NOVEL HEREGULIN-MEDIATED PATHWAYS TO MAMMALIAN TARGET OF RAPAMYCIN COMPLEX 1 ACTIVATION

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NOVEL HEREGULIN-MEDIATED PATHWAYS TO MAMMALIAN TARGET OF RAPAMYCIN COMPLEX 1 ACTIVATION

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Heregulin (HRG) is a growth factor that mediates the activation of ErbB2/ErbB3 receptors. Aberrant signaling of HRG and the ErbB receptors give rise to human cancer. Our laboratory has previously identified mammalian target of rapamycin (mTOR), an essential hub for growth factor and nutrient sensing, as an important intermediate in HRG-signaling. This thesis focuses on the pathways that lead to the activation of mTORC1 (mTOR complex 1) in response to HRG.

First, I identified the importance of mTORC2 signaling to mTORC1 in ErbB2/HRG-mediated cellular transformation in SKBR3 breast cancer cells. mTORC2 was initially identified to play a role in actin cytoskeletal remodeling, but with the discovery of novel mTORC2 targets, mTORC2 has been implicated in cellular functions such as cell proliferation, survival, and metabolism. By utilizing rapamycin and an ATP-competitive inhibitor of mTOR, INK128, I was able to differentiate between mTORC1 and mTORC2 activation by HRG. In HRG/ErbB2-mediated signaling to AKT, mTORC2 is required for the phosphorylation on AKT (S473) and this precedes the activating PDK1 phosphorylation at AKT (T308). AKT phosphorylates TSC2, making TSC2 unable to function on Rheb. Rheb remains in its GTP-bound form and activates mTORC1. By performing a Rictor knock-down, which decreased mTORC2 availability in the cell, the HRG-mediated transforming capability of SKBR3 cells was reduced.

Next, I took a mechanistic approach to identify how small GTPases, namely Rheb, Rac, and

Cdc42, are playing a role in HRG-mediated mTORC1 activation. Using a knock-down and rescue approach, I was able to delineate that Rac and Cdc42 are upstream of Rheb, and that they signal independently of one another to mTORC1 in this context. Additionally, I found that Dock7, a GEF for Rac and Cdc42, serves as a unique scaffold for the G-proteins and mTORC1. The most intriguing finding, however, is that Dock7 also possesses properties of a Rheb GEF. It has long been hypothesized that only a GAP is needed for the regulation of Rheb, so the identification of a putative Rheb GEF is of significant interest to the field.

BIOGRAPHICAL SKETCH

The author was born in Taipei, Taiwan to a family of five. When she was 10 years old, the family moved to Eugene, Oregon in order for her father to pursue his dream of getting a Ph.D. and becoming a professor in economics. It was a struggle at the beginning, since the only English phrase she knew how to say was, "I don't know". But thankfully over the four years in Eugene, her English improved. She made friends, joined two orchestras, and even managed to make it onto the math team. It was during the time spent in Eugene that she fell in love with the sciences and the experience may have planted the seed causing her to come back to the United States. The move back to Taiwan was another interesting journey when the joint high school entrance exam was a year away and the author crammed four years of forgotten Mandarin, history, geography, all into one year of study. She insisted on entering Taipei Municipal Neihu Senior High School, a school known to cultivate freedom and independent-thinking. It also meant she had to spend two years living away from her family, as they were living in central Taiwan at the time. However, she learned to enjoy the freedom and independence and the weekends roaming around book stores. After graduating from high school, she went through another round of entrance exams and entered National Taiwan University. She knew she wanted to study basic science but was not sure whether to major in math, physics, or chemistry. She is grateful that she ended up in the Chemistry Department and later found her love in the life sciences. She was fascinated by the fact that all life forms are giant machines with countless chemical reactions. She did her undergraduate research with Dr. Inn-Ho Tsai at the Institute of Biological Chemistry, Academia Sinica, Taiwan, on serine proteases found in rattlesnake venom. After graduating from college, she wanted to affirm her interest in doing research and took a position as research assistant at the

President's Laboratory, National Health Research Institutes in Taiwan (NHRI), under the guidance of Dr. Ken Chen-Wen Wu and Dr. Wen-Shaing Wayne Chang. For two years she worked on proteases and protease inhibitors involved in tumor invasion and metastasis. Working at the NHRI strengthened her desire to pursue an advanced degree and also widened her perspective of biomedical research. She entered the field of Biochemistry, Molecular and Cell Biology at Cornell University in the fall of 2005 and eventually joined Dr. Richard Cerione's Laboratory in 2006. The Ph. D. has truly been a journey of not only scientific-discovery but also an unexpected journey of self-discovery.

To my family, especially my parents

美妙長遠，

美妙發達，

美妙的事物永遠聚集在一起

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Joe Druso, my best friend and partner, who kept my spirits up when it felt like the end of the world. Thank you for all the encouragement and positive thinking. I couldn't have done this without you.

And lastly, my family, for giving me unconditional love and support, letting me know they will always be on my side no matter what I decide to do.

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LIST OF ABBREVIATIONS

4E-BP1	eIF-4E binding protein 1
AKT	Thymoma viral proto-oncogene, also known as protein kinase B
ADP	Adenosine-5’-diphosphate
AGC family	Protein kinase A, protein kinase G, protein kinase C family
AMP	Adenosine-5’-monophosphate
AMPK	AMP kinase
Arf	ADP-ribosylation factor
Atg13	Autophagy-related 13
ATP	Adenosine-5’-triphosphate
CBC	Nuclear cap-binding complex
Cbl	Casitas B-lineage lymphoma
Cdc42	Cell division cycle 42
cDNA	Complementary DNA
Cip1	Cyclin-dependent kinase inhibitor 1A
Cool-1	Cloned out of library 1
Dbl	Diffuse B-cell lymphoma
Deptor	DEP domain-containing mTOR-interacting protein
DH	Dbl-homology
DHR1/2	Dock-homology region 1/2
DMSO	Dimethyl sulfoxide
Dock	Dedicator of cytokinesis

ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
eIF3	Eukaryotic translation initiation factor 3
eIF4E	Eukaryotic translation initiation factor 4E
ErbB	Erythroblastic leukemia viral oncogene
ERK	Extracellular signal-regulated kinase
FBS	Fetal bovine albumin
FKBP12	12 kDa FK-506 binding protein
FKBP38	FK506-binding protein 38
FRB	FKBP12-rapamycin binding
GAP	GTPase activating protein
GDP	Guanosine-5’-diphosphate
GEF	Guanine nucleotide exchange factor
GTP	Guanosine-5’-triphosphate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HER2	Human epidermal growth factor 2
HRG	Heregulin
hVPS34	Human vacuolar protein sorting 34
hVPS39	Human vacuolar protein sorting 39
IP	Immunoprecipitation
MEF	Mouse embryonic fibroblast

mLST8	Mammalian lethal with SEC13 protein 8
mSin1	Mammalian stress-activated protein kinase interacting protein
mTOR	Mammalian target of rapamycin
mTORC1/C2	Mammalian target of rapamycin complex 1/2
P-REX1	Phosphatidylinositol-3,4,5-triphosphate-dependent Rac-GEF
PA	Phosphatidic acid
PBD	P21-binding domain of PAK
PC	Phosphatidylcholine
PDK1	Phosphoinositide-dependent kinase 1
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PIKK	Phosphatidylinositol-4,5-bisphosphate 3-kinase-like kinase
PIP2	Phosphatidylinositol-4,5-bisphosphate
PIP3	Phosphatidylinositol-3,4,5-triphosphate
PH	Pleckstrin-homology
PLD1	Phospho-lipase D1
PKC	Protein kinase C
PRAS40	Proline-rich AKT substrate 40 kDa
Pten	Phosphatase and tensin homolog
Rab	Ras-related proteins in brain
Rac	Ras-related C3 botulinum toxin substrate
Rag	Ras-related GTP binding
Ral	Ras-like protein
Ran	Ras-related nuclear protein

Raptor	Regulatory associated protein of mTOR
Ras	Rat sarcoma
Rheb	Ras-homology enriched in brain
Rho	Ras-homology
Rictor	Rapamycin-insensitive companion of mTOR
rpS6	Ribosomal S6 protein
RTK	Receptor tyrosine kinase
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SGK	Serum- and glucocorticoid-induced protein kinase
shRNA	Short-hairpin RNA
siRNA	Small-interfering RNA
SREBP	Sterol-regulatory-element binding protein
TCTP	Translationally controlled tumor protein
TSC1/TSC2	Tuberous sclerosis complex 1/2
ULK1/2	Unc-51-like kinase 1/2
WCL	Whole cell lysate

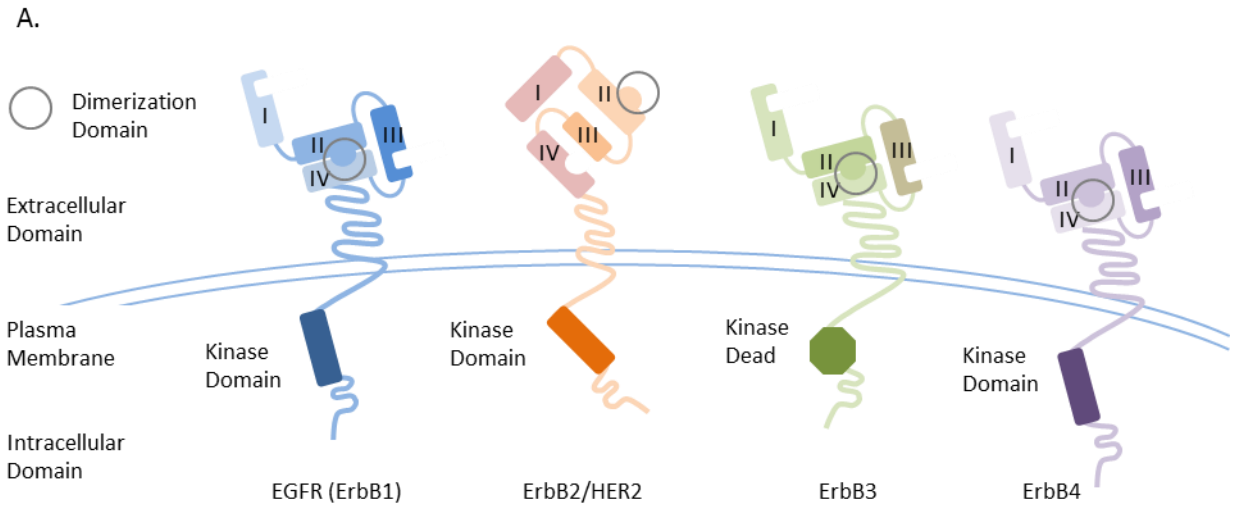
Chapter 1

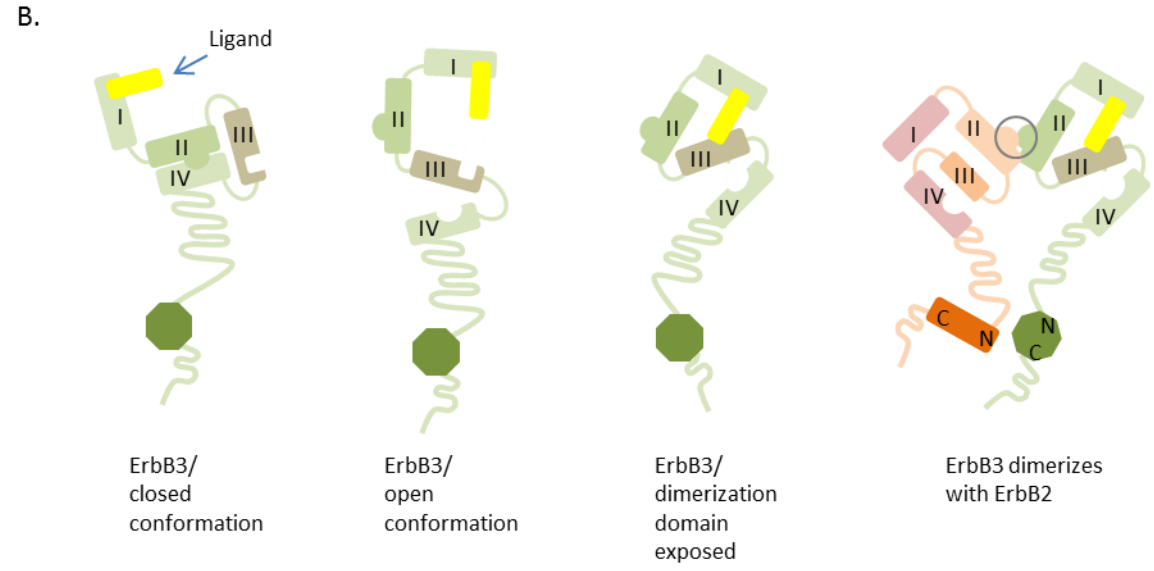
Introduction

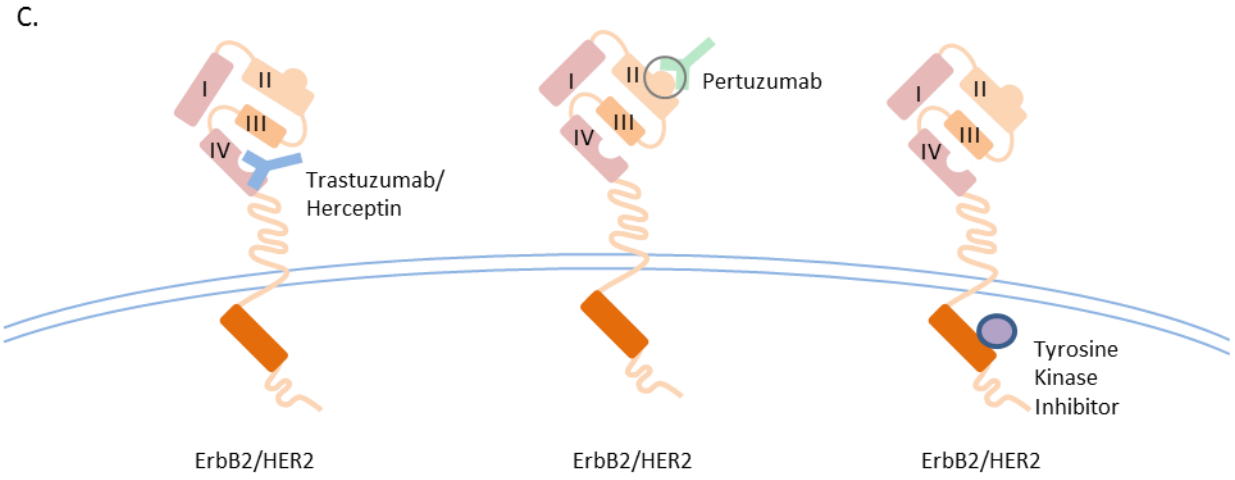
ErbB2/HRG Signaling in Cancer

Growth factor receptor tyrosine kinases (RTKs) have been under extensive study due to the fact that anomalies either in the regulation of the growth factors that signal through these receptors or in the receptors themselves can give rise to human cancer [1]. The EGF (epidermal growth factor) family of receptor tyrosine kinases includes four members, EGF receptor/ErbB1, ErbB2/HER2, ErbB3 and ErbB4 (Figure 1.1A). This family of RTKs signals by forming homo- or heterodimers with other members of the family [1] (Figure 1.1B). One growth factor-induced signaling pathway of particular interest to our laboratory is the heregulin (HRG)-stimulated activation of ErbB2 through the initial binding of HRG to either ErbB3 or ErbB4 and its subsequent heterodimerization with ErbB2 [2, 3]. What is most interesting about ErbB2-ErbB3 heterodimers is that ErbB2 has no known ligand and ErbB3 is incapable of kinase activity [4]. However, the ErbB2-ErbB3 heterodimer gives rise to the most potent signaling among the ErbB family members in terms of cell growth and transformation [5, 6]. Over-expression of ErbB2 is found in 20-30% of breast and ovarian tumors, and this is correlated with poor prognosis and

Figure 1.1 The ErbB family of receptor tyrosine kinases. A. The ErbB family of receptor tyrosine kinases consists of four members, epidermal growth factor receptor (EGFR/ErbB1), ErbB2/HER2, ErbB3, and ErbB4. Each is comprised of an extracellular domain for ligand binding (i.e. EGF, HRG), a transmembrane domain, and an intracellular kinase domain. ErbB2 is an orphan receptor and has no known ligand whereas ErbB3 lacks kinase activity. **B.** The ErbB receptors are activated through the formation of homo- or heterodimers. Depicted in this diagram is ErbB3 binding to a ligand (e.g. HRG) which then opens up the dimerization domain of the receptor, allowing it to heterodimerize with ErbB2. This allows the autophosphorylation of ErbB2 and also the transphosphorylation of ErbB3 by ErbB2, enabling the transmission of downstream signals. **C.** Examples of ErbB2 inhibitors used as anti-cancer reagents. Trastuzumab/Herceptin, a monoclonal antibody targeting the ErbB2 receptor, suppresses ErbB2 signaling activity, prevents cleavage of the extracellular domain and targets the cancer cells that over-express ErbB2 for immunological attack. Pertuzumab is also a monoclonal antibody, however, it specifically targets the dimerization domain of ErbB2 preventing the formation of ErbB2-ErbB3 heterodimers, and thus preventing signaling by ErbB2-ErbB3. Various tyrosine kinase inhibitors have been developed to inhibit ErbB2-dependent signaling (e.g. Lapatinib). This figure is adapted from Baselga et al. [7]


A.
Dimerization Domain
Extracellular Domain
Plasma Membrane
Intracellular Domain
Kinase Domain
Kinase Domain
Kinase Dead
Kinase Domain
EGFR (ErbB1)
ErbB2/HER2
ErbB3
ErbB4


B.
Ligand
ErbB3/ closed conformation
ErbB3/ open conformation
ErbB3/ dimerization domain exposed
ErbB3 dimerizes with ErbB2


C.
Trastuzumab/ Herceptin
Pertuzumab
Tyrosine Kinase Inhibitor
ErbB2/HER2
ErbB2/HER2
ErbB2/HER2

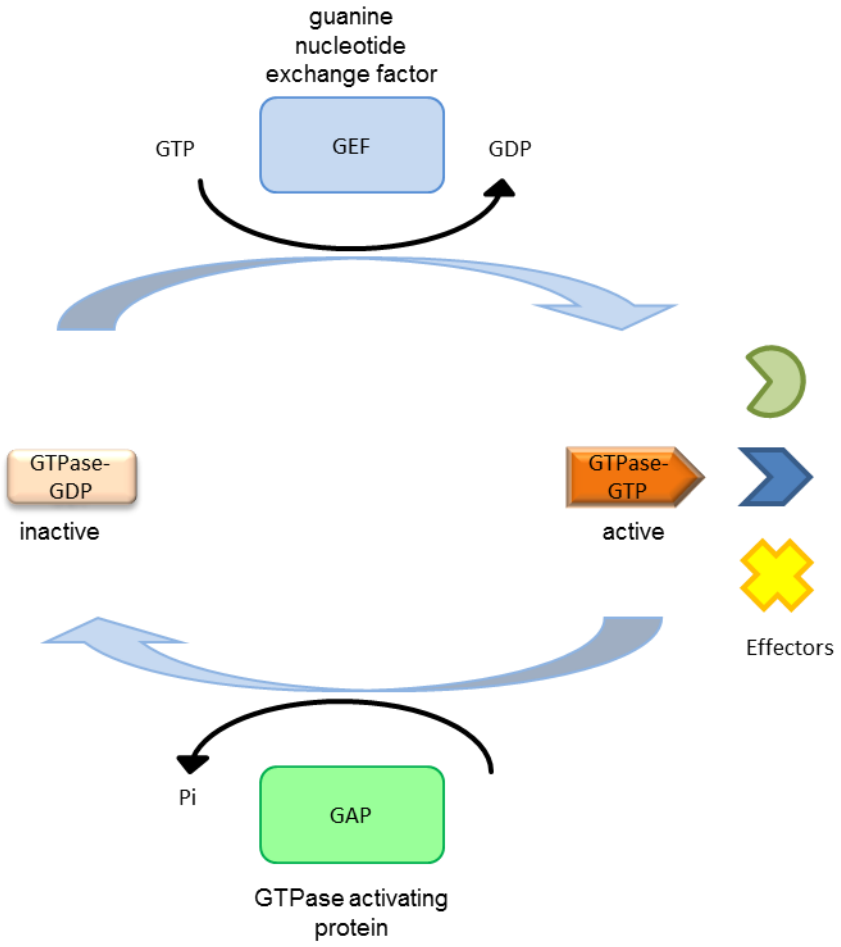
tumor chemoresistance in these patients [8-10]. Moreover, HRG is also found to be over-expressed in breast, ovarian, and prostate cancers (reviewed in [11]) and can drive cellular transformation by the activation of ErbB2, independent of the expression status of the receptor [12]. Inhibitors of ErbB2, such as trastuzumab (also known as Herceptin, a monoclonal antibody that recognizes the ErbB2 receptor) and Lapatinib (a tyrosine kinase inhibitor) have been developed as anti-cancer agents [7] (Figure 1.1C). More recently, however, many ErbB2-overexpressing breast cancers have been shown to exhibit resistance to such therapy [13, 14]. Therefore, understanding the molecular mechanisms responsible for ErbB2/HRG-signaling, and identifying novel targets in the ErbB2 pathways, could yield strategies for overcoming the resistance to standard anti-ErbB2/HRG therapies.

Previous studies performed in our laboratory have established that HRG can stimulate both cell growth and cellular differentiation, thus distinguishing it from EGF, whose interaction with the EGFR primarily results in mitogenic signaling [15]. Furthermore, HRG is able to signal though an mTOR- (mammalian target of rapamycin) and S6 kinase-dependent pathway to alter the RNA splicing and transport properties of the nuclear cap-binding complex (CBC) and Ran, a nuclear small GTPase [16, 17].

Ras-like GTPases

When considering the architecture of cellular signaling pathways, it becomes important to understand the structure and function of a class of molecular switches, namely, the members of the super-family of Ras-like GTPases (also known as small GTPases or small G-proteins). The Ras super-family consists of 5 subfamilies: Ras, Rho, Arf, Rab, and Ran [18]. They are capable of binding guanine nucleotides, and cycle between an inactive GDP-bound state and an active GTP-bound state [18] (Figure 1.2). The small GTPases can bind to specific regulators or effectors in their inactive and active states, thus transmitting cell signals from cell-cell contacts, growth factors, and the extracellular matrix [19-21]. Ras-like GTPases are activated by guanine nucleotide exchange factors (GEFs) [22]. These regulatory proteins, upon binding to the inactive forms (GDP-bound) of the GTPase in response to a stimulus, destabilize the binding of GDP, allowing GTP (which is at a higher concentration in the cell) to gain access to the nucleotide binding pocket of the small G-protein, rendering it active [22]. Small G-proteins have intrinsic GTP-hydrolytic activity, but GTPase activating proteins (GAPs) can greatly accelerate the hydrolysis of GTP to GDP [23]. Of particular interest in our laboratory are the Rho family of GTPases, specifically Cdc42 and Rac, as our laboratory was the first to clone Cdc42 and discover Cool-1/Cool-2 (cloned out of library), a Cdc42/Rac GEF [24-26]. Also, Cdc42 and Rac have been implicated in mTOR signaling downstream of HRG (unpublished data).

Figure 1.2 The GDP/GTP cycle of small GTPases. Small GTPases in their inactive state are bound to GDP. Upon stimulation by extracellular cues, such as growth factors, guanine nucleotide exchange factors (GEFs) facilitate the release of GDP. GTP, which is 10-fold higher in concentration than GDP in the cell, will then occupy the nucleotide binding pocket, rendering the GTPase active. The GTP-bound small G protein undergoes conformational changes which enables it to bind to downstream effectors. The GTPase activating protein (GAP) accelerates the hydrolysis of GTP to GDP, returning the GTPase back to its inactive state.


guanine
nucleotide
exchange factor
GEF
GTP
GDP
GTPase-
GDP
inactive
GTPase-
GTP
active
Effectors
Pi
GAP
GTPase activating
protein

Rho GTPases have been implicated in many cellular functions such as cytoskeleton remodeling and vesicular trafficking [27, 28]. Additionally, Cdc42 is known to be activated in response to EGF and has been found to play a role in EGFR homeostasis (reviewed in [28]). The activated EGFR signals downstream to Src, which phosphorylates Cool-1, a GEF for Cdc42, thus activating Cdc42. The activated Cool-1/Cdc42 complex then sequesters Cbl, an adaptor protein that exhibits E3 ubiquitin ligase activity, from binding to the EGFR. Once Cdc42 is inactivated by GTP hydrolysis, the Cdc42-Cool-1-Cbl complex dissociates, allowing Cbl to then catalyze EGFR ubiquitination, targeting it for degradation upon endocytosis [29, 30]. Cool-1 highlights an intriguing role for certain GEFs: Not only can they act as activators, but also as effectors for their target small GTPases. More examples (i.e. Dock11) of this dual function will be discussed later in this chapter.

HRG is more potent than EGF in its ability to activate Rac in the breast cancer cell lines MCF-7 and T-47D [31]. The HRG-mediated Rac activation in these cells is PI3K-dependent and signals through ERK to control cell proliferation via the cell cycle regulators cyclin D1 and p21Cip [31, 32]. Moreover, the phosphatidylinositol 3,4,5-trisphosphate-dependent Rac-GEF (P-REX1) has been found to be over-expressed in certain breast cancers and can regulate Rac activation downstream of HRG signaling [33-35]. P-REX1 has also been shown to be activated through mTORC2 (mTOR complex 2) to promote Rac activation and cell migration [36].

mTOR

The mammalian target of rapamycin (mTOR) is a member of the PI-3 kinase-like kinase family (PIKK) and forms two functionally distinct complexes, mTORC1 and mTORC2 [37-39]. mTORC1 is involved in numerous cellular processes such as cell proliferation, lipid biogenesis, autophagy, and metabolism [37-42]. It is now highly recognized to be a master regulator of a number of cellular functions, being activated in response to the nutrient and energy status of the cell and also by growth factors [37-39]. mTORC2, a more recently identified mTOR complex, has been implicated in cytoskeletal remodeling and more recently to be involved in cellular transformation [43-45]. The cellular regulation of the mTOR complexes is critical as their aberrant activation can lead to cancer progression [38, 46, 47].

mTORC1

mTORC1 consists of Raptor, PRAS40, mLST8, and Deptor. Raptor (Regulatory associated protein of mTOR) is an mTORC1-specific scaffold protein and has been implicated in recruiting downstream effectors of mTOR (i.e. S6 kinase and 4E-BP1) [48-50]. PRAS40 (proline-rich AKT substrate 40 kDa) negatively regulates mTORC1, and this inhibition can be released by AKT-catalyzed phosphorylation and subsequent binding of the scaffolding protein, 14-3-3 [51]. The function of mLST8 (mammalian lethal with SEC13 protein 8) in mTORC1 still remains elusive [52], whereas Deptor (DEP domain-containing mTOR-interacting protein) appears to

function as a negative regulator of mTORC1 kinase activity [53].

The discovery of rapamycin, an anti-fungal agent and immuno-suppressant found in the bacteria *Streptomyces hygroscopicus* from soil samples on Rapa Nui (Easter Island), contributed greatly to our initial understanding of mTORC1 function [54, 55]. Rapamycin works as an allosteric inhibitor which interacts with FKBP12 (12 kDa FK-506 binding protein) and the FRB (FKBP12-rapamycin binding) domain of mTOR to bring FKBP12 and mTOR together [54, 56]. Once the FKBP12-rapamycin complex binds to mTOR, it destabilizes the binding of mTOR to Raptor, thus disrupting the recruitment of mTORC1 targets [57]. Raptor is not present in mTORC2, therefore, mTORC2 has often been regarded as the rapamycin-insensitive mTOR complex [43, 44].

