity is similar, but not identical, in each assay. Common GBD probes are described based on the amino acid residues and species (when specified) of the downstream effector from which they were derived. Fluorophores that have been attached to the GBD are also listed. The tested specificity for GTPases in each assay is given. *=SMART database alignment for GBDs.

			Pulldown assay		Recruitment assay		
GBD probe	**Amino acid residues (species)**	**Fluorophore**	**GTPase binds**	**GTPase does NOT bind**	**GTPase recruits**	**GTPase does NOT recruit**	**Notes**
Raf-1 RBD	51-131 (human)(347-349) 1-149(341, 395)	GFP(348, 349), RFP(250)	N-Ras(347), K-Ras(341, 347), H-Ras(341, 347, 395), R-Ras(396, 397)	Rap1A(347), Rit1(381), Rit2(381)	N-Ras(250, 347, 349), K-Ras(347, 349), H-Ras(250, 347-349)	Rap1A(349)	Rap1A binding affinity 50x less than Ras(398)
RalBP1 RBD	397-518(399)		RalA(399), RalB(400)				
RalGDS RBD	786-883 (human)(335)	GFP(335)	Rap1(396), Rap2(396), Ras(335)		Rap1(265, 335)	Ras(335)	Ras binding affinity 50x less than Rap(398)
N-WASP CRIB	230-288(401) 215-295(344) 142-276(402) 201-321 (human)(384)	GFP(344, 345, 401), RFP(345)	Cdc42(344), Rac1(344), Rac2(384), Rac3(384), TCL(384), TC10(384), Chp(383)	RhoA(344, 384), RhoB(384), RhoC(384), RhoD(384), RhoG(384), RhoH(384), Ras(344), Chp(384),	Cdc42(344, 345, 401)	RhoA(401), Rac1(401)	Rac1/2/3 and TC10 binding affinity lower than Cdc42(344, 402); binds N-terminally truncated but not full length Chp(383)

				Wrch-1(384), Rnd1(384), Rnd2(384), Rnd3(384), RhoBTB1(384), RhoBTB2(384), Rif(384)			
PAK1 PBD	67-150 (human)(51) 65-150 (human)(385) 69-150(355) 75-118 (human)(321) 56-267 (human)(384) 1-252 (rat)(337) 135-227 (yeast)(51)	YFP(385), GFP(321)	Rac1(51, 384), Rac2(51, 111, 384), Rac3(355, 384), Cdc42(51, 337, 384), TC10(323, 384, 394), Wrch1(321, 384), TCL(384), Rnd1(384), Rnd2 (slightly)(384), RhoBTB2(384), Rif(384)	RhoA(337, 384), RhoB(384), RhoC(384), RhoD(384), RhoG(384), RhoH(384), Chp(384), Rnd3(384), RhoBTB1(384),	Rac1(385, 397), Wrch1(321)	Cdc42(385)	
POSH GBD	292-362 (mouse)(336) 292-396(393)		Rac1(336)	RhoA(336), Cdc42(336)			Has been used as a FRET probe for TC10(393)
Rhotekin RBD	7-89 (mouse)(50) 1-90(403) 1-89(384) 40-107 (mouse)*	GFP	RhoA(50, 384), RhoB(111, 384), RhoC(111, 384, 403), TC10(384), (slightly TCL,	Rac1(50, 384), Rac2(384), Rac3(384), Cdc42(50, 384), RhoG(384), Wrch-1(384), Chp(384),	RhoA(345, 353, 397)		

			RhoH, RhoD, Rif, Rnd1(384))	Rnd2(384), Rnd3(384), RhoBTB1(384), RhoBTB2(384)			

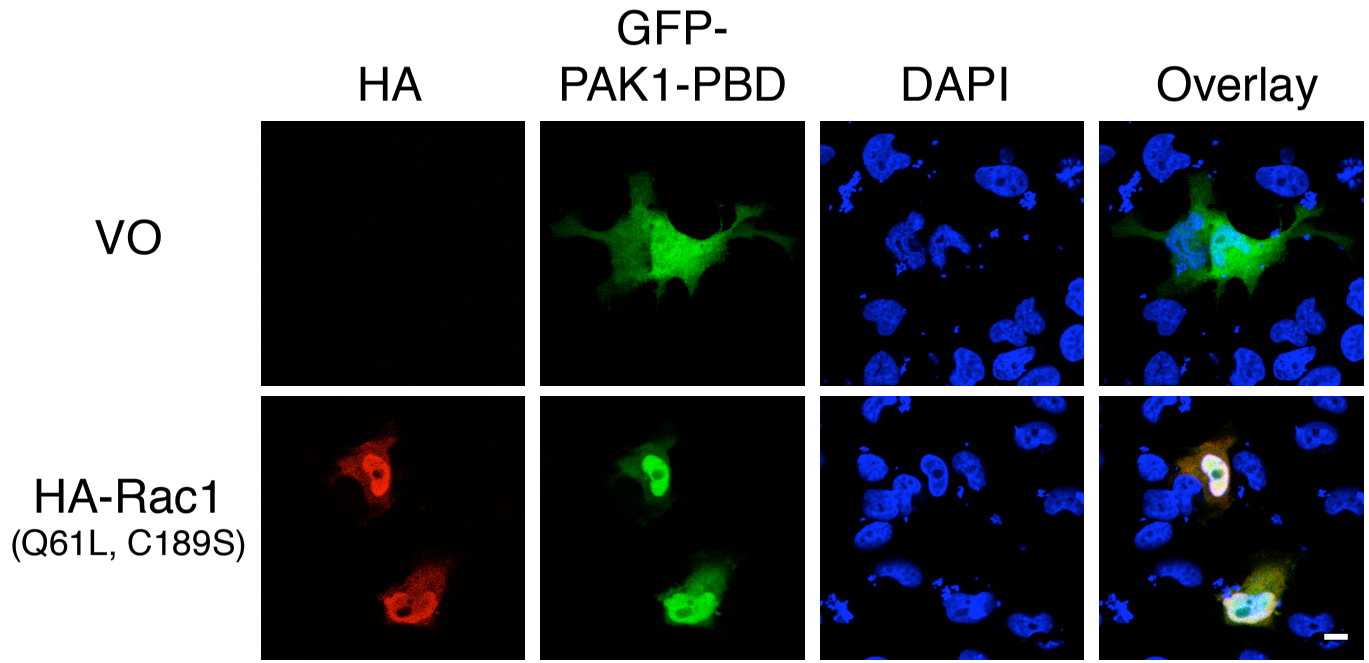

Figure 3.1. Nuclearly localized, constitutively active Rac1 (Rac1-Q61L-C189S) recruits GFP-PAK1-PBD to the nucleus. An example of conditions described in the methods section is shown. GFP-PAK1-PBD (green) is localized diffusely throughout the cell in cells co-expressing only empty pCGN vector. In contrast, when HA-tagged Rac1-61L-C189S (red) is ectopically expressed and localizes to the nucleus, GFP-PAK1-PBD is then observed highlighting the nucleus. This acts as easily visualized proof-of-principle that GFP-PAK1-PBD is recruited to areas of active, GTP-bound Rac1. DAPI serves as a nuclear marker. Images were taken using confocal microscopy at a magnification of 40x. Scale bar represents 10 microns.

CHAPTER 4: THE ROLE OF ECT2 NUCLEAR RHOGEF ACTIVITY IN OVARIAN CANCER CELL TRANSFORMATION[3]

OVERVIEW

Ect2, a Rho guanine nucleotide exchange factor (RhoGEF), is atypical among RhoGEFs in its predominantly nuclear localization in interphase cells. One current model suggests that Ect2 mislocalization drives cellular transformation by promoting aberrant activation of cytoplasmic Rho family GTPase substrates. However, in ovarian cancers, where Ect2 is both amplified and overexpressed at the mRNA level, we observed that the protein is highly expressed and predominantly nuclear, and that nuclear but not cytoplasmic

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[3]This chapter has been accepted for publication in Genes & Cancer and will be published by SAGE. The author list is as follows: Huff LP, DeCristo MJ, Trembath D, Kuan P, Yim M, Liu J, Cook DR, Miller CR, Der CJ, Cox AD. I wrote the chapter, and the work described was performed by me except for the aspects indicated below. All authors participated in editing the manuscript. Molly J. DeCristo performed some of the soft agar assays averaged in Figure 4.3, and contributed greatly through discussions about Ect2 and Rho GTPase function in ovarian cancer cells that enhanced my experimental approaches. Dimitri Trembath scored the ovarian TMA for Ect2 expression in the nucleus and cytoplasm, and taught me about pathology during the process. Pei Fen Kuan performed all the statistics related to the TMA and growth curves, and taught me how to perform the correct statistics for the other data. Margaret Yim performed some of the soft agar assays averaged in Figure 4.3. Jinsong Liu provided the ovarian TMA. Danielle R. Cook performed site-directed mutagenesis to generate NLS-mutant and GEF-deficient (DH-mutant) Ect2 constructs, which I then cloned into the expression vectors used here. C. Ryan Miller wrote the IRB covering this work and supervised the immunohistochemical staining of the TMA. Channing J. Der and Adrienne D. Cox initiated this project. Channing J. Der also initiated a collaboration with Millipore to generate a novel Ect2 antibody that I characterized and demonstrated to be suitable for immunofluorescence and immunohistochemistry. Adrienne D. Cox oversaw the project. She also edited this chapter down from twice its initial length and rendered it suitable for journal submission.

Ect2 increases with advanced disease. Knockdown of Ect2 in ovarian cancer cell lines impaired their anchorage-independent growth without affecting their growth on plastic. Restoration of Ect2 expression rescued the anchorage-independent growth defect, but not if either the DH catalytic domain or the nuclear localization sequences (NLS) of Ect2 were mutated. These results suggested a novel mechanism whereby Ect2 could drive transformation in ovarian cancer cells by acting as a RhoGEF specifically within the nucleus. Interestingly, Ect2 had an intrinsically distinct GTPase specificity profile in the nucleus versus the cytoplasm. Nuclear Ect2 bound preferentially to Rac1, while cytoplasmic Ect2 bound to RhoA but not Rac. Consistent with nuclear activation of endogenous Rac, Ect2 overexpression was sufficient to recruit Rac effectors to the nucleus, a process that required a functional Ect2 catalytic domain. Further, expression of active nuclearly targeted Rac1 rescued the defect in transformed growth caused by Ect2 knockdown. Our work suggests a novel mechanism of Ect2-driven transformation, identifies subcellular localization as a regulator of GEF specificity, and implicates activation of nuclear Rac1 in cellular transformation.

INTRODUCTION

Rho family GTPases are molecular switches that act as signaling nodes to integrate extracellular signals and propagate intracellular signals. They control many normal cellular processes, including actomyosin remodeling, cell polarity, gene expression, and cell cycle progression (338, 404). The best-studied members of this family are RhoA, Rac1, and Cdc42. These Rho GTPases are frequently overexpressed or dysregulated in tumors (47, 186, 405).

Aberrant Rho GTPase activity regulates transformation, invasion, metastasis, and angiogenesis (47, 186, 405).

GDP-bound Rho proteins are in the off-state, whereas GTP-bound proteins are in the on-state, in which they bind to their effector targets and transmit downstream signals. Guanine nucleotide exchange factors (GEFs) catalyze release of guanine nucleotides from GTPases, thereby enhancing binding of the more abundant cellular GTP (85). While some GEFs activate only specific GTPases, others are more promiscuous.

Ect2 (epithelial cell transforming sequence 2) is a Rho family GEF capable of activating RhoA, Rac1, and Cdc42 *in vitro* (76), but *in cellulo* it is more selective, in a context-dependent manner (111, 121, 123, 125, 126, 138, 159). Atypically for RhoGEFs, Ect2 contains two nuclear localization signals (NLSs), and has a prominent nuclear localization in interphase cells (76). In contrast, Rho proteins are found outside the nucleus (47). It has been proposed that Ect2 is autoinhibited and sequestered from Rho GTPases in the nucleus of normal interphase cells, but becomes mislocalized to the cytoplasm in tumor cells, where its autoinhibition is lost, and where it then activates Rho family GTPases to drive transformation (111, 137). However, the subcellular localization of Ect2/Rho GTPases interactions has never been directly investigated in tumor cells.

Here we utilized ovarian tumor cells to further examine the role of Ect2 in transformation. Aberrant Rho GTPase activity has been implicated in this tumor type (223, 224, 229, 230). Ect2 is located on chromosome 3q26.1-26.2 (152), a common amplicon in ovarian tumors (151, 157); indeed, ovarian cancer has the second highest frequency of Ect2 amplification among human cancers to date (149). Ect2 is also overexpressed at the mRNA level (45, 151, 218). However, the protein expression of Ect2 and its functional

consequences have not been studied in ovarian tumors. We examined a patient tissue microarray (TMA) and observed that Ect2 protein was strongly expressed predominantly in the nucleus of ovarian cancer cells. We have identified a requirement for Ect2 in ovarian cancer cell transformation, and a novel mechanism whereby Ect2 can activate Rac1 and can drive ovarian tumor cell transformation from within the nucleus.

RESULTS

Nuclear localization of Ect2 correlates with advanced disease in human serous epithelial ovarian cancers

Protein expression and subcellular distribution of Ect2 in ovarian cancers has not been evaluated previously. We evaluated these properties in a previously validated ovarian TMA (406-409) containing approximately 400 full-faced cores from ovarian tumors and non-matched normal ovarian cysts. We optimized the immunohistochemical protocol such that <5% of OVCAR8 cells with Ect2 knockdown stained positive for Ect2, using an Ect2 antibody that we had previously validated for specificity by immunoblot analysis (Supplementary Information (SI) Figs. 4.1A & B). Nuclear and cytoplasmic Ect2 expression were scored independently for each core. Unexpectedly, we found that higher scores for nuclear expression correlated with more advanced serous epithelial tumors (p=0.0001516, Fig. 4.1A), whereas those for cytoplasmic expression correlated with less advanced tumors (p=0.0007163, Fig. 4.1B). Indeed, in serous cysts Ect2 was expressed at low levels in the cytoplasm but undetectable in the nucleus, whereas for most cells in advanced tumors, Ect2 was concentrated in nuclei (Fig. 4.1C). These results suggested that nuclear rather than

cytoplasmic localization of Ect2 may be important for its oncogenic functions in this tumor type.

Ect2 is expressed predominantly in the nucleus of ovarian cancer cell lines

We turned to cell lines to study the localization and function of Ect2 in ovarian tumor cells directly. Ect2 was easily detected by immunoblotting in all of our panel of 8 ovarian cancer cell lines (Fig. 4.2A). To assess its subcellular localization, we performed immunofluorescence, staining for endogenous Ect2, co-staining for DAPI as a nuclear marker, and imaging the cells using confocal microscopy. Specificity of the Ect2 antibody immunofluorescence signal was confirmed using Ect2 knockdown cells (SI Fig. 4.1C). We observed a direct overlay of the vast majority of the Ect2 and DAPI signals in all cell lines examined (Fig. 4.2B and data not shown), indicating that Ect2 is predominantly nuclear, and supporting our observation from the TMA that nuclear Ect2 may be more relevant than cytoplasmic Ect2 for ovarian cancer cells.

Ect2 is required for transformed growth of ovarian cancer cell lines

Anchorage-independent growth is a hallmark of the transformed phenotype and is classically measured by soft agar colony formation (410). To determine the requirement for Ect2, we stably knocked down Ect2 using two independent shRNAs in cell lines that grow vigorously in an anchorage-independent manner. Both shRNAs robustly decreased expression of Ect2 in all cell lines, with Ect2 shRNA#3 showing more complete knockdown than shRNA#2 (98% and 84%, respectively, in OVCAR8 cells) (Fig. 4.3A). We then compared anchorage-independent growth of cells expressing non-targeted (NT) shRNA or

Ect2 shRNA. Knockdown of Ect2 decreased soft agar colony formation of multiple cell lines, with the most striking effects seen in OVCAR8 cells (Fig. 4.3B, $*p<0.05$). Thus, Ect2 is necessary for anchorage-independent growth of multiple ovarian cancer cell lines. We selected OVCAR8 for further study.

MTT proliferation assays revealed no significant differences between NT and Ect2 knockdown ovarian cancer cells (Fig. 4.3C). This indicates that the observed Ect2 dependence of anchorage-independent growth was not due simply to a general proliferation defect. And although Ect2 is known to play a critical role in cytokinesis of normal cells through activation of RhoA (49, 125), its role in some tumor cells is less clear (121, 137). Here, the level of multinucleated cells remained low upon knockdown of Ect2 (SI Figs. 4.2 & 4.4), indicating that these ovarian tumor cells can still undergo cytokinesis efficiently when Ect2 is depleted.

RhoGEF activity is required for Ect2-mediated transformed growth

Ect2 is a RhoGEF with other functional domains (85) that may also play a role in its ability to drive transformed growth. To determine if the GEF activity itself is required, we first generated an shRNA-resistant, putatively GEF-deficient Ect2 mutant. Residues E428 and N608 in the catalytic Dbl homology (DH) domain of Ect2 are conserved among RhoGEFs, interact with the switch I and switch II regions of Rho GTPases respectively, and are important for nucleotide exchange (85). We mutated these residues to alanines (E428A, N608A). Both wild type (WT) and the DH-mutant Ect2 were tagged with the HA epitope so they could be monitored separately from endogenous Ect2. The localization of the DH-mutant was indistinguishable from WT (SI Fig. 4.3). Using standard pulldown assays for

active Rho GTPases, we then observed in 293T cells that these mutations decreased by 90% the ability of Ect2 to activate RhoA, and completely ablated Rac1 activation (Fig. 4.4A), validating that this Ect2 mutant is GEF-deficient on these substrates. Interestingly, these mutations did not prevent Ect2-induced activation of Cdc42, indicating that this activation is not dependent on these catalytic residues, and is likely indirect.

We next assessed whether GEF-deficient Ect2 could rescue anchorage-independent growth. Ect2 expression was rescued in OVCAR8 Ect2 knockdown cells using either WT or GEF-deficient Ect2 (Fig. 4.4B). As expected, WT Ect2 rescued soft agar colony numbers to levels similar to those seen in cells without Ect2 knockdown, whereas the GEF-deficient mutant only partially rescued the phenotype (Fig. 4.4C). Thus, the RhoGEF activity of Ect2 is required to support full anchorage-independent growth in OVCAR8 cells.

We then sought to determine which Rho GTPases are activated by Ect2 in these cells. We compared the levels of active RhoA, Rac, and Cdc42 in whole cell lysates with or without Ect2 knockdown, utilizing standard Rhotekin-RBD (for RhoA) and PAK-RBD (for Rac and Cdc42) pulldown assays. Upon Ect2 knockdown, RhoA activity decreased by about a third (*p<0.05, Fig. 4.4D). We also saw a clear but more variable decrease in active Rac, despite the continued presence of multiple other RhoGEFs capable of activating these GTPases. Cdc42 activity remained constant (data not shown), consistent with the lack of effect of Ect2 GEF deficiency on Cdc42-GTP levels. These results indicate that Ect2 GEF activity is required for full RhoA and Rac activation in OVCAR8 cells at steady state and that other GEFs cannot fully compensate for loss of Ect2.

Predominantly nuclear localization is required for Ect2-mediated transformed growth

In ovarian cancer cells, Ect2 is a predominantly nuclear RhoGEF. Although RhoA and Rac1 are predominantly cytosolic proteins (47), both have been observed to lesser extents in the nucleus (61-64, 68). Loss of Ect2 GEF activity impaired ovarian cancer cell transformed growth, and also impaired full RhoA and Rac activation. Collectively, these findings suggest that Ect2 could activate nuclear rather than cytoplasmic pools of RhoA and Rac to drive transformation. If so, depleting Ect2 selectively from the nucleus should impair anchorage-independent growth.

To address this possibility, we mutated the arginines in both NLSs of the shRNA-resistant Ect2 to alanines (R348,349,350,370,372A). We first evaluated subcellular localization using immunofluorescence and confocal microscopy. Co-staining for the HA-epitope tag and DAPI confirmed that the NLS-mutant had a greatly decreased nuclear localization and greatly enriched cytoplasmic localization compared to WT Ect2 (Fig. 4.5A). To quantitate the subcellular distribution of NLS-mutant Ect2, we then fractionated the cells and performed immunoblotting analyses. The results (Fig. 4.5B) demonstrated that only about 10% of the NLS-mutant was still localized to the nuclear fraction. The NLS-mutant was well-expressed and well-tolerated, such that cells lines stably expressing NLS-mutant Ect2 were easily generated (SI Fig. 4.4A), without apparent effects on cell cycle distribution (SI Fig. 4.4D) or multinucleation (SI Figs. 4.4B & C).

Despite higher levels of expression than WT Ect2 (Fig. 4.5C), the NLS-mutant was not capable of rescuing the anchorage-independent growth defect of Ect2 knockdown cells (Fig. 4.5D). Interestingly, its overexpression alone in cells retaining endogenous Ect2 was sufficient to lead to decreased anchorage-independent growth. This suggests that simply

localizing Ect2 to the cytoplasm in the presence of nuclear Ect2 is not sufficient to drive transformation in these ovarian cancer cells, but rather may have the opposite effect, consistent with our TMA findings in which higher levels of cytoplasmic Ect2 were found preferentially in the less-advanced lesions.

Some nuclear Ect2 is present in an active conformation that has enhanced specificity for Rac1

It has been proposed that nuclear Ect2 is autoinhibited (111, 115), whereas our results suggest at least a portion is active. To test this directly, we fractionated OVCAR8 cells and performed a pulldown assay (343, 411) for active Ect2 using a constitutively nucleotide-free form of RhoA(17A), which binds with high affinity to the active form of RhoGEFs (411). Using tubulin as a cytoplasmic marker and PARP as a nuclear marker, we confirmed effective fractionation (Fig. 4.6A, left). Immunoblotting of endogenous Ect2 demonstrated that, while the vast majority was observably nuclear (NUC), some Ect2 was detectable in the non-nuclear fraction (CYT). This is unlikely to be due to nuclear contamination of the CYT fraction because PARP was not detectable there. In both fractions, Ect2 bound with notably higher affinity to GST-RhoA(17A) compared to the GST-only control (Fig. 4.6A, right), revealing that some active Ect2 is present in both compartments.

The ability of a GEF to interact with nucleotide-free mutant GTPases is dependent on both the concentration of active GEF (343) and the specificity of the GEF for the GTPase (411-413). To determine the ability of Ect2 to interact with other Rho GTPases within each subcellular compartment, we extended these results with GST-RhoA(17A), GST-Rac1(15A) and GST-Cdc42(15A). Equal amounts of GST-Rho proteins were added in excess to each

Ect2-containing lysate (Fig. 4.6B Coomassie), so the relative amounts of endogenous RhoA and Rac in each cellular compartment were irrelevant and could not contribute to any observed differences in Ect2 interactions. Additionally, to avoid complications from the vastly different amounts of Ect2 present in the nuclear versus non-nuclear compartments, here we pulled down Ect2 from lysates containing equal amounts of total Ect2 rather than equal amounts of total protein.

Unexpectedly, the Rho GTPase interactions of Ect2 in the nucleus were different from those in the cytoplasm (Fig. 4.6B). In the nucleus, Ect2 interacted with both Rac1(15A) and RhoA(17A), with a distinct preference for Rac1 despite equal exposure to both. In the cytoplasm, Ect2 interacted almost exclusively with RhoA. The minimal levels of Ect2 pulled down with Cdc42(15A) from either compartment are consistent with our proposal that activation of Cdc42 via Ect2 is indirect in these cells. Using densitometry to quantify the amount of Ect2 from each compartment that was pulled down by Rac versus Rho, we calculated that the ratio of Ect2 pulled down by Rac to that of Ect2 pulled down by Rho was 1.7 in the nucleus but only 0.4 in the non-nuclear compartment (Fig. 4.6C). Because equal amounts of ectopic Rac and Rho were available for Ect2 binding in each case, these different ratios suggest the possibility of an intrinsic conformational difference between nuclear and cytoplasmic Ect2.

We detected both endogenous Rac and endogenous Rho in the nucleus (Fig. 4.6D), but with unequal distribution: a greater proportion of Rac was distributed to the nucleus (16%) than was Rho (6%) (Fig. 4.6E). Thus, intrinsic differences detected by the pulldowns using ectopic nucleotide-free GTPases are likely to be amplified by the relative availability of endogenous Rac and Rho in each compartment during cellular signaling.

Ect2 activates endogenous Rac in the nucleus and endogenous RhoA in the cytoplasm

To determine if Ect2 is capable of activating endogenous Rac and/or Rho in the nucleus, we first performed standard Rho binding domain (RBD) recruitment assays (321, 348, 349, 414, 415). We used POSH, a downstream effector specific to Rac (336), to detect active Rac-GTP. Under basal conditions, GFP-POSH-RBD was expressed diffusely throughout the cytoplasm and nucleus, but was recruited to the nucleus in the presence of exogenous WT Ect2 (Fig. 4.7A; quantified in Fig. 4.7C). Nucleolar exclusion of GFP-POSH-RBD was evident by confocal microscopy (Fig. 4.7A); thus, GFP-POSH-RBD was recruited into the nucleus and not onto the nuclear envelope. Recruitment was dependent on both GEF activity and nuclear localization of Ect2, because the GEF-deficient and the NLS-mutant each failed to recapitulate the effects of WT Ect2. Consistent with these results, GFP-PAK1-RBD (205) was also recruited to the nucleus by Ect2, in a GEF- and NLS-dependent manner (SI Figs. 4.5A & B). These results indicate that WT Ect2 activates endogenous Rac1, and the site of activation is primarily in the nucleus.

Notably, the cytoplasmically localized NLS-mutant did not activate Rac as measured either by recruitment assay (Fig. 4.7A, SI Fig. 4.5A) or by pulldown assay (Fig. 4.4A). In contrast, we observed that the cytoplasmically localized NLS-mutant Ect2 strongly increased RhoA-GTP levels (50.5 fold, Fig. 4.4A). These results indicate that Ect2 activity in the cytoplasm preferentially results in activation of Rho. However, GFP-Rhotekin-RBD, a surrogate for RhoA activity (416) was not recruited either to the cytoplasm or to the nucleus (Fig. 4.7B, quantified in Fig. 4.7C). One reason for this may be that any shift of the smaller pool of nuclear GFP-Rhotekin-RBD into the cytoplasm may not be detectable in the larger pool of cytoplasmic Rhotekin-RBD. Therefore, to determine if the elevated RhoA-GTP that

we detected by pulldown led to a biological consequence in the cytoplasm, we performed phalloidin staining to examine the actin cytoskeleton of OVCAR8 cells exogenously expressing WT or mutant Ect2. The canonical cytoskeletal consequence of RhoA activity is the formation of stress fibers (33), and indeed we found that only the cytoplasmically localized NLS-mutant Ect2 significantly enhanced stress fiber formation, whereas the nuclearly localized WT and GEF-deficient Ect2 did not (Figs. 4.7D & E). These results indicate that cytoplasmically localized Ect2 can functionally activate RhoA. In contrast, and consistent with its inability to activate Rac (Fig. 4.4A), NLS-mutant Ect2 did not increase membrane ruffling, a canonical consequence of Rac activity (31) (Figs. 4.7D & E). WT Ect2 also did not enhance membrane ruffling, consistent with a previous report that overexpression of a nuclearly localized, constitutively active Rac1 mutant enhanced cell proliferation without inducing membrane ruffles (62). Collectively, our results indicate that Ect2 preferentially activates Rac1 in the nucleus and RhoA in the cytoplasm. The larger pool of cytoplasmic Rho family GTPases (Fig. 4.6E) may explain why we observed more striking changes in RhoA activation compared to Rac activation upon Ect2 overexpression (Fig. 4.4A) and knockdown (Fig. 4.4D) in whole cell lysate.

Nuclear Rac1 activity is sufficient to rescue defects in Ect2-mediated transformed growth

Since we have demonstrated that both nuclear localization and GEF activity are required for Ect2-mediated transformed growth in ovarian cancer cell lines and that Ect2 preferentially activates Rac1 in the nucleus, our results suggest that Ect2 activation of nuclear Rac may be an important contributor to its transforming ability.

We first attempted to determine directly whether Rac1 is necessary for Ect2-driven anchorage-independent growth by asking whether Rac1 knockdown impaired colony formation in soft agar. However, it was impossible to generate cells lacking Rac1, as transfection with any of four independent Rac1-targeted siRNAs (Dharmacon/Thermo Scientific, Pittsburgh, PA) induced widespread cell death (data not shown), leaving us with insufficient Rac1-knockdown cells for analysis and indicating that Rac1 is an essential protein for viability of these cells. Although it is likely that the non-nuclear functions of Rac1 were critical, we were unable to selectively knock down only nuclear Rac1. Another approach to testing our model is to determine whether nuclear Rac1 can overcome the defect in transformed growth conferred by loss of Ect2 expression. To test this, we generated HA-tagged activated Rac1 mutants that were preferentially localized in the nucleus (Fig. 4.8A). We used mutants of Rac1 that were activated independently of Ect2 by either the traditional GTP-locked G12V mutation (31) or by the fast-cycling F28L mutation (173). Prenylation of Rac1 is required for its plasma membrane association (47) and impairs its nuclear import. Non-prenylated Rac1 localizes strongly to the nucleus when GFP-tagged (13, 62). However, we determined that an additional N-terminal NLS was required to concentrate unprenylated HA-tagged Rac1 in the nucleus. Therefore, to preferentially localize the activated Rac1 mutants to the nucleus, we both mutated the Rac1 prenylated cysteine to serine (C189S, “SAAX” mutation) and added the classical NLS from SV40T antigen (417) to the N-terminus to generate "NLS-Rac1-SAAX" mutants. When expressed stably in cells with Ect2 knocked down (Fig. 4.8B), each of these nuclearly concentrated, active mutants of Rac1 was able to rescue anchorage-independent growth to the same extent as re-expression

of WT Ect2 (Fig. 4.8C). These results support our model that Ect2 activation of Rac1 in the nucleus is a key component of its transforming function.

