Cytotoxic and Cytostatic Properties of Rapamycin: Implications for Antitumor Efficacy

by

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Abstract

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mTOR (mammalian target of rapamycin) is a central regulator of cell growth and proliferation. Frequently dysregulated in cancer cells, it is an attractive therapeutic target, named for its first inhibitor, rapamycin. Low (nanomolar) doses of rapamycin treatment inhibit phosphorylation of mTORC1 (mTOR complex 1) substrate, S6 kinase, thwarting protein synthesis and subsequently, proliferation. Though highly potent and specific, rapamcyin has lacked clinical success because it lacks universal anti-tumor effects and only a fraction of patients respond.

We have revealed rapamycin as a site-specific, cytotoxic anticancer drug in the absence of serum. Our studies demonstrate that high-dose rapamcyin induces apoptosis because it effectively inhibits all phosphorylation sites on 4E-BP1, subsequently inactivating eIF4E. Importantly, we demonstrate that cancer cells are resistant to high-dose rapamycin upon 4E-BP1 knockdown, confirming that the drug retains its site-specific property regardless of dose. Furthermore, we show that high-dose rapamycin irreversibly compromises the integrity of the mTORC1 complex, as it pertains to mTOR-raptor association. We acknowledge the inefficacy of rapamycin in cancer cells that activate Akt upon mTORC1 inhibition. Under these circumstances, an

inhibitor of both mTORC1 and mTORC2 complexes is necessary and sufficient to induce apoptosis

Our current study is to further investigate the surprising observation that while high-dose rapamycin treatment (indirect inactivation of eIF4E) induces apoptosis only in the absence of serum, knockdown of eIF4E (direct inactivation of eIF4E) induces apoptosis in both the presence and absence of serum. Thus, there is a mechanistic difference between the indirect and direct inactivation of eIF4E.

Since high-dose rapamycin treatment also inhibits phosphorylation of S6K and does not induce apoptosis in the presence of serum, we reasoned that protection from apoptosis induced by high-dose rapamycin could be due to suppression of S6K. Consistent with this hypothesis, dual S6K and eIF4E knockdown prevents cell death otherwise induced by knockdown of eIF4E alone.

We hypothesize that simultaneous inhibition of both S6K and eIF4E results in coordinate induction of transforming growth factor-β (TGF-β) signaling that is sufficient to induce arrest. This is predicated on prior work in the lab that shows high-dose rapamycin causes G1 arrest rather than apoptosis, if and only if, TGF-β signaling is intact. TGF-β mediates a cytostatic response by activation of Smad signaling and cyclin-dependent kinase inhibitor (CDK) p27.

Altogether, these data reveal the complexity of high-dose rapamycin, with implications for both cytotoxic and cytostatic effectiveness in the absence and presence of serum, respectively.

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4E-BP1 eIF-4E binding protein-1

Akt protein kinase B/PKB

Cl. PARP cleaved PARP

CDK cyclin-dependent kinase

DMEM Dulbecco's Modified Eagle Medium

DSP dithiobis(succinimidyl) propionate

eIF-4E eukaryotic initiation factor 4E

FBS fetal bovine serum

FKBP12 FK506-binding protein of 12kDa

FRB FKBP12-rapamcyin binding

HEK human embryonic kidney

Ins insulin

IRS1 insulin receptor substrate-1

MTD maximum tolerated dose

MEF mouse embryonic fibroblast

mTOR mammalian target of rapamycin

mLST8 mammalian lethal with SEC13 protein 8

mTORC1 mTOR complex 1

mTORC2 mTOR complex 2

PARP poly-ADP-ribose polymerase

PRAS4 praline-rich Akt substrate of 40kDA

PI3K phosphoinositide 3-kinase

p70S6K p70 ribosomal protein subunit kinase

rap./rapa. rapamycin

raptor regulatory associated protein of mTOR

rictor rapamcyin insensitive companion of tor

RTK receptor tyrosine kinase

S6 ribosomal protein subunit S6

S6K ribosomal protein subunit S6 kinase

sin1 stress-activated protein kinase-interacting protein 1

siRNA small interfering ribonucleic acid

TGF-β transforming growth factor-β

TOR target of rapamycin

TSC1/2 tuberous sclerosis complex (TSC) hamartin (1) or tuberin (2)

Wort wortmannin

Chapter 1

Introduction

gure 1.1 mTORC1 is a central regulator in the cell and is dysregulated in many seases.

dapted from Huang J and Manning B.D., *Biochem Soc Trans* 2009)

obesity diabetes

1.1 Mammalian target of rapamycin (mTOR): a central regulator in the cell

aging

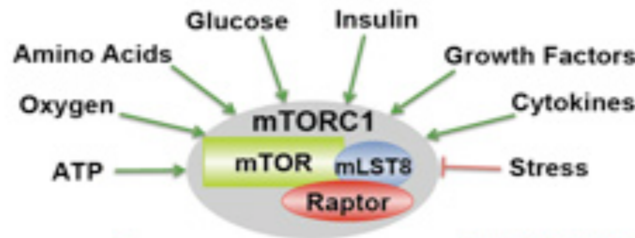
neurological disorders

cancer

Normal cells respond appropriately to diverse environmental cues, operating via tightly controlled signal transduction networks to achieve homeostasis. The protein kinase TOR (target of rapamycin) functions as one such environmental sensor. In unicellular eukaryotes, such as yeasts, TOR responds primarily to nutrient levels. Evolution of multicellular organisms provided TOR with the ability to respond to a diverse array of environmental cues, such as hormones and growth factors, which has placed this serine/threonine kinase as a central regulator of cellular functions.

aging

Mammalian TOR (mTOR) complex I (mTORC1) is frequently dysregulated in a wide variety of complex diseases, including cancer, diabetes, obesity, aging and neurological disorders (Huang and Manning, 2009) **(Figure 1.1)**. Its subsequent hyperactivity has driven the development of an array of mTOR inhibitors **(1.6b, 1.7)**. The work presented here serves to expand the mechanistic attributes of one such potent and specific mTOR inhibitor, rapamycin, through investigation of two breast cancer lines, MDA-MB-231 and MCF-7.

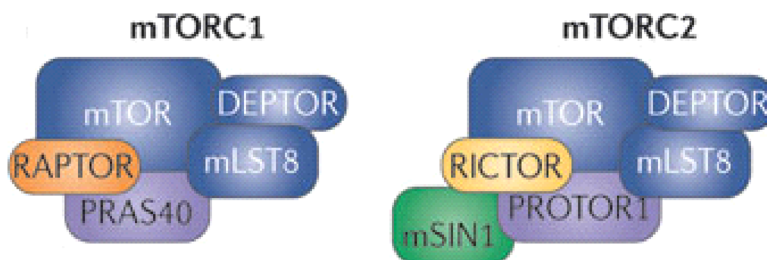


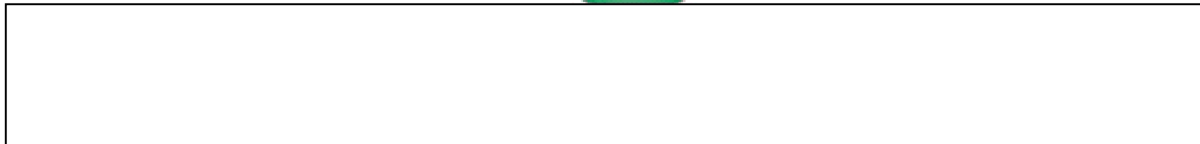
gure 1.2 Components of mTOR complexes, mTORC1 and mTORC2

ɩoue et al., *Nature Reviews Neuroscience*, 2011)

1.2 mTORC1 and mTORC2 complexes: subunit composition

mTORC1 is composed of several subunits: mTOR, raptor (regulatory associated protein of mTOR), mLST8 (mammalian lethal with SEC13 protein 8, known as GβL) **(Figure 1.1)** and PRAS4 (proline-rich Akt substrate of 40kDa). PRAS40 functions as a negative regulator when bound within the complex, dissociating in response to insulin. mTORC1 regulates many cellular processes including cell growth, proliferation through effectors such as ribosomal protein p70 S6 kinase (S6K) and eukaryotic initiation factor 4E binding protein-1 (4E-BP1). mTOR also exists in another complex, mTOR complex 2 (mTORC2) which contains mTOR, rictor, sin1 (stress-activated protein kinase-interacting protein 1), mLST8 and Protor (Guertin and Sabatini, 2007). It regulates the pro-survival kinase Akt/PKB by phosphorylating it on serine 473 (S473) (Sarbassov et al., 2005). Full Akt activation occurs only upon phosphorylation of S473 which is preceded by phosphorylation on threonine 308 (T308) (Alessi et al., 1996) **(Figure 1.4)**. Thus, the readout for mTORC2 activity is phosphorylation at S473. The complete composition of mTOR complexes is shown in **Figure 1.2** (Boue et al., 2011). Because raptor and rictor are specific to mTORC1 and mTORC2, respectively, they serve as signature components and readouts of complex stability.





The best understood mTORC1 substrates are the S6K and 4E-BP1 proteins, both of which are coordinately involved in translation of key mRNAs of proteins required for cell cycle progression, and thus cell growth and proliferation **(Figure 1.3.1)** (Ma and Blenis, 2009). As such, mTORC1 is a critical regulator of translation initiation (Coleman et al., 2004).