Upstream of mTORC1: Regulation by growth factors, amino acids, and energy status of the cell

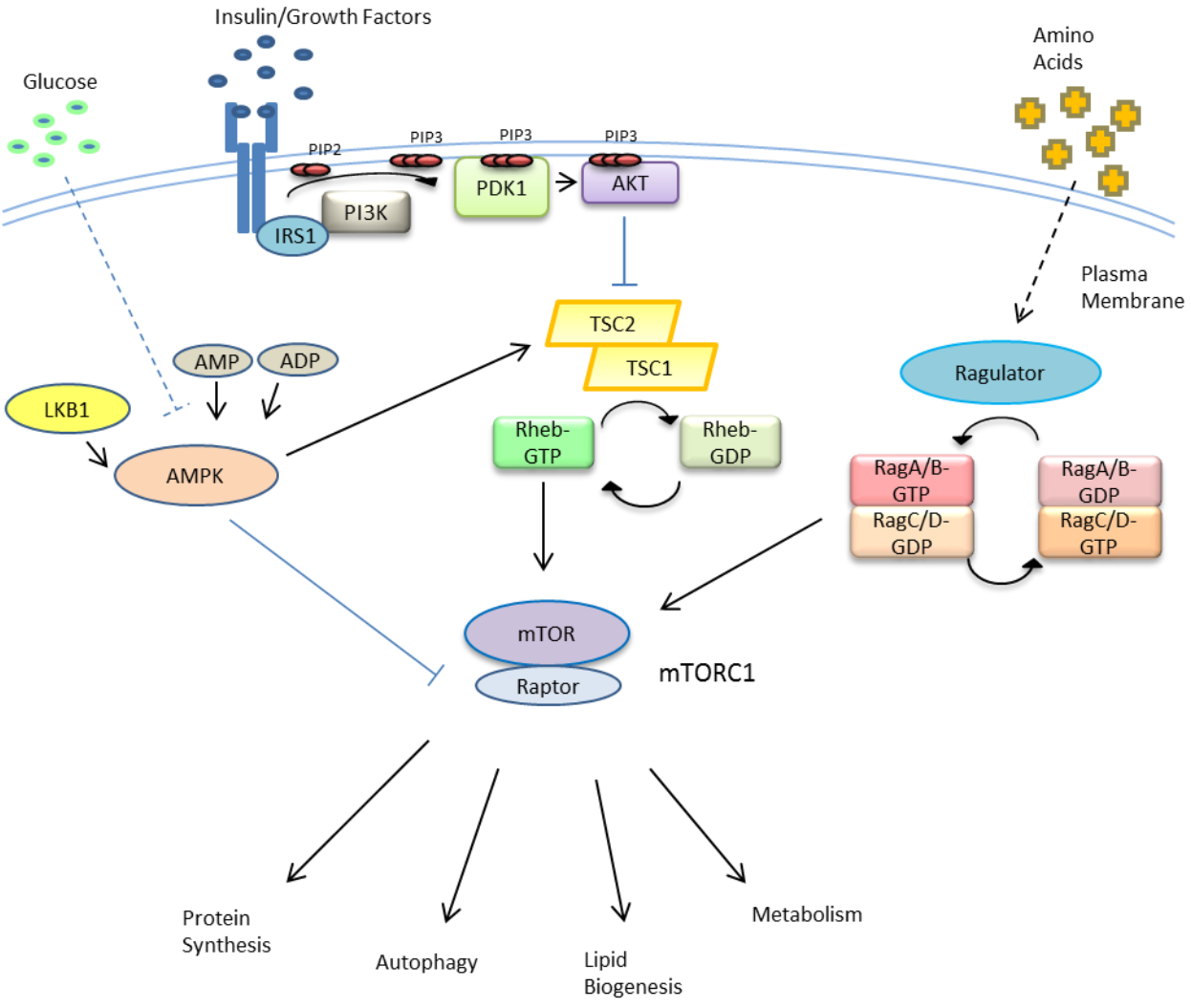
The best-studied growth factor-stimulated pathway leading to the activation of mTORC1 is insulin-mediated signaling, which is often regarded as the canonical pathway [37, 46, 58]. Insulin signals through PI3K to catalyze the conversion of PIP2 to PIP3 [37, 46, 58]. PDK1 (phosphoinositide-dependent kinase 1) is then recruited to the plasma membrane by PIP3, where it phosphorylates AKT at threonine 308 (reviewed in [59]). AKT achieves maximal activation when it is phosphorylated on both threonine 308 in its activation loop and serine 473 within its

hydrophobic motif [60]. Once activated, AKT phosphorylates multiple sites on TSC2 (tuberous sclerosis complex 2), a GAP for the small GTPase Rheb (reviewed in [61]). Phosphorylation of TSC2 inhibits its ability to associate with TSC1 at the endo-membrane where Rheb resides, thereby preventing TSC2 from turning off Rheb [62]. Rheb-GTP binds and activates mTORC1, although the molecular basis for this activation remains poorly defined [63] (Figure 1.3).

Amino acid sensing provides another important input toward mTORC1 activation. Previous studies have shown that growth factor signaling to mTORC1 requires the presence of amino acids [64-66]. Various mechanisms have been proposed for how amino acids can regulate the activation of mTORC1. One involves the hVps34 (human vacuolar protein sorting 34) protein, a class III PI3K [67, 68]. The evidence for the involvement of hVps34 in amino acid sensing has been indirect [67, 68]. However, PLD1 (phospho-lipase D1) has been shown to bind to hVps34 on the lysosomal membrane, where Rheb resides. PLD1 is responsible for the production of phosphatidic acid which has been suggested to promote the activation of mTORC1 [69].

The identification of Rag proteins greatly advanced the understanding of how amino acids signal to mTORC1 [64]. Rag proteins are a unique class of small GTPases existing in heterodimeric forms in the cell [64]. They localize at the lysosomal membrane and, upon stimulation with amino acids, recruit mTORC1 through a protein complex called the ‘Ragulator’, allowing mTORC1 to be activated by Rheb on the lysosome [70-72] (Figure 1.3). The details of

Figure 1.3 Upstream signaling to mTORC1. mTORC1 can be activated by several extracellular signals such as growth factors, amino acids, and glucose. Insulin-mediated mTORC1 activation is PI3K-dependent. Amino acids activate mTORC1 through a Rag-dependent mechanism. Glucose levels, or the cellular energy status as indicated by AMP and ADP levels, can be sensed through AMP kinase (AMPK). Details of the pathways are described in the text.


Insulin/Growth Factors
Glucose
Amino Acids
PIP2
PIP3
PIP3
PIP3
PDK1
AKT
PI3K
IRS1
Plasma Membrane
TSC2
TSC1
AMP
ADP
LKB1
AMPK
Rheb-GTP
Rheb-GDP
Ragulator
RagA/B-GTP
RagC/D-GDP
RagA/B-GDP
RagC/D-GTP
mTOR
Raptor
mTORC1
Protein Synthesis
Autophagy
Lipid Biogenesis
Metabolism

how Rag proteins regulate mTORC1 will be discussed later in this chapter.

mTORC1 senses the energy status of the cell through AMP-activated kinase (AMPK) [73, 74]. ATP is produced from cellular processes such as glycolysis and the tri-carboxylic acid cycle, ultimately being broken down to ADP and AMP. AMPK contains nucleotide binding pockets for AMP/ADP, thereby allowing it to sense the increase in AMP/ADP, and the need for more ATP production [73]. AMPK activation results in a phosphorylation on TSC2 which augments TSC2 function and thus suppresses mTORC1 activity [75]. AMPK can also phosphorylate the mTORC1 scaffold, Raptor, to inhibit mTORC1 from phosphorylating its downstream substrates [76] (Figure 1.3).

Downstream of mTORC1: Cellular effects of mTORC1 (Protein synthesis, Lipid biogenesis, Autophagy)

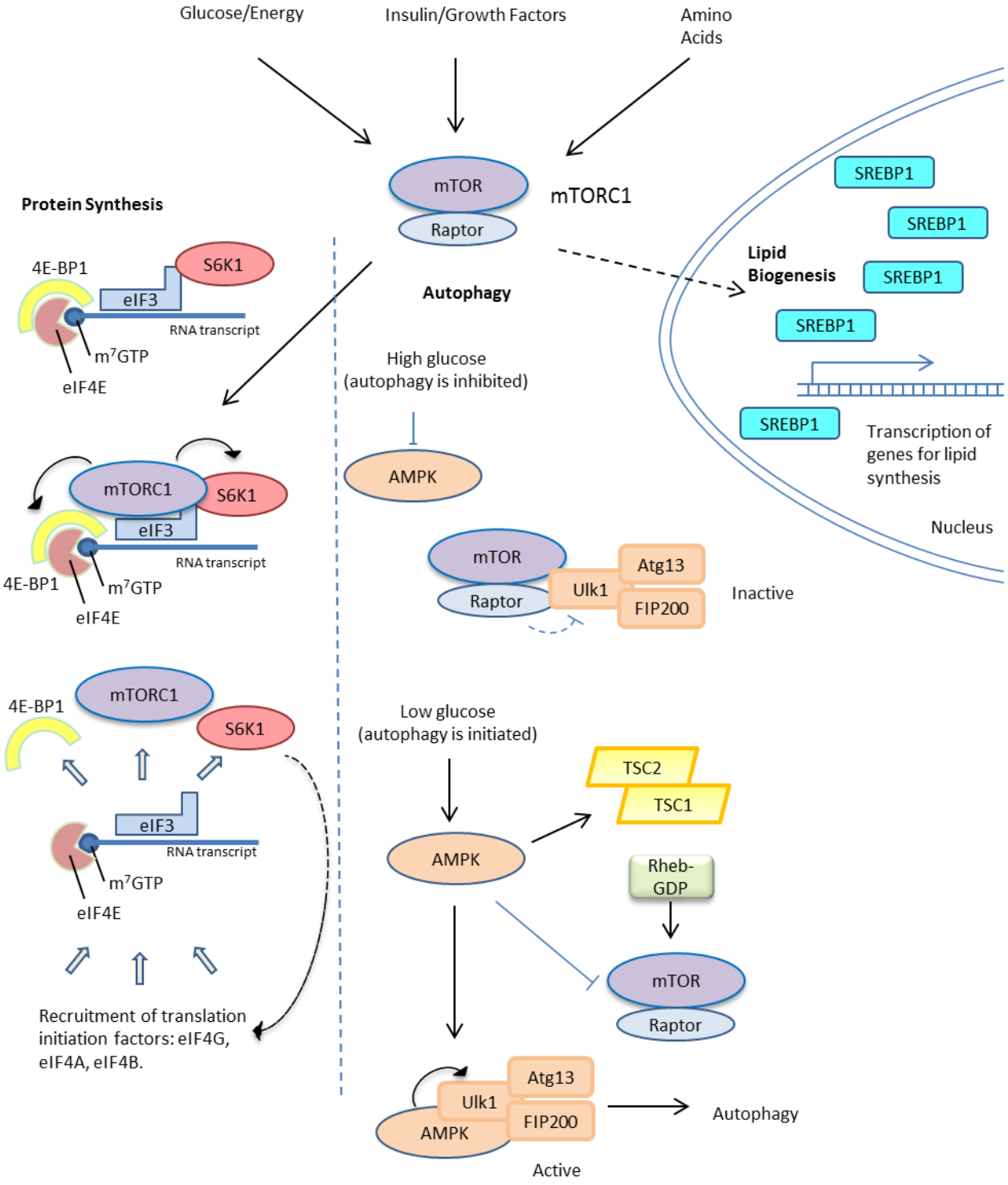
The best-characterized downstream effectors of mTORC1 are S6 kinase and 4E-BP1 [77, 78]. Since both proteins play major roles in protein synthesis, this is the area of mTORC1 signaling that has been extensively explored [39, 77, 78]. mTORC1 phosphorylates S6 kinase on T389, which together with the phosphorylation of T229 by PDK1, renders S6 kinase active [78]. For over a decade, ribosomal S6 protein (rpS6) was the only known substrate for S6 kinase, however, how the phosphorylation of rpS6 promotes protein synthesis is still unclear [78]. Numerous other substrates for S6 kinase have since been identified, and have provided insight

into the individual steps involved in protein translation [78]. One way that mTORC1-S6 kinase regulates protein synthesis is through the dynamic interaction with the eIF3 (eukaryotic translation initiation factor 3) scaffold of the translation initiation complex [79]. S6 kinase forms a stable complex with eIF3 when inactive [79]. Upon mTORC1 activation, the S6 kinase-eIF3 complex is recruited to mTORC1 and the phosphorylation of S6 kinase on T389 causes a dissociation of eIF3 from S6 kinase [79]. mTORC1 subsequently binds to eIF3 which can then interact with eIF-4E (the cytoplasmic cap-binding protein), bringing the mTORC1-eIF3 complex in close proximity to another mTORC1 substrate, 4E-BP1 (eIF-4E binding protein 1) ([79], reviewed in [77]). Phosphorylation of 4E-BP1 releases its inhibition of eIF-4E (eukaryotic translation initiation factor 4E), allowing eIF-4E to recruit other translation initiation factors to start protein synthesis [77, 80] (Figure 1.4).

mTORC1 regulates lipogenesis through SREBPs (sterol-regulatory-element binding proteins) that function as transcription factors to up-regulate the expression of genes required for fatty acid and cholesterol synthesis [41, 81]. It is still unclear if mTORC1 can directly phosphorylate SREBPs to alter either their binding properties or localization in the cell [81]. However, rapamycin inhibits the nuclear accumulation of SREBPs and also the transcription of SREBP-dependent genes [82] (Figure 1.4).

Autophagy, a process of recycling intracellular components, is initiated to maintain cellular

Figure 1.4 Cellular effects of mTORC1. Shown here are three cellular outcomes downstream of mTORC1. Two important targets of mTORC1 in regulating protein synthesis are S6 kinase and 4E-BP1. mTORC1 can regulate the initiation of autophagy in concert with AMPK activation. mTORC1 has also been shown to modulate lipid biogenesis through the sterol-regulatory-element binding proteins (SREBPs). Details of these are described in the text. This figure is a compilation of [77, 82, 83].


Glucose/Energy
Insulin/Growth Factors
Amino Acids
mTOR
Raptor
mTORC1
Protein Synthesis
4E-BP1
S6K1
eIF3
RNA transcript
m7GTP
eIF4E
Autophagy
High glucose
(autophagy is inhibited)
AMPK
mTOR
Raptor
Ulk1
Atg13
FIP200
Inactive
SREBP1
SREBP1
SREBP1
SREBP1
SREBP1
Lipid Biogenesis
Transcription of genes for lipid synthesis
Nucleus
mTORC1
S6K1
eIF3
RNA transcript
4E-BP1
m7GTP
eIF4E
mTORC1
4E-BP1
S6K1
eIF3
RNA transcript
m7GTP
eIF4E
Recruitment of translation initiation factors: eIF4G, eIF4A, eIF4B.
Low glucose
(autophagy is initiated)
AMPK
TSC2
TSC1
Rheb-GDP
mTOR
Raptor
Atg13
Ulk1
FIP200
AMPK
Active
Autophagy

homeostasis when a cell is deprived of nutrients or energy [84, 85]. mTORC1 has been shown to have a key role in the initiation of autophagy [42, 86]. mTORC1, along with AMPK, coordinates the phosphorylation of ULK1/2 (unc-51-like kinase), a serine/threonine kinase acting as an initiator of autophagic processes [83]. Under nutrient-sufficient conditions, mTORC1 phosphorylates ULK1/2 and Atg13 (an accessory protein stably bound to ULK1/2 in mammalian cells), thus suppressing ULK1/2 kinase activity and inhibiting autophagy [83]. Under starvation conditions, these sites on ULK1/2 and Atg13 are quickly dephosphorylated through unknown phosphatases, causing the dissociation of ULK1/2-Atg13 from mTORC1 [83]. Once this dissociation occurs, AMPK can then phosphorylate ULK1/2 to initiate autophagy [73, 83, 87] (Figure 1.4).

mTORC2

mTORC2 consists of Rictor, mLST8, mSin1, Protor, and Deptor [37-39, 47]. Rictor (rapamycin-insensitive companion of mTOR) and mSin1 (mammalian stress-activated protein kinase interacting protein) are necessary for mTORC2 kinase activity and, therefore, are often used as targets for genetic manipulation to study mTORC2-specific functions [43, 44, 52, 88, 89]. mLST8 and Deptor are components found in both mTORC1 and mTORC2, however, knock-down of mLST8 expression only affects mTORC2 function but not mTORC1 [52]. Deptor is an inhibitor of both mTORC1 and mTORC2 activity [53]. The function of Protor

(protein observed with Rictor) is not yet defined.

Upstream of mTORC2

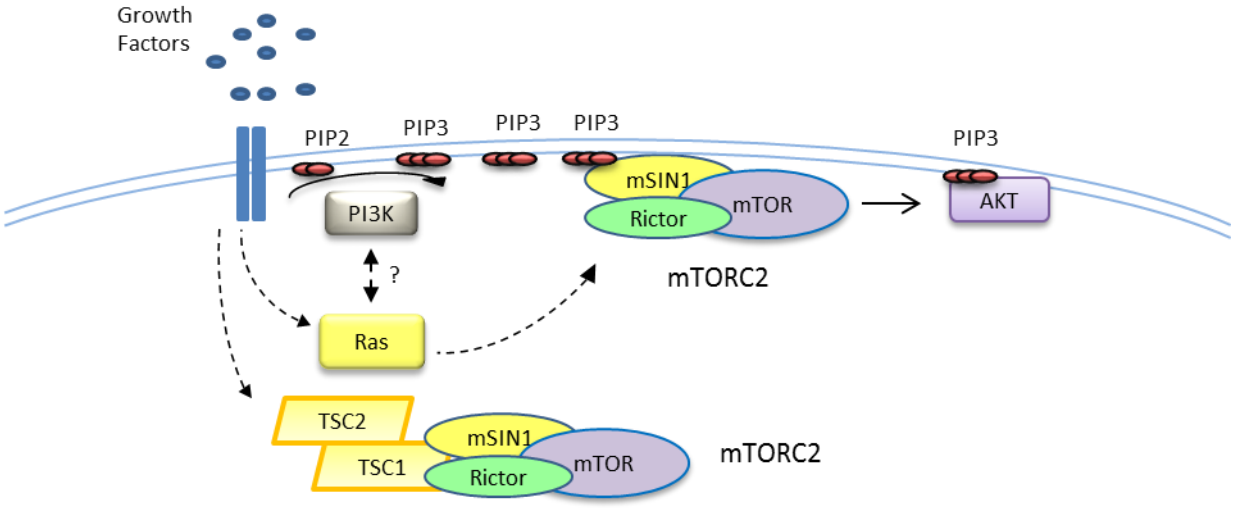
Since the discovery of mTORC2 is relatively recent, the upstream regulators of mTORC2 are still under investigation. It has been suggested that PI3K can activate mTORC2 in response to growth factors [89-91]. PIP3 has been shown to directly activate mTORC2 kinase activity *in vitro* and *in vivo* [92]; in addition, the PH domain of mSin1 binds to PIP3 which recruits mTORC2 into close proximity to its substrate, AKT [93, 94]. Ras has also been shown to interact with mSin1 [93], however, it is still unclear whether there is some degree of cross-talk between PI3K and Ras in the regulation of mTORC2 function. Interestingly, the negative regulator for mTORC1, the TSC1/2 complex, is necessary for mTORC2 activity [95]. TSC1/2 has been shown to interact with mTORC2 and regulates mTORC2 function independently of TSC2 GAP activity [95]. These findings are summarized in Figure 1.5A.

mTORC2 targets

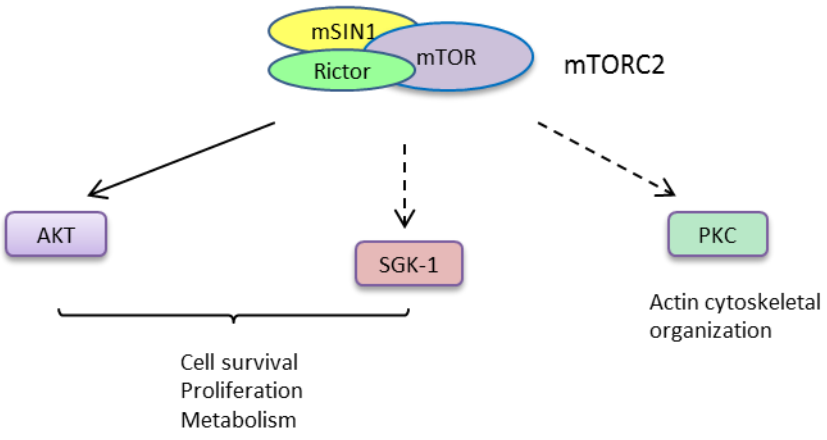
AKT was the first direct kinase target identified for mTORC2, with S473 on the hydrophobic motif of AKT being the site of phosphorylation [96]. This provided a great deal of insight into how AKT is regulated. There are two critical phosphorylation sites on AKT, Threonine 308 and Serine 473, and AKT achieves maximal activation when both sites are phosphorylated [60]. Before the identification of mTORC2 as the bona fide kinase for

Figure 1.5 Up- and downstream of mTORC2. **A.** Upstream regulators of mTORC2. Growth factors, namely insulin, have been shown to activate mTORC2 through a PI3K-dependent mechanism. The direct addition of PIP3 also activates mTORC2. TSC1/TSC2 shows direct binding to mTORC2 and is required for the activity of mTORC2. **B.** Downstream effects of mTORC2. This figure is adapted from [97].

A.


Growth
Factors
PIP2
PIP3
PIP3
PIP3
PIP3
PI3K
?
mSIN1
Rictor
mTOR
mTORC2
AKT
Ras
TSC2
TSC1
mSIN1
Rictor
mTOR
mTORC2

B.


mSIN1
Rictor
mTOR
mTORC2
AKT
SGK-1
PKC
Actin cytoskeletal
organization
Cell survival
Proliferation
Metabolism

AKT (S473), PDK1 was thought to be responsible for phosphorylating both T308 and S473, and the two phosphorylation sites have often been used interchangeably to read out AKT activation [47]. The discovery of mTORC2 has added another dimension to the regulation of AKT and its role in cell proliferation and survival [98, 99]. This also provides an intriguing possibility for cross-talk between mTORC2 and mTORC1, given that AKT can act upstream of mTORC1 and downstream of mTORC2 [47].

Other kinase targets of mTORC2 include the SGK proteins (serum- and glucocorticoid-induced protein kinase) and PKC (protein kinase C) [52, 90, 100, 101]. These, along with AKT, all belong to the AGC family of protein kinases [47]. The direct phosphorylation of SGK1 (S422) by mTORC2 activates SGK1 [90]. It has been shown that various cancer cell lines with PI3K-activating mutations rely on SGK proteins instead of AKT to confer their oncogenic properties [102, 103]. There is still no *in vitro* evidence that the phosphorylation of PKC is directly catalyzed by mTORC2. However, mTORC2 does regulate the phosphorylation and stability of PKCα, which in turn alters the organization of the actin cytoskeleton [44] (Figure 1.5B).

mTORC1 and mTORC2

For a number of years, researchers have treated mTORC1 and mTORC2 as having separate signaling networks [44, 47, 104]. As more studies have emerged for mTORC2, the two mTOR

complexes have shown more similarities than was initially appreciated. Even though the mTOR complexes have different downstream effectors, both have now been implicated to play a role in cancer progression [38, 46, 47]. Rapalogs (rapamycin analogs) were developed to specifically inhibit mTORC1 function [105, 106]. However, inhibiting mTORC1 alone also disrupts the negative-feedback regulation that S6 kinase imparts upon PI3K, causing cancer cells to have aberrant PI3K activity [105, 106]. With the discovery of mTORC2 as an upstream regulator of AKT, and a downstream effector of PI3K, ATP-competitive inhibitors for mTOR (inhibiting both mTOR complexes) have proven to be more effective in restricting cancer cell growth [47, 107, 108].

Regulation of mTORC1/mTORC2 by Small GTPases

Rheb and mTORC1

Ras-homology enriched in brain (Rheb) belongs to the Ras-family of small GTPases [109]. There are two Rheb genes in mammals, Rheb1 and Rheb2 [110]. Rheb1 is ubiquitously expressed whereas Rheb2 is mostly expressed in brain [109-111]. Far less is known about Rheb2 (aka RhebL) and I will focus on Rheb1 (aka Rheb) for its role as a critical regulator of mTORC1. Like other GTPases, Rheb shuttles between the inactive GDP-bound form and an active GTP-bound form [109]. The mechanism responsible for how Rheb activates mTORC1 remains

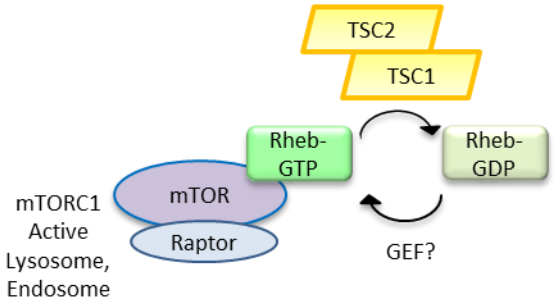
elusive but it is generally recognized that the activation is due to the direct binding of Rheb to the catalytic domain of mTOR [112]. However, while Rheb-GDP binds with higher affinity to mTORC1, it is only the GTP-bound form of Rheb that can activate mTORC1 [112] (Figure 1.6A).

Various effectors of Rheb have also been identified to play a role in mTORC1 activation, e.g. PLD1 and FKBP38 (FK506-binding protein 38) [69, 113]. Rheb-GTP can bind to and activate PLD1 to catalyze the conversion of phosphatidylcholine (PC) to phosphatidic acid (PA) and choline [69]. PA can act as a second messenger which binds directly to and activates mTORC1 [114]. The PLD1 pathway, as aforementioned, appears to function in parallel with the Rags to sense amino acids [115, 116] (Figure 1.6B). FKBP38 is a mitochondrial protein which also binds to Rheb-GTP with higher affinity than Rheb-GDP [113, 117, 118]. FKBP38 is regarded as a negative regulator of mTORC1 [113]. Upon binding to Rheb-GTP, FKBP38 releases its inhibition of mTORC1, allowing it to be active [113] (Figure 1.6C). However, *in vitro* biochemical assays have argued against a direct interaction occurring between FKBP38 and Rheb [119, 120].

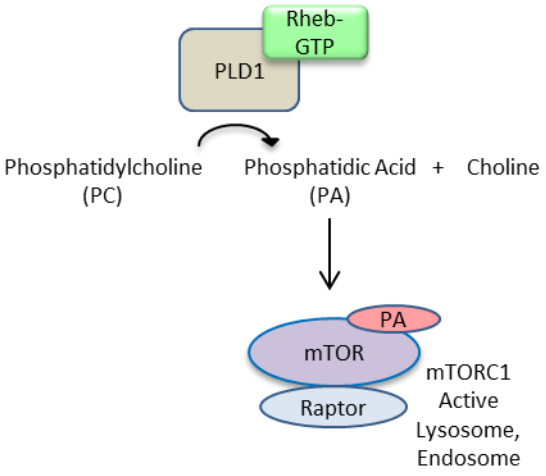
Rheb is regulated upstream by its GAP, TSC2, which forms a stable complex with TSC1 [121-124]. TSC1 stabilizes TSC2 and prevents it from ubiquitin-dependent degradation [125, 126]. There are multiple sites on both TSC1/TSC2 that can be phosphorylated in response to

Figure 1.6 Rheb in mTORC1 activation. A. GTP-bound Rheb directly binds to and activates mTORC1. **B.** GTP-bound Rheb activates its effector, PLD1, which produces phosphatidic acid to activate mTORC1. **C.** Rheb-GTP binds to a negative regulator of mTORC1, FKBP38, and sequesters FKBP38, thereby activating mTORC1.

