Investigating Rac1 Subcellular Localization, Phosphorylation and Novel role in the Nucleus

by

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Abstract

Rac1 is a small GTPase that belongs to the Rho family. Like other Rho family GTPases, it mediates a plethora of cellular effects, including regulation of cytoarchitecture, cell size, cell adhesion, cell polarity, cell motility, proliferation, apoptosis/survival, and membrane trafficking. The Activity of Rac1 is controlled by three classes of regulatory: Guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs), and guanine-nucleotide-dissociation inhibitors (GDIs). However, accumulated evidence indicates that Rac1 activity and function are also regulated by other modifications, including RNA splicing and microRNAs, various post- translational modifications. Among various post-translational modifications, phosphorylation has been shown to play an important role in regulating the function of Rac1. Rac1 is phosphorylated at S71, Y64, and T108 by various kinases. The objective of my research is to understand how the phosphorylation of Rac1 regulates its function. I first studied the role of S71 phosphorylation in mediating Rac1 interaction with 14-3-3 proteins. Using Western blots, GST pull-down assays, co-immunoprecipitation, and phosphorylation assays. I studied and characterized novel interaction of Rac1 protein with each of the seven 14-3-3 isoforms in response to EGF. In this thesis, I showed that that 14-3-3s interact with Rac1. Rac1 S71 mediates this interaction in both phosphorylation-dependent and -independent manners, but the phosphorylation-dependent interaction is much stronger. EGF strongly stimulates the phosphorylation of Rac1 S71 and the interaction between 14-3-3 and Rac1. Mutating S71 to A completely abolished both phosphorylation-dependent and -independent interactions

between 14-3-3 and Rac1. The interaction between 14-3-3 and Rac1 mostly serves to regulate the activity and subcellular localization of Rac1. Among the seven 14-3-3 isoforms, 14-3-3η, -γ, -σ, and -θ interact with Rac1. I then studied the effects of T108 phosphorylation on the nuclear localization and nuclear function of Rac1. I showed that in response to EGF, Rac1 was targeted to nuclear speckles (NS) and co-localized with the NS marker SRSF2. Rac1 was also partially colocalized with A' protein of U2 snRNP (U2A') that localizes to the actual splicing sites at the peripheral region of NS. I also showed that the NS localization of Rac1 was dependent on its T108 phosphorylation by EGF. In addition to T108 phosphorylation, Rac1 PBR and GTPase activity also contributed to its NS localization. Moreover, Rac1 interacts with various proteins involved in pre-mRNA splicing, including SRSF2, U2A', and hnRNPA1, as indicated by co-IP. These interactions are also dependent on T108 phosphorylation and affected by Rac1 PBR and GTPase activity. Finally, I showed that Rac1 regulated EGF-induced pre-mRNA splicing, and this is mediated by T108 phosphorylation.

Preface

Chapter 1 of this thesis has been published as "Abdrabou, A.; Wang, Z. Post-Translational Modification and Subcellular Distribution of Rac1: An Update. *Cells* **2018**, *7*, 263. I wrote the review. Z. Wang was the supervisory author and was involved in manuscript composition.

Chapter 2 of this thesis has been published as Abdrabou, A.; Brandwein, D.; Liu, C.; Wang, Z. Rac1 S71 Mediates the Interaction between Rac1 and 14-3-3 Proteins. *Cells* **2019**, *8*, 1006. I was responsible for experiments performance, data collection, and analysis as well as the manuscript composition. D Brandwein. assisted with the data collection for figures 7 and 8. Z. Wang was the supervisory author and was involved and manuscript composition.

Parts of Chapter 3 of this thesis are “under review” as Abdrabou, A., Wang, Z. "Nuclear Speckle localization of Rac1 and its role in pre-mRNA splicing". I was responsible for experiments performance, data collection, and analysis as well as the manuscript composition. Z. Wang was the supervisory author and was involved in manuscript composition.

Dedication

To

Mom, Dad, and my sisters

Acknowledgments

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Abbreviations

14-3-3 protein	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein
α/β, ε, γ, η, σ, τ/θ, δ/ζ	Alpha/beta, epsilon, gamma, beta, sigma, tau/theta,
delta/zeta AEBSF	4-(2-aminoethyl)-benzenesulfonyl fluoride
Akt	Protein kinase B
APS	Ammonium persulfate
ARHGEF2	Rho guanine nucleotide exchange factor 2
ARP2/3	Actin-related protein 2/3
ATCC	American type culture collection
β1PIX	P21-activated kinase-interacting exchange factor beta
BAD	Bcl-2-associated death promoter
BES	*N*, *N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid

BSA	Bovine serum albumin
BSE	Bovine spongiform encephalopathy
BV02	2-(2,3-Dihydro-1,5-dimethyl-3-oxo-2-phenyl-1H-pyrazol-4-yl)- 2,3-dihydro-1,3-dioxo-1H-isoindole-5-carboxylic acid
COS-7	CV-1 (simian) in origin, and carrying the SV40 genetic material-7
DAPI	4’,6-diamidino-2-phenylindole
DLC1	Deleted in liver cancer 1
DMEM	Dulbecco’s modified eagle medium
ECM	Extracellular matrix
EDTA	Ethylenediaminetetracetic acid
EGF	Epidermal growth factor
EGTA	Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
ER	Endoplasmic reticulum
ERK	Extracellular regulated kinase
FAM65B	Family with sequence similarity 65, member B
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate

G2/M	Second gap phase to mitosis DNA damage checkpoint
GAP	GTPase-activating protein
GDI	Guanine nucleotide dissociation inhibitor
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
GTP	Guanosine triphosphate
HEK293T	Human embryonic kidney 293T
HnRNPA1	Heterogeneous nuclear ribonucleoprotein A1
HSP60	Heat shock protein 60
hTERT	Human telomerase reverse transcriptase
IRDyes	Infrared fluorescent dyes
JAK/STAT	Janus kinase/signal transducer and activator of transcription
kDa	Kilodalton
LB broth base	Luria Bertani broth base medium
LIMK	LIM domain kinase
LKB1	Liver kinase B1
MAPT	Microtubule-associated protein tau
$MgCl_2$	Magnesium chloride
MOC	Manders' overlap coefficient
MS	Multiple sclerosis
NaCl	Sodium chloride

NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
NaF	Sodium fluoride
Na_2HPO_4	Sodium phosphate dibasic
NaN_3	Sodium azide
Na_3VO_4	Sodium orthovanadate
NEAAs	Non-essential amino acids
NFTs	Neurofibrillary tangles
NLS	Nuclear localization signal
NP-40	Nonidet P40
NtCDPK1	*Nicotiana tabacum* Ca^{2+}-dependent protein kinase 1
Opti-MEM	Reduced-serum minimal essential medium
p21Waf1/Cip1	Cyclin-dependent kinase inhibitor 1/CDK-interacting protein 1
PAK1	P21 activated kinase 1
Pan 14-3-3	Total 14-3-3
Par3	Partitioning-defective protein 3
PBR	Polybasic region
PBS	Phosphate buffered saline
PC3	Human prostate cancer
PCC	Pearson's correlation coefficient
pEGFR	Phospho-epidermal growth factor receptor
PI3K	Phosphoinositide 3-kinase

PM	Plasma membrane
PML	Promyelocytic leukemia protein
PP1α	Protein phosphatase 1 alpha
PPI	Protein-protein interaction
pSX1–2–COOH	14-3-3 consensus binding motif extreme C-terminus (mode III)
Q61L	Glutamine to leucine substitution at position 61
Rac1	Ras-related C3 botulinum toxin substrate 1
Rac2	Ras-related C3 botulinum toxin substrate 2
Rac3	Ras-related C3 botulinum toxin substrate 3
RGS14	Regulator of G Protein Signaling 14
RhoA	Ras homolog gene family, member A
RhoGDIα	Rho guanine dissociation inhibitor alpha
Rnd3	Rho family GTPase 3
ROCK	Rho-associated coiled-coil containing protein kinase
ROR1	Receptor tyrosine kinase-like orphan receptor 1
RSXpSXP	14-3-3 consensus binding motif mode I
RXY/FXpSXP	14-3-3 consensus binding motif mode II
S71A	Mutation of serine 71 to alanine
S71E	Mutation of serine 71 to glutamic acid
SDS	Sodium dodecyl sulfate

SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SGK1	Serum- and glucocorticoid-induced protein kinase 1
siRNA	Small interfering ribonucleic acid
SRC	Sarcoma viral oncogene
SRSF2	**Splicing factor, arginine/serine-rich 2**
T108E	**Mutation of threonine 108 to glutamic acid**
T17N	**Mutation of threonine 17 to asparagine**
TBS	**Tris-buffered saline**
TBS-T	**Tris-buffered saline-Tween 20**
TcdA	**Clostridium difficile toxin A**
TCL	**Total cell lysate**
TCR	**T-cell receptor**
TEMED	**Tetramethylethylenediamine**
TGF-β1	**Transforming growth factor-beta 1**
Tiam1	**T-cell lymphoma invasion and metastasis-inducing protein 1**
Tir	Translocated intimin receptor
TM	Trabecular meshwork
Tris-HCl	Tris (hydroxymethyl) aminomethane hydrochloride
TRITC	Tetramethylrhodamine isothiocyanate
UTKO1	Cell migration inhibitor
WAVE	WASP-family verprolin-homologous
WT	Wild type

Y64F/D	Mutation of tyrosine 64 to phenylalanine/aspartic acid

Chapter 1:
Introduction

1.1 Overview

The Rho family of GTPases mediates a plethora of cellular effects, including regulation of cytoarchitecture, cell size, cell adhesion, cell polarity, cell motility, proliferation, apoptosis/survival, and membrane trafficking[1,2]. The Rho family of GTPases accounts for as many as 23 members[3]. Among them, the archetypes RhoA, Rac1, and Cdc42 have been the best characterized. Like all members of the small GTPases superfamily, Rho proteins act as molecular switches to control cellular processes by cycling between active, GTP-bound and inactive, GDP-bound states. A significant function of Rho proteins is to control cell cytoskeleton remodeling and cell migration[4,5]. Rac1 regulates actin polymerization and the formation of lamellipodia and membrane ruffles, presumably through interaction with the WASP-family verprolin- homologous (WAVE) complex. Endothelial-specific knockout of Rac1 causes embryonic lethality in mid-gestation (around E9.5). [6]

As a consequence of a large number of critical functions assigned to Rho proteins, it is not surprising that they play essential roles in many human diseases[7,8]. Accumulating evidence has implicated Rho GTPases in many aspects of cancer development, especially in cancer cell invasion and metastasis[9,10]. Deregulated Rho GTPases have been discovered in many human tumors, including colon, breast, lung, myeloma, and head and neck squamous cell carcinoma[11,12]. Thus, Rho GTPases and the signal pathways regulated by them have been proposed as potential anticancer therapeutic targets[13]. Rac1 is overexpressed or mutated in breast cancer and many other cancers and is linked to many other diseases[14]. Due to its well-established role in controlling cytoskeleton dynamics, studies have been focused on revealing the role of Rac1 in cancer metastasis.

Until recently, Rac1 itself was considered "undruggable" by small molecules due to the lack of any deep hydrophobic pockets on the surface and earlier failure in finding effective pharmacologic inhibitors. The primary approach was to target its downstream effectors of kinases, such as PAK[15].

The cycling of Rac1 between the GTP- and GDP-bound states is essential for sufficient signal flow to elicit downstream biological functions[16]. Other modifications of Rac1 include RNA splicing and microRNAs; various post-translational modifications have also been shown to regulate the activity and function of Rac1[17,18]. The reported post- translational modifications include lipidation, ubiquitination, phosphorylation, glutathionylation and adenylylation. Additionally, the Rac1 activity and function are regulated by its subcellular distribution and translocation[19].

1.2 Rho Family of GTPases

Rac1 is a small GTPase (M 21,450 kDa) that belongs to the Rho family of GTPases. The Rho family of small GTPases is a subfamily of the Ras superfamily[3,20]. The Ras superfamily is comprised of five subfamilies based on their sequence homologies, which includes the Ras GTPase subfamily, Rho GTPases, the Rab subfamily, Arf subfamilies, and the Ran subfamily (Figure 1)[21]. Each subfamily functions mostly at distinct subcellular localizations and is regulated Guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs) [22,23,24].

The Rho family of GTPases is composed of 20 members in mammalian cells. The most studied members are the canonical proteins Rho, Rac, and Cdc42. Rho GTPases signaling to the cytoskeleton and vesicular traffic, notably by regulating the dynamics of actin[25,26]. Moreover, Rho GTPases regulate cell size, cell proliferation, cell survival, membrane trafficking, cell motility, polarity, and adhesion. Like other small GTPases, most Rho family proteins function as switches by cycling between inactive (GDP-bound) and active (GTP-bound) forms[27]. The cycle between inactive and active forms is controlled by three classes of regulatory proteins: GEFs that activate Rho GTPases by promoting the release of GDP to allow binding of GTP; GAPs that inactivate Rho GTPases by stimulating its intrinsic GTP-hydrolysis activity; and guanine-nucleotide- dissociation inhibitors (GDIs), which interact with the GDP-bound inactive Rho to prevent its exchange to the GTP-bound form and prevent its translocation to the membrane for action[2,8,29,30].

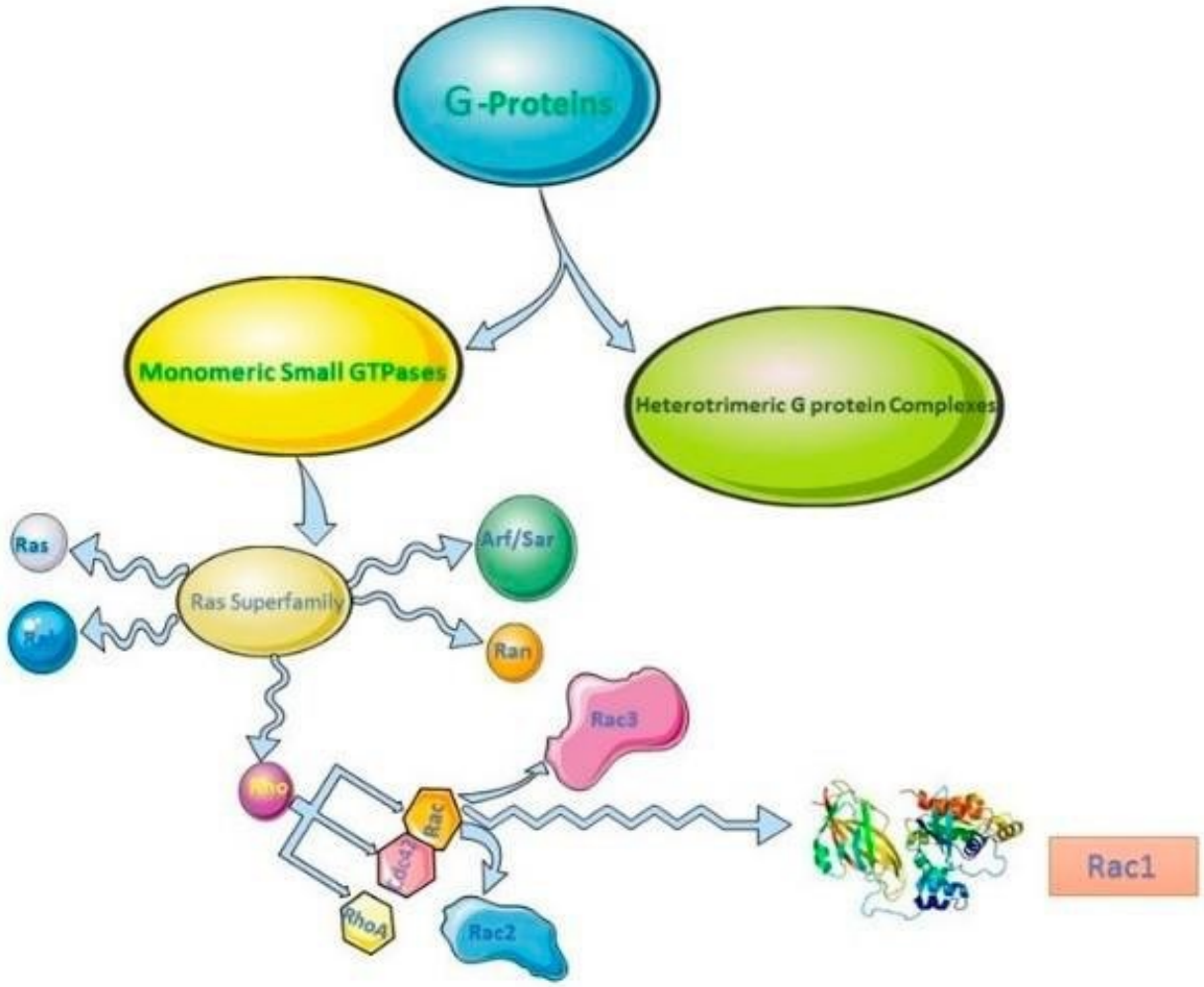

Figure 1. The diagram illustrates the composition of the Ras superfamily. (I've designed the figure)

1.2.1 Rho mediators

Rho GEFs are divided into two unrelated families; one family contains a ~200 amino acid (aa) Dbl-homology domain (DH), and the other family contains a ~400 aa Dock homology region (DHR)[31]. Dbl-homology family is the largest and best-studied Rho GEF family with at least 70 members. The Dbl family of Rho GEFs contains both DH and pleckstrin homology (PH) domains[32]. Most Rho GEF activity is mediated by the catalytic DH domains. A primary mechanism in the regulation of GEFs is the relief of the intramolecular inhibition caused by the PH domain[33]. GEFs also contains several other functional domains, many of which couple to upstream receptors. Thus, it is not surprising that Rho GEFs and Rho are regulated by receptors, including the epidermal growth factor (EGF) receptor (EGFR)[1,34–36]. Recent studies indicate that the cycling between the GTP- and GDP-bound states might be essential for adequate signal flow through Rho GTPases to elicit downstream biological functions, and this could involve the concerted action of all classes of the regulatory proteins [37]. Like most of the typical small GTPases, the regulatory cycle of Rac1 is exerted by three distinct families of proteins: GEFs, GAP, and GDIs[38–40]. So far, more than twenty GEFs have been identified for Rac1, some of them specifically for the Rac family, some less selective.
Most of the GEFs that act on Rac1 are in the 60-member Dbl family, including members of the Tiam, Vav, PIX, SWAP-70, and P-Rex families. DOCK180, a member of the DOCK family of GEFs, has also been shown to regulate Rac1[41]. DOCK proteins contain a conserved catalytic domain, a phospholipid domain, and a DOCK homology region 2[4,22,4]. It is generally accepted that the membrane localization of Rac is a prerequisite for Rac activation. Several Rho GAPs were already known in 1992; however, since then, the number of known genes encoding a Rho-GAP domain has increased to about 80[16,43–45]. The functions of most of these proteins are still unknown, though the variations in additional domains found in these proteins indicate that they act in a wide variety of signaling pathways in different tissues. The GDIs are pivotal regulators of the Rho GTPase function. GDIs control the access of

1.2.2 Rac subfamily

Rac is also known as Ras-related C3 botulinum toxin substrate. There are three members in the Rac subfamily, including Rac1, Rac2, and Rac3, which share a significant sequence identity (~88%)[48–50]. These three diverge primarily in the C- terminal 15 residues. All the Rac-related proteins stimulate the formation of lamellipodia and membrane ruffles [51–53].

1.2.3 Rac1

Rac1 promotes lamellipodia formation by stimulating the polymerization of branched actin at the leading edge of the cell [54]. Activated Rac1 binds to the WASP-family verprolin-homologous (WAVE) proteins through association with its downstream effector insulin receptor tyrosine kinase substrate p53 (IRSp53)[55,56]. The WAVE proteins then bind to and activate the protein complex composed of actin nucleating protein and actin-related protein 2/3 (Arp2/3), which leads to the enhanced polymerization of branched actin at the leading edge and promote lamellipodia formation. Additionally, Rac1 has also been shown to regulate the expression of various matrix metalloproteinases (MMPs), which are required for the proteolytic degradation of the extracellular matrix [57,58]. The *in vivo* and *in vitro* studies in the last decades have firmly established the role of Rac1 in cancer cell invasion and metastasis[59]. Rac1 can stimulate MMP-1 or MT1-MMP production in lung cancer cell lines and enhance invasion in vitro. When adherens junctions are weakened by EGF or hepatocyte growth factor (HGF), Rac is required to promote cell migration and invasion[60,61].