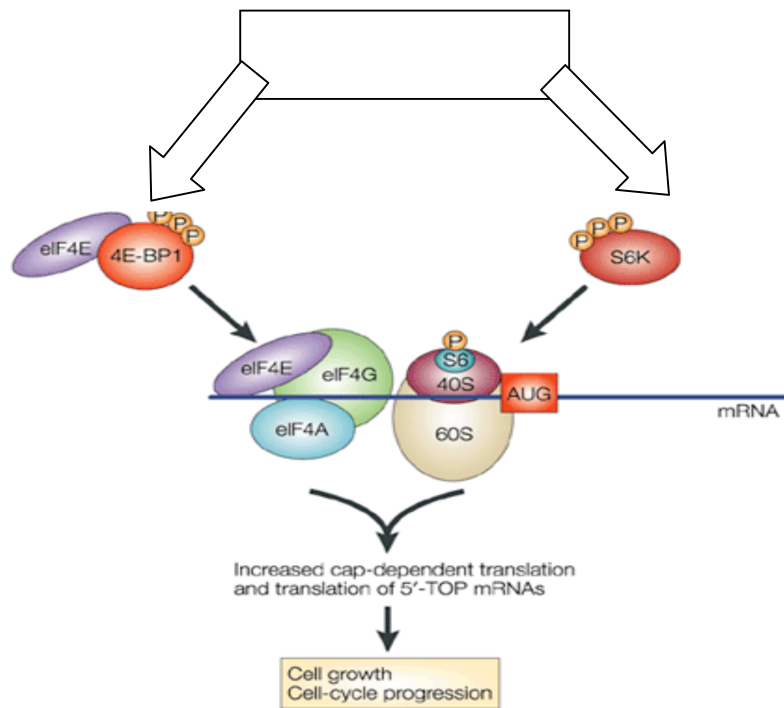


Figure 1.3.1 S6 kinase ar

mTORC1 phosphorylates
molecule for further phosp
4E-BP1 and S6K, respecti
recruited into the translation-initiation-factor complex (eIF4F) that includes eIF4A and eIF4G. Assembly of this complex promotes translation that is dependent on the 5'-cap structure of mRNA.
S6K phosphorylates the S6 subunit of the 40S ribosome, which enhances translation of mRNAs (representing as many as 200 genes, including components of the translational apparatus).

Enhanced protein synthesis allows cell growth and, in turn, cell-cycle progression.

(Adapted from Coleman et al., *Nature Reviews Molecular Cell Biology*, 2004).

<u>S6 kinase (S6K)</u>

S6K activation requires mTORC1-mediated phosphorylation. In turn, S6K phosphorylates its own targets, several of which are involved in protein synthesis (Ma and Blenis, 2009). Its most studied substrate, ribosomal protein S6 (S6) is a component of the 40S ribosome (Meyuhas, 2008; Meyuhas and Dreazen, 2009). mTORC1

phosphorylates S6K on threonine 389 (T389) which is particularly important because substitution of this residue with alanine blocks the activation of the kinase domain (Dennis et al., 1998). As such, phosphorylation status on T389 has become a principal readout for mTORC1 activity.

4E-BP1/eIF4E

Though both S6K and 4E-BP1 regulate translation, only 4E-BP1's role is well-elucidated. 4E-BP1 belongs to a family of repressor proteins that bind to eukaryotic-initiation factor 4E (eIF4E) and inhibit cap-dependent translation. Binding to eIF4E is determined by the phosphorylation state of 4E-BP1. Hypo-phosphorylated 4E-BP1 binds to the eIF4E mRNA cap-binding protein and prevents its assembly into the eIF4F cap-binding complex, thereby blocking cap-dependent translation (Beretta et al., 1996a). However, in the presence of adequate growth factors, amino acids and energy, mTORC1 phosphorylates 4E-BP1, hierarchically on threonine 37 (T37), threonine 46 (T46), threonine 70 (T70) and serine 65 (S65) (Gingras et al., 2001a; Gingras et al., 2001c). In this, its hyper-phosphorylated state, 4E-BP1 releases eIF4E; eIF4E then binds to eIF4G and recruits the helicase (eIF4A) which is necessary to unwind and inhibitory structure in the 5' region of mRNA. Ultimately, a new complex is formed (eIF4E/eIF4G/eIF4A) on the 5' cap which then recruits the 40S ribosome to the 5' cap to form a translation pre-initiation complex. A general schematic (sufficient for understanding our work) is shown in **Figure 1.3.2** (Kasinath et al., 2006).

Thus, S6K is a translational activator and 4E-BP1 is a translational repressor.

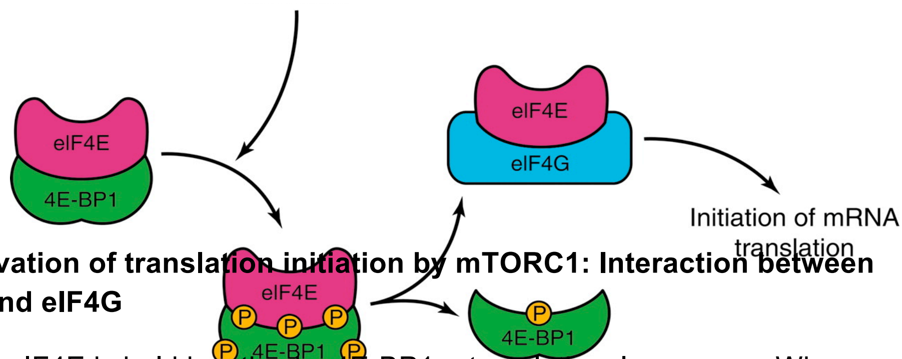


Figure 1.3.2 Activation of translation initiation by mTORC1: Interaction between eIF4E, 4E-BP1, and eIF4G

In the basal state, eIF4E is held inactive by 4E-BP1-a translational repressor. When a stimulus is received for protein synthesis, 4E-BP1 is phosphorylated and dissociates from eIF4E. Free eIF4E binds to eIF4G and facilitates onset of the initiation phase of mRNA translation.

(Adapted from Kasinath et al., *mRNA Translation: Unexplored Territory in Renal Science*, 2006)

1.4 mTOR signaling and cancer

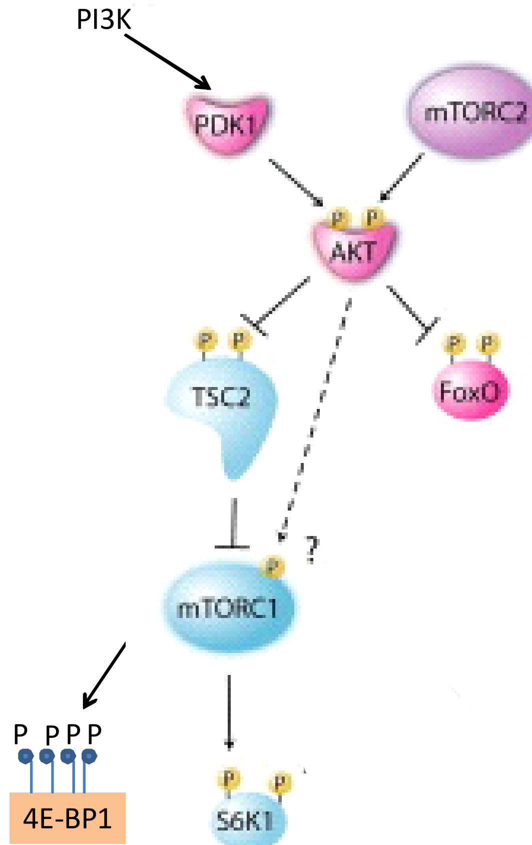
As activation of S6K and 4E-BP1 are well-regulated in normal cells, so it follows that growth and proliferation is as well. The dysregulation of these substrates by hyper- or constitutively activated mTOR signaling leads to aberrant growth and proliferation, a hallmark of cancer cells. Hyper-activation can occur by mutations in the phosphoinositide 3-kinase (PI3K) signaling pathway which functions upstream of both mTORC1 and mTORC2. Additionally, the loss of tumor suppressor p53 promotes mTORC1 activation (Feng et al., 2005). Mutations in genes encoding proteins upstream of mTORC1 such as TSC1/2 (tuberous sclerosis complex (TSC) hamartin (1) or tuberin (2)) are often mutated which permits constitutive mTORC1 signaling (Choo et al., 2010).

The evidence linking dysregulated mTOR signaling to cancer has generated significant interest in targeting the PI3K/Akt/mTORC1pathway for cancer therapy **(Figure 1.4)**.

Figure 1.4 mTOR functions both upstream and downstream of Akt.

Full activation of Akt requires phosphorylation on two sites, T308 and S473. Activated Akt facilitates growth (inhibiting TSC2, thus activating mTORC1) and survival (ex. inhibiting apoptotic genes, such as FoxO).

(Adapted from Guertin et al., *Cancer Cell*, 2007)



1.5 4E-BP1/eIF4E is a necessary target for effective mTORC1 inhibition.

The overall role of mTORC1 in the regulation of mRNA translation is very significant because complete inhibition of mTORC1 function significantly reduces rates of protein synthesis in proliferating cells (Thoreen et al., 2009). Several studies have demonstrated the role of S6K in tumor invasiveness (Skinner et al., 2004; Zhou and Wong, 2006). For instance, Zhou showed that constitutively active S6K increases invasiveness in ovarian cancer cells. Nonetheless, the contribution of S6K and S6 to oncogenic action is limited. In fact, 4E-BP1 appears to promote cell cycle progression and cell proliferation independent of S6K.

Much evidence supports dysregulation of protein synthesis at the level of 4E-BP1/eIF4E as playing a central role in tumor formation. De-phosphorylated 4E-BP1 can inhibit cell growth and reverse the transformed phenotype of eIF4E over-expressing cells (Rousseau et al., 1996). Constitutively active 4E-BP1 mutants (all phosphorylated sites mutated to alanine) are not candidates for phosphorylation by mTORC1. This 4E-BP1 mutant also blocks myc-induced transformation (myc is an immortalizing gene) and prevents tumor growth (Fingar et al., 2004; Fingar et al., 2002; Lynch et al., 2004). In both instances, the 4E-BP1/eIF4E interaction is enhanced; this leads to a decrease in cell size and inhibition of cell cycle progression (Fingar et al., 2004). These data reveal that 4E-BP1 is a major cellular effector of mTORC1-driven cell cycle progression and cell proliferation.

While mutating 4E-BP1 stalls growth, deleting 4E-BP1 promotes growth. As discussed above, eIF4E is sequestered and permanently held inactive by a mutant 4E-

BP1. By contrast, it is always “free” by absent 4E-BP1. Otherwise low in normal cells, eIF4E levels are over-expressed by loss of 4E-BP1 and translation is dysregulated. Mouse embryonic fibroblasts (MEFs) lacking 4E-BP1 resist inhibition of cell cycle progression by raptor knockdown (Dowling et al., 2007). This indicates that mTORC1 does not directly impact on eIF4E and supports our data **(3.10, Figure 3.10)**. Such over-expression has been shown to promote translation of mRNAs implicated in cell growth, proliferation and survival (Mamane et al., 2004). Over-expression of eIF4E also accelerates G1/S-phase progression and confers partial protection from rapamycin (Fingar et al., 2004).