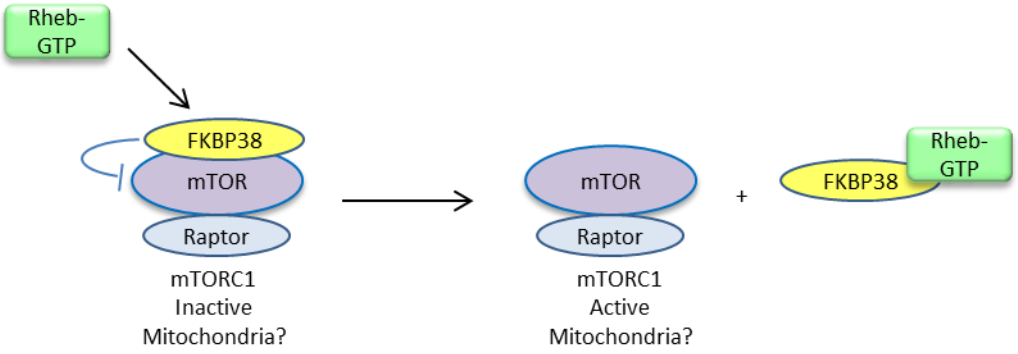
A.


TSC2
TSC1
Rheb-
GTP
Rheb-
GDP
mTOR
Raptor
mTORC1
Active
Lysosome,
Endosome
GEF?

B.


Rheb-
GTP
PLD1
Phosphatidylcholine
(PC)
Phosphatidic Acid
(PA)
+
Choline
PA
mTOR
Raptor
mTORC1
Active
Lysosome,
Endosome

C.


Rheb-
GTP
FKBP38
mTOR
Raptor
mTORC1
Inactive
Mitochondria?
mTOR
Raptor
mTORC1
Active
Mitochondria?
+
FKBP38
Rheb-
GTP

growth factors, amino acids, and the energy status of the cell (reviewed in [124]). Under favorable growth conditions or upon stimulation by growth factors, these phosphorylation sites create binding sites for the scaffolding protein 14-3-3 on TSC2, thus preventing the inactivation of Rheb [62, 127]. When the cell is deprived of nutrients, an alternative set of sites are phosphorylated on the TSC1/TSC2 complex that enhance and stabilize the complex and subsequently enable TSC2 to exert its GAP activity towards Rheb [123, 124].

Rheb shows a few unique properties as a small GTPase. It has very low intrinsic GTP hydrolysis activity and exhibits an abnormally high GTP-bound/GDP-bound ratio (over 20%) in cells compared to other Ras superfamily members [128]. Furthermore, Rheb that is ectopically expressed in cells is mainly in the GTP-bound state [128]. Another interesting property of Rheb is that it has one known GAP, whereas thus far, no GEF has been identified [110, 123, 124]. The current hypothesis is that perhaps Rheb, with its low intrinsic GTP hydrolysis activity and high GTP-bound ratio in the cell, only needs the regulation of a GAP but not a GEF. However, this hypothesis does not explain how Rheb becomes activated within the cell. TCTP (translationally controlled tumor protein) was identified from a *Drosophila* screen to regulate Rheb/mTORC1 function [129], however, biochemical assays performed by various groups including our laboratory have questioned whether TCTP is a bona fide Rheb GEF ([120, 130], unpublished results). Thus, the search for a Rheb GEF continues to be an ongoing effort in the field.

Rheb has a farnesyl tail on the C-terminus [131, 132] and can localize to various endo-membranes such as the ER, Golgi, lysosome, and mitochondria [64, 133-137]. This also implies that mTORC1 can be activated on the membranes of these different cellular compartments [64, 133, 135, 137]. The reasoning would be that mTORC1 has to activate different downstream targets for its many functions, therefore, the spatial activation of mTORC1 is critical.

Rheb and mTORC2

Rheb is well-established as a direct upstream activator of mTORC1, however, it has been demonstrated to have opposite effects on mTORC2 [138]. Knock-downs of Rheb in both *Drosophila* and mammalian cells show increased phosphorylation on AKT (S473), an indicator of mTORC2 activation [138]. It has been suggested that the inhibition of mTORC2 by Rheb may not be direct but potentially through the activation of S6 kinase which then gives rise to a feed-back inhibition of the PI3K pathway [138]. It has also been suggested that Rheb increases the ratio of mTORC1 to mTORC2 in cells [138]. Along the lines of these findings, the TSC1/TSC2 complex has also been shown to be necessary and positively regulate mTORC2 activation independent of TSC2's GAP activity [95].

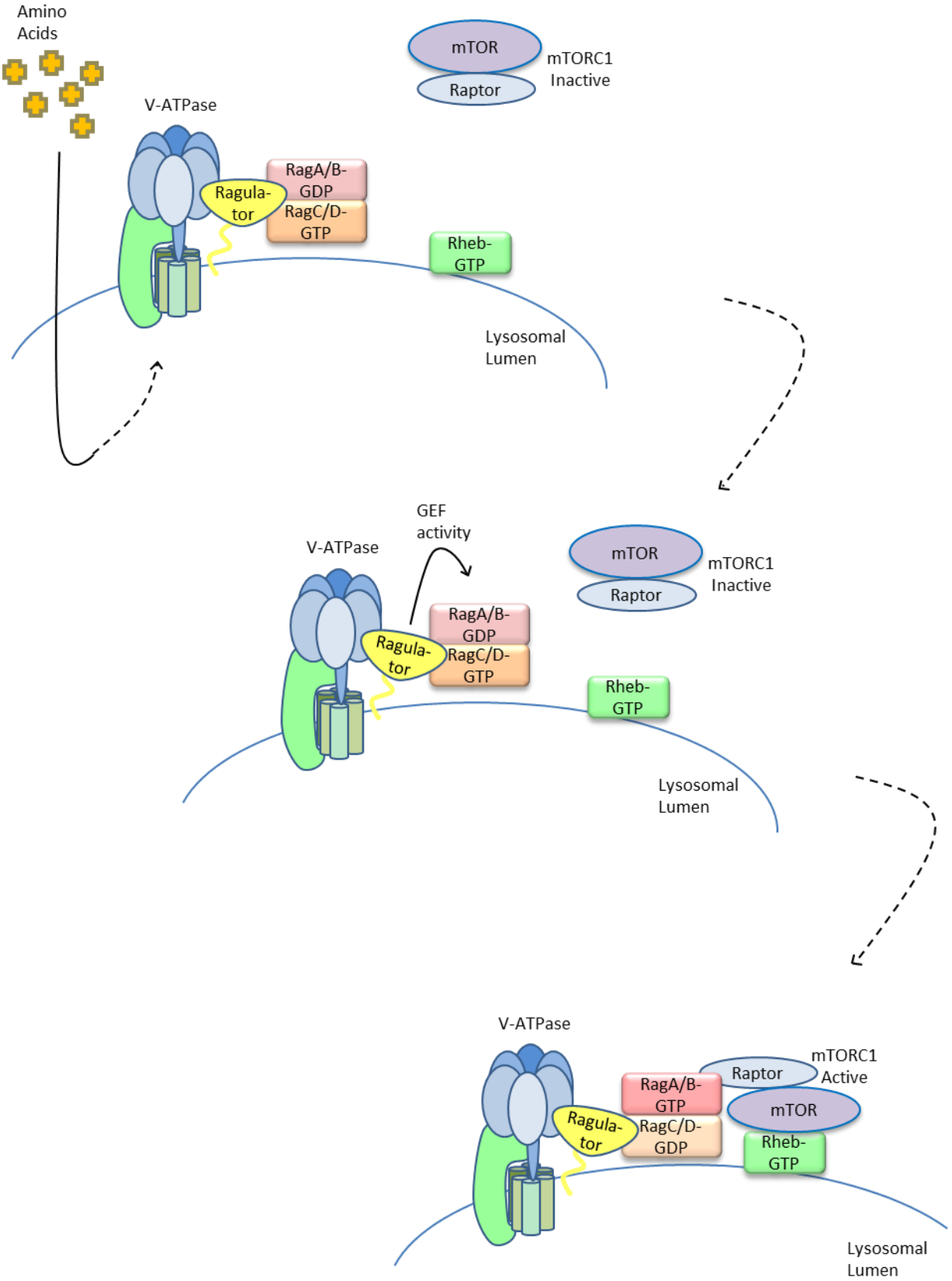
Rags and mTORC1

Rags are newly identified regulators for mTORC1 in amino acid sensing [64, 139]. There

are four Rag proteins in mammals, RagA, RagB, RagC and RagD [140, 141]. The Rag GTPases are non-conventional in that they are larger (40 kDa) than other Ras-related GTPases (25 kDa) and exist as dimers in the cell [141]. RagA/B forms a heterodimer with RagC/D and the nucleotide binding status of the heterodimer is often found to be in alternate GTP- or GDP-bound states [64]. Rags have been identified to bind to Raptor, the mTORC1-specific scaffold [64, 142]. Of the heterodimers tested to bind to Raptor, the strongest interaction is RagB-GTP/RagC-GDP [64]. Rags have been shown to tether to the lysosomal membrane through the interaction of Ragulators [70]. Ragulator is composed of five proteins: p14, MP1, p18, HBXIP, and C7orf59 [70, 71]. The Ragulator complex as a whole serves as a GEF for RagA and RagB and has no enzymatic function when the individual components are not in complex with each other [71].

Rags can sense amino acids through a lysosomal v-ATPase-dependent, inside-out mechanism [72]. When cells are stimulated with amino acids, an accumulation of amino acids occurs in the lysosome [143]. The v-ATPase resides on the lysosomal membrane and can sense the amino acids through an unknown mechanism presumably independent of its ability to form a proton gradient [72]. The Ragulator then senses an activating signal from the v-ATPase to turn on RagA or RagB [72]. Once RagA or RagB is activated, it can then interact with Raptor to bring mTORC1 to the lysosomal surface to be activated by Rheb [64, 70-72] (Figure 1.7). It is

Figure 1.7 Rags in amino acid sensing and mTORC1 activation. Rags can sense amino acids through a lysosomal v-ATPase-dependent, inside-out mechanism. The details of this mechanism are described in the text. Adapted from [71].


Amino
Acids
V-ATPase
mTOR
Raptor
mTORC1
Inactive
RagA/B-
GDP
Ragula-
tor
RagC/D-
GTP
Rheb-
GTP
Lysosomal
Lumen
GEF
activity
V-ATPase
mTOR
Raptor
mTORC1
Inactive
RagA/B-
GDP
Ragula-
tor
RagC/D-
GTP
Rheb-
GTP
Lysosomal
Lumen
V-ATPase
Raptor
mTORC1
Active
RagA/B-
GTP
mTOR
Ragula-
tor
RagC/D-
GDP
Rheb-
GTP
Lysosomal
Lumen

interesting to note that Rags do not activate mTORC1 directly but work as an intermediate to alter the localization of mTORC1 to its activator, Rheb, upon amino acid stimulation.

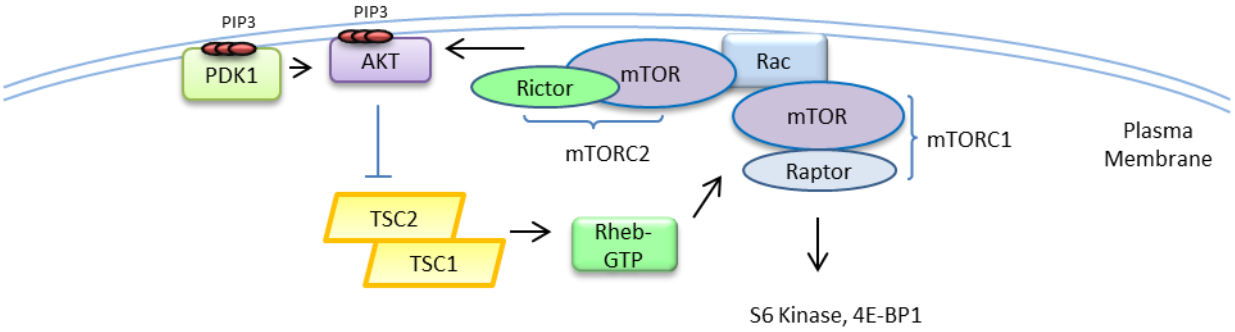
Rac and Cdc42 on mTOR regulation

Rac and Cdc42 are Rho GTPases known for their roles in cytoskeletal remodeling [27, 28]. Over the years, Rac and Cdc42 have also been shown to directly regulate S6 kinase, the mTORC1 downstream substrate, even though this has been controversial and much less is known in this regard [144]. More recently, it was reported that knock-outs as well as knock-downs of Rac in mouse embryonic fibroblasts (MEFs) and HeLa cervical carcinoma cells showed a decrease in cell size, a hallmark of mTORC1 inhibition. The authors then went on to show that Rac can interact with both mTORC1 and mTORC2, regardless of its nucleotide binding state, to localize the mTOR complexes to the plasma membrane [145] (Figure 1.8A). However, the specific mechanism of how this interaction regulates mTOR function is still unclear.

mTORC2 was initially identified for its role in cytoskeletal remodeling [43, 44], making Rac one of the first downstream effectors investigated [43]. Knock-down of mTORC2-specific components showed a decrease in GTP-bound Rac in NIH3T3 cells [43]. This correlation of mTORC2-Rac activity was later found to be through P-REX1 [36], a GEF for Rac that has also shown activity towards Cdc42 *in vitro* [146]. P-REX1 interacts with both mTORC1 and

Figure 1.8 Rac and Cdc42 in mTOR regulation. A. Rac binds to both mTORC1 and mTORC2 and localizes the complexes to the plasma membrane. Adapted from [145]. **B.** In the P19 embryonic carcinoma cell line, Cdc42 activates mTOR to call up transcription factors important for the maintenance of neural progenitor status. **C.** Cdc42 activates PLD1, a Rheb effector, which in turn activates mTORC1.

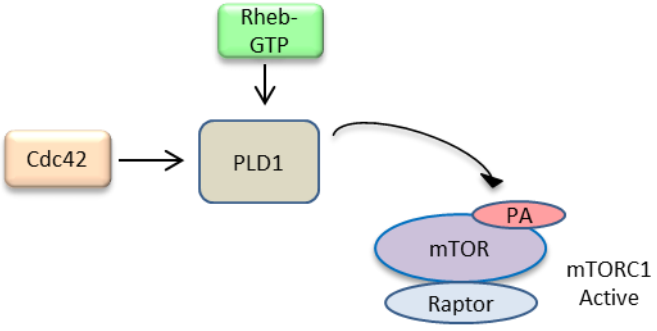
A.


PIP3
PIP3
PDK1
AKT
Rictor
mTOR
Rac
mTOR
Raptor
mTORC1
mTORC2
Plasma
Membrane
TSC2
TSC1
Rheb-
GTP
S6 Kinase, 4E-BP1

B.


Retinoic
Acid
Cdc42
?
?
mTOR
Hes5
Pax6
(Maintenance of Neural
Progenitor Status)

C.


Rheb-
GTP
Cdc42
PLD1
PA
mTOR
Raptor
mTORC1
Active

mTORC2, but is only active when bound to mTORC2 [36]. Taken together, Rac can act as a direct upstream regulator of mTORC1 [145]. Rac can also act both up- and downstream of mTORC2, albeit not as a direct effector of mTORC2 [36, 43, 145].

The known regulation of mTORC1 by Cdc42 is less direct than Rac. A study from our laboratory identified the importance of Cdc42 for activating mTOR in P19 cells, a pluripotent embryonic carcinoma cell line, in order to promote the differentiation of these cells into neural progenitors [147] (Figure 1.8B). However, the mechanism of how this activation is achieved is still not understood. Another study suggested that Cdc42 can regulate the activation of mTORC1 through PLD1 [148]. Cdc42 has been shown to activate PLD1 through its Rho-insert region, i.e. a stretch of residues unique to Rho, Rac, and Cdc42 but missing in Ras [149]. A mutation in the Rho-insert region of Cdc42 lowered PLD1 and S6 kinase activities but was rescued by the PLD1 product, phosphatidic acid [148]. As aforementioned, PLD1 has been shown to be an effector of Rheb, and PA can activate mTORC1 directly [69, 114]. This raises the possibility of a novel mechanism by which Cdc42 signals to mTORC1 through its ability to activate PLD1 (Figure 1.8C). However, this does not shed light on the relationship between Cdc42 and Rheb with regard to PLD1 signaling to mTORC1.

TSC2 (-/-) fibroblasts and colon cancer cells show a defect in cell polarization and migration due to lowered Rac and Cdc42 activation [150]. It was hypothesized that this may be

due to the over-activation of S6 kinase in TSC2 (-/-) cells which then results in the feedback inhibition of PI3K, decreasing PI3K activity and subsequently Rac and Cdc42 downstream [150]. However, the TSC complex has also been shown to be important in regulating mTORC2 activity [95], creating another possible mode of regulation for Rac and Cdc42 by mTORC2.

Other small GTPases in mTOR signaling (Rab, Arf, and RalA)

Various other small GTPases have also been implicated in mTORC1 signaling. Over-expression of constitutively active Rab5 or the knock-down of hVps39, a GEF for Rab7, inhibits the maturation of early endosomes to late endosomes, creating a hybrid endosome that contains both early and late endosomal markers. Under such circumstances, mTORC1 is still recruited to the late endosome/lysosome but cannot be activated [151]. It is interesting to note that over-expression of exogenous Rheb rescues the mTORC1 inhibition but not the activation of endogenous Rheb. This suggests that the local environment is critical for Rheb localization and furthermore, mTORC1 activation [151]. A large-scale RNAi screen of *Drosophila* small GTPases revealed that the Rab and Arf proteins, which are important for intracellular trafficking, are necessary for mTORC1 activation [152]. RalA, a Ras-like small GTPase, has also been shown to be downstream of Rheb in the amino acid- and glucose-sensing pathway of mTORC1 [153]. Most of these studies were performed using RNAi [151-153]. However, while some of these small GTPases have been shown to be necessary for mTORC1 signaling, their exact mechanisms

of action are still unknown.

What I have summarized here are the various modes of regulation that small GTPases can have on mTORC1/mTORC2 signaling. Rheb acts as a direct activator of mTORC1, whereas Rags and Rac are important for mTORC1 localization upon stimulation. Rabs, on the other hand, are important for the local environment of where mTORC1 activation takes place in the cell. The involvement of so many small GTPases emphasizes the importance of the spatial and temporal regulation of mTOR activation.

Dock Family of GEFs – Dock7

A member of the Dock180 family of GEFs, Dock7, has emerged as a novel player in mTOR signaling. The Dock180 family is one of two classes of GEFs discovered so far, the other being the Dbl-homology-pleckstrin-homology domain (DH-PH) containing family [154-156]. Dock180 family members differ from the classical Dbl-like GEFs in that they contain DHR1 (dock homology region 1) and DHR2 domains, instead of the canonical DH-PH domain [155, 156]. Even though the DHR1-DHR2 domains of Dock family members and the DH-PH domains of Dbl family members do not show any sequence similarities, the functions of the domains remain similar [157]. The DHR2 domain functions like the DH domain in that it catalyzes the guanine-nucleotide exchange activity of Rho GTPases [158]. The DHR1 domain has been

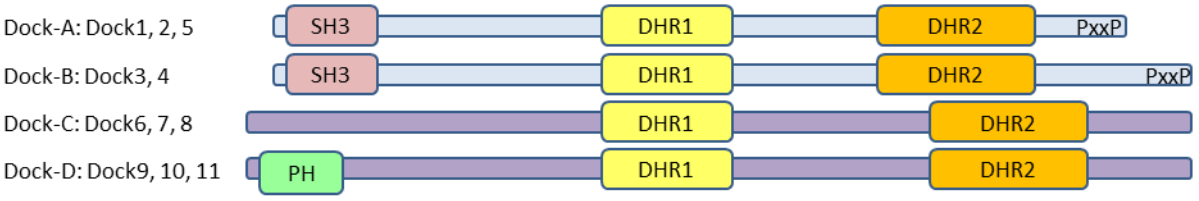
implicated in cell membrane binding (e.g. PIP3) similar to the function of PH domains [159, 160]. The Dock180 family of GEFs consists of 11 members that have only been shown to exhibit GEF activity towards the Rho GTPases Cdc42 and Rac. Depending on their activity and sequence domains, Dock family members can be further divided into four sub-groups, Dock-A, B, C, and D. Dock-A and B group members are Rac-specific, whereas Dock-D members are Cdc42-specific. Dock-C can activate both Rac and Cdc42, with Dock7 being a member of this sub-family (reviewed in [155], Figure 1.9A).

The GEF activity of Dock7 towards Cdc42 or Rac is not yet clearly defined. The Van Aelst group demonstrated that Dock7 can act as a Rac-specific GEF in hippocampal neuronal cells and can contribute to axon formation [161]. It was inferred from this study that Dock7 can activate Rac to further phosphorylate Stathmin/Op18, a microtubule destabilizing protein, in a localized manner to promote axon formation. The over-expression of Dock7 promoted the growth of multiple axons whereas the knock-down of Dock7 prevented axon formation [161].

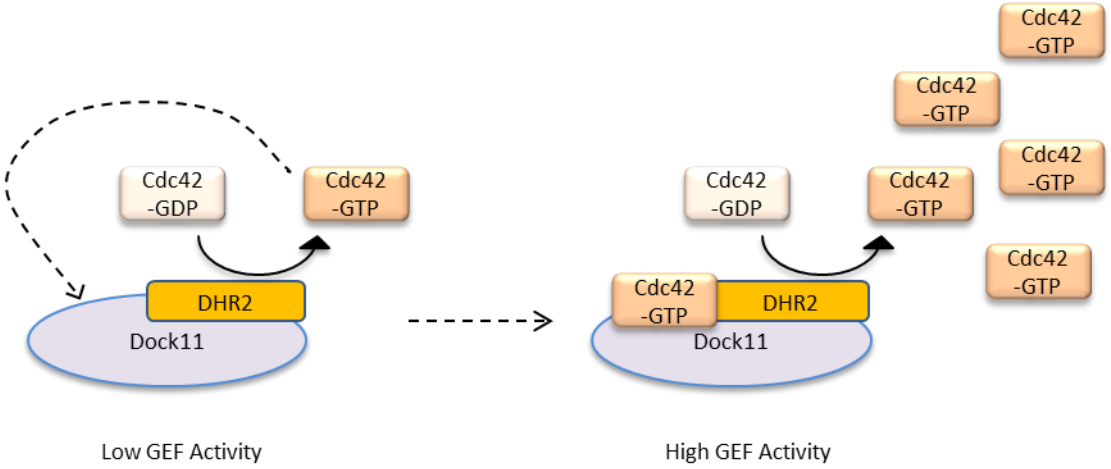
Yamauchi et al. have shown in Schwann cells that ErbB2 can be activated through HRG stimulation to phosphorylate Dock7 [162]. This enhances the GEF activity of Dock7 towards Cdc42 and Rac to promote Schwann cell migration [162]. In a follow-up study, the authors showed that Dock7 needs to be down-regulated to promote Schwann cell differentiation and myelination once the cells have migrated down the axon to their final destination to form myelin

Figure 1.9 Dock180 family of guanine nucleotide exchange factors. A. The DHR1-DHR2 domains are conserved amongst Dock180 family members. Adapted from [155]. **B.** A novel form of regulation for Dock11. Activated Cdc42 allosterically interacts with Dock11 to enhance its catalytic activity. Adapted from [163].

A.


Dock-A: Dock1, 2, 5
SH3
DHR1
DHR2
PxxP
Dock-B: Dock3, 4
SH3
DHR1
DHR2
PxxP
Dock-C: Dock6, 7, 8
DHR1
DHR2
Dock-D: Dock9, 10, 11
PH
DHR1
DHR2

B.


Cdc42
-GDP
Cdc42
-GTP
DHR2
Dock11
Low GEF Activity
Cdc42
-GDP
Cdc42
-GTP
Cdc42
-GTP
Cdc42
-GTP
Cdc42
-GTP
Cdc42
-GTP
DHR2
Dock11
High GEF Activity

sheaths [164]. Furthermore, a report published in 2005 demonstrated that Dock7 can form a complex with the Rheb GAP, TSC1/TSC2, suggesting a potential role for Dock7 in Rheb regulation [165].

So far, Dock7-related studies have predominantly focused on the nervous system, where Dock7 is highly expressed [161, 162, 164, 166]. However, we have evidence that Dock7 is expressed in SKBR3, HeLa, and 293T cells (unpublished results). We are particularly interested in Dock family members because we have previously shown that Dock11, a Cdc42-specific GEF in the Dock-D subfamily, can act both as an activator and effector for Cdc42. Activated Cdc42 can bind to Dock11 at an allosteric site to augment its GEF activity toward Cdc42 [163] (Figure 1.9B). We have also observed that Dock7 shows similar properties to Dock11 where the allosteric binding of activated Cdc42 enhances its GEF activity (Zhou et al., submitted). Given the ability of Dock7 to act both as an activator and effector for Cdc42, coupled with its ability to interact with mTOR signaling components, we have been very interested in gaining a better understanding of how Dock7 coordinates Rac/Cdc42 activation and mTORC1 signaling.

Overview of the Thesis

In this thesis, I have taken advantage of our laboratory's experience in HRG-signaling as a means to understand growth factor signaling to mTOR. In Chapter 2, I investigated how

HRG/ErbB2-signaling contributes to the transformed properties of SKBR3 breast cancer cells. The transformed growth characteristics of these cancer cells are largely due to signaling to mTORC1 in an mTORC2-dependent manner. When inhibiting mTORC2 by knocking-down Rictor, the mTORC2 scaffold, mTORC1 activation was blocked as was the ability of SKBR3 cells to exhibit anchorage-independent growth and form colonies in soft agar.

In Chapter 3, the HeLa cervical carcinoma cell line was used as a model system to delineate the signaling interplay between Rac, Cdc42, and Rheb upstream of mTORC1. Using a knock-down and rescue approach, I have shown that Rac and Cdc42 are upstream of Rheb in a signaling pathway that leads to mTORC1 activation. However, Rac and Cdc42 appear to provide independent inputs into Rheb. Interestingly, I have also discovered that Dock7 not only acts as a GEF for Rac and Cdc42, but it also serves as a scaffolding protein for mTOR and TSC1/TSC2. More surprisingly, Dock7 also shows properties of a Rheb GEF, leading to the idea that Dock7 serves as a novel mTOR scaffold that may coordinate the activation of Rac, Cdc42, and Rheb upon HRG-stimulation.

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Chapter 2

Identification of an mTORC2-mTORC1 signaling cascade necessary for HRG/ErbB2-dependent cellular transformation

Abstract

ErbB2 is both a prognostic indicator and a target for therapy in breast cancer. The over-expression of heregulin (HRG), a growth factor that activates the ErbB2 receptor, has also been shown to be critical for patient responsiveness to ErbB2 inhibitors. Although ErbB2 inhibitors offer a major advancement in the treatment of ErbB2-dependent breast cancers, patients are susceptible to developing clinical resistance to these inhibitors. Therefore, understanding the molecular mechanism of HRG/ErbB2-induced tumorigenesis is critical for the development of effective therapeutic strategies for this cohort of breast cancer patients. We demonstrate that HRG promotes anchorage-independent growth more potently than EGF in the breast cancer cell line, SKBR3, and determine that both PI3K and mTORC1 are necessary for this transformation event. Surprisingly, we find that the activation of mTORC1 in response to HRG is dependent upon the upstream activation of mTORC2. HRG signaling to PI3K bifurcates

to activate both PDK1 and mTORC2 and then converges at the level of AKT/TSC2, where we observe a dominance of mTORC2 over PDK1 in the activation of AKT. We further demonstrate that eliminating the mTORC2 component, Rictor, is detrimental to both the activation of mTORC1 and HRG-mediated cellular transformation. This study highlights a previously unappreciated role for mTORC2 in ErbB2-dependent breast cancer and suggests benefits for targeting mTORC1 and mTORC2 in these cancers.