1.3 Regulation of Rac1 by EGFR and other Membrane Receptors

The activity and the function of Rac1 are ultimately regulated by environmental cues, among which are the membrane receptors, including many receptor tyrosine kinases (RTKs) [62,63]. The most studied membrane receptor in this regard is EGFR. It is well-

established that EGFR activates multiple signaling pathways and regulates many cellular functions, including cell proliferation, differentiation, survival, adhesion, and migration [64,65]. Many EGFR-mediated cell functions are partially mediated by Rac1 and other Rho family proteins[66].

The most common mechanism by which EGFR regulates Rac1 activity is through the regulation of Rac1 GEFs[39,67,68]. GEFs implicated in EGF-stimulated Rac1 activation include members of Tiam, Sos, DOCK, Vavs, and Asef family. EGF stimulates Tiam1 through the activation of Phosphoinositide 3-kinases (PI3K)[3,6,34]. Moreover, EGF stimulates two waves of Rac1 activation, first at 5 min and the second at 12 h following the addition of EGF invitro. Cell migration is driven by the second wave of EGF-induced Rac1 activation. It was further shown that EGF activates Rac1 through the activation of Tiam1, either via 14-3-3ζ or through 5-lipoxygenase-leukotriene C4[6,9,70].

Recent data further supported the role of EGFR in the regulation of Rac1 activity and cell migration by controlling Rac1 GEF. EGFR exerts its effects mostly by phosphorylating Rac1 GEF, but also by ubiquitination and controlling the level and availability[6,71]. For example, treatment of cells with either EGF or platelet-derived growth factor stimulate tyrosine phosphorylation and activation of Vav2, which promotes cell migration through the activation of Rac1, Cdc42, and RhoA. EGF stimulates the phosphorylation of Try94 of Asef, which activates Asef[72,73]. It was further shown that EGF, basic fibroblast growth factor, and hepatocyte growth factor all activate Asef through PI3K, which promotes cell migration[74,75,76]. It was later reported that EGFRvIII, a constitutively active EGFR mutant, stimulates the phosphorylation of Dock180 via Src, which stimulates Rac1 signaling, glioblastoma cell survival, and migration[77,78]. Moreover, EGFRvIII induces the phosphorylation of Dock180 via protein kinase A, which activates Rac1 and promotes glioma tumor growth and invasion[42].

EGFR may also regulate Rac1 GEF by controlling it's level/availability. It was shown that EGFR activates Rac1 by increasing the accumulation of Tiam1 in colon cancer and non-small-cell lung cancer cells[79,80]. Moreover, the effects of EGFR on the accumulation of

Tiam1 are through the activation of Akt. Activated Akt promotes the interaction between the Tiam1 and 14-3-3 protein by phosphorylating Tiam1. Recently, it was reported that Vav3.1, a shorter member of the Vav family, was downregulated by the addition of EGF and Transforming growth factor β(TGFβ) in the pathogenesis of oral squamous cell carcinoma. Down-regulation of Asef by siRNA inhibits the EGF- induced activation of Rac1[52,81–83].

EGF stimulates the phosphorylation of Try94 of Asef, which activates Asef. The activated EGFR disrupts adherens junctions in human mammary epithelial cells by Vav2 and Rac1/Cdc42 activation. It was further shown that EGF, basic fibroblast growth factor, and hepatocyte growth factor all activate Asef through PI3K, which promotes cell migration. EGF enhances the ubiquitination of Dock180 by stimulating the translocation of Ubiquitinated Dock180 to the plasma membrane (PM), whereas Dock180 is ubiquitinated[41,77,84]. It was also reported that the Mer-family tyrosine kinase activation stimulates a post-receptor signaling cascade involving Src-mediated FAK phosphorylation, which promotes the formation of a p130CAS/CrkII/Dock180 complex to activate Rac1. Most recently, it was shown that EGFR overexpression or expression of EGFRvIII stimulates Rac1 activation and promotes glioblastoma cell migration through activating the MLK3-JNK signaling axis[77,84,85].

Much less is known about the role of EGFR and other membrane receptors in the regulation of Rac1 GAPs. It is known that some Rac-GAPs are stringently regulated by receptor stimulation. For example, the chimeric Rac-GAPs are activated by diacylglycerol (DAG) generated in response to growth factor receptor activation[86]. DAG binds to chimaerins to promote their redistribution to the PM, where they inactivate Rac1. This regulation is via PKC-mediated phosphorylation and other protein-protein interactions, which modulates the subcellular localization and membrane association of chimaerin. It was shown recently that EGFR and Src signaling regulates FilGAP through association with RBM10[87,88].

Recently, more studies have demonstrated the role of EGFR-stimulated activation of Rac1 in cancer cell migration, invasion, and survival. EGF stimulates head and neck squamous cell carcinoma by activating PKD1-fibronectin-Rac1/Cdc42 and metalloproteinases (NMP)[54,89]. Previously, It was shown that Rac1 could stimulate

MMP-1 production in lung cancer cell lines and enhance invasion in vitro. EGF also stimulates PI3K-Akt-Rac1 signaling cascades through the activation of Giα2, which promotes the migration and invasion of prostate cancer cells[79,90]. Periodic mechanical stress stimulates nucleus pulpous cell proliferation by activating EGFR-Rac1-ERK cascades. Ligand-specific EGFR signaling regulates apicobasal polarity via PI3K and Rac1, which controls cell fate and pancreatic organogenesis. T-cadherin translocation to cell-cell contact is sensitive to EGFR-mediated activation of Rac1 and p38MAPK [91,9293]. In human epidermal growth factor receptor 2 (HER2)-positive breast cancer cells, two distinct mTORC2-dependent pathways converge on Rac1, either through Akt-Tiam1 or

hrough PKC-RhoGDI2[94].

1.3 Regulation of Rac1 Activity by Post-Translational Modification

The cycling of Rac1 between the GTP- and GDP-bound states is essential for effective signal flow to elicit downstream biological functions. Other modifications, including RNA splicing, microRNAs, and various post-translational modifications, have also been shown to regulate the activity and function of Rac1[9,95]. The reported post- translational modifications include lipidation, ubiquitination, glutathionylation phosphorylation, and adenylylation, which were all shown to play essential roles in the regulation of Rac1 and other Rho GTPases.

1.3.1 Regulation of Rac1 Activity by Lipidation

Protein prenylation involves the attachment of either a 15-carbon farnesyl or 20-carbon geranylgeranyl isoprenoid to a cysteine residue four amino acids from the C-terminus of a protein[96,97]. Prenylation is the first and most reported post-translational modification of Rac1 and other Rho GTPases, which plays a critical role in the

regulation of Rac1 by targeting Rac1 to the PM and facilitating Rac1 interaction with GEFs [46,98,99]. Like many other small G proteins, Rac1 has a unique C-terminal amino acid sequence of a CAAL, where C is cysteine, A is an aliphatic amino acid, and L is leucine (Figure 2). Small G proteins having this C-terminal structure were post-translationally processed[66,100,101]:(1) geranylgeranylation of the cysteine residue; (2) removal of the A- A-Leu portion; and (3) carboxyl methylation of the exposed cysteine residue. Rac1 is geranylgeranylated at its C-terminal Cysteine residue[102,10346].

Figure 2. Rac1 sequences, various motifs, and amino acid residues subjected to post-translational modification.

It was initially thought that the newly synthesized Rho GTPases such as Rac1 is quickly prenylated in the cytosol and then moved to Endoplasmic reticulum for further modifications. However, later research indicates that the entrance and passage of small GTPases, including Rac1 through the prenylation pathway, are regulated by two splice variants of SmgGDS. SmgGDS-558[10,49,7,105] which selectively associates with prenylated small GTPases and facilitates their trafficking to the PM, whereas SmgGDS-607 associates with non-prenylated GTPases and regulates the entry of Rho small GTPases into the prenylation pathway. These results indicated that SmgGDS splice variants could regulate the entrance and passage of small GTPases through the prenylation pathway[106–108].

Interestingly, we recently showed that Rac1 contains an ERK docking site located at its C-terminal 183KKRKRKCLLL192[32,109]. The coreconsensusmotif of ERKD-sites is (K/R)1-3-X1-6-ϕ-X-ϕ. The D-site is critical for the interaction between Rac1 and ERK present following its geranylgeranylation. Thus, the interaction between Rac1 and ERK should occur before the geranylgeranylation. The regulated entry into the prenylation pathway by SmgGDS provides a mechanism to allow the interaction between ERK and non-prenylated Rac1[110,111].

It was later reported that Rac1 is also palmitoylated. Palmitoylation is the post-translational covalent binding of the 16-carbon fatty acid palmitate through a thioester bond[97,112]. Different from prenylation, palmitoylation is reversible. There is no conserved motifidentified for palmitoylation. In Rac1, among the two possible Cys for palmitoylation, Cys6 (N-terminus), or Cys178 (C-terminus), Cys178 was shown to be palmitoylated[45,113,114]. Blocking Rac1 palmitoylation by mutation significantly reduces PM localization and the GTP loading of Rac1, which leads to reduced activation of PAK at the PM[97].

1.3.2 Rac1 Ubiquitination

The expression level of Rho GTPase is also an important factor in regulating its signaling and function. Like most proteins, the expression level of Rho GTPases is determined by both its synthesis and degradation[71,115]. The synthesis of Rac1 and the

other Rho GTPases are mostly regulated by various external stimuli. The degradation of Rac1 and the other Rho GTPases are regulated through their ubiquitination and subsequent proteasomal degradation. In Rho GTPases, the positions of ubiquitinate

Lysines are not conserved[71,116,117]. While the most studied ubiquitinated lysines are located in a region on the opposite side of the GTP-binding and switch regions, many additional ubiquitinated lysines are scattered around the protein surface. It has been well documented that Rac1 is ubiquitylated by E3 proteins HACE, XIAP, and c-IAP1 on K147, and by FBXL19 on K166 dependent on S71 phosphorylation. Rac3 is ubiquitylated on K166 by the E3 protein FBXL19[118,119].

The significance of Rac1 ubiquitylation was revealed by the discovery of HACE1 ubiquitin ligase as a tumor suppressor[120,121]. While HACE1 has multiple targets, it has been shown that loss of HACE1 increases Rac1 activity, which induces reactive oxygen species generation and cell migration that likely contribute to Rac-mediated tumor progression[122].

1.3.3 Phosphorylation of Rac1 and other Rho GTPases

Recent findings have suggested that additional regulatory mechanisms such as phosphorylation might further contribute to the tight regulation of Rho GTPases. Rac1 is phosphorylated on S71 by Akt[119,123]. This phosphorylation of Rac1 inhibits its GTP binding activity without any significant change in GTPase activity. Both the GTP- binding and GTPase activities of the mutant Rac1 S71A are abolished regardless of the activity of Akt[124–126]. It was later reported that the phosphorylation of Rac1 S71 decreases the pathogenic effect mediated by Clostridium difficile toxin A (TcdA)[127,128]. Moreover, phosphorylation of Rac1 at S71 represents a reversible mechanism to determine the binding specificity of Rac1/Cdc32 to their downstream substrates. Besides, Rac1 is phosphorylated at Y64 by FAK and Src kinases; Y64 phosphorylation targets Rac1 to focal adhesions[129]. Rac1-Y64F displayed increased GTP-binding, increased association with βPIX, and reduced binding with RhoGDI as compared with wild-type Rac1[129].

Rac1-Y64D had less binding to PAK than Rac1-WT or Rac1-64F. In vitro, assays

demonstrated that Y64 in Rac1 is a target for FAK and Src[51,65]. These findings demonstrate that both Serine/Threonine and tyrosine phosphorylation of Rac1 are common phenomena and regulate multiple aspects of Rac1 functions. So far, there is no pathogenic evidence to support the role of these phosphorylation of these residues in the development of cancer and other diseases. Further research is needed Recently, we identified that PLC-γ1 is a Rac1 GEF, both in vitro and in vivo. We showed that the interaction between PLC-γ1 and Rac1 is mediated by PLC-γ1 SH3 domain and Rac1 proline-rich motif 106PNTP109. Moreover, we showed that EGF- induced interaction between the PLC-γ1 SH3 domain and the Rac1 106PNTP109 motif resulted in the activation of Rac1 and enhanced EGF-induced cytoskeleton reorganization and cell migration [130]. Interestingly, sequence analysis of Rac1 shows that Rac1 T108 within the 106PNTP109 motif is likely an ERK phosphorylation site; Rac1 has the ERK docking site 183KKRKRKCLLL192 (D-site) at C-terminus. We showed that both transfected and endogenous Rac1 interacts with ERK, and this interaction is mediated by its D-site. GFP-Rac1 is threonine (T) phosphorylated in response to EGF, and EGF-induced Rac1 threonine phosphorylation is dependent on the activation of ERK[131,132]. Furthermore, mutant Rac1 with the mutation of T108 to alanine(A) is not threonine phosphorylated in response to EGF In vitro ERK kinase assay further shows that pure active ERK phosphorylates purified Rac1, but not mutant Rac1T108A. Additionally, we showed that Rac1 T108 phosphorylation decreases its activity, partially due to inhibiting its interaction with PLC-γ1. T108 phosphorylation targets Rac1 to the nucleus, which isolates Rac1 from other GEFs and hinders Rac1's role in cell migration. We concluded that Rac1 T108 is phosphorylated by ERK in response to EGF, which plays a vital role in regulating Rac1[130,133].

As many of the kinases, such as Akt, ERK, and Src, that phosphorylates Rac1 are activated by EGFR and other RTKs, it is not surprising that EGFR and other RTKs play essential roles in the regulation of Rac1 phosphorylation. We have shown that EGF stimulates the Rac1 T108 phosphorylation.

1.4 Regulation of Subcellular Localization of Rac1

It has been well documented that Rac1 and other Rho GTPases distribute between

multiple cellular compartments. At various subcellular locations, Rho GTPases may interact with different effectors, and thus, produce spatially complex signaling outputs[48,134]. Besides the well-documented localization in the cytosol and the PM, Rac1 has been shown to localize to the other subcellular compartments, including endosomes and the nucleus[135].

1.4.1 Regulation of the PM Localization of Rac1

In addition to cycling between GDP bound inactive form to GTP-bound active form, Rho GTPases also cycle between PM and cytosol, which regulates their activities and function in actin cytoskeleton remodeling and cell migration. The lipidation of Rac1 is

important for its membrane-binding [136,137]. It was shown that membrane bound Rac1 is geranylgeranylated, and the soluble Rac1 is not geranylgeranylated. In the aged mouse brain, the reduced geranylgeranylation of Rac1 due to lower geranylgeranyltransferase activity decreases the PM association of Rac1 [138,139].

The subcellular localization of Rac1 is also regulated by its C-terminal polybasic region (PBR). Many small GTPases in the Ras and Rho families have a PBR comprised of multiple lysines or arginines. The PBR controls diverse functions of these small GTPases, including their ability to associate with membranes, interact with specific proteins, and localize in subcellular compartments. A significant function of the PBR is to promote the interactions of small GTPases with the guanine nucleotide exchange factor SmgGDS[109,111].

Palmitoylation of Rac1 targets Rac1 to the lipid raft and ordered membrane region of the PM [140]. This is a significant finding, as Rac1 and RhoA are localized in caveolar, and the cholesterol-enriched lipid rafts are the primary sites of signaling by Rac1 and RhoA. On the other hand, palmitoyl modification are known to target proteins to lipid rafts. For example, H-Ras is targeted to lipid rafts due to its palmitoylation. Thus, the reported palmitoylation of Rac1 provides a mechanism for the localization of Rac1 in lipid rafts. The cycle between palmitoylation and depalmitoylation allows proteins to associate with membranes transiently, thereby regulating their sorting, localization, and function [141,142]. Rac1 palmitoylation requires its prior prenylation and the presence

1.4.2 Regulation of the Nuclear Localization of Rac1

Rac1 also localizes to endosomes and the nucleus. Rac1 activation on early endosomes and subsequent recycling of Rac1 to the PM ensure polarized signaling which means Rac1 signal transduction pathways that spatially segregate different regions of the cell, especially the cell membrane, are reinforced and maintained by positive-feedback loops., leading to localized actin-based migratory protrusions and spatial restriction of Rac1 mitogenic signals, which promotes mesenchymal motility[145,146]. In the nucleus, the accumulation of Rac1 has been linked to the regulation of Rac1 proteasomal degradation. Additionally, the cell-cycle-dependent accumulation of nuclear Rac1 promotes mitotic progression. These findings suggest that nucleocytoplasmic shuttling is important for the spatial control of specific Rac1 functions[147,148].

Both Rac1 and RhoA are localized to the nucleus. The data regarding the mechanisms

regulating Rac1 nuclear localization are very controversial. It was shown that the PBR of Rac1 has a functional nuclear localization signal (NLS)[149]. However, PBR has also been shown to target Rac1 to the PM [150]. It was found that the Rac1 NLS is cryptic in the sense that it is inhibited by the adjacent geranylgeranyl modification. However, the same research showed that the endogenous nuclear Rac1 is prenylated. It was also reported that the nuclear import of Rac1 is mediated by the direct interaction with karyopherin α2, and Rac1 activation is required for its nuclear localization. In a study of Rac1 palmitoylation, it is found that C6 is not palmitoylated, but mutation of C6 to S reduced nuclear localization of Rac1. It is not clear how C6 mutation alters the nuclear localization of Rac1[1,12,17,151].

We have shown that Rac1 T108 phosphorylation by ERK targets Rac1 to the nucleus. We show by both fluorescence microscopy and Western blotting that a significant amount of GFP-Rac1 is translocated from cytosol to the nucleus in response to EGF. Inhibition of T108 phosphorylation by mutating T to A blocks the nuclear localization of Rac1. Moreover, the T108 phosphorylation mimic GFP-Rac1T108Ewas almost exclusively localized to the nucleus with or without EGF stimulation[9,32,109].

Although the majority of RhoA is localized in the cytosol and at the PM of cells, a fraction of the total RhoA pool is distributed to the nucleus and regulates downstream signaling. We showed that RhoA is phosphorylated by ERK at S88. While this phosphorylation increases the PM translocation of RhoA, it does not target RhoA to the nucleus. Like other members of the Rho proteins family, both Rac1 and RhoA have a PBR in their C-termini[48,150].

The PBR is adjacent to and immediately precedes the C-terminal CAAX sequence. In addition to the prenylation of the CAAX motif and the interaction with RhoGDI, the subcellular localization of Rac1and RhoA is also regulated by its PBR. It has been shown that the Rac1 PBR (PVKKRKRK) contains an NLS, and thus, promotes Rac1 nuclear accumulation, whereas the RhoA PBR (RRGKKKSG) lacks an NLS and sequesters RhoA in the cytosol[102,149,152,153].

Recently, we examined the relative contribution of ERK-induced phosphorylation and the PBR on the subcellular localization of Rac1 and RhoA. We showed that the PBR is the determining factor for the subcellular localization of both Rac1 and RhoA. Replacing RhoA PBR with Rac1 PBR resulted in significant nuclear localization of RhoA regardless of the phosphorylation status of RhoA[1,5,4]. On the other hand, RhoA PBR was able to significantly reduce the nuclear localization of Rac1 regardless of the phosphorylation status of Rac1. EGF-induced nuclear translocation of Rac1 is dependent on ERK-induced Rac1 T108 phosphorylation. However, EGF-induced nuclear translocation of Rac1 also requires the presence of Rac1 PBR. It has been shown that the switching of the PBRs between Rac1 and RhoA alters their nuclear accumulation[109].

While it is well documented that a portion of Rac1 is localized to the nucleus and some mechanisms underlying the nuclear localization of Rac1 have been revealed, very little is known regarding the function and biological significance of nuclear Rac1. Nuclear localization of Rac1 could serve as a fundamental mechanism regulating the activity and function of Rac1. Localization into the nucleus will isolate Rac1 from its regulatory proteins localized in the cytoplasm and the PM [134,155]. Currently, there is no report on the nuclear localization of Rac1 GEFs. Most of the known Rac1 GEFs, including PLC-γ1, Vav proteins, and Tiam1, are outside of the nucleus–, and thus, will not be able to

activate the nuclear Rac1.