Indeed, eIF4E was the first translation factor shown to cause malignant transformation when over-expressed in cultured rodent cells (Lazaris-Karatzas et al., 1990). These studies defined eIF4E as an oncogene as it cooperates with myc to transform primary embryo fibroblasts (Lazaris-Karatzas and Sonenberg, 1992). In fact, increased eIF4E expression has been associated with tumor formation and progression in cancer including leukemia, lymphoma, and cancers of several tissues (breast, colon, bladder, lung, prostate).

Collectively these studies implicate that impairing 4E-BP1/eIF4E complex stability plays an important role in cancer by controlling translation of various transcripts that promote cell proliferation and tumorigenesis.

1.6 Rapamycin

Naturally produced by the bacterium, *Streptomyces Hygroscopicus*, rapamycin is a macrolide antibiotic discovered in Easter Island in the South Pacific. It was originally defined as an antifungal compound in 1975 because it induced G1 cell cycle arrest (Guertin and Sabatini, 2009) and later found to possess immunosuppressant properties (Heitman et al., 1991). Its broad anti-proliferative properties led to its discovery as an inhibitor of S6 kinase phosphorylation at site T389. Nahum Sonenberg's group also found that rapamycin inhibited phosphorylation of 4E-BP1 (Beretta et al., 1996b). Only later was it found that mTOR phosphorylates S6K; thus, rapamycin was the first defined inhibitor of mTOR.

1.6a Mechanism of action

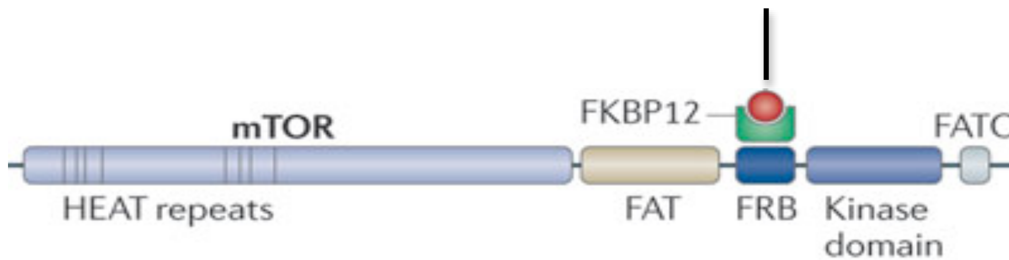
In contrast to more traditional kinase inhibitors, rapamycin is an allosteric inhibitor of mTORC1, working through a gain-of-function mechanism **(Figure 1.6a)** in which it associates with its intracellular receptor, FKBP12 (FK506-binding protein of 12 kDa). The resulting complex interacts with the FRB (FKBP12–rapamycin binding) domain, a short sequence lying upstream of the carboxyl terminus of mTOR (Guertin D.A. and Sabatini D.M., 2009). Binding of rapamycin–FKBP12 to the FRB domain of mTOR disrupts the association of mTOR from raptor, uncoupling mTORC1 from its substrates, and subsequently blocking mTORC1 signaling.

Because FKBP12-rapamycin does not bind to mTORC2 (Jacinto et al., 2004) rapamycin is thought to inhibit only mTORC1. In fact, rictor is an acronym that defines its response to rapamycin: (R) rapamycin (I) insensitive (C) companion of (T) TOR.

acting as an allosteric inhibitor. **Rapamycin**

(Adapted from Boue et al., *Nature Reviews Neuroscience*, 2011).

While this holds true for acute treatments, it is worthy to note that Sabatini's group has shown "prolonged rapamycin treatment inhibits mTORC2 assembly and Akt/PKB" (publication title within quotations) (Sarbassov et al., 2006). In agreement, we have also generated data showing the loss of Akt phosphorylation at S473 after 48 and 72 hour rapamycin treatment. At first glance, this reveals a mechanism that elevates the potential for rapamycin as a cancer therapeutic. But, it may only prove relevant or beneficial in some cancer cell types because rapamcyin treatment activates Akt in some cells, promoting cell survival **(1.6d, 3.2, 3.5, 5.4)**. Therefore, elucidating rapamcyin's mechanism of action has implications for clinical efficacy and effectiveness.



1.6b Efficacy

Initial clinical trials in any area of medicine are primarily designed to test efficacy (is the drug or device beneficial under ideal conditions?) rather than to test effectiveness or relevance (is the drug or device beneficial in everyday practice?).

Although rapamycin, clinically known as sirolimus, has demonstrated anti-proliferative effects and apoptosis in head and neck squamous cell carcinoma (Vivanco et al., 2002), overall it has proven only modestly efficacious. Because it has been reported to have poor solubility, several analogs (rapalogs) have been developed and are being tested in the clinic. Rapamycin and rapalogs have been approved as immunosuppressive agents to blunt organ transplant rejection, in cardiology to reduce restenosis following angioplasty, and in oncology to treat renal cell carcinoma or are in various stages of clinical trials (Chueh and Kahan, 2005). In 2007, the Food and Drug Administration (FDA) approved the rapalog temsirolimus for the treatment of advanced-stage renal cell carcinoma, making it the first mTOR inhibitor approved for cancer therapy. Recently, the rapalog everolimus was approved for the treatment of tuberous sclerosis, an autosomal dominant disorder characterized by hamartomas (benign tissue growth resembling a tumor) in various organs, including the brain (Chan et al., 2010).

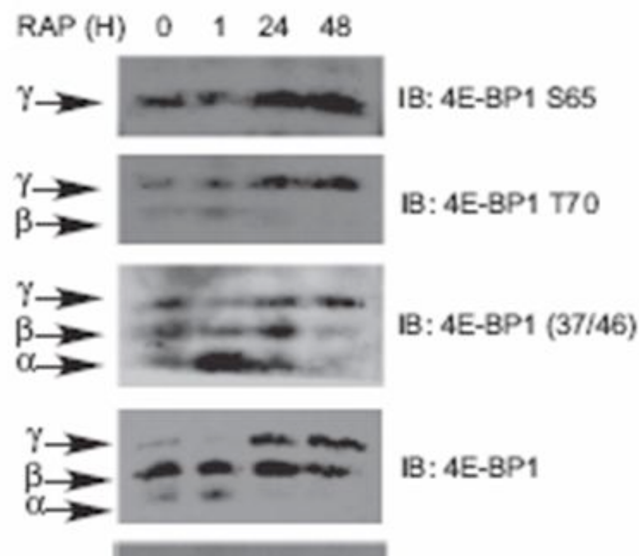
Overall, rapamycin and rapalogs have been unsuccessful cancer therapeutics, unable to kill cancer cells or stop their growth. Failure has been attributed to two problems: (1) inability to inhibit 4E-BP1 phosphorylation and (2) potential Akt activation. Below **(1.6c)** we describe the research that underlies Problem 1 because it not only sets the stage for our initial observation (high doses of rapamycin are cytotoxic) but underscores the relevance of our results.

1.6c Rapamyin does not inhibit 4E-BP1 phosphorylation

Dr. Anne-Claude Gingras found that rapamycin increases the interaction between eIF4E and 4E-BP1 by inhibiting 4E-BP1 phosphorylation (Gingras et al.,

2001b; Grolleau et al., 2002). As such, it was concluded that rapamycin treatment mimics the effects observed by the 4E-BP1 mutant **(1.5)** (Avdulov et al., 2004; Fingar et al., 2002). The work suggested that the ability of rapamycin to inhibit cancer cell growth is in part mediated through the de-phosphorylation of 4E-BP1.

However, what Gingras actually found was that while rapamycin effectively inhibited 4E-BP1 phosphorylation on sites S65 and T70, it did not inhibit phosphorylation on sites T37/46. That rapamcyin only partially inhibits the phosphorylation of 4E-BP1 has been well-supported (Feldman et al., 2009; Thoreen et al., 2009). In **Figure 1.6c**, sites T37/46 are insensitive to rapamycin (Choo et al., 2008). The phosphorylation-induced gel shifts of 4E-BP1 are correlative with increases in known phosphorylation sites on 4E-BP1. The data indicates that although phosphorylation is inhibited after one hour rapamycin treatment, it re-emerges. They do note that differential inhibition of 4E-BP1 phosphorylation occurs depending on cell type (the blot shown below is representative of human embryonic kidney (HEK) cells). Already, this suggests a variable mechanism for phosphorylation/de-phosphorylation of 4E-BP1. Nevertheless, it is purported that rapamycin can not de-phosphorylate 4E-BP1 on all sites.



e 1.6c Rapamycin does not inhibit all phoshorylation sites on 4E-BP1.

93 cells were treated with rapamycin fo24, 48 h or ethanol for 48 h. Lysates from ıycin-treated samples were blotted for the phosphorylation status of 4E-BP1. The sites zed were S65, T70, T37/46 and total 4E-BP1. Gel shifts can be observed in samples d with rapamycin for 24 or 48 h. The α-β-γ isoforms represent the phosphorylation ; of 4E-BP1 with α being hypo-phosphorylated and γ being hyper-phosphorylated.

› et al., *PNAS*, 2008).

Importantly, these results reflect the use of low doses of rapamycin (20nM). As aforementioned **(1.6)**, rapamycin was originally found to inhibit S6K activity. Phosphorylation at site T389 is blocked universally (in all cell lines tested thus far), quickly (minutes) and in low (20 ng/ml) concentrations. [20ng/ml rapamycin is equivalent to 21 nanomolar]. As such, phosphorylation status of S6K is considered a solid indicator of rapamycin sensitivity. As is vital to our investigation, it is important to

note that the same concentrations of rapamycin that have been used to evaluate the effect on S6K have been used to evaluate phosphorylation of 4E-BP1.

Yet, the coordinative activation of S6K and 4E-BP1 is necessary for protein synthesis and thus growth and proliferation. Thus, it is necessary to inhibit phosphorylation of both proteins. It appears that while effectively inhibiting 4E-BP1 is challenging, inhibiting S6K activity is easier. Paradoxically, the inhibition of S6K phosphorylation stimulates cell survival in some cancer cells as it activates Akt.