Introduction

ErbB2 over-expression characterizes 20 – 30 percent of all breast cancers and correlates with a poor prognosis for patients presented with this key biomarker [1, 2]. Additionally, heregulin (HRG), an EGF-like growth factor that binds to the ErbB3/ErbB4 receptor and induces ErbB2 dimerization and activation [3, 4], is also found over-expressed in breast, ovarian and prostate cancers (reviewed in [5]) and can drive cellular transformation by the activation of ErbB2, independent of the over-expression status of the receptor [6]. A major advancement in the treatment of ErbB2-positive cancers came with the development of monoclonal antibodies against ErbB2 (trastuzumab/Herceptin) and more recently, ErbB2 kinase inhibitors (e.g. lapatinib) (reviewed in [7]). These strategies have offered significant clinical value but, as is now being appreciated for many forms of targeted therapy in cancer, patients treated with either trastuzumab

or lapatinib are susceptible to the development of clinical resistance to these therapies [8-10]. As new treatment options are considered for ErbB2-positive cancers, a molecular understanding of the signaling events that underlie HRG/ErbB2-dependent cellular transformation will be critical.

We have found previously that HRG, but not the closely related growth factor, EGF, signals to the RNA processing machinery to impact cell growth [11]. Specifically, the activation of ErbB2 at the cell surface translates into the nuclear activation of the small GTPase Ran [11]. Ran, together with importin α and β, regulates the binding and processing of capped mRNAs by the nuclear cap-binding complex (CBC) to promote mitogenesis [11-13]. The over-expression of wild-type Ran or constitutively-active Ran mutants is sufficient to transform NIH-3T3 fibroblasts and non-invasive R37 mammary cells [14, 15], as well as accelerate the transforming potential of the breast cancer cell line, SKBR3 [14], thus underscoring the significance of this signaling endpoint in HRG/ErbB2-dependent transformation.

The ability of HRG to signal to Ran and the CBC is dependent upon the mammalian target of rapamycin (mTOR, [11, 12]). mTOR is a 280 kDa Ser/Thr kinase that forms two functionally distinct complexes in mammalian cells, mTORC1 and mTORC2. The rapamycin-sensitive mTORC1 consists of mTOR, Raptor, mLST8, and PRAS40. mTORC1 controls cell size, proliferation, lipid biogenesis, metabolism, and autophagy by sensing growth factors and the nutrient availability of the cell (reviewed in [16-18], [19], [20]). mTORC2 is insensitive to

short-term rapamycin treatment and is comprised of mTOR, Rictor, mSin1 and mLST8 ([21-23]). Raptor and Rictor are commonly used as markers to discern the two complexes [22, 24]. Significantly less is understood regarding the functions and regulation of mTORC2, with the exception of a role for mTORC2 in cytoskeletal remodeling [22, 25]. There is, however, emerging evidence for the involvement of mTORC2 in growth factor signaling and tumor progression [26, 27].

Many growth factors signal to mTORC1 by activating PI3K, which converts PIP2 to PIP3 at the cell membrane (reviewed in [16-18]). PDK1 (phosphoinositide-dependent kinase 1) is then recruited to the membrane, where it phosphorylates AKT at threonine 308 (reviewed in [28]). AKT achieves maximal activation when it is phosphorylated on both threonine 308 in its activation loop and serine 473 within the hydrophobic motif [29]. Once activated, AKT phosphorylates an inhibitory site on TSC2 (tuberous sclerosis complex 2), a GTPase-activating protein (GAP) for the small GTPase Rheb (reviewed in [30]). Rheb binds and activates mTORC1 although the molecular basis for this activation remains poorly defined [31].

In this study we sought to better understand the cellular signals that underlie the transforming potential of HRG, with an emphasis on HRG signaling to mTORC1. We demonstrate that HRG promotes colony formation more efficiently than EGF in the SKBR3 breast cancer cell line, and that the differential activation of mTORC1 is necessary for the

enhanced potency. Surprisingly, the ability of HRG to signal to mTORC2 plays a critical role in the ability of HRG to activate mTORC1 and promote cellular transformation. Pharmacological studies contrasting rapamycin and an ATP-competitive inhibitor of mTOR, INK-128 [32], reveal that the phosphorylation of AKT at serine 473 by mTORC2 is critical for downstream TSC2 phosphorylation and mTORC1 activation in response to HRG. The specific disruption of mTORC2 signaling by the introduction of Rictor shRNA, not only attenuated the activation of mTORC1 and its upstream signaling activators but also had a deleterious effect on HRG-mediated colony formation. Taken together, these data highlight mTORC2 as a previously unappreciated signaling endpoint for HRG, demonstrate that mTORC2 is necessary for the activation of mTORC1 by HRG, and provide evidence for an important role for mTORC2 in HRG- and ErbB2-dependent cellular transformation.

Materials and Methods

Antibodies and reagents

The antibodies used for this study were purchased from Cell Signaling Technology with the exception of anti-pan-mTOR (Millipore), anti-pan-S6 kinase (Millipore), anti-actin (NeoMarker), and anti-Myc (Covance). Rapamycin and LY294002 were purchased from Calbiochem. INK-128 was a generous gift from Dr. Kevan Shokat (UCSF). HRG (Heregulin β, EGF domain, residues

178-241) was obtained from Sigma. EGF and Protein G beads were purchased from Invitrogen.

DNA constructs and shRNAs

Rictor (Addgene plasmid 1860) and Raptor (Addgene plasmid 1859) DNA constructs were obtained from Addgene [22]. The shRNAs targeting Rictor were purchased from Sigma (TRCN0000074288, TRCN0000074290). The lenti-viral constructs expressing Rictor shRNAs were generated according to the manufacturer's protocol (Sigma).

Cell culture conditions

The breast cancer cell line, SKBR3, was maintained in RPMI 1640 (Invitrogen) containing 10% FBS (Invitrogen) at 37℃, 5% CO2. For growth factor stimulation, SKBR3 cells were seeded at 5-7x10^5 on 100 mm cell culture plates (Corning), followed by serum-starvation with RPMI for 40-48 hours, replenishing with fresh RPMI 24 h after initiation of starvation. SKBR3 cells were then stimulated with HRG at the concentration and times indicated, followed by cell lysis. For inhibitor analysis, SKBR3 cells were pre-treated with 50 nM rapamycin, 50 nM INK-128, or 10 μM LY294002 for 30 min followed by the addition of HRG. 293T cells were maintained in 10% FBS-containing DMEM (Invitrogen) at 37℃, 5% CO2.

Transfection

HEK 293T cells were seeded at 1x10^6 cells on 100 mm cell culture plates. Four μg of DNA was transfected into the cells the next day using Lipofectamine and Plus Reagent, following the

manufacturer's protocol (Invitrogen).

Immunoblot analysis

Cells were lysed with cell lysis buffer (50 mM Hepes, 150 mM NaCl, 1 mM $MgCl_2$, 25 mM NaF, 1 mM Na_3VO_4, 50 mM β-glycerophosphate, 10 μg/ml Leupeptin, 10 μg/ml Aprotinin, 1% Triton X-100). The lysates were resolved by SDS-PAGE, and then the proteins were transferred to polyvinylidene fluoride membranes. The membranes were incubated with the indicated primary antibodies diluted in 20 mM Tris, 135 mM NaCl, and 0.02% Tween-20. The primary antibodies were detected with horseradish peroxidase-conjugated secondary antibodies (GE Healthcare) followed by exposure to ECL reagent (Perkin Elmer).

Soft-agar assays

SKBR3 cells, or SKBR3 cells infected with the various Rictor shRNAs as described, were seeded at a density of $5x10^3$ cells/well in complete medium (10% FBS, RPMI) containing 0.3% agarose, onto underlays composed of growth medium containing 0.6% agarose in 6-well dishes. The corresponding growth factors or inhibitors were added in the cell mixture. The cultures were fed with complete medium containing 0.3% agarose along with their respective growth factors or inhibitors every three days. Colonies were counted after 13 days.

Immunoprecipitation

293T cells were lysed with cell lysis buffer (50 mM Hepes, 150 mM NaCl, 1 mM $MgCl_2$, 25 mM

NaF, 1 mM Na_3VO_4, 50 mM β-glycerophosphate, 10 μg/ml Leupeptin, 10 μg/ml Aprotinin, 0.3% CHAPS). Lysates were precleared with Protein G beads on a rotator at 4℃ for 10 min. The supernatant was collected and added with anti-Myc antibody for 2 h followed by the addition of Protein G beads for 1 h at 4℃. Immunoprecipitates were washed 3 times with the lysis buffer followed by the addition of 5X Laemilli buffer.

ImageJ quantification

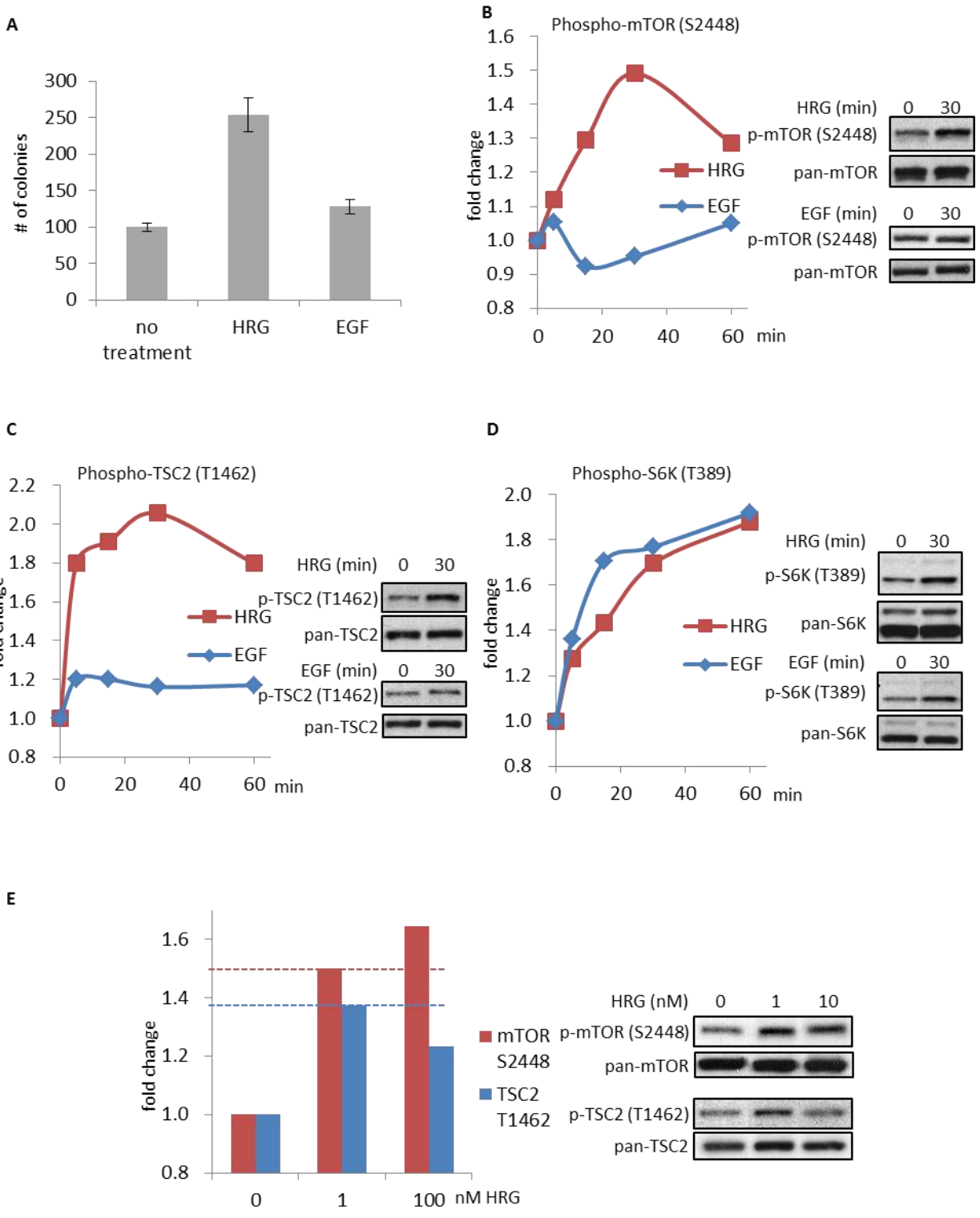
The Western blots were quantified using ImageJ (http://rsbweb.nih.gov/ij/) under the Gel Analysis Tool. The intensity of the different lanes was then normalized to the control lane, which was set to one. In Figure 2.4, the difference in intensity was obtained by subtracting the intensity of the control (untreated) samples from the HRG-stimulated samples. The percentage of inhibition is calculated by (1-(Difference in $Intensity_{knock\text{-}down}$)/(Difference in $Intensity_{control}$)) x100%.

Results

To investigate important aspects of HRG/ErbB2-dependent transformation, we started by comparing the relative effectiveness of HRG and EGF to stimulate mitogenesis in breast cancer cells and then attempted to understand what signaling components contribute to any differences observed. The SKBR3 cell line is a low-grade breast cancer cell line that expresses EGFR and

over-expresses ErbB2, and as such is a useful model for comparisons between HRG- and EGF-dependent signaling. We first compared the abilities of HRG and EGF to enhance the anchorage-independent growth of SKBR3 cells. Cells were seeded in soft agar either in the presence of regular growth media (no treatment), or media supplemented with the addition of 100 nM HRG, or 100 ng/ml EGF, and colonies were then counted after 13 days. As shown in Figure 2.1A, treating the SKBR3 cells with HRG significantly enhances the ability of these cells to form colonies in soft agar while EGF does not. Previous studies indicate that mTOR is a necessary component in the HRG-specific activation of the Ran GTPase and the CBC in SKBR3 cells [11]. Thus we next examined the differential abilities of HRG and EGF to activate mTORC1. SKBR3 cells were serum-starved for two days and stimulated with 100 nM HRG or 100 ng/ml EGF for the times indicated. Despite a relatively high basal level of mTOR activity (we observe that both the over-expression of ErbB2 and the presence of amino acids contribute to this background mTOR activity; data not shown), the treatment of SKBR3 cells with HRG leads to a time-dependent increase in the phosphorylation of mTOR as determined by Western blotting using a phospho-mTOR (S2448) antibody as quantified in Figure 2.1B (left). An example of the 30 minute time-point stimulation by HRG, compared to the background mTOR activity at time zero, is shown on the right panel of Figure 2.1B. In contrast, EGF was relatively ineffective in its ability to activate mTOR.

Figure 2.1 HRG is more effective than EGF at promoting colony formation in SKBR3 cells and does so in an mTOR-dependent signaling pathway. A. SKBR3 cells were seeded in 0.3% agarose-containing complete medium with the addition of 100 nM HRG or 100 ng/ml EGF. Cells were fed every three days with the growth factor- containing medium and colonies were counted on day 13. The experiment was done in triplicate and the results were averaged and graphed. **B.** SKBR3 cells were serum-starved for 40-48 h followed by 0-60 min treatment of 100 nM HRG or 100 ng/ml EGF. Whole cell lysates were collected and subjected to Western blotting with phospho-mTOR (S2448) and pan-mTOR antibody. The blots were quantified using ImageJ. Relative intensities of the bands were plotted against the zero-minute time-point of each individual blot, which was normalized to one. Two time points, 0 and 30 minutes, are shown on the right as an example of the Western blots. **C.** SKBR3 cells were treated as stated above. Whole cell lysates were collected and subjected to Western blotting with phospho-TSC2 (T1462) and pan-TSC2 antibody. The blots were quantified as described above. Two time points, 0 and 30 minutes, are shown on the right as an example of the Western blots. **D.** SKBR3 cells were treated as stated above. Whole cell lysates were collected and subjected to Western Blotting with phospho-S6 kinase (T389) and pan-S6 kinase antibody. The blots were quantified as described above. Two time points, 0 and 30 minutes, are shown on the right as an example of the Western blots. **E.** SKBR3 cells were serum-starved for 40-48 h followed by 0, 1, or 100 nM HRG stimulation for 30 minutes. Whole cell lysates were collected and subjected to Western blotting with phospho-mTOR (S2448), phospho-TSC2 (T1462), pan-mTOR, and pan-TSC2. The blots were quantified as described above.


A
# of colonies
300
250
200
150
100
50
0
no treatment
HRG
EGF
B
Phospho-mTOR (S2448)
fold change
1.6
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0
20
40
60
min
HRG
EGF
HRG (min) 0 30
p-mTOR (S2448)
pan-mTOR
EGF (min) 0 30
p-mTOR (S2448)
pan-mTOR
C
Phospho-TSC2 (T1462)
fold change
2.2
2.0
1.8
1.6
1.4
1.2
1.0
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0
20
40
60
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HRG
EGF
HRG (min) 0 30
p-TSC2 (T1462)
pan-TSC2
EGF (min) 0 30
p-TSC2 (T1462)
pan-TSC2
D
Phospho-S6K (T389)
fold change
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0
20
40
60
min
HRG
EGF
HRG (min) 0 30
p-S6K (T389)
pan-S6K
EGF (min) 0 30
p-S6K (T389)
pan-S6K
E
fold change
1.6
1.4
1.2
1.0
0.8
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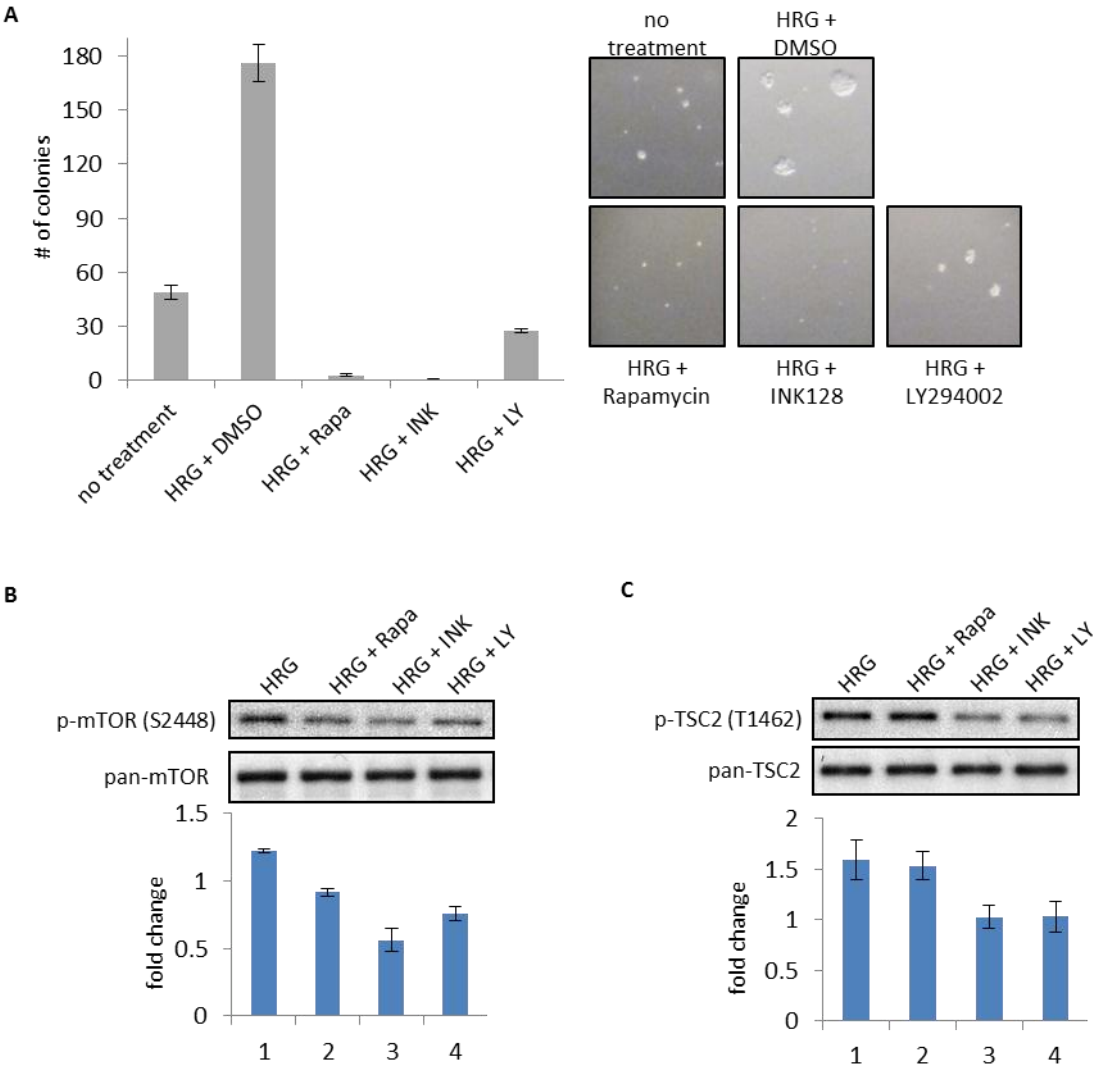
1
100
nM HRG
mTOR S2448
TSC2 T1462
HRG (nM) 0 1 10
p-mTOR (S2448)
pan-mTOR
p-TSC2 (T1462)
pan-TSC2

We next examined the effects of HRG and EGF on other constituents of mTORC1 signaling. TSC2 functions upstream of mTORC1 by regulating the GTP-binding activity of Rheb (reviewed in [30]). Phosphorylation of TSC2 by AKT at threonine 1462 disrupts the ability of TSC2 to regulate Rheb [30], resulting in enhanced mTORC1 function. Probing for the phosphorylation of TSC2 at T1462 indicated that HRG potentiates this phosphorylation to a greater extent than does EGF (Figure 2.1C), similar to what was observed for mTOR (S2448). Interestingly, S6 kinase, a downstream target of mTORC1 that is known to have a complex mode of activation [33], showed little difference in the ability of HRG versus EGF to induce its phosphorylation at threonine 389 (Fig. 2.1D).

Additionally, we examined the HRG dose-dependence of these signaling events by stimulating SKBR3 cells with 1 nM and 100 nM HRG, as read-out by Western blotting with phospho-mTOR (S2448) and phospho-TSC2 (T1462) antibodies. 1 nM HRG was sufficient to achieve near maximal phosphorylation of mTOR and TSC2 (Figure 2.1E), and also to support the enhanced ability of SKBR3 cells to grow in soft agar (Figure 2.2A).

Given that HRG is better at promoting anchorage-independent growth in SKBR3 cells than EGF, and is similarly more effective at activating components of mTORC1 signaling (i.e., TSC2 and mTOR), we wanted to probe the necessity of mTORC1 for HRG-induced cellular transformation. To this end, we utilized inhibitors for mTOR (rapamycin and INK-128) as well

Figure 2.2 Inhibitors of PI3K and mTOR inhibit colony growth and HRG-mediated signaling to mTOR and TSC2. A. SKBR3 cells were seeded in 0.3% agarose with complete medium containing either DMSO (vehicle), DMSO + 1 nM HRG, 50 nM Rapamycin + 1 nM HRG, 50 nM INK-128 + 1 nM HRG, or 10 μM LY294002 + 1 nM HRG. Cells were replenished with the inhibitor + HRG every three days. Colonies were counted on day 13. The experiment was done in triplicate and the results were averaged and graphed. **B.** SKBR3 cells were serum-starved for 40-48 h. Cells were then pretreated with 50 nM rapamycin, 50 nM INK-128, or 10 μM LY294002 for 30 min followed by the corresponding inhibitor plus 1 nM HRG for 30 min. Whole cell lysates were collected and subjected to Western blotting with phospho-mTOR (S2448) and pan-mTOR antibodies. The bar graph was generated by quantifying blots from three independent experiments using ImageJ and normalizing the intensity of the bands to the untreated lane. **C.** SKBR3 cells were treated as described above, followed by blotting with phospho-TSC2 (T1462) and pan-TSC2 antibodies. The bar graph was generated by quantifying blots from three independent experiments using ImageJ and normalizing the intensity of the bands to the untreated lane.


A
# of colonies
180
150
120
90
60
30
0
no treatment
HRG + DMSO
HRG + Rapa
HRG + INK
HRG + LY
no treatment
HRG + DMSO
HRG + Rapamycin
HRG + INK128
HRG + LY294002
B
HRG
HRG + Rapa
HRG + INK
HRG + LY
p-mTOR (S2448)
pan-mTOR
fold change
1.5
1
0.5
0
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2
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4
C
HRG
HRG + Rapa
HRG + INK
HRG + LY
p-TSC2 (T1462)
pan-TSC2
fold change
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0.5
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4

as a conventional PI3K inhibitor (LY294002) since the mitogenic activation of mTORC1 is classically described as occurring as a result of PI3K/AKT signaling. Rapamycin is a specific allosteric inhibitor of mTORC1, although prolonged treatments with rapamycin have also been suggested to inhibit mTORC2 [34], while INK-128 is a novel ATP-competitive mTOR inhibitor and as such, does not distinguish between mTORC1 and mTORC2 [32].

SKBR3 cells were seeded in soft agar in the presence of 1 nM HRG, followed by the addition of either DMSO (vehicle), rapamycin, INK-128 or LY294002. These treatments were repeated every three days until colonies were counted on day 13. As shown in Figure 2.2A, 1 nM HRG markedly augments the ability of SKBR3 cells to form colonies in soft agar, allowing for an increase in colony size as well as colony number. Both mTOR inhibitors were quite potent in their ability to block HRG-stimulated colony formation. INK-128 in particular was striking for its ability to limit the growth of cells beyond the single cell state. The inhibition of PI3K also blocks HRG-mediated colony formation, albeit to a lesser extent both in number and colony size. Taken together, these data demonstrate that mTOR signaling, as well as PI3K signaling, is essential for HRG-induced cellular transformation.