On the other hand, localization of Rac1 to the nucleus allows its interaction with a very different set of molecules and carry out different functions. It was shown that Rac1 regulates the activity of nuclear factor-κB (NF-κB). Rac1 also regulates gene transcription by interacting with STAT3 and STAT5[156]. In the nucleus, the accumulation of Rac1 has been shown to regulate the proteasomal degradation of Rac1. It was also reported that Rac1 is localized to the nucleus during the G2 phase of the cell cycle and promotes cell division. Recently, nucleolar phosphoprotein B23 was identified as a nuclear binding partner of Rac1; this binding depends on the N-terminal 88 amino acids of Rac1[37]. Rac1 nuclear exit depends on two nuclear export signals (NES) and interaction with B23. Activated Rac1 in the nucleus modulates nuclear actin polymerization and nuclear membrane shape, which controls cell invasion. However, our understanding of the function and biological significance of nuclear Rac1 is stil minimal, and further research is needed.

1.3 Conclusions

As shown in Figure 3, while Rac1 activity is primarily regulated by GEF, GAPs, and GDIs, post-translational modification provides additional mechanisms for the regulation of Rac1 activity and function. Recent research on Rac1 and other Rho GTPases has been concentrated on their post-translational modification and subcellular distribution. Recent progress includes the discovery of multiple novel post-translational modifications of Rac1 such as the palmitoylation, phosphorylation, and ubiquitination of Rac1. Besides shuttling between PM and cytoplasm, Rac1 also shuttles between the nucleus and cytoplasm in response to EGF and other stimuli. The nuclear localization of Rac1 is regulated by both the NES and NLS motifs and the various post-translational modifications. Very little is known regarding the function and biological significance of Rac1, which needs further research (Figure 3).

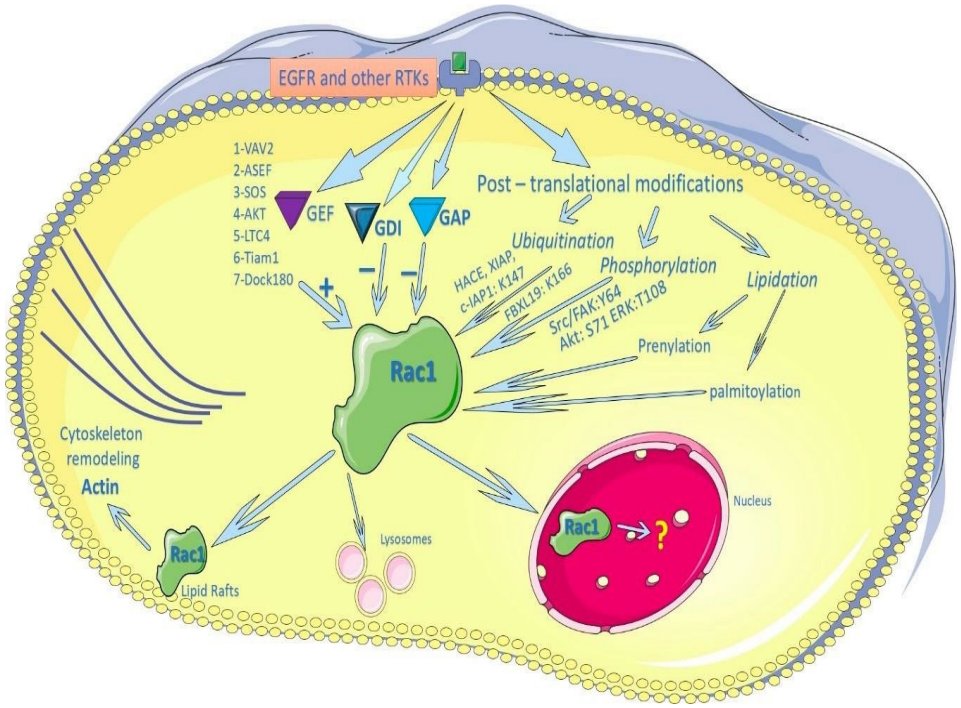

Figure 3. Summary of Rac1 regulation, subcellular distribution, and function.(I've designed the figure)

Chapter 2: Results I – Rac1 S71 Mediates the Interaction between Rac1 and 14-3-3 Proteins

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2.1 Chapter Abstract

Both 14-3-3 proteins (14-3-3s) and Rho proteins regulate cytoskeleton remodeling and cell migration, which suggests a possible interaction between the signaling pathways regulated by these two groups of proteins. Indeed, more and more emerging evidence indicates the mutual regulation of these two signaling pathways. However, all of the data regarding the interaction between Rac1 signaling pathways and 14-3-3 signaling pathways are through either the upstream regulators or downstream substrates. It is not clear if Rac1 could interact with 14-3-3s directly. It is interesting to notice that the Rac1 sequence ^{68}RPLSYP^{73} is likely a 14-3-3 protein binding motif following the phosphorylation of S71 by Akt. Thus, we hypothesize that Rac1 directly interacts with 14-3-3s. By using mutagenesis, co-immunoprecipitation (co-IP), Rac1 activity assay, immunoblotting, and indirect immunofluorescence, we demonstrate that 14-3-3s interact with Rac1. This interaction is mediated by Rac1 S71 in both phosphorylation-dependent and -independent manners, but the phosphorylation-dependent interaction is much more durable. Epidermal growth factor (EGF) strongly stimulates the phosphorylation of Rac1 S71 and the interaction between 14-3-3s and Rac1. Mutating S71 to A completely abolishes both phosphorylation-dependent and-independent interactions between 14-3-3s and Rac1. The interaction between 14-3-3s and Rac1 mostly serves to regulate the activity

and subcellular localization of Rac1. Among the seven 14-3-3 isoforms, 14-3-3η, -σ, and -θ showed interactions with Rac1 in both Cos-7 and HEK 293 cells. 14-3-3γ also binds to Rac1 in HEK 293 cells, but not in Cos-7 cells. We conclude that 14-3-3s interact with Rac1. This interaction is mediated by Rac1 S71 in both phosphorylation-dependent and -independent manners. The interaction between 14-3-3 and Rac1 mostly serves to regulate the activity and subcellular localization of Rac1. Among the seven 14-3-3 isoforms, 14-3-3η, -γ, -σ, and
-θ interact with Rac1.

2.2 Introduction

Rac1 is a small GTPase (molecular mass 21,450 Da) that belongs to the Rho family of GTPases. There are three members (Rac1, Rac2, and Rac3) in the Rac subfamily of the Rho family, and they share significant sequence similarity (~88%)[2,157]. These three Rac proteins diverge primarily in the C-terminal 15 residues[13]. Similar to other Rho GTPases, the most studied function of Rac1 in the regulation of cytoskeleton remodeling through the regulation of actin and microtubules [158]. In particular, Rac1 stimulates the formation of lamellipodia and membrane ruffles. Rac1 promotes lamellipodia formation by stimulating the polymerization of branched actin at the leading edge of the cell[51,159]. Rac1 is also involved in the regulation of cell size, cell proliferation, cell survival, membrane trafficking, cell motility, polarity, and adhesion[102,135,160]. Similar to other small GTPases, Rac1 functions as a switch by cycling between inactive (GDP-bound) and active (GTP-bound) forms. The cycle between inactive and active forms is controlled by three classes of regulatory proteins: guanine nucleotide exchange factors (GEFs) that activate Rho GTPases by promoting the release of GDP to allow binding of GTP; GTPase-activating proteins (GAPs) that inactivate Rho GTPases by stimulating their intrinsic GTP-hydrolysisactivity; and guanine-nucleotide-dissociation inhibitors(GDIs), which interact with the GDP-bound inactive Rho to prevent its exchange to the GTP-bound form and prevent its translocation to the membrane for action[22,70,161] . The cycling of Rac1 between the GTP- and GDP-bound states is essential for effective signal flow to elicit downstream biological functions . Other modifications including RNA splicing , microRNAs , and various post-translational modifications have also been shown to regulate the activity and function of Rac1. The reported post-translational modifications include lipidation, ubiquitination, phosphorylation, and adenylylation . All these post-translational modifications have been shown to play important roles in the regulation of Rac1

and other Rho GTPases[39,46,71,101,162] . Recent findings suggest that Rac1 is phosphorylated at multiple sites and the phosphorylation may play significant roles in regulating Rac1

functions . Rac1 is phosphorylated on S71 by Akt[74,163] . This phosphorylation of Rac1 inhibits its GTP binding activity without any significant change in GTPase activity[164]. Both the GTP-binding and GTPase activities of the mutant Rac1 S71A (Rac1 with the replacement of S71 by A)are abolished regardless of the activity of Akt . It was later reported that the phosphorylation of Rac1 S71 decreases the pathogenic effect mediated by Clostridium difficile toxin A (TcdA)[127] . Moreover, phosphorylation of Rac1 at S71 represents a reversible mechanism to determine the binding specificity of Rac1/Cdc42 to their downstream substrates[165] . In addition, Rac1 is phosphorylated at Y64 by FAK and SRC kinases. Y64 phosphorylation targets Rac1 to focal adhesions[129]. Rac1-Y64F displayed increased GTP-binding, increased association with βPIX, and reduced binding with RhoGDI as compared with wild-type Rac1 (Rac1-WT). Rac1-Y64D had a less binding activity to PAK than Rac1-WT or Rac1-Y64F [166,167]. We have shown that ERK phosphorylates Rac1 T108 in response to EGF stimulation. This phosphorylation alters the Rac1 activity, its interaction with PLC-γ1, and its subcellular localization and affects the Rac1 function in mediating cell migration [12,133]. These findings demonstrate that both serine/threonine and tyrosine phosphorylation of Rac1 are common phenomena and regulate multiple aspects of Rac1 functions.

The family of 14-3-3 proteins (14-3-3s) comprise seven isoforms and exist as homo- and/or heterodimers in cells. 14-3-3s are 28–33 kDa, acidic, evolutionarily conserved, widely expressed proteins that bind to a vast number of intracellular proteins in normal and cancer cells[168–170]. 14-3-3s bind to two phosphorylation-dependent high affinity binding motifs: RSXpSXP (mode I) and RXY/FXpSXP (mode II) [171,172]. In addition to the two binding motifs, 14-3-3s exhibit binding to the extreme C-terminus ($pSX_{1–2}$–COOH) of many proteins, lately defined as mode III [173]. By binding with serine/threonine

phosphorylated intracellular proteins, they alter the conformation, activity, and subcellular localization of their binding partners[174,175]. 14-3-3s interact with a wide range of proteins involved in cell signaling, cytoskeleton remodeling, DNA repair, and transcription regulation. Therefore, 14-3-3s regulate diverse cellular functions, including cell cycle, cell development, cell proliferation, apoptosis, and cell motility[176–178]. Recently, a growing number of proteins involved in actin remodeling have been identified as 14-3-3 binding partners . Global downregulation of 14- 3-3 expression causes tumor suppression, while overexpression of 14-3-3s is often seen in many cancers. [175,179,180] .

Both 14-3-3s and Rho proteins regulate cytoskeleton remodeling and cell migration, which suggests a possible interaction between the signaling pathways regulated by these two groups of proteins. Indeed, more and more emerging evidence indicates the mutual regulation of these two signaling pathways [181]. Most findings support the role of 14-3-3s in the regulation of Rho GTPases by interacting with Rac1 regulators including GEFs, GAPs, and GDIs. Some new data also suggest that 14-3-3s could act downstream of Rac1 by modulating Rac1 substrates[70,17,3] . Thus far, all data regarding the interaction between Rac1 signaling pathways and 14-3-3 protein signaling pathways are through either the upstream regulators or downstream substrates. However, it is possible that Rac1 could interact with 14-3-3s directly. Here, we show that Rac1 interacts with 14-3-3s directly in response to EGF, and this interaction is mediated by the phosphorylation of Rac1 S71. This interaction was not dependent on Rac1 activity but slightly modulates the Rac1 activity.

Moreover, this interaction alters the subcellular localization of Rac1. Among the seven 14-3-3 isoforms, 14-3-3η, -σ, and -θ showed interactions with Rac1 in both Cos-7 and HEK 293T cells. 14-3-3γ also binds to Rac1 in HEK 293T cells, but not in Cos-7 cells.

2.3 Materials and Methods

2.3.1 Cell Culture and Treatment

COS-7 and 293T cells were cultured in DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 10% fetal bovine serum (FBS), 100 IU/mL penicillin and 100 μg/mL streptomycin, and were maintained in a 5% CO_2 atmosphere at 37 ˚C. For the EGF treatments, COS-7 cells were serum-starved for 12–16 h followed by addition of EGF to a final concentration of 50 ng/mL for 15 min or as indicated. Serum starvation in Cos-7 cells was done by adding the medium without antibiotics, and FBS for 12 hours and adding the medium without antibiotic and 1% FBS for 12 hours before treatment. The serum starvation was doen to get the sharpest possible increase from EGF treatment. For the treatment with BV02, a 14-3-3 PPI that inhibits the 14-3-3 scaffolding proteins docking site. cells were incubated with BV02 at the indicated concentration for 24 h. For wortmannin treatment, the cells were incubated with wortmannin at 100 nM for 30 min before EGF treatment. Wortmannin is a potent and specific phosphatidylinositol 3-kinase (PI3-K) inhibitor with an IC_{50} of 2-4 nM that Inhibits PI3-K/Akt signal transduction cascade

2.3.2 Transient Transfection

COS-7 and 293T cells were grown to 70–80% confluency in 6-cm dishes before the transfection. The transfection was performed using the calcium phosphate transfection method with BES buffer (40 mM NaCl, 0.75 mM sodium phosphate dibasic (Na_2HPO_4), 25 mM BES, pH 6.95). Cells were typically analyzed 36–48 h post-transfection.

2.3.3 Antibodies and Chemicals

Mouse monoclonal anti-Rac1 antibody was purchased from Cytoskeleton Inc. (Denver, CO, USA). Rabbit anti-GFP antibody was from Clontech (Mountain View, CA, USA). Mouse monoclonal anti-pRac1S71, anti-14-3-3s, 14-3-3η, -γ, -σ, and -θ, and anti-GST antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Rabbit monoclonal anti- α-tubulin antibody was from Abcam (Abcam Inc, Toronto, ON, Canada). FITC- and TRITC- conjugated donkey anti-mouse and anti-rabbit antibodies were from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA). Glutathione cross-linked to 4% agarose, goat anti-mouse IgG conjugated with agarose, and protein A conjugated with agarose were purchased from Sigma-Aldrich (St. Louis, MO, USA). Mammalian Protein Extraction Reagent (M-Per) was purchased from Thermo Fisher Scientific Inc. (Rockford, IL USA). Unless otherwise specified, all chemicals were purchased from Sigma-Aldrich.

A plasmid encoding GFP-Rac1 was a gift from Dr. Mark R. Philips (New York University, New York, NY, USA). A plasmid encoding GST-PAK was a gift from Dr. Gary Eitzen (University of Alberta, Edmonton, AB, Canada). Plasmids encoding GST-Rac1, GFP-tagged constitutively active Rac1 L61 (GFP-L61), and dominant-negative Rac1 N17 (GFP- N17) were generated previously in the laboratory. GFP- and GST-tagged mutant Rac1 S71A were created with the QuikChange Multiple Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) using GFP-Rac1 as a template.

2.1.1 Expression and Purification of GST-Fusion Proteins

To purify various GST-fusion proteins, the pGEX plasmids containing GST alone, GST- Rac1, GST-PAK, and GST-S71 constructs were transformed into *Escherichia coli* DH5α. Bacteria were grown to an optical density $(OD)_{600}$ of 0.6–0.8 at 37 °C and induced with 0.2 mM isopropyl-1-thio-β- d-galactopyranoside (IPTG) and incubated for four h at 30 °C with shaking. After pelleting, bacterial cells were lysed by sonication in PBS in the presence of protease inhibitors (0.1 mM) 4-(2-aminoethyl)-benzenesulfonyl fluoride, tenμg/mLaprotinin, and one μM pepstatin A). After sonication, 1% Triton X-100 was added to enhance solubilization. Particulates were removed by centrifugation for 15 min at

10,000 rpm, and the cleared supernatant was incubated with 50:50 glutathione-agarose beads (Sigma-Aldrich) in PBS for two h at 4 ˚C. The beads were washed three times with ice-cold PBS and stored. The immobilized GST fusion proteins on the beads were used for GST pull-downassays.

COS-7 cells were lysed into BOS buffer (50 mM Tris-HCl, pH 7.4, 200 mM NaCl, 1% Nonidet P-40, 10% glycerol, 10 mM NaF, 2.5 mM $MgCl_2$, and 1 mM EDTA) with protease inhibitors. The lysates were centrifuged at 21,000× *g* at 4 ˚C for 15 min. Supernatants were used in the pull-down assay. GST-fusion proteins bound to glutathione-agarose beads were added to the supernatant and incubated at 4 ˚C for two h with shaking. Beads were collected by centrifugation and washed three times with a BOS buffer, after which the 2× sample loading buffer was added. The pull-down proteins were resolved on SDS-PAGE and analyzed by Western blotting. protease inhibitors (Sigma Aldrich). The lysates were centrifuged at 21,000× *g* at 4 ˚C for 15 min. Supernatants were used in the binding assay. GST-PAK fusion proteins bound to glutathione-agarose beads in GST-PAK buffer were added and incubated at 4 ˚C for two h. Beads were collected by centrifugation, washed three times with GST-PAK buffer, after which SDS loading buffer was added. The pull-down active Rac1 was resolved on SDS-PAGE and analyzed by Western blotting.

2.1.2 Immunoprecipitation

. Briefly, cells were lysed with IP buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.1% sodium deoxycholate, 100 mM NaF, 5 mM $MgCl_2$, 0.5 mM Na_3VO_4, 0.02% NaN_3, 0.1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 10 µg/mL aprotinin, and 1

µM pepstatin A). Cell lysates were centrifuged at 22,000× *g* for 30 min to remove debris. The supernatants, containing approximately 1 mg of total protein, were pre-cleared with the agarose beads and then were used to incubate with one µg of specific antibody at 4 ˚C overnight with gentle mixing. Then, goat anti-mouse IgG conjugated with agarose or protein A conjugated with agarose was added to each fraction and incubated for two h at

4 °C with agitation. Both the agarose beads and the non-precipitated supernatant were collected by centrifugation. For the controls, mouse or rabbit IgG was used to replace the primary antibodies. The agarose beads were washed three times with IP buffer and then mixed with 2× sample loading buffer. The sample was boiled for 5 min and subjected to the Western blot assay.

2.1.3 Immunoblotting

The protein content of cell lysates was determined by Bradford analysis, and approximately 20 μg of total protein was used for each sample. Protein samples were resolved by SDS-PAGE 12% gel and electrophoretically transferred onto nitrocellulose membranes. After blocking in 3% milk for 60 min, membranes were incubated with primary antibody at 4 °C overnight. The primary antibodies were detected with their corresponding horseradish peroxidase-conjugated secondary antibodies followed by enhanced chemiluminescence development (Pierce Chemical, Rockford, IL, USA) and light detection with Fuji (Tokyo, Japan) Super RX film.

2.1.4 Fluorescence Microscopy

Cells were cultured on glass coverslips for 48 h before treatment. After treatment, the cells were rinsed in tris-buffered saline (TBS; 6% tris, 8.8% NaCl, 85.2% dH_2O, pH 7.6) and were fixed by cold methanol for 4 min. Cells were permeabilized with TBS containing 0.2%

Triton X-100 for 10 min, followed by blocking with TBS containing 1% BSA and 0.1% Triton X-100 for an hour. After blocking, the coverslips were incubated in 1 µg/mL primary antibody in TBS with 0.1% Triton X-100 as indicated for an hour. Afterward, the coverslips were rinsed in TBS with 0.1% Triton X-100 three times each for 5 min and then incubated in 1 µg/mL solution of FITC- and/or TRITC-conjugated secondary antibody in TBS with 0.1% Triton X- 100 with for an hour in the dark. After that, the coverslips were entirely washed in TBS and incubated in 1 µg/mL of 4، 6-diamidino-2-phenylindole (DAPI) solution in TBS for 5 min at room temperature in the dark. The coverslips were then mounted on glass slides and observed using a DeltaVision fluorescence microscopy system 1.42 NA lens & 0.2 µm (Applied Precision Inc., Mississauga, ON, Canada).