1.6d Inhibition of S6 kinase phosphorylation stimulates pro-survival kinase Akt

Chronic mTORC1-mediated activation of S6K, as in Tsc1$^{-/-}$ or Tsc2$^{-/-}$ cells (Choo et al., 2010) that lack tumor-suppressive function, induces a state of cellular insulin resistance by a mechanism termed the ‘negative-feedback loop’ (Harrington et al., 2004; Shah et al., 2004). When activated by mTORC1, S6K directly phosphorylates the insulin receptor substrate-1 (IRS1), which ultimately leads to reduced signaling to downstream PI3K effectors such as Akt, (Carracedo et al., 2008). It has been shown that rapamycin’s potent effect on mTORC1 inhibits this feedback loop. This is illustrated in **Figure 1.6d.1**.

gure 1.6a.1 Schematic representation of mTORC1 negative feedback loop with and ithout rapamcyin treatment

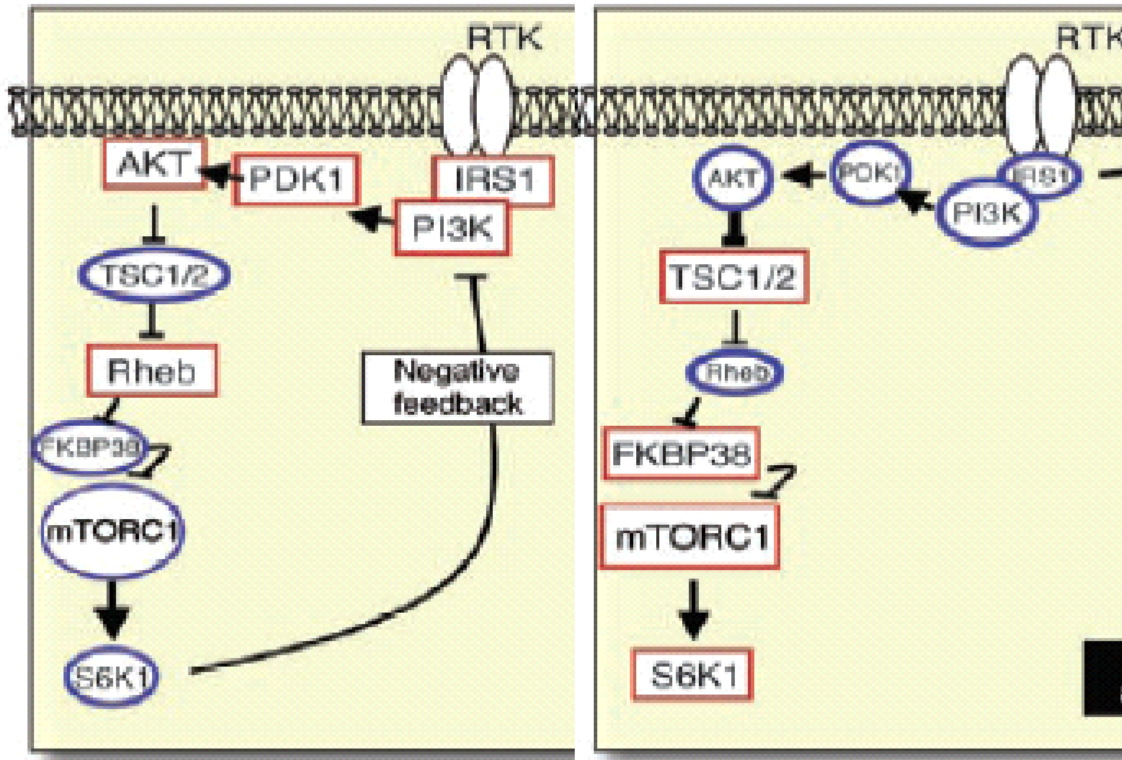
mTORC1 activation leads to PI3K inhibition through a negative feedback loop emming from S6K (left panel).

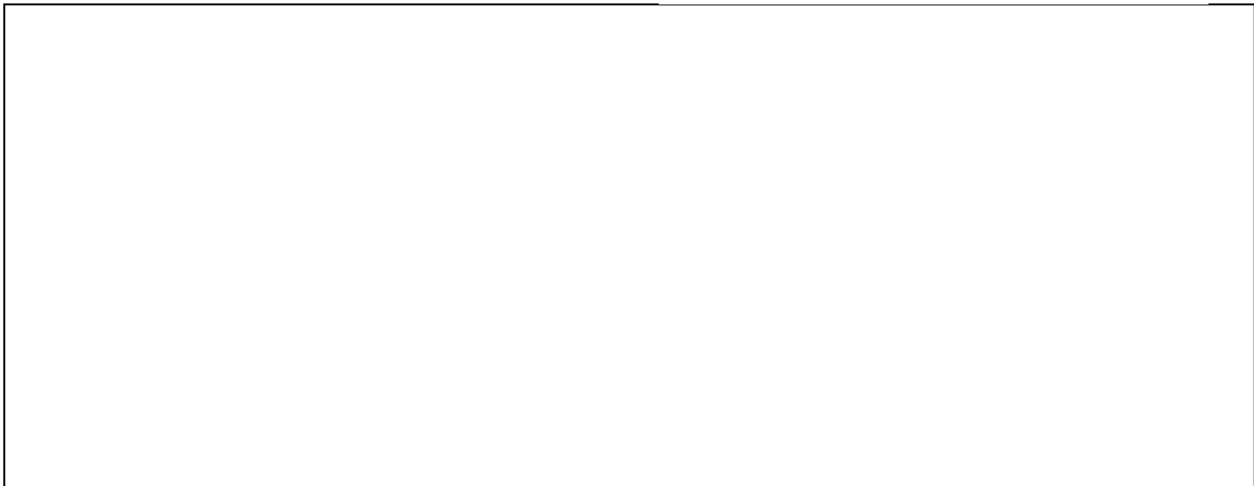
Treatment with mTORC1 inhibitors results in a hyperactive RTK (receptor-tyrosine nase)/IRS-1/PI3K/Akt pathway.

Rapamycin-untreated **Rapamycin-treated**

ctivated protein

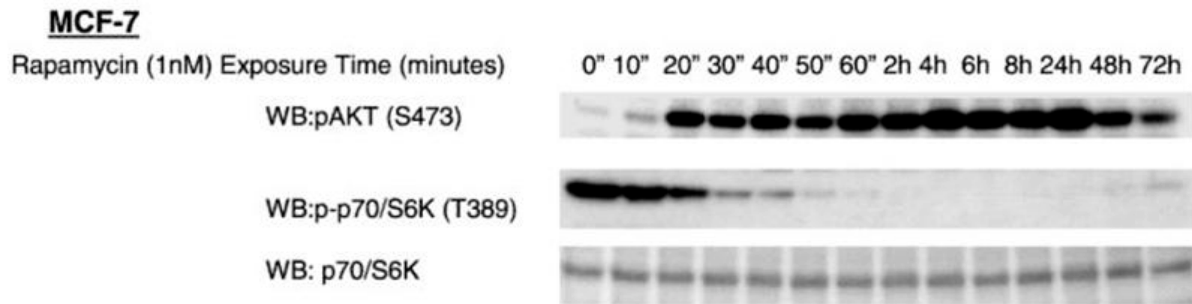
dapted from C

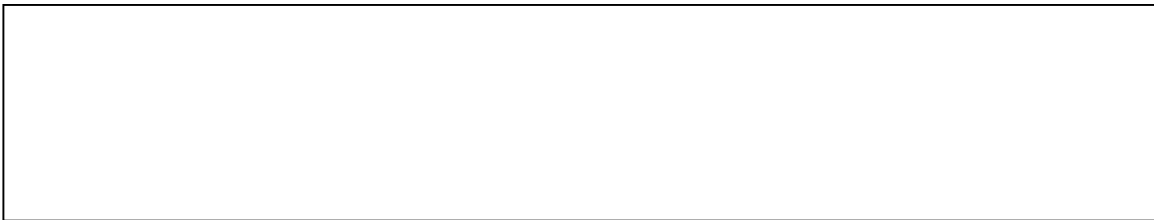




1 nmol/L rapamycin treatment induced S473 Akt in vitro in a MCF-7 cancer cell line. Phosphorylation of p70/S6K decreased with rapamycin treatment whereas total levels of p70/S6K did not change.

Data demonstrating this effect is shown below (O'Reilly et al., 2006) **(Figure 1.6d.2)**. Rapamycin inhibits S6K activity (western blot shows inhibition of phosphorylation of S6K at T389) and results in activation of Akt (western blot shows increase in phosphorylation of Akt at S473). Importantly, the data is similar to our own work in MCF-7 cells (**Figure 3.5a)**.





Thus, the presence of a negative feedback loop in the mTOR pathway may also contribute to limit the therapeutic efficacy of rapamycin. The inability to effectively inhibit total mTORC1 activity as well as the activation of mTORC2 activity may in part explain why rapamycin and rapalogs have failed to perform in anti-cancer clinical trials as well as originally hoped (Liu et al., 2009b). It also promoted the development of a different type of mTOR inhibitor.

1.7 Second-generation of mTOR inhibitors

Advantages

With the rationale that inhibition of both mTORC1 and mTORC2 would have a greater impact on cancer cells, came the recent development of ATP-competitive mTOR catalytic inhibitors (i.e. Torin1, PP242, Ku-0063794, WAY600, AZD8055) (Liu et al., 2009a). These small molecules bind directly to the ATP-binding site in the mTOR kinase that is required for the functions of both mTOR complexes, thereby blocking phosphorylation of all known downstream targets of mTORC1 and mTORC2 (Chresta et al., 2010). AZD8055 **(3.6)** is currently in Phase I/II clinical development in patients with advanced solid tumors (Chresta et al., 2010).

As anticipated, these inhibitors do impair cell growth and proliferation in vitro and tumor growth in vivo to a much greater degree than rapamycin (Falcon et al., 2011; Janes et al., 2010; Yu et al., 2010). Unlike rapamycin (note: low doses), these inhibitors completely block 4E-BP1 phosphorylation which results in a stronger inhibition of cap-dependent translation. Moreover, mTOR kinase inhibitors induce a significantly broader transcriptional response compared with rapamycin; many genes with roles in tumor biology and metabolism are only affected by complete mTOR inhibition (Wang et al., 2011).