DISCUSSION

Despite the largely nuclear localization of the RhoGEF Ect2 in interphase cells (76, 124, 137, 138, 145), previous studies have focused on its actions at non-nuclear locations. For example, the most prominent role for Ect2 in normal cells is its requirement during cytokinesis, when the nucleus is not intact and Ect2 activates RhoA at the cleavage furrow and midbody (45, 76, 121, 125, 131). But the function of Ect2 in cytokinesis of normal cells can be uncoupled from its role in promoting transformed growth of tumor cells (121, 137, 161), leaving the mechanisms whereby it promotes transformation less well understood. One model suggests that Ect2 must be cytoplasmically mislocalized to drive transformation (111, 137). In contrast, our study indicates a prominent role for a nuclear pool of Ect2 in the transformed phenotype of ovarian cancer cells. Further, we have identified a requirement for Ect2 to act as a RhoGEF within the nucleus to promote growth transformation in ovarian cancer cells.

In both ovarian cancer patient tumor tissues and established cell lines, we found that Ect2 protein was expressed robustly, consistent with its published genomic amplification and mRNA overexpression in ovarian tumors (45, 151, 218). Ect2 was predominantly localized to the nucleus, particularly in advanced disease. Interestingly, we did not observe significant Ect2 localization to the cytoplasm in advanced tumors; instead, benign ovarian cysts had the most prominent cytoplasmic staining. Although Ect2 was reported to be strongly mislocalized from the nucleus to the cytoplasm in non-small cell lung cancers (NSCLC)

compared to normal lung (137), our observations of increased nuclear expression in ovarian cancers are more consistent with other reports where inspection of the data shows that Ect2 increases globally upon tumor progression, with extensive increases in nuclear expression and less dramatic increases in cytoplasmic Ect2 (138, 145-147). We show here that both Ect2 nuclear localization and its RhoGEF activity are required for Ect2-driven anchorage-independent growth of ovarian cancer cells, suggesting that Ect2 acts as a GEF from within the nucleus to drive transformation. Indeed, our observations that cytoplasmic Ect2 localization was inversely correlated with advanced disease, and that a cytoplasmically localized Ect2 NLS-mutant reduced anchorage-independent growth, indicate that mislocalization of Ect2 to the cytoplasm does not drive ovarian transformation but rather might oppose the effect of nuclear Ect2. That this NLS-mutant Ect2 was able to act as a GEF to increase RhoA-GTP and promote stress fiber formation, suggest the intriguing possibility that Ect2 activation of RhoA in the cytoplasm might even counteract its actions in the nucleus.

In NSCLC cell lines, transformed growth can be driven by Ect2-induced activation of Rac1 (137). Rac1 activation was proposed to occur in the cytoplasm, although this was not tested directly (137, 161). Since the BRCT domains of Ect2 can autoinhibit its activity (111, 112, 115), nuclear Ect2 was proposed to be autoinhibited and sequestered from Rho GTPases (111). Here, we found that active Ect2 was present in the nucleus. There has been almost no prior investigation of the subcellular distribution of active Ect2, but one study did identify active Ect2 in the nucleus of HEK293 cells (68).

While Rho GTPases are described as cytoplasmic proteins (47), this is not the entire story. In particular, Rac1 (62-64) and, to a lesser extent, RhoA (61, 68) and Cdc42 (65) have

all been detected within the nucleus. We were able to detect both endogenous Rac and some endogenous RhoA in the nucleus of OVCAR8 cells; therefore, both are available in the nucleus as substrates for Ect2 RhoGEF activity. Our work also provides further evidence for nuclear activation of Rho GTPases. Although early reports simply described nucleocytoplasmic shuttling (63, 64), there are recent reports that RhoA can be activated from within the nucleus in some contexts (61, 68).

We observed that Ect2 preferentially activates Rac in the nucleus. We also identified at least two likely contributors to this finding: 1) the presence of more Rac than RhoA in the nuclear compartment, and 2) an intrinsic substrate specificity, such that nuclear Ect2 preferentially binds to Rac1 whereas cytoplasmic Ect2 preferentially binds to RhoA. Consistent with the latter, the NLS-mutant, which is defective for nuclear localization and is distributed well into the cytoplasm, robustly activated RhoA but not Rac.

Modulation of intrinsic substrate specificity may be complex. Phosphorylation is required to optimally activate Rac both *in vitro* (76) and in cells (134). We have observed that bacterially expressed, i.e., unphosphorylated, full-length Ect2 can activate RhoA but not Rac1 *in vitro* (131). It is tempting to speculate that nuclear Ect2 is appropriately phosphorylated; however, despite repeated attempts we have not observed mobility differences between nuclear and cytoplasmic Ect2 (as observed previously for phosphorylated versus unphosphorylated Ect2 (76, 125)). Alternatively, as-yet-undefined protein-protein interactions that occur in the nucleus may promote interaction with Rac, perhaps involving the Ect2 C-terminus, which is required to activate Rac in cells (94). Similarly, cytoplasmic scaffolding may promote RhoA but not Rac activation. Protein-protein interactions have been speculated to explain reports of varying Ect2 specificity

throughout cytokinesis/mitosis (234). Regardless of the mechanism, to our knowledge, this study is the first to demonstrate altered specificity of a RhoGEF based on its subcellular localization.

Our work is also the first to show that active Rac1 is capable of driving transformation when concentrated in the nucleus (and excluded from the plasma membrane). The precise function of nuclear Rac in these cells is unknown. Forced expression of a constitutively active but unprenylated Rac1 mutant in the nucleus of NIH3T3 cells accelerated cell cycle progression (62). However, this mutant did not induce lamellipodia formation, a primary function of cytoplasmic Rac1 (62), suggesting that nuclear and cytoplasmic functions of active Rac may be distinct. Similarly, although overexpression of Ect2 was capable of recruiting the RBD of Rac effectors to the nucleus, we did not observe an increase in lamellipodia or membrane ruffling upon expression of Ect2. Still, negative regulators (81, 84) and downstream signaling molecules (72, 227, 228, 418, 419) of Rac can be detected in the nucleus, suggesting that some cytoplasmic signaling pathways could remain intact there, albeit with distinct stoichiometry. Some Rac effectors directly regulate transcription from the nucleus (72, 418, 419), but we did not observe Ect2-mediated alterations in transcriptional activation (data not shown). The speckled pattern of Ect2 localization in the nucleus suggests that Ect2 localizes to distinct subnuclear structures. Nuclear speckles regulate mRNA splicing (420), and Ect2 interacts with the SNRNP200 gene product, a component of the spliceosome (114), so it is possible that Ect2 signaling to Rac plays a role in this process.

In conclusion, here we identified Ect2 as necessary for cellular transformation in ovarian tumor cells. We also identified a novel mechanism through which Ect2 can drive

cellular transformation by acting as a GEF from within the nucleus, in a manner distinct from its actions in the cytoplasm. We showed that Ect2 preferentially activates Rac in the nucleus of ovarian cancer cells and RhoA in the cytoplasm. In addition to Ect2, the RhoGEFs Net1, LARG and Vav3 have also been identified in the nucleus (78, 421, 422). It would be interesting to determine if subcellular localization also alters the specificity of these and other RhoGEFs. Our results suggest that nuclear Rac1 activity is biologically relevant for transformed growth, and implicate activation of nuclear Rac as a mediator of Ect2-driven transformation. In the future it may become possible to selectively ablate Ect2-activated nuclear Rac. If so, this would allow more rigorous testing of a requirement for nuclear Rac activity in Ect2-driven ovarian cancer cell transformation. As physiologically relevant substrates of nuclear Ect2 and Rac1 are identified, it will also be interesting to uncover their roles in transformation.

MATERIALS AND METHODS

Molecular constructs and transfections

Short hairpin RNA (shRNA) against human Ect2, non-targeted (NT) shRNA, and shRNA-insensitive full length Ect2, were generously provided by Alan Fields (Mayo Clinic, FL) (137). Quikchange II site-directed mutagenesis kit (Stratagene, Santa Clara, CA, USA) was used to create NLS- and DH-mutant Ect2 from shRNA-insensitive full length Ect2 (primers, SI Table 4.1). NLS-Rac1-SAAX mutants were generated using PCR (primers, SI Table 4.1) to amplify Rac1(F28L) (131) and Rac1(G12V) (gift of Peter Hordijk, Sanquin). All Ect2 and Rac1 expression constructs were cloned into pFugW-HA-blasticidin (gift of Jeran Stratford, UNC-CH). Viral psPAX2 (packaging) and pMDG.2 (envelope) vectors were

from Addgene. pGEX-2T and RBD constructs (321, 345, 423) were gifts from Keith Burridge (UNC-CH). Transfections were performed using TransIT-LTI (Mirus, Madison, WI, USA) according to the manufacturer's instructions.

Cell culture, lentiviral infection, and generation of stable transfectants

Human ovarian epithelial cancer cell lines were grown in RPMI-1640 with 10% fetal bovine serum (FBS, Sigma, St. Louis, MO, USA). 293T cells were grown in DMEM-H + 10% FBS. Lentiviruses were generated as described (424), except virus was collected 24 and 48 h post transfection. Lentivirus-infected cells (424) were selected in puromycin (1 μg/ml, Cellgro, Manassas, VA, USA) or blasticidin (10 μg/ml, Invitrogen, Grand Island, NY, USA).

Flow cytometry

Flow cytometry was performed exactly as we have described previously (131).

Immunoblotting, immunofluorescence, phalloidin staining and microscopy

Immunoblotting was performed from RIPA cell lysates by our standard methods (321). For immunofluorescence and phalloidin staining, cells were plated on fibronectin, fixed in paraformaldehyde, exposed to antibodies or to Alexa Fluor568-conjugated phalloidin (Invitrogen), and confocal microscopy imaging performed as we have done previously (321, 322). A Nikon (Melville, NY, USA) Eclipse TS100 microscope was used to image cells and colonies in bright field.

Ovarian tumor tissue microarray (TMA) immunohistochemistry

A previously validated TMA (406-409), containing formalin-fixed, paraffin-embedded primary epithelial ovarian carcinomas or benign cysts, was stained for Ect2 by the UNC-Translational Pathology Laboratory Core. We used a standard method of scoring the TMA (409), except each core was scored separately for nuclear and cytoplasmic staining. Each compartment was scored separately on a scale of 0-100 for percent cyst or tumor cells stained, and on a scale of 0-3 for average intensity of staining, where 0 was no staining and 3 was maximal staining. The score for each compartment was the product of percent cells staining in the nucleus/cytoplasm multiplied by the average intensity of nuclear/cytoplasic staining for each core.

Previously collected patient data (406-409) allowed us to classify the serous tumors by degree of malignancy (cysts, serous borderline tumors-SBT, serous low grade tumors, and serous high grade tumors). A Kruskal-Wallis test was performed to compare both nuclear and cytoplasmic scores among these groups; $p<0.05$ was considered significant. IRB approval was through UNC-CH IRB 08-0242.

Anchorage-dependent and -independent growth assays.

Soft agar assays to assess anchorage-independent growth were performed and counted as we have described (321). A two-tailed Student's *t*-test assuming unequal variance was performed on colony counts. The *p*-values were adjusted for multiple tests using the Bonferroni correction, and $p<0.05$ was considered significant. Standard MTT proliferation assays were performed (425). Cells were normalized to the average value for day 0 and growth curves were generated from the average of all experiments. Slopes of the log-

transformed growth curves were compared using likelihood ratio tests to determine statistical significance and a Bonferroni corrected p-value <0.05 was considered significant.

Cell fractionation

Cell fractionation into nuclear and postnuclear compartments was performed by hypotonic swelling, Dounce homogenization, centrifugation through 28.5% iodixanol (Sigma), and sonication as described in detail previously (343).

Pulldown and recruitment assays for active Ect2 and Rho GTPases

Active Ect2 was assessed by incubating fractionated cell lysates with nucleotide-free Rho GTPases isolated from bacteria and bound to agarose beads, resolved on SDS-PAGE and immunoblotted for Ect2 (343, 411). Prior to performing pulldowns, fractions were subjected to Bradford analysis for total protein content. For some experiments (Fig. 4.6A), equal amounts of total protein were added to the beads. Alternatively (Fig. 4.6B), loading of fractionated cell lysates was normalized for equal expression of Ect2. This was done by comparing the distribution of Ect2 in nuclear versus cytosolic fractions using densitometry of western blots of the fractionated lysates, which showed that, loading 2.6x more of the cytosolic fraction resulted in approximately equal loading of Ect2 from each fraction. Loading of Ect2 was examined by western blot following each of these assays. Beads were blocked in 5% BSA for 30 min to minimize non-specific binding of Ect2 to GST. Equal loading of the beads was confirmed by SDS-PAGE and Coomassie blue staining.

To determine levels of active, GTP-bound RhoA, Rac, and Cdc42, pulldowns using GST-Rhotekin-RBD or -PAK-PBD, respectively (423), were performed from whole cell

lysates. Densitometry on immunoblots was performed using ImageJ and normalized as described in the Results section.

Recruitment assays (321, 348, 349, 414, 415) using GFP-tagged RBDs for Rhotekin, POSH and PAK were utilized to monitor changes in the subcellular distribution of active endogenous Rho GTPases. Fixed cells expressing HA-Ect2 in each specific location (vector: diffuse; WT and DH-mutant: nucleus; NLS-mutant: cytoplasm) that co-expressed GFP-RBD were scored for GFP-RBD localization based on the following categories: nuclear-excluded, plasma membrane-highlighted, diffuse nuclear and cytoplasmic localization, nuclear-highlighted. The average percentage of cells nuclear-highlighted out of total cells counted is shown. Statistical significance was determined as described for the soft agar assays.

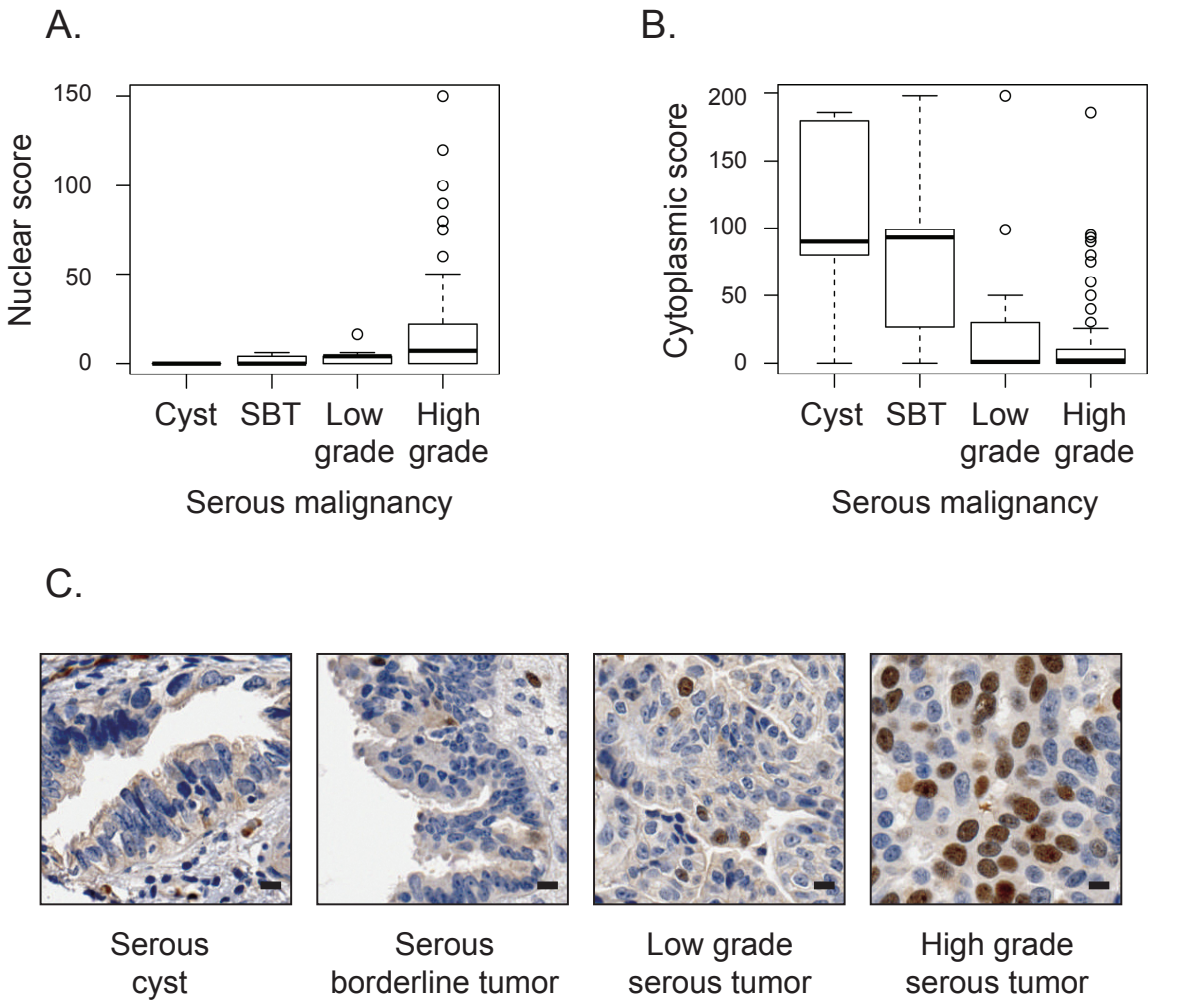


Figure 4.1. Nuclear localization of Ect2 correlates with advanced disease in human serous epithelial ovarian cancers. Ect2 subcellular localization was analyzed by immunohistochemistry (IHC) using a well-validated (406-409) ovarian tissue microarray (TMA). Tumor cores were stained with Ect2 antibody (1: 350, Millipore, Billerica, MA, USA) and scored for percentage cells stained as well as intensity of staining in each location. Separate nuclear scores and cytoplasmic scores were calculated as described in Materials & Methods, and binned according to disease severity. *Quantification of Ect2 localization showed (A) significantly greater nuclear expression (p=0.0001516) and (B) significantly lower cytoplasmic expression (p=0.0007163) in advanced disease.* SBT, serous borderline tumor. Box and whisker plots include horizontal lines corresponding to median, first and third quartiles (hinges), and extreme points excluding outliers (whiskers). *C. Representative images of a benign serous cyst, a serous borderline tumor, a low grade and a high grade serous epithelial tumor stained for Ect2 illustrate increased nuclear staining upon disease progression.* Scale bars represent 10 microns.

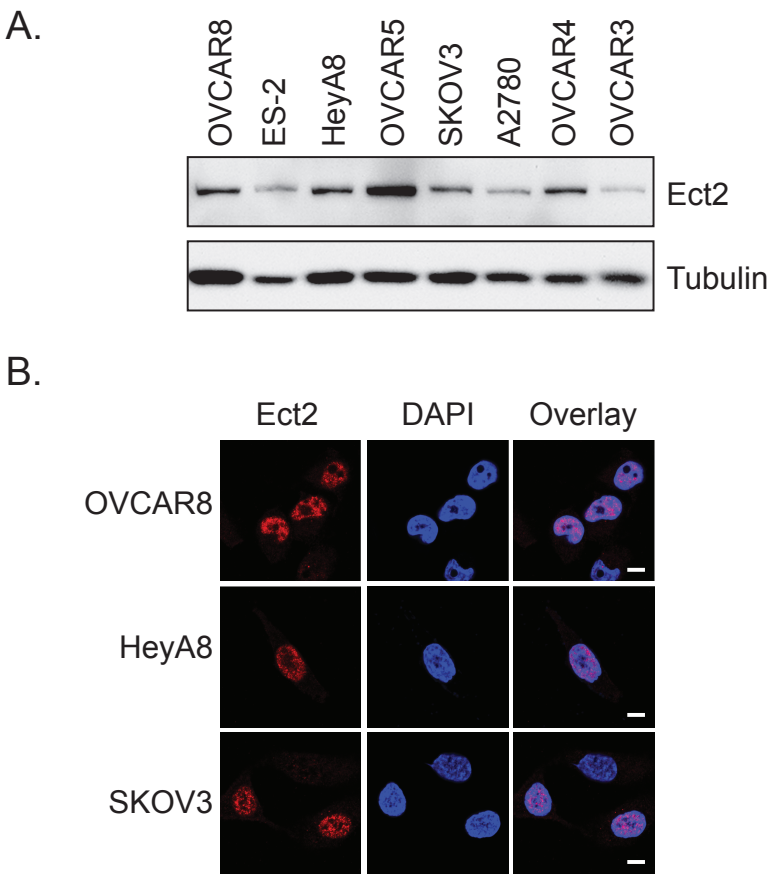


Figure 4.2. Ect2 is expressed predominantly in the nucleus of ovarian cancer cell lines. *A. Ect2 is highly expressed in ovarian cancer cell lines.* Cell lysates from a panel of 8 human ovarian cancer cell lines were probed by immunoblotting with the anti-Ect2 antibody (1: 1 000, n=4). Equal amounts of cell lysate were loaded onto the gel; tubulin (1: 1 000 clone DM1A Sigma-Aldrich) served as a loading control. *B. Ect2 is predominantly nuclear in established ovarian cancer cell lines.* Cells were stained with anti-Ect2 antibody (1: 1 000) and the subcellular distribution of endogenous Ect2 was visualized by confocal immunofluorescence microscopy to detect the Alexafluor 594-coupled secondary antibody. Nuclei were stained with DAPI. Predominantly nuclear staining was seen across cell lines (images representative of n≥4). Scale bars represent 10 microns.

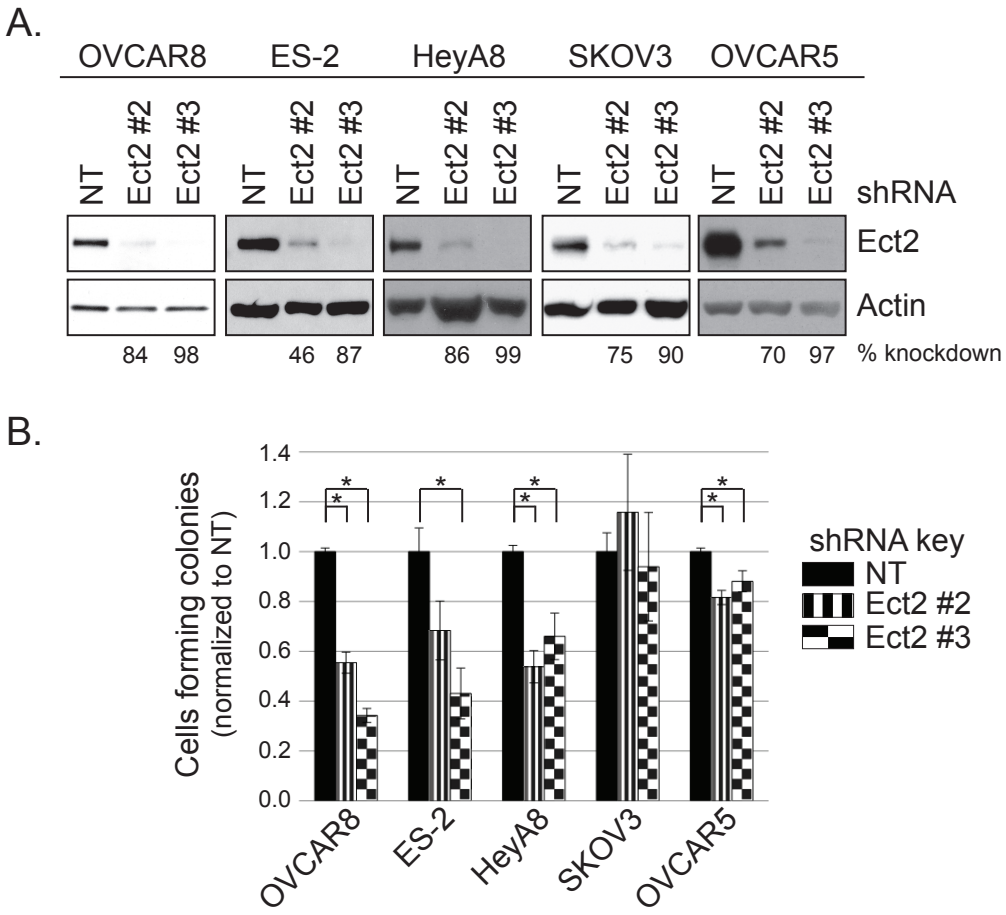
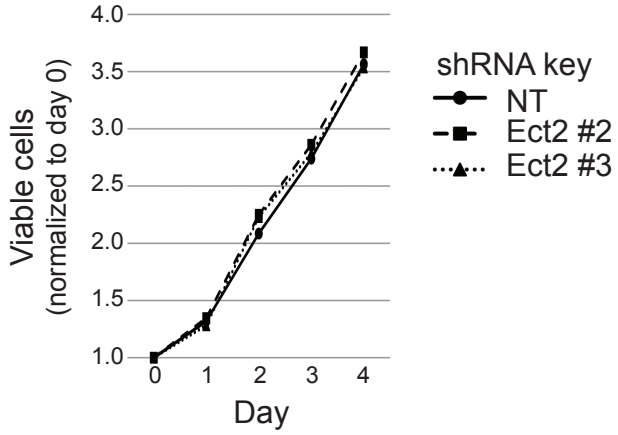


Figure 4.3. Ect2 is required for transformed growth of ovarian cancer cell lines. Our ovarian cancer cell lines that grow robustly in an anchorage-independent manner were evaluated for the importance of Ect2 in transformed growth. Cells were infected with lentiviruses expressing one of two independent shRNAs directed against Ect2, or non-targeted (NT) shRNA. Infected cells were selected in puromycin and colonies were pooled for further use. *A. Ect2 knockdown using two independent shRNAs was confirmed by immunoblotting.* Equal amounts of cell lysate were loaded onto the gel; actin (1: 30 000, clone AC-74 Sigma-Aldrich) served as a loading control (n≥3). Percent knockdown shown is based on densitometry performed on each Ect2 blot and normalized to actin. *B. Ect2 knockdown impairs anchorage-independent growth.* Cells with or without Ect2 knockdown were grown in soft agar, and colonies were counted after two to five weeks (n≥3). Statistical significance was evaluated using Student's *t*-tests, with the Bonferonni correction (**p*<0.05); error bars represent standard error (SEM).

C.



C. Reduced anchorage-independent growth is not primarily due to a general proliferation defect. Cell growth in monolayer culture was not statistically different whether Ect2 was (Ect2 shRNA#2, #3) or was not (NT shRNA) knocked down, as determined by MTT assay over 4 days (shown: OVCAR8 cells). Error bars represent SEM (n=3).

A.