2.1.5 Artificial Network Analysis

Using the predictions generated from the Barton group 14-3-3 Pred, data were integrated from support vector machines (SVM), position-specific scoring matrices, and support vector machines (SVM) and artificial neural network (ANN) classification methods were trained to discriminate experimentally determined 14-3-3-binding motifs from non-binding phosphopeptides. Afterward, we used Netphos to predict the molecule that is likely to be involved in the phosphorylation of S71. Then, we used R-studio to generate a blot graph representing the sites that are most likely in genuine interaction with Rac1. Rac1 S71 phosphorylation by PKB (AKT) showed the highest consensus among all other motifs and phosphorylation kinases.

2.1.6 Quantification and Statistical analysis

All protein bands were quantitated by densitometry using ImageJ software (ImageJ2, NIH, Bethesda, MD, USA). The values shown represent average and S.E. of at least 3 independent biological replicates and *p* values were calculated by student *t* test. unpaired Student's t test *$p<0.05$; **$p<0.01$; ***$p<0.001$ with Ms Excel software

2.1 Results

2.1.1 Association between Rac1 and 14-3-3s in Response to EGF-Induced Akt Phosphorylation

More and more emerging evidence indicates the mutual regulation of Rac1 signaling pathways and 14-3-3 signaling pathways in terms of the regulation of cytoskeleton remodeling and cell migration in response to growth factor stimulation. Here, we first determined whether Rac1 and 14-3-3s physically associate in response to EGF by co-immunoprecipitation (co- IP). Cos-7 cells were treated with EGF for the indicated time, and Rac1 was immunoprecipitated with mouse anti-Rac1 antibody. The co-IP of 14-3-3s was examined by immunoblotting with antibodies to pan 14-3-3 protein. As shown in Figure 4A, 14-3-3s co- immunoprecipitated (co-IPed) with Rac1 following EGF stimulation, and the amount of co- immunoprecipitated 14-3-3s reached a maximum at 5–15 min following EGF stimulation. This indicates that EGF stimulates the association between Rac1 and 14-3-3s.

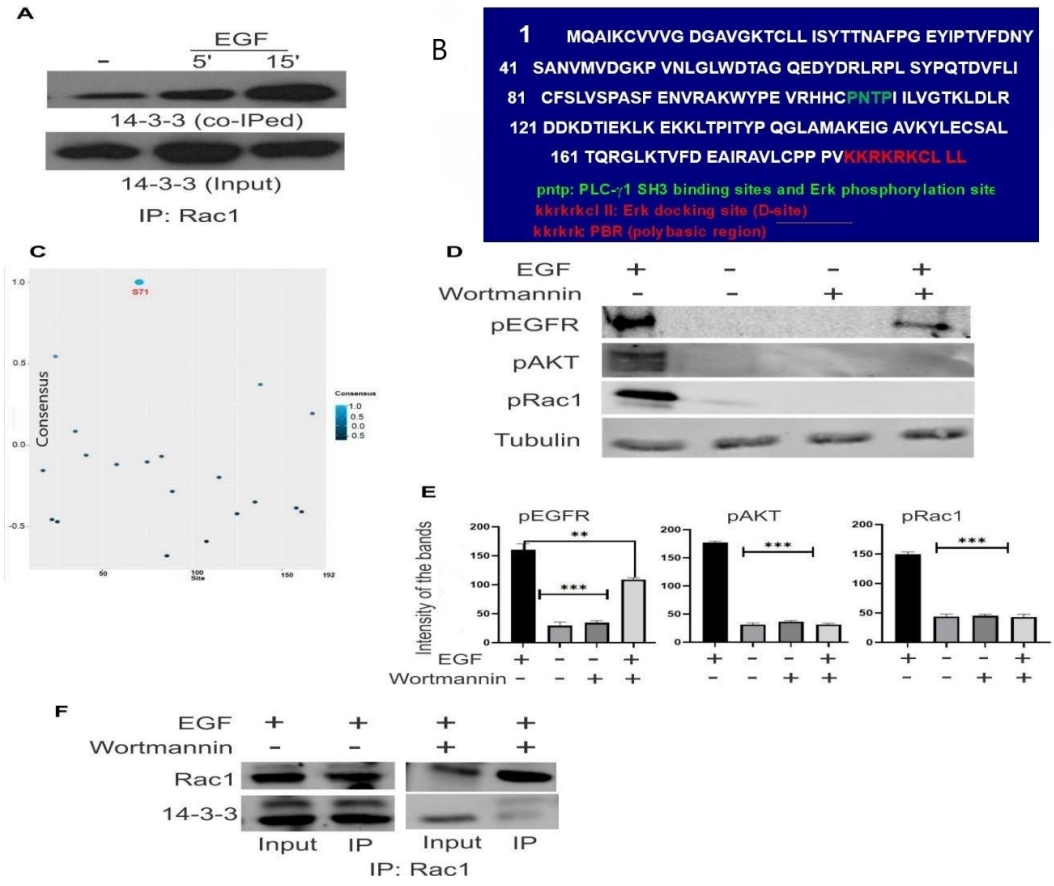


Figure 4. 14-3-3s interact with Rac1 in response to EGF in Cos-7 cells. (**A**) 14- 3-3s interact with Rac1 in response to EGF in Cos-7 cells. Cos-7 cells were treated with EGF for the indicated time, and the interaction between 14-3-3s and Rac1 was determined by co-immunoprecipitation (co-IP) of 14-3-3s upon immunoprecipitation (IP) of Rac1. (**B**) Sequence analysis of Rac1 reveals the presence of the mode I 14-3-3 binding motif ^{68}RPLSYP^{73} if S71 is phosphorylated.

(**C**) The highest consensus between 14-3-3 and Rac1 (value 1.0) is within the S71-motif as predicted by ANN 14-3-3 Prediction software. Each point represent a possible interaction of Rac1 with 14-3-3 proteins, not all 192 amino acids are shown as some are predicted to have no interaction (below -1.0)(**D**) The effects of EGF and wortmannin on the phosphorylation of EGFR, Akt, and Rac1 S71. Cos-7 cells were treated with EGF and/or wortmannin. The phosphorylation of EGFR, Akt, and Rac S71 was revealed by immunoblotting with phospho-specific

antibodies. (**E**) Quantification of the data in (**D**). The level of protein phosphorylation was quantitated by densitometry. Each value is the average of at least three biological replicates , P-Rac1 antibody was specifically designed for S71 motif and the error bar is the standard error. ** $p < 0.01$, *** $p < 0.001$. (**F**) The effects of EGF and wortmannin. Cos-7 cells were treated with EGF and wortmannin, as indicated. The interaction between 14-3-3s and Rac1 was determined by a co-IP of 14- 3-3s upon the IP of Rac1.

To understand the mechanisms underlying this interaction, we analyzed the amino acid sequence of Rac1 to identify potential binding motifs for 14-3-3 protein. The mode I consensus 14-3-3 protein binding motif is RSXpSXP, and the Rac1 sequence ^{68}RPLSYP^{73} is likely a 14-3-3 protein binding motif following the phosphorylation of S71 (Figure 4B). It is well established that Rac1 is phosphorylated at S71 by Akt. Moreover, ANN 14-3-3 Prediction software has predicted the highest consensus between 14-3-3 and Rac1 to be within the S71-containing motif, where S71 has a value of approximately 1 (Figure 4C). Thus, we propose that in response to EGF, Akt is phosphorylated by activated EGFR via PI3K, which results in the phosphorylation of Rac1 S71 and the interaction between Rac1 and 14- 3-3s.

To test this hypothesis, we treated the cells with EGF and showed that EGFR, Akt, and Rac1 S71 are all phosphorylated in response to EGFR (Figure 4D). However, inhibition of PI3K with wortmannin blocked the phosphorylation of both Akt and Rac1 S71 (Figure 4D). We then examined if wortmannin can inhibit the interaction between Rac1 and 14-3-3s. As shown in Figure 4F, wortmannin treatment inhibited the EGF-induced interaction between Rac1 and 14-3-3s. These data strongly suggest that EGF-induced Rac1 S71 phosphorylation through Akt leads to the interaction between Rac1 and 14-3-3s.

2.1.1 Rac1 and 14-3-3s Interaction is Mediated by Rac1 S71

To confirm the role of Rac1 S71 in mediating the interaction between Rac1 and 14-3-3s, we generated a GFP-tagged mutant Rac1 with the mutation of S71 to A (GFP-S71A). Our

previously generated GFP-tagged wild type Rac1 (GFP-Rac1) was used as a control. We transfected Cos-7 cells with both GFP-Rac1 and GFP-S71A. As shown in Figure 5A, wild type Rac1 co-IPed with 14-3-3s. Intriguingly, the S71A mutant failed to co-IP with 14-3-3s, which supports the prediction that S71 phosphorylation is critical in mediating the interaction between Rac1 and 14-3-3s as previously described. To further validate our results, we also transfected 293T cells with GFP-Rac1 and GFP-S71A, and we showed that GFP-Rac1, but not GFP-S71A, co-IPed with 14-3-3s (Figure 7C), which confirmed our observations in Cos-7 cells.

To further examine the interaction between Rac1 and 14-3-3s, we performed a GST pull-down experiment in Cos-7 cells. We generated GST-tagged Rac1 with the mutation of S71 to A (GST-S71A). We have generated GST-tagged wild type Rac1 (GST-Rac1) previously. Lysates of COS-7 cells were incubated with glutathione-agarose beads charged with GST, GST-S71A, or GST-Rac1. Following the incubation, the proteins associated with the agarose beads were immunoblotted with antibodies to pan 14-3-3s. As shown in Figure 5B, while the detectable amount of 14-3-3s was pulled down by GST-Rac1, no 14-3-3s were pulled down by GST-S71A. This result indicates that the mutation of S71 to A completely inhibits the binding between Rac1 and 14-3-3s.

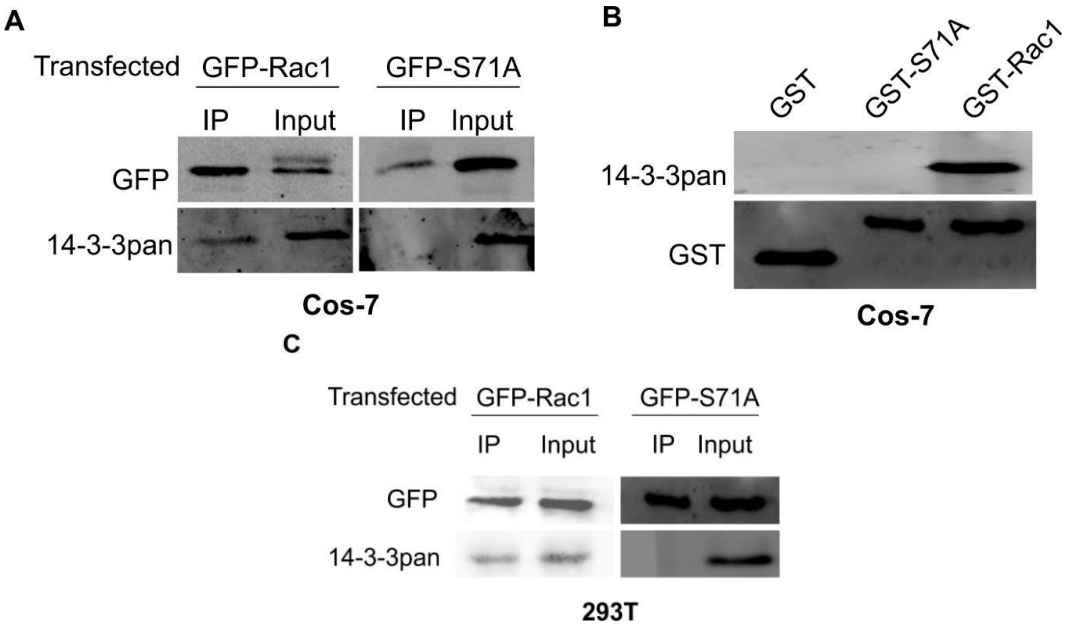


Figure 5

Figure 5. The effects of Rac1 S71 mutation to A on the interaction between 14- 3-3s and Rac1 in both Cos-7 and 293T cells. (**A**) The effects of Rac1 S71 mutation to A on the interaction between 14-3-3s and Rac1 in Cos-7 cells. Cos-7 cells were transfected with wild type GFP-Rac1 and mutant GFP-S71A. The expressed Rac1 was immunoprecipitated (IP) with an antibody to GFP, and the co-IPed 14-3-3s were examined by immunoblotting. (**B**) The effects of Rac1 S71 mutation to A on the interaction between 14-3-3s and Rac1 in Cos-7 cells by GST pulldown. The Cos-7 lysates were incubated with glutathione-agarose beads charged with GST, GST- Rac1, or mutant GST-S71A. The agarose beads were then separated and subjected to immunoblotting analysis with an antibody against 14-3-3 and GST. (**C**) The effects of Rac1 S71 mutation to A on the interaction

2.1.2 The Relationship between Rac1 Activity and Its Interaction with 14-3-3s

To examine if Rac1 activity has any effects on its interaction with 14-3-3s, we transfected Cos-7 cells with two commonly known GFP-tagged mutants; the constitutively active Rac1 (GFP-L61, with the replacement of 61Q by L) and the dominant-negative Rac1 (GFP-N17, with the replacement of 17T by N) that were generated previously. The interaction between transfected Rac1 mutants and 14- 3-3s was first examined by co-IP experiments. As shown in Figure 6A, both GFP-L61 and GFP-N17 co-IPed with 14-3-3s, similar to wild type GFP-Rac1 (Figure 4A), which indicates that Rac1 activity does not affect its interaction with 14-3-3s. We then examined if the interaction between Rac1 and 14-3-3 affects the activity of Rac1. As the mutation of S71 to A disrupts the interaction between Rac1 and 14-3-3, we examined the activity of GFP-S71A. We transfected Cos-7 cells with GFP-Rac1 and GFP-S71A, stimulating cells with EGF for 15 min, subjected the cell lysate to Rac1 activity assay using GST fusion Rac-binding domain of PAK (GST-PAK) or GST bound to glutathione-agarose beads. As shown in Figure 6B, the S71 mutation prevented both, the 14-3-3 interaction and its activation in response to EGF. It is really only the BVO2 experiment that says the activation depends on 14-3-3 . We then examined if disruption of the interaction between Rac1 and 14- 3-3s, by inhibiting 14-3-3 docking sites, also affects Rac1 activity. BV02 is an inhibitor of the 14-3-3 scaffolding protein docking sites. We treated Cos-7 cells with BV02 and examined the effects on Rac1 activity with or without EGF stimulation. As shown in Figure 6C, D, EGF stimulated the activation of Rac1 and BV02 inhibited Rac1 activity both with and without EGFR stimulation. Together, these data suggest that Rac1 activity has no effects on its interaction with 14-3-3; however, the interaction between Rac1 and 14-3-3 protein is essential for EGF-induced Rac1 activation.

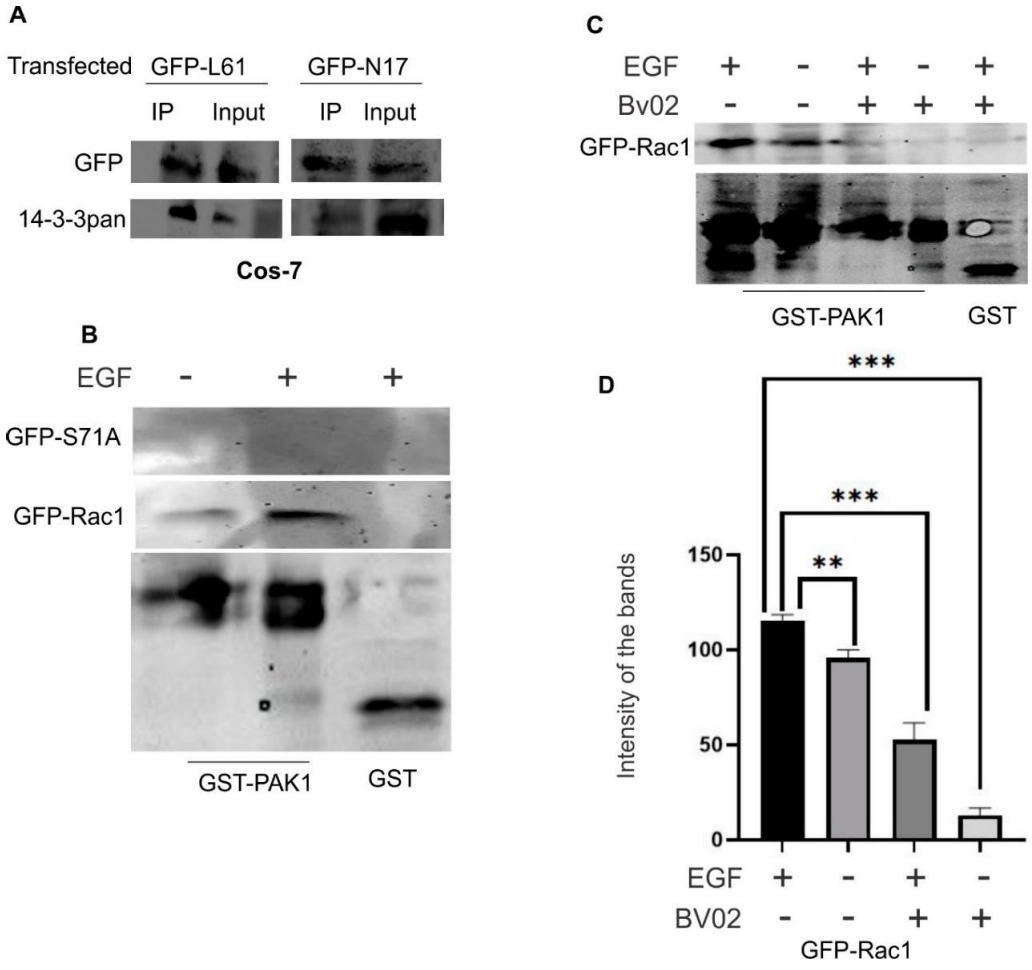


Figure 6. The relationship between Rac1 activity and its interaction with 14-3-3s in Cos-7 cells. (**A**) The effects of Rac1 activity on the interaction between 14-3-3s and Rac1. Cos-7 cells were transfected with either constitutively activated Rac1 (GFP-L61) or dominant-negative Rac1 (GFP-N17). The interaction of these two mutants with 14-3-3s was examined by co-IP, as described above. (**B**) The effects of Rac1 S71 mutation to A on the activation of Rac1. Cos-7 cells were transfected with either GFP-Rac1 or mutant GFP- S71A, incubated with or without EGF for 15 min, and lysed. The cell lysates were incubated with the GST fusion Rac-binding domain of PAK (GST-PAK) or GST bound to glutathione-agarose beads. The active Rac1 that binds to GST-PAK was determined by immunoblotting with an antibody to GFP; GST-PAK and GST were detected with an antibody to GST. (**C**) The effects of BV02 on the activation of Rac1. Cos-7 cells were transfected with GFP-Rac1 and incubated with BV02 at a concentration of 30 μM for 24 h. The cells were then incubated with or without EGF for 15 min. Cells

were then lysed, and the cell lysates were incubated with GST-PAK or GST bound to glutathione-agarose beads. The active Rac1 that binds to GST-PAK was determined by immunoblotting with an antibody to GFP; GST-PAK and GST were detected with an antibody to GST. (**D**) Quantification of the data in (**C**). The pulled-down active Rac1 bands were quantitated by

densitometry. Each value is the average of at least three experiments, and the error bar is the standard error. ** $p < 0.01$, *** $p < 0.001$.

2.1.1 The Interaction between Rac1 and Various 14-3-3 Isoforms

There are seven 14-3-3 isoforms in mammalian cells. These isoforms have distinct subcellular localizations and functions. We next examined which 14-3-3 isoform interacts with Rac1. We transfected Cos-7 cells with GFP-Rac1. Following the immunoprecipitation (IP) with an antibody to GFP, we examined which 14-3-3 isoforms co-IPed with GFP-Rac1 by immunoblotting with antibodies to various 14-3-3 isoforms. As shown in Figure 7A, 14-3-3η,

-σ, and -θ co-IPed with GFP-Rac1, but none of 14-3-3β, -ε, -γ, and -ζ co-IPed with GFP-Rac1. To confirm these interactions and to determine if Rac1. S71 is responsible for these interactions. We performed GST-pulldown experiments with GST-Rac1 and GST-S71A. As shown in Figure 7B, GST-Rac1 was able to pull down all three identified isoforms, including 14-3-3η, -σ, and -θ. However, GST-S71A was unable to pull down these isoforms. These data indicate the essential role of S71 in the interaction.