Disadvantages

It was originally thought that the inhibition of the mTORC2-Akt axis would account for the success of these compounds. Recently an mTOR-specific kinase inhibitor, Torin1, was developed in David Sabatini's lab (Thoreen et al., 2009). The investigators showed that Torin1 was much more effective than rapamycin in inhibiting

translation and proliferation. Consistent with the recovery in 4E-BP1 phosphorylation by rapamycin (Choo et al., 2008, Thoreen et al., 2009), Torin1 was also much more effective in de-phosphorylation of 4E-BP1 phosphorylation than rapamycin. Surprisingly, even in mTORC2-deficient cells, these inhibitors cause a greater reduction in proliferation than rapamycin (Thoreen et al., 2009). It was reasoned that mTOR kinase inhibitors may exert their anti-proliferative effects through suppression of rapamycin-resistant functions of mTORC1. In effect, this rationale strengthens the results in our study **(Chapter 3)**. Firstly, it places an importance on inhibiting all of mTORC1 activity (S6K and 4E-BP1) and it also highlights the requirement of high-doses in order to do so **(3.3, 3.4a)**.

Another reason why ATP-competitive inhibitors have not been considered a worthy treatment option is due to widespread cytoxicity. The concentrations that ensure efficacy are simply not tolerable to the patient. Thus, their promising therapeutic mechanism observed at the bench is rendered ineffective in the clinic. In contrast, our study demonstrates an important characteristic of rapamycin: its cytotoxic effect is not global. Rapamycin treatment retains its specificity on mTORC1 regardless of dose **(3.10)**.

Altogether, this raises questions: has the mechanism of rapamycin, as a specific mTORC1 inhibitor, been fully elucidated? In turn, has its potential as an anticancer agent, been fully exploited?

1.8 Project rationale

The information provided thus far characterizes rapamcyin as a cytostatic drug. However, previous work in the (Foster) lab demonstrated that rapamycin induces apoptosis in several cancer cell lines, albeit at high (μM) doses (Chen et al., 2005; Chen et al., 2003; Gadir et al., 2008). Thus, we set forth to determine the mechanism by which this occurs with no reservation that the cytotoxicity was due to a global effect on the cell (we had also previously shown that high-dose rapamycin had no effect on cells with a mTOR kinase-dead mutant). Our recently published work **(Chapter 3)** establishes that such high doses are required to effectively inhibit all 4E-BP1 phosphorylation sites. Knockdown of eIF4E but not 4E-BP1 also caused cell death, indicating that high-dose rapamycin is cytotoxic by an indirect effect of inhibiting eIF4E.

Importantly, this cytotoxic effect occurs only in the absence of serum because in the presence of serum, high-dose rapamycin causes a cytostatic effect, with cells arresting in G1 (Gadir et al., 2008). As high-dose rapamycin indirectly inactivates eIF4E **(Chapter 3)**, we were surprised to observe cell death upon knockdown of eIF4E in the presence of serum. Curiously, simultaneous knockdown of S6K and eIF4E does not cause cell death. This observation is the basis for the work begun in **Chapter 4**. From the data in **Figure 3.1**, it can be deduced that inhibiting S6K is not sufficient to induce G1 arrest. From the apoptotic effect of eIF4E knockdown in serum, it is apparent that inhibition of eIF4E is necessary but not sufficient to induce arrest. Thus, the aforementioned observation (knockdown of both proteins does not cause cell death)

suggests that both inhibition of S6K and inhibition of eIF4E is necessary to induce arrest.

We hypothesize that dual knockdown of S6K and eIF4E results in coordinate induction of transforming growth factor-β (TGF-β) signaling that is sufficient to induce arrest. This is predicated on Gadir's work that shows high-dose rapamycin causes G1 arrest rather than apoptosis, if and only if, TGF-β signaling is intact (Gadir et al., 2008). TGF-β mediates a cytostatic response by activation of Smad signaling and cyclin-dependent kinase inhibitor (CDK) p27.

Chapter 2

Materials and Methods

2.1 Cell culture

The human breast cancer cell lines MDA-MB-231 and MCF-7 were obtained from the American Tissue Type Culture Collection (ATCC) and cultured in Dulbecco's modified Eagle medium (DMEM supplemented with 10% Fetal Bovine Serum (Sigma).

2.2 Antibodies and reagents

The following antibodies were used: PARP, cleaved PARP, p- P70 S6 kinase T389, P70 S6 kinase, p-4E-BP1 T37/46, p-4E-BP1 S65, p-4E-BP1 T70, 4E-BP1,p-Akt S473, Akt (Cell Signaling); α-actin (Sigma); eIF4E (Santa Cruz Biotechnology). Negative control siRNA (Dharmacon), siRNAs targeted against S6 kinase, 4E-BP1, eIF4E (Santa Cruz Biotechnology) were purchased. Lipofectamine RNAiMax (Invitrogen) was used for transient transfections. Rapamycin and wortmannin were purchased from Calbiochem. Insulin was purchased from Sigma. [3H]-thymidine was from Perkin Elmer; the catalytic mTOR inhibitor AZD8055 was from Axonblot analysis.

2.3 Cell cycle synchronization and progression

Cell cycle progression under different concentrations of rapamycin was studied using thymidine incorporation assay. Cells were plated, synchronized in M phase using nocodazole (Sigma) and then treated with the indicated concentration of rapamycin along with[3H]-thymidine (1 mCi/ml, 20 Ci/mMole) (Perkin Elmer) label as described in the figure legend. Twenty-four hours later, the cells were washed twice with PBS and then precipitated twice with 10% trichloroacetic acid. The precipitates were solubilized

in 0.5 ml of 0.5% SDS/0.5 M NaOH solution. The extent of thymidine incorporation was quantified using 75μl of sample and 3 ml of scintillation fluid.

2.4 Cell viability

Cell viability was determined by trypan blue exclusion or by counting viable attached cells. For trypan blue exclusion, cells were harvested, washed and treated with trypan blue at a concentration of 0.4% v/v. After 5 min, trypan blue uptake (dead cells) was scored using a hemocytometer.

Cell viability was also determined by counting adherent cells 24 h after treatment, using a hemocytometer or Coulter counter.

2.5 Microscopy

Cell images were taken using a Nikon DXM camera on a Nikon Optiphot 2 microscope.

2.6 Protein gel blot analysis

Extraction of proteins from cultured cells and protein gel blot analysis of extracted proteins was performed using the ECL system (Amersham).

2.7 Transient transfection

Cells were plated in 6-well plates in medium containing 10% FBS. The next day (50% confluence), transfections with siRNAs (75nM) in Lipofectamine RNAiMax were performed. After 6 h, reagents were replaced with fresh 10% FBS and cells were allowed to incubate for an additional 48 h.

2.8 Cross-linking assay and immunoprecipitation

The crosslinking of mTOR with raptor by dithiobis(succinimidyl) propionate (DSP) (Sigma) and co-immunoprecipitation was performed. Quantitative changes in protein levels were analyzed by densitometry using Image-Jsoftware.

Chapter 3

High-dose rapamycin induces apoptosis in human cancer cells by dissociating mTORC1 and inhibiting 4E-BP1 phosphorylation

3.1 High-dose rapamycin treatment establishes critical effect on cell cycle progression

Although it had been initially reported that low doses of rapamycin (nM) were sufficient to cause arrest (Albers et al., 1993; Fingar and Blenis, 2004; Neshat et al., 2001; Podsypanina et al., 2001), subsequent research suggested that the effect only delayed progression through the cell cycle (Choo and Blenis, 2009; Choo et al., 2008). Previously, we reported that high doses of rapamycin (µM) causes G1 cell cycle arrest in the presence of serum (Gadir et al., 2008). Here, we examined the effect of increasing rapamycin doses on progression through S-phase in two breast cancer cell lines, MDA-MB-231 and MCF-7 **(Figure 3.1)**. We synchronized the cells by nocodazole treatment, a chemical inhibitor of cell cycle progression in G2/M phase (Jackman and O'Connor, 2001). The cells were tested for progression into S-phase (as measured by [3H]-thymidine uptake) after collection and re-plating into fresh media with varying concentrations of rapamycin for 24 hours. In MDA-MB-231 cells, we observe a very modest reduction in S-phase with rapamycin concentrations up to 200 nM but a dramatic reduction upon the high (20 µM) dose. In contrast, the MCF-7 cells respond with substantial reduction of S-phase content with low (20 nM) dose rapamycin. Altogether, complete G1 arrest (corresponding to substantial reduction of S-phase) occurs at 20µM in both cell lines. The data indicate that while low doses of rapamycin retard progression through the cell cycle in a manner that varies with cell type, high doses of rapamycin ensure G1 arrest. We also note that there is an undefined, yet pronounced effect of rapamycin on cell cycle progression between 2 and 20µM.

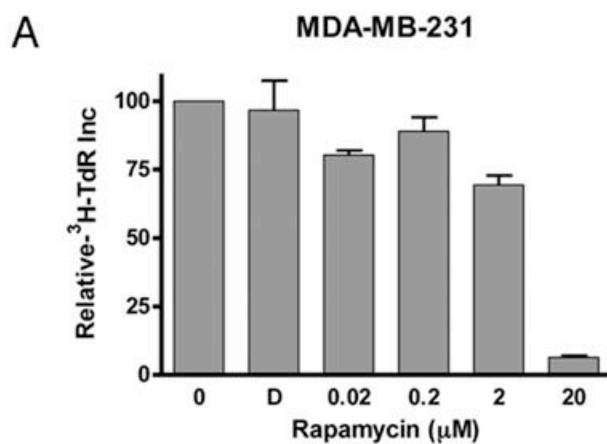
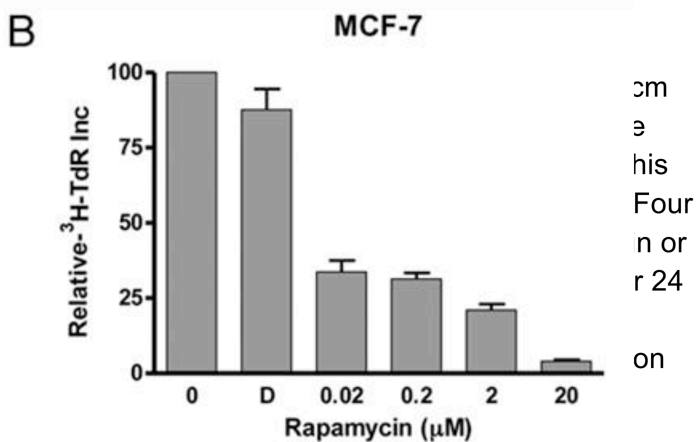