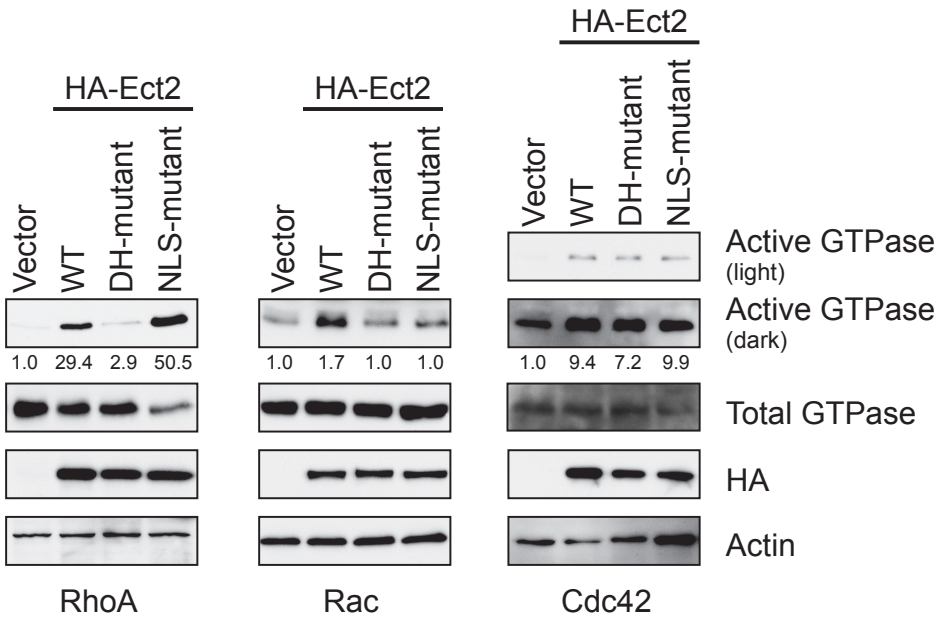
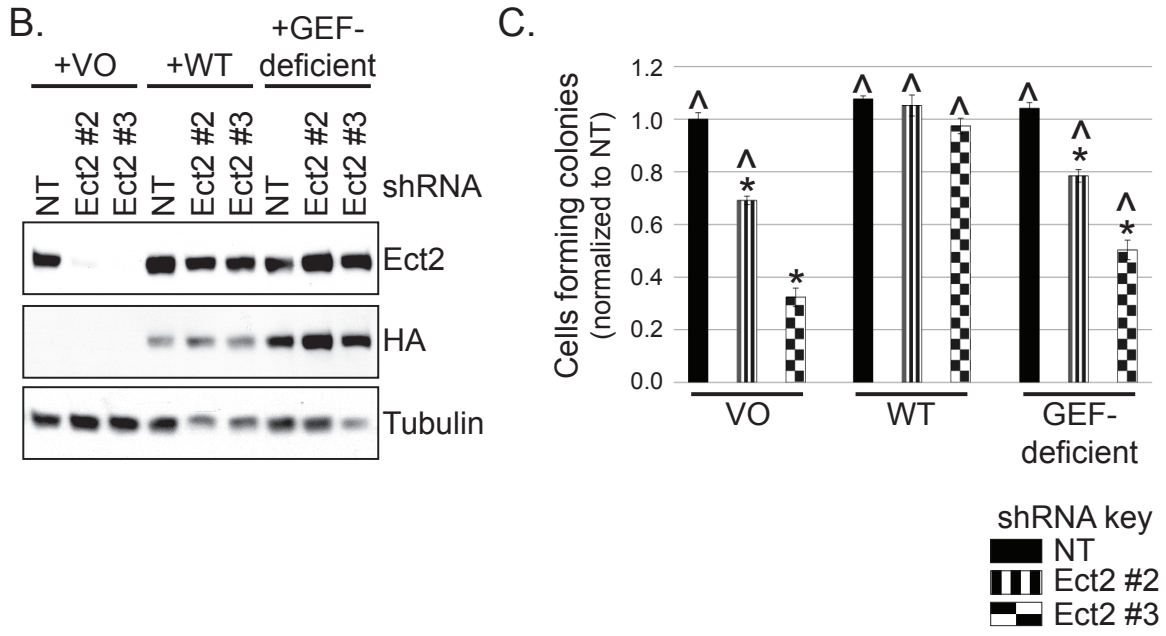
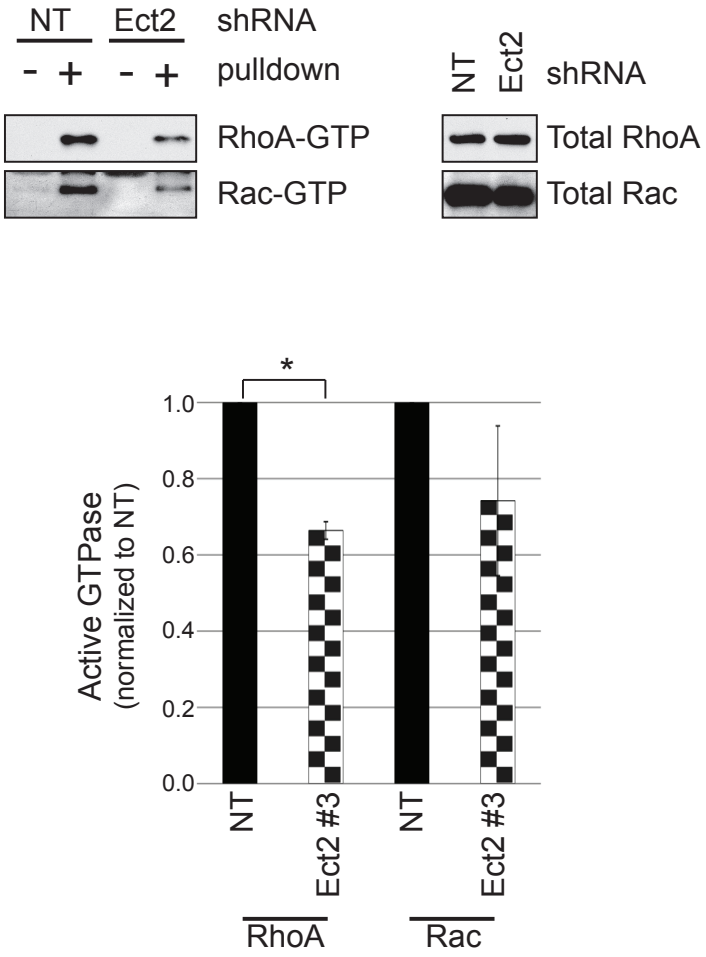


Figure 4.4. RhoGEF activity is required for Ect2-mediated transformed growth. *A. A catalytic DH-domain mutant (E428A/N608A) of Ect2 is impaired in RhoGEF activity on RhoA and Rac1 but not Cdc42.* Ectopic expression of HA-tagged wild type (WT) but not mutant Ect2 increased active RhoA-GTP or Rac1-GTP in whole cell lysates, as shown by pulldown assays using the Rho binding domain (RBD) of effectors that selectively bind the active, GTP-bound form of each GTPase (293T cells; anti-HA, 1: 1 000 clone 3F10 Roche, Indianapolis, IN, USA; anti-RhoA, 1: 1 000 clone 67B9 Cell Signaling, Danvers, MA, USA; anti-Rac1, 1: 1 000 clone 23A8 Millipore; anti-Cdc42, 1: 500 clone B-8 Santa Cruz, Dallas, Texas, USA). In the same assay, overexpression of Ect2 with mutated nuclear localization signals (NLS) caused a dramatic increase in RhoA activity, but not Rac activity. Ect2 activation of Cdc42 was unaffected by either DH or NLS mutations. Percent activation is shown based on densitometry performed on each active GTPase blot and normalized to total GTPase (representative of n≥3).



B. Re-expression of WT or the GEF-deficient DH-mutant Ect2 in knockdown cells restores Ect2 levels to similar degrees. The shRNA-resistant mutants indicated above were stably expressed in the Ect2 knockdown OVCAR8 cells and immunoblotted with anti-HA antibody for ectopic Ect2 and with anti-Ect2 antibody for endogenous + ectopic Ect2. Tubulin served as a loading control. *C. Re-expression of WT but not GEF-deficient Ect2 rescues anchorage-independent growth*. Error bars represent SEM. The baseline of colony counts was considered to be those seen in cells where Ect2 was not knocked down (NT) and expression was "rescued" with vector-only (VO). Colony counts statistically significant from this baseline are marked with an asterisk * (p<0.05, with Bonferroni correction). The ability of ectopic Ect2 to rescue Ect2 knockdown was evaluated by comparison to cells expressing Ect2 shRNA#3 and "rescued" with vector-only (^, p<0.05, with Bonferroni correction; n=5).

D.



D. Ect2 knockdown results in decreased levels of active RhoA and Rac in whole cell lysate. Standard pulldown assays were performed using the Rho binding domain (RBD) of effectors that selectively bind the active, GTP-bound form of RhoA (Rhotekin-RBD) and Rac (PAK-RBD). Upper panel, representative pulldown; lower panel, average of all assays (n≥4). Error bars represent SEM and * represents $p<0.05$, based on a paired *t*-test in which values were log-transformed for normality.

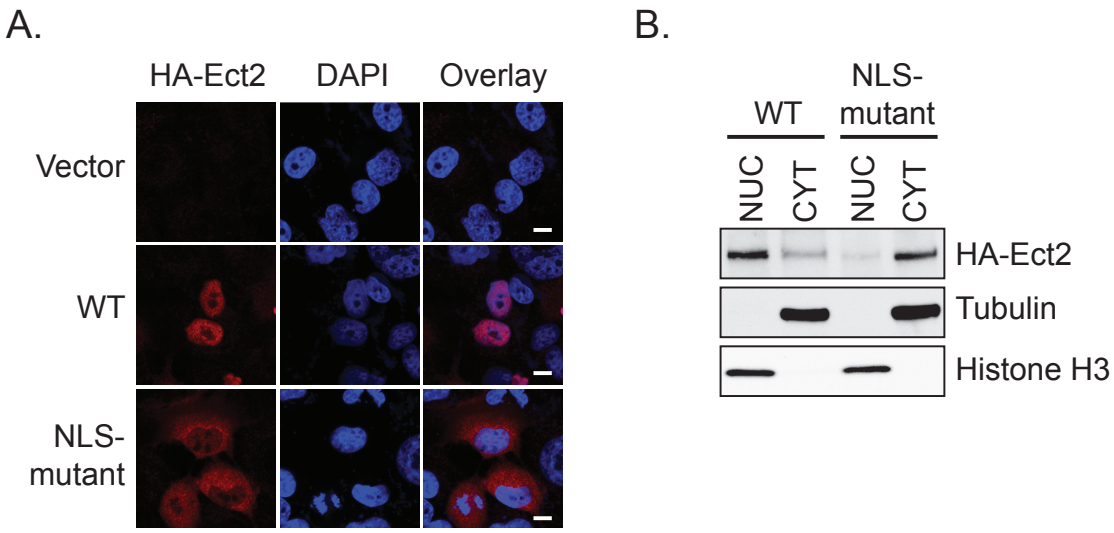
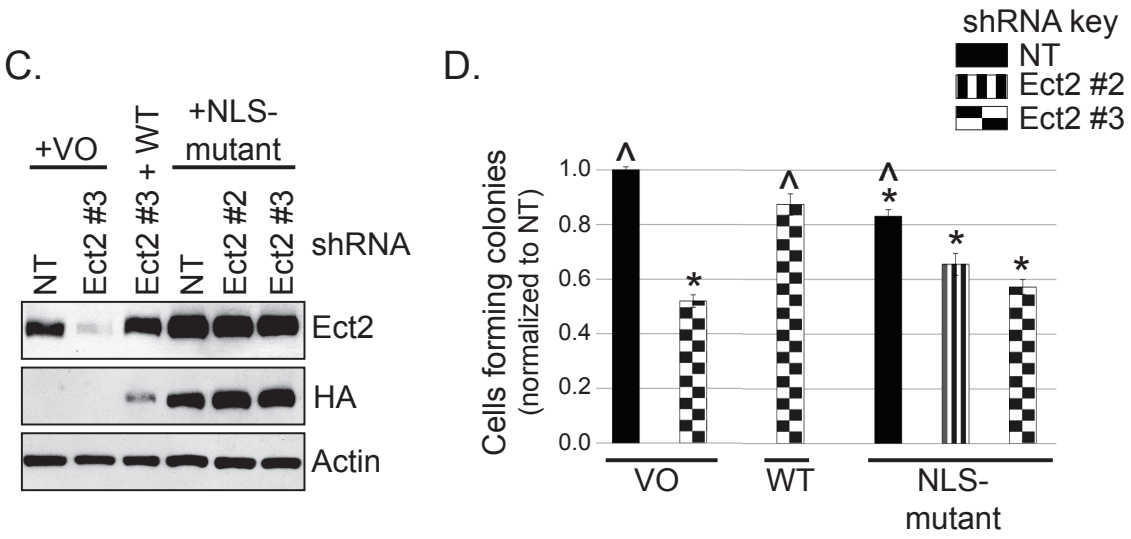


Figure 4.5. Predominantly nuclear localization is required for Ect2-mediated transformed growth. *A. Disruption of the nuclear localization signals (NLSs) of Ect2 impairs its nuclear localization compared to WT Ect2.* Localization of an Ect2 NLS-mutant (R348,349,350,370,372A) in which both NLSs have been disrupted was determined by confocal immunofluorescence microscopy, probing for HA-tagged Ect2 (red, 1: 500 clone 16B12 Covance, Princeton, NJ, USA) and the nuclear marker DAPI (blue), n=3. Scale bars represent 10 microns. *B. NLS-mutant Ect2 is localized predominantly in the cytoplasm.* The shift in localization of Ect2 upon NLS mutation was confirmed by fractionating cells expressing either NLS-mutant or WT Ect2, and blotting for HA-tagged Ect2 (1: 1 000 Covance). Purity of each fraction was confirmed via immunoblot using anti-histone H3 antibody (1: 12 000 Ab1791 AbCam, Cambridge, MA, USA) as a nuclear marker and tubulin as a cytoplasmic marker, with equal amounts of the nuclear and cytoplasmic fractions loaded onto the gel. Average percentage of Ect2 in the nucleus was calculated from densitometry values. Whereas 72.3% of WT Ect2 was observed in the nucleus, only 10.4% of NLS-mutant Ect2 was nuclear (n=2).



C. NLS-mutant Ect2 restored Ect2 expression in knockdown cells to a higher degree than WT. The shRNA-resistant mutants indicated above were stably expressed in Ect2-knockdown OVCAR8 cells. Cell lysates were immunoblotted as in Fig. 4.4B except that actin served as a loading control. *D. Ect2 NLS-mutant failed to rescue colony formation in soft agar*. WT but not NLS-mutant Ect2 rescued the impairment of soft agar colony formation caused by Ect2 knockdown (n=5). Error bars represent SEM. Statistically significant differences are marked with * and ^ as in Fig. 4.4C.

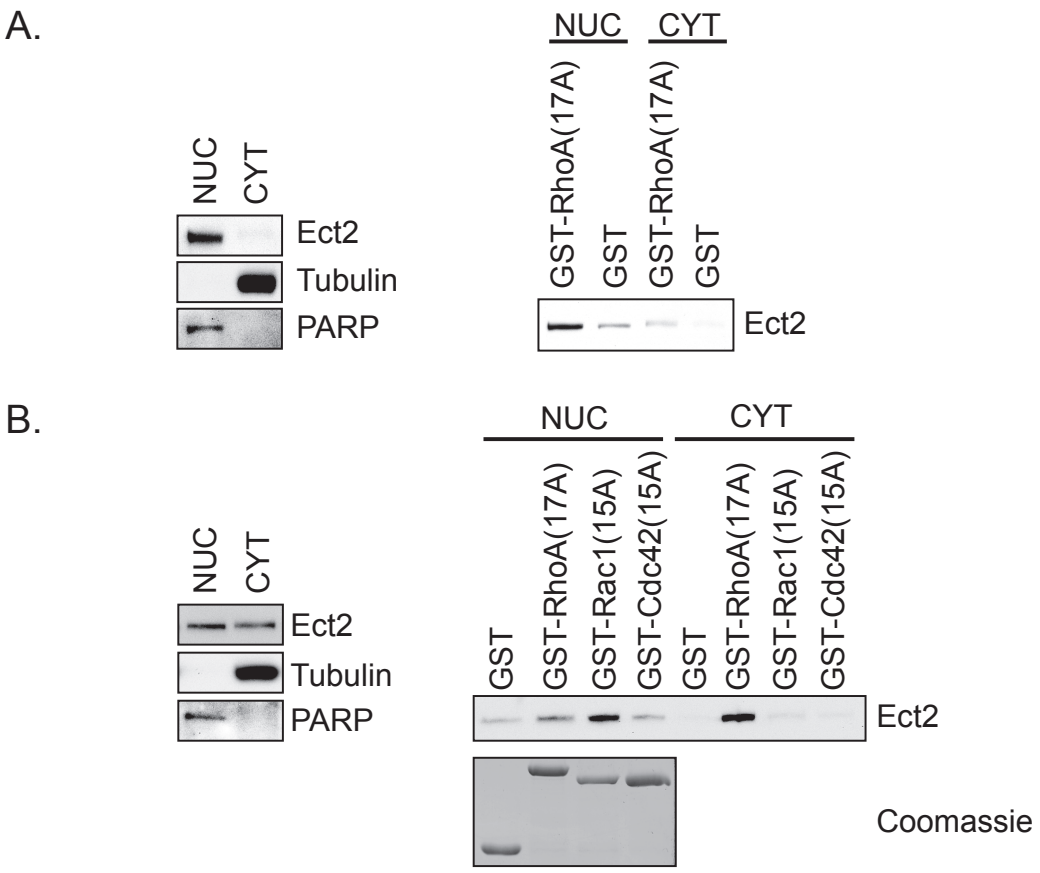
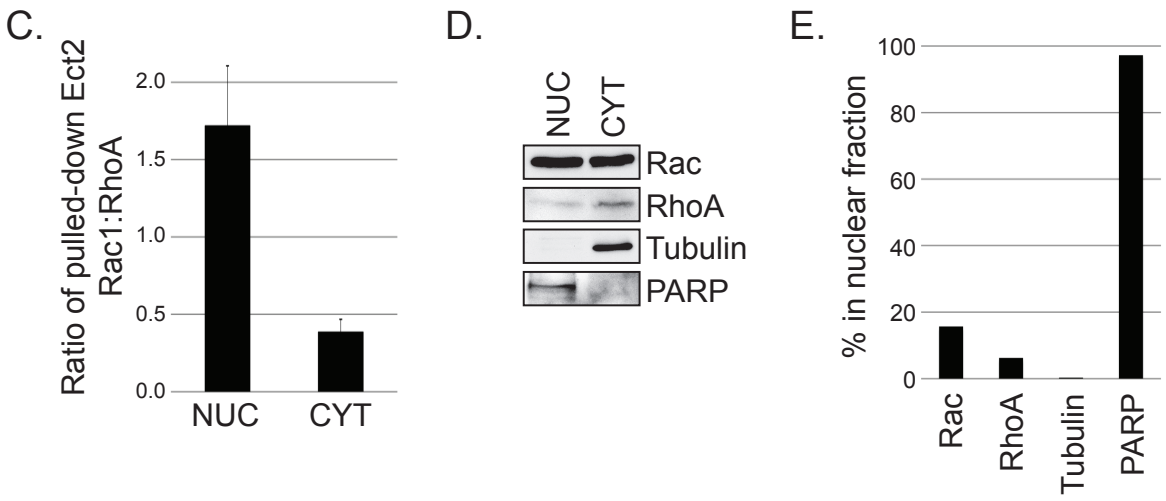


Figure 4.6. Some nuclear Ect2 is present in an active conformation that has enhanced specificity for Rac. *A. At least some nuclear Ect2 is present in an active conformation.* OVCAR8 cells were fractionated into nuclear (NUC) and non-nuclear (CYT) compartments. Purity of each fraction was confirmed via immunoblot using PARP (1: 2 000 clone C2-10 BD Bioscience, San Jose, CA, USA) as a nuclear marker and tubulin as a cytoplasmic marker, with equal amounts of the nuclear and cytoplasmic fractions loaded onto the gel. As expected, Ect2 was predominantly nuclear (left panel). Pulldowns (right panel) were then performed on equal concentrations of each fraction. GST-RhoA(17A) pulled down active Ect2 from each location. GST alone served as a control for nonspecific binding (n=5). *B. Ect2 preferentially interacts with Rac1 in the nucleus, and with RhoA in the cytoplasm.* As above, cells were fractionated, but here the pulldowns were performed on fractions distributed for equal expression of Ect2 as described in Materials and Methods (confirmed by immunoblot, left panel). Pulldown analyses using GST alone, GST-RhoA(17A), GST-Rac1(15A) or GST-Cdc42(15A) were performed on each fraction to determine the GTPase specificity of Ect2 within each fraction (right panel, n=8). Equal bead loading was confirmed by SDS-PAGE and Coomassie staining.



C. The ratio of Ect2 pulled down by Rac to Ect2 pulled down by Rho in the nucleus is greater than the same ratio in the cytoplasm. The amount of Ect2 pulled down by nucleotide-free Rac1 or RhoA in each subcellular compartment (as shown in Figure 4.6B) was quantified by densitometry, and the ratio of the amount of Ect2 pulled down by Rac1 to the amount pulled down by RhoA in the nuclear (NUC) or non-nuclear compartments (CYT) is shown. This ratio switches from >1 in the NUC to <1 in the CYT, indicating that Ect2 associates preferentially with Rac1 in the nucleus and with RhoA in the cytoplasm. The graph shows the average of all experiments (n=8), and error bars represent SEM. *D. Endogenous Rac and RhoA are present in the nucleus of OVCAR8 cells.* Cells were fractionated and immunoblotted for RhoA and Rac1. To allow for detection of the less abundant nuclear GTPases, 6.5x more protein was loaded from the NUC compartment, while still retaining the purity of each compartment. *E. A higher proportion of total Rac is nuclear, compared to RhoA.* Densitometry was performed on the immunoblot shown in Figure 4.6D and the percentage of nuclear protein was calculated as: (nuclear intensity) / (nuclear intensity + 6.5x cytoplasmic intensity) to account for the unequal loading of each compartment.

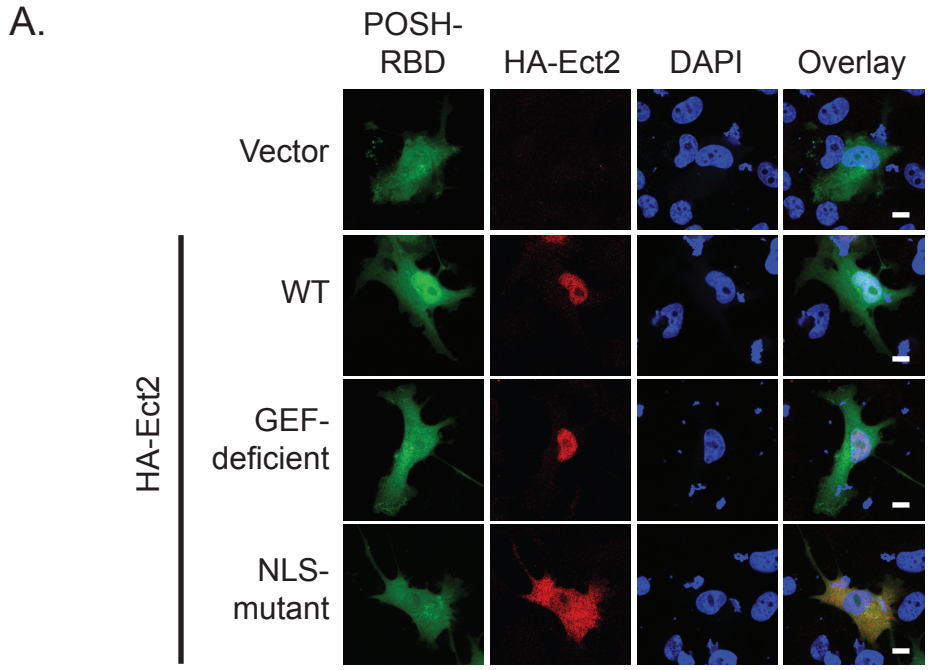
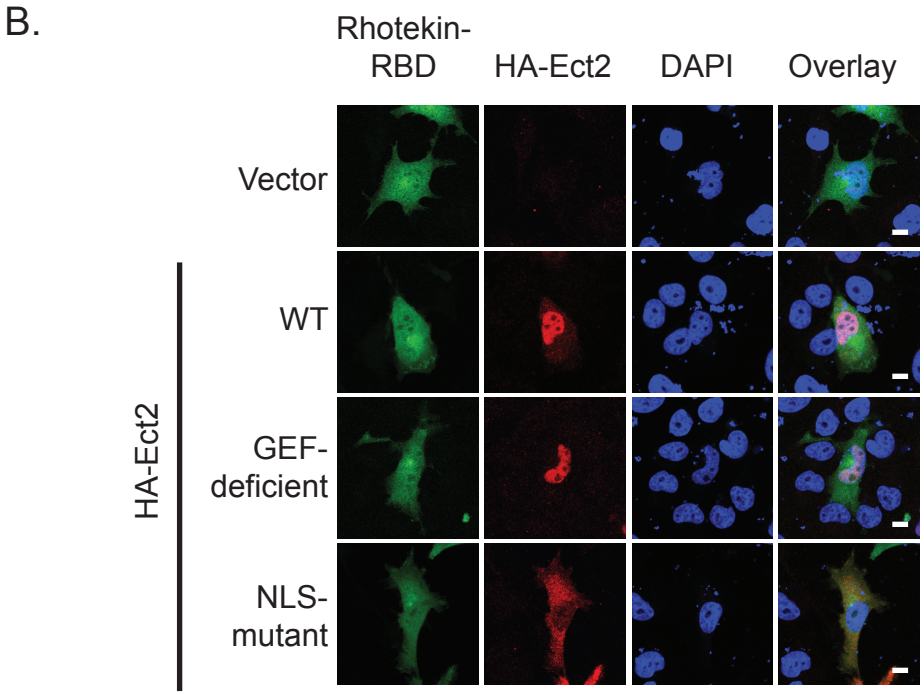
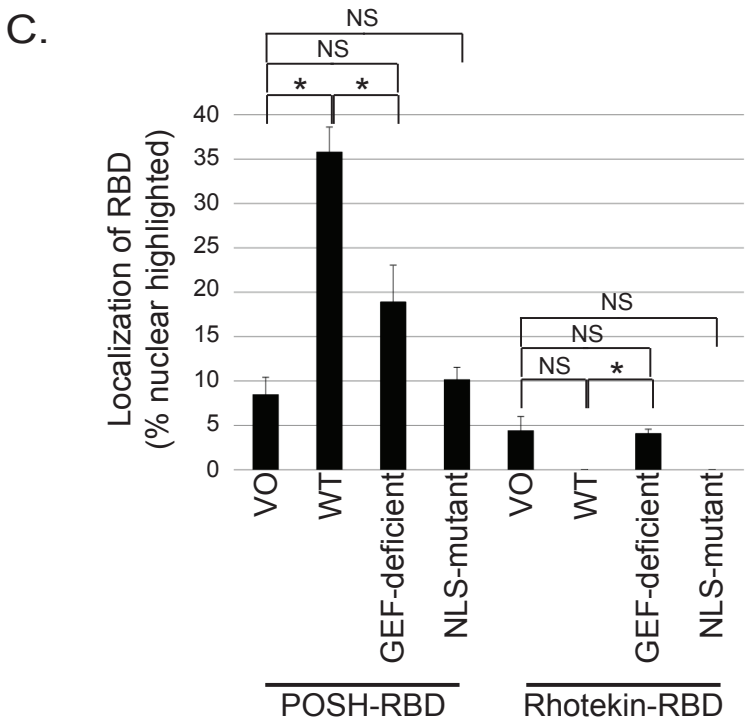


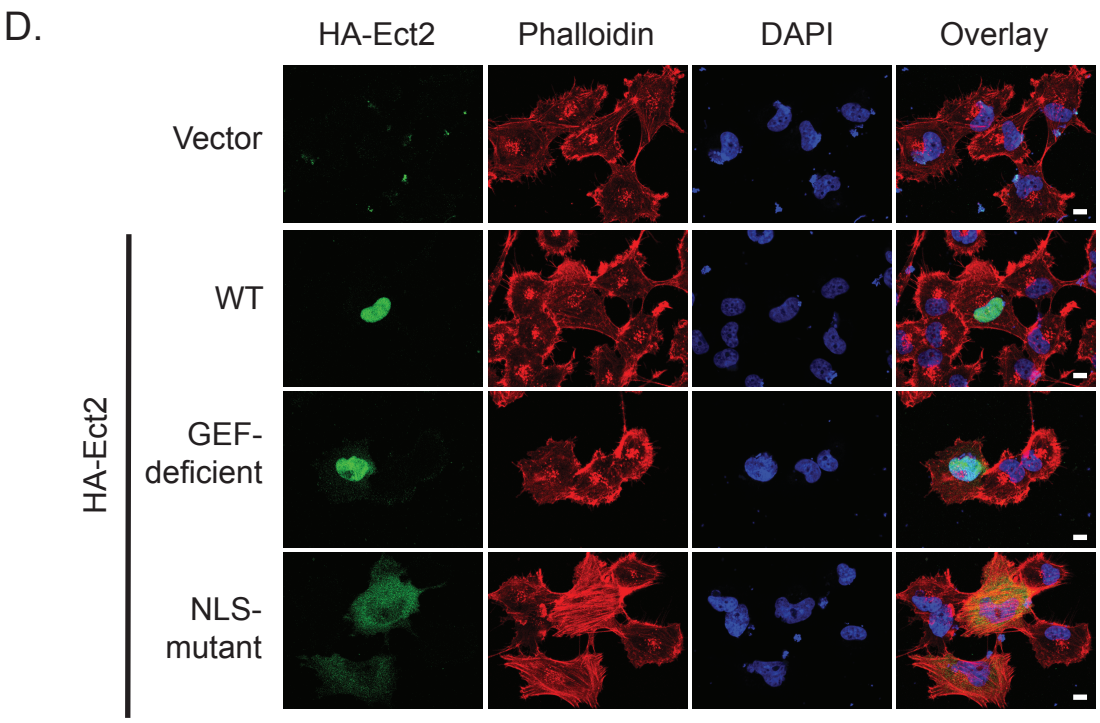
Figure 4.7. Ect2 recruits downstream effectors of Rac to the nucleus and initiates canonical RhoA signaling in the cytoplasm. To determine if Ect2 was capable of activating endogenous Rho GTPases in the nucleus, we examined GFP-RBD recruitment in OVCAR8 cells expressing empty vector versus exogenous HA-tagged WT, GEF-deficient or NLS-mutant Ect2. Confocal immunofluorescence microscopy was used to image cells expressing both HA-Ect2 (red) and each GFP-RBD (green); nuclei were stained using DAPI (blue). *A. Ect2 recruits POSH-RBD to the nucleus in a GEF-dependent manner.* POSH is a Rac-specific effector, and recruitment of POSH-RBD reveals endogenous Rac activation in the nucleus (n=7, with an average of 40 cells/condition in each replicate). Scale bars represent 10 microns.



B. Ect2 does not recruit Rhotekin-RBD to the nucleus. Rhotekin is a Rho-specific effector which was not detectably recruited (n=6, with an average of 30 cells/condition in each replicate); see Results section. Scale bars represent 10 microns.

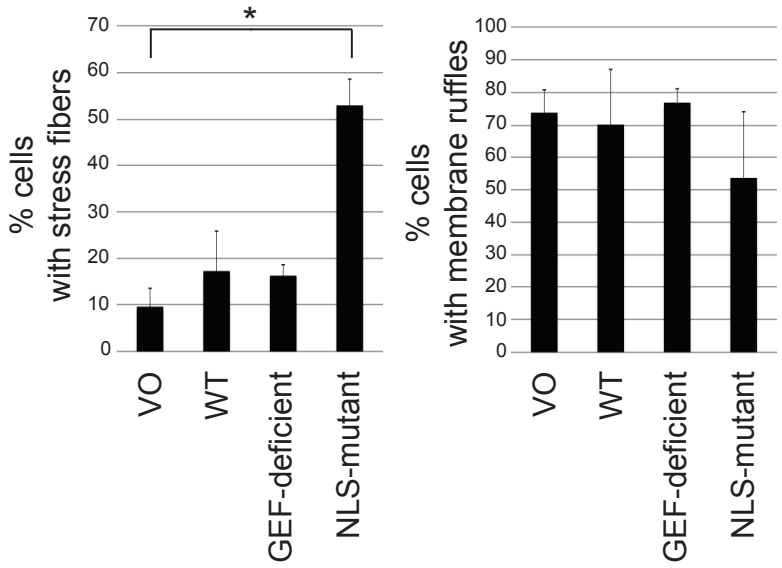


C. Ect2 recruitment of POSH to the nucleus is robust and statistically significant. Quantification of GFP-RBD localization was performed for each condition, as described in Materials and Methods. The percent of cells with nuclear-highlighted expression of the GFP-RBD is shown (*p<0.05 difference from vector with Bonferonni correction; error bars represent SEM).