As HRG-promoted transformation was sensitive to both mTOR and PI3K inhibitors, it appeared that this event was most likely an outcome of HRG-stimulated PI3K/PDK1/AKT activation, which in turn would lead to the phosphorylation and deactivation of TSC2 and a

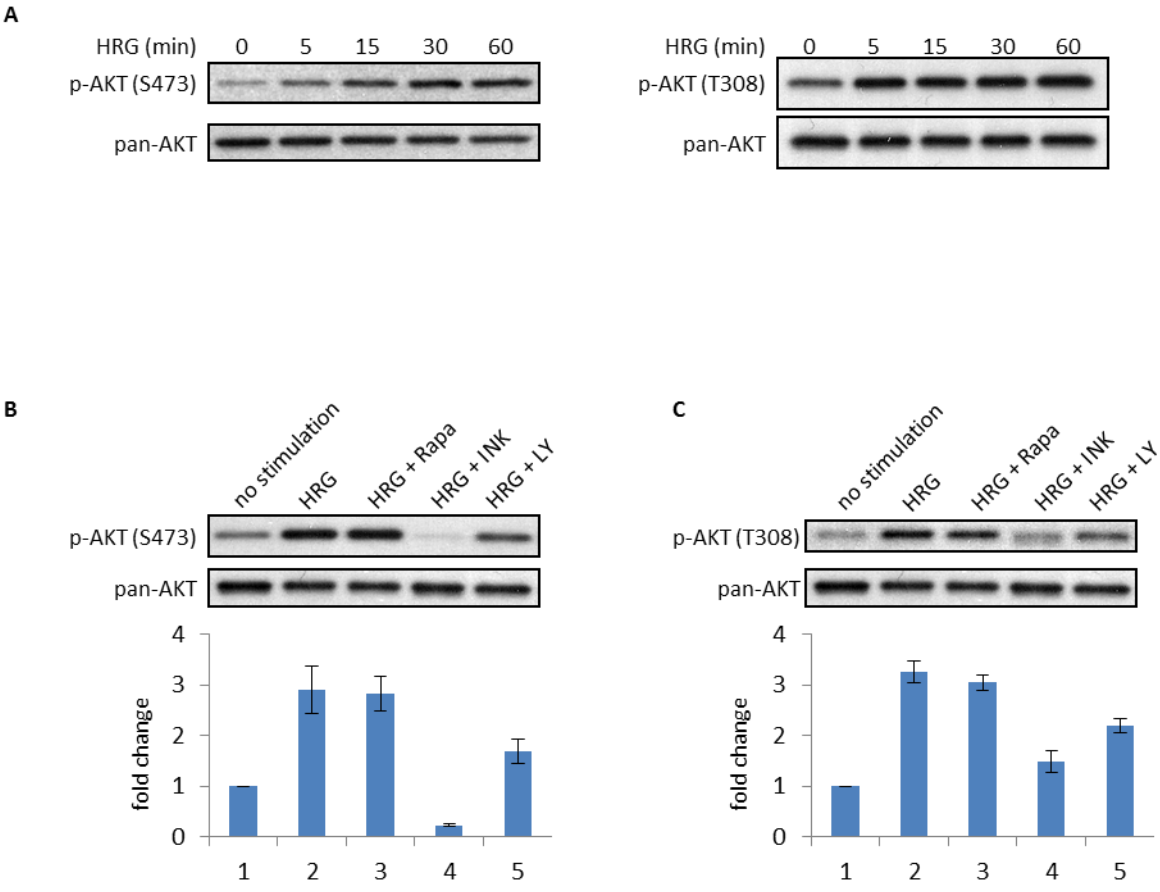
corresponding stimulation of mTORC1 activity due to increased Rheb-GTP levels, similar to what has been described for insulin-stimulated signaling [16]. To test this hypothesis, we examined the impact of rapamycin, INK-128 and LY294002 on phospho-mTOR (S2448) and phospho-TSC2 (T1462). SKBR3 cells were serum-starved for two days and then stimulated with HRG in the presence or absence of these inhibitors. Cell lysates generated from these cells were then analyzed by Western blotting. The expectation was that all three inhibitors should impact the phosphorylation of mTOR at S2448, whereas TSC2, as an up-stream regulator of mTOR, would be expected to be sensitive to PI3K inhibition. Indeed, this is the case for the phosphorylation of mTOR at S2448 (Fig. 2.2B). Each of the inhibitors was able to reduce the HRG-stimulated phosphorylation of mTOR. Also as anticipated, Figure 2.2C shows that rapamycin did not affect the ability of HRG to stimulate the phosphorylation of TSC2 at T1462, whereas the PI3K inhibitor, LY294002, inhibited TSC2 phosphorylation. What was surprising, however, was that INK-128 was as effective as LY294002 at inhibiting the phosphorylation of TSC2 (T1462) in response to HRG.

Since the short-term treatment of HRG-stimulated SKBR3 cells with rapamycin did not affect the phosphorylation of TSC2, whereas treatment with INK-128 reduced the phosphorylation, we could only reconcile these data by attributing the effects of INK-128 to the inhibition of mTORC2. This then raised the question of how mTORC2 might be feeding into the

mTORC1 pathway. As mentioned earlier, T1462 has been identified as an AKT-dependent phosphorylation site on TSC2 [30]. It is known that the phosphorylation of two amino acid residues on AKT (T308 and S473) are important for its activation [35], and often these sites have been used interchangeably as read-outs for PDK1-catalyzed AKT phosphorylation [36]. More recently it has been demonstrated that mTORC2 is a bona fide kinase for AKT by phosphorylating serine 473 [37], thus distinguishing these two phosphorylation sites on AKT. The time course for HRG-stimulation in SKBR3 cells revealed that the levels of both phospho-AKT (S473) and phospho-AKT (T308) were increased in response to the growth factor (Fig. 2.3A), suggesting that HRG not only promotes the phosphorylation of AKT at the PDK1 site as would be anticipated with the classical activation of the mTORC1 pathway, but it also is able to send signals to AKT via mTORC2.

To further understand how HRG is directing signals to AKT, we probed for the effects of rapamycin, INK-128 and LY294002 on the HRG-stimulated phosphorylation of AKT (S473) and AKT (T308). As shown in Figure 2.3B, rapamycin does not inhibit the phosphorylation of AKT (S473), similar to what we observed for TSC2 (T1462). In contrast, INK-128 leads to a dramatic decrease in AKT (S473) phosphorylation. LY294002 reduces the phosphorylation of AKT (S473) to approximately basal levels, consistent with suggestions that PI3K may play a role in signaling upstream of mTORC2 [27]. All three inhibitors showed some ability to impact the

Figure 2.3 mTORC2 is an intermediate in HRG-signaling. A. SKBR3 cells were serum-starved for 40-48 h followed by 0-60 min of 1 nM HRG stimulation. Whole cell lysates were collected and subjected to Western blotting with phospho-AKT (S473), phospho-AKT (T308) and pan-AKT antibodies. **B.** SKBR3 cells were serum-starved for 40-48 h. Cells were then pretreated with 50 nM rapamycin, 50 nM INK-128, or 10 μM LY294002 for 30 min followed by the corresponding inhibitor plus 1 nM HRG for 30 min. Whole cell lysates were collected and subjected to Western blotting with phospho-AKT (S473) and pan-AKT antibodies. The bar graph was generated by quantifying blots from three independent experiments using ImageJ and normalizing the intensity of the bands to the untreated lane. **C.** SKBR3 cells were treated as described above, followed by blotting with phospho-TSC2 (T1462) and pan-TSC2 antibodies. The bar graph was generated by quantifying blots from three independent experiments using ImageJ and normalizing the intensity of the bands to the untreated lane.


A
HRG (min) 0 5 15 30 60
p-AKT (S473)
pan-AKT
HRG (min) 0 5 15 30 60
p-AKT (T308)
pan-AKT
B
no stimulation
HRG
HRG + Rapa
HRG + INK
HRG + LY
p-AKT (S473)
pan-AKT
fold change
0 1 2 3 4
1 2 3 4 5
C
no stimulation
HRG
HRG + Rapa
HRG + INK
HRG + LY
p-AKT (T308)
pan-AKT
fold change
0 1 2 3 4
1 2 3 4 5

phosphorylation of AKT (T308) in response to HRG, although only INK-128 was able to reduce the phosphorylation at this site to near basal levels (Fig 2.3C), suggesting that phosphorylation of AKT at S473 may influence the phosphorylation at T308.

The experiments outlined in Figures 2 and 3 provide pharmacological evidence to suggest that mTORC2 may be playing a pivotal role in relaying signals arising from the interactions between HRG and ErbB receptors to mTORC1 in the promotion of the transformed phenotype. While INK-128 certainly appears to be having effects on signaling events which are distinct from rapamycin, thereby suggesting an involvement of mTORC2 in the regulation of the mTORC1 pathway, we cannot rule out the possibility that INK-128 is simply a more potent inhibitor of mTORC1 under the conditions used.

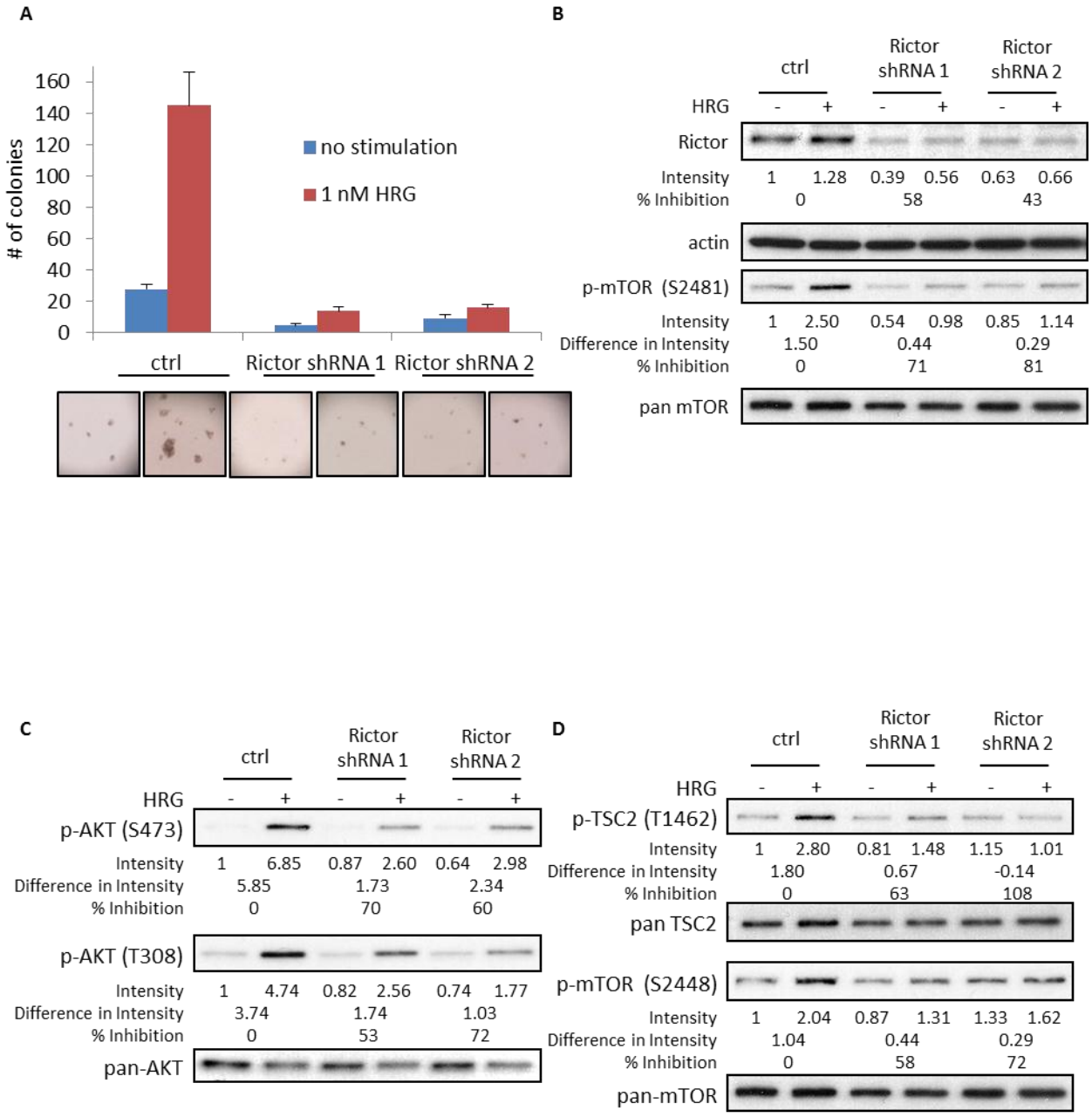
To distinguish these possibilities, we complimented the inhibitor studies with a genetic approach. Rictor is a key component of mTORC2 assembly and function, yet it is not present within mTORC1 [22]. Thus, by specifically targeting Rictor using an shRNA knock-down approach, we can specifically assess the role of mTORC2 in the HRG-stimulated transformation of SKBR3 cells and activation of mTORC1. The importance of mTORC2 in the transforming capability of HRG was examined in soft agar assays. SKBR3 cells were infected twice, one day apart, with a Rictor shRNA-carrying virus or a control virus, and cells were then selected with puromycin for 48 hours. Following selection, cells were seeded in soft agar and fed every three

days with regular growth medium in the presence or absence of 1 nM HRG until colonies were scored on day 13. As seen in Figure 2.4A, the cells in the control-infected plates formed colony growth in response to HRG. In contrast, colony formation did not occur in cells where Rictor had been knocked down, demonstrating that Rictor, and by extension mTORC2, is necessary for HRG to promote the transformed features of SKBR3 cells.

We next investigated the role of Rictor in relaying HRG-promoted signaling events. Cells were infected and selected as described above and then serum-starved for 2 days. After serum-starvation, cells were treated with or without 1 nM HRG for 30 minutes. The top panel of Figure 2.4B shows that the Rictor shRNAs achieved an approximately 50% knock-down of the Rictor protein as compared to the control samples. Examination of phospho-mTOR (S2481), a specific phosphorylation site on mTOR which has been shown to both be a marker for intact mTORC2 [38], as well as a rapamycin-insensitive autophosphorylation site [39], was next examined to determine how much intact mTORC2 is available to the cells following the knock-down of Rictor. There is a 70-80% inhibition of the phosphorylation of mTOR at S2481 between the two different sets of Rictor shRNAs (Fig. 2.4B, middle panel), indicating a significant loss of mTORC2 assembly without affecting total levels of mTOR protein (Fig. 2.4B, lower panel).

Having confirmed the efficacy of the Rictor knock-down on mTORC2 function in response

Figure 2.4 Rictor is essential for HRG-mediated colony formation and HRG-signaling to mTORC1 in SKBR3 cells. A. SKBR3 cells were infected with virus containing Rictor shRNA twice, one day apart, followed by 48 h selection with 2 μg/ml puromycin. Cells were then seeded in 0.3% agarose in complete medium with or without 1 nM HRG. Cells were fed every three days and colonies were counted on day 13. The experiment was done in triplicate and the results were averaged and graphed. **B.** SKBR3 cells were infected and selected as described above. Cells were then serum-starved for 40-48 h followed by stimulation with 1 nM HRG for 30 min. Whole cell lysates were collected and subjected to Western blotting. Blots were probed for Rictor, actin, phospho-mTOR (S2481) and pan-mTOR. Detailed quantification and calculation is described in Materials and Methods. **C.** SKBR3 cells were treated as described in Figure 2.4B. Blots were probed for phospho-AKT (S473), phospho-AKT (T308), and pan-AKT. **D.** SKBR3 cells were treated as described in Figure 2.4B. Blots were probed for phospho-TSC2 (T1462), pan-TSC2, phospho-mTOR (S2448), and pan-mTOR.


A
# of colonies
160
140
120
100
80
60
40
20
0
no stimulation
1 nM HRG
ctrl
Rictor shRNA 1
Rictor shRNA 2
B
ctrl
Rictor shRNA 1
Rictor shRNA 2
HRG - + - + - +
Rictor
Intensity 1 1.28 0.39 0.56 0.63 0.66
% Inhibition 0 58 43
actin
p-mTOR (S2481)
Intensity 1 2.50 0.54 0.98 0.85 1.14
Difference in Intensity 1.50 0.44 0.29
% Inhibition 0 71 81
pan mTOR
C
ctrl
Rictor shRNA 1
Rictor shRNA 2
HRG - + - + - +
p-AKT (S473)
Intensity 1 6.85 0.87 2.60 0.64 2.98
Difference in Intensity 5.85 1.73 2.34
% Inhibition 0 70 60
p-AKT (T308)
Intensity 1 4.74 0.82 2.56 0.74 1.77
Difference in Intensity 3.74 1.74 1.03
% Inhibition 0 53 72
pan-AKT
D
ctrl
Rictor shRNA 1
Rictor shRNA 2
HRG - + - + - +
p-TSC2 (T1462)
Intensity 1 2.80 0.81 1.48 1.15 1.01
Difference in Intensity 1.80 0.67 -0.14
% Inhibition 0 63 108
pan TSC2
p-mTOR (S2448)
Intensity 1 2.04 0.87 1.31 1.33 1.62
Difference in Intensity 1.04 0.44 0.29
% Inhibition 0 58 72
pan-mTOR

to HRG, we went on to examine the role of Rictor/mTORC2 in other HRG- stimulated signaling events. AKT phosphorylation is attenuated upon the loss of Rictor from HRG-stimulated cells (Figure 2.4C). Not only is there a decrease in AKT phosphorylation at the mTORC2 site (i.e., AKT (S473)), but phosphorylation at Thr308 of AKT (i.e. the PDK1 site) is significantly impacted as well. This again suggests the need for the priming of phospho-AKT (S473) in SKBR3 cells before AKT can be phosphorylated on threonine 308. Figure 2.4D shows the effects of the loss of Rictor on TSC2 (T1462) (top panel) and mTOR (S2448) (lower panel) phosphorylation in response to HRG. Destabilization of mTORC2 resulted in the abrogation of mTORC1 function as read out by the decrease in phosphorylation of TSC2 (T1462) and mTOR (S2448).

In total, these results indicate that mTORC2 plays a previously unappreciated role in HRG-promoted transformation via its ability to signal to mTORC1. While mTORC1 is described in the literature as a master signal-integrating complex due to its ability to receive signals from mitogens, nutrients and energy sources (reviewed in [16-18]), the role of mTORC2 is less well understood and largely limited to a role in regulating the cytoskeleton [22, 25].

One potential clue to understanding this cross-talk between mTORC2 and mTORC1 is a reported interaction between mTOR and TSC2 [40]. Given that we have shown TSC2 phosphorylation to be regulated by mTORC2 in response to HRG, and that TSC2 is an upstream

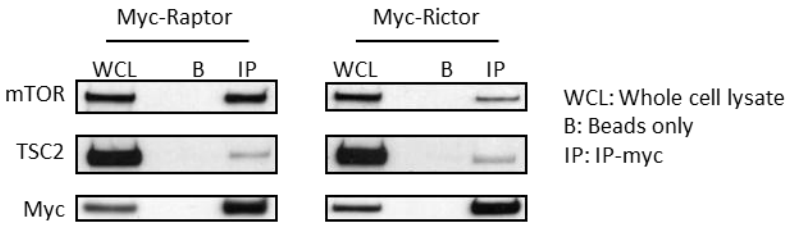
regulator of mTORC1, might TSC2 function as a scaffold to bring together the two mTOR complexes under certain cellular contexts? To investigate this possibility we utilized HEK 293T cells, a cell line which has been indispensable for the characterization of the different mTOR complexes [22, 24, 40], to determine whether both mTORC1 and mTORC2 might be capable of complexing with TSC2. HEK-293T cells were transiently transfected with either Myc-tagged Rictor or Myc-tagged Raptor (a distinct component of mTORC1) [24], and then these proteins were isolated from the resulting cell lysates by immunoprecipitation with an anti-Myc antibody. As expected, Myc-Raptor and Myc-Rictor were both able to precipitate mTOR (Figure 2.5A, top panel), representing the formation of mTORC1 and mTORC2, respectively. Further probing with an antibody targeting TSC2 revealed the ability of both complexes to bind to TSC2 (Figure 2.5A, middle panel), suggesting the possibility that TSC2 may provide a point of convergence between mTORC2 and mTORC1.

Discussion

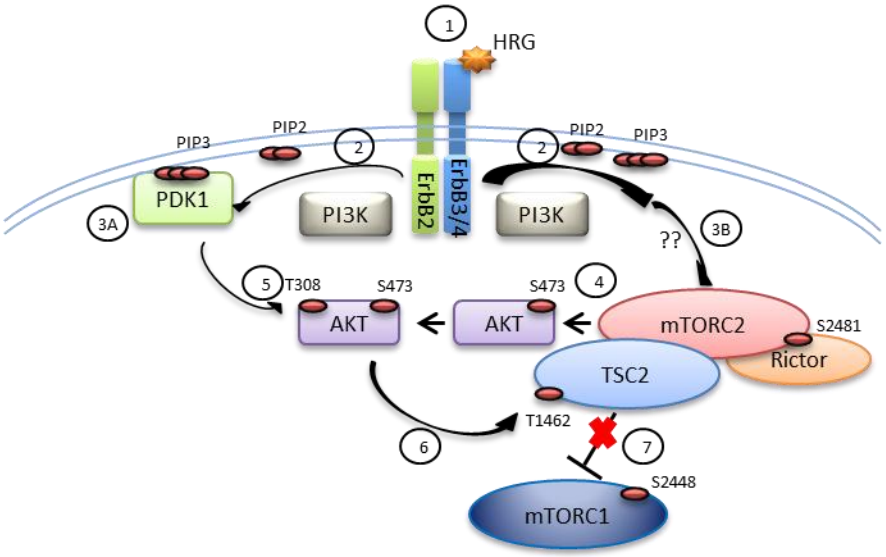
In the present study, we identify mTORC2 as a novel target of HRG/ErbB2 signaling that is critical for the ability of HRG to activate mTORC1. We originally anticipated that the activation of mTORC1 in response to HRG would be achieved via the canonical PI3K/PDK1/AKT pathway, but the use of mechanistically distinct mTOR inhibitors (i.e.

Figure 2.5 HRG signals through mTORC2 to mTORC1. A. HEK-293T cells were transfected with either Myc-Raptor or Myc-Rictor. Cells were then lysed in lysis buffer containing 0.3% CHAPS and subjected to immunoprecipitation using anti-Myc antibody to pull-down Raptor or Rictor. The precipitated samples were subjected to Western blotting probing for TSC2, mTOR, and Myc. **B.** Model for HRG-mediated signaling via mTORC2 to mTORC1. HRG/ErbB2 signals through an mTORC2-dependent pathway to phosphorylate AKT (S473). This phosphorylation precedes the phosphorylation at AKT (T308) by PDK1. Once AKT is fully activated, it phosphorylates TSC2 on multiple sites, sequestering TSC2 away from Rheb, allowing Rheb to stay active. Thus, mTORC1 is activated.

A



B



1. HRG binding to the ErbB3/4 receptor, heterodimerizes with ErbB2
2. PI3K gets activated and converts PIP2 to PIP3
3A. PDK1 gets recruited to the membrane
3B. mTORC2 gets activated through a PI3K-dependent mechanism
4. mTORC2 phosphorylates AKT (S473)
5. PDK1 phosphorylates AKT (T308), AKT becomes fully active
6. AKT phosphorylates TSC2 (T1462) and other sites on TSC2, making TSC2 unable to inactivate Rheb
7. Rheb stays in the GTP-bound state, allowing mTORC1 to be active

rapamycin and INK-128) and a PI3K inhibitor revealed a more complex signaling paradigm. The model in Figure 2.5B shows the HRG signal bifurcating at PI3K and then converging again at AKT where PI3K/PDK1/AKT (as indicated by the phosphorylation of AKT at T308) represents one branch of the HRG signal, and PI3K/mTORC2/AKT (i.e., phospho-AKT S473) delineates the other. Disruption of the mTORC2 arm of the pathway via the knock-down of Rictor significantly attenuates the activation of TSC2/mTORC1 by HRG (Figure 2.4D), arguing that the mTORC2 pathway is dominant to the PDK1 pathway in this HRG-invoked response.

The phosphorylation and activation of AKT, which in turn phosphorylates TSC2, enabling it to regulate Rheb, is an essential aspect of the activation of mTORC1 by growth factors [30, 36]. It is well known that both T308 and S473 are critical phosphorylation sites for AKT and that having both sites phosphorylated has a synergistic effect on AKT activation [29]. For many years the detection of phosphorylation at these two sites has been used interchangeably to interpret the activation of AKT by PDK1 [36]. The discovery by the Sabatini group that AKT (S473) is in fact a preferred mTORC2 substrate [37] opens the door for an important role for mTORC2 in mitogenic signaling [27]. Indeed we find the S473 site of AKT to be more effectively phosphorylated in response to HRG than the T308 site (Figure 2.3C). The phosphorylation of S473 is exquisitely sensitive to INK-128 but not rapamycin (Figure 2.3B) demonstrating the significance of mTORC2 activation by HRG. Also, the effectiveness of INK-128 in inhibiting the

phosphorylation of AKT at the PDK1 site (T308) suggests that phosphorylation of S473 is necessary to allow efficient phosphorylation at T308. This interpretation is consistent with the observed dominance of the mTORC2 pathway over the PDK1 pathway in the activation of mTORC1.

How is HRG signaling to mTORC2? Certainly how mTORC2 becomes activated by HRG and other growth factors is a question that will be garnering acute attention as the appreciation for the role of mTORC2 in mitogenic signaling grows. mTORC2 regulation and function is poorly characterized relative to mTORC1. The discrepancy in the understanding between the two complexes most likely stems from the long-standing use of rapamycin to specifically probe mTORC1 function. Prior to the realization that mTORC2 functions as an AKT kinase, mTORC2 was primarily noted for its role in cytoskeletal remodeling [22, 25]. Our data point to a role for PI3K in the activation of mTORC2, consistent with emerging suggestions that PI3K, as well as Ras, are upstream regulators of mTORC2 (reviewed in [16, 27], [41]).

The association of TSC2 with both Raptor and Rictor reported here makes TSC2 an attractive point of convergence for mTORC1 and mTORC2, especially given reports of distinct modes of regulation for mTORC1 and mTORC2 by TSC2. While the loss of TSC1-TSC2 complex from cells gives rise to the activation of mTORC1, consistent with a role for these proteins as negative regulators of mTORC1 [30, 42], TSC1-TSC2 deficiency attenuates

mTORC2 function, suggesting that the tuberous scleroses complex positively influences mTORC2 function [40]. In addition to our findings, it has also been shown that the N-terminus of TSC2 can interact with the C-terminus of Rictor [43]. Thus, TSC2 may serve as a biological bidirectional switch to bring the two complexes in close proximity to achieve signaling and feedback in an efficient manner, both temporally and spatially.

The observation that mTORC2 is necessary for HRG signaling to mTORC1 is underscored by the necessity of mTORC2 for HRG/ErbB2-dependent cellular transformation. Along with an emerging appreciation for the role of mTORC2 in mitogenic signaling is a nascent understanding of the importance of mTORC2 in tumorigenesis. mTORC2 was shown to be necessary for prostate cancer development in *Pten* deficient mice [26], as well as for the transformation of other cancer cells (i.e. glioma, breast cancer, colorectal cancer), while being less important to normal cells [27]. The fact that we find functional mTORC2 to be required for HRG to potentiate the transformation of SKBR3 cells, raises questions as to whether mTORC2 should be considered as a potential therapeutic target when addressing ErbB2-positive cancers (see below). While this study provides evidence for mTORC2 functioning as a signaling intermediary in a pathway from HRG to mTORC1, might mTORC2 play distinct roles that contribute to tumorigenesis? mTORC2 has the potential to promote cell migration and invasion of SKBR3 and other breast cancer cells in response to HRG through its function as a cytoskeletal remodeler [44,

45]. The Rac GTPase, well known for its participation in cell migration and cytoskeletal events, has been observed by us and others to become activated in response to HRG (data not shown, [46, 47]) and to associate with mTORC1 and mTORC2 to mediate the localization of both these complexes [48]. Additionally, a guanine nucleotide exchange factor for Rac, P-Rex1, which can function downstream of mTORC2 [49] has been implicated in breast cancer [50]. Future efforts will be directed toward distinguishing the different contributions of mTORC2 to cellular transformation.

The data presented in this study describe a pivotal role for mTORC2, as well as mTORC1, in the ability of HRG/ErbB2 to stimulate oncogenesis in the SKBR3 breast cancer cell line. Additionally, we show that the mTOR kinase inhibitor, INK-128 is effective not only at inhibiting mTOR (within the context of mTORC1 and mTORC2), but also at blocking PI3K inputs into AKT. Interestingly, the use of mTOR inhibitors (both rapalogs and kinase inhibitors) as a co-therapy with either trastuzumab or lapatinib is currently being investigated for cancers that are refractory to ErbB2-directed monotherapies [8, 51, 52], as aberrant PI3K/AKT/mTOR activity is one hallmark of ErbB2 therapy resistance [8, 9]. Our findings support the rationale of this therapeutic approach and would point to a greater efficacy with the use of dual mTORC1 and mTORC2 inhibitors.