Figure 3.1 Reduction in S-p



(A) MDA-MB-231 and **(B)** M(cm
dish in complete medium cor e
treated with nocodazole at 2(his
point, mitotic cells were colle Four
hours later, after the cells rea n or
DMSO vehicle (D) were adde r 24
hours, lysates were collectec
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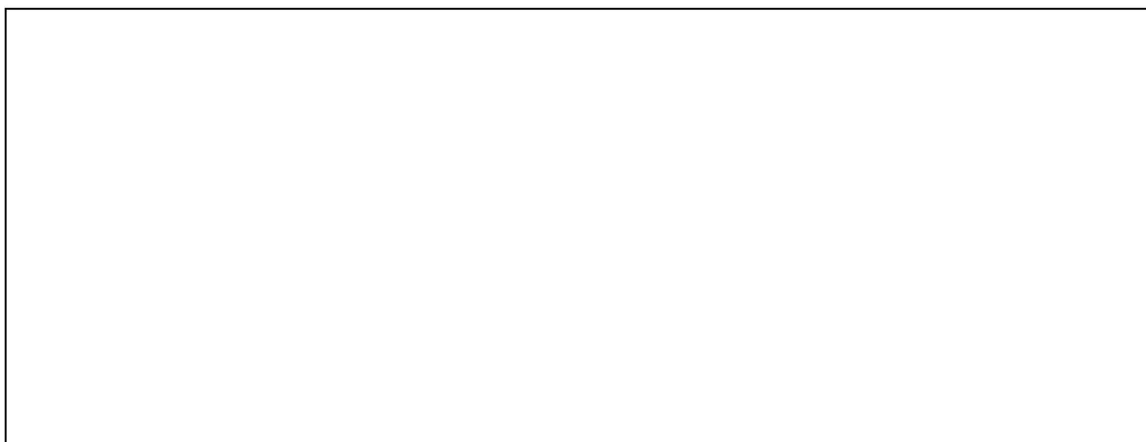


3.2 Differential effects of low- and high-dose rapamycin treatment on cell viability

Having established a cytostatic effect of high-dose rapamycin in serum, we investigated the effect of the drug in the absence of serum. Again, we observe a differential response between MDA-MB-231 and MCF-7 cells at the high dose. Cell viability **(Figure 3.2a)** was measured both quantitatively by cell count and qualitatively by cleavage of the caspase 3 substrate poly-ADP-ribose polymerase (PARP) which is an indicator of apoptotic cell death. At ninety-percent confluence, the cells were treated with rapamycin under serum-free conditions for twenty-four hours. A substantial 10-fold increase in percent non-viable cells is observed in MDA-MB-231 between the control and 20μM (compare 5% to nearly 50%). On the other hand, the MCF-7 cells remain viable, with less than 20% measurable cell death upon 20μM rapamycin treatment. These effects correspond with the induction of cleaved PARP which occurs only in the MDA-MB-231 cells at high-dose rapamycin. The absence of any cleaved PARP in the MCF-7 cells indicates a resistance to rapamycin. Note: Cleaved PARP was measured at 4 hours rapamcyin treatment because a time course revealed that this is the minimum time required to produce a robust response **(Figure 3.2b)**.

3.2a Differential effect of high-dose rapamycin in MDA-MB-231 and MCF-7 cells

igure 3.2b Time course to measure onset of cleaved PARP upon 20 μM rapamycin

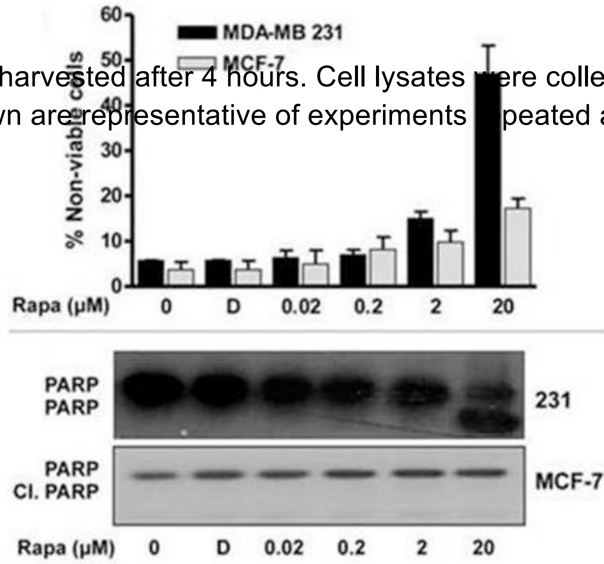
A

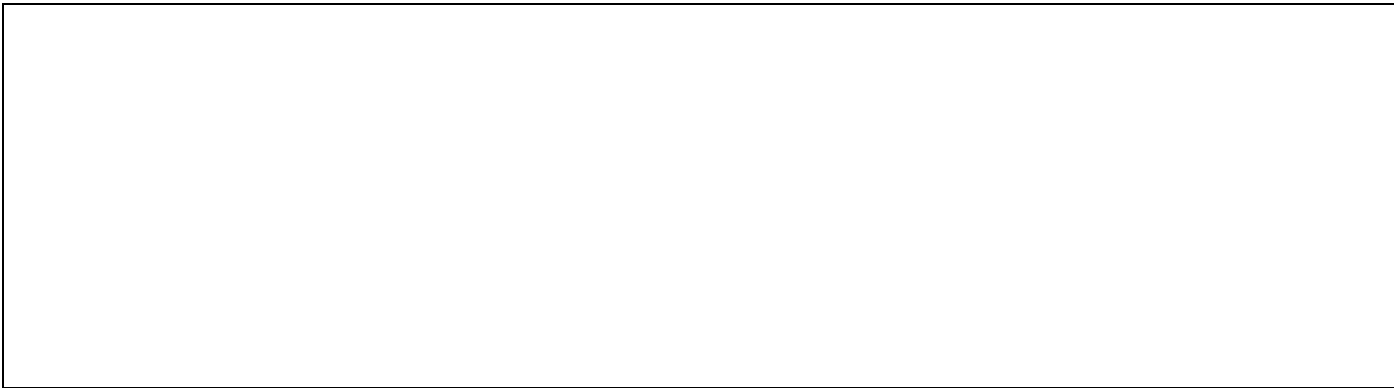
cell lines were plated in regular (10% serum) medium. Twenty-four hours later, at 90%
ice, cells were exposed to 0% serum and treated with DMSO (D) or rapamycin (Rapa) at
d doses and cell viability (as described in Materials and Methods) was determined after 24

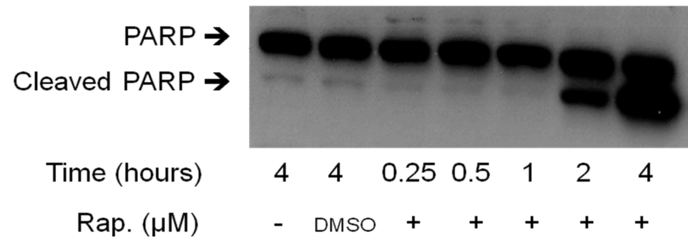
IDA-MB-231 cells were plated at 90% confluence, exposed to 0% serum and treated with
apamycin. After indicated time points, cell lysates were collected and immunoblotted with
ARP antibody.

s were plated and treated as in out harvested after 4 hours. Cell lysates were collected and
blotted with PARP antibody. Data shown are representative of experiments repeated at least
es.

B



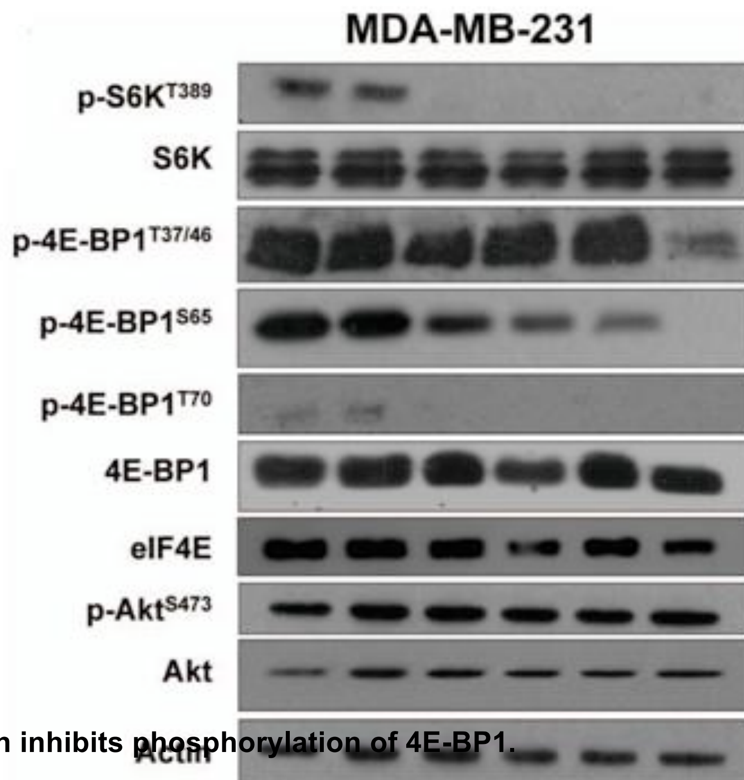




The data **(Figures 3.1, 3.2)** reveal both cytotoxic and cytostatic properties of rapamycin in MDA-MB-231 cells, notably occurring at doses between 2 and 20 μM. While rapamcyin does not cause cell death in the MCF-7 cells, it does cause cell cycle arrest. Overall, the data establish a critical effect of rapamycin at high doses. Of significance, the data implicate an extended and as-of-yet unrealized potential for rapamycin as a cytotoxic drug.

3.3 mTORC1 substrates are differentially sensitive to rapamycin doses in MDA-MB-231 cells.

We next examined the sensitivity of mTORC1 substrates to rapamycin in MDA-MB-231 cells. In accord with its well-established effect on S6 kinase phosphorylation, rapamycin effectively prevents phosphorylation of S6 kinase at T389 at 20 nM rapamycin. Inactivity of S6 kinase does not correspond with cleaved PARP, but inhibition of 4E-BP1 phosphorylation does, as is indicated by corresponding induction of cleaved PARP with its de-phosphorylated state **(Figure 3.3)**. Specifically, phosphorylation at T70 is sensitive to the low dose rapamycin sufficient to inhibit S6 kinase phosphorylation. However, phosphorylation of T37/T46 and S65 is only sensitive to rapamycin doses that induce apoptosis. Thus, rapamycin induces apoptosis at the same concentration that suppress 4E-BP1 phosphorylation, namely at T37/46 and S65.