D. NLS-mutant Ect2 enhances stress fiber formation but not membrane ruffling. Extra-nuclear Rho GTPase signaling was investigated by examining the actin cytoskeleton. Canonical RhoA signal transduction induces stress fiber formation, whereas Rac1 drives membrane ruffling. We compared organization of the actin cytoskeleton in OVCAR8 cells expressing empty vector versus exogenous HA-tagged WT, GEF-deficient or NLS-mutant Ect2. Confocal immunofluorescence microscopy was used to image cells expressing HA-Ect2 (green, Covance); actin was stained using Alexa Fluor 568-conjugated phalloidin (red, Invitrogen), and nuclei were stained using DAPI (blue).

E.



E. Cells expressing each construct as shown in panel D were quantified for presence or absence of stress fibers or membrane ruffles (n=3, with an average of 35 cells/condition in each replicate). Statistical significance was evaluated using Student's *t*-test, with the Bonferonni correction (*p<0.05); error bars represent standard error (SEM).

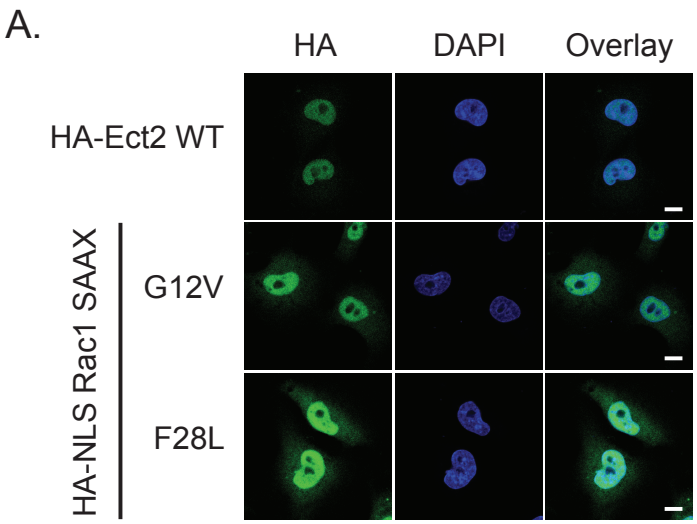
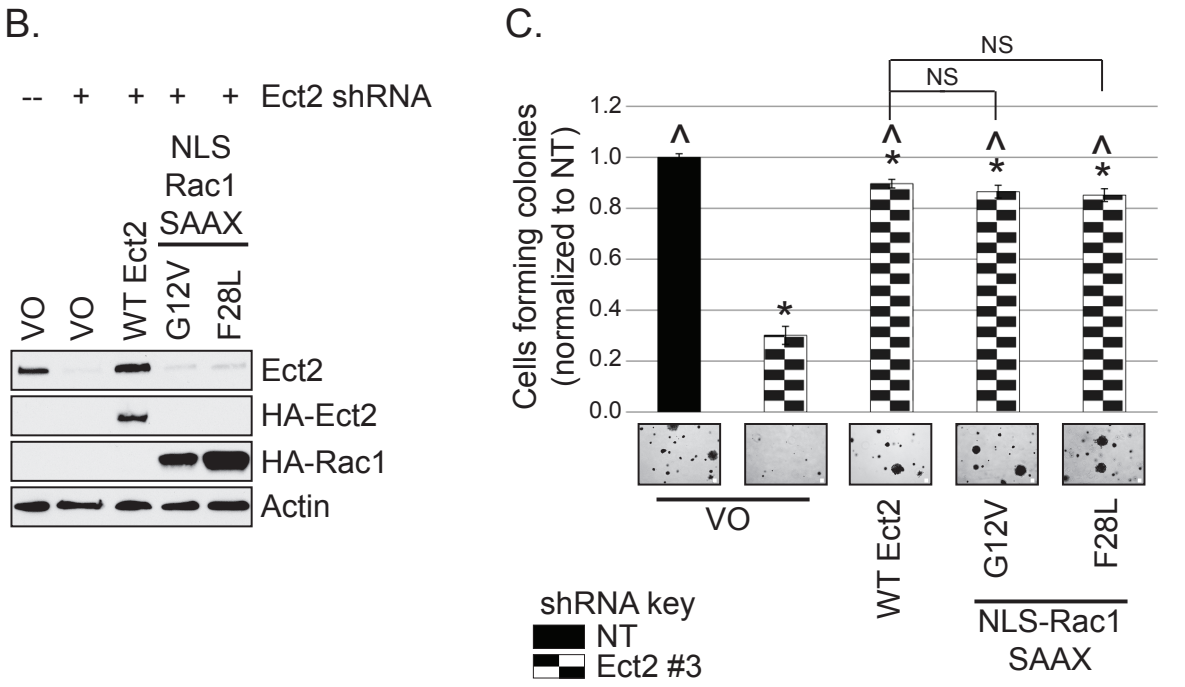


Figure 4.8. Nuclear Rac1 activity is sufficient to rescue defects in Ect2-mediated transformed growth. *A. Rac1 is targeted to the nucleus upon disruption of its CAAX box and addition of an NLS.* The localization of Ect2, NLS-Rac1-G12V-SAAX, and NLS-Rac1-F28L-SAAX was detected by confocal immunofluorescence microscopy, probing for HA-tagged Rac1 or Ect2 (green, Covance) and the nuclear marker DAPI (blue), n=3. Scale bars represent 10 microns.



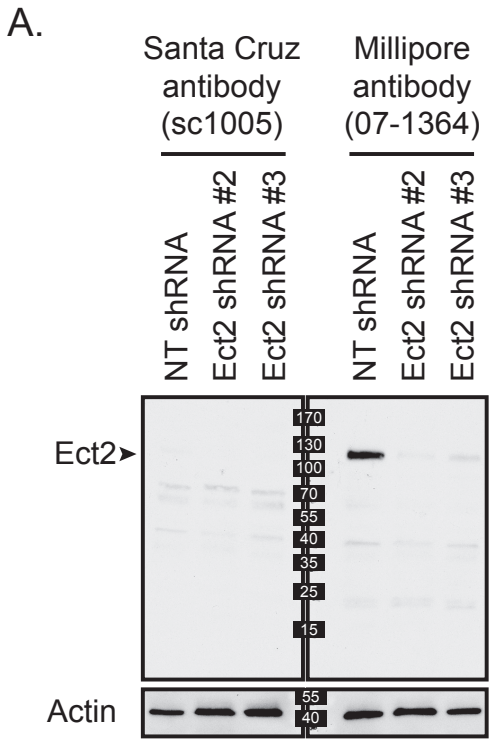
B. Stable expression of nuclearly targeted Rac1 mutants in Ect2 knockdown OVCAR8 cells. Lysate was immunoblotted with anti-HA (Covance) for ectopic Rac1 and Ect2 or with anti-Ect2 for endogenous + ectopic Ect2. Actin served as a loading control. *C. Rac1 mutant expression rescued the defect in anchorage-independent growth caused by Ect2 knockdown.* Both constitutively active and fast-cycling nuclearly localized Rac1 mutants were able to rescue the defect in soft agar colony formation caused by Ect2 knockdown, to the same extent as re-expression of WT Ect2 (n=3). Representative images (4x magnification) of colonies are shown. Scale bars represent 100 microns. Error bars represent SEM. Statistically significant differences are marked with * and ^ as in Fig. 4.4C.

Supplemental Table 4.1. Primer sequences used to generate NLS-mutant Ect2, GEF-deficient Ect2, and NLS-Rac1-SAAX. *A.* The Quikchange II site-directed mutagenesis kit (Stratagene) was used to create NLS-mutant and GEF-deficient (DH-mutant) Ect2 from shRNA-insensitive full length Ect2, using the following primers.

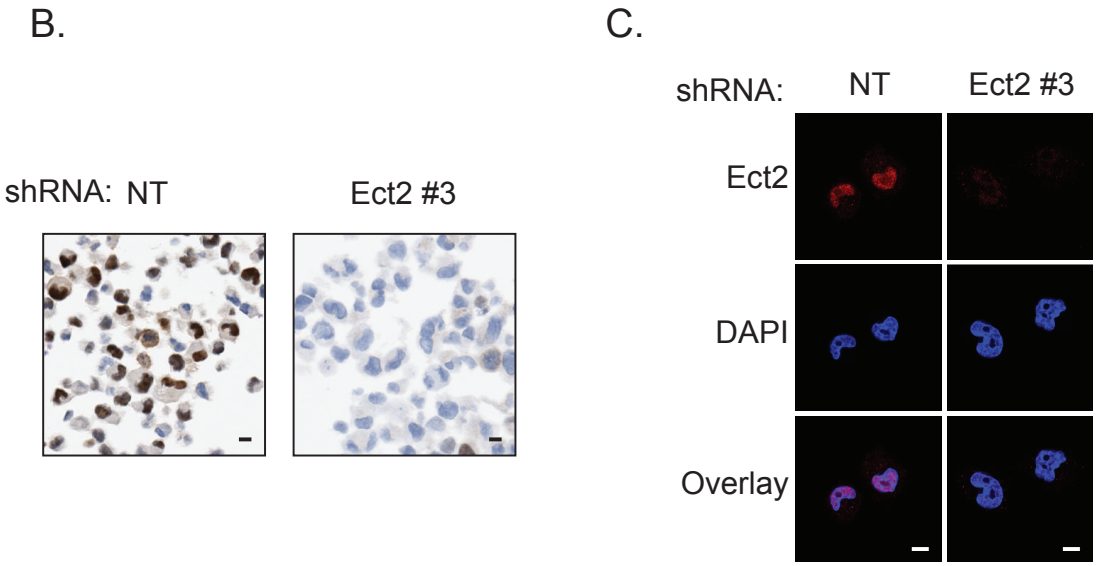
Domain	Mutation	Primer sequence
NLS	R348A, R349A, R350A	5'CTAACAGCAATCGCAAAGCAGCTGCTTTAAAAGAAA CACTTG3', 5'CAAGTGTTTCTTTTAAAGCAGCTGCTTTGCGATTGCT GTTAG3'
NLS	R370A, R372A	5'CACCATTTCCACCCGCTAAGGCCCCATCAGCTGAG3', 5'CTCAGCTGATGGGGCCTTAGCGGGTGGAAATGGTG3'
DH	E428A	5'GTGGCAAGTTGCAAAAGCGCTTTATCAAACTGAAAG 3', 5'CTTTCAGTTTGATAAAGCGCTTTTGCAACTTGCCAC3'
DH	N608A	5'GTAATGACGCATATTGCTGAGGATAAGAG3', 5'CTCTTATCCTCAGCAATATGCGTCATTAC3'

B. PCR was used to add the NLS of SV40T antigen to the N-terminus of Rac1(G12V) or Rac1(F28L) and to mutate their C-terminal CAAX sequence to SAAX (C189S). The mutant versions of Rac1 were then cloned into pFugW-HA-blasticidin.

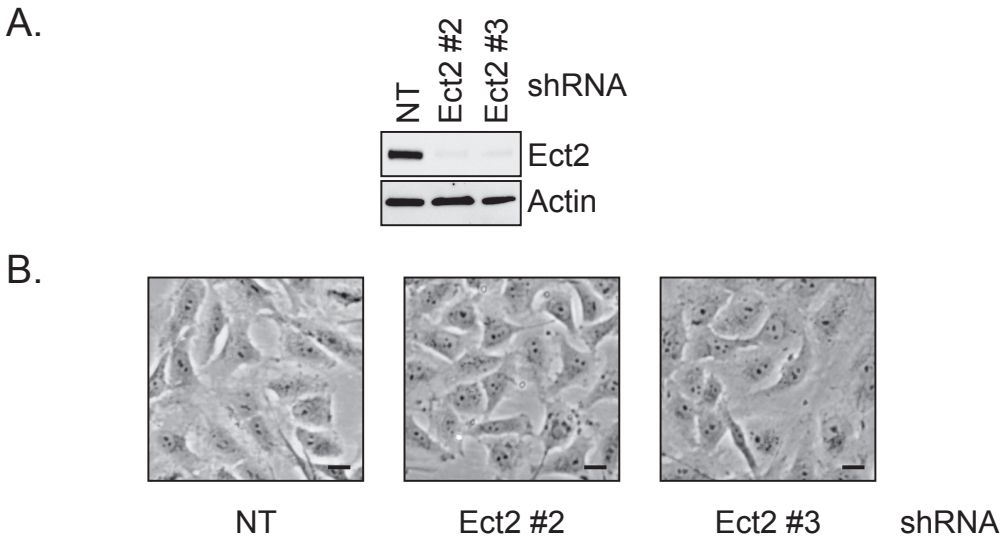
Addition/ Mutation	Primer sequence
SV40T antigen NLS	5'GCGGGATCCCCCAAGAAGAAGAGGAAGGTGATGCAGGCCAT CAAGTGTGTG3'
C189S	5'CGCGGATCCTCTAGATTACAACAGCAGGGATTTTCTCTT3'



Supplemental Figure 4.1. Specificity of Ect2 antibody in immunoblot, immunohistochemistry, and immunofluorescence analyses *A. Specific recognition of Ect2 by immunoblot analysis.* To validate the specificity of commercially available Ect2 antibodies, we first performed immunoblot analyses using lysate from OVCAR8 cells stably expressing either non-targeted (NT) or Ect2 shRNA. Each cell lysate was loaded into multiple lanes of the same gel, separated by standard molecular weight markers. Following protein separation by SDS-PAGE and transfer to PVDF-membrane, the membrane was then cut within the ladder and incubated with anti-Ect2 antibodies from either Millipore (1: 1 000, 07-1364) or Santa Cruz (1: 1 000, sc-1005). To assure equal exposure times, the membranes were then re-aligned at the ladder prior to development as a single unit. Equal loading was confirmed by stripping and re-probing the membrane halves for actin. The Millipore antibody was then selected for subsequent analyses.

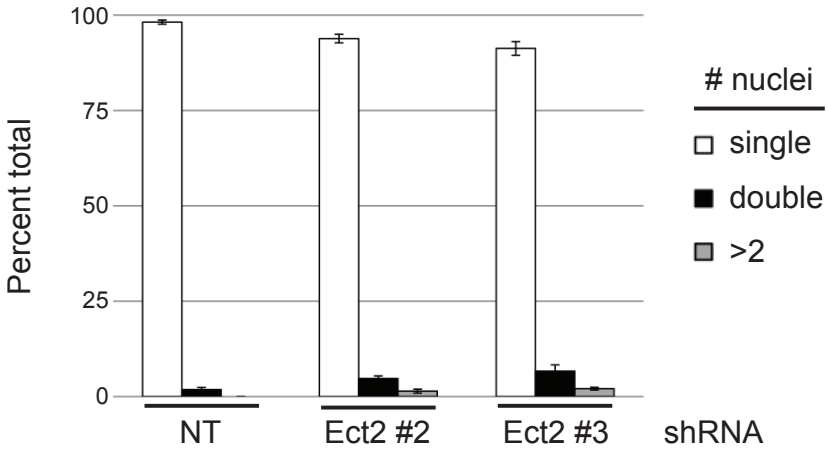


B. Specific recognition of Ect2 by immunohistochemistry (IHC). Validation and optimization of the Millipore antibody for use in IHC was performed using formalin-fixed, paraffin-embedded human ovarian cancer cell lines (shown: OVCAR8) in which Ect2 was knocked down stably. Conditions were determined such that staining was easily visible in NT shRNA cells, but in less than 5% of cells expressing Ect2 shRNA, as shown here. IHC was performed by the UNC-Translational Pathology Laboratory Core using Leica's (Buffalo Grove, IL, USA) Bond IHC Autostainer and detected with Leica's Bond Polymer Refine Detection kit. The optimal conditions determined for use on the TMA cores were a 1: 350 dilution of Ect2 antibody (Millipore) for 4 h, followed by heat-induced citrate base (pH6) antigen retrieval for 30 min. Nuclei were co-stained with haematoxylin. *C. Specific recognition of Ect2 by immunofluorescence.* Immunofluorescence was performed by staining the above-described cells, fixed in 4% paraformaldehyde, with the Millipore antibody at a dilution of 1: 1 000, then detected by secondary anti-rabbit antibody coupled to Alexa Fluor 594.



Supplemental Figure 4.2. OVCAR8 cells can undergo efficient cytokinesis upon Ect2 knockdown. *A. Ect2 knockdown is nearly complete in cell lines utilized for soft agar assays.* Equal amounts of cell lysate were loaded onto the gel and immunoblotted with anti-Ect2; actin served as a loading control. *B. The vast majority of cells have single nuclei regardless of their Ect2 status.* Bright-field images of the cells whose lysates are shown in panel A were taken at 10x magnification; scale bars represent 20 microns.

C.



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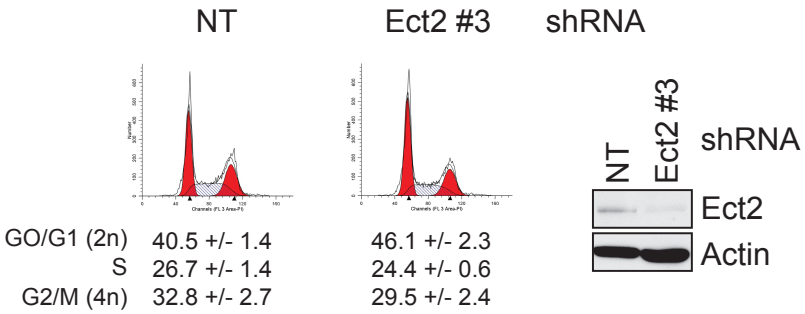
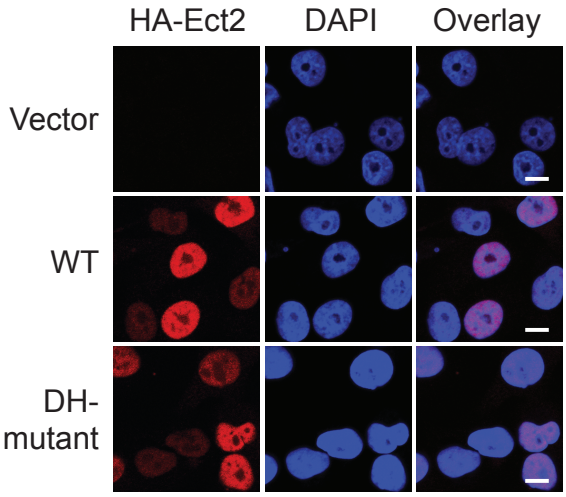
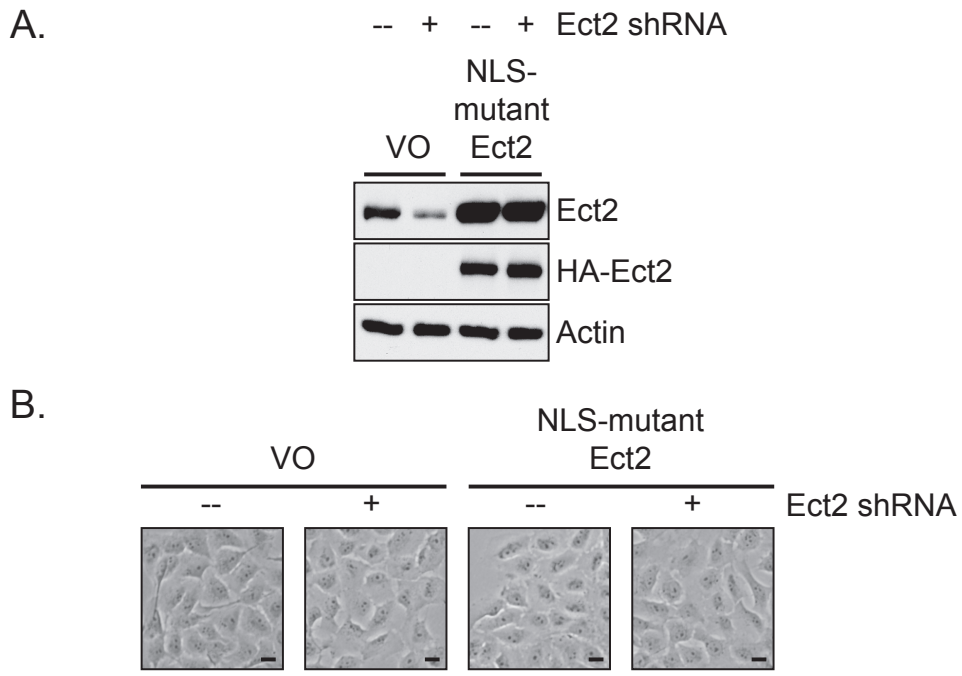


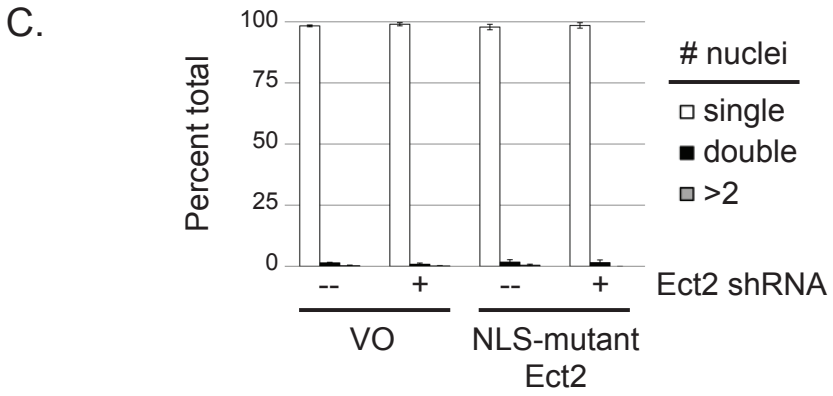
C. Cell nuclei were quantitated and cells were binned according to whether they displayed single, double, or >2 nuclei. The percentages of cells in each category are shown; error bars represent SEM (n=7, with >100 cells/condition in each replicate). *D. Ect2 knockdown does not alter the number of nuclei in suspended cells.* Flow cytometric analysis of OVCAR8 cells suspended in culture medium overnight did not reveal any increase in ≥4n DNA content upon Ect2 knockdown. Mod fit curves of cellular DNA content and cell cycle distribution for each condition are shown. The average percentage of cells in GO/G1 (2n) phase, S phase, or G2/M (4n) phase for each condition is shown, and "+/-" indicate SEM (n=4, 10 000 events/condition in each replicate).

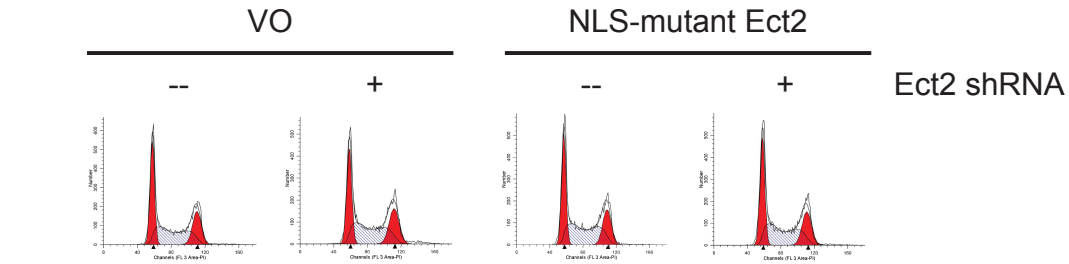


Supplemental Figure 4.3. GEF-deficient (DH-mutant, E428A/N608A) Ect2 localizes to the nucleus in a manner indistinguishable from that of WT Ect2. To confirm that the point mutations E428A and N608A rendering Ect2 GEF-deficient for RhoA and Rac did not have unintended consequences on Ect2 subcellular localization, we performed confocal immunofluorescence microscopy to detect the ectopic HA-tagged Ect2. Co-staining for the nuclear marker DAPI showed that both WT and GEF-deficient Ect2 localized predominantly in the nucleus (OVCAR8, n=3). Scale bars represent 10 microns.



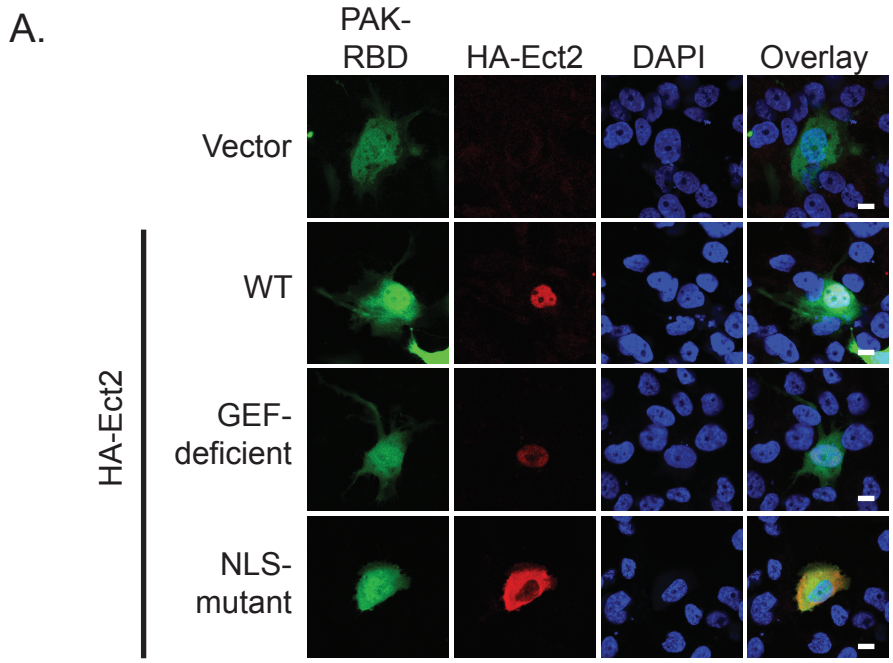
Supplemental Figure 4.4. Expression of NLS-mutant Ect2 does not affect cytokinesis of OVCAR8 cells. *A. The NLS-mutant was highly expressed in OVCAR8 cells.* Equal amounts of lysate from cells expressing NLS-mutant Ect2 in the presence of endogenous Ect2 (- Ect2 shRNA) or not (+ Ect2 shRNA) were loaded onto the gel; actin served as a loading control. *B. Expression of NLS-mutant Ect2 did not alter the frequency of cellular multinucleation.* Brightfield images of cells were taken at 10x. Scale bars represent 20 microns.



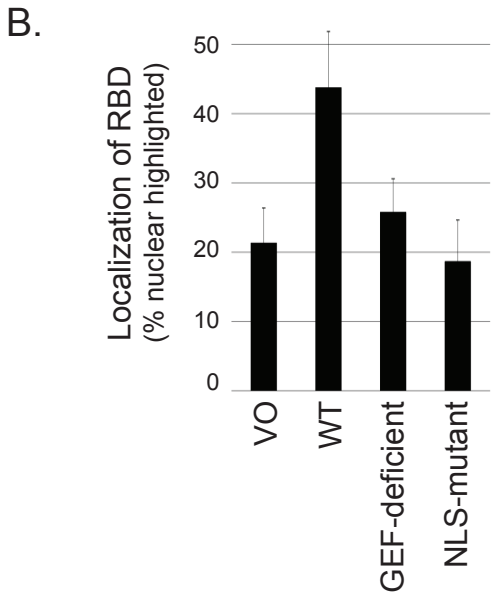


GO/G1 (2n)	39.3 +/- 1.7	37.3 +/- 2.3	39.0 +/- 2.1	40.5 +/- 2.02
S	21.4 +/- 0.6	24.5 +/- 0.6	20.3 +/- 0.4	21.5 +/- 0.7
G2/M (4n)	39.3 +/- 0.5	38.2 +/- 2.4	40.7 +/- 1.8	38.1 +/- 1.8

C. Overexpression of NLS-mutant Ect2 does not increase the percentage of multinucleated cells. To confirm that high expression of the NLS-mutant did not have unintended effects on cytokinesis, cells were binned according to display of single, double, or >2 nuclei (n=3, >100 cells/condition in each replicate). Percentages of each are shown; error bars represent SEM. *D. No changes in cellular DNA content / cell cycle were observed upon overexpression of NLS-mutant Ect2.* Mod fit curves from flow cytometry performed on the above-described cells are shown, with the average percentage of cells in GO/G1 (2n) phase, S phase, or G2/M (4n) phase shown for each stable cell line; "+/-" indicate SEM (n=3, 10 000 events/condition in each replicate).



Supplemental Figure 4.5. Ect2 activates Rac in the nucleus: Ect2 recruits PAK-RBD to the nucleus in a GEF-dependent manner. GFP-RBD recruitment assays were performed as in Fig. 4.7, but here using GFP-PAK1-RBD instead of GFP-POSH-RBD. PAK1 is downstream of both Rac and Cdc42 and multiple other Rho GTPases; its RBD was used to corroborate the Rac1 effector recruitment seen with the Rac-specific POSH-RBD in Fig. 4.7. Here, PAK-RBD was also recruited to the nucleus upon Ect2 overexpression, in a GEF- and NLS-dependent manner. These results are consistent with Ect2 activation of Rac in the nucleus. Since numerous GTPases in addition to those regulated by Ect2 can also regulate the localization of PAK-RBD, PAK-RBD recruitment is less apparent than for POSH-RBD. Representative images (*A*) and quantitation of the percentage of cells with GFP-PAK-RBD highlighting the nucleus in each condition (*B*) are shown (n=9, average of 35 cells/condition in each replicate); error bars represent SEM.



Representative images (*A*) and quantitation of the percentage of cells with GFP-PAK-RBD highlighting the nucleus in each condition (*B*) are shown (n=9, average of 35 cells/condition in each replicate); error bars represent SEM.