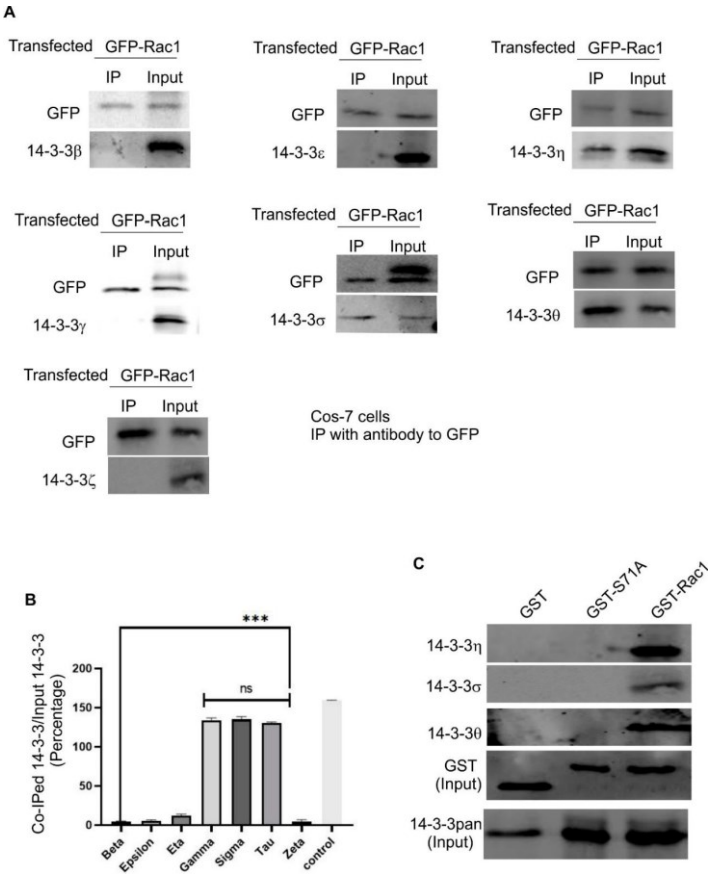


Figure 7. The interaction between Rac1 and seven 14-3-3 isoforms in Cos-7 cells.

(A) Co-IP experiments to determine the interaction between Rac1 and seven 14- 3-3 isoforms in Cos-7 cells. Cos-7 cells were transfected with GFP-Rac1. The expressed GFP-Rac1 was IPed with the anti-GFP antibody. The co-IP of various 14- 3-3 isoforms was determined by immunoblotting. (**B**) Quantification of the data in (**A**). The levels of co-IPed 14-3-3s were quantitated by densitometry. Each value is the average of at least three experiments, and the error bar is the standard error. *** $p < 0.001$, ns: $p > 0.1$. (**C**) GST pull-down experiments to determine the interaction between three 14-3-3 isoforms, including 14-3-3η, -σ, and -θ and GST-Rac1 and GST-S71A. The Cos-7 lysates were incubated with glutathione agarose beads charged with GST, GST-Rac1, or mutant GST-S71A. The agarose beads were then separated and subjected to immunoblotting analysis with antibodies against 14-3-3 η, -σ, and

We next examined the interaction between Rac1 and various 14-3-3 isoforms in 293T cells. We transfected 293T cells with GFP-Rac1. Following the IP with an antibody to GFP, we examined which 14-3-3 isoforms co-IPed with GFP-Rac1 by immunoblotting with antibodies to various 14-3-3 isoforms. As shown in Figure 8, we confirmed that, in 293T cells, 14-3-3η, -σ, and -θ also co-IPed with GFP-Rac1 as in Cos-7 cells; however, we observed that, differently from Cos-7 cells, 14-3-3γ co-IPed with GFP-Rac1 in 293T cells. As in Cos-7 cells, none of 14-3-3β, -ε, and -ζ co-IPed with GFP-Rac1. These data further indicate that Rac1 selectively interacts with a subset of 14-3-3 isoforms.

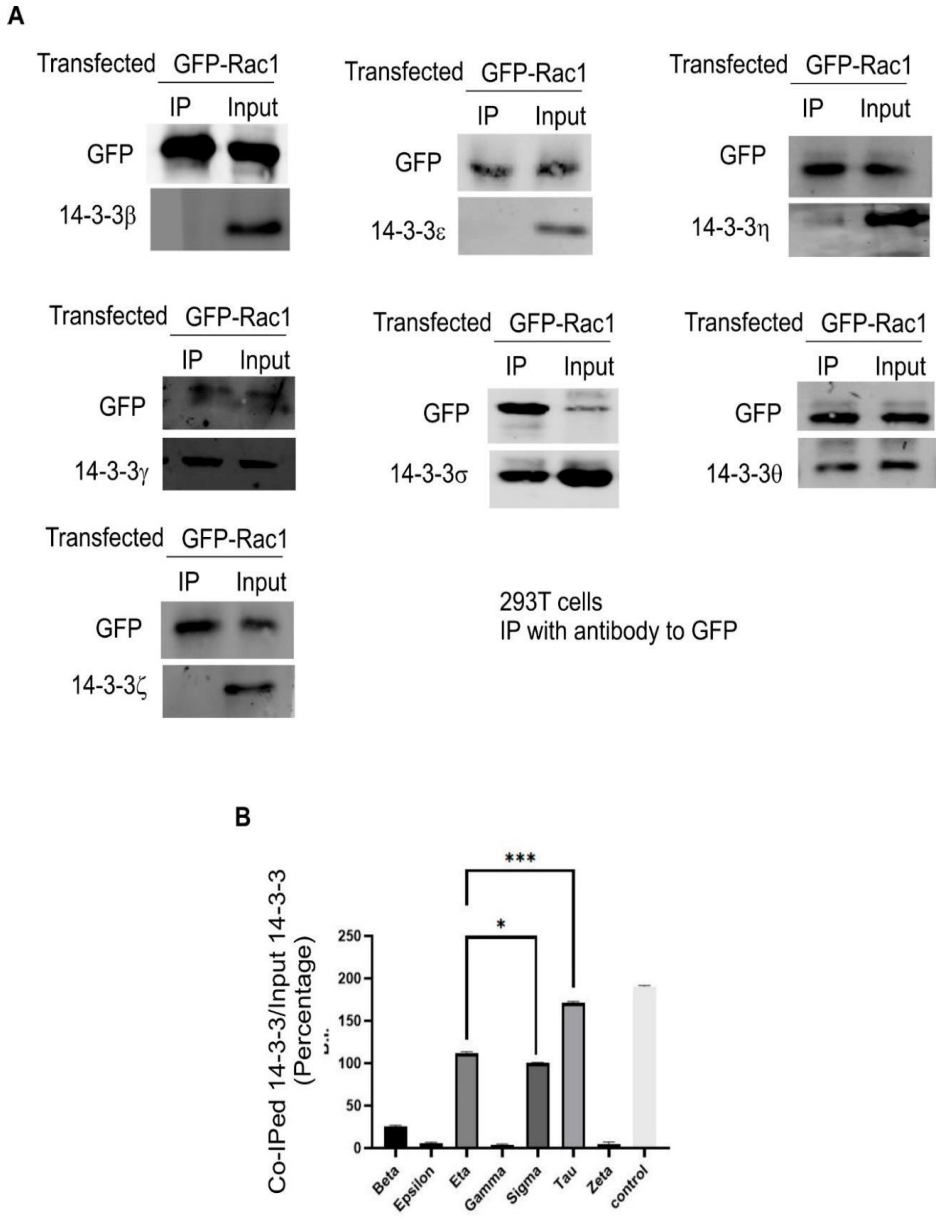


Figure 8. The interaction between Rac1 and seven 14-3-3 isoforms in 293T cells.

(**A**) 293T cells were transfected with GFP-Rac1. The expressed GFP-Rac1 was IPed with the anti-GFP antibody. The co-IP of various 14-3-3 isoforms was determined by immunoblotting. (**B**) Quantification of the data in (**A**). The levels of co-IPed 14- 3-3s were quantitated by densitometry. Each value is the average of at least three experiments, and the error bar is the standard error. * $p < 0.1$, *** $p < 0.001$.

2.1.2 The Effects of Rac1 and 14-3-3 Protein Interaction on the Subcellular Localization of Rac1 and 14-3-3s

We finally examined if the interaction between Rac1 and 14-3-3s has any effect on the subcellular localization of both proteins. We first examined the effects on the subcellular localization of Rac1 following the disruption of the interaction between Rac1 and 14-3-3s. We transfected the Cos- 7 cells with GFP-Rac1 and GFP-S71A, and the localization of these two proteins was revealed by their intrinsic fluorescence. As shown in Figure 9A, GFP-Rac1 is mostly diffusively localized to the cytoplasm, with visible plasma membrane localization; however, GFP-S71A was mostly localized to the plasma membrane and associated with punctate structures with a much weaker cytosolic distribution. We also transfected the Cos-7 cells with GFP-Rac1 and then treated the cells with BV02 to disrupt the interaction between Rac1 and 14-3-3s. As shown in Figure 9A, in the presence of BV02, GFP-Rac1 showed similar subcellular localization as the mutant GFP-S71A as it mostly associated with the plasma membrane and showed punctate structures with a much weaker cytosolic distribution. These data strongly indicate that the interaction between Rac1 and 14-3-3 protein plays a vital role in regulating the subcellular localization of Rac1.

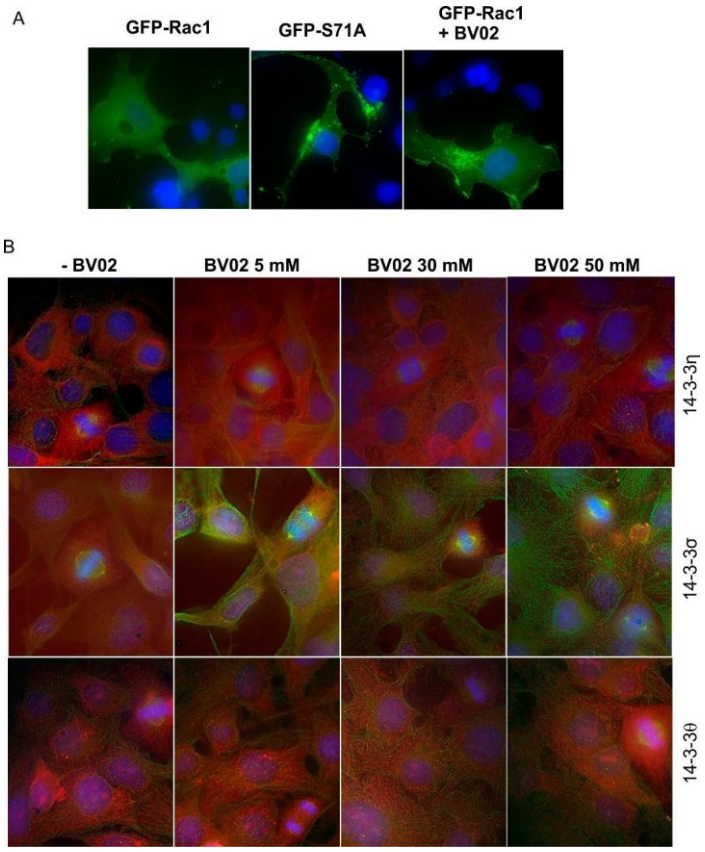


Figure 9. Disruption of the interaction between 14-3-3s and Rac1 and the effects on the subcellular localization of Rac1 and three 14-3-3 isoforms, including 14- 3-3η, -σ, and -θ. (**A**) The effects on the subcellular localization of Rac1. COS-7 cells were transfected with GFP-Rac1 or GFP-S71A. With or without treatment with BV02 for 24 h, the subcellular localization of GFP-Rac1 and GFP-S71A was revealed by the intrinsic fluorescence (green). The cells were counterstained with DAPI (blue). Size bar = 10 μm. (**B**) The effects on the subcellular localization of 14- 3-3 isoforms. Cos-7 cells were treated with BV02 at the indicated concentration for 24 h. The cells were stained with mouse antibodies to 14-3-3η, -σ, and -θand a rabbit antibody to α-tubulin, followed by incubation with TRITC-conjugated anti-mouse IgG and FITC-conjugated anti-rabbit IgG. DNA was stained by DAPI. The localization of 14-3-3 isoforms is shown as red, α-tubulin as green, and chromosome/nucleus as blue. Size bar = 10 μm.

We also examined the effects of BV02 on the subcellular localization of the three 14-3-3 isoforms. As shown in Figure 9B, there are no significant changes in the subcellular localization of these 14-3-3 isoforms following the treatment of the cells with BV02 at various concentrations. Thus, disrupting the interaction between 14-3-3 and Rac1 did not alter the subcellular localization of 14-3-3s. It is noteworthy that the specificity of the antibodies tested using synthetic peptides supplied by SCBT.

3. Discussion and future directions

It is well-established that 14-3-3 protein signaling pathways and Rac1 signaling pathways co-regulate essential cell functions, including cytoskeleton remodeling and cell migration [182]. While the data may suggest there's an interaction between 14-3-3s and Rac1 upstream regulators or downstream substrates[183] , we can't firmly argue this fact as the interaction may have been possible through other modification such as phosphorylation Here, we demonstrated that a subset of 14-3-3s could interact directly with Rac1, which provides an additional regulation of Rac1 signaling pathways by 14-3-3 protein.

Both sequence analysis and ANN 14-3-3 Prediction software suggest that Rac1 S71 constitutes a 14-3-3 binding motif following its phosphorylation. The Rac1 sequence ^{68}RPLSYP^{73} is likely the mode I 14-3-3 consensus binding motif following the phosphorylation of S71 (Figure 4). It is well established that Rac1 is phosphorylated at S71 by Akt. Weshowed in this research that EGF stimulated the phosphorylation of Akt and Rac1 S71, which leads to the interaction between 14-3-3 and Rac1. Inhibition of Akt phosphorylation by wortmannin inhibited the EGF-induced interaction between 14-3-3s and Rac1 (Figure4). Moreover, we showed that the mutation of S71 to A inhibited the interaction between 14-3-3s and Rac1. These data strongly indicate the importance of S71 and its phosphorylation in mediating the interaction between 14-3-3s and Rac1 (Figure5).

While it has been shown that Akt phosphorylates Rac1, little is known about the functional significance of this interaction. Our findings here indicate that the phosphorylation of S71 creates a robust binding motif for 14-3-3s, which allows for the direct interaction between 14-3-3s and Rac1. As both 14-3-3s and Rac1 regulate many standard cell functions, our findings suggest the presence of another layer of regulation.

The GST pull-down experiments suggest that 14-3-3s may interact with Rac1 in the absence of S71 phosphorylation as GST-Rac1 pull down 14-3-3s(Figures 5B, 7B). as GST - WT was generated from bacteria and it's unphosphorylated. The inability to pulldown 14-3-3s by GST-S71A indicates the requirement of S71 for the interaction between 14-3-3s and Rac1. Although most interactions between 14-3-3s and their binding partners are mediated by phosphorylated motifs

of the binding partners, a significantnumber of proteins have been shown to interact with 14-3-3s in a phosphorylation- independent manner. These proteins include human telomerase (hTERT), SGK1 and tau, amyloid β-protein precursor intracellular domain fragment, HSP60, and PrP^C the enteropathogenic *Escherichia coli* Tir protein, exoenzyme S, NtCDPK1, non-muscle myosin II liver kinase B1(LKB1), and RGS14. Among these proteins, SGK1, LKB1, NtCDPK1, and RGS14 interact with 14-3-3s in both phosphorylation-dependent and -independent manners. The existence of both phosphorylation-dependent and -independent interactions could serve to enhance the interaction, maintain the interaction, or regulate the different functions of their binding partners. Therefore, it is likely that 14-3-3 may interact with Rac1 in both phosphorylation-dependent and phosphorylation-independent manners; however, both interactions require the presence of S71. Mutating S71 to A completelyabolishes its interaction with 14-3-3s, and phosphorylation of S71 strongly enhances its interaction with 14-3-3s.

It is well established that 14-3-3s are scaffold proteins and function to regulate the activity of their binding partners. On the other hand, Rac1 is a GTPase and functions as a switch to control many signaling pathways directly. Thus, it is likely that 14-3-3s serve to regulate the activity and function of Rac1. Indeed, we showed that disruption of the interaction between 14-3-3 and Rac1 alters Rac1 activity and Rac1 subcellular localization (Figures 6 and 9).

However, the activity of Rac1 does not affect the interaction between 14-3-3 and Rac1, and the disruption of the interaction between 14-3-3 and Rac1 does not change the subcellular localization of 14-3-3. There are seven 14-3-3 isoforms in mammalian cells. These isoforms may have different subcellular localizations and functions. We determined which isoforms interact with Rac1 and showed that three isoforms, including 14-3-3η, -σ, and -θ, bind to Rac1 in Cos-7 cells and 293T cells. Surprisingly, 14-3-3γ binds to Rac1 in 293T cells but not in Cos-7 cells. We do not fully understand

what causes this difference. Cos-7 cells are isolated from the kidney epithelial cells of African green monkeys, and HEK-293T cells are isolated from human embryonic kidney cells. Human Rac1 and African green monkey Rac1 have an identical amino acid sequence (Figure 11). Thus, this difference may be due to other factors, such as expression levels and post-translational modifications. We have shown recently that 14-3-3η, -σ, and -θ are all associated with the cytoskeleton, while 14-3-3ηalso showed a strong association with the mitochondria and 14-3-3σshoweda strong association with the centrosome during mitosis. 14-3-3γ mostly localized to the nucleus. Rac1 has been shown to strongly associate with the cytoskeleton and regulate cytoskeleton remodeling and cell migration, in addition to the nucleus and the mitochondria.

The colocalization and interaction of Rac1 with specific 14-3-3 isoforms provides interesting clues and a strong basis for studying the role of this interaction in regulating specific and critical cell functions. For example, both 14-3-3 protein and Rac1 have been shown to regulate apoptosis. The interaction between 14-3-3η and Rac1 and the localization of both proteins in the mitochondria suggests that they may co-regulate mitochondria-mediated apoptosis. The colocalization of 14-3-3γ and Rac1 in the nucleus and our observed interaction between 14-3-3γ and Rac1 in 293T cells suggests that they may co-regulate certain nuclear functions with 14-3-3s directly. Here, we show that Rac1 interacts with 14-3-3s directly in response to EGF, and this interaction is mediated by the phosphorylation of Rac1 S71. This interaction was not dependent on Rac1 activity but slightly modulates the Rac1 activity.

Moreover, this interaction alters the subcellular localization of Rac1. Among the seven 14-3-3 isoforms, 14-3-3η, -σ, and -θ showed interactions with Rac1 in both Cos-7 and HEK 293T cells. 14-3-3γ also binds to Rac1 in HEK 293T cells, but not in Cos-7 cells.

I demonstrated here that 14-3-3s interact with Rac1. This interaction is mediated by Rac1 S71 in both phosphorylation-dependent and -independent manners, but the phosphorylation-dependent interaction is much stronger. EGF strongly stimulates the phosphorylation of Rac1 S71 and the interaction between 14-3-3 and Rac1. Mutating S71 to A completely abolishes both phosphorylation-dependent and -independent

interactions between 14-3-3 and Rac1. The interaction between 14-3-3 and Rac1 mostly serves to regulate the activity and subcellular localization of Rac1. Among the seven 14-3-3 isoforms, 14-3-3η, -γ, -σ, and -θ interact with Rac1.

Figure 10. The amino acid sequence alignment of human Rac1 and African green monkey Rac1.

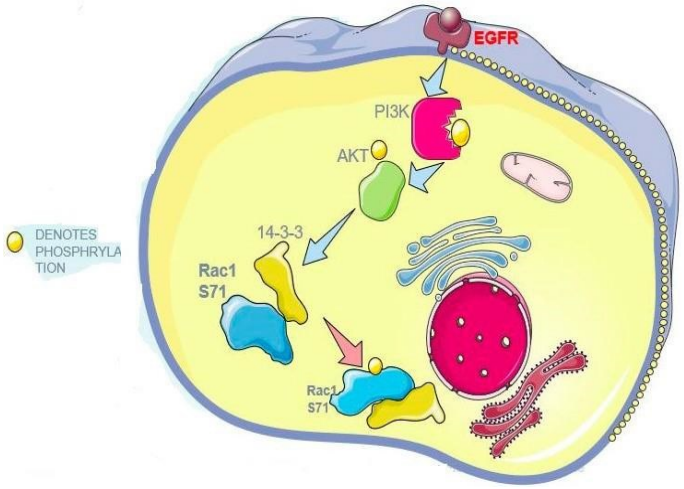


Figure 11. The diagram depicts the interaction between Rac1 and 14-3-3s.