ıure 3.3 High-dose rapamycin inhibits phosphorylation of 4E-BP1.

)A-MB-231 cells were plated in regular medium. Twenty-four hours later, at 90% confluence, ls were exposed to 0% serum and treated with DMSO (D) or rapamycin (Rapa) at the indicated ıcentrations for 4 h. Cells were harvested and lysates were immunoblotted with the indicated ibodies.

3.4 High-dose rapamycin irreversibly compromises the integrity of the mTORC1 complex.

It has been reported that nanomolar rapamycin concentrations disrupt the association of mTOR with raptor (Kim et al., 2002; Toschi et al., 2009). As we revealed that high doses of rapamycin are required to inhibit phosphorylation sites of all 4E-BP1 phosphorylation sites, we tested the degree of dissociation of raptor from mTOR with varying doses of rapamycin **(Figure 3.4a)**. The bar graph indicates that low-dose

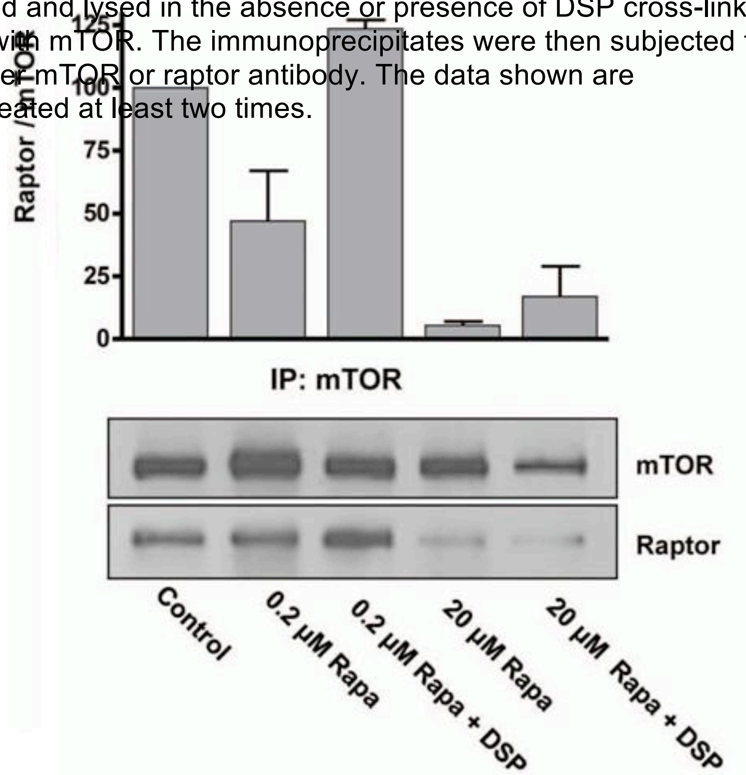
rapamycin treatment causes an approximate fifty percent reduction in raptor/mTOR association while high-dose rapamycin treatment nearly obliterates the association altogether.

To better elucidate the necessity of raptor-/mTOR association for activation of mTORC1 downstream targets, we tested the effect of a cross-linking reagent (DSP) in the presence of low and high-dose rapamycin. We find that upon co-treatment of low-dose rapamycin with DSP, raptor regains or even retains association with mTOR, suggesting that low-dose rapamycin treatment only weakens the association between mTOR and raptor. This finding suggests that low-dose rapamycin treatment disrupts the structure of mTORC1 sufficiently to inhibit phosphorylation of S6 kinase but not 4E-BP1.

Conversely, the cross-linking reagent fails to rescue raptor-mTOR complex association upon high-dose rapamycin treatment. Thus, high-dose rapamycin causes an irreversible dissociation of raptor from mTOR. If raptor-mTOR association is a sufficient indicator of mTOR substrate activity (S6 kinase and 4E-BP1 phosphorylation), then this effect correlates well with the rapamycin dose effects shown in **Figure 3.3**.

ıre 3.4a High-dose rapamycin irreversibly dissociates raptor from mTORC1.

s were exposed to 0% serum and treated with indicated rapamycin concentrations for 4 hour. sequently, they were harvested and lysed in the absence or presence of DSP cross-linking ent and immunoprecipitated with mTOR. The immunoprecipitates were then subjected to ein gel blot analysis using either mTOR or raptor antibody. The data shown are esentative of experiments repeated at least two times.

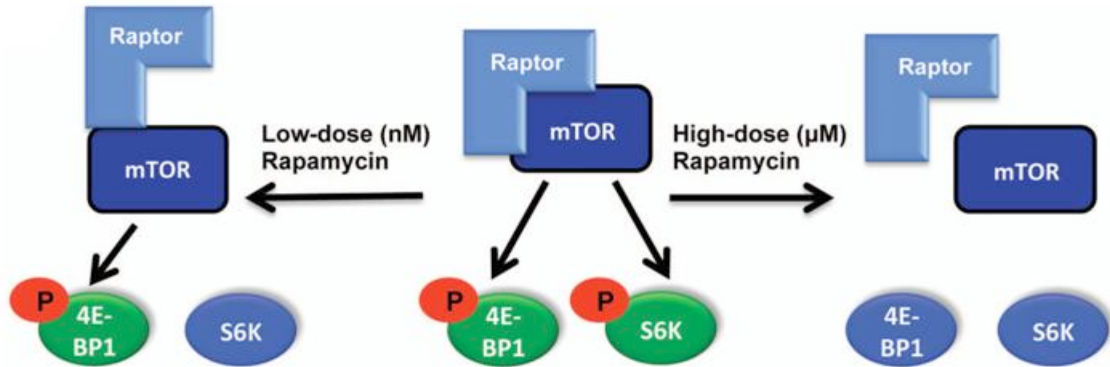


This concept is shown as a model in **Figure 3.4b**. We suggest that the differential effects of high and low-dose rapamycin treatment on mTORC1 are due to differential disruption of the mTORC1 complex. Low doses loosely disrupt the structure of mTORC1 such that S6 kinase is no longer recognized as a substrate and high doses completely dissociate the complex such that 4E-BP1 (in addition to S6 kinase) is no

longer recognized. Importantly, this indelible mark on mTORC1 complex stability by high-dose rapamycin correlates with loss of substrate functionality.

Figure 3.4b A model depicting the differential sensitivity of mTORC1 substrate phosphorylation by rapamycin

Low-dose rapamycin weakly dissociates raptor from mTOR, resulting in loss of S6K phosphorylation but maintenance of 4E-BP1 phosphorylation. High-dose rapamycin strongly dissociates raptor from mTOR, resulting in loss of both S6K and 4E-BP1 phosphorylation.



3.5 Hyper-phosphorylation of Akt at S473 prevents rapamycin-induced apoptosis.

In a similar manner, we also examined the sensitivity of mTOR substrate phosphorylation to rapamycin in the MCF-7cells. As shown in **Figure 3.5a**, these cells do not undergo apoptosis in response to high-dose rapamycin. Interestingly, phosphorylation status of both mTORC1 substrates mimicked that of MDA-MB-231 cells in response to rapamycin, with de- phosphorylation of S6 kinase and 4E-BP1 at nM and

μM doses, respectively. Thus, while high-dose rapamycin treatment also suppressed 4E-BP1 phosphorylation in the MCF-7 cells, this treatment does not induce apoptosis.

As was previously reported, (O'Reilly et al., 2006; Sun et al., 2005), nanomolar rapamycin does cause a modest increase in Akt phosphorylation. However, at 20 μM rapamycin, we observe a dramatic hyper-phosphorylation of Akt at S473 in the MCF-7 cells **(Figure 3.5a)**. This is in direct contrast to the observation made in the MDA-MB-231 cells, in which rapamycin has no effect on the phosphorylation of Akt at site S473 **(Figure 3.3)**. From this, we eliminate the possibility that the apoptotic effect of rapamycin in MDA-MB-231 cells could in part by attributed to dysfunctional mTORC2 and instead name a compromised mTORC1 as the sole effector.

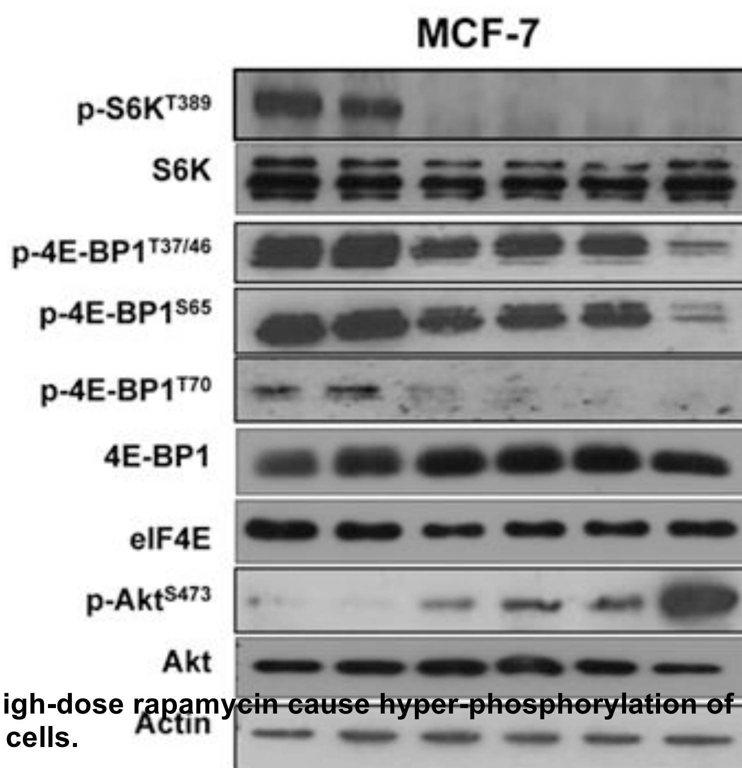


Figure 3.5a High-dose rapamycin cause hyper-phosphorylation of Akt in MCF-7 cells.

MCF-7 cells were plated and treated as in Figure 3.3. Cells were harvested, and lysates were immunoblotted with the indicated antibodies.