CHAPTER 5: RHOGEF INHIBITORS: IDENTIFYING THE GTPASE SELECTIVITY OF POTENTIAL MOLECULARLY TARGETED THERAPEUTICS[4]

OVERVIEW

Rho family GTPases (especially RhoA, Rac1, and Cdc42) are well-validated as potential cancer drug targets. They act as molecular switches within the cell, "on" while GTP-bound and "off" while GDP-bound, to regulate a variety of signaling pathways that drive tumorigenesis, invasion, and metastasis when misregulated. Unfortunately these GTPases are currently considered "un-druggable". Thus, research has begun to focus on upstream activators and downstream kinase effectors of Rho GTPases. Their upstream activators, Rho guanine nucleotide exchange factors (RhoGEFs), are frequently even more transforming than GTPases. Importantly, they are often aberrantly upregulated in human cancers and thereby drive excess Rho activity. Inhibitors of RhoGEFs have been designed; however, they have not been effective at therapeutic doses. Canonical RhoGEFs share a common catalytic DH/PH domain that promotes nucleotide exchange to enhance GTPase activation. We screened a library of 20,000 small molecules *in vitro* for their ability to inhibit DH/PH-enhanced nucleotide exchange on RhoA. We validated the *in vitro* specificity of hits for RhoA, Rac1, and Cdc42 GEF-induced exchange, using additional nucleotide

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[4]I wrote this chapter to summarize my studies of a novel series of putative RhoGEF inhibitors. We plan to submit it for publication when combined with the screening and *in vitro* work performed in the laboratories of Drs. Saïd M. Sebti (Moffitt) and John Sondek (UNC-Chapel Hill). The graphs shown are averages of many experiments performed by Kelly A. Gewain, Cercina Onesto (laboratory of Channing J. Der, UNC-CH), and myself.

exchange assays, and selected 3 putative RhoGEF inhibitors to examine *in cellulo*. RhoGEFs induce distinct, GTPase-specific cytoskeletal structures. Therefore by quantifying cytoskeletal responses to our compounds we determined the *in cellulo* specificity of each inhibitor towards GEFs that activate the three major classes of Rho family GTPases. Consistent with its *in vitro* selectivity, our lead compound, RPM811, specifically inhibited the cytoskeletal effects of RhoA at doses as low as 1 μM and also Rac1 signaling at 10 μM. To determine its efficacy against cellular transformation, we performed assays to measure anchorage-independent growth and observed an inhibition of transformed growth upon treatment with RPM811. Thus, we report here evidence of a RhoGEF inhibitor with anti-transformation activity and a dose-dependent selectivity for Rho GTPase signaling.

INTRODUCTION

The role of Rho GTPases in tumor cell transformation and invasion/metastasis has been well established and frequently reviewed (47, 186, 426). In brief, Rho GTPases are often overexpressed, misregulated, and (as recently discovered (168-170)) mutated in a variety of tumor types. Since Rho GTPases control a plethora of critical cellular functions, including cytoskeletal rearrangement, the cell cycle, gene expression, and cell polarity, this altered activity drives many tumor phenotypes. Thus, inhibition of these GTPases has been proposed as a mechanism of targeted chemotherapy (427). Yet GTPases have been considered "undruggable" due to their globular protein structure that lacks sufficient pockets for high-affinity binding to small molecules (428). Additionally, GTPases bind GTP with extremely high (picomolar) affinity that cannot be easily outcompeted by a drug (2).

Therefore, efforts to inhibit Rho GTPase activity have focused on upstream and downstream signaling molecules. Rho GTPases act as molecular switches within the cell. They are active and able to interact with downstream effectors while GTP-bound, and inactive while GDP-bound. This cycle is regulated by two types of proteins: GTPase activating proteins (GAPs) and guanine nucleotide exchange factors (GEFs). GTPases have low intrinsic GTPase activity, so GAPs catalyze their ability to hydrolyze GTP and inactivate the GTPase. GEFs activate the GTPase by enhancing release of bound nucleotide and allowing for the more prevalent cellular guanine nucleotide, GTP, to bind to the GTPase.

RhoGEFs are particularly attractive cancer drug targets for a variety of reasons. First, RhoGEFs themselves act as potent oncogenes (99). Many RhoGEFs were first identified in screens for proteins capable of inducing cellular transformation (190), and since then active RhoGEFs have even proven more transforming than active Rho GTPases when overexpressed in fibroblasts (46). Secondly, inhibition of RhoGEFs allows for more targeted control of cell signaling when compared to Rho GTPases. Currently 20 Rho GTPases have been identified, whereas approximately 80 RhoGEFs have been found to activate these GTPases (28, 85). GTPases control a wide variety of cellular functions, and inhibition of RhoGEFs allows for inhibition of GTPase activation due to specific stimuli. Finally, RhoGEFs are potentially more "druggable", since a few inhibitors directed towards specific RhoGEF activity have been identified (429-432). Still, no current inhibitor is effective at therapeutic doses. We set out to remedy this situation.

The largest family of RhoGEFs is the Dbl family. All Dbl family RhoGEFs contain a Dbl homology (DH) domain followed by a pleckstrin homology (PH) domain. The DH domain is the catalytic domain, which binds the GTPase to shift its conformation and induce

release of the nucleotide and its binding co-factor magnesium. Although this domain has low sequence identity across Dbl family RhoGEFs, its structure is well-conserved. The function of the RhoGEF PH domain varies from enabling proper localization to acting as an allosteric modulator of the DH domain (85). The crystal structure of the DH/PH domain of many RhoGEFs has been solved (95, 97, 433). Interestingly, the orientation of the DH and PH domains of LARG (RhoA GEF) and distantly related RhoGEFs Trio-N (RhoG/Rac GEF) and Dbs (Cdc42/Rho/Rac GEF) are very similar. It is likely that direct contact of the PH domain with the GTPase helps to promote nucleotide exchange by these GEFs (95).

LARG (leukemia-associated RhoGEF) was originally identified in acute myeloid leukemia cells as a fusion partner of MLL (mixed lineage leukemia). In these fusions it was expressed as an N-terminally truncated mutant that retained functional DH/PH domains (434) but lacked autoinhibitory domains. Overexpression of a distinct C-terminally truncated mutant of LARG (435), or full-length LARG in combination with activated Raf-1 (187) is sufficient to transform fibroblasts. LARG signaling is required for head and neck cancer cell growth/migration (436), prostate cancer cell migration/invasion (437), breast cancer cell invasion (438), and has been implicated in colorectal cancer cell migration and invasion (439). Additionally, G protein-coupled receptor (GPCR) signaling through Rho GTPases is known to cause cellular transformation, and LARG is a regulator of G-protein signaling (RGS) domain-containing RhoGEF that is driven by GPCR signaling to activate RhoA (440-442). Although there has been one report of LARG functioning as a tumor suppressor in breast and colorectal tumors (443), most data suggest that an inhibitor of LARG signaling would be clinically beneficial in tumor treatment in the appropriate context.

We screened a library of 20,000 small molecules for their ability to inhibit activation of RhoA induced by the DH/PH domain of LARG. From this initial screen 6 compounds were identified for further study *in vitro* and then 3 were studied *in cellulo*. RPM811 was identified as the most potent inhibitor. It showed significant effects on RhoA-driven cell morphology at a dose as low as 1 μM, the lowest effective dose that has been described *in cellulo* for potential RhoGEF inhibitors. RPM811 was also effective at inhibiting cellular transformation at 10 μM, suggesting that it may be a good prototype for future chemotherapeutics.

RESULTS

Screen and hits

The screen was performed in the lab of Dr. Saïd M. Sebti and will be further described prior to manuscript submission. In brief... To identify RhoGEF inhibitors, we utilized fluorescently labeled GTPγS to monitor GTP binding to purified RhoA in the presence of the DH/PH domain of LARG. We screened the ChemDiv library of 20,000 compounds for their ability to decrease fluorescent-GTPγS bound to Rho, as previously described with a smaller library (444). Six compounds were chosen for further study.

In vitro selectivity

The in vitro work was performed in the lab of Dr. John Sondek and will be further described prior to manuscript submission. In brief... RhoA, Rac1, and Cdc42 are the best studied Rho family GTPases. We chose three GEFs (LARG, Trio, and Dbs respectively) to represent

each of these signaling pathways *in vitro*, and performed radioactive exchange assays in the presence of drug. Each of the 6 compounds was able to inhibit GEF-induced nucleotide exchange on RhoA and Rac1 to varying degrees *in vitro*. Three compounds (RPM8111, RPM1052, RPM598b) were chosen for further study. These compounds showed selectivity towards inhibition of exchange from RhoA, yet still weakly inhibited exchange from Rac1. RPM811 had the most dramatic effect on RhoA nucleotide exchange, followed by RPM1052. RPM598b only weakly inhibited nucleotide exchange on RhoA *in vitro*.

RPM compounds dose-dependently inhibit Rho GTPase signaling in cellulo

The described *in vitro* assays demonstrate that each identified compound is capable of inhibiting nucleotide exchange from purified, unprenylated RhoA or Rac1 in the presence of truncated GEFs. While useful for initial characterization of the compounds, these assays do not address the ability of each drug to inhibit full-length protein and to regulate Rho GTPases signaling in a cellular context. To determine the efficacy and selectivity of the lead compounds *in cellulo*, we examined their effects on the actin cytoskeleton.

Each of the major Rho family GTPases controls distinct cellular morphologies. Microinjection of constitutively active RhoA into fibroblasts induces stress fiber formation (30), whereas Rac1 causes membrane ruffles (31), and overexpression of Cdc42 increases filopodia formation (32). To activate each pathway through upstream GEFs, we treated NIH 3T3 cells with lysophosphatidic acid (LPA), platelet-derived growth factor (PDGF), or bradykinin (BDK). Compared to vehicle-treated cells, the fraction of cells with stress fibers, lamellipodia, or filopodia increased approximately 2-fold in cells stimulated with LPA, PDGF, or bradykinin, respectively (Fig. 5.1A). This confirmed that the anticipated signaling

cascades were activated, as previously published (31-33).

To determine the effect of the inhibitors on stimulated Rho GTPase signaling, we scored cells stimulated with LPA, PDGF, or BDK for presence or absence of stress fibers, membrane ruffles, or filopodia respectively, after treatment with increasing concentrations of RPM compound (0, 0.3, 1, 3, 10, 30 μM). Each RPM compound had a dose-dependent effect on stimulus-induced stress fibers and lamellipodia, but did not affect BDK-induced filopodia formation (Fig. 5.1B). This indicates that these compounds inhibit RhoA and Rac1 signaling, but do not affect Cdc42 signaling *in cellulo*. All three compounds have similarly shaped dose response curves with respect to inhibition of stress fiber formation (Fig. 5.1B), indicating that they have similar potencies for inhibiting RhoA signaling. However, as can be seen from the difference in amplitude (Fig. 5.1B) and in accordance with the *in vitro* assays (data not shown), RPM811 and RPM1052 are more efficacious than RPM598b at inhibiting stress fiber formation. At submaximal doses, RPM811 is slightly more efficacious at inhibiting stress fibers than RPM1052 (Fig. 5.1B), and RPM811 is much more efficacious than RPM1052 at inhibiting lamellipodia (Fig. 5.1B). At a dose as low as 1 μM, RPM811 reduced the fraction of LPA-stimulated cells displaying stress fibers by a statistically significant margin ($p<0.05$), and when used at 10 μM RPM811 also significantly reduced PDGF-induced membrane ruffles (Fig. 5.1C & D). Still, treatment with 10 μM of RPM811 was not as effective at inhibiting Rac1 signaling as 5 μM of RPM947 (also referred to as EHT-1864), which was used as a positive control for inhibition of Rac1 signaling (445). RPM1052 was effective at inhibiting LPA-induced RhoA signaling when used at 10 μM; however, at the doses tested it did not significantly inhibit PDGF-induced Rac1 signaling (Fig. 5.1C & D). No toxicity was apparent upon addition of any of the compounds.

In addition to observing the effect of the RPM compounds on stimulated Rho GTPase activity, we also examined their effect on basal Rho GTPase signaling. Starved and un-stimulated cells were exposed to the same titration of RPM compounds (0, 0.3, 1, 3, 10, 30 μM) and scored for presence or absence of stress fibers, membrane ruffles, and filopodia. As expected, due to a lower signal-to-noise ratio in the absence of external stimuli, the effects of the compounds on basal signaling was less dramatic, and RPM1052 and RPM598b displayed minimal effects on the basal cytoskeleton (data not shown). However, RPM811 strikingly decreased stress fibers and membrane ruffles in unstimulated cells (Fig. 5.1E & data not shown), indicating again that RPM811 is the strongest inhibitor of RhoA and Rac1 signaling in our panel. Unexpectedly, RPM811 also dose-dependently increased filopodia formation (Fig. 5.1E). This suggests that the compound may enhance basal signaling to Cdc42. The effect was not observed in cells stimulated with BDK (Fig. 5.1B), indicating that it can be masked by a strong stimulus of Cdc42 activity.

RPM811 inhibits anchorage-independent growth of colorectal adenocarcinoma cells

The ultimate goal of the performed drug screen was to identify compounds that could be used in treatment of tumor cells via inhibition of Rho GTPase signaling. We identified RPM811 as our lead compound for inhibiting RhoA and Rac1 activity both *in vitro* and *in cellulo*. To determine if RPM8111 may be useful as an inhibitor of cellular transformation we evaluated the ability of tumor cells to grow anchorage-independently when treated with RPM811. Although non-transformed epithelial cells require adhesion to a surface prior to proliferation, many transformed tumor cells lack this checkpoint and proliferate even while suspended (410). Thus, soft agar assays are used to measure anchorage-independent growth

of tumor cells, and have been described as "the best *in vitro* correlate to *in vivo* growth potential" (446).

Colon tumors overexpress both RhoA and Rac1, and these GTPases regulate many aspects of cellular transformation (426). Therefore we utilized LS174T colon adenocarcinoma cells to perform soft agar assays. We compared anchorage-independent growth of these cells in the presence of DMSO vehicle, 1 μM, or 10 μM of RPM811. Although no effect was seen with 1 μM of RPM811, 10 μM of RPM811 significantly ($*p<10^{-7}$) inhibited anchorage-independent growth (Fig. 5.2). Thus at concentrations that attenuate both RhoA and Rac1 signaling, RPM811 inhibits cellular transformation.

DISCUSSION

Rho GTPases have been well validated as signaling molecules essential for tumor cell proliferation, invasion, and metastasis (47, 186, 426). Yet, attempts to inhibit their signaling have been largely unsuccessful due to their high affinity for GTP (not easily outcompeted) and globular structure (not easily bound) (2, 428). In 2004, the first Rho GTPase inhibitor, NSC23766, was identified. NSC23766 binds to Rac1 and inhibits its activation by Tiam1 or Trio (429). In the decade since then, only 5 other inhibitors have been identified that are capable of preventing Rho GTPase activation or Rho GEF activity (428, 430-432, 447). While most of these inhibitors bind to the GTPase or GEF with high affinity (nM Kd), to inhibit cellular activities they must be used at much higher doses. Our lead compound, RPM811, significantly reduced LPA-induced stress fibers at a dose as low as 1 μM, which is the most potent inhibition *in cellulo* of Rho signaling described to date.

Additionally, we have shown that RPM811 attenuates anchorage-independent growth of tumor cells. The only other Rho inhibitor previously examined in this assay is NSC23766 (429), which was used at a dose 2.5x higher than RPM811 to attenuate tumor cell growth. Rhosin (Rho activity specific inhibitor) (428) and Y16 (an inhibitor of LARG) (432) have also both recently been utilized to inhibit mammary sphere formation of breast cancer cells. Our experiments serve as further proof-of-principle that Rho GEFs are good cancer drug targets for some tumors. Interestingly, RPM811 was effective only at doses that inhibited both RhoA and Rac1 signaling. This indicates that, in these cells, inhibiting RhoA activity is not sufficient to attenuate anchorage-independent growth, and also suggests that, analogously to the recent emphasis on "multi-kinase" inhibitors, the less selective RhoGEF inhibitors may be more desirable for tumor treatment. The utility of inhibiting multiple Rho GTPases is consistent with previous studies. For example, when RhoA-specific siRNA and dominant-negative RhoA (inhibitory to multiple Rho GTPases) were each utilized in a variety of transformation assays, the less selective dominant-negative appeared to be slightly more effective at inhibiting anchorage-independent growth and significantly more effective at inhibiting migration/invasion (448). It has been previously proposed that RhoA is required for tumor initiation whereas RhoC is required for tumor metastasis (192). Certainly there are a variety of Rho family GTPases associated with numerous functions within tumor cells (47, 186, 426), and it is likely that by inhibiting multiple Rho GTPases we could simultaneously attenuate both tumorigenesis and metastasis/invasion.

Still, the relative lack of specificity of our lead inhibitor is perhaps surprising given the design of the screen used to identify it. In the screen, the isolated DH/PH domain of LARG and purified, unprenylated RhoA were used. However, *in vitro* the compound can

also inhibit the activation of purified Rac1 by the DH/PH domain of Trio, as well as Rac1-driven functions *in cellulo*. This suggests that the compound binds to a similar region of RhoA and Rac1 or a similar region of the two DH/PH domains. Sequence divergence is great among DH domains, but their structures are very similar (85). Therefore it is possible that the compound binds to a conserved fold within the DH domain. Although DH/PH conformation differs greatly among RhoGEFs (95), and inclusion of the PH domain theoretically generated a more selective screen, the DH/PH domain of LARG and Trio have been described as two of the "most similar" RhoGEFs with respect to the positioning of their DH/PH domains (85). Thus it is likely that, while RPM811 can block LARG and Trio activation of Rho GTPases, it cannot inhibit signaling from many other RhoGEFs. Indeed, BDK-induced filopodia were not affected by RPM811, suggesting that RPM811 does not inhibit Cdc42 activation by RhoGEFs.

Interestingly, RPM811 actually increased filopodia formation in starved cells, indicating that it can have some effect on Cdc42 signaling, possibly by loss of negative feedback. However, since it is masked by BDK stimulation, the activation is minor compared to that of stimulus induced Cdc42 activation, and is likely indirect. As recently reviewed by Guilluy et al. (449), signaling through a Rho GTPase frequently regulates the activity of other Rho GTPases. Thus, it is likely that inhibition of RhoA or Rac1 allows for signaling to Cdc42 that is normally inhibited by these other GTPases. Alternatively, multiple other Rho GTPases regulate filopodia formation (including RhoF, TC10, TCL, Wrch-1, and Chp (338)). If RPM811 directly or indirectly activates one of these GTPases, the effect could be observed in un-stimulated cells. Similarly, the effects on stress fibers and membrane ruffles may be due to inhibition of a variety of Rho GTPases. However, all

effects may well be due ultimately to direct inhibition of RhoA and Rac1 signaling, given that RPM811 inhibits nucleotide exchange on these GTPases *in vitro*.

It is interesting to compare the effects of RPM811 in stimulated and un-stimulated conditions. As would be expected, due to the larger signal-to-noise ratio, RPM811 inhibited stress fiber formation and membrane ruffles of stimulated cells more dramatically than un-stimulated cells. In other words, RPM811 had a larger effect on RhoA and Rac1 signaling in stimulated cells. In contrast, RPM811 had a larger effect on Cdc42 signaling in un-stimulated cells. Since tumor cells overexpress both RhoGEFs and Rho GTPases (47, 186, 426), it is tempting to speculate that their signaling would be more similar to the stimulated cells, whereas non-tumor cells would basally be in an un-stimulated state. Therefore it is possible that at certain doses RPM811 could selectively inhibit misregulated Rho GTPase signaling in tumor cells, while having little effect on normal cells. Indeed, even if normal Rho GTPase signaling is slightly attenuated, the model of oncogene addiction (450) suggests that their inhibition in tumor cells may be more detrimental than in normal cells. It is also promising that no toxicity was apparent in NIH 3T3 fibroblasts when exposed to 30 μM of each RPM compound. However, assays for cell death should be performed.

In summary, we have identified an inhibitor of Rho GTPase signaling that is capable of attenuating certain aspects of tumor cell transformation. Although RPM811 is the most potent *in cellulo* single Rho GTPase inhibitor currently described, continued modification to increase its potency will enhance its clinical relevance. Additionally, since Rho GTPase activating mutants have recently been identified in human tumors (168-170), it will be important to determine if RPM811 is capable of inhibiting mutant Rho GTPase signaling in addition to GEF-activated signaling.

MATERIALS AND METHODS

Cell culture

NIH 3T3 mouse fibroblasts were grown in DMEM-H (GIBCO/Invitrogen) supplemented with 10% calf serum (GCS, GIBCO). LS174T human colon adenocarcinoma cells were grown in RPMI-1640 medium (GIBCO/Invitrogen) supplemented with 10% fetal bovine serum (FBS, Sigma). All media contained 1% penicillin-streptomycin and cells were maintained at 37°C and 5% CO_2.

Actin cytoskeleton assays

The effect of each RPM compound on cell morphology was determined similarly to previously described protocols (445, 451). Briefly, NIH 3T3 cells were seeded onto glass coverslips (Fisherbrand) at a density of 40,000 cells/well in a 12-well plate (Corning). The following day, cells were serum-starved for 16 h in media containing 0.5% serum. During the final 4 hours of starvation, cells were treated with RPM811, RPM1052, or RPM598b at the concentrations of: 0, 0.3, 1, 3, 10, and 30 μM in DMSO. 15 minutes prior to fixation, cells were treated with LPA (40 ng/ml, Sigma-Aldrich), PDGF (5 ng/ml, Sigma-Aldrich), or BDK (100 ng/ml, Sigma-Aldrich) to stimulate formation of stress fibers, membrane ruffles, or filopodia, respectively. Cells were rinsed repeatedly in DPBS (GIBCO) prior to fixation and between each of the subsequent steps. Cells were fixed by application of paraformaldehyde (3.7%, Electron Microscopy Services) for 20 min. The cells were then permeabilized with 0.2% Triton X-100 (Sigma-Aldrich) for 5 min, and incubated with phalloidin-conjugated Alexa fluor 594 (1:40 dilution, Invitrogen) for 30 min. Coverslips were rinsed with ddH_20 prior to mounting on slides using Prolong Gold anti-fade mounting

medium (Invitrogen). Images of cells were taken using a LeicaSP2-AOBS confocal microscope with a 63x oil lens.

For each condition, 100 cells were scored (per repeat). Un-stimulated cells were scored for presence or absence of stress fibers, membrane ruffles, and filopodia. LPA-stimulated, PDGF-stimulated, and BDK-stimulated cells were scored for their respective structures. Dose-response curves were generated by normalizing the fraction of cells with each morphology to its corresponding non-drug treated control and averaging the repeats. The intermediate doses of 1 μM and 10 μM were chosen to compare the effect of RPM compounds to vehicle. Two-tailed Student's *t*-tests assuming unequal variance were used to determine statistical significance. Differences were considered significant when $p<0.05$. Results are the compilation of assays completed by at least two independent reviewers.

Anchorage-independent growth assays

Soft agar assays were performed similarly to our previous publications (321, 425). In short, cells were suspended in agar and plated in 6-well plates (Corning). The bottom agar (2 ml) consisted of 0.6% agar (BD Biosciences), 1X RPMI, 10% fetal bovine serum, and 1% penicillin-streptomycin. The top agar (1 ml) consisted of the bottom agar diluted to 0.4% agar with a single-cell suspension of 5,000 LS174T cells/well. RPM811 was added to both the top and bottom agar for a final concentration of 0, 1, or 10 μM. After approximately 2-3 weeks viable colonies were stained with 500 μl of 2 mg/ml 3-(4-5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich). The number of colonies was quantified using ImageJ software. The quantified colonies were normalized to vehicle-treated. A two-

tailed Student's *t*-test assuming unequal variance was performed on these values to determine if the observed effects were statistically significant. $p < 0.05$ was considered significant.

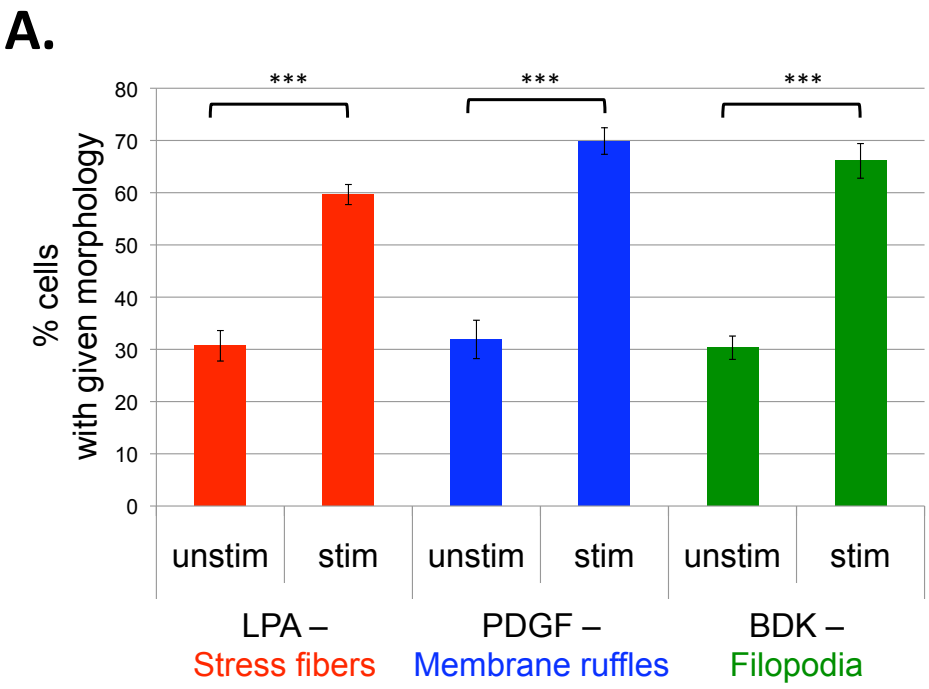
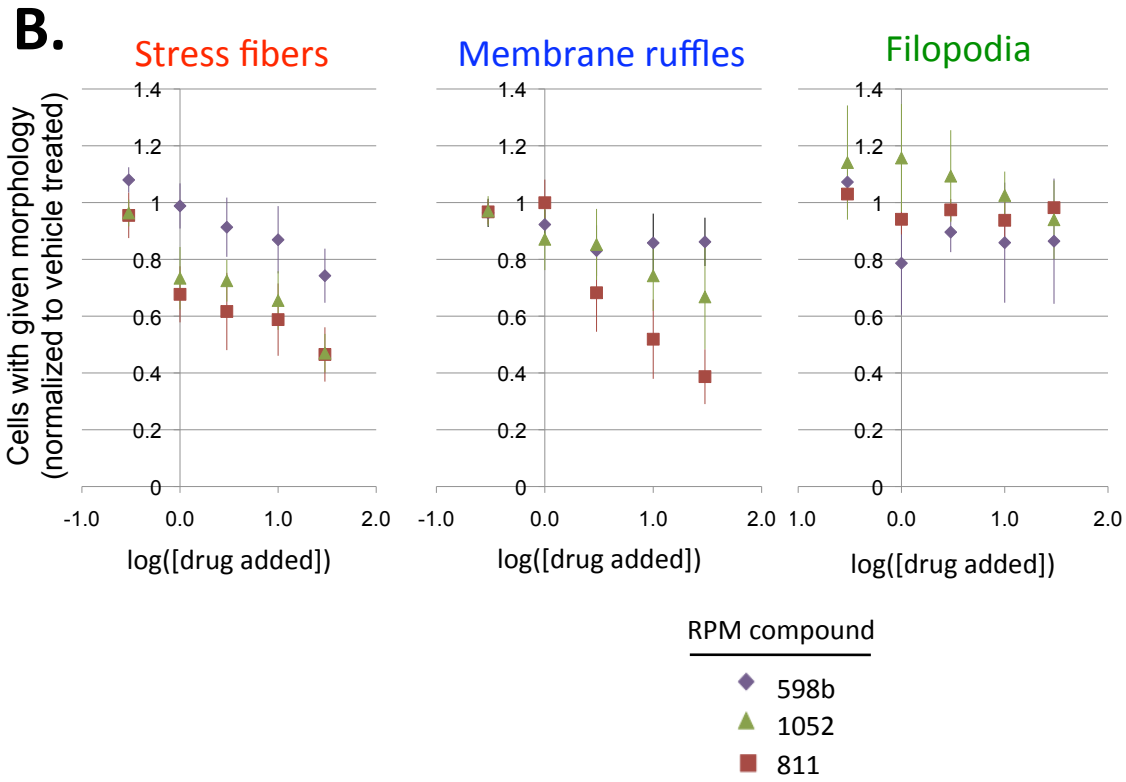
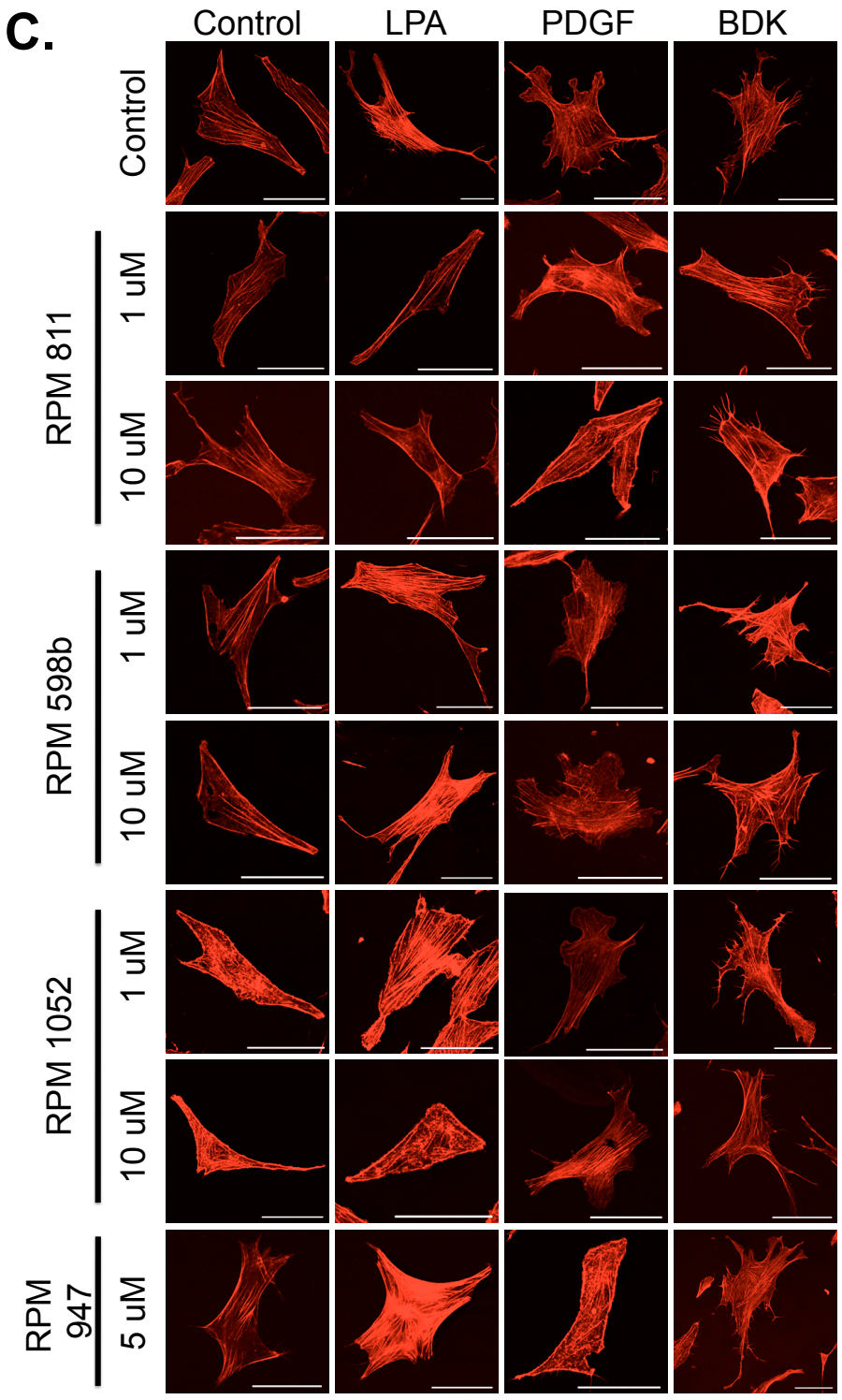