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Chapter 3

Interplay of Small GTPases in Heregulin-Mediated Signaling to mTOR/S6 Kinase

Abstract

Heregulin-initiated signal transduction plays a role not only in the differentiation of normal cells, but has also been shown to be aberrantly activated in various types of human cancer. We have shown previously that HRG can activate both the nuclear cap-binding protein and the nuclear GTPase Ran in a mammalian target of rapamycin/S6 kinase-dependent manner. However, the processes that translate the interaction of HRG-activated receptors into the activation of mTOR/S6 kinase have yet to be elucidated. A number of GTP-binding proteins have been implicated in different aspects of mTOR signaling. Rheb (Ras-homology enriched in brain) is a GTPase thought to bind directly to and activate mTOR. The involvement of the Rag proteins is important for the localization of mTOR in amino acid sensing. More recently, Rac has been shown to interact with mTOR and localize mTOR to the leading edges of the cell. Here, we show that Rheb, Rac, and Cdc42 are necessary and sufficient participants in the HRG-mediated activation of mTOR/S6 kinase in the HeLa cervical carcinoma cell line. Using a knock-down and rescue approach, we have determined that Rac and Cdc42 each signal independently to the key

mTOR regulator, Rheb, in response to the stimulation of cells with HRG. These events are being coordinated by a unique mTORC1 scaffold, Dock7, which modulates the interplay between these GTPases and mTOR/S6 kinase. More surprisingly, we have identified Dock7 as a novel guanine nucleotide exchange factor for Rheb.

Introduction

In Chapter 2, we demonstrated that the HRG-dependent activation of mTORC2 plays a necessary role for the activation of mTORC1 in SKBR3 cells. In this chapter, we continue to investigate the signaling mechanisms that contribute to the HRG-dependent activation of mTORC1, focusing in particular on the roles of the Cdc42 and Rac GTPases in this event.

Small GTPases act as molecular switches in the cell [1]. They are activated by guanine nucleotide exchange factors (GEFs) in response to growth factor signaling, cell-extracellular matrix interactions, and cell-cell contact [1-4]. The intrinsic rate of GTP hydrolysis for small GTPases is slow but can be greatly enhanced by a GTPase activating protein (GAP) to turn-off signaling in a timely manner [5, 6]. Our laboratory has been interested in the Rho family of GTPases, namely, Cdc42 and Rac. De-regulation of Cdc42 causes cellular transformation due to prolonged signaling of the epidermal growth factor receptor (EGFR) [7, 8] . Moreover, we have also unveiled a Cdc42-mTOR link in the development of neural progenitor cells where Cdc42

signals through mTOR to up-regulate the expression of Hes5 and Pax6, which are transcription factors important for the maintenance of neuroepithelial and radial glial cells [9].

The mammalian target of rapamycin (mTOR) is a member of the PI-3 kinase-like kinase family (PIKK) and forms two functionally distinct complexes, mTORC1 and mTORC2 [10-12]. mTORC1 is involved in numerous cellular processes such as cell proliferation, autophagy, and metabolism [10-15]. It has often been regarded as the master regulator of the cell, as it can be activated according to the nutrient and energy status of the cell and also in response to growth factors [10-12]. mTORC2 is the other mTOR complex that was initially identified for its role in cytoskeletal remodeling [16, 17]. More recently, mTORC2 has also been shown to be involved in growth factor signaling and cellular transformation [18, 19]. The proper regulation of the mTOR complexes is critical in cells and aberrant activation of either mTORC1 or mTORC2 can lead to cancer progression [11, 19, 20].

Numerous small GTPases have been implicated in mTORC1 signaling. Rheb (Ras-homology enriched in brain), is a key activator of mTORC1 and is thought to bind mTOR directly, leading to the activation of its kinase activity through a poorly defined mechanism [21]. The Rag proteins are responsible for mTORC1 localization to the lysosome upon amino acid stimulation [22-26]. Rac has recently been shown to bind to both mTORC1 and mTORC2 to localize the complexes to the plasma membrane [27]. Also, the knock-out of Rac in mouse

embryonic fibroblasts (MEFs) causes a decrease in cell size, a hallmark of mTORC1 inactivation [27]. Previous work from our laboratory has suggested that Cdc42, as well as Rac, may play a role in propagating signals to mTORC1 in response to the treatment of cells with HRG (unpublished results). In this study, we have taken a systematic approach to define the roles of three GTPases, Rheb, Rac, and Cdc42, in the HRG-mediated activation of mTORC1.

First, we show that Rheb, Rac, and Cdc42 are necessary and sufficient participants in the HRG-mediated activation of mTOR/S6 kinase in the HeLa cervical carcinoma cell line. Using a knock-down and rescue approach, we find that Rac and Cdc42 each signal independently to the key mTORC1 regulator, Rheb, in response to the stimulation of cells with HRG. Dock7, a Rac and Cdc42 GEF, also plays a necessary role upstream of mTORC1 and is found in a complex with the Rheb GAP, TSC1/TSC2, and mTORC1. Unexpectedly, Dock7 binds to nucleotide-free forms of Rheb, and promotes the guanine nucleotide exchange of Rheb, as well as Cdc42 and Rac, providing evidence for Dock7 as a novel guanine nucleotide exchange factor for Rheb. Together, our results suggest that Dock7 is a unique scaffold that brings together the upstream activators of mTORC1 (i.e. Rheb, Rac and Cdc42), as well as the negative regulators of mTORC1 (i.e. TSC1/TSC2), to achieve the regulated activation of mTORC1 in response to HRG.

Materials and Methods

Antibodies and reagents

Phospho-mTOR (S2448), phospho-S6 kinase (T389), phospho-ERK, Rheb, Raptor, Rictor, and phospho-S6 antibodies were purchased from Cell Signaling Technology. Pan-mTOR, pan-S6 kinase, Cool-1, Rac, and Cdc42 antibodies were obtained from Millipore. Dock7 and Flag antibodies were purchased from Sigma. Myc and HA antibodies were from Covance and anti-actin was purchased from NeoMarker. HRG (Heregulin β, EGF domain, residues 178-241) was obtained from Sigma. Rapamycin was purchased from Calbiochem. Protein G beads were purchased from Invitrogen.

DNA constructs and siRNAs

Various Rac, Cdc42, and Rheb wild-type and point mutation constructs were cloned in our laboratory. Full-length Dock7 construct was cloned from SKBR3 cDNA into pcDNA3.1. TSC1, TSC2, and mTOR DNA constructs were obtained from Addgene [28]. siRNAs of Rheb, Rac, Cdc42, and Dock7 were purchased from Invitrogen.

Cell culture conditions

The cervical carcinoma cell line, HeLa, was maintained in RPMI 1640 (Invitrogen) containing 10% FBS (Invitrogen) at 37℃, 5% CO_2. For growth factor stimulation, HeLa cells were seeded at $7x10^5$ cells on 100 mm cell culture plates (Corning), followed by serum-starvation with RPMI

for 20-24 hours. HeLa cells were then stimulated with HRG at the concentration and times indicated, followed by cell lysis. HEK-293T cells were maintained in 10% FBS-containing DMEM (Invitrogen) at 37℃, 5% CO_2.

Transfection

HeLa cells were seeded at $7x10^5$ on 100 mm cell culture plates (Corning). Four μg of DNA were transfected into the cells the next day using Lipofectamine and Plus Reagent following the manufacturer's protocol (Invitrogen). The cells were recovered in complete medium for 3 hours followed by serum starvation for 20-24 hours. For knock-down experiments, HeLa cells were seeded as described above, with 2.5 nM siRNA being transfected into the cells the next day using Lipofectamine2000 following the manufacturer's protocol (Invitrogen). For rescue experiments, cells were immediately split onto 60 mm cell culture plates ($2.5x10^5$ cells) following siRNA transfection and allowed to recover overnight. One ug of DNA construct was then transfected as described above using Lipofectamine and Plus Reagent. HEK 293T cells were seeded at $3x10^6$ cells on 100 mm cell culture plates. Four μg of DNA was transfected into the cells the next day using Lipofectamine and Plus Reagent following the manufacturer's protocol (Invitrogen).

PBD assays

PBD beads were purchased from Millipore. Cells were cultured as described above. PBD assays were performed according to the manufacturer's protocol using a magnesium-containing lysis

buffer.

Immunoblot analysis

Cells were lysed with cell lysis buffer (50 mM Hepes, 150 mM NaCl, 1 mM $MgCl_2$, 25 mM NaF, 1 mM Na_3VO_4, 50 mM β-glycerophosphate, 10 μg/ml Leupeptin, 10 μg/ml Aprotinin, and 1% Triton X-100). The lysates were resolved by SDS-PAGE, and then the proteins were transferred to polyvinylidene fluoride membranes. The membranes were incubated with the indicated primary antibodies diluted in 20 mM Tris, 135 mM NaCl, and 0.02% Tween-20. The primary antibodies were detected with horseradish peroxidase-conjugated secondary antibodies (GE Healthcare) followed by exposure to ECL reagent (Perkin Elmer).

Immunoprecipitation

293T cells were lysed with cell lysis buffer (50 mM Hepes, 150 mM NaCl, 1 mM $MgCl_2$, 25 mM NaF, 1 mM Na_3VO_4, 50 mM β-glycerophosphate, 10 μg/ml Leupeptin, 10 μg/ml Aprotinin, 0.3% CHAPS). Lysates were pre-cleared with Protein G beads on a rotator at 4℃ for 15 min. The supernatant was collected and added with anti-tag (Myc, HA, or Flag) antibody for 2 h followed by the addition of Protein G beads for 1 h at 4℃. Immunoprecipitates were washed 3 times with the lysis buffer followed by the addition of 2X Laemilli buffer. For nucleotide binding to HA-tagged Cdc42, Rac, and Rheb, cells were transfected and lysed as described above. Cell lysates were then treated with 10 mM EDTA, 10 mM EDTA + 1 mM GDP, or 10 mM EDTA +

100 μM GTPγS at RT for 15 min followed by immunoprecipitation as described above.

GEF activity assays

Full-length Dock7 was transfected into Cos7 cells, followed by purification using Cobalt beads according to the manufacturer's protocol (Talon). Recombinant Rheb was purified from BL21(DE3) using 1x HMN buffer (20 mM Hepes, 5 mM $MgCl_2$, 100 mM NaCl). Final concentrations used for the reaction were as follows: 500 nM RhebWT, 5 μM GTPγS, ~100 nM/~200 nM Dock7 (estimated) to a total of 400 μl in 1x HMN buffer. For the EDTA control, 20 mM EDTA and 125 mM $MgCl_2$ were added immediately before starting the reaction. 50 μl samples were taken from the following time points: 2, 5, 10, 20, 30, 45, 60 min and filtered through nitrocellulose membranes (Schleicher & Schuell, BA85) followed by HMN buffer rinses. The amount of [^{35}S]GTPγS-incorporated Rheb was quantified by scintillation counting using a LS 6500 Scintillation System (Beckman). The graph was then normalized to EDTA positive control as 100% GTPγS incorporation.

ImageJ quantification

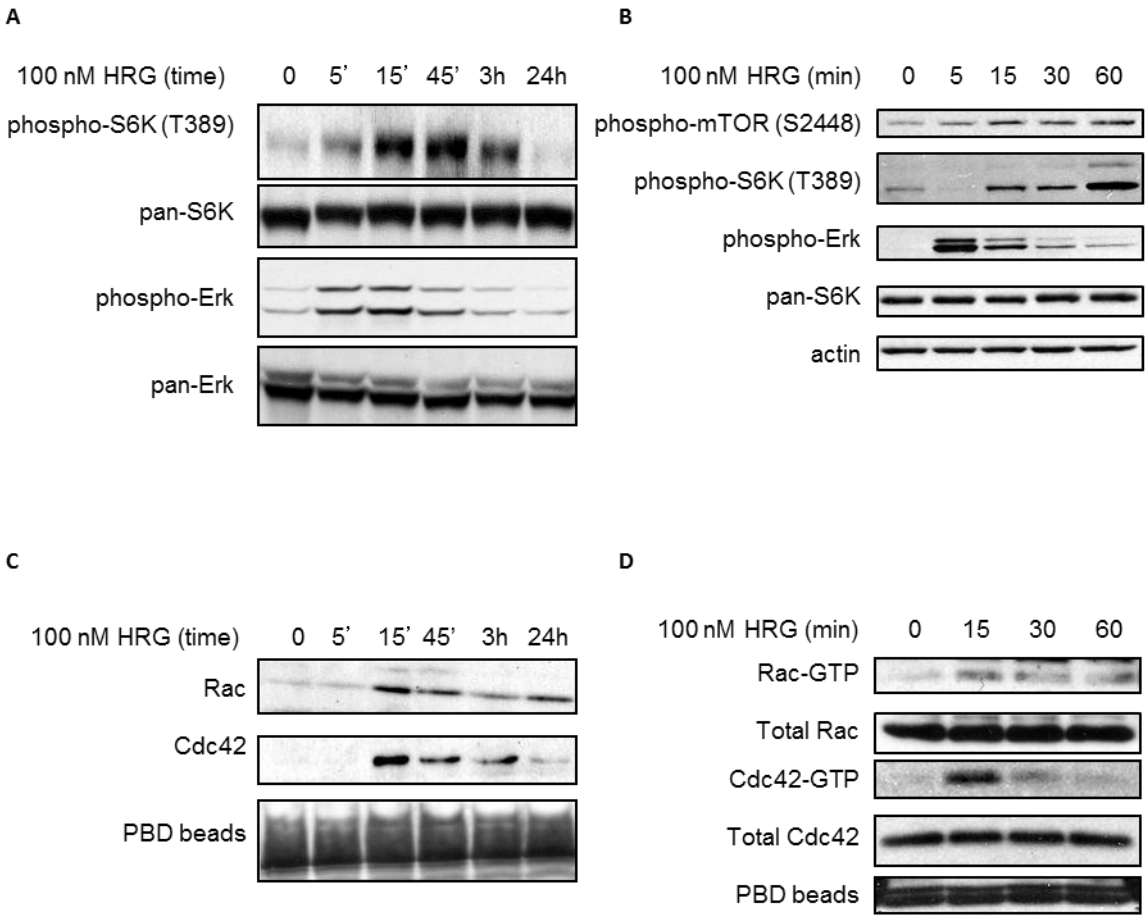
The Western blots were quantified using ImageJ (http://rsbweb.nih.gov/ij/) under the Gel Analysis Tool. The intensity of the different lanes was then normalized to the control lane, which was set to one.

Results

As discussed in Chapter 2, we observe the activation of participants in mTOR signaling, including S6 kinase, when the SKBR3 breast cancer cell line is stimulated with HRG. An example for this is shown in Figure 3.1A, where SKBR3 cells were serum-starved and then stimulated with HRG for the times indicated. Erk is rapidly phosphorylated in response to HRG, while S6 kinase phosphorylation (an indicator of mTORC1 activation (reviewed in [29])) is more gradual and sustained. A similar pattern can also be observed in HeLa cells, another HRG-sensitive cell line, where both S6 kinase and mTOR phosphorylation are gradual and sustained in contrast to the rapid and transient activation of ERK (Figure 3.1B).

We, and others have demonstrated that Rac and Cdc42 can signal to S6 kinase (unpublished results, [30, 31]), and Rac has been shown to bind to mTORC1 and mTORC2 [27]. Thus, we were interested in determining if these GTPases are involved in the ability of HRG to activate mTORC1 signaling. As a first step, we sought to determine whether Cdc42 and/or Rac could be activated in response to HRG, as read-out by a PBD (p21-binding domain of the Cdc42/Rac effector, Pak) assay that specifically detects the GTP-bound forms of Cdc42 and Rac [32]. SKBR3 and HeLa cells were serum-starved and then stimulated with HRG for the times indicated (Figures 3.1C and 3.1D). The cells were then lysed in a HEPES-based, magnesium-containing lysis buffer followed by a PBD pull-down. The precipitated proteins were

Figure 3.1 mTOR/S6 kinase and Rac/Cdc42 are activated in response to HRG in SKBR3 and HeLa cells. A. SKBR3 cells were serum-starved for 40-48 h and stimulated with 100 nM HRG for the times indicated. Whole cell lysates were collected and the activation of mTOR, S6 kinase, and ERK was detected by performing Western blotting with phospho-specific antibodies for mTOR (Ser2448), S6 kinase (Thr389), and ERK (Tyr202/Tyr204). **B.** HeLa cells were serum-starved overnight and stimulated with 100 nM HRG for the times indicated. The activation of mTOR, S6 kinase, and ERK was detected by performing Western blotting as described above. **C.** SKBR3 cells were serum-starved 40-48 h and stimulated with 100 nM HRG for the times indicated. Whole cell lysates were collected using a Mg^{2+}-containing buffer to preserve the nucleotide binding states of Rac and Cdc42. GST-PBD (p21 binding domain of PAK) beads were then used to pull-down only the activated forms of Rac and Cdc42. The pulled-down products were then subjected to Western blotting for Rac and Cdc42. **D.** HeLa cells were serum-starved overnight and stimulated with 100 nM HRG for the times indicated. PBD assays were performed as described above. **E.** HeLa cells were transfected with either wild-type Rheb, constitutively active RacQ61L, or constitutively active Cdc42Q61L followed by overnight serum-starvation. Whole cell lysates were collected and subjected to Western blotting with phospho-mTOR (S2448), phospho-S6 kinase (T389), pan-S6 kinase, Rheb, Rac, and Cdc42 antibodies.


A
100 nM HRG (time)
0
5'
15'
45'
3h
24h
phospho-S6K (T389)
pan-S6K
phospho-Erk
pan-Erk
B
100 nM HRG (min)
0
5
15
30
60
phospho-mTOR (S2448)
phospho-S6K (T389)
phospho-Erk
pan-S6K
actin
C
100 nM HRG (time)
0
5'
15'
45'
3h
24h
Rac
Cdc42
PBD beads
D
100 nM HRG (min)
0
15
30
60
Rac-GTP
Total Rac
Cdc42-GTP
Total Cdc42
PBD beads
E
mock
mock + HRG
RhebWT
RacQ61L
Cdc42Q61L
phospho-mTOR (S2448)
phospho-S6K (T389)
pan-S6K
Rheb (short exposure)
Rheb (long exposure)
Rac
Cdc42

subjected to Western blotting, probing for Rac and Cdc42. In both cell types, Rac and Cdc42 were activated within 15 minutes of HRG treatment. The timing and the level of Rac and Cdc42 activation in response to HRG appeared to be consistent with the activation of mTOR and S6 kinase.

We next wanted to confirm that Cdc42 and Rac are sufficient to activate mTOR and S6 kinase. To this end, we introduced constitutively active forms of Rac (RacQ61L) and Cdc42 (Cdc42Q61L) into HeLa cells. As a positive control, we also expressed a wild-type allele of the Rheb GTPase. Introducing exogenous, wild-type Rheb is sufficient to activate mTOR, and in turn, S6 kinase [21, 33, 34]. Following transfection, cells were serum-starved for 16-20 hours, and as an additional positive control, mock control cells were stimulated with HRG for 60 minutes prior to harvesting. Figure 3.1E shows that individually over-expressing either RhebWT, RacQ61L, or Cdc42Q61L in HeLa cells is sufficient to activate mTOR and S6 kinase under serum-starved conditions. It also shows that under such conditions, the degree of S6 kinase activation by RhebWT, RacQ61L, and Cdc42Q61L is comparable to the treatment of cells with HRG.

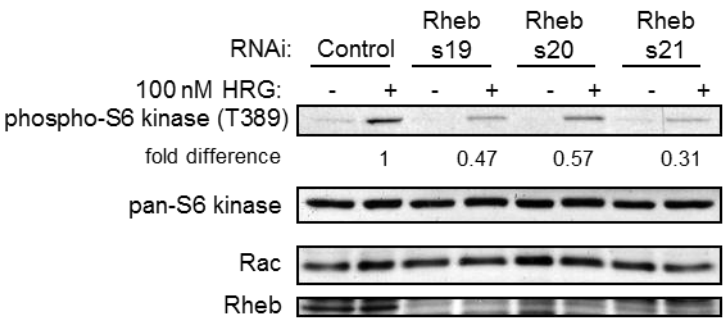
Having demonstrated that Rheb, Cdc42 and Rac are sufficient to activate mTOR and S6 kinase, we determined whether these GTPases play a necessary role in transducing the HRG signal to mTORC1. To do this, HeLa cells were transfected with small-interfering RNA (siRNA)

for either Rheb, Rac, or Cdc42. Five hours after the transfection, cells were recovered in full serum for 24 hours and then serum-starved, followed by HRG stimulation. Scrambled siRNA was used as negative control. Figure 3.2A shows that the siRNA successfully knocked-down Rheb expression in HeLa cells. In the lanes where Rheb was knocked-down, a decrease in S6 kinase activation by HRG was also observed, confirming that Rheb is necessary for the activation of mTOR and S6 kinase by HRG. The knockdowns of Rac and Cdc42 showed similar results, indicating that both Rac and Cdc42 are also necessary for HRG's ability to activate mTOR and S6 kinase (Figures 3.2B and 3.2C). Curiously, despite the high degree of efficiency of each of the individual knockdowns, none of the knockdowns were able to dampen the HRG effect by more than approximately fifty percent, suggesting the possibility of an inter-play amongst these three GTPases in mediating the HRG response.

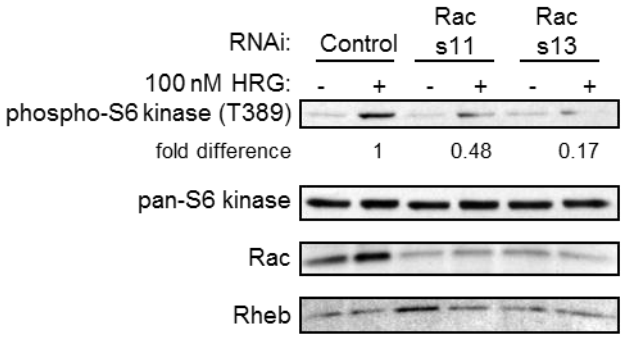
We designed a series of knock-down/rescue experiments to methodically dissect what relationships might exist between these GTPases in this pathway. First, we started by knocking-down Rac or Cdc42, followed by transfection of RhebWT (rescue). In short, HeLa cells were transfected with either scrambled siRNA, Rac or Cdc42 siRNA for 5 h, and after an overnight recovery period, were transfected with RhebWT. Cells were then serum-starved for 16-20 h and whole cell lysates were collected and subjected to Western blotting. Figures 3.3A and 3.3B show that RhebWT is able to rescue both the Rac and Cdc42 knocked-down in cells,

Figure 3.2 Rheb, Rac, and Cdc42 are necessary for the HRG-mediated activation of mTOR/S6 kinase. A. HeLa cells were transfected with either control or Rheb siRNA. Twenty-four hours after the transfections, cells were serum-starved overnight and then treated with or without 100 nM HRG for 60 minutes. Whole cell lysates were collected and subjected to Western blotting with phospho-S6 kinase, pan-S6 kinase, Rac, and Rheb antibodies. The phospho-S6 kinase blot was quantified using ImageJ. Relative intensities of the bands were calculated relative to the control siRNA/HRG-stimulated lane, which was normalized to one. **B.** HeLa cells were transfected with either control or Rac siRNA. Cells were treated as described above. The phospho-S6 kinase blot was quantified as described above. **C.** HeLa cells were transfected with either control or Cdc42 siRNA. Cells were treated as described above. Phospho-S6 kinase blot was quantified as described above.

A


RNAi:
Control
Rheb s19
Rheb s20
Rheb s21
100 nM HRG:
- + - + - + - +
phospho-S6 kinase (T389)
fold difference
1
0.47
0.57
0.31
pan-S6 kinase
Rac
Rheb

B


RNAi:
Control
Rac s11
Rac s13
100 nM HRG:
- + - + - +
phospho-S6 kinase (T389)
fold difference
1
0.48
0.17
pan-S6 kinase
Rac
Rheb

C

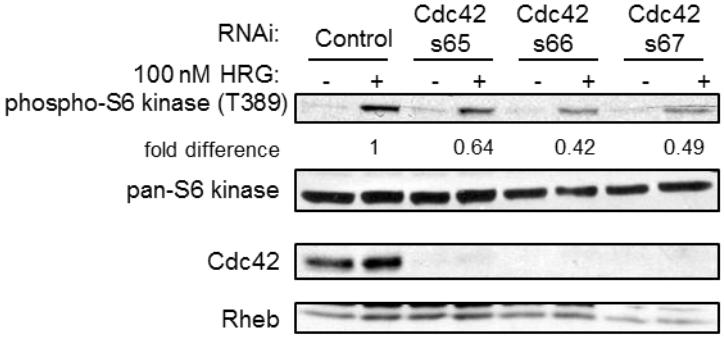
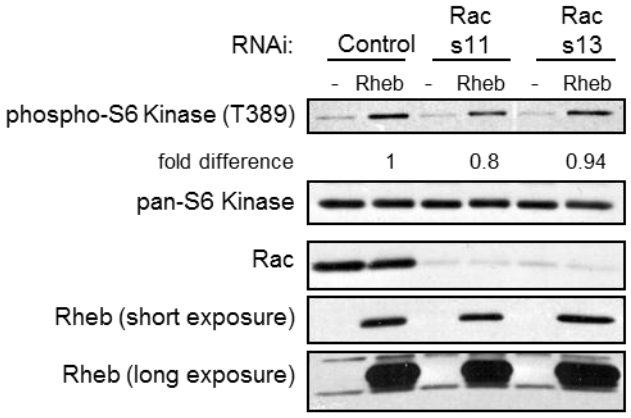

RNAi:
Control
Cdc42 s65
Cdc42 s66
Cdc42 s67
100 nM HRG:
- + - + - + - +
phospho-S6 kinase (T389)
fold difference
1
0.64
0.42
0.49
pan-S6 kinase
Cdc42
Rheb

Figure 3.3 Knock-down and over-expression of the small GTPases show that Rac and Cdc42 are mutually exclusive in their ability to signal to mTOR/S6 kinase via Rheb. A. HeLa cells were transfected with either control or Rac siRNA. Twenty hours after siRNA transfections, RhebWT DNA was transfected into the cells. Cells were then serum-starved overnight and whole cell lysates were collected and subjected to Western blotting for phospho-S6 kinase, pan-S6 kinase, Rac, and Rheb. The phospho-S6 kinase blot was quantified using ImageJ. Relative intensities of the bands were calculated relative to the control siRNA/RhebWT-over-expression lane, which was normalized to one. **B.** HeLa cells were transfected with either control or Cdc42 siRNA. Twenty hours after siRNA transfection, RhebWT DNA was transfected into the cells. Cells were then serum-starved overnight and whole cell lysates were collected and subjected to Western blotting probing for phospho-S6 kinase, pan-S6 kinase, Cdc42, and Rheb. The phospho-S6 kinase blot was quantified using ImageJ. Relative intensities of the bands were calculated relative to the control siRNA/RhebWT-over-expression lane, which was normalized to one. **C.** HeLa cells were transfected with either control or Rheb siRNA. Twenty hours after siRNA transfection, RacQ61L DNA was transfected into the cells. Cells were treated, probed, and quantified as described above. Relative intensities of the bands were calculated relative to the control siRNA/RacQ61L-over-expression lane, which was normalized to one. **D.** HeLa cells were transfected with either control or Rheb siRNA. Twenty hours after siRNA transfection, Cdc42Q61L DNA was transfected into the cells. Cells were treated, probed, and quantified as described above. Relative intensities of the bands were calculated relative to the control siRNA/Cdc42Q61L-over-expression lane, which was normalized to one. **E.** HeLa cells were transfected with either control or Rac siRNA. Twenty hours after siRNA transfection, Cdc42Q61L DNA was transfected into the cells. Cells were treated, probed, and quantified as described above. Relative intensities of the bands were calculated relative to the control siRNA/Cdc42Q61L-over-expression lane, which was normalized to one. **F.** HeLa cells were transfected with either control or Cdc42 siRNA. Twenty hours after siRNA transfection, RacQ61L DNA was transfected into the cells. Cells were treated, probed, and quantified as described above. Relative intensities of the bands were calculated relative to the control siRNA/RacQ61L-over-expression lane, which was normalized to one.