(I've designed the figure)

Chapter 3: Results II – Nuclear Speckle localization of Rac1 and its role in pre-mRNA splicing

3.1 Chapter Abstract

Despite significant evidence that Rac1 is localized to the nucleus, studies have focused on the extranuclear function of Rac1, very little is known regarding the function and biological significance of nuclear Rac1. Here, we studied the subnuclear localization of Rac1 and the potential function related to its subnuclear localization. We showed that in response to EGF, Rac1 was targeted to nuclear speckles (NS) and co-localized with the NS marker SRSF2. Rac1 was also partially colocalized with A' protein of U2 snRNP (U2A') that localizes to the actual splicing sites at the peripheral region of NS. We also showed that the NS localization of Rac1 was dependent on its T108 phosphorylation by EGF. In addition to T108 phosphorylation, Rac1 PBR and GTPase activity also contributed to its NS localization.

Moreover, Rac1 interacts with various proteins involved in pre-mRNA splicing, including SRSF2, U2A', and hnRNPA1, as indicated by co-IP. These interactions are also dependent on T108 phosphorylation and affected by Rac1 PBR and GTPase activity. Finally, we showed that Rac1 regulated EGF-induced pre- mRNA splicing, and this is mediated by T108 phosphorylation. We conclude that in response to EGF, T108 phosphorylated Rac1 is targeted to NS, interacts with NS proteins involved in pre-mRNA splicing, and regulates EGF-induced pre-mRNA splicing.

Rac1 regulates multiple cellular processes and cell functions. Rac1 is overexpressed or mutated in many cancers and other diseases. The cycling of Rac1 between the GTP- and GDP- bound states is essential for effective signal flow to elicit downstream biological functions. Rac1 activity and function are also regulated by various post-translational modifications and subcellular translocation. Despite significant evidence of the nuclear localization of Rac1, studies have been focused on the extranuclear function of Rac1.

Almost all of the known Rac1 functions are linked to the extranuclear Rac1. It was shown that Rac1 is accumulated in the nucleus at the G2 phase. Nuclear Rac1 modulates actin polymerization in the nucleus, functioning to fine-tune cytoplasmic Rac1 activity. We showed that Rac1 ^{106}PNTP^{109} motif mediates its interaction with PLC-γ1 SH3 domain . Rac1 T108 within this motif is phosphorylated by ERK in response to EGF, which, together with its C- terminal polybasic region (PBR), targets Rac1 to the nucleus. Here, we investigated the subnuclear localization of Rac1 and its function related to its subnuclear localization. We show that in response to EGF, Rac1 is localized to NS, interacts with NS proteins involved in pre-mRNA splicing, and regulates EGF-induced pre-mRNA splicing, all of which is dependent on Rac1 T108 phosphorylation. The identification of this novel nuclear function of Rac1 in NS-associate pre-mRNA processing may profoundly change our understanding of Rac1.

3.2 Introduction

Like other Rho GTPases, Rac1 is involved in the acquisition of all the hallmarks of cancer. Rac1 is overexpressed or mutated in breast cancer and many other cancers and is linked to many other diseases. Due to its well-established role in controlling cytoskeleton dynamics, studies have focused on revealing the role of Rac1 in cancer metastasis[91,115].

The cycling of Rac1 between the GTP- and GDP-bound states is essential for effective signal flow to elicit downstream biological functions. The cycle between inactive and active forms is controlled by three distinct families of proteins: guanine nucleotide exchange factors (GEFs) that activate Rac1 by promoting uptake of free nucleotide, GTPase-activating proteins (GAPs) that negatively regulate Rac1 by stimulating its intrinsic GTPase activity leading to an inactive GDP-bound state, and guanine nucleotide dissociation inhibitors (GDIs) that inhibit the dissociation of GDP from Rac1. GDIs. Other modifications, including RNA splicing, microRNAs, and various post-translational modifications, have also been shown to regulate the activity and function of Rac1. The reported post-translational modifications include lipidation, ubiquitination, phosphorylation, and adenylylation, which are all shown to play important roles in the regulation of Rac1[432].

Rac1 is reported to be phosphorylated on S71 by Akt[184] . This phosphorylation of Rac1 inhibits its GTP binding activity without any significant change in GTPase activity. It was later reported that the phosphorylation of Rac1 S71 decreases the pathogenic effect mediated by Clostridium difficile toxin A (TcdA). Moreover, phosphorylation of Rac1 at S71 represents a reversible mechanism to determine the binding specificity of Rac1/Cdc32 to their downstream substrates. We recently showed that Rac1 S71 phosphorylation enhances the interaction between Rac1 and 14-3-3 proteins[176,185]. Moreover, Rac1 may be phosphorylated at Y64 by FAK and SRC kinases, Y64 phosphorylation targets Rac1 to focal adhesions. Rac1-Y64F displayed increased GTP-binding, increased association with βPIX, and reduced binding with Rho GDI as compared with wild-type Rac1. We showed that Rac1 has an ERK docking site [183]KKRKRKCLLL[192] (D-site) at C-terminus, which mediates the interaction between Rac1 and ERK.

Moreover, Rac1 T108 is phosphorylated in response to EGF, and EGF-induced Rac1 threonine phosphorylation is dependent on the activation of ERK. T108 phosphorylation targets Rac1 to the nucleus[135].

The most well-established function of Rac1 in the regulation of the cytoskeleton remodeling, especially the regulation of actin polymerization and the formation of lamellipodia and membrane ruffles [186,187]. Like other Rho GTPases, Rac1 is also cycling between PM and cytosol, which regulates their activities and functions in actin cytoskeleton remodeling and cell migration. The lipidation of Rac1 is important for its membrane-binding. It was shown that membrane-bound Rac1 is geranylgeranylated, and the soluble Rac1 is not geranylgeranylated.

Rac1 also localizes the nucleus. The data regarding the mechanisms regulating Rac1 nuclear localization are very controversial. It was shown that the PBR of Rac1 has a functional nuclear localization signal (NLS). Indeed, Rac1 PBR can target RhoA to the nucleus[109]. It was found that the Rac1 NLS is cryptic in the sense that it is inhibited by the adjacent geranylgeranyl modification[150]. However, the same research showed that the endogenous nuclear Rac1 is prenylated. It was also reported that the nuclear import of Rac1 is mediated by the direct interaction with karyopherin α2, and Rac1 activation is required for its nuclear localization. We have shown that Rac1 T108 phosphorylation by ERK targets Rac1 to the nucleus[133] . Inhibition of T108 phosphorylation by mutating T to A blocks the nuclear localization of Rac1.

Moreover, the T108 phosphorylation mimic GFP-Rac1T108E (T108E) was almost exclusively localized to the nucleus with or without EGF stimulation. We further studied the relative contribution of Rac1 PBR and T108 phosphorylation to the nuclear localization of Rac1. It is well-established that RhoA PBR lacks NLS. We showed that regardless of the phosphorylation status of the RhoA and Rac1, the substitution of the RhoA PBR with the Rac1 PBR targets RhoA to the nucleus and substitution of Rac1 PBR with RhoA PBR significantly reduces the nuclear localization of Rac1[132].

Despite significant evidence that Rac1 is localized to the nucleus, studies have focused on the extranuclear function of Rac1, very little is known regarding the function and biological significance of nuclear Rac1. It was suggested that localization into the nucleus would isolate Rac1 from its regulatory proteins localized in the cytoplasm and the

PM. For example, most of the known Rac1 GEFs, including PLC-γ1, Vav proteins, and Tiam1 are outside of the nucleus**,** and thus will not be able to activate the nuclear Rac1[15,22].

Whether Rac1 carries out any active nuclear function is an open question. Our previous immunofluorescence images showed that nuclear Rac1, especially nuclear T108E, is associated with nuclear bodies, which suggests that Rac1 may play an active role in regulating nuclear functions. In this research, we aimed to determine the subnuclear localization of Rac1 and the potential function related to its

subnuclear localization. We showed that while wild type Rac1 (Rac1) was localized in the nucleus in a small population of the Cos7 cell in the absence of EGF, Rac1 was strongly translocated to the nucleus and associated with nuclear bodies resemble the morphology of nuclear speckles (NS). Indeed, Rac1 was colocalized with the NS marker SRSF2 (SC-35). Rac1 was also colocalized with other nuclear proteins involved in RNA splicing, including U2 snRNP and hnRNPA1, to a less extent. Moreover, phosphomimetic mutant Rac1T108E (T108) was almost exclusively localized to the nucleus and co-localized with SRSF2 with or without EGF stimulation. However, a portion of Rac1T108A (T108A) was localized to the nucleus with EGF stimulation, but it failed to co-localize with SRSF2. More interestingly, we showed that substitution of the RhoA PBR with the Rac1 PBR targets RhoA to the nucleus, and the nuclear RhoARac1PBR was colocalized with SRSF2 with or without EGF stimulation like Rac1T108E. Moreover, we showed that both constitutively active mutant Rac1Q61L (L61) and dominant-negative mutant Rac1T17N (N17) were translocated to the nucleus. While L61 showed strong co-localization with SRSF2 like wild type Rac1, N17 only showed weak colocalization with SRSF2. We also showed by co- immunoprecipitation (co-IP) that Rac1, Rac1T108E, RhoARac1PBR, and L61 interacts all interact with SRSF2, U2 snRNP, and hnRNPA1. On the other hand, Rac1T108A, Rac1RhoAPBR, and N17 did not or only weakly interacted with SRSF2, U2 snRNP, and hnRNPA1. Finally, we showed that Rac1 plays a role in alternative RNA splicing. We showed that Rac1, Rac1T108E, RhoARac1PBR, and L61 all positively regulate alternative RNA splicing.

3.3 Methods and Materials

3.3.1 Cell culture and treatment

COS-7 cells (purchased from American Type Culture Collection, ATCC) were cultured in DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 10% fetal bovine serum (FBS) and antibiotics including penicillin (100U/ml) and streptomycin (100µg/ml). The cells were maintained in a 5% CO2 atmosphere at 37 °C. For the EGF treatments, COS-7 cells were incubated with EGF (50 ng/ml) for 30 min or as indicated following serum starvation for 16 h in DMEM medium.

3.3.2 Transient transfection

Plasmid DNA for transfection was prepared by using a Qiagen midiprep kit (Qiagen Inc., Chatsworth, CA) according to the manufacturer's instructions. COS-7 cells were grown to 70-80% confluence in 6-cm dishes before the transfection. Transfections were performed using Lipofectamine 2000 (Thermo-Fisher) according to the manufacturer's instructions. Cells were typically analyzed 48 h post-transfection.

3.3.3 Antibodies and chemicals

Mouse monoclonal antibodies against SRSF1 (pSC35), U2 snRNPA1, hnRNPA1, and goat polyclonal antibody against phosphorylated EGFR (p1086) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit anti-GFP antibody was a gift from Dr. Luc Berthiaume (University of Alberta, AB). FITC- and TRITC (Rhodamine)-conjugated secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, PA). Goat anti-mouse IgG conjugated with agarose, protein A conjugated with agarose, were purchased from Sigma-Aldrich (St. Louis, MO). Unless otherwise specified, all chemicals were purchased from Sigma-Aldrich.

3.3.4 Plasmids

GFP-Rac1 is a gift from Dr. Mark R. Philips (NYU School of Medicine). GFP-Rac1RhoAPBR, which was constructed by replacing the Rac1 PBR (181PVKKRKRK188) with RhoA PBR (182RRGKKKSG189), and GFP-RhoARac1PBR, which was constructed by replacing RhoA PBR (182RRGKKKSG189) with Rac1 PBR (181PVKKRKRK188), were generously provided by Dr. Carol Williams (Medical College of Wisconsin, WI, USA). Phosphomimetic mutant GFP-Rac1T108E (with the replacement of T108 by E), GFP-Rac1T108A (with the replacement of T108 by A) that is unable to be phosphorylated at 108, constitutively active GFP-Rac1L61 (with the replacement of Q61 by L), and dominant-negative GFP-Rac1N17 (with the replacement of T17 by N) were generated previously in the laboratory [32,109].

3.3.5 Immunoprecipitation

Cells were lysed with IP buffer [20 mM Tris, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.1% sodium deoxycholate, 100 mm NaF, 5 mM $MgCl_2$, 0.5 mM Na_3VO_4, 0.02% NaN_3, 0.1 mM 4-(2-aminoethyl)- benzenesulfonyl fluoride, 10 μg/ml aprotinin, and 1 μM pepstatin A]. Cell lysates were centrifuged at 21,000 × *g* for 15 min to remove debris. The supernatants, containing approximately 1 mg of total protein, were pre-cleared with agarose beads and then were incubated with 1 μg of specific antibody at 4°C overnight. Secondary antibodies or protein A conjugated with agarose was then added to each supernatant/antibody mixture. Following two h incubation at 4°C with agitation, the supernatant/antibody mixture was centrifuged, and the pelleted agarose beads and the non-precipitated supernatant were collected. The agarose beads were washed three times with IP buffer and then mixed with a 2x sample loading buffer. The sample was boiled for 5 min and subjected to SDS-PAGE followed by immunoblotting.

3.3.6 Immunoblotting

Protein samples were prepared by boiling in half volume of 4X laemmli buffer for 5 min. Twenty μg of total protein was run in 8% polyacrylamide gel by vertical electrophoresis at 100V electric potential for 100 minutes and then transferred on nitrocellulose membrane at 15V electric potential for 90 minutes using a semi-dry protein transfer system (Bio-Rad

Laboratories, Berkeley, USA). The membranes were blocked by incubation in Odyssey® Blocking Buffer (TBS) (LI-COR biotechnology Inc., Lincoln, NE, USA) for 60 minutes and then were incubated overnight in 0.2 µg/ml primary antibody solution. After washing with Tris-buffered saline (TBS) containing 0.05% tween-20, the membranes were incubated in 25 ng/ml RDye® primary antibody solution for 60 minutes and then after washing were visualized by using Odyssey® CLx imaging system (LI-COR Biotechnology Inc., Lincoln, NE, USA). Query protein bands' intensity was quantified and normalized to the intensity of relevant loading control protein bands.

3.3.7 Subcellular localization of proteins by fluorescence microscopy

Approximately 10^5 cells were seeded on 15 mm round cover glass in 24 well-plates and were cultured in standard culture condition for 48 h and then were starved overnight in 1% FBS culture medium. After treatment, the coverslips were washed with ice-cold PBS, and the cells were fixed by incubation in - 20 ºC methanol for 5 minutes, followed by Triton 0.1 X permeabilization for 15 min . Then, the coverslips were washed with TBS and were blocked in 1% bovine serum albumin (BSA) solution in TBS for 60 minutes. After blocking, the coverslips were incubated in 2 µg/ml of primary antibody for 60 minutes. The coverslips were washed and then were incubated in 1 µg/ml FITC-conjugated and one µg/ml rhodamine-conjugated secondary antibodies solutions for 60 minutes in the dark. Afterward, the coverslips were washed with TBS and then were incubated in 1 µg/ml DAPI solution for 5 minutes. The coverslips were mounted on microscope slides and were observed by using a GE Healthcare DeltaVision Deconvolution Microscope system (GE Healthcare Life Science, Mississauga, ON, Canada). All of the images were deconvolved.

3.3.8 RNA isolation:

Using at least 10^6 cells, Cos7 Cells were washed with PBS 1X, and 1 ml of Trizol was added. Cells were scraped into an Eppendorf tube and 250 µl chloroform. Then, Centrifuged at 10,000 rpm for 5 min. The clear top layer was carefully removed & then placed in a new 1.5 ml Eppendorf tube. 550 µl of isopropanol was added to the aqueous phase and mixed gently then centrifuged at 8,000 g for 30 min and then again recentrifuged at 6000 g for 5 min after g ethanol. Ethanol was poured off, and the pellets were left to
air-dry. After pouring off the bulk of the ethanol wash, the absorbance was measured at 260 nm using Nanodrop. The 260/280 ratio was greater than 1.8.

3.3.9 RT-PCR and Pre- mRNA Splicing assay:

Reverse transcription was performed with the High Capacity cDNA Reverse Transcription Kit (applied biosystems) using 1µg total RNA and ten pmol reverse sequence-specific primer, and The reaction was incubated at 25℃ for 10 min, 120min at 37℃and 85℃ for 5 min. 2ul of the reaction was used for endpoint PCR with Phusion™ High–Fidelity DNA Polymerase (Thermofisher) 10 mM dNTPs, and 0.2 primers in 5X Phusion HF buffer. PCR products were resolved in 2.5%(wt/vol) agarose gels and stained by Sybersafe. The products (amplicons) generated from PCR will correlate with the splcing efficiency in each experiment which will give us more insights on how strong or weak the splicing efficiency is in each.. The band expected to see are 0.3kbp, 0.4kbp, 0.6kbp. 1.2kbp and 1.5 kbp.

F' Strand	GGAAAGGCAACAGCAAACTC
R' Strand	CGGGTGTCTTGAAAAACGTC

3.3.10 Co-transfection protocol:

Co-transfection was performed according to manufacturer protocol. Briefly, one day before transfection, the 70-80%cells were plated in 100x21mm Dish, Thermofisher, Catalogue no. 172931 a serum-free DMEM media without antibiotics. For each transfection experiment, E2F7minigene-Rac1 mutant Lipofectamine 2000 complexes were prepared as follows: The two plasmids constructs were diluted in Opti-MEM medium without serum. Then Lipofectamine 2000 was diluted in Opti-MEM medium without serum. The two tubes were mixed gently and incubated for 10 minutes at room temperature. Then both were combined with the Lipofectamine 2000 and incubated for 30 minutes at room temperature to allow the complex formation to occur. Then the full complex was added to each plate containing cells and mixed gently. The cells were incubated at 37°C in a CO_2 incubator for 72hrs, and then RNA was harvested. The growth medium was replaced after 6 hours to avoid the toxicity of Lipofectamine 2000.

3.4 Results

3.4.1 The association of Rac1 with nuclear speckles

Determination of the subcellular localization of Rac1 will help us to understand the nuclear function of Rac1. We showed previously that nuclear Rac1, including both GFP, tagged wild type Rac1 (Rac1), and the phosphomimic mutant T108E formed specific punctate patterns, rather than diffusely distributed, through the nucleus, which suggest that nuclear Rac1 may be associated with nuclear bodies. It is well- documented that the nucleus is a complex and highly dynamic environment with many functionally

specialized regions of substructure that are formed and maintained in the absence of membranes[189]. These specialized regions or subnuclear domains include nucleoli, nuclear speckles, Cajal bodies, gems, promyelocytic leukemia (PML) bodies, and chromosomes. Careful examination of the IF images of both Rac1 and T108E indicated that the morphology, the size, and the number of Rac1-positive nuclear bodies are very similar to that of nuclear speckles (Fig. 12A). Nuclear speckles are nuclear domains enriched in pre-mRNA splicing factors that are located in the interchromatin regions of the nucleoplasm in mammalian cells.

To determine if Rac1 is indeed localized to the nuclear speckles, we examined the co-localization of Rac1 with SRSF2 (also called phosphorylated SC35). SRSF2 is the core component and a constitutive protein of nuclear speckles. We transfected Cos-7 cells with GFP tagged wild type Rac1 (GFP-Rac1). Following EGF stimulation for 30 min, the co-localization of Rac1 and Nuclear speckles was determined by immunofluorescence (Fig. 12B). As shown in Fig. 12B, Rac1 is mostly cytosolic without EGF stimulation, but following EGF stimulation, a portion of Rac1 was translocated to the nucleus and showed the strong co-localization with SRSF2. We further studied EGF-induced nuclear speckle localization of Rac1 by time-course experiments. Cos-7 cells transfected with GFP-Rac1 were stimulated with EGF for 5, 15, 30, 60, and 120 min. The localization of Rac1, SRSF2, and the co-localization of Rac1 with SRSF2 was examined by IF. As shown in Fig. 12C, SRSF2 was always localized to the nuclear speckle regardless of EGF stimulation. However, the SRSF2 positive nuclear speckles changed in size and morphology. In the absence of EGF, SRSF2-positive nuclear speckles were relatively smaller and round. With longer EGF stimulation, SRSF2-positive nuclear speckles became larger and irregular in shape
. As for Rac1, in the absence of EGF, Rac1 was primarily localized to the PM and cytoplasm. Following EGF stimulation for 5 min, EGFR was phosphorylated and mostly localized to the plasma membrane and small vesicles near the plasma membrane

. A visible portion of Rac1 was translocated to the nucleus and colocalized with SRSF2 in the nuclear speckles. After EGF stimulation for 15 and 30 min, pEGFR is localized to larger vesicles resemble endosomes, and more Rac1 was translocated to the nucleus and colocalized with SRSF2 in the nuclear speckles. With longer EGF stimulation for 60 and 120 min, pEGFR was gradually localized to larger and perinuclear vesicles that resemble late endosomes and lysosomes. The nuclear localization and co-localization with SRSF2 in nuclear speckles remained similar to that at 30 min (Fig. 12C).