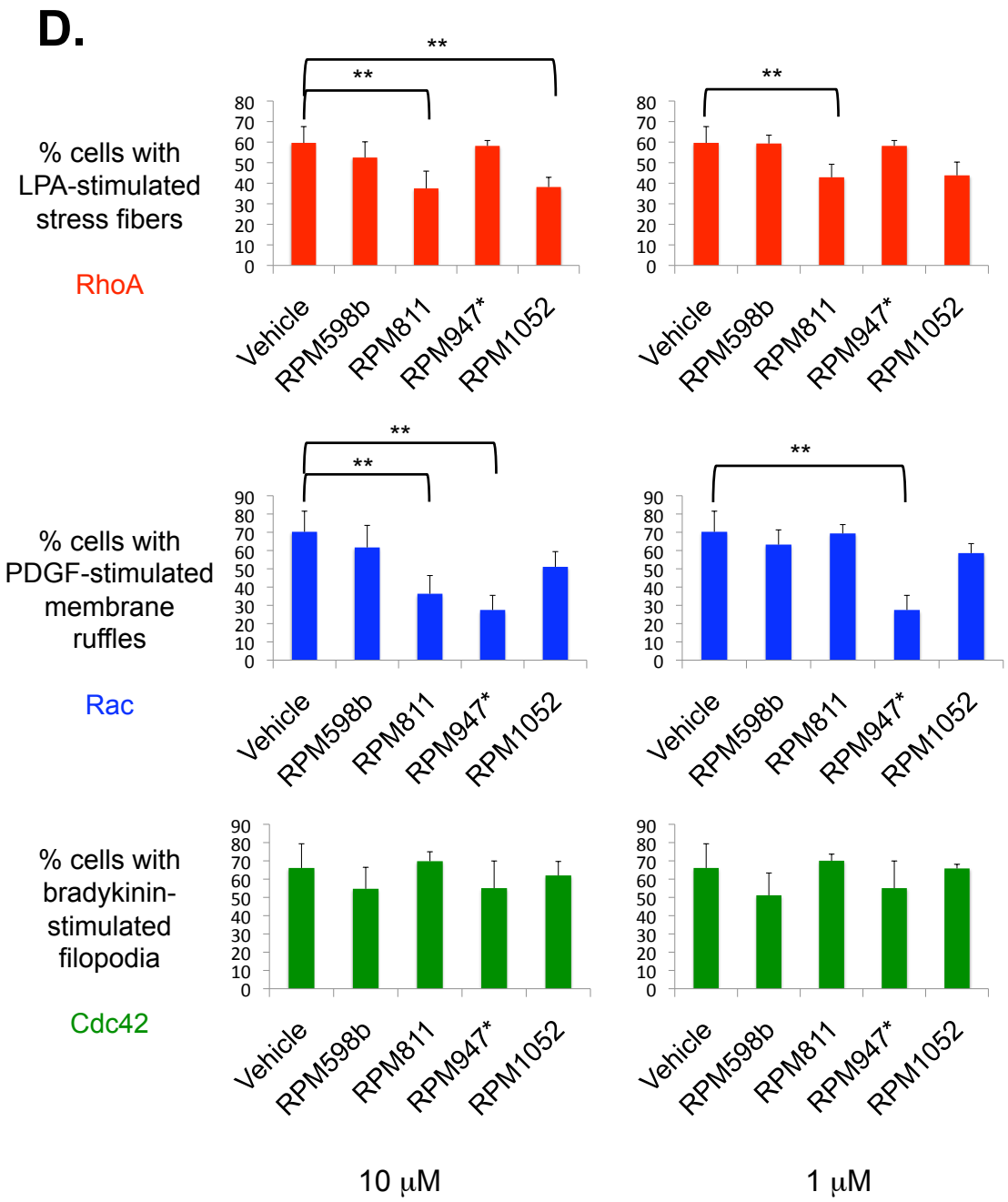
Figure 5.1. RPM compounds dose-dependently inhibit Rho GTPase signaling *in cellulo*. To determine the efficacy and selectivity of RPM compounds *in cellulo*, serum starved NIH 3T3 cells were treated with lysophosphatidic acid (LPA), platelet-derived growth factor (PDGF), or bradykinin (BDK) to stimulate RhoA, Rac1, or Cdc42 activity, respectively. Cells were then fixed and their actin structures were visualized using phalloidin-conjugated Alexa Fluor 594. These cells were quantified for presence or absence of stress fibers, membrane ruffles, and filopodia (described in Materials and Methods). All error bars represent standard error of the mean (SEM) and Student's *t*-test was used to calculate-values. *A. As expected, LPA induced stress fibers, PDGF increased membrane ruffling, and BDK caused filopodia formation.* The percentage of LPA-stimulated (stim) cells with stress fibers, PDGF-stimulated cells with membrane ruffles, and BDK-stimulated cells with filopodia is graphed and compared to vehicle (unstim) treated cells (n=16-19). ***$p<10^{-8}$



Prior to stimulation cells were treated for 4 h with a range of doses of RPM compounds (0, 0.3, 1, 3, 10, 30 μM). *B. RPM811 and RPM1052 dramatically reduced LPA-induced stress fiber formation and PDGF-induced membrane ruffling.* Separate dose-response curves are shown for LPA-induced stress fibers, PDGF-induced membrane ruffles, and BDK-induced filopodia. Dose-response curves were generated by normalizing the percentage of cells with a given stimulated morphology post-drug treatment to its corresponding stimulated, vehicle-treated control. The effect of RPM811, RPM1052, and RPM598b on each morphology is shown (n=3-6).

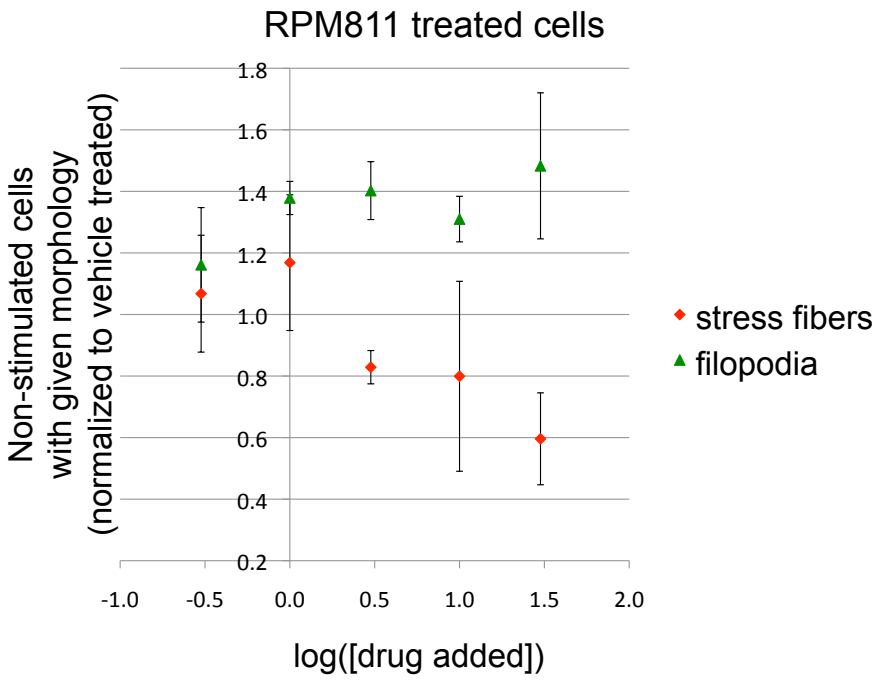


C. RPM811 inhibition of LPA-induced stress fibers is observed at doses as low as 1 μM, and RPM811 and RPM1052 both inhibit stimulated Rho GTPases at 10 μM. Representative images of stimulated (LPA, PDGF, or BDK) and un-stimulated cells treated with 0, 1 and 10 μM of each RPM compound are shown. Images show the actin cytoskeleton as stained with phalloidin-conjugated Alexa Fluor 594. Cells were imaged via confocal microscopy. The scale bars represent 50 microns.



D. RPM811 and RPM1052 effects are statistically significant. The average percentage of LPA-, PDGF-, or BDK- stimulated cells with the expected morphology is graphed separately for treatment with 1 or 10 μM of RPM compounds, to show graphically the conditions imaged above (n=3-6). *RPM947 (also referred to as EHT-1864) was used at 5 μM as a positive control for inhibition of PDGF-induced membrane ruffling. **p<0.05.

E.



E. RPM811 reduces stress fibers and enhances filopodia formation of non-stimulated cells in a dose-dependent manner. As with stimulated cells, non-stimulated cells were also subjected to the panel of RPM compounds at a range of doses. The effect of RPM811 on basal stress fibers and filopodia is shown.

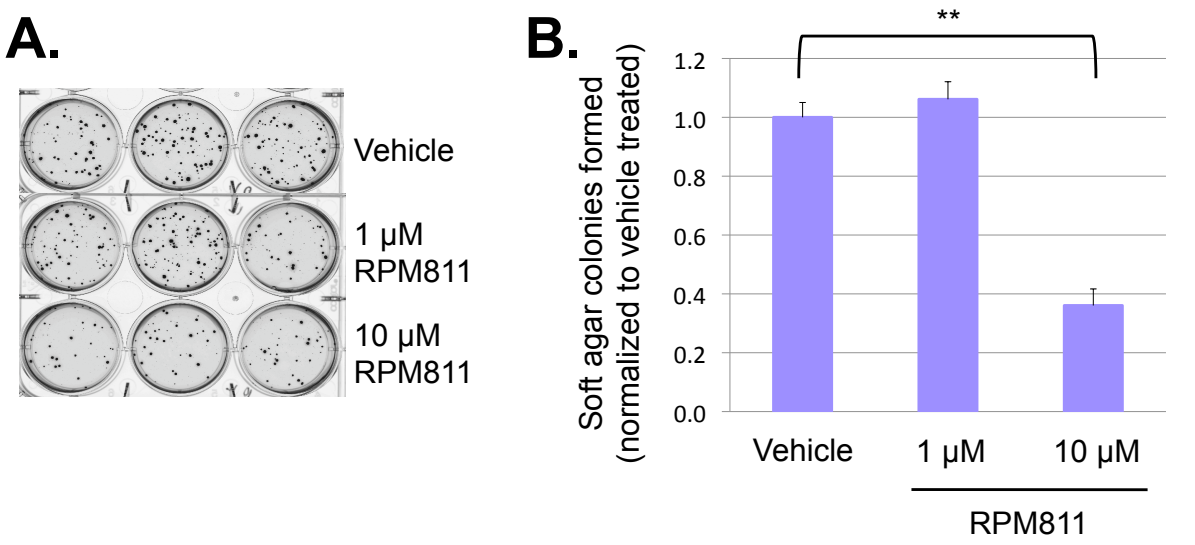


Figure 5.2. RPM811 inhibits anchorage-independent growth of colorectal adenocarcinoma cells. To determine the effect of RPM811 on anchorage-independent growth of tumor cells, soft agar assays were performed on LS174T cells in presence of 0, 1, or 10 μM of RPM811. Colonies of metabolically active cells were stained with MTT, scanned, and counted using ImageJ. A representative scan is shown (*A*), and the average of all assays is graphed (*B*, n=4 in triplicate). Student's *t*-test was used to calculate statistical significance, **$p<10^{-7}$. Error bars represent SEM.

CHAPTER 6: CONCLUSIONS AND FUTURE DIRECTIONS

CONCLUSIONS

My graduate work has focused on the RhoGEF Ect2 and its role in ovarian cancer cells. I have determined that Ect2 is an important regulator of ovarian cancer cell transformation, as it is in other tumor types (137, 138, 145-148, 159). More importantly, my work has also uncovered a novel mechanism by which Ect2 can drive cancer cell transformation, that does not require cytoplasmic mislocalization as previously thought (111, 137). I have shown that Ect2 can act as a GEF from within the nucleus, and that this activity is required for transformation. Further, I found that nuclearly localized, active mutants of Rac1 can rescue the defects in anchorage-independent growth caused by Ect2 knockdown. While others have described the existence of a pool of nuclear Rac1 (63, 64, 67) and its role in mitosis (62) and transcription (44, 69, 70), my work now also implicates nuclear Rac1 (and possibly other nuclear Rho family GTPases) in cellular transformation. I have also uncovered a change in Ect2 specificity between the nucleus and cytoplasm that is the first description of an intrinsic difference in GEF specificity depending on the subcellular compartment to which it is localized.

My work on Ect2 suggests that utilizing kinase inhibitors to prevent cytoplasmic mislocalization of Ect2 is not a tractable anti-cancer therapy for the treatment of ovarian cancer. Instead, RhoGEF inhibitors themselves may be more useful, although the extent of RhoGEF "druggability" is currently unclear (452). As part of a collaboration to identify

novel RhoGEF inhibitors, I validated lead compounds for specificity towards distinct Rho GTPase pathways and their ability to inhibit transformed cell growth. Further modifications will be necessary to create an inhibitor that is useful at clinically relevant doses.

Additionally, I described in detail the use of effector recruitment assays to monitor the localization of GTPase activity, an important technique that I used to visualize where within the cell Ect2 activates endogenous Rac1. Finally, I reviewed the literature on C-terminal phosphorylation as an alternative regulator of Rho GTPase localization and activity. Still there are many questions remaining with regard to the effects of subcellular localization on Rho GTPases and their regulators. I focus on questions about Ect2 and Rac1 activity below.

FUTURE DIRECTION 1 – HOW ARE LOCALIZATION AND SPECIFICITY OF ECT2 REGULATED?

As described in Chapter 4, I have observed that the specificity of Ect2 changes with its subcellular localization. Nuclear Ect2 is capable of activating both Rac1 and RhoA, while cytoplasmic Ect2 is more specific to RhoA. This is likely due to intrinsic differences between the pools of Ect2 present in the nucleus and in the cytoplasm, as it is apparent using bacterially expressed, nucleotide-free GTPases to pull down the Ect2 capable of acting on each GTPase in each compartment. The ratio of GTPases available in each compartment may further amplify the effect *in cellulo*. What regulates this correlation between specificity and localization is unknown. Below I speculate on how these properties are determined.

1.1 Does phosphorylation regulate the specificity of Ect2?

When the GEF activity of Ect2 was first identified *in vitro*, Tatsumoto and colleagues showed that VHR phosphatase treatment of Ect2 inhibited Ect2-induced nucleotide exchange on Rac1(76). They then proposed that Ect2 must be phosphorylated to catalyze nucleotide exchange (76). While this was assumed to be the case not only for Rac1 but also for RhoA and Cdc42, only Rac1 was tested experimentally, and the specific phosphorylation site(s) were not determined. Since then phosphodeficient mutants of Ect2 have been used to demonstrate that T327 and T814 are required for Ect2 to optimally activate Rac1 (134, 161). And Dr. Kent Rossman has observed that bacterially expressed, i.e., unphosphorylated, full length Ect2 can activate RhoA but not Rac1 *in vitro* (Dr. Kent Rossman, personal communication). Based on these results, the simplest hypothesis is that phosphorylation of Ect2 is required for activation of Rac1 but not RhoA, and therefore phosphorylation of Ect2 drives the specificity of Ect2 towards Rac1 versus RhoA. Yet, Ect2 T412 (which is phosphorylated by Cdk-1, see Introduction) has been shown to be necessary for optimal activation of RhoA by Ect2 (134). And it has been proposed, although not convincingly proven, that phosphorylation of Ect2 at T341 releases autoinhibition of Ect2, allowing it to activate Rho GTPases (115). Based on this literature, it appears that there are many phosphorylation sites that can regulate the ability of Ect2 to catalyze nucleotide exchange. In light of my work, it is possible that there are also multiple states of Ect2 activation, perhaps conformations in which Ect2 is most active on Rho and others in which it preferentially activates Rac. Therefore we should consider the role of phosphorylation in determining the specificity of Ect2.

The studies described above examined the role of phosphorylation of certain Ect2 residues on activation of individual GTPases; each was very focused on a single GTPase and did not examine if/how Ect2 phosphorylation may regulate specificity. To determine if the Ect2 that activates Rac1 is differentially phosphorylated from the Ect2 that activates RhoA, I used bacterially expressed nucleotide-free GTPases to pulldown Ect2 capable of activating each of these GTPases. The nucleotide-free GTPases interact strongly with GEFs capable of activating them (411-413), and I have previously observed that nuclear Ect2 is pulled down by nucleotide-free Rac and Rho, while cytoplasmic Ect2 is pulled down only by nucleotide-free Rho (Chapter 4). Although mobility shifts have been previously described for Ect2 phosphorylation changes during the cell cycle (76, 125). I did not observe any mobility differences between Ect2 pulled down by Rac when compared to Ect2 pulled down by Rho using standard SDS-PAGE techniques.

Addition of Zn^{2+}-phos-tag to acrylamide gels can "capture" the highly negatively charged phosphorylated proteins, slow their migration through gels, and create more dramatic mobility shifts upon phosphorylation (453). Running the nucleotide-free pulldown samples on these modified gels, I was able to observe differences in mobility between Ect2 pulled down by RhoA vs. Ect2 pulled down by Rac1 (Fig. 6.1A). Since phos-tag alters apparent molecular weight, it is especially important to confirm specificity of the antibody. Performing the pulldowns in cells with Ect2 knocked down, I confirmed that the observed bands were in fact Ect2, because their signal decreased upon knockdown while the loading control, tubulin, remained constant (Fig. 6.1B). This suggests that there are intrinsic differences between the Ect2 pools interacting with each GTPase.

Unfortunately, I was unable to observe consistent, reproducible banding patterns for Ect2 pulled down by Rho versus Rac. It is known that Ect2 is phosphorylated during the cell cycle (76, 125), and I may see more consistent results if I synchronized the cells prior to performing the pulldown. However, if the differences observed were due to phosphorylation events, then calf intestinal phosphatase (CIP) treatment of the pulldowns should reduce the ratio of high mobility (highly phosphorylated protein) bands to low mobility (less phosphorylated protein) bands. Although CIP treatment did reduce staining by pan-phospho-threonine, it did not reduce levels of high mobility Ect2 bands (data not shown). Therefore these high mobility bands may represent another post-translational modification to Ect2, or represent phosphorylation events that are resistant to CIP treatment based on protein structure. To identify specific residues that are differentially modified (phosphorylated or otherwise) when Ect2 is interacting with RhoA compared to Rac1 in an unbiased manner, I could perform the nucleotide-free pulldowns, isolate Ect2 using immunoprecipitation, and use mass spectrometry to compare the modifications on Ect2 pulled down by each GTPase.

The methods described above can be used to identify correlations. However, to determine if phosphorylation of Ect2 is differentially required for activation of RhoA vs. Rac1, I compared whole cell lysates subject to phosphatase (CIP) treatment or phosphatase inhibition (HALT, phosphatase inhibitor cocktail) for 1 hour prior to performing the nucleotide-free pulldowns. Phosphatase treatment reduced the amount of Ect2 pulled down by RhoA(17A), while not dramatically affecting the amount of Ect2 pulled down by Rac1(15A) (Fig. 6.2). This suggests that phosphorylation is required for interaction with RhoA, but not Rac1, and thus may help to drive differential specificity of Ect2.

These conclusions about the possible role of phosphorylation on Ect2 specificity for Rho versus Rac are in contrast to my original hypothesis (described above), that was based on multiple studies in which phosphorylation was required for Ect2 to optimally activate Rac (76, 134, 161). In the conditions used in Fig. 6.2, Rac pulled down only low levels of Ect2. Therefore, it is possible that in these conditions Ect2 is already dephosphorylated on residues required for optimal Rac activation and thus no further decrease could be observed upon CIP treatment. Additionally, the buffers used for the CIP/HALT treatment may not be optimal for pulldown of Ect2 by Rac.

To further explore how phosphorylation of Ect2 regulates its specificity, phosphomimetic and phosphodeficient mutants of Ect2 can be utilized. As mentioned above T327, T341, T412, and T814 are all residues of Ect2 that have been previously implicated in regulating its activity (115, 134, 161) and which should be examined for their potential role in substrate specificity. In addition, I can perform mass spectrometry to uncover other sites.

1.2 Does the C-terminus regulate the specificity of Ect2?

The C-terminus of Ect2 (a.a. 775-882) does not contain any known functional domains. Still, it is required, in addition to the catalytic DH/PH domain of Ect2, for activation of Rac1 and Cdc42 and for optimal activation of RhoA by Ect2 (94). This makes the C-terminus of Ect2 of interest as an uncharacterized regulator of Ect2 specificity. Solski et al. (94) utilized a truncated form of Ect2, lacking the N-terminus, when they observed that GEF activity was optimal in the presence of the C-terminus. It would be interesting to determine if the C-terminus of Ect2 is similarly required for optimal GEF activity using full length Ect2, and to determine if there is a hierarchy of GTPase dependency on the C-

terminus. This could be determined by ectopically expressing HA-tagged full length Ect2 or HA-tagged Ect2 with a truncated C-terminus, performing nucleotide-free GTPase pulldowns for Ect2, and blotting for HA. Comparing the Rac:Rho ratio of Ect2 pulled down with and without the C-terminus should help define the relative GTPase dependency on the C-terminus. When Solski et al. attempted this in 2004 they were unable to pulldown Ect2 with Rac(15A) (independent of the C-terminus) (94). Since full length Ect2 is pulled down by nucleotide-free Rac in our system whereas they used N-terminally truncated Ect2, there is likely a difference in specificity between full length and N-terminally truncated Ect2. Additionally, rhotekin and PAK pulldowns for Rho and Rac activation upon overexpression of full length versus truncated Ect2 provide another method to answer the question.

The C-terminus has also been implicated in GTPase activation because it is required for Ect2 to increase cyclin D1 transcription (46), and it is necessary for cellular transformation (46, 94, 111). In each of these papers, "the C-terminus" is defined slightly differently. Collectively, it appears that somewhere between a.a. 775-831 of human Ect2 is required for transformation. It would be interesting to further truncate the C-terminus of Ect2 and determine a smaller region that is required for GEF activity. It is possible that certain regions of the C-terminus are required for Rac activity, while other areas allow for optimal Rho activity.

Interestingly, 32% of the C-terminus is a serine or threonine, while no tyrosines are present. ELM (454) lists 4 known phosphorylation sites in this area and nearly all C-terminal serine and threonines are predicted as possible phosphorylation sites by NetPhos (455), suggesting that at least some of the phosphorylation events described above may occur in the C-terminus to regulate specificity.

1.3 How is Ect2 specificity regulated?

There are many predictions in the literature as to how phosphorylation of Ect2 regulates its activation. It is likely that at least some of these same mechanisms are utilized to control its specificity for individual GTPases. As one mechanism, phosphorylation of Ect2 is proposed to regulate Ect2 via creating binding sites for other proteins. For example, Cdk1 phosphorylation of Ect2 at T412 creates a binding site for Plk1, which is required for Ect2 activation of RhoA (134). In another example, phosphorylation of Ect2 at T341 inhibits binding to MgcRacGAP (120). Since MgcRacGAP catalyzes GTP hydrolysis and thus directly counters Ect2 activity on Rac, binding between MgcRacGAP and Ect2 should cause an apparent increase in specificity for RhoA over Rac. While this would not affect nucleotide-free GTPase mutants and thus cannot explain the observed intrinsic differences in Ect2 specificity, *in cellulo* this binding could have a large effect on GTPase specificity. To determine if other proteins are necessary for phosphorylation-driven Ect2 specificity, *in vitro* exchange assays on RhoA and Rac1 can be performed. These assays would use bacterially isolated Ect2 that contains a phosphomimetic mutation at the residue of interest. The ability of Ect2 to activate RhoA and Rac1 should be compared when Ect2 is exposed to cellular lyate or exposed to lysis buffer. If differences are observed, then it is likely due to other eukaryotic proteins interacting with Ect2 to drive activity. Phosphodeficient Ect2 can be used in the assay instead to determine if interactions with these other proteins are due to phosphorylation.

Phosphorylation may also control GTPase specificity by regulating the localization of Ect2. For example, dephosphorylation of T341 within Ect2 and subsequent MgcRacGAP

binding may allow for Ect2 co-localization with RhoA near the central spindle during anaphase (120).

1.4 What determines the localization of Ect2?

As described in Chapter 1, Ect2 has been observed at many subcellular locations. I have observed both nuclear and cytoplasmic Ect2 in ovarian tumor cells, but it is unclear what mechanisms control the nuclear vs. cytoplasmic localization of Ect2.

It is possible that the same residues that drive specificity also regulate localization of Ect2. It has been proposed that phosphorylation of Ect2 T327 (T328 in reference, see Fig. 1.2 legend) by PKCi is necessary for Ect2 to activate Rac1 and also to drive Ect2 from the nucleus into the cytoplasm of non-small cell lung cancer (NSCLC) cells (137, 161). It is clear that PKCi regulates phosphorylation of Ect2 at residue T327 and that this phosphorylation is required for optimal activation of Rac (161), yet the localization data is lacking. The most striking localization data shows a decrease in cytoplasmic Ect2 upon PKCi knockdown via immunofluorescence (137). However, my validation experiments showed that the antibody used in this work (Santa Cruz) was not specific to Ect2 (Chapters 1 & 4). Data on the same phenomenon obtained via fractionation was also not convincing (137). Using a well-validated Ect2 phospho-T327 antibody they were able to show higher amounts of phospho-T327 Ect2 in the cytoplasm compared to the nucleus. However, total Ect2 was also much higher in the cytoplasmic vs. nuclear fraction, and the total Ect2 signal was over-exposed, such that a proper comparison could not be determined regarding percentage of Ect2 that is phosphorylated at T327 in each compartment (161). Thus it is unclear if residue T327 regulates the subcellular localization of Ect2 via phosphorylation. To

examine the importance of this residue towards subcellular localization myself, I created GFP-tagged phospho-mimetic (T327D) and phospho-deficient (T327A) mutants of Ect2 at this residue. I expressed them in OVCAR8 cells, and used WT Ect2 as a control for predominantly nuclear localization, and DH/PH/C Ect2 (lacking the NLSs) as a control for predominantly cytoplasmic localization. I fixed the cells, blinded myself to the mutant expressed, and counted the number of cells in the following categories of Ect2 localization: plasma membrane-highlighted, nuclear-excluded, nucleus/cytoplasm equal, and nuclear-highlighted. I determined the fraction of cells with cytoplasmic Ect2 as the sum of all categories except nuclear-highlighted, divided by the total number of cells counted. As expected, in 97% of cells expressing DH/PH/C Ect2, fluorescence was cytoplasmic, whereas this was only true for 30% of WT Ect2-expressing cells (Fig. 6.3). Surprisingly, T327D was also mostly confined to the nucleus, with only 38% of cells expressing it showing cytoplasmic Ect2, and instead T327A was more highly expressed in the cytoplasm (71% of cells showing cytoplasmic Ect2) (Fig. 6.3). These results were confirmed by two other independent and blinded reviewers (Molly J. DeCristo and Tim Rose). This suggests that WT Ect2 is basally phosphorylated at T327 in OVCAR8 cells and that phosphorylation of this residue is required for proper nuclear localization. It would be interesting to attempt this assay in NSCLC cells, and to immunoblot OVCAR8 fractions with the Ect2 phospho-T327 antibody generated by the Fields lab, to determine if the observed differences are cell- or tumor type-dependent, or assay-dependent.

Another region of Ect2 that may drive specificity and also regulate localization of Ect2 is the C-terminus. As discussed above, the C-terminus of Ect2 is known to be important for optimal GTPase activation. While much of the C-terminus is a possible phosphorylation

site (32% of the C-terminus is a serine or threonine), another 20% of the C-terminus is composed of basic amino acids. Basic (positively charged) amino acids are commonly utilized in the cell to regulate localization relevant for Ect2, for example in NLSs (66) and for plasma membrane association (9). Therefore, I hypothesize that the positively charged residues in the C-terminus of Ect2 help assign its subcellular localization, while phosphorylation events negate the positive charges and allow finer and more dynamic regulation.