A


RNAi:
Control
Rac s11
Rac s13
- Rheb - Rheb - Rheb
phospho-S6 Kinase (T389)
fold difference
1
0.8
0.94
pan-S6 Kinase
Rac
Rheb (short exposure)
Rheb (long exposure)

B

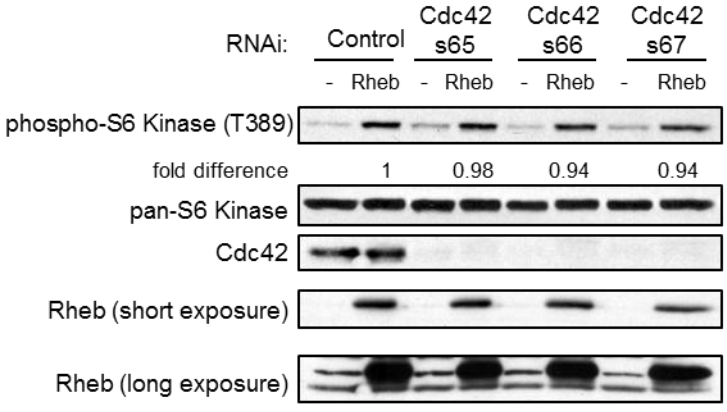
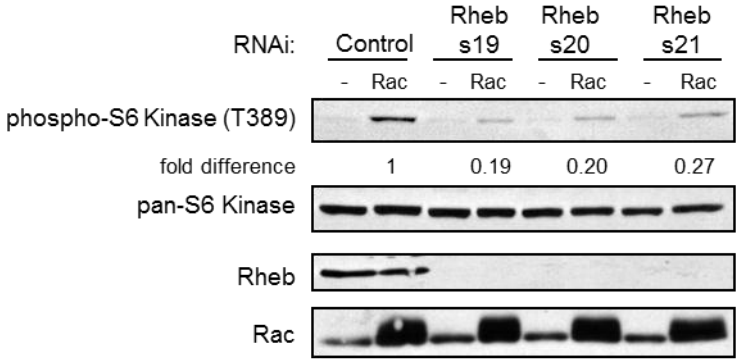

RNAi:
Control
Cdc42 s65
Cdc42 s66
Cdc42 s67
- Rheb - Rheb - Rheb - Rheb
phospho-S6 Kinase (T389)
fold difference
1
0.98
0.94
0.94
pan-S6 Kinase
Cdc42
Rheb (short exposure)
Rheb (long exposure)

Figure 3.3 (continued)

C



D

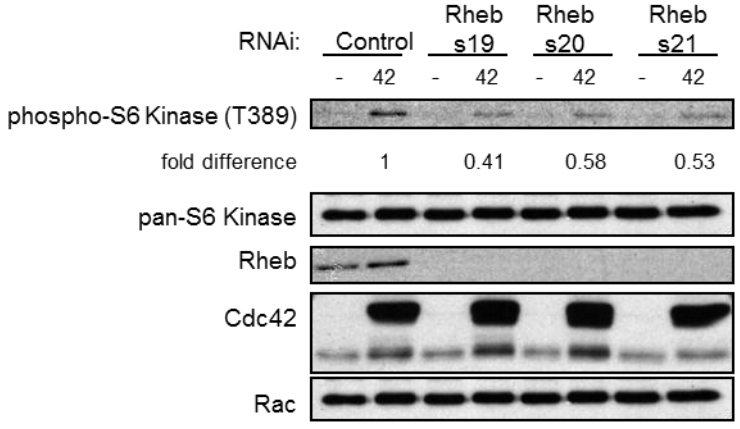
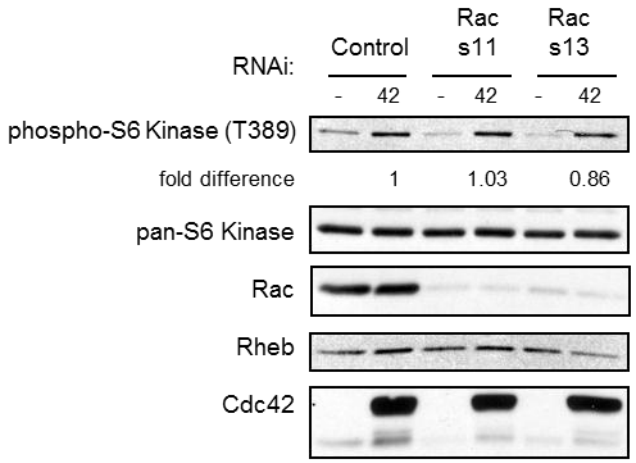
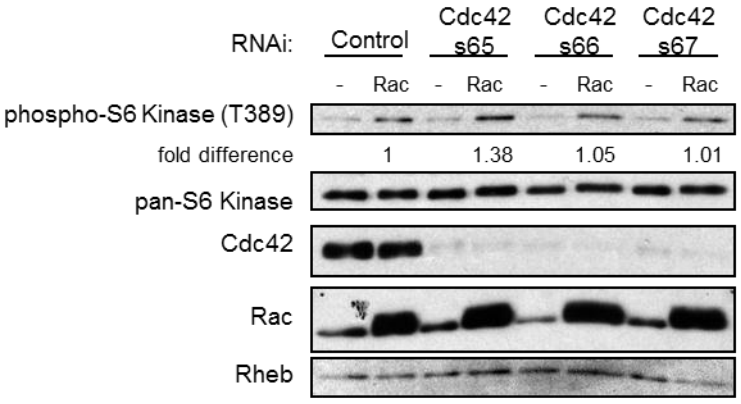


Figure 3.3 (continued)

E



F



demonstrating that Rheb is able to rescue the ability of Rac and Cdc42 to activate mTOR/S6 kinase.

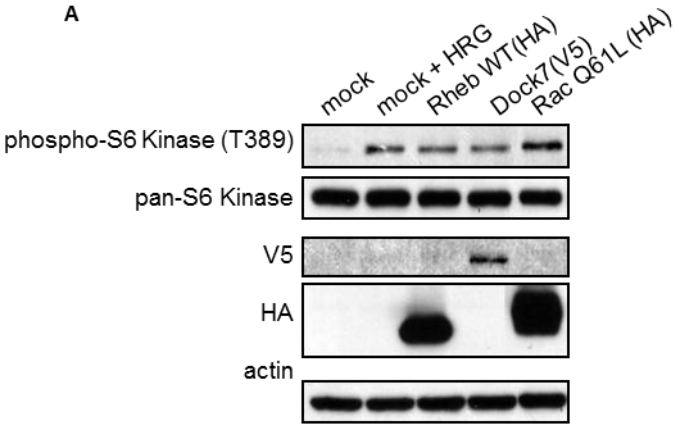
We then performed the reciprocal experiment where we knocked down Rheb and then attempted a rescue with either RacQ61L or Cdc42Q61L (Figures 3.3C and 3.3D, respectively). The knock-down of Rheb significantly attenuated the ability of both RacQ61L and Cdc42Q61L to signal to S6 kinase, again suggesting that Rheb is the immediate activator of mTOR and that Rac and Cdc42 are working through Rheb in this activation event. It is interesting to note that over-expression of activated-Cdc42 was less impaired by Rheb knock-down, suggesting that Rac and Cdc42 may have independent feed-ins to Rheb.

Having determined that Rac and Cdc42 function upstream of Rheb, we then wanted to distinguish between the possibilities that Rac and Cdc42 signal to mTOR/S6 kinase independently of one another or that they function in the same pathway. First we knocked-down Rac, and then over-expressed Cdc42Q61L. As shown in Figure 3.3E, Cdc42 is able to rescue the Rac knock-down. This raises two possibilities: Cdc42 is either downstream of Rac or that Cdc42 can work independently of Rac and compensate for its knock-down to activate mTOR/S6 kinase. We went on to knock-down Cdc42 and examine the effects of RacQ61L over-expression and found that activated Rac was sufficient to rescue the ability of Cdc42 to activate S6 kinase (Figure 3.3F). Taking Figures 3.3E and 3.3F together, it appears that Cdc42 and Rac are mutually

exclusive in their ability to signal to mTOR/S6 kinase via Rheb under conditions of over-expression, and that each GTPase can compensate for the loss of the other.

Since both Cdc42 and Rac can signal through Rheb to activate mTORC1, we next set forth to understand the mechanism underlying these relationships. We found a potential clue in the guanine nucleotide exchange factor (GEF), Dock7, which has been reported to be phosphorylated and activated by ErbB2 (downstream of HRG) to function on Cdc42 and Rac [35, 36]. To test a possible role for Dock7 in our system, we first examined whether full-length Dock7 is sufficient to activate S6 kinase. HeLa cells were transiently transfected with RhebWT, Dock7 or RacQ61L and then serum-starved for 20 hours. As an additional positive control, mock-transfected cells were stimulated with HRG for 1 hour prior to harvesting. Cell lysates were generated and then probed for the phosphorylation of S6 kinase (Figure 3.4A). Dock7 alone is able to activate S6 kinase to an extent similar to HRG, Rheb and Rac. We also tested whether Dock7 is necessary in activating S6 kinase in response to HRG stimulation. HeLa cells were transfected with either scrambled siRNA or Dock7 siRNA followed by recovery and serum-starvation. Cells were stimulated with HRG for 1 h before harvesting and lysates were subjected to Western blotting. Figure 3.4B shows that knocking-down Dock7 expression inhibits the HRG-mediated activation of S6 kinase as well as its downstream target, S6. Together, these data suggest an involvement of Dock7 in HRG-mediated activation of mTORC1.

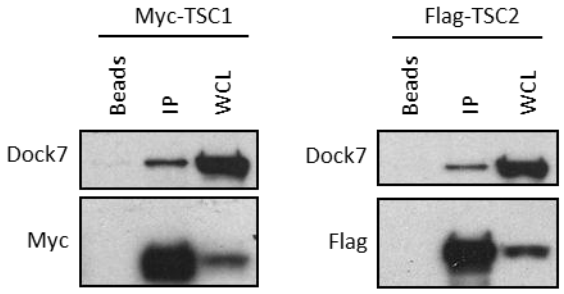
Figure 3.4 Dock7 is sufficient and necessary for HRG-mediated mTOR/S6 kinase activation. **A.** HeLa cells were transfected with either mock, wild-type Rheb, Dock7, or constitutively active RacQ61L followed by overnight serum-starvation. Mock-transfected cells stimulated with 100 nM HRG for 30 min before harvesting were used as a positive control for S6 kinase activation. Whole cell lysates were collected and subjected to Western blotting with phospho-S6 kinase (T389), pan-S6 kinase, V5, HA, and actin antibodies. **B.** HeLa cells were transfected with either control or Dock7 siRNA. Twenty-four hours after the transfection, cells were serum-starved overnight and then treated with or without 100 nM HRG for 60 minutes. Whole cell lysates were collected and subjected to Western blotting with Dock7, phospho-S6 kinase, pan-S6 kinase, phospho-S6, and actin antibodies.


A
mock
mock + HRG
Rheb WT(HA)
Dock7(V5)
Rac Q61L (HA)
phospho-S6 Kinase (T389)
pan-S6 Kinase
V5
HA
actin
B
RNAi:
control
Dock7
100 nM HRG
-
+
-
+
Dock7
phospho-S6K
pan-S6K
phospho-S6
actin

We then wanted to determine how Dock7 may be playing a role in the context of mTORC1 signaling. Dock7 was previously identified as a novel binding partner for TSC1/TSC2, the Rheb GAP [37]. We proceeded to validate the interaction between Dock7, TSC1, and TSC2 in HEK-293T cells, as these cells are routinely used for dissecting the components of mTOR complexes [17, 38, 39]. 293T cells were transfected either with Myc-TSC1 or Flag-TSC2. Cell lysates were then collected and the expressed proteins were immunoprecipitated with either Myc- or Flag- antibodies followed by Western blotting and probing for Dock7. In Figure 3.5A, Dock7 is clearly found associated with both TSC1 and TSC2. Based on our observation in Chapter 2 that TSC2 is able to bind to mTOR, we suspected that Dock7 may also associate within a larger TSC1/TSC2/mTOR complex. As shown in the left panel of Figure 3.5B, Myc-mTOR, when transfected into and immunoprecipitated from HEK-293T cells, is able to associate with Dock7. To investigate whether these Dock7 interactions existed within the context of mTORC1, we probed for an interaction between Dock7 and the mTORC1-specific scaffold, Raptor [38]. Myc-tagged Raptor was transfected into HEK-293T cells, followed by immunoprecipitation with anti-Myc antibody to precipitate Raptor. As shown in the right panel of Figure 3.5B, Dock7, as well as mTOR, is able to bind to Raptor, indicating that Dock7 is a component of mTORC1. However, the interaction of Dock7 with Raptor appears to be weaker than the interaction between Dock7 and mTOR. This suggests that the interaction of Raptor with

Figure 3.5 Dock7 and nucleotide-free Rac and Cdc42 form complexes with TSC1/TSC2 and mTORC1. A. HEK-293T cells were transfected with either Myc-TSC1 or Flag-TSC2. Twenty-four hours after the transfection, cells were collected with buffer containing 0.3% CHAPS. Cells were then immunoprecipitated using anti-Myc or anti-Flag antibodies. The immunoprecipitates were subjected to Western blotting for Dock7, Myc-TSC1, or Flag-TSC2. IP: immunoprecipitate. WCL: whole cell lysate. **B.** HEK-293T cells were transfected with either Myc-mTOR or Myc-Raptor. Twenty-four hours after the transfection, cells were collected with buffer containing 0.3% CHAPS. Cells were then immunoprecipitated using anti-Myc antibodies. The immunoprecipitates were subjected to Western blotting for Dock7, Myc-mTOR, Myc-Raptor, or endogenous mTOR. **C.** HEK-293T cells were transfected with Myc-Raptor. Twenty-four hours after the transfection, cells were subjected to a second round of transfections with either mock, RacT17N, or Cdc42T17N. Whole cell lysates were collected the following day with buffer containing 0.3% CHAPS and then immunoprecipitated using anti-Myc antibody. The immunoprecipitates were subjected to Western blotting for HA-RacT17N, HA-Cdc42T17N, mTOR, and Myc-Raptor.

A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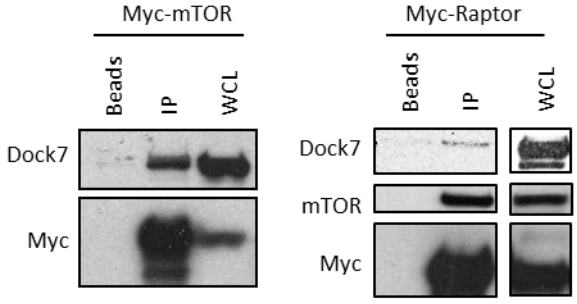
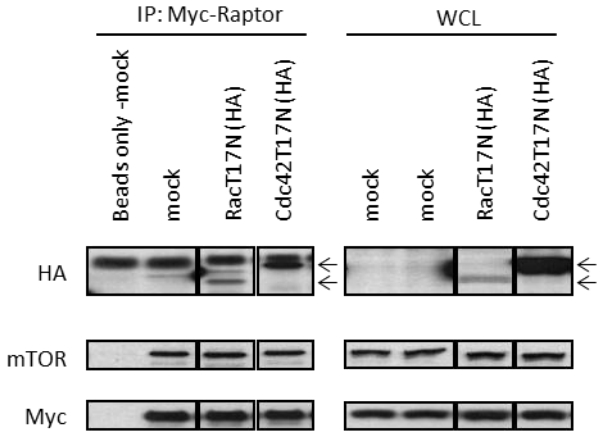
Flag-TSC2
Beads
IP
WCL
Beads
IP
WCL
Dock7
Myc
Dock7
Flag

B


Myc-mTOR
Myc-Raptor
Beads
IP
WCL
Beads
IP
WCL
Dock7
Myc
Dock7
mTOR
Myc

C

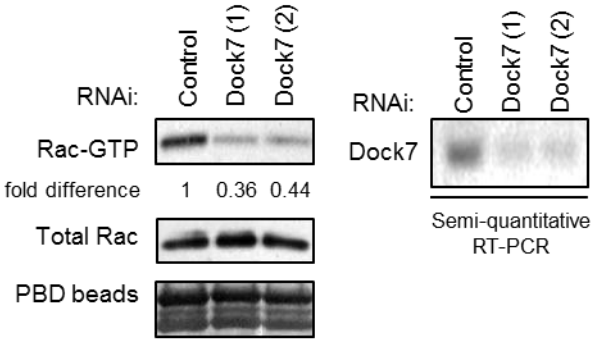

IP: Myc-Raptor
WCL
Beads only -mock
mock
RacT17N (HA)
Cdc42T17N (HA)
mock
mock
RacT17N (HA)
Cdc42T17N (HA)
HA
mTOR
Myc

Dock7 may be occurring predominantly in an mTOR-dependent manner. Finally, we examined whether Rac and Cdc42 are binding to the Dock7-mTOR-TSC1/2 complex in an mTORC1-dependent manner. We co-transfected Raptor, along with either RacT17N or Cdc42T17N into 293T cells. Given that Dock7 is a Cdc42/Rac GEF, we expected the nucleotide-free forms of Rac and Cdc42 to interact with mTORC1. Indeed, shown in Figure 3.5C, Rac and Cdc42 are binding to immunoprecipitated Raptor in a nucleotide-free manner.

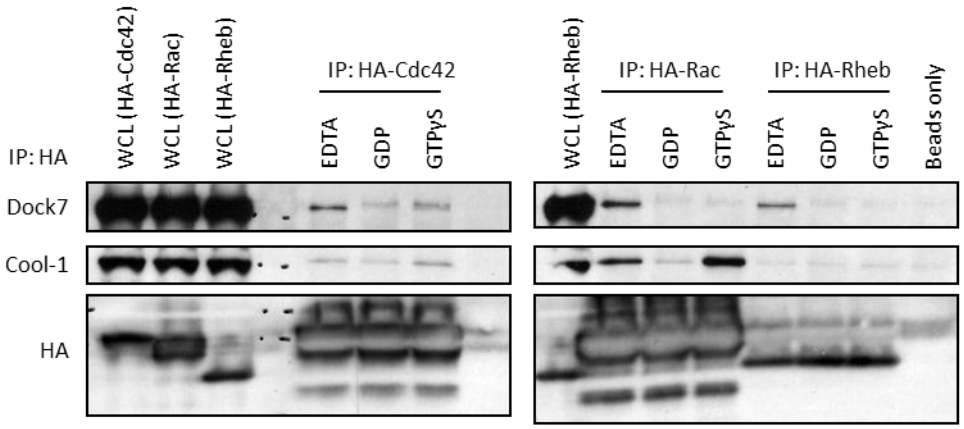
Thus far, we have shown that Dock7 is necessary in HRG-signaling to mTORC1. It also shows scaffolding properties for mTORC1 signaling components, including TSC1/2, mTOR, and Raptor, and can recruit Rac and Cdc42 to mTORC1. Dock7 has been shown to act as a GEF for Rac [36], or Cdc42 and Rac [35], and our laboratory has shown that Dock7 acts as a GEF specifically for prenylated Cdc42 and Rac using *in vitro* liposome systems (Zhou et al., submitted). To examine the GEF function of Dock7 in cells, we started by knocking-down Dock7 in SKBR3 cells and examining the effect on the activation of Rac using the PBD assay. As shown in Figure 3.6A, knocking-down Dock7 significantly attenuated the basal activity of Rac in these cells. We also have *in vitro* data suggesting that a recombinant limit domain of Dock7 can serve as a target for Cdc42 (as evidenced by the ability of Cdc42-GTP to bind to Dock7 at an allosteric site) (Zhou et al., submitted). This is similar to the dual functions that have been reported for other GEFs, including Cool-1 and Dock11 [8, 40]. In these cases, it is thought

Figure 3.6 Dock7 is a guanine nucleotide exchange factor for Rac, Cdc42, and Rheb. A. SKBR3 cells were transfected either with control or Dock7 siRNA followed by serum-starvation for 40 h. Whole cell lysates were collected using a Mg^{2+}-containing buffer to preserve the nucleotide binding states of Rac. GST-PBD (p21 binding domain of PAK) beads were then used to pull-down the activated forms of Rac. The pulled-down product was then subjected to Western blotting for Rac. Dock7 knock-down was monitored by semi-quantitative RT-PCR with primers specific for Dock7. **B.** HEK-293T cells were transfected with either HA-Cdc42, HA-Rac, or HA-Rheb. Twenty-four hours after the transfection, cells were collected with buffer containing 1% Igepal and MgCl. Cells either remained in nucleotide-free buffer (EDTA) or incubated with GDP or GTPγS. GTPases were then immunoprecipitated using anti-HA antibody. The immunoprecipitates were subjected to Western blotting for Dock7, Cool-1, and HA. IP: immunoprecipitate. WCL: whole cell lysate. **C.** Guanine nucleotide exchange assays were performed using recombinant Rheb purified from *E.* coli and Dock7 purified from Cos7 cells as described in Materials and Methods. Nucleotide exchange was determined by the percentage of GTPγS bound to Rheb in a time-dependent manner.

A


RNAi:
Control
Dock7 (1)
Dock7 (2)
Rac-GTP
fold difference
1
0.36
0.44
Total Rac
PBD beads
RNAi:
Dock7
Semi-quantitative RT-PCR

B


IP: HA
WCL (HA-Cdc42)
WCL (HA-Rac)
WCL (HA-Rheb)
IP: HA-Cdc42
EDTA
GDP
GTPγS
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IP: HA-Rac
EDTA
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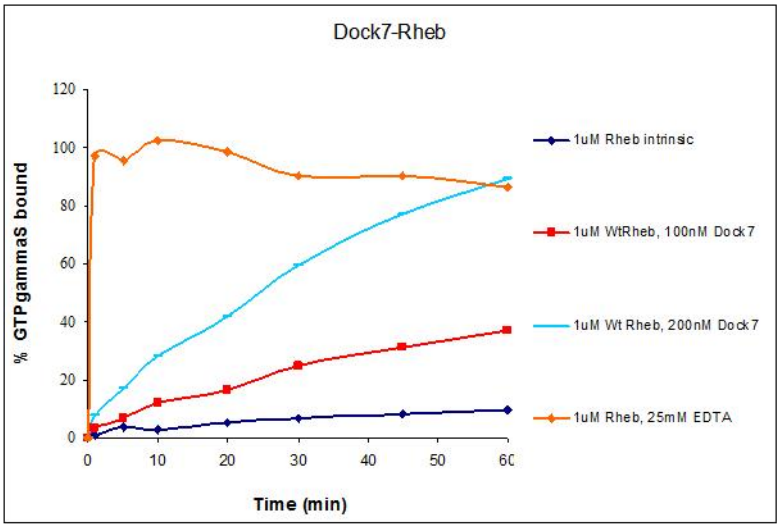
GDP
GTPγS
Beads only
Dock7
Cool-1
HA

C


Dock7-Rheb
% GTPgammaS bound
Time (min)
1uM Rheb intrinsic
1uM WtRheb, 100nM Dock7
1uM Wt Rheb, 200nM Dock7
1uM Rheb, 25mM EDTA

that the differential binding interactions can either facilitate a GTPase signaling cascade and/or provide a positive feedback mechanism to enhance signaling.

To demonstrate that endogenous Dock7 can function as a target for activated forms of Cdc42 as well as a GEF for Cdc42 and Rac, HA-tagged Cdc42WT, RacWT, and RhebWT (negative control) were transfected into HEK 293T cells. Cell lysates were then collected and stripped of the nucleotide by adding EDTA to the lysates, and then supplemented with GDP/Mg^{2+} to drive the GDP-inactive state of the GTPases, GTP/Mg^{2+} (GTP-active state), or left in EDTA to achieve the nucleotide-depleted state which is known to preferentially bind GEFs. The HA-tagged proteins were then immunoprecipitated from the lysates and subjected to Western blotting for either Dock7 or Cool-1, as a control. Shown in the left panel of Figure 3.6B, we can detect Dock7 being immunoprecipitated together with Cdc42 from treated lysates with the following preferences: EDTA>GTP>GDP; consistent with cellular Dock7 not only functioning as a Cdc42 GEF but also being able to bind to activated forms of Cdc42. A similar pattern of interaction between Cdc42 and Cool-1 is also observed, consistent with previous work [8]. On the right-hand panel of Figure 3.6B, we show that Dock7 interacts specifically with Rac from EDTA-treated lysates but not from GTP- or GDP-loaded lysates. This is consistent with the interpretation that Dock7 predominantly engages Rac as a GEF but not as an effector. In contrast, Cool-1 binds to nucleotide-free Rac, as well as GTP-bound Rac, as demonstrated previously [41].

Together, these data suggest that while Dock7 can interact with Cdc42 and Rac as a GEF, the activated form of Cdc42 additionally can associate with Dock7. The most unanticipated finding, however, was that Rheb can also bind to Dock7, and it does so specifically in the nucleotide-free state.

Rheb has been shown to bind to mTOR in its nucleotide-free state [21]. Since we find Dock7 associating with mTOR, it is possible that the interaction we detect between Dock7 and nucleotide-free Rheb is being bridged by mTOR. However, an alternate explanation is that Dock7 is functioning directly to promote nucleotide exchange on Rheb. To examine the possibility of the latter, *in vitro* nucleotide exchange assays were performed using Rheb and Dock7. Recombinant, wild-type Rheb was purified from *E. coli* in GDP-containing buffers. His-tagged Dock7 was transfected into Cos7 cells and purified using Cobalt resin. Guanine nucleotide exchange was initiated by the addition of [^{35}S]GTPγS to recombinant Rheb in the absence or presence of EDTA (positive control), or two different concentrations of Dock7. The nucleotide exchange catalyzed by Dock7 was read-out by the incorporation of [^{35}S]GTPγS into Rheb, as a function of time. As shown in Figure 3.6C, RhebWT alone (dark blue) showed very little intrinsic nucleotide exchange activity over the course of one hour, an indication that a GEF is needed for a more rapid activation of this GTPase. In the presence of EDTA, we observed a rapid exchange of [^{35}S]GTPγS onto Rheb verifying that the GTPase is functional, i.e. EDTA

chelates the Mg^{2+} from the GTPase, destabilizing the binding of GDP and allowing excess [^{35}S]GTPγS to bind. In the presence of Dock7, a dose-dependent increase in the formation of Rheb-[^{35}S]GTPγS is observed. Indeed, at the higher concentration of Dock7, complete nucleotide exchange on Rheb is observed over time. Together these data show that Rheb, like Cdc42 and Rac, can be activated by Dock7.