We further examined the co-localization of Rac1 with U2 snRNP by a time-course experiment with EGF stimulation for 0, 5, 15, 30, 60, and 120 min. U2 is an essential protein for RNA splicing and is localized to the actual RNA splice sites that partially overlap with nuclear speckles. We showed that the nuclear localization pattern of U2 snRNP remained the same throughout the time course. Rac1 was co-

localized with U2 in response to EGF, but to a less extent than SRSF2 (Fig. 13A). Frequently, Rac1 partially co-localized with U2 snRNP-positive nuclear structures. This suggests that Rac1 may not localize to the actual RNA splice site but remain in the nuclear speckles. We finally examined the co-localization of Rac1 with hnRNP A1. hnRNPs assist in controlling the maturation of newly formed nuclear RNAs (hnRNAs/pre- mRNAs) into mRNAs, stabilize mRNA during their transport, and control their translation. hnRNP A1 also shuttles between cytoplasm and nucleus. Indeed, we showed that hnRNP A1 was much broadly distributed in the cell (Fig. 2B). In the nucleus, hnRNPA1 did not localize to nuclear speckle-like structures but distributed in the inter-chromatin region in a web-like pattern. Most interestingly, hnRNPA1 nuclear localization significantly changed with EGF stimulation. In the absence of EGF, hnRNPA1 was localized to both cytoplasm and nucleus, with EGF stimulation from 5 min to 1h, and the cytoplasm-associated hnRNPA1 gradually translocated to the nucleus. However, at 2h EGF stimulation, a significant portion of hnRNPA1 translocate back from the nucleus to the cytoplasm. It was localized to the nuclear speckle as well as other smaller sized nuclear structures. As some nuclear Rac1 was also distributed in the inter- chromatin region, we observed the co-localization of Rac1 and hnRNPA1 in these inter-chromatin regions (Fig. 13B).

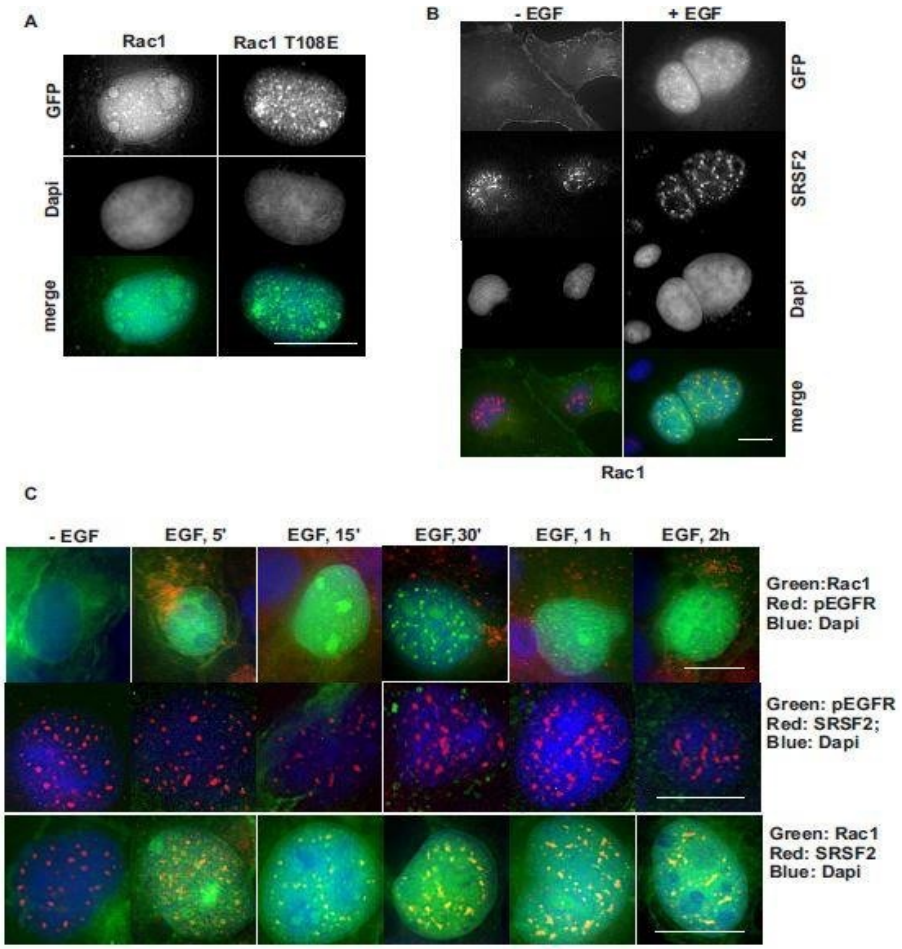


Fig 12

Nuclear translocation and Subnuclear localization of Rac1 in response to EGF revealed by IF. Cos-7 cells were transfected with GFP-tagged wild type or mutant Rac1. A) The localization of GFP-Rac1 and GFP- Rac1T108E to the nuclear speckles. Cells were stimulated with EGF for 30 min and counterstained with Dapi. Arrow indicates nuclear speckles. B) Co-localization of GFP-Rac1 and SRSF2. The cell was either not treated or treated with EGF for 30 min. The colocalization of Rac1 (green) and SRSF2 (red) was revealed by immunofluorescence. Cells were count stained with Dapi. C) Time-course of the co-localization of GFP-Rac1 and SRSF2 in response to EGF. Cells were stimulated with EGF for the indicated time. Upper panel: the co-localization of GFP-Rac1 (green) and pEGFR (red). Middle panel: co-localization of SRSF2 (red) and pEGFR (green). Lower Panel: co-localization of GFP-Rac1 (green) and SRSF2 (red). Cells were counterstained with Dapi. Size bar = 10 μm.

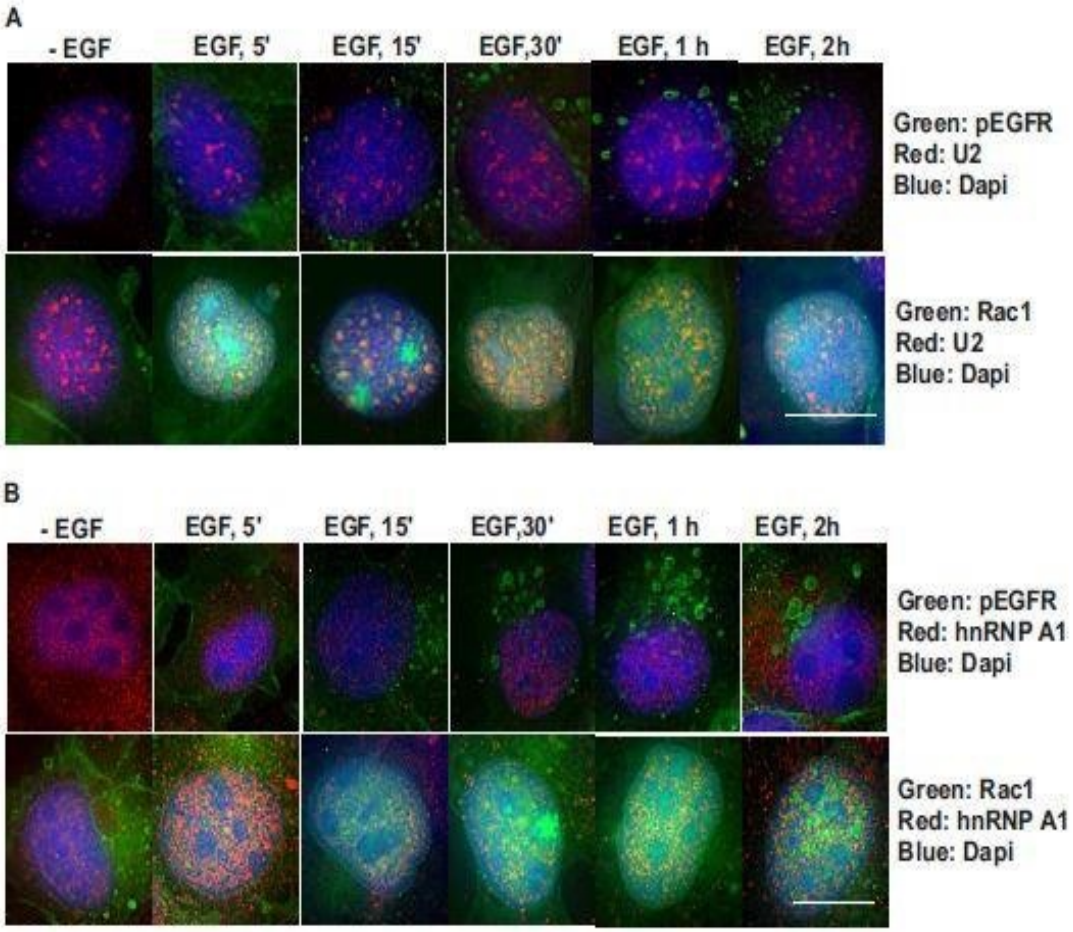


Fig. 13. Time-course of the co-localization of GFP-Rac1 and U2 snRNP or hnRNPA1 in response to EGF. Cells were stimulated with EGF for the indicated time**. A)** Upper panel: The co-localization of U2 snRNP (red) and pEGFR (green). Lower Panel: co-localization of GFP-Rac1 (green) and U2 snRNP (red).

B) Upper panel: The co-localization of hnRNPA1 (red) and pEGFR (green). Lower Panel: co-localization of GFP-Rac1 (green) and hnRNPA1 (red). Cells were counterstained with Dapi. Size bar = 10 μm.

3.4.2 The structure requirements for nuclear speckle localization of Rac1

Next, we examined what Rac1 motifs contribute to the nuclear speckle localization of Rac1. It is shown that the Rac1 C-terminal PBR domain contains NLS. We and others have shown previously that the PBR is critical for the nuclear localization of Rac1[134]. Here we examined the role of T108 phosphorylation and PBR in the nuclear speckle association of Rac1. We first examine the nuclear speckle localization of two Rac1 mutants: GFP tagged phosphomimic T108E, and GFP tagged T108A (T108A) that is unable to be phosphorylated at amino acid 108. We showed that T108E was almost exclusively associated with nuclear bodies and very little distribution in the inter-chromatin regions with or without EGF stimulation (Fig. 13A&B). All of the SRSF2-positive nuclear bodies were positive for Rac1T10E. However, some of the Rac1T108E-positive nuclear bodies were not positive for SRSF1. These data indicated that T to E mutation greatly increased the nuclear localization and nuclear speckle association of Rac1. On the other hand, the localization and translocation of T108A were quite different. In the absence of EGF, T108A is mostly localized to the cytoplasm, which is similar to Rac1 (Fig. 14A&B). Following EGF stimulation for 30 min, a portion of T108A was translocated to the nucleus, which is again similar to Rac1. However, nuclear T108A seemed not to be associated with any nuclear bodies but diffusely distributed within the nucleus (Fig. 14A&B). The small proportion of T108A Rac1 that's till translocate could be attributed to PBR region that still exists in that mutant.

To further study the role of Rac1 PBR in the nuclear speckle localization of Rac1, we examine the nuclear speckle association of two previously generated Rac1 and RhoA mutants including $RhoA_{Rac1PBR}$ (The PBR of GFP tagged RhoA was replaced by Rac1 PBR) and $Rac1_{RhoAPBR}$ (The PBR GFP-tagged Rac1 was replaced by RhoA PBR). As shown in Fig. 14C&D, $RhoA_{Rac1PBR}$ was mostly localized to nucleus with or without EGF stimulation, which is consistent with our previous data[32]. The subnuclear localization of $RhoA_{Rac1PBR}$ was very similar to Rac1T108E. $RhoA_{Rac1PBR}$ was almost exclusively associated with nuclear bodies and very little distribution in the inter-chromatin regions with or without EGF stimulation (Fig. 14C&D). All of the SRSF2-positive nuclear bodies were positive for $RhoA_{Rac1PBR}$.

However, some of the $RhoA_{Rac1PBR}$ -positive nuclear bodies were not positive for SRSF1. However, $Rac1_{RhoAPBR}$ was exclusively localized to the cytosol and the plasma membrane (Fig. 14C).

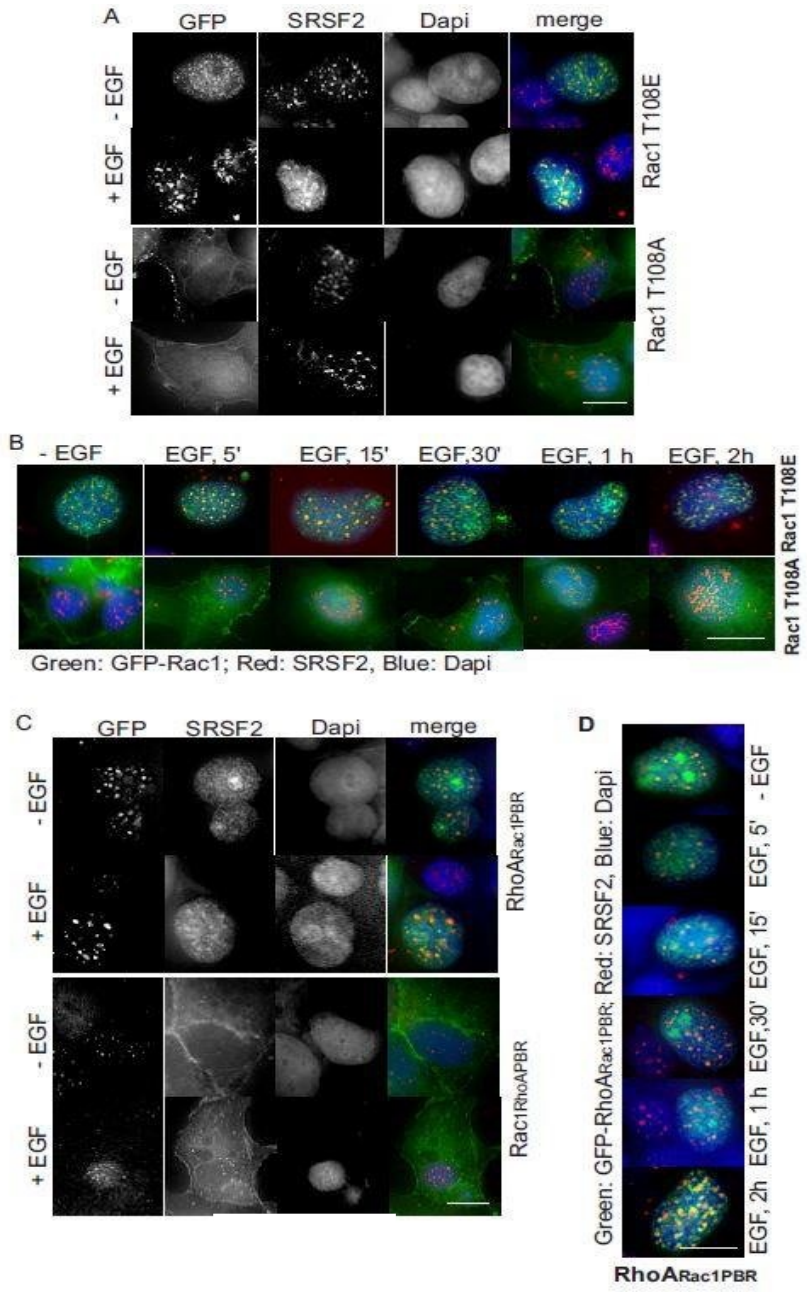


Figure 14

The effects of Rac1 T108 phosphorylation and PBR on the nuclear and nuclear speckle localization of Rac1. Cos-7 cells were transfected with various GFP-tagged Rac1 mutants. Their co-localization with SRSF2 in response to EGF was revealed by IF. A) Co-localization of GFP-tagged Rac1T108E/Rac1T108A and SRSF2. The cell was either not treated or treated with EGF for 30 min. The colocalization of mutant Rac1 (green) and SRSF2 (red) was revealed by immunofluorescence. Cells were count stained with Dapi.

3.4.3 The role of Rac1 activity in its nuclear speckle localization

We next examined if Rac1 GTPase activity plays a role in its nuclear speckle localization. We transfected Cos-7 cells with two GFP-tagged Rac1 mutants, including a constitutively active mutant Rac1L61 (with the replacement of Q61 by L) and a dominant-negative mutant Rac1N17 (with the replacement of T17 by N). The localization of these two mutants and their co-localization with SRSF2 with or without EGF stimulation was examined by IF. As shown in Fig. 15, the localization and EGF-induced nuclear translocation of these two mutants were very similar to wild type Rac1. In the absence of EGF, both mutants are mostly localized to the plasma membrane and cytoplasm; however, following EGF stimulation, both mutants were translocated to the nucleus. However, the nuclear speckle localization of the two mutants is different. The nuclear Rac1Q61L was strongly localized to the nuclear speckles and co-localized with SRSF2. On the other hand, Rac1T17N was only weakly localized to the nuclear speckles and co-localized with SRSF2 (Fig. 15). These data indicated that the Rac1 GTPase activity did not affect its nuclear localization, affected its association with nuclear speckles.

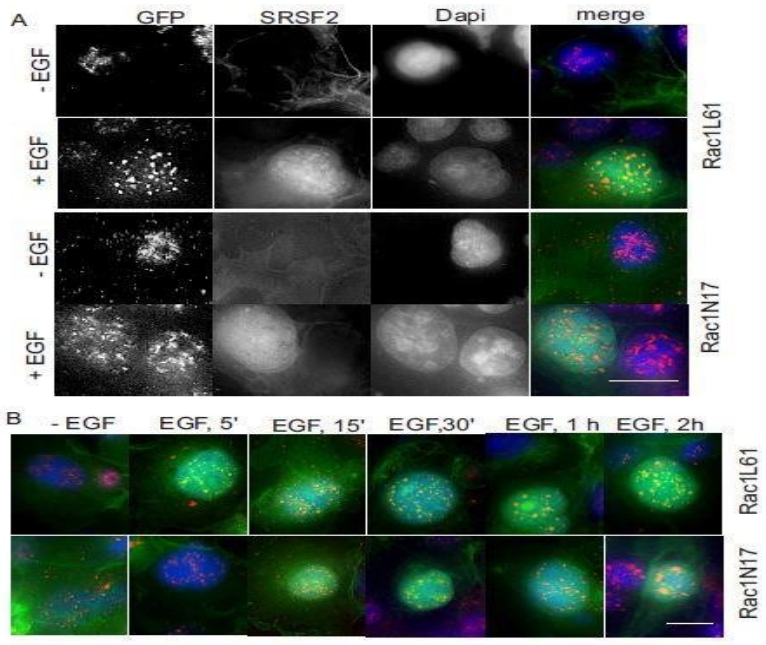


Figure 15

Figure 15 The effects of Rac1 GTPase activity on its nuclear and nuclear speckle localization in response to EGF. Cos-7 cells were transfected with GFP-tagged constitutive active mutant Rac1L61 and dominant-negative mutant Rac1N17. Their co-localization with SRSF2 in response to EGF was revealed by IF. **A)** Co-localization of GFP-tagged Rac1L61/Rac1N17 and SRSF2. The cell was either not treated or treated with EGF for 30 min. The colocalization of mutant Rac1 (green) and SRSF2 (red) was revealed by immunofluorescence. Cells were count stained with Dapi. **B)** Time-course of the co-localization of GFP- tagged Rac1L61/Rac1N17 and SRSF2 in response to EGF. Cells were stimulated with EGF for the indicated time**.** Upper panel: The co-localization of Rac1L61 (green) and SRSF2 (green). Lower Panel: co- localization of Rac1N17 (green) and SRSF2 (red).