Saito et al. described an NLS in the C-terminus of Ect2, and expression of just their GFP-tagged C-terminus (a.a. 753-882) was nuclearly localized (111). Yet, the described experiment could have been hindered by the fact that GFP is larger than the C-terminus of Ect2 and also concentrates in the nucleus (456). Instead, other evidence suggests that the C-terminus is actually responsible for nuclear exclusion. In a study by Solski et al., localization of the HA-tagged DH/PH and DH/PH/C domains of Ect2 was visualized by immunofluorescence (94). These images show Ect2 DH/PH highlighting the nucleus while also being expressed diffusely in the cytoplasm, whereas Ect2 DH/PH/C is visible mostly in the cytoplasm and plasma membrane. These constructs were truncated, beginning at residue 415, and thus lack both central NLSs. Truncations of Ect2 have not been identified in tumors, thus for a more accurate understanding of how the C-terminus regulates Ect2 in tumors, I propose examining the localization of full length Ect2 and comparing that to C-terminally truncated Ect2. I have previously observed that neither N-terminal HA- nor N-terminal Myc-tags alter the subcellular localization of WT Ect2 when compared to endogenous Ect2 (data not shown). Therefore, either of these tags can be used to label the protein for immunofluorescence.

1.5 A comprehensive model of specificity and localization of Ect2

The precise mechanism(s) that regulate the correlation I observed between Rac/Rho specificity and subcellular localization remains to be determined. As diagrammed in Figure 6.4, I envision three options for how they may be related: 1) a single mechanism regulates both the localization and specificity of Ect2; 2) Ect2 localization to one cellular compartment allows for other modifications affecting its specificity; 3) changes in Ect2 specificity drive its change in localization. To differentiate between scenarios 1 and 2, I could mutate NLS sequences to drive Ect2 to the cytoplasm and additionally create phosphomimetic or phosphodeficient mutations on the same construct. If a residue is important for specificity while in the cytoplasm (for example) then the phosphomimetic NLS mutant should drive activation of that GTPase when in the cytoplasm, whereas phosphodeficient NLS mutant will not. But if another residue is important for specificity and this residue drives only localization, then both phosphomimetic and phosphodeficient NLS mutants will have the same effect on active GTPase levels.

Combined the data from Future direction 1 would allow me to uncover the players that regulate Ect2 subcellular localization/specificity, and how they do so. Since I have shown that Ect2 is required for ovarian cancer cell transformation through its activation of GTPases in the nucleus (Chapter 4), this information could be valuable to help target Ect2 for treatment of ovarian tumors. Although RhoGEF inhibitors (430-432, 457) and compounds that bind GTPases to prevent GEF interactions (428, 429) have been identified, none have been proven potent enough for clinical use. Thus, understanding the regulation of Ect2 may produce more tractable drug targets. For example, there are a variety of kinase inhibitors that are approved for clinical use (458), which may be effective in preventing Ect2 from entering

the nucleus and/or activating Rac (or any other GTPases required for ovarian cancer transformation).

FUTURE DIRECTION 2 – HOW DOES NUCLEAR ECT2 RHOGEF ACTIVITY DRIVE TRANSFORMATION?

As described in Chapter 4, I have observed that both GEF activity and nuclear localization of Ect2 are required for optimal Ect2-driven anchorage-independent growth in ovarian cancer cells. This suggests that Ect2 activates GTPases from within the nucleus to drive transformation. I speculate on how this occurs in the following section.

2.1 Is nuclear Rac activity required for Ect2-driven transformation?

In Chapter 4 I show multiple lines of data that suggest that Ect2 preferentially activates Rac (over RhoA and Cdc42) while in the nucleus. Therefore, I hypothesized that the observed nuclear GEF-dependent effect on transformation is mediated through Rac. This is consistent with previous studies that have noted correlations between Ect2-driven cellular transformation and Rac1 activation (see Chapter 1). However, it is still unknown if nuclear Rac1 activity is required for Ect2-driven cellular transformation. To determine this, I would first have to determine which residues are required for Ect2 to specifically activate Rac1 (see Chapter 6.1), create a version of Ect2 that is deficient at activating Rac only, and then determine if it is capable of rescuing the defect in anchorage-independent growth caused by Ect2 knockdown. Since the residues that confer substrate specificity for Ect2 are currently unknown, and indeed the substrate specificity remains controversial, this process will not be straightforward. Beyond phosphorylation sites at various locations throughout Ect2 (Chapter

6.1), there are also amino acids in the DH domain that are likely necessary for conferring its specificity. Although, the crystal structure of Ect2 has not been solved, analysis of the crystal structures of other RhoGEFs in complex with GTPases suggests that isoleucine 1187 of Tiam1 is key for its ability to activate Rac1 (459). This isoleucine is conserved among Rac GEFs, but is more variable among RhoA and Cdc42 specific GEFs (459). Using the ClustalW alignment tool, I have determined that an I562L mutant of Ect2 may no longer be able to activate Rac1, yet still able to activate RhoA. However, this remains to be tested.

2.2 Does Ect2 regulate mRNA splicing?

The speckled pattern of Ect2 localization in the nucleus suggests that Ect2 localizes to distinct subnuclear structures. Nuclear speckles and paraspeckles have a similar appearance to that of Ect2 (460). Nuclear speckles are best-known for storing, assembling, and modifying mRNA splicing factors (420). Paraspeckles are primarily known for retaining edited mRNA in the nucleus (420), but also contain proteins involved in splicing (461, 462). Using mass spectrometry, Ect2 has been identified as a binding partner of the protein encoded for by SNRNP200 (114), U5 SnRNA component of the spliceosome, so it is possible that Ect2 regulates splicing.

To further examine this, I can determine if Ect2 localizes in nuclear speckles or paraspeckles, by looking for co-localization with SRSF1, SRSF2, Malat1 (nuclear speckles componenets) or PSP1, p54nrb, Neat1 (paraspeckle components). I can also confirm the interaction of Ect2 with U5 by performing co-immunoprecipitations. If Ect2 is confirmed to localize to nuclear speckles and/or to interact with U5 then I should proceed to determine if Ect2 has a role in splicing.

To examine a role for Ect2 in splicing in an unbiased manner (since the genes that Ect2 may regulate are unknown) I can utilize a splice junction microarray. These arrays allow identification of relative amounts of different splice variants for a variety of genes (463). Alternatively, I could use RT-PCR to amplify genes and their splice variants (464) or utilize different splice reporters (464, 465). I could then compare splicing profiles in cells with Ect2 expressed vs. knocked down, or Ect2 expressed in the nucleus vs. the cytoplasm. These would help me determine if Ect2 is general regulator of splicing or is specific to a subset of genes, and could inform further studies on the process.

FUTURE DIRECTION 3 – HOW AND WHY DOES COMPARTMENTALIZATION OF ECT2 AFFECT PATIENT PROGNOSIS?

As described in Chapter 4, we used IHC to stain for Ect2 in an ovarian tissue microarray (TMA), and generated both a nuclear and cytoplasmic Ect2 score for each core, based on the intensity of staining and percentage of cells staining in each compartment. We observed that nuclear score increased with increasing tumor malignancy whereas cytoplasmic score decreased (Fig. 4.1). This suggested that nuclear, but not cytoplasmic, Ect2 may be an important driver of ovarian tumors. Not described in Chapter 4, we also generated Kaplan-Meier plots to look for potential correlations between nuclear or cytoplasmic score and patient survival. Although we observed trends suggesting that a high cytoplasmic score correlated with good patient outcome (Fig. 6.5A), whereas a high nuclear score correlated with poor patient outcome (Fig. 6.5B), neither of these trends was significant.

To more fully describe Ect2 distribution in the tumor cores, while scoring the TMA we also binned the tumors into the following categories: 0) no staining; 1) only nuclear

staining; 2) only cytoplasmic staining; 3) both stain, but in different cells; 4) both stain in the same cells; 5) cells with nuclear staining also stain in the cytoplasm, but not vice versa; 6) cells with cytoplasmic staining also stain in the nucleus, but not vice versa; 7) independent staining with approximately half the cells staining in only one compartment and half the cells staining in both. We then compared patient survival between these groups using a Kaplan-Meier curve, but since many of these categories contained only a few tumor cores, there was insufficient data to reach statistical significance (data not shown). Still, the trends suggested that there may be a difference in patient survival when Ect2 is restricted to one compartment or expressed in both compartments. The above categories were further simplified to define two groups of nuclear/cytoplasmic overlap: compartmentalized/restricted expression (subcategories 1,2,3 above) and non-compartmentalized/unrestricted expression (subcatebories 4,5,6,7 above). Interestingly, these categories did display statistically significant differences in patient survival ($p=0.0314$, Fig. 6.6A). Patients whose tumor cores displayed Ect2 expression in both the nucleus and the cytoplasm survived longer than patients whose tumor cores displayed Ect2 expression restricted to one subcellular compartment. This observation was even more robust when focusing solely on serous tumors ($p=0.0268$, Fig. 6.6B).

We found the described correlation surprising. Since the restricted and unrestricted categories were defined because of observed trends with this TMA, our data represented a training set. It would be interesting to apply the same definitions of nuclear/cytoplasmic overlap to a new set of TMA data and determine if the same correlation is observed (validation set). If this observation is validated, many questions arise as to why restriction of Ect2 to a subcellular compartment results in poor prognosis. These are discussed below.

3.1 Does Ect2 need to be able to shuttle between the nucleus and cytoplasm to prevent transformation?

Ect2 has been previously predicted to shuttle between the nucleus and cytoplasm (137), and this shuttling has been observed in cells prior to nuclear envelope breakdown during mitosis (119). Since restriction of Ect2 to either the nucleus or the cytoplasm is linked to poor prognosis, possibly Ect2 needs to shuttle between the nucleus and the cytoplasm to prevent tumor growth, angiogenesis, or tumor metastasis.

3.2 Does Ect2 regulate opposing functions in the nucleus and the cytoplasm?

The correlation shown in Figure 6.6 suggests that the role of Ect2 in the nucleus and the role of Ect2 in the cytoplasm are detrimental to patient outcome, yet combined they somehow oppose their separate detrimental effects. In support of this, I have observed that nuclear expression of Ect2 is required for anchorage-independent growth; however, expression of Ect2 in the cytoplasm (NLS-mutant Ect2) can reduce anchorage-independent growth of cells expressing endogenous (mostly nuclear) Ect2 (Fig. 4.5).

The role of nuclear Ect2 in anchorage-independent growth is GEF-dependent (Fig. 4.4). To determine if the antagonizing role of Ect2 in the cytoplasm is GEF-dependent as well, I could perform soft agar assays on cells expressing endogenous Ect2 and also a mutant Ect2 that combines the NLS- and DH-mutations.

Anchorage-independent growth is tightly correlated with *in vivo* tumor growth (410) and as such may be related to patient survival. I have also observed that Ect2 regulates both directed and random migration of ovarian cancer cells (Fig. 6.7), which are key functions related to tumor metastasis (466) and especially important in ovarian cancer, which tends to

disseminate locally but widely within the peritoneum. Although the effect of Ect2 knockdown on migration was consistent between assay types, the results were very cell line-dependent for reasons not currently understood. Determining the required localization and function of Ect2 to regulate migration, using the NLS mutant and GEF-deficient mutant, may shed light on why restriction to either location results in poor patient outcome.

Still, if Ect2 drives migration (and by extension possibly invasion/metastasis) from the cytoplasm, it is unclear why high cytoplasmic score correlates with decreased tumor malignancy (Chapter 4) and trends with good patient outcome (Fig. 6.5). As observed by the cell line dependence on Ect2 for migration, it may be that in certain genetic backgrounds cytoplasmic Ect2 can have a negative effect, but in most it does not.

FUTURE DIRECTION 4 – OTHER QUESTIONS RAISED

Numerous other questions are raised by my experiments. Some are described below.

4.1 Is activation of Rac1 by Ect2 direct?

It has been previously suggested that Ect2 activation of Rac1 is indirect: the result of Ect2 activation of Cdc42 then leading to activation of the RacGEF Trio (159). I have shown that Ect2 activates Rac in a GEF-dependent manner in the nucleus, whereas activation of Cdc42 was GEF-independent (Fig. 4.4). Further, Ect2 did not bind to nucleotide-free Cdc42 (Fig. 4.6), Therefore, I hypothesize that Ect2 directly activates Rac1. If my hypothesis is correct, DH-mutant Ect2 will be unable to interact with nucleotide-free Rac1.

4.2 Does Rac1 nucleotide binding affect its localization?

As described in Chapter 4, creating a mutant of Rac1 that was predominantly nuclear required both abolishing prenylation of Rac1 (SAAX mutation) and adding a nuclear localization signal (+NLS). In the process of determining this, Dr. James Fiordalisi and I created G12V and F28L mutants of HA-tagged Rac1 with only a SAAX mutation, only an additional NLS, or with neither modification. Interestingly, the single SAAX mutation or the additional NLS caused a more dramatic shift towards nuclear accumulation in the F28L background than the G12V background (Figs. 6.8A & B). This implies that the fast-cycling mutant of Rac1 more freely enters the nucleus than constitutively active Rac1, and that nucleotide binding may affect the localization of Rac1.

One possible explanation for the enhanced nuclear entry of the F28L mutant is that it has an enhanced ability to bind and release a chaperone at appropriate times for nuclear entry compared to Rac1(G12V). Prenylation of Rac1 is irreversible, and although prenylation inhibits nuclear entry of Rac1, endogenous nuclear Rac1 has been determined to be prenylated (62). Therefore Rac1 nuclear entry likely requires a chaperone protein to cover the geranylgeranyl group on Rac1. Conversely, the G12V mutant may exhibit poorer nuclear entry if it permanently binds to an extranuclear effector (or other protein) and thus not be available for entry into the nucleus. For example, it has previously been shown that RhoGDIs inhibit nuclear entry of RhoA (68). It is debated as to whether the nucleotide state of a GTPase affects RhoGDI binding (see Chapter 1). However, if GTP-bound Rac1 binds more strongly to RhoGDI than fast-cycling Rac1, this may explain why NLS-Rac1(G12V) shows less nuclear accumulation than NLS-Rac1(F28L). Still, neither SAAX mutant interacts with RhoGDI, because GDIs bind prenyl groups, which SAAX mutants lack.

Therefore RhoGDI binding cannot be the full explanation, and likely Rac1 effector proteins are involved as well. A more trivial explanation related to protein:protein interactions is based on stoichiometry. The F28L mutants were more highly expressed compared to G12V mutants (Fig. 6.8C). Therefore, there may be more Rac1(F28L) to freely move into the nucleus, while Rac1(G12V) is sequestered away from the nucleus by effectors. Alternatively, fast-cycling and constitutively active Rac1 may be modified differently. A recent study found that palmitoylation of Rac1 at C178 is required for nuclear entry of prenylated Rac1. It also suggested that this palmitoylation enhances GTP-loading, but did not investigate the role of GTP- vs. GDP-binding on palmitoylation (467).

4.3 How does Rac1 localization correlate with Rac1 activity and cellular transformation?

Localization of the Rac mutants described above (Chapter 6.4.2) was determined by expressing each of the Rac mutants in OVCAR8 cells with Ect2 knocked down, selecting for stably expressing cells, and staining for the HA epitope tag on the mutants. During generation of the stable transfectants I noticed that many of the Rac-expressing cells showed enhanced ruffling, as is common upon Rac1 activation (see Chapter 1). However, most strikingly, NLS-Rac1(G12V) expression caused a large number of cells to become filled with vacuolar structures (Fig. 6.9). A milder but similar phenotype was observed with NLS-Rac1(F28L) as well. This type of vesicle formation has been described previously upon Rac1 activation in fibroblasts, and is thought to result from macropinocytosis (31). It is interesting that addition of an NLS to Rac1(G12V) enhanced this phenotype in OVCAR8 cells, especially since in basal conditions the localization of NLS-Rac1(G12V) and Rac1(G12V) are indistinguishable (Figs. 6.8A & B). Perhaps, synchronizing the cells prior

to staining would reveal differences in the localization of Rac1(G12V) +/- the NLS and enhance our understanding of this phenotype.

To better understand the role of Rac1 subcellular localization on Rac1 activity, I also generated HA-tagged Rac1(G12V) with the K-Ras tail. The GFP-tagged version of this Rac1 mutant is excluded from the nucleus (62). Transient expression suggested that the HA-tagged mutant is similarly localized; however, this could not be confirmed in the same conditions as the cells described above, because all of the cells died during selection (data not shown). The observed lethality suggests that active Rac1 must be able to cycle into the nucleus for cell viability. The cycling into the nucleus could be to turn on nuclear signaling, or to attenuate signaling at the membrane, since Rac1 signaling has been previously associated with apoptosis (202). Alternatively, adding the K-tail to Rac1 may cause Rac1 to outcompete K-Ras in microdomains, where K-Ras signaling is required for cell viability. To rule out this more trivial explanation, I would exclude Rac1 from the nucleus by another mechanism (e.g. by mutation of its NLSs) and determine if a similar lethality is observed, and/or determine if knockdown of K-Ras causes cell death in this cell type.

To further examine the role of Rac1 subcellular localization on transformation, I performed soft agar assays with the above-described cells. NLS-Rac1(G12V) with the NLS from Ect2 was used, since the high number of vesicles slowed cell growth upon expression of NLS (SV40T antigen)-Rac1(G12V). Additionally, Rac1(G12V) K-tail could not be used, because the cells died during selection. All trends described below were also true with Rac1(F28L) (data not shown), but the rescues were not as robust with F28L, suggesting that constitutively active Rac1 is more effective at driving transformation than fast-cycling Rac1. As expected, Rac1(G12V) partially rescued anchorage-independent growth and Rac1(G12V)

SAAX did not (Fig. 6.10). This is consistent with our previous understanding of the role of Rac1 and prenylation on cellular transformation (13). Additionally, Rac1(G12V)+NLS rescued anchorage-independent growth, which was not surprising because it appeared to localize the same as Rac1(G12V) (Figs. 6.8A & B). Consistent with our hypothesis that Ect2 activates Rac1 in the nucleus to drive transformation, the nuclearly localized NLS-Rac1(G12V) SAAX mutant also rescued anchorage-independent growth (Chapter 4). However, it was still surprising that there was such a dramatic difference between the ability of the SAAX mutant and SAAX+NLS mutant to rescue. The SAAX mutant alone stains strongly in the nucleus, like the SAAX+NLS mutant (Figs. 6.8A & B). The main difference caused by the additional NLS, is that there is less cytoplasmic Rac1 accompanying the nuclear Rac1. This suggests that cytoplasmic Rac1 activity may have contradictory effects to its nuclear activity (similar to Chapter 6.3.2). Alternatively, the SAAX+NLS mutant may cycle in and out of the nucleus differently from the SAAX mutant and this could drive the dramatic differences in their ability to rescue transformation (similar to Chapter 6.3.1). It would be interesting to examine the subcellular localization of both mutants more precisely (for example: throughout the cell cycle and in more detailed microdomains) to help determine exactly where Rac1 activity is required to drive transformation.

FIGURES

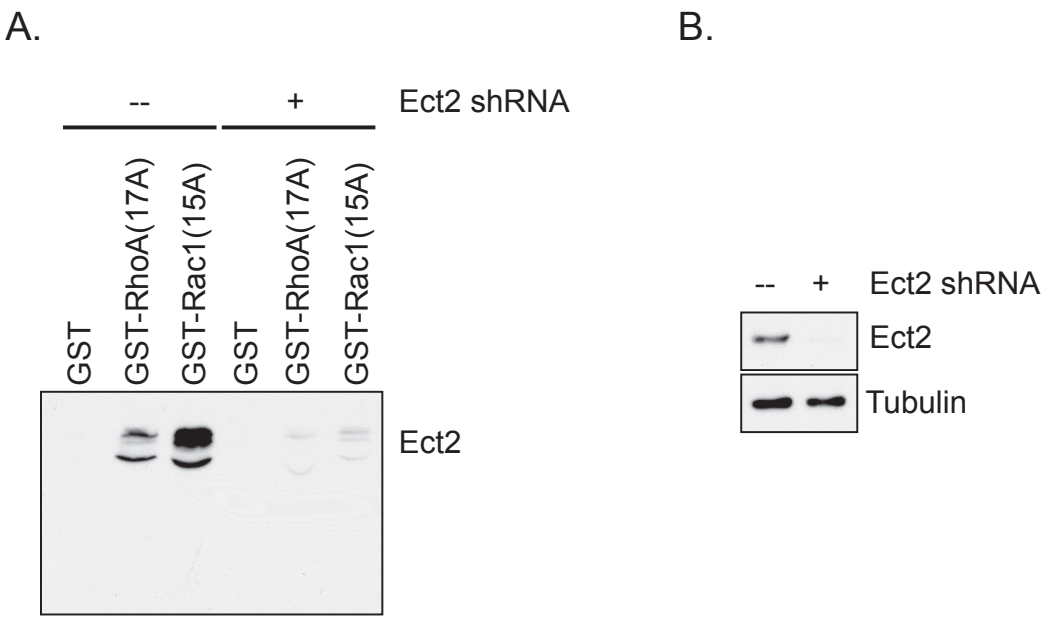


Figure 6.1. Ect2 that interacts with RhoA has a different mobility than Ect2 that interacts with Rac1. To examine the mechanism of Ect2 substrate specificity, Ect2 that interacts with either RhoA or Rac1 was isolated by performing GST-RhoA(17A) and GST-Rac1(15A) pulldowns from whole cell lysates. To enhance any mobility differences between isolated Ect2 that could be caused by phosphorylation of Ect2, pulldown products were separated on an acrylamide gel containing Zn^{2+}-phos-tag. *A. Upon immunoblotting for Ect2, differences in mobility between Ect2 pulled down by nucleotide-free RhoA vs. Rac1 could be observed.* Differences in mobility between Ect2 pulled down by RhoA vs. Rac1 were observed repeatedly, however, the banding patterns differed upon repetition (n=3). GST was used as a negative control. To confirm specificity of the signal, pulldowns were also performed on cells with Ect2 knocked down. *B. Ect2 knockdown was confirmed by western blot.* Tubulin served as a loading control.

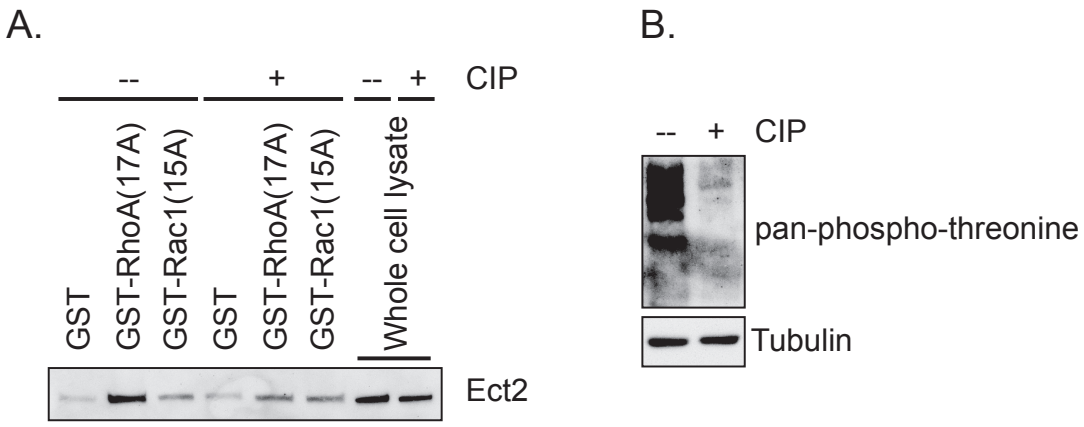


Figure 6.2. Phosphorylation events regulate the interaction of Ect2 and nucleotide-free RhoA, but not Rac1. To determine if phosphorylation of Ect2 is differentially required for activation of RhoA vs. Rac1, lysates were treated with phosphatase (+ CIP) or phosphatase inhibitior (-- CIP, HALT phosphatase inhibitor cocktail) for 1 hour prior to performing the nucleotide-free pulldowns. *A. The amount of Ect2 pulled down by RhoA(17A) was decreased by CIP treatment, yet, the amount of Ect2 pulled down by Rac1(15A) did not differ dramatically between conditions* (n=3). GST was used as a negative control. Equal loading of the pulldown (+/-- CIP) was confirmed by blotting the whole cell lysate loaded for each. *B*. Effectiveness of phosphatase treatment was confirmed by immunoblotting the whole cell lysates with a pan-phospho-threonine antibody. Phosphatase treatment reduced the staining observed with this antibody, confirming effective phosphatase treatment. Tubulin served as loading control.

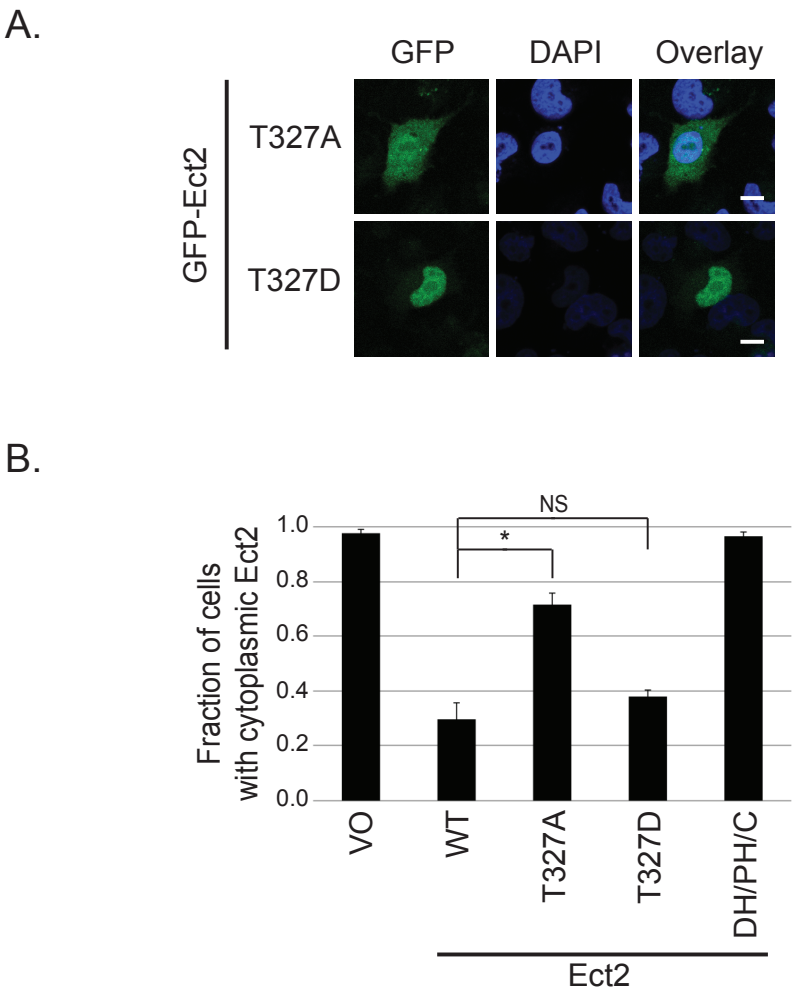


Figure 6.3. Abolition of the potential phosphorylation site T327 in Ect2 enhances its cytoplasmic localization. *A.* To determine if phosphorylation of Ect2 at T327 regulates the localization of Ect2, GFP-tagged phospho-mimetic (T327D) and phospho-deficient (T327A) mutants of Ect2 were expressed in OVCAR8 cells. Representative confocal images are shown. Scale bars represent 10 microns. *B.* Three independent reviewers, expressed these constructs in OVCAR8 cells, fixed the cells, and while blinded to the identity of the construct expressed binned cells into the following categories of GFP-Ect2 localization: plasma membrane highlighted, nuclear excluded, nucleus/cytoplasm equal, nuclear highlighted. The percentage of cells with cytoplasmic Ect2 was determined as the sum of all categories except "nuclear highlighted", divided by the total number of cells counted. WT Ect2 (expressed in serum starved cells) and DH/PH/C Ect2 served as controls for nuclear and cytoplasmic localization respectively. As shown in the bar graph, phosphomimetic (T327D) Ect2 localized similarly to WT Ect2, while phosphodeficient (T327A) Ect2 was more highly expressed in the cytoplasm ($n \geq 3$). Error bars represent SEM. Significance was determined using Bonferroni corrected Student's t-tests, *$p<0.05$, NS=not significant.