Discussion

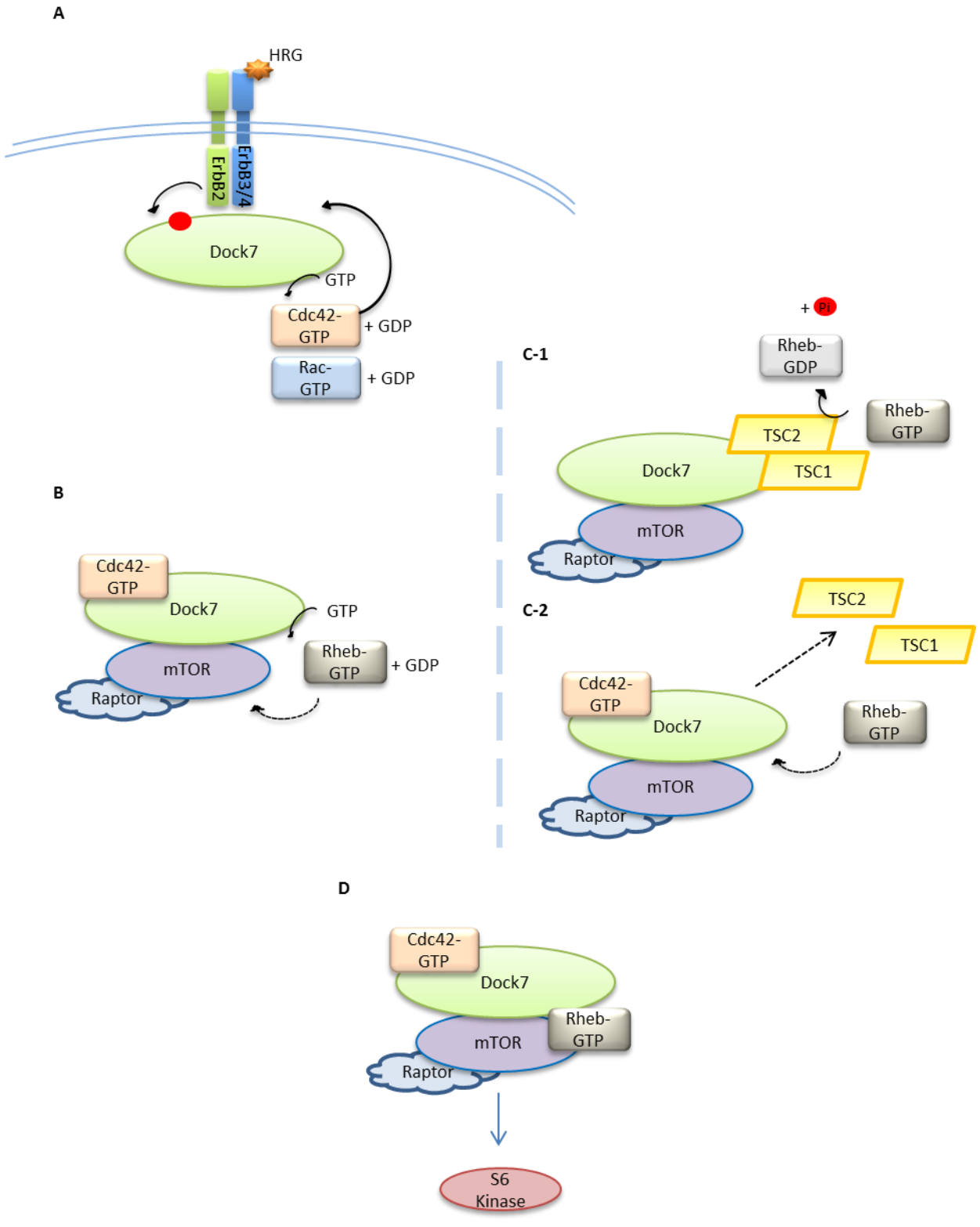
In this study, we initially set out to determine the relationship of the small GTPases, namely Rac, Cdc42, and Rheb, in HRG to mTORC1 signaling. Using a knock-down and over-expression approach, we identified Rac and Cdc42 to work in parallel upstream of Rheb to activate mTORC1. We have also discovered that Dock7 not only acts as a GEF for Rac and Cdc42 but also as a novel binding partner of mTORC1. The most unexpected finding, however, is that Dock7 displays properties of a Rheb GEF.

The search for a Rheb GEF is an ongoing effort in the field. It has always been debated whether a Rheb GEF is truly needed [42, 43]. Since the cellular Rheb-GTP levels are high compared to other small GTPases [33], one might hypothesize that only a GAP is required for turning-off Rheb. However, to achieve activation in a timely manner, a GEF is necessary, especially considering the slow intrinsic nucleotide exchange exhibited by wild-type Rheb

(Figure 3.6C, dark blue line). The fact that Dock7 interacts with TSC1/2 and mTORC1 (Figures 3.5A and 3.5B) makes it an attractive candidate as a Rheb GEF. This finding is also supported by a previous observation that mTOR preferentially interacts with nucleotide-free and GDP-bound Rheb but not GTP-bound Rheb, even though GTP-bound Rheb is necessary for the activation of mTORC1 [21]. Dock7, a Rheb GEF that stably interacts with mTOR, would provide a plausible explanation for this observation. Additionally, the interaction with the Rheb GAP suggests that spatially in the cell, Dock7 has access to inactive Rheb. Furthermore, once Rheb is activated by Dock7, mTORC1 is in close proximity to be stimulated by the freshly generated Rheb-GTP. There is also the possibility that TSC and Dock7 are in a complex to ensure the activity of the GAP and GEF for Rheb are tightly regulated. The activation of the GAP may result in the inactivation of the GEF, and vice versa, thus achieving the fine-tuning required for mTORC1 signaling.

It is also interesting that Dock7 not only acts as a Rac and Cdc42 GEF but also a target for activated Cdc42. This adds another level of complexity to how Dock7 may be regulating mTORC1 signaling. Two possibilities arise: First, we have *in vitro* evidence showing the binding of Cdc42-GTP enhances Dock7's GEF activity toward GDP-bound Cdc42 (Zhou et al., submitted). It may be that not only the GEF activity of Dock7 toward Cdc42 is augmented but also its GEF activity toward Rheb (as depicted in Figure 3.7B). This hypothesis is in agreement

Figure 3.7 Model depicting Rac, Cdc42, Rheb, and Dock7 are regulating HRG-mediated mTORC1 activation. A. HRG binds to and activates ErbB3/4 receptors which in turn activates ErbB2. Activated ErbB2 phosphorylates Dock7, activating its GEF activity towards Cdc42 and Rac. **B.** One hypothesis is that activated Cdc42 binds to Dock7 at an allosteric site which enhances Dock7's ability to act as a Rheb GEF. **C.** Another hypothesis is that Dock7 forms a stable interaction with mTORC1 and the TSC complex and this complex enables Rheb to be turned off (C-1). Once Cdc42 is activated, it binds to the allosteric site on Dock7, which then destabilizes the TSC complex allowing Rheb to remain active (C-2). **D.** Rheb activates mTORC1 and its downstream substrate S6 kinase.

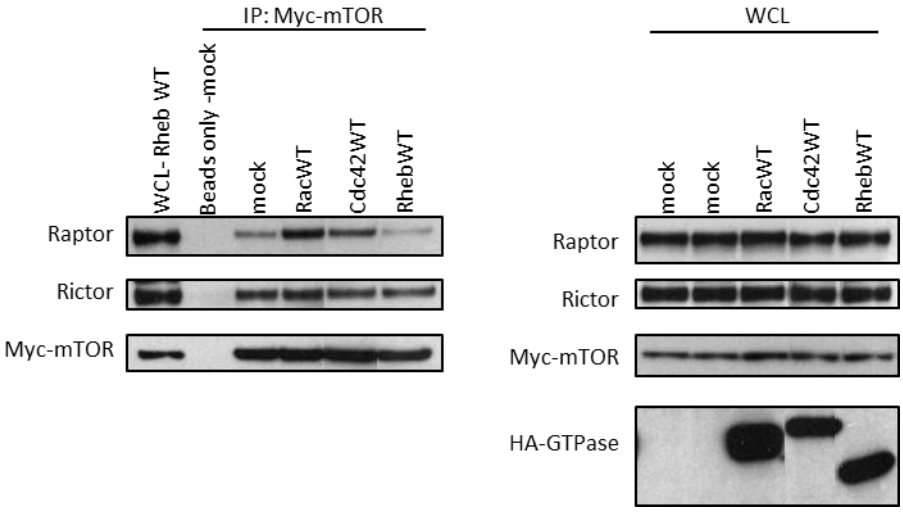

A
HRG
ErbB2
ErbB3/4
Dock7
GTP
Cdc42-GTP
+ GDP
Rac-GTP
+ GDP
B
Cdc42-GTP
Dock7
GTP
mTOR
Rheb-GTP
+ GDP
Raptor
C-1
+ Pi
Rheb-GDP
Rheb-GTP
TSC2
TSC1
Dock7
mTOR
Raptor
C-2
TSC2
TSC1
Cdc42-GTP
Dock7
Rheb-GTP
mTOR
Raptor
D
Cdc42-GTP
Dock7
Rheb-GTP
mTOR
Raptor
S6 Kinase

with the requirement that Cdc42 acts upstream of Rheb for mTORC1 activation. The other possibility is whether the allosteric binding of Cdc42-GTP to Dock7 changes its interacting partners. We observed that Dock7 forms a stable complex with TSC1/2 and mTORC1 under full serum/growing conditions (Figure 3.5). It would be interesting to see if the activated form of Cdc42 alters the equilibrium of this interaction. As illustrated in Figure 3.7C, the allosteric binding of Cdc42-GTP weakens the interaction of TSC1/2 with Dock7 and mTORC1, allowing Rheb to remain active. Further investigation is needed to prove this hypothesis. We do, however, have preliminary data showing wild-type Rac and Cdc42 enhancing the formation of mTORC1 (Figure 3.8).

The enhancement of mTORC1 formation by wild-type Rac and Cdc42 provides yet another clue as to how the small GTPases are regulating mTORC1 activity. By bringing Raptor and mTOR together, Rac and Cdc42 are increasing the pool of mTORC1 in the cell and as a consequence, recruitment of mTORC1 substrates such as S6 kinase and 4E-BP1 [44]. As a control, wild-type Rheb was unable to enhance mTORC1 formation but was still needed for its activation, suggesting differential roles for Rac and Cdc42 compared to Rheb.

We have concluded from Figure 3.3 that Rac and Cdc42 are working through independent pathways in Rheb/mTORC1 activation. However, it has been unclear as to how Rac contributes to this activation apart from mTORC1 recruitment, as shown in Figure 3.8. A previous report

Figure 3.8 Over-expression of RacWT and Cdc42WT enhances mTORC1 formation.
HEK-293T cells were transfected with Myc-mTOR. Twenty-four hours after the transfection, cells were subjected to a second round of transfections with either mock, RacWT, Cdc42WT, or RhebWT. Whole cell lysates were collected the following day with buffer containing 0.3% CHAPS and then immunoprecipitated using anti-Myc antibody. The immunoprecipitates were subjected to Western blotting for Raptor, Rictor, and Myc-mTOR.


IP: Myc-mTOR
WCL
WCL- Rheb WT
Beads only -mock
mock
RacWT
Cdc42WT
RhebWT
mock
mock
RacWT
Cdc42WT
RhebWT
Raptor
Rictor
Myc-mTOR
Raptor
Rictor
Myc-mTOR
HA-GTPase

shows a direct binding of Rac to mTOR through its C-terminal domain regardless of its nucleotide-binding status [27]. We have also shown in Figure 3.5C that nucleotide-free Rac and Cdc42 bind to Raptor and mTOR. However, the stoichiometry of this interaction differs from the amount of Dock7 immunoprecipitating with Raptor (Figure 3.5B). This suggests either Raptor itself is acting as a pool for inactive Rac and Cdc42, or there is another GEF in the complex that has not yet been identified. One possible candidate is Cool-1. We have unpublished data showing the knock-down of Cool-1 inhibited HRG-mediated activation of mTORC1 in SKBR3 cells. Also, shown in Figure 3.6B, Cool-1 exhibits the same properties toward Rac, as Dock7 displays for Cdc42, in that Cool-1 can be an activator and an effector for Rac. As for the direct binding of Rac and Cdc42 to Raptor, this would not be the first instance of a small GTPase binding to Raptor. The Rag proteins, namely RagA and RagB, directly interact with Raptor in a GTP-dependent manner upon amino acid stimulation to recruit mTORC1 to the lysosomal surface [22-26, 45]. It will therefore be interesting to see whether Rac and Cdc42 have the same ability to interact with Raptor and affect the localization of mTORC1.

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Chapter 4

Conclusions and Perspectives

Over the past two decades, our laboratory has been interested in HRG-signaling: From the discovery of ErbB3 as the bona fide receptor for HRG, to an end-point of its signaling, the nuclear cap-binding complex. Over the years, we have identified mTOR, an essential hub for nutrient and growth factor sensing, as an important intermediate in HRG-signaling. mTOR has gained much interest since its discovery in the 1990's. Research on the myriad of functions and the complexity of regulation for mTOR has been central in understanding cell homeostasis and proliferation. Here in this thesis, I have shown two alternative pathways in which mTORC1 can be activated by HRG.

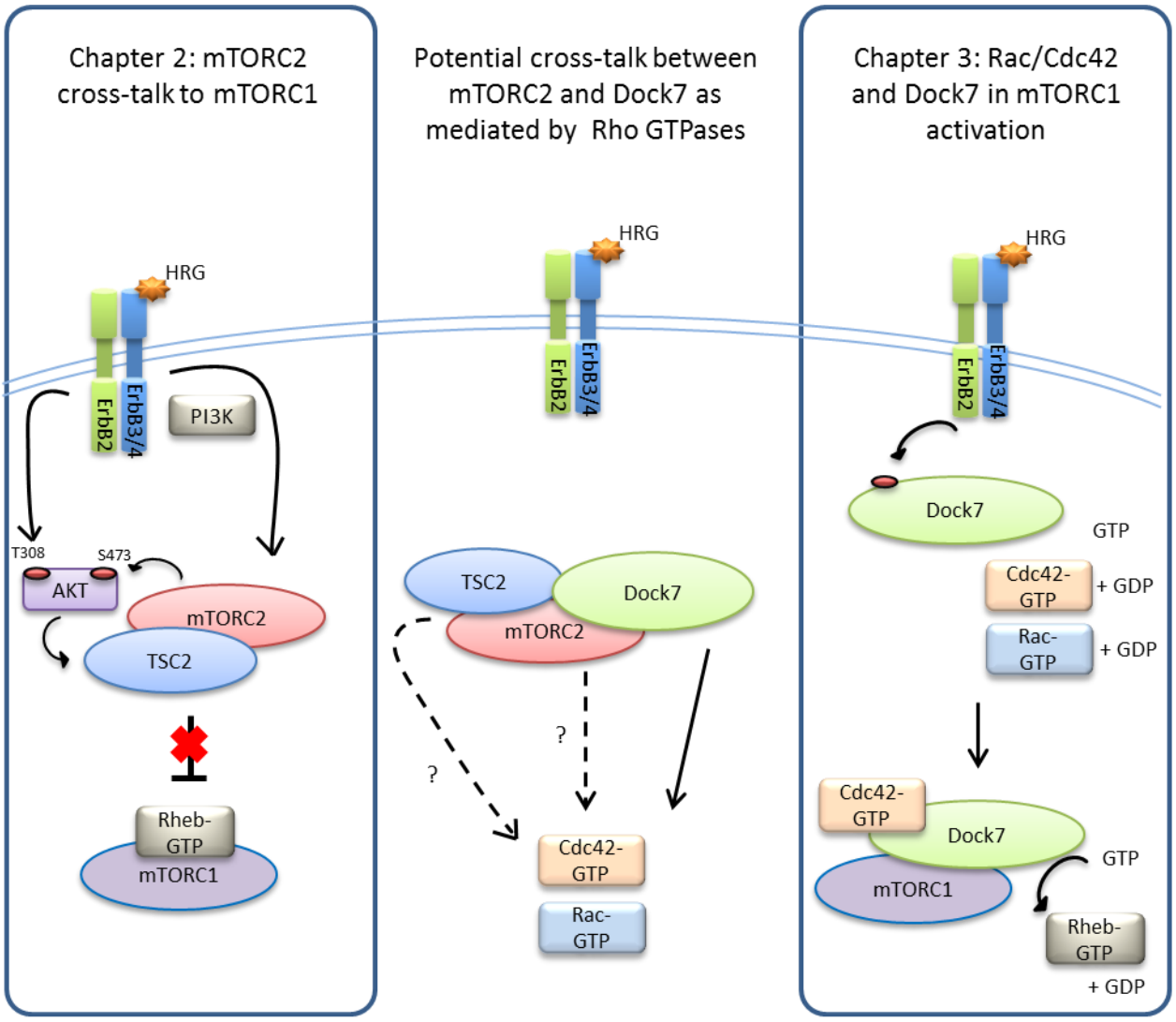
In Chapter 2, I identified the importance of mTORC2 signaling to mTORC1 in ErbB2/HRG-mediated cellular transformation in SKBR3 breast cancer cells. mTORC2 was initially identified to play a role in actin cytoskeletal remodeling [1, 2], but with the discovery of novel mTORC2 targets, mTORC2 has been implicated in cellular functions such as cell proliferation, survival, and metabolism [3]. By utilizing rapamycin and an ATP-competitive inhibitor of mTOR, INK128, I was able to differentiate between mTORC1 and mTORC2

activation by HRG. In HRG/ErbB2-mediated signaling to AKT, mTORC2 is required for the phosphorylation on AKT (S473) and this precedes the activating PDK1 phosphorylation at AKT (T308). AKT phosphorylates TSC2, making TSC2 unable to function on Rheb. Rheb remains in its GTP-bound form and activates mTORC1 (summarized in Figure 4.1, left panel). By performing a Rictor knock-down, which decreased mTORC2 availability in the cell, the HRG-mediated transforming capability of SKBR3 was reduced.

In Chapter 3, I took a mechanistic approach to identify how small GTPases, namely Rheb, Rac, and Cdc42, are playing a role in HRG-mediated mTORC1 activation. Using a knock-down and rescue approach, I was able to delineate that Rac and Cdc42 are upstream of Rheb, and that they signal independently of one another to mTORC1 in this context. At this time, it is not clear as to the individual roles of Rac and Cdc42 in achieving mTORC1 activation, and whether under more physiologic conditions, the two GTPases might show functional redundancy. Additionally, I found that Dock7, a GEF for Rac and Cdc42, serves as a unique scaffold for the G-proteins and mTORC1. The most intriguing finding, however, is that Dock7 also possesses properties of a Rheb GEF (summarized in Figure 4.1, right panel). It has long been hypothesized that only a GAP is needed for the regulation of Rheb, so the identification of a putative Rheb GEF will be of significant interest to the field.

The focus of this thesis is on the pathways that lead to the activation of mTORC1 in

Figure 4.1 Summary and model of mTORC2-Rac/Cdc42-Dock7 cross-talk. As described in Chapter 2, HRG activates mTORC1 through an mTORC2-dependent pathway in SKBR3 cells (left panel). In Chapter 3, Rac/Cdc42 and Dock7 are necessary for mTORC1 activation in HeLa cells. Dock7 can act as a scaffold for mTORC1 and also a Rheb GEF (right panel). mTORC2 has been shown to regulate the Rho GTPases. TSC2 is also necessary for Rac and Cdc42 activation. Dock7 interacts with mTORC2, TSC2, and mTORC1, serving as scaffold, thus creating a potential cross-talk between mTORC2 and Dock7/Rho GTPases in regulating the activation of mTORC1 (middle panel).


Chapter 2: mTORC2 cross-talk to mTORC1
Potential cross-talk between mTORC2 and Dock7 as mediated by Rho GTPases
Chapter 3: Rac/Cdc42 and Dock7 in mTORC1 activation
HRG
ErbB2
ErbB3/4
PI3K
T308
S473
AKT
mTORC2
TSC2
Rheb-GTP
mTORC1
Dock7
GTP
Cdc42-GTP
+ GDP
Rac-GTP
?
mTORC2 regulates Rheb activation through AKT and TSC2.
mTORC2 and TSC2 can both be upstream of Rho GTPases. Dock7 interacts with TSC2 and mTORC2, thus bridging the two models.
Dock7 activates Cdc42 and Rac. Allosteric binding of Cdc42-GTP may increase Dock7's GEF activity towards Rheb. Rac and Cdc42 enhance mTORC1 formation.
mTORC1 active

response to HRG. One obvious question arises: Are the pathways described in Chapter 2 and Chapter 3 interconnected? As previously mentioned, mTORC2 was initially discovered to play a role in cytoskeletal remodeling [1, 2]. Constitutively active forms of Rac and RhoA have been shown to rescue the actin defects due to the loss of functional mTORC2 components such as mLST8 and Rictor [2]. Also, a decrease in Rac-GTP levels has been observed in NIH3T3 cells following a Rictor knock-down [2]. This suggests that AKT and TSC2 may not be the only point of convergence between the mTOR complexes. From what we observed in Chapter 3, and the implication that Rho GTPases can signal downstream of mTORC2, suggests another junction in the pathway. Additional evidence for the potential crosstalk between mTORC2 and mTORC1, as mediated by the Rho GTPases is a Rac-specific GEF, P-REX1. P-REX1 interacts with both mTORC1 and mTORC2, however, P-REX1 only displays GEF activity when bound to mTORC2 [4]. Moreover, Rac has also been shown to bind to both mTOR complexes at the plasma membrane [5]. We observed similar properties for Dock7 in that it interacts with both mTOR complexes (data not shown). However, we do not have evidence yet as to whether Dock7 can regulate the activity of both mTOR complexes. Another piece of evidence for the mTORC2-Rac/Cdc42-mTORC1 crosstalk is the necessity for TSC2 in mTORC2 activation [6]. As shown in Figure 2.5 of Chapter 2, we observed that TSC2 co-immunoprecipitates with both mTOR complexes, suggesting TSC2 serves as a node for mTOR cross-talk. In addition, TSC2

has also been shown to control cell polarity and migration through Rac and Cdc42 [7]. It is still unclear if this regulation is through mTORC2. However, data from our laboratory and others suggests that mTORC1 and mTORC2 may share more players than initially accounted for, i.e. Dock7, Rac/Cdc42, TSC1/TSC2. Therefore, it is of great interest to decipher the role of each of these players in achieving the fine-tuning that is necessary for mTOR signaling (Figure 4.1).

As described in Chapter 2, HRG-mediated mTORC2-mTORC1 activation is important for the ability of SKBR3 breast cancer cells to exhibit colony formation. If such crosstalk is potentially mediated by Rac/Cdc42 and Dock7 as hypothesized above, another logical question to ask would be whether Dock7 contributes to the tumorigenic capability of cancer cell lines. We have evidence that Dock7 is not only expressed in neuronal systems but is also expressed in the P19 embryonic carcinoma cell line (data not shown), 293T human embryonic kidney cells, breast cancer SKBR3 cells, and the HeLa cervical carcinoma cell line. It will be interesting to determine whether Dock7 expression and/or its GEF activity is up-regulated in cancer cell lines in comparison to normal cells. Considering Dock7's potential to function as a Rheb GEF, and that Rheb has been shown to be over-expressed in certain cancers [8, 9], this is a plausible hypothesis. Furthermore, if Dock7 is up-regulated in tumor cells, what aspect of tumorigenesis is Dock7 affecting? Is it controlling cell size and proliferation by its ability to act as an mTORC1 regulator? Or is it affecting the migration and invasion capability of the cancer cells as an

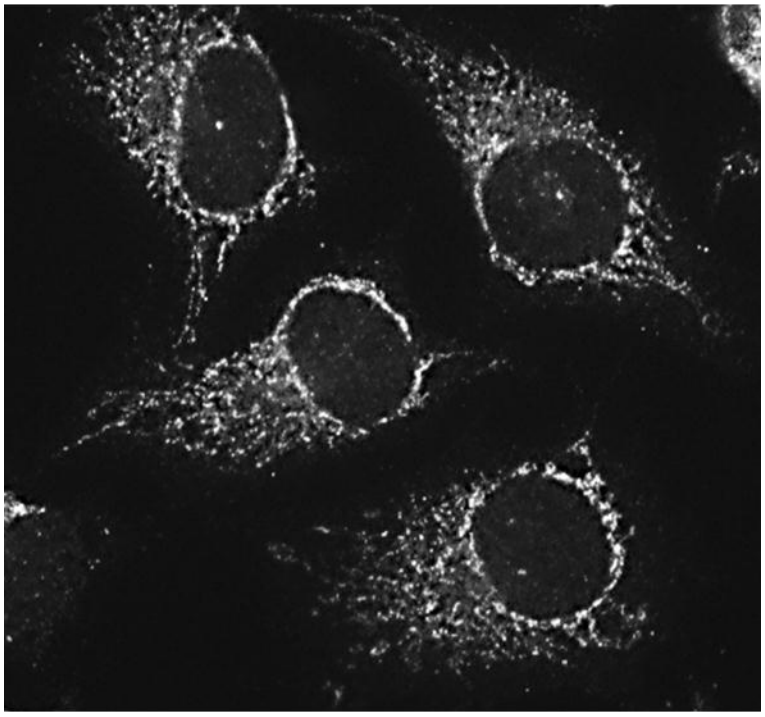
activator of Rac and Cdc42? These are all questions that we are very interested in answering.

Additional clues as to what cellular functions Dock7 is performing may be garnered by determining its localization. The localization of the various signaling components is crucial in understanding the functions of the different signaling pathways. For example, the Rag proteins are important in sensing amino acids and localizing mTORC1 to the lysosome to be activated by Rheb [10-12]. We have preliminary data showing that Dock7 localization resembles that of the mitochondria in HeLa cells (Figure 4.2, cell fractionation data not shown). This is very surprising, as Dock7 can be activated by the ErbB2 receptor [13], and PIP3 has been shown to bind to the DHR1 region of Dock180 [14], leading to the hypothesis that Dock7 localizes to the plasma membrane. Is it possible that Dock7 is regulating a mitochondrial-specific function of mTORC1? mTORC1 has been shown to localize to the outer membrane of the mitochondria [15, 16]. Inhibition of mTORC1 by rapamycin results in a general decrease of mitochondrial metabolism, as exhibited by lowered oxygen consumption and tri-carboxylic acid cycle intermediates in Jurkat cells [16, 17]. Understanding the regulation of Dock7 may provide novel insights into how mTOR may be playing a role in mitochondrial functions.

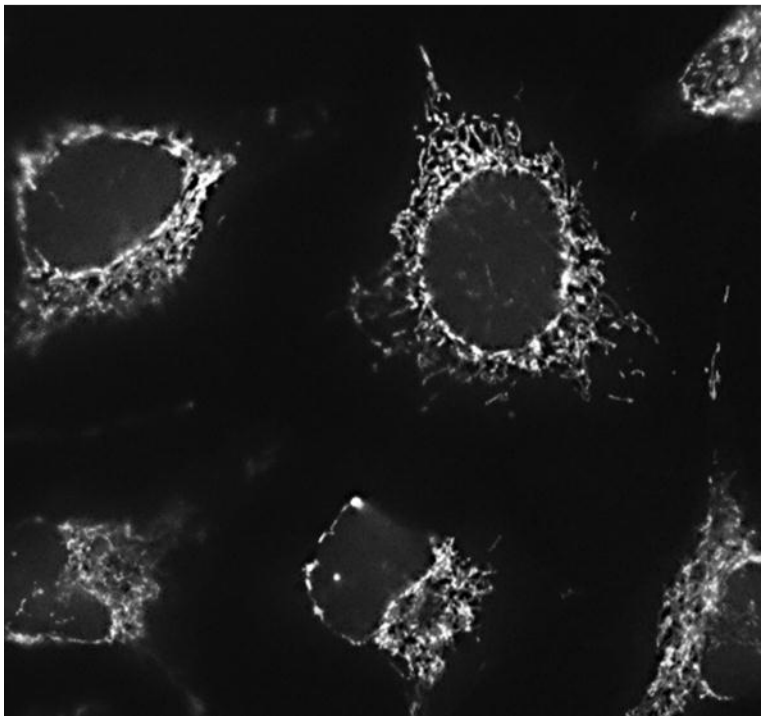
In this thesis, I have furthered our understanding of how HRG signals to mTORC1, by identifying two pathways that lead to the activation of mTORC1, and by demonstrating the ability of Dock7 to function as a novel Rheb GEF. The long term goal will be to understand

Figure 4.2 Dock7 localization resembles that of mitochondria. Top: HeLa cells were fixed in 4% paraformaldehyde and stained with anti-Dock7 antibody (Sigma) and Texas Red-conjugated secondary antibody (Invitrogen). Bottom: HeLa cells were treated with 25 nM MitoTracker (Invitrogen), a mitochondrial probe, for 15 min before fixing with 4% paraformaldehyde. Cells were visualized using fluorescence microscopy

Dock7



MitoTracker



whether these signaling pathways and players are unique to HRG signaling to mTORC1, or if they are also responsible for other aspects of mTORC1 signaling. For example, do other growth factors share these signaling pathways? Also, are Rac, Cdc42, and Dock7 important for the amino acid-sensing capabilities of mTORC1? In addition, what is the broader implication of this signaling in other cell types? What cellular functions can be achieved through this signaling in either normal cells or cancer cells? Answering these questions will greatly add to what is known about mTOR signaling and function.

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