3.4.4 The association of Rac1 with nuclear proteins involved in pre-mRNA splicing

Next, we determined if Rac1 is associated with proteins involved in mRNA splicing. We transfected Cos7 cells with GFP-Rac1 and examined if SRSF2, U2, and hnRNPA1 were co-immunoprecipitated with Rac1 before and after EGF stimulation. As shown in Fig. 16A, in the absence of EGF, SRSF2, U2, and hnRNPA1 only weakly co-IPed with Rac1, however, following EGF stimulation for 30 min, SRSF2, U2 and hnRNPA1 showed much stronger co-IP with Rac1.

We then determined the association between various Rac1 mutants and SRSF2, U2, and hnRNPA1. Two GFP tagged Rac1 T108 mutant T108E and T108A were expressed in Cos-7 cells, and Rac1 mutants were IPed with GFP antibody and the co-IP of SRSF2, U2 and RNPA1 were examined by immunoblotting. As shown in Fig. 16, T108E showed strong co-IP with SRSF2, U2, and hnRNPA1; however, T108A did not co-IP with SRSF2, U2, and hnRNPA1. We did not treat the cells with EGF when the T108 was mutated.

We also expressed $RhoA_{Rac1PBR}$, and $Rac1_{RhoAPBR}$ in Cos7 cells and examined their co-IP with SRSF2, U2, and hnRNPA1 with or without EGF (Fig. 16). SRSF2, U2, and hnRNPA1 co-IPed with $RhoA_{Rac1PBR}$ with or without EGF stimulation, but did not co-IP with $Rac1_{RhoAPBR}$, with or without EGF stimulation (Fig. 16).

To determine if the Rac1 GTPase activity is involved in the association between Rac1 and SRSF2, U2, and hnRNPA1, we expressed Rac1L61 and Rac1N17 in Cos-7 cells. Co-IP experiments showed that like wild type Rac1, Rac1L61 strongly complexed with SRSF2, U2, and hnRNPA1 in response to EGF.

However, Rac1C17N only weakly interacted with SRSF2, U2, and hnRNPA1 following EGF stimulation (Fig. 16).

3.4.5 The role of Rac1 in mRNA splicing

The data below indicated that Rac1 was not only localized to the nuclear speckles but also complexed with RNA splicing proteins. We then examined if Rac1 is involved in mRNA splicing. To determine the role of Rac1 in mRNA splicing, we employed a generally accepted method for studying mRNA splicing. An E2F7minigene was used as a reporter for pre-mRNA splicing assay. As shown in Fig. 16, in the cells expressing Rac1,

EGF stimulated strong pre-mRNA splicing; however, this splicing activity is inhibited when Rac1 was substituted by Rac1T108A. Moreover, pre-mRNA splicing activity is high with or without EGF in cells expressing Rac1T108E (Fig, 16). These data support the role of Rac1 in EGF-induced pre-mRNA splicing

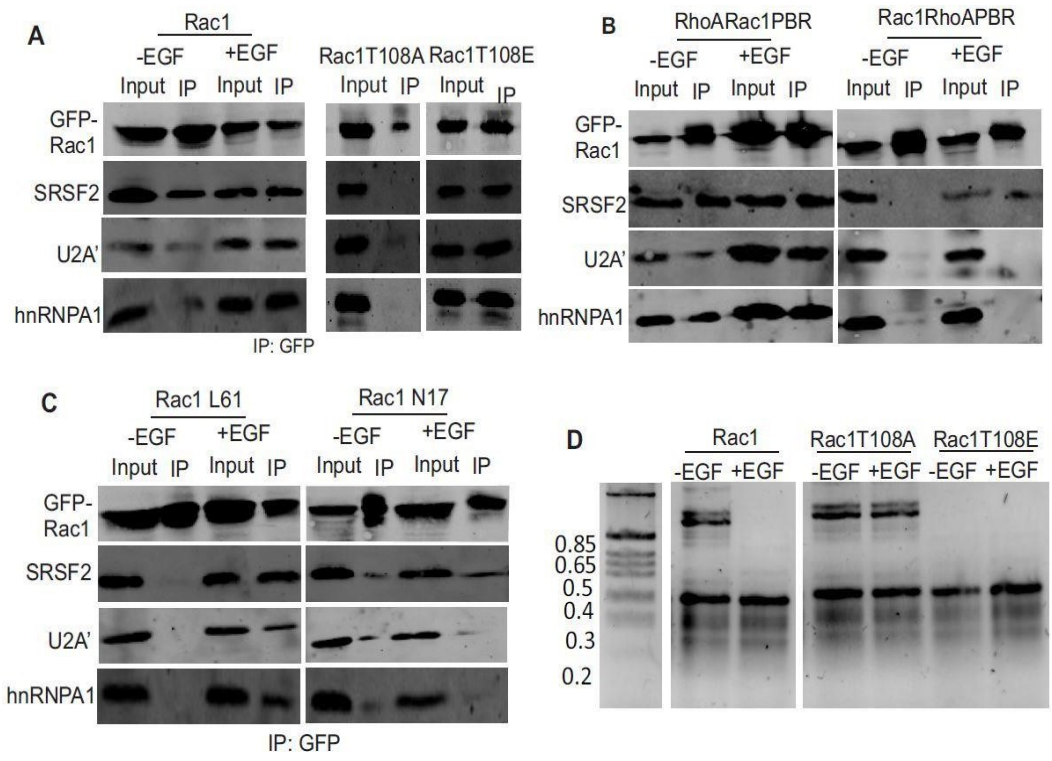


Fig 16

The interaction of Rac1 with SRSF2, U2A', and hnRNPA1 and the effects of Rac1 on EGF- induced alternative pre-mRNA splicing. A-C) Cos-7 cells were transfected with GFP-tagged wild type and mutant Rac1. The cells were stimulated with EGF for 30 min or not stimulated. Rac1 and mutants were IPed with antibody to GFP. The co-IP of SRSF2, U2A', and hnRNPA1 was determined by immunoblotting with respective antibodies as indicated. **D)** pre-mRNA splicing assay with E2F7 Minigene as a reporter. Cos-7 cells were co-expressed with E2F7 minigene, and GFP tagged Rac1, Rac1T108A, or Rac1T108E. The effects of Rac1 and mutants on EGF-induced E2F7 splicing was examined as described.

3.5 Discussion

Rac1 is a small GTPase and has been shown to play an essential role in many different cellular functions, including regulation of cytoarchitecture, cell size, cell adhesion, cell polarity, cell motility, proliferation, apoptosis/survival, and membrane trafficking [101,188]. Rac1 has been implicated in many aspects of cancer development, especially in cancer cell invasion and metastasis. Deregulated Rho GTPases have been discovered in many human tumors, including colon, breast, lung, myeloma, and head and neck squamous cell carcinoma.

Despite significant evidence that Rac1 is localized to the nucleus, studies have focused on the extranuclear function of Rac1, very little is known regarding the function and biological significance of nuclear Rac1. It was suggested that localization into the nucleus would isolate Rac1 from its regulatory proteins localized in the cytoplasm and the PM [101]. A recent study suggests that nuclear Rac1 modulates actin polymerization in the nucleus, functioning to fine- tune cytoplasmic Rac1 activity [189]. No significant and active nuclear function of Rac1 has been identified. We showed here that nuclear Rac1 was significantly associated with nuclear speckles (Fig. 12-16). Rac1 was co-localized with proteins involved in mRNA splicing, including SRSF1, U2 snRNP, and hnRNPA1 (Fig. 15). Rac1 actively regulates the alternative mRNA splicing (Fig. 16). Identification of this significant novel nuclear function of Rac1 will likely change the paradigm of Rac1 research.

Nuclear localization of Rac1 has been well-documented, and the PBR that contains an NLS has been demonstrated as the most important sequence for the nuclear localization of Rac1 by multiple pieces of research[32, 133, 149]. We have shown previously that the nuclear localization of Rac1 is significantly increased in

response to EGF[109,190]. We showed here that GFP-tagged wild type Rac1 once entered the nucleus there's a significant colocalization nuclear speckles and co-localize with SRSF2, the most accepted marker for nuclear speckles[29]. While the nuclear speckle regulates the multiple steps of nascent mRNA processing, including transcription, storage, splicing, and transportation, the actual mRNA splicing site is likely in the peripheral region of nuclear speckles[160,191]. U1 snRNP is directly regulated RNA splicing. We observed the partial

overlap between Rac1 and U2 snRNP. Rac1-positive nuclear speckles were frequently surrounded by U2 snRNP (Fig.13). These data suggest that Rac1 may not directly localize to RNA splicing sites. We did show the interaction between Rac1 and U2 snRNP (Fig. 5), and this interaction could be in the nuclear speckle as U2 snRNP is stored there.

We also observed the co-localization of Rac1 with hnRNPA1 in the interchromatin region, not in the nuclear speckles. We showed that Rac1 interacted with hnRNPA1 (Fig.13). However, we are not sure about the location of this interaction. They could interact in the cytoplasm, nucleus, or both. One interesting observation is that both Rac1 and hnRNPA1 translocated from cytoplasm to nucleus in response to EGF. It is not clear if their translocation is coupled together. However, at 2h of EGF stimulation, a large portion of hnRNPA1 translocated back to the cytoplasm, but most nuclear Rac1 remained in the nucleus.

To gain insight into the regulation of Rac1 nuclear localization, we examine two Rac1T108 mutants, Rac1T108E and T108A. We showed that Rac1T108A was not localized to nuclear speckles while in the nucleus, but Rac1T108E was strongly localized to the nuclear speckles even in the absence of EGF. These data suggest that T108 phosphorylation is critical for the Rac1 nuclear speckle localization. It is believed that nuclear speckle localization requires nuclear speckle localization signals (NSLS), and several NSLS have been identified or suggested

. 1) The SR domain of some SR proteins is necessary and sufficient for the targeting of these factors to nuclear speckles [192]. 2) The threonine-proline repeats of SF3B1 [155]. 3) the polyHis- repeat were proposed to act as a speckle-targeting signal that functions by acting as an interaction surface for resident nuclear speckle constituents [191]

. Rac1 PBR domain is a central region and thus could be important for its nuclear speckle targeting. However, Rac1 PBR is essential for the nuclear localization of Rac1; Rac1RhoAPBR was not able to enter the nucleus (Fig. 14C). Thus, we are not sure if Rac1 PBR is the NSLS. It is interesting to notice that RhoARac1PBR was strongly localized to the nuclear speckles (Fig. 14), which support the role of Rac1 PBR as the NSLS. Sequences surrounding T108 could also serve as NSLS. We showed previously that the Rac1 ^{106}PNTP^{109} motif is responsible for binding PLC-γ1[19], and T108 was phosphorylated by ERK in response to EGF, which enhances the nuclear localization of Rac1 [175]. As TP repeats have been shown as NSLS, besides ^{108}TP^{109}, Rac1 also contains another TP at ^{135}TP^{136}, as well as multiple YP and SP. Those dipeptides, especially ^{108}TP^{109} could serve as NSLS. Further research is needed to define the NSLS of Rac1.

We showed that Rac1 was co-IPed with SRSF2, U2 snRNP, and hnRNPA1, and this co- IP was dependent on EGF stimulation. Moreover, all the Rac1 mutants studied co-IPed with SRSF2, U2 snRNP, and hnRNPA1 as long as they showed localization to nuclear speckles (Fig. 12-13). We are not sure if Rac1 directly interacted with all these three proteins, or Rac1 just interacted with one protein, but all of them were in the same complex.

The most significant finding is that Rac1 played a positive role in the regulation of alternative mRNA splicing. We showed that Rac1 & Rac1T108E all positively regulate alternative RNA splicing.

In conclusion, we showed that in response to EGF, Rac1 is localized to the nucleus, and nuclear Rac1 showed significant localization to SRSF2-positive nuclear speckles. Rac1 T108 phosphorylation and PBR domain contributed to the nuclear speckle localization of Rac1. Rac1 interacts with proteins involved in RNA splicing, including SRSF2, U2 snRNP, and hnRNPA1, in response to EGF. Most importantly, Rac1 positively regulates alternative mRNA splicing. Identification of this novel nuclear function of Rac1 suggests that the function of Rac1 is not limited to the cytoplasm, and Rac1 may be an important player of nascent RNA processing, which could significantly impact future Rac1

Chapter 4: Discussion & Future Directions

I mainly focused my research on characterizing and understanding how Rac1phosphorylation regulates its function in the cytoplasm and in the nucleus. Rac1 is a RhoGTPase involved in a plethora of cellular pathways, and some of its key functions are, but not limited to, regulation of cytoarchitecture, cell polarity, and apoptosis/survival, which requires crosstalk between Rac1 and a subset of proteins to accomplish coordinated signaling. The first part looked at colocalization and interaction of Rac1 with specific 14-3-3 isoforms, which provides interesting clues and a strong basis for studying the role of this interaction in regulating specific and critical cell functions. For example, both 14-3-3 protein and Rac1 have been shown to regulate apoptosis. The interaction between 14-3-3η and Rac1 and the localization of both proteins in the mitochondria suggests that they may co-regulate mitochondria-mediated apoptosis. The colocalization of 14-3-3γ and Rac1 in the nucleus and our observed interaction between 14-3-3γ and Rac1 in 293T cells suggests that they may co-regulate certain nuclear functions.

I also demonstrated here that 14-3-3s interacts with Rac1. This interaction is strongly enhanced by Rac1 S71 Phosphorylation. However, given that GFP WT Rac1 which is an unphosphorylated form produced by bacteria can pull down 14-3-3, it means interaction can be phosphorylation independent, but the phosphorylation-dependent interaction is much stronger. EGF strongly stimulates the phosphorylation of Rac1 S71 and the interaction between 14-3-3 and Rac1. Moreover, I also showed that mutating S71 to A completely abolishes both phosphorylation-dependent and - independent interactions between 14-3-3 and Rac1. Although, the data may suggest a possible interaction between Rac1 and 14-3-3. However, more invitro experiments that will allow us to examine if both the proteins interact or not such as Proximity Ligation assay or Y2H are needed to confirm there's an actual direct interaction between the two of them The

interaction between 14-3-3 and Rac1 mostly serves to regulate the activity and subcellular localization of Rac1. Among the seven 14- 3-3 isoforms, 14-3-3η, -γ, -σ, and -θ interact with Rac1.

The second part of my work looked more closely at the nucleus. Nuclear localization post- phosphorylation of Rac1 protein has been reported, but the functional aspect was never thoroughly investigated, which we thought may carry a potential role in regulating gene

expression. In our study, we demonstrated that Rac1 could translocate to the nucleus speckles post-EGF stimulation and that translocation is dependent on both the T108 motif and the PBR region. While it has been shown that Rac1 can translocate to the nucleus, little is known about the functional significance of this cellular process. Our immunofluorescence data suggests a possible role of Nuclear Rac1 in gene expression as it shows a clear co-localization with certain distinctive splicing and Nucleus speckles markers, hnRNPA1, U2, and SRSF2, respectively.

Interestingly, our co-IP experiments confirmed that both Rac1 and hnRNPa1, SRSF2, and U2snRNP, are co-precipitate in the same complex which could suggest that Rac1 may have a role in the pre- mRNA splicing process in the nucleus. However, the Rac1 T108 A mutant did not pull down these markers, which suggests a decisive role of Rac1 T108 domain phosphorylation in mediating this interaction. The inability to pull down splicing and nuclear speckles markers indicate the requirement of T108 Phosphorylation for the interaction between them and Rac1.

Overexpressing WT Rac1 has resulted in halting of the splicing process and retaining of the introns. However, upon stimulation with EGF, there was no introns retention, and the splicing process efficiently proceeds with overexpression of the band around 400bp that represents the splice variant with full exon 12 included.

The T108A mutant showed a splicing pattern as WT Rac1 without EGF stimulation, whether T108A was stimulated or not. On the other hand, T108E showed overexpression of a 400 bp band, which includes the full sequence of exon 12 and the same pattern of splicing as wtRac1 with EGF stimulation.

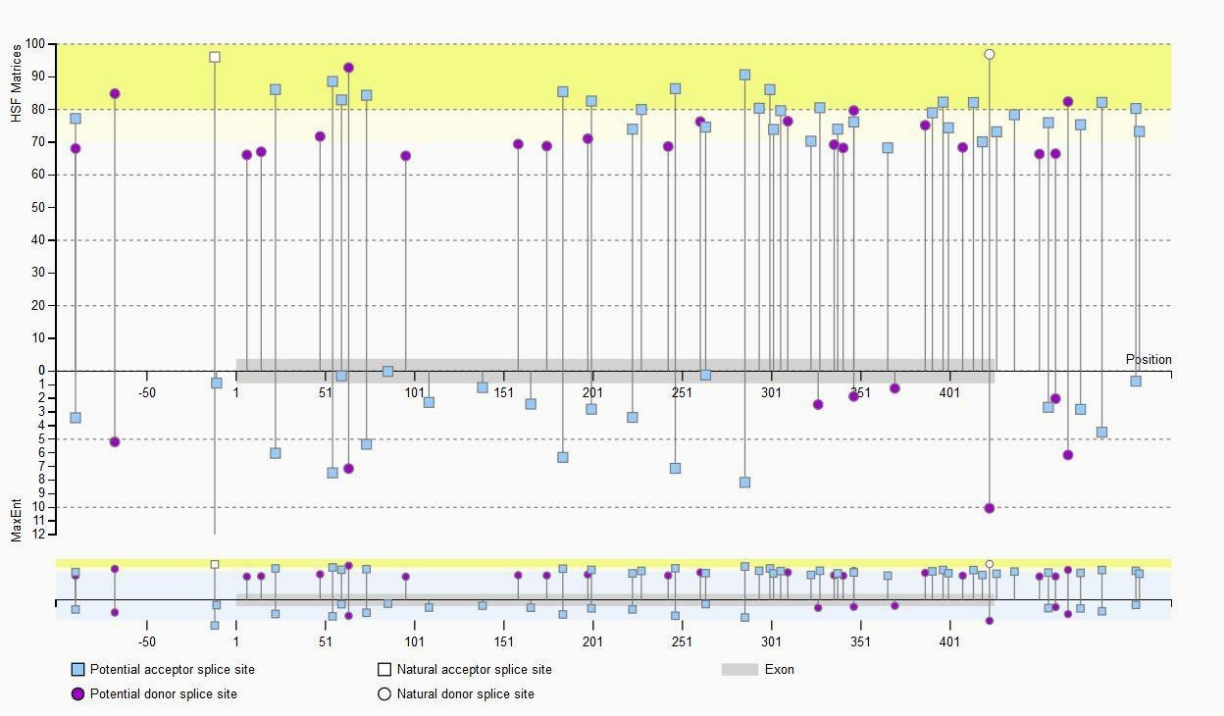
The colocalization interaction and splicing analysis of Rac1 with our reporter gene provide interesting findings and a strong argument for studying the role of this interaction in regulating specific and critical Nuclear functions related to gene expression.

While the significance of Rac1 translocation to the nucleus is not fully understood and requires further research, our data suggest that nuclear Rac1 may have a role in the process of "exon definition" by aiding the cells to choose the appropriate splice sites. The spliceosome can choose

homogenously sized exon sequences using "exon definition." The SR- proteins assemble on the exon sequence to help in the process of marking off each 3' and 5' splice site, starting at the 5' site. Then U1 snRNA and U2 snRNA are recruited to mark the downstream and upstream, respectively. Therefore, the uniform size of the exons increases the cell accuracy in recruiting splicing machinery on nascent RNA avoiding "near miss" splice sites. However, the mechanistic insights behind how SR proteins differentiate between intron and exon sequences are not fully understood[193].

The alternative splicing regulation of E2F7 is quite important for the protein to function correctly as any change in the process affect the reading frame and modify the c-terminus of the protein which is crucial for the transcription factor to activate its target genes, Rac1 overexpression is common in human cancers, and Rac1 changes in cancer may affect E2F7- Dependent cell cycle regulation hindering the anti-proliferative properties of E2F7 [194]. Whether Rac1 interacts directly with the splicing component and whether EGF plays a role in that will require us to perform a sort of " a global analysis" where we incorporate thousands of genes in order to understand the effect of this novel observation such as RASL-seq[1]

A graphical representation of E2F7 exon 12 splicing patterns generated using Human Splicing Finder algorithms



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