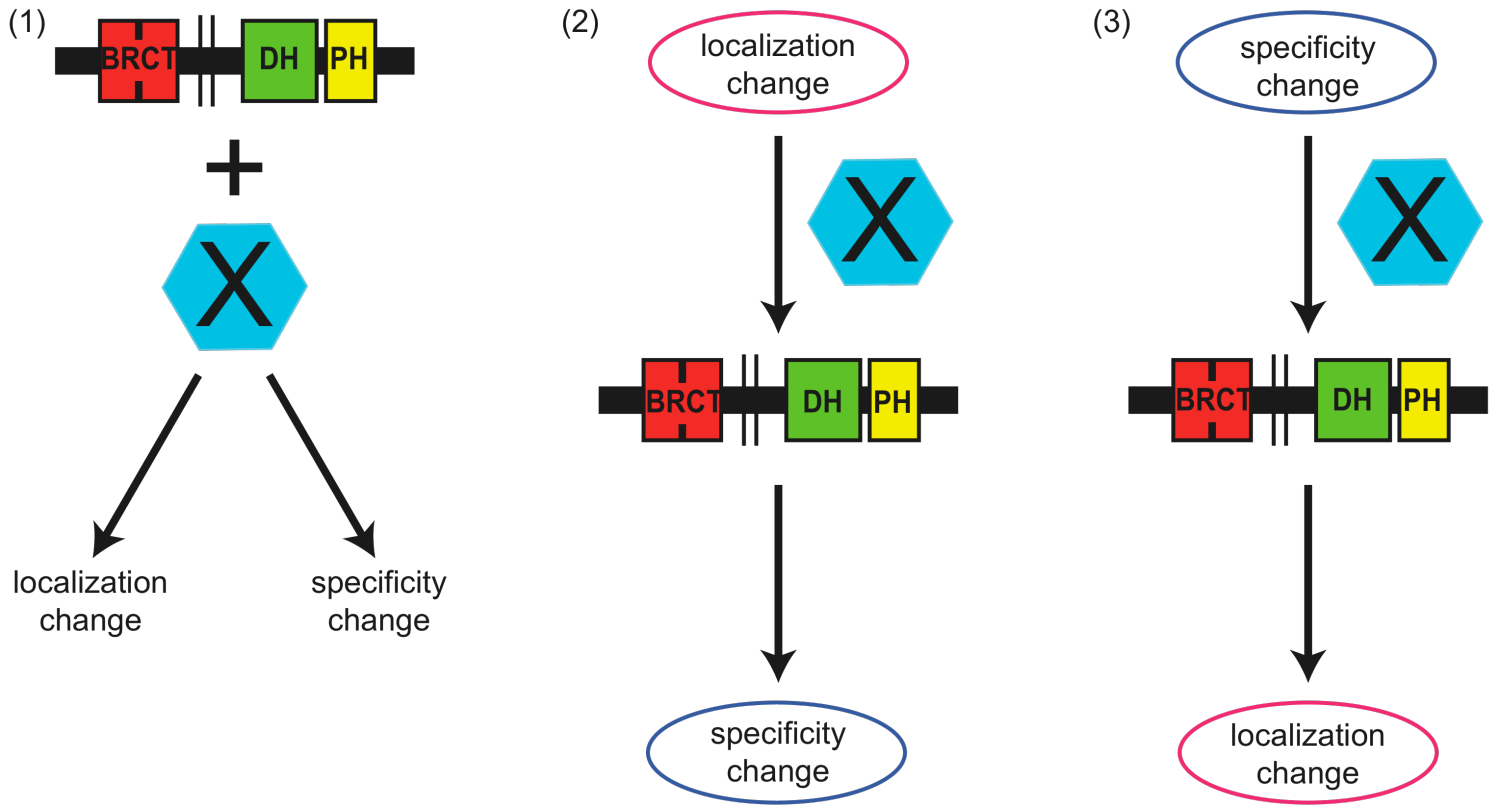


Figure 6.4. Three mechanisms by which localization and specificity may be related. As determined in Chapter 4, Ect2 specificity varies with its subcellular localization. There are 3 mechanisms by which localization and specificity may be related to each other: (1) a single mechanism regulates both the localization and specificity of Ect2; (2) Ect2 localization to one cellular compartment allows for other modifications affecting its specificity; (3) changes in Ect2 specificity drive its change in localization. X=unknown regulator of Ect2

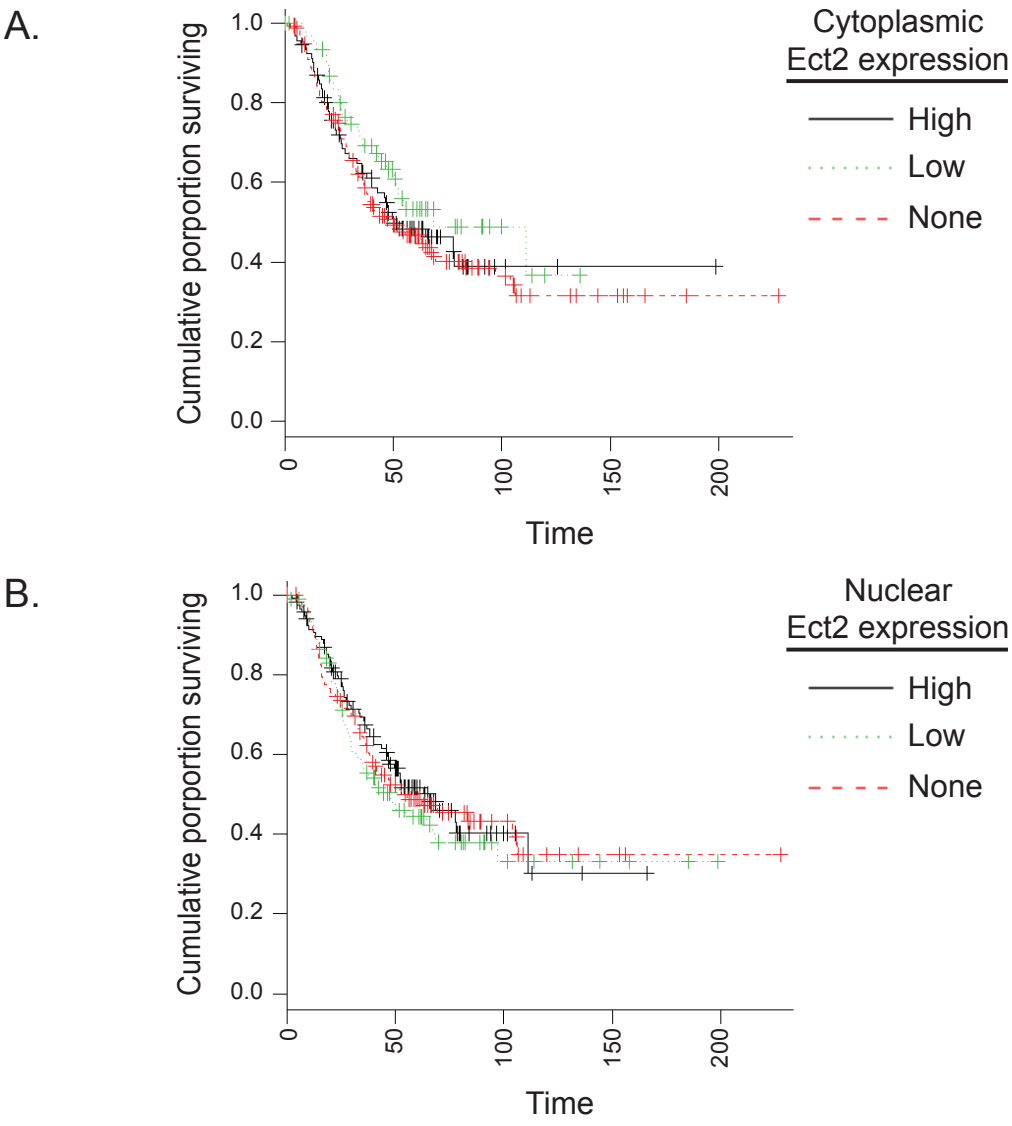


Figure 6.5. High cytoplasmic Ect2 expression trends with positive patient outcome, whereas high nuclear Ect2 expression trends with poor patient outcome. As described in Chapter 4, hundreds of ovarian tumors were stained for Ect2 and given both nuclear and cytoplasmic scores for Ect2 expression. *A.* To determine if there was a relationship between cytoplasmic score and patient survival, scores were binned into 3 categories of expression (none (0), low (1-10), and high (>10)) and a Kaplan-Meier plot was derived. There was no significant difference between the categories; however, high cytoplasmic score trended with positive patient outcome. *B.* To determine if there was a relationship between nuclear score and patient survival, scores were binned into 3 categories of expression (none (0), low (1-15), and high (>15)) and a Kaplan-Meier plot was derived. There was no significant difference between the categories, however, high nuclear score trended with poor patient outcome.

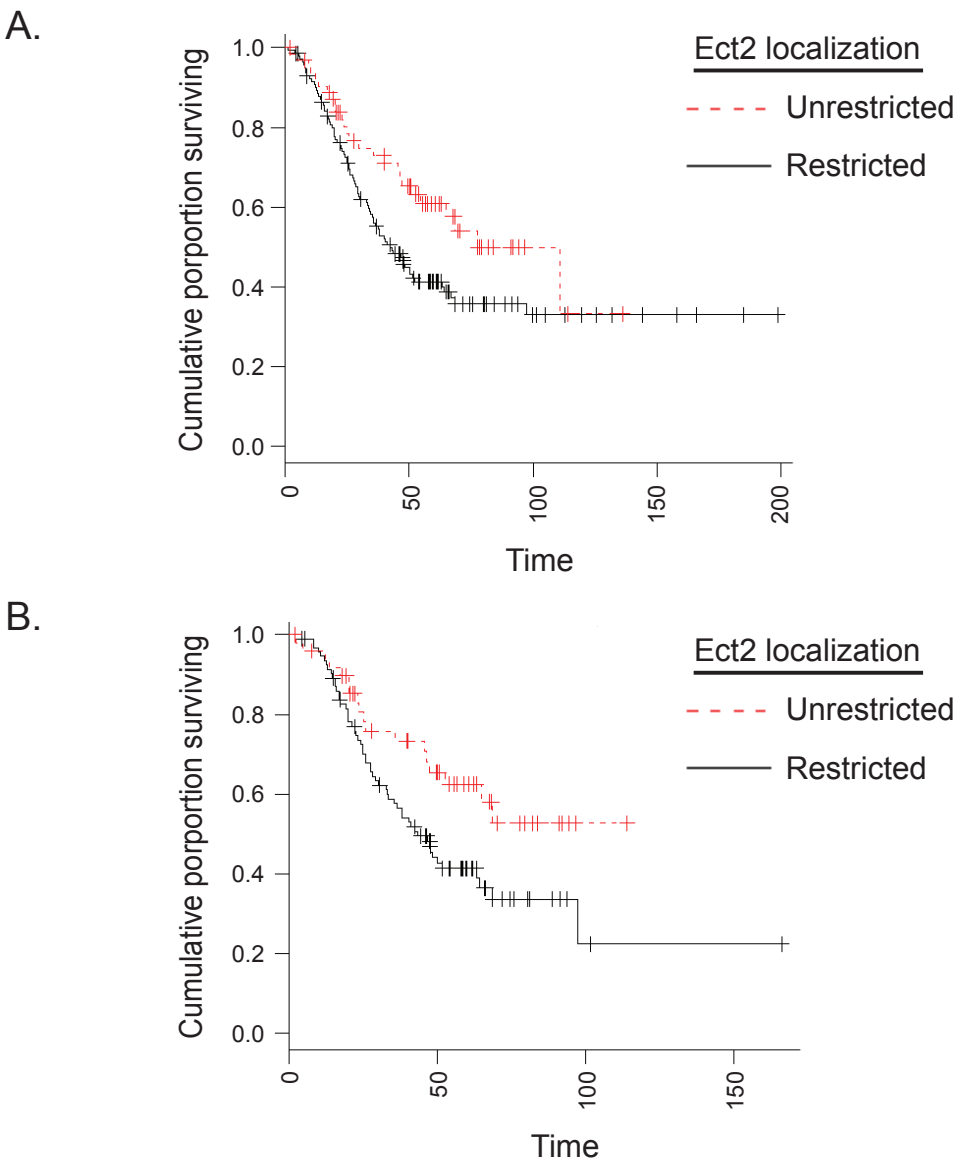


Figure 6.6. Restriction of Ect2 to the nucleus or cytoplasm correlates with poor patient outcome. To further describe the expression of Ect2 in ovarian tumors, while scoring the TMA the tumors were binned into the following categories: 0) no staining; 1) only nuclear staining; 2) only cytoplasmic staining; 3) both stain, but in different cells; 4) both stain in the same cells; 5) cells with nuclear staining also stain in the cytoplasm, but not vice versa; 6) cells with cytoplasmic staining also stain in the nucleus, but not vice versa; 7) independent staining with approximately half the cells staining in only one compartment and half the cells staining in both. The above categories were further simplified to define two groups of nuclear/cytoplasmic overlap: compartmentalized/restricted expression (subcategories 1,2,3 above) and non-compartmentalized/unrestricted expression (subcatebories 4,5,6,7 above). *A. Patients whose tumors had "restricted" expression had a worse outcome than those with "unrestricted" expression* (p=0.0314). *B. This correlation was even more apparent upon confining analysis to serous tumor cores* (p=0.0268).

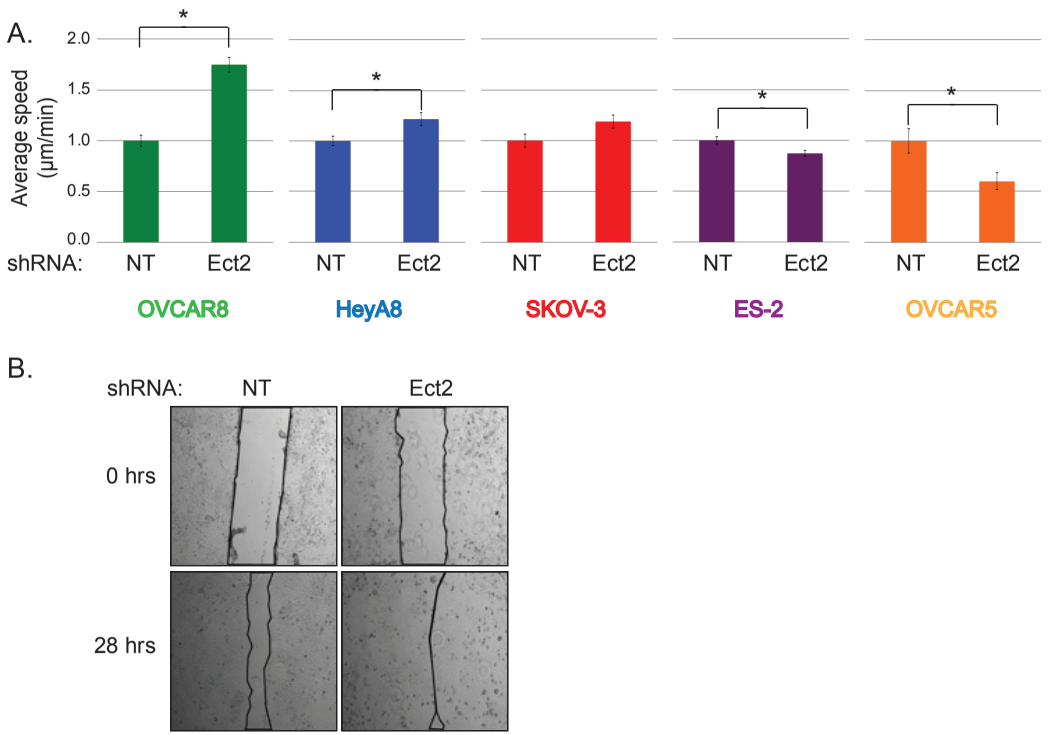


Figure 6.7. Ect2 regulates both random and directed migration of ovarian cancer cells. *A*. To examine the role of Ect2 in random cellular migration, the rate of migration of a panel of ovarian cancer cell lines stably expressing non-targeted (NT) or Ect2 shRNA was compared. A Nikon BioStation-IM microscope was used to image approximately 30 cells/condition at 5 minute intervals over a two hour period. The rate of migration was determined using ImageJ (Manual Tracking). In some cell lines knockdown of Ect2 reduced speed of migration, while in other cell lines it enhanced migration. Western blots confirming knockdown are shown in Chapter 4. Error bars represent SEM. Statistical significance was determined using Student's t-test and combining p-values with Fisher's method (*$p<0.05$, n=6 OVCAR8, n=4 HeyA8, n=2 SKOV-3, n=3 ES-2, n=1 OVCAR5). *B*. To examine the role of Ect2 in directed cellular migration, scratch assays were performed on a panel of ovarian cancer cell lines. Cells were allowed to grow to confluency, scratched, and imaged over time as the cells migrated to fill the wound. To inhibit the confounding factor of cell proliferation, cells were treated with 10μg/ml of mitomycin C prior to scratching. The same trends as seen for random migration were observed for directed migration. Representative images of a scratch assay performed on OVCAR8 cells are shown (n=2). The wound is outlined in black.

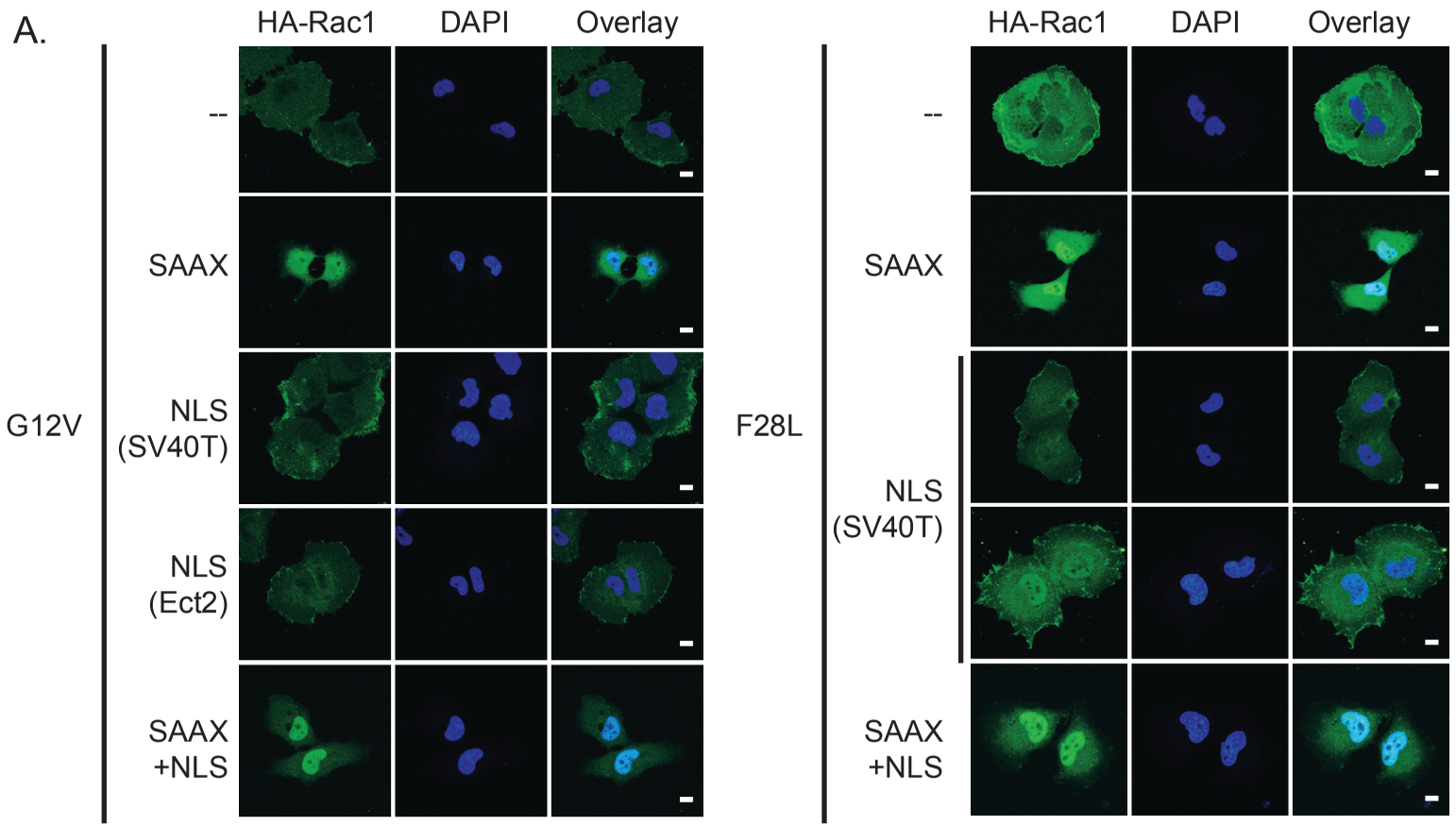
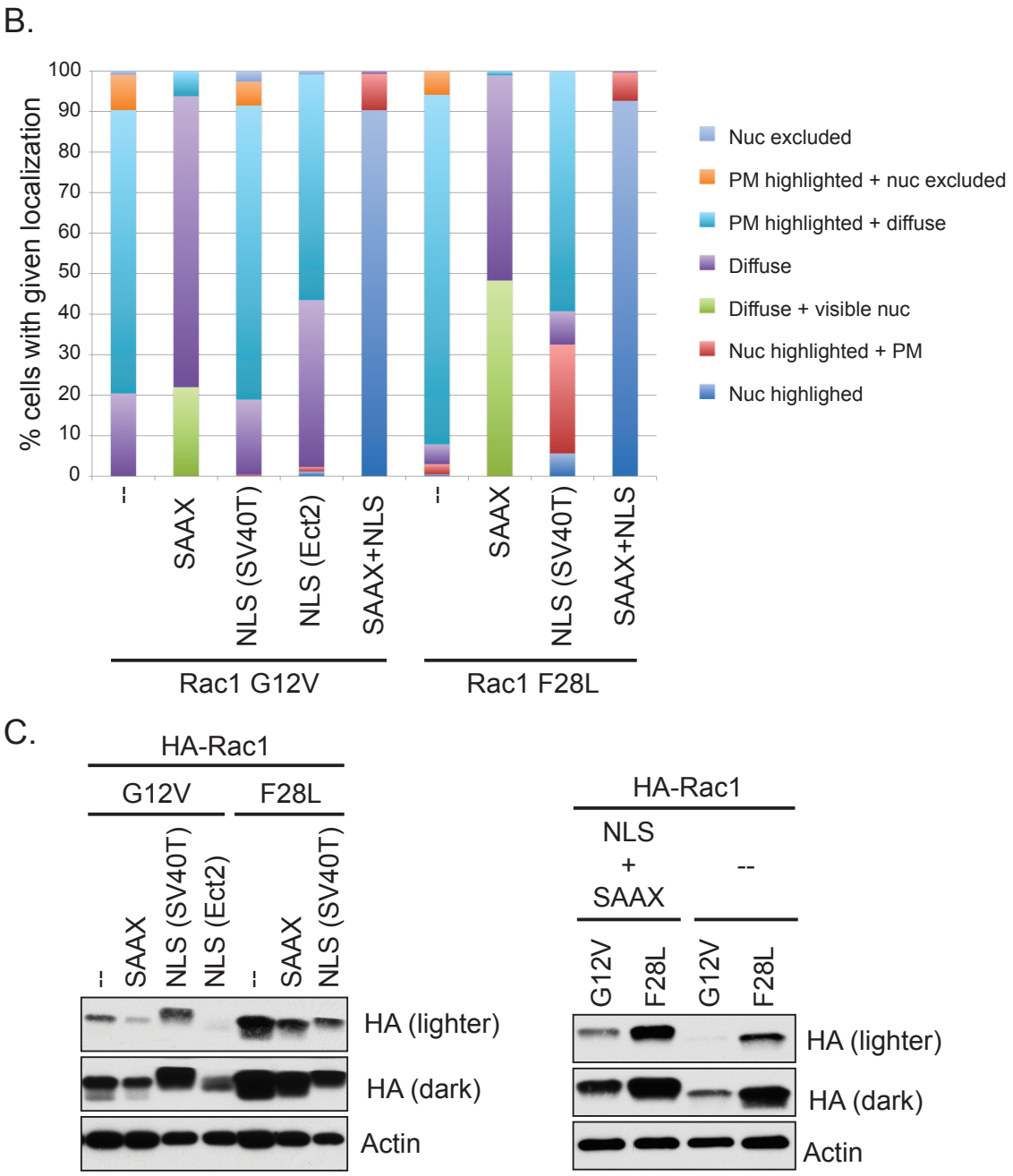


Figure 6.8. Rac1 F28L mutants concentrate in the nucleus more freely and are expressed more highly than G12V mutants. To target active HA-Rac1 to the nucleus, G12V and F28L HA-tagged Rac1 were modified in a variety of ways, including: the SAAX mutation, addition of a NLS (from Ect2 or SV40T antigen), both modifications, or neither modification. OVCAR8 Ect2 knockdown cells were infected with each of these constructs and selected for stable selection. Cells were then fixed, stained with HA antibody, and the localization of each construct was visualized with Alexa fluor 488 secondary antibody. *A.* Cells were imaged using confocal microscopy. Representative images are shown. Scale bars represent 10 microns.



B. The localization of Rac1 in the cell population was quantified by counting cells with the following subcellular localization patterns: nuclear (nuc) excluded, plasma membrane (PM) +nuc excluded, PM+diffuse, diffuse, diffuse with visible nuc, nuc highlighted+PM, nuc highlighted. The fraction of cells in each location is shown in the bar graph ($n \geq 3$, with approximately 100 cells counted per condition per n). Interestingly, the SAAX mutation or the addition of an NLS caused a more dramatic shift towards nuclear accumulation in the F28L background than the G12V background. *C.* Relative expression levels of each mutant was determined by immunoblot. HA-Rac1(G12V) and HA-Rac1(F28L) are shown in both blots to allow relative comparisons across blots. Actin served as a loading control.

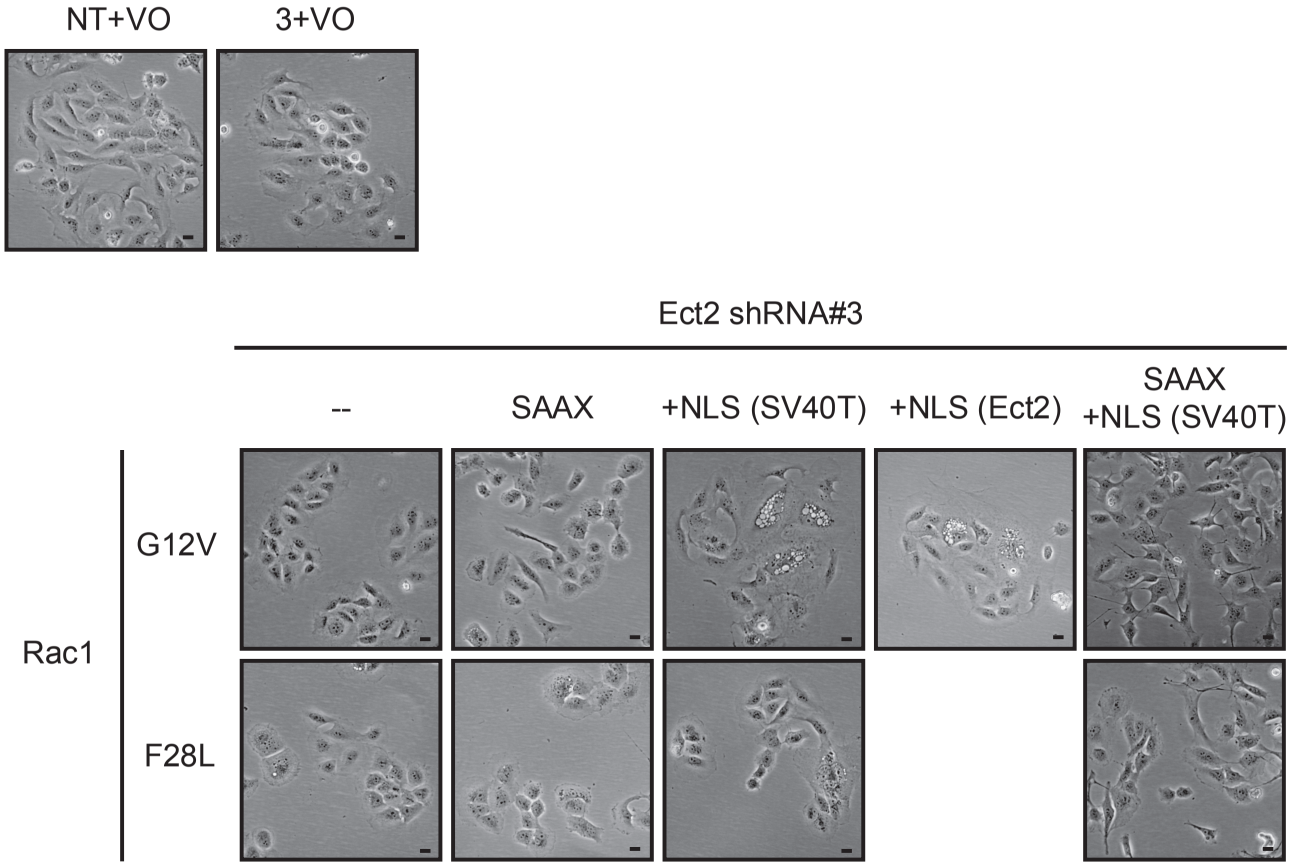


Figure 6.9. Expression of NLS-HA-Rac1 induces vesicle formation in OVCAR8 cells. The OVCAR8 cells described in Figure 6.8 were imaged while undergoing stable selection. Representative images are shown. Cells expressing non-targeted shRNA and vector (NT+VO) and Ect2 shRNA#3 and vector (3+VO) are included as controls. Scale bar represents 20 microns.

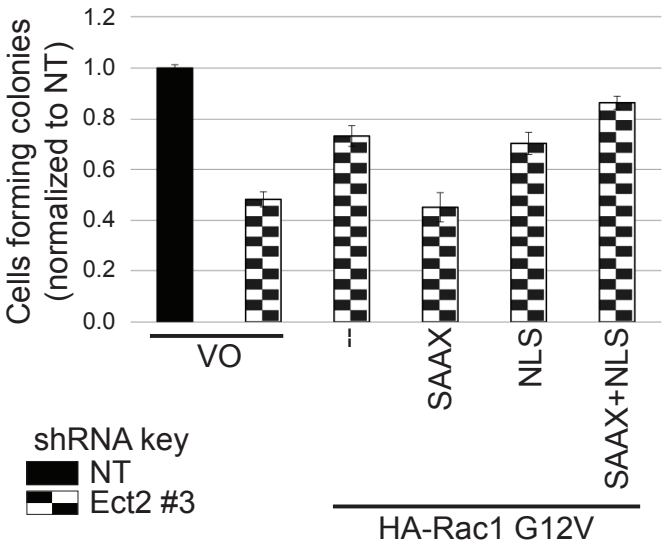


Figure 6.10. Expression of active Rac1 mutants rescues the defect in anchorage-independent growth caused by Ect2 knockdown, except when Rac1 contains a SAAX mutation and no NLS. To further examine the role of Rac1 subcellular localization on transformation, the cells described above were used for soft agar assays. Cells expressing non-targeted (NT) shRNA and vector (VO) were used as controls for anchorage-independent growth and cells expressing Ect2 shRNA and VO were used as controls for inhibited cell growth. NLS-Rac1(G12V) with the NLS from Ect2 was used, since the high number of vesicles (Fig. 6.9) correlated with slowed cell growth upon expression of NLS (SV40T antigen)-Rac1(G12V). Cells were grown in agar for approximately 2.5 weeks and then counted for colony formation. The fraction of cells forming colonies, normalized to NT+VO is shown. Error bars represent SEM.

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