Since Akt phosphorylation at S473 has been implicated in survival signaling, (Manning and Cantley, 2007) we examined whether suppression of Akt phosphorylation sensitized the MCF-7 cells to high-dose rapamycin. As phosphorylation of Akt at S473 is dependent on PI3K (Manning and Cantley, 2007), treatment with wortmannin, a PI3K inhibitor, suppressed Akt phosphorylation at S473 and sensitized the MCF-7 cells to high-dose rapamycin. Cleaved PARP is observed by Western blot **(Figure 3.5b)**.

These data indicate that the hyper-phosphorylation of Akt in MCF-7 cells is sufficient to protect against the apoptotic effects of high-dose rapamycin.

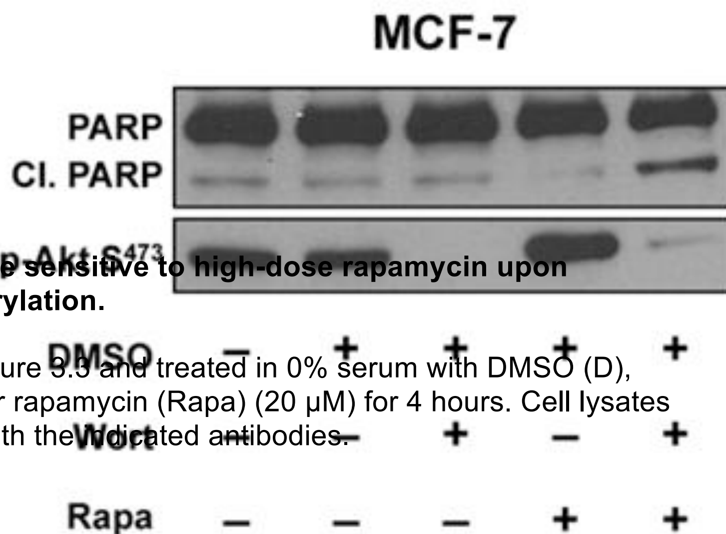


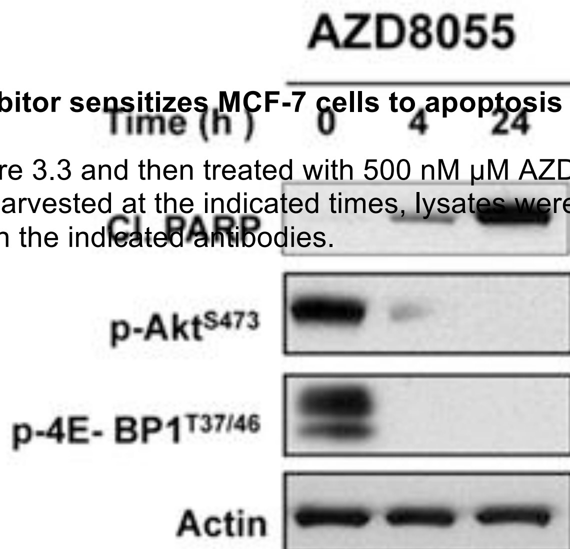
Figure 3.5b MCF-7 cells are sensitive to high-dose rapamycin upon inhibition of Akt phosphorylation.

MCF-7 cells plated as in Figure 3.5 and treated in 0% serum with DMSO (D), wortmannin (Wort) (1 μM) or rapamycin (Rapa) (20 μM) for 4 hours. Cell lysates were then immunoblotted with the indicated antibodies.

3.6 mTOR catalytic inhibitor sensitizes MCF-7 cells to apoptosis

We next investigated the effect of a catalytic mTOR inhibitor, AZD8055, with the hypothesis that a small molecule ATP-competitive inhibitor of mTOR would induce apoptosis in MCF-7 cells. mTOR is the catalytic subunit of both mTORC1 and mTORC2 complexes; mTORC2 is the kinase that phosphorylates Akt at S473, that confers its full activation. Thus, it would be expected that the catalytic inhibitor would

inhibit both mTORC1 and mTORC2 and consequently, induce apoptosis (Liu et al., 2009b). As shown in **Figure 3.6**, AZD8055 induces PARP cleavage in the MCF-7cells, (Chresta et al., 2010) providing further evidence that the hyper-phosphorylation of Akt at S473 mediates resistance to high-dose rapamycin treatment.



igure 3.6 mTOR catalytic inhibitor sensitizes MCF-7 cells to apoptosis

ICF-7 were prepared as in Figure 3.3 and then treated with 500 nM µM AZD8055 for the ndicated times. The cells were harvested at the indicated times, lysates were then repared and immunoblotted with the indicated antibodies.

3.7 Activation of Akt by insulin confers rapamycin-resistance in MDA-MB-231 cells.

Reasoning that Akt activation was sufficient to prevent cell death in the presence of high-dose rapamycin, we next examined whether or not inducing Akt phosphorylation would impart rapamycin-resistance to MDA-MB-231 cells. Previously, we reported that

insulin stimulates Akt phosphorylation at S473 in this cell line (Toschi et al., 2009). As shown in **Figure 3.7**, insulin treatment causes transient stimulation of Akt phosphorylation upon which no cleaved PARP is observed. Collectively, these data **(Figures 3.6, 3.7)** reveal that stimulating Akt phosphorylation at S473 prevents rapaymcin-induced apoptosis.

gure 3.7 Insulin confers rapamycin-resistance in MDA-MB-231 ells.

DA-MB 231 were plated as in Figure 3.2 and treated in 0% serum with sulin or rapamcyin (20 μM) for indicated times. Cell lysates were munoblotted with the indicated antibodies. All data are representative at least two independent experiments.

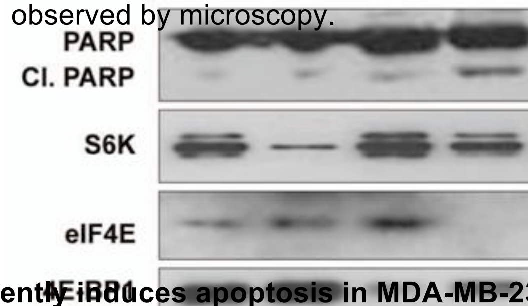
3.8 Inhibition of eIF4E expression leads to apoptosis in MDA-MB-231 cells.

De-phosphorylated 4E-BP1 binds eIF4E, effectively preventing eIF4E activity (Bjornsti and Houghton, 2004; Richter and Sonenberg, 2005). Upon its phosphorylation by mTORC1, 4E-BP1 and eIF4E dissociate, enabling "free" eIF4E to facilitate translation of RNAs encoding proteins involved in cell cycle progression (Bjornsti et al., 2004, Richter and Sonenberg, 2005) **(1.3)**. Having established that the cytotoxic effect of rapamycin occurs upon inhibition of S6 kinase and 4E-BP1 phosphorylation, **(Figure**

3.3) we investigated the effect on cell viability upon small interfering ribonucleic acid (siRNA) targeted against eIF4e, 4E-BP1 and S6 kinase **(Figure 3.8a)**. Likening protein expression levels to phosphorylation status, we predicted that knockdown of S6 kinase would mimic low-dose rapamycin treatment. This is the case. Cells remain viable and no cleaved PARP is observed upon S6 kinase knockdown. Presumably, knockdown of 4E-BP1 results in unbound eIF4E; thus, we did not expect cell death. In fact, only ablation of eIF4E expression caused cell death, mimicking the effect of high-dose rapamycin treatment. We do observe modest (about twenty percent) cell death upon S6 kinase and 4E-BP1 knockdown but knockdown of eIF4E results in a substantially higher percentage of non-viable cells (nearly fifty percent).

We observe similar effects by microscopy **(Figure 3.8b)**. As evident by loss of mesenchymal structure and adherence to the plate, MDA-MB-231 cells die upon eIF4E knockdown. In contrast, S6 kinase and 4E-BP1 knockdown cells are comparable to the control. These data further support our model **(Figure 34b)** whereby the apoptotic effect of high-dose rapamycin is due to suppression 4E-BP1 phosphorylation and subsequent inhibition of eIF4E.

MDA-MB-231



re 3.8a Knockdown of eIF4E sufficiently induces apoptosis in MDA-MB-231 cells.

-MB-231 cells were transfected at 50% confluence with negative control siRNA or IA targeted for S6 kinase, 4E-BP1 or eIF4E. 48 hours later, immunoblotting was used to yze the indicated proteins. The percentage of non-viable cells was determined at 48 s as described in Materials and Methods.

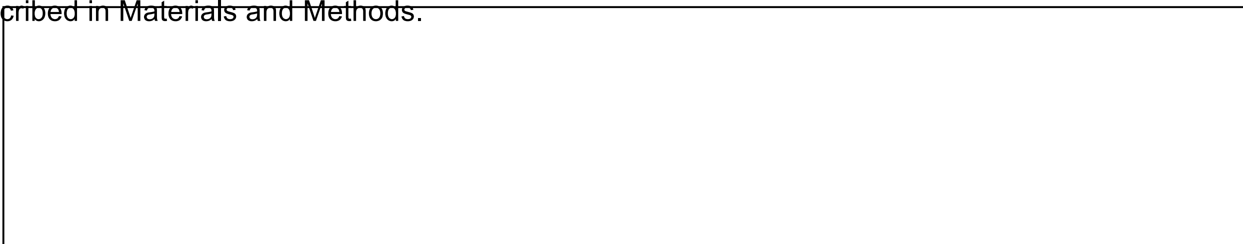
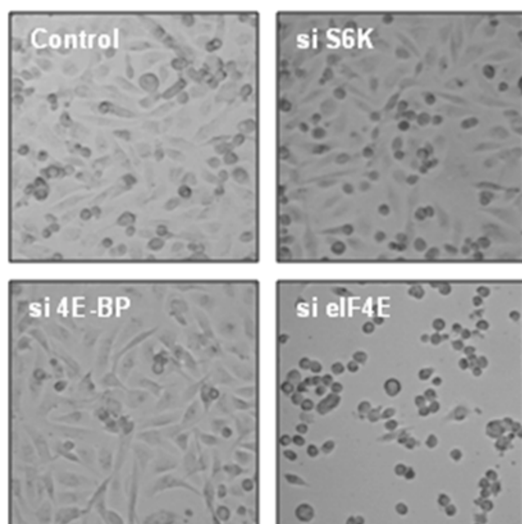


Figure 3.8b Morphology of control, S6K, 4E-BP1 and eIF4E knockdown in MDA-MB-231 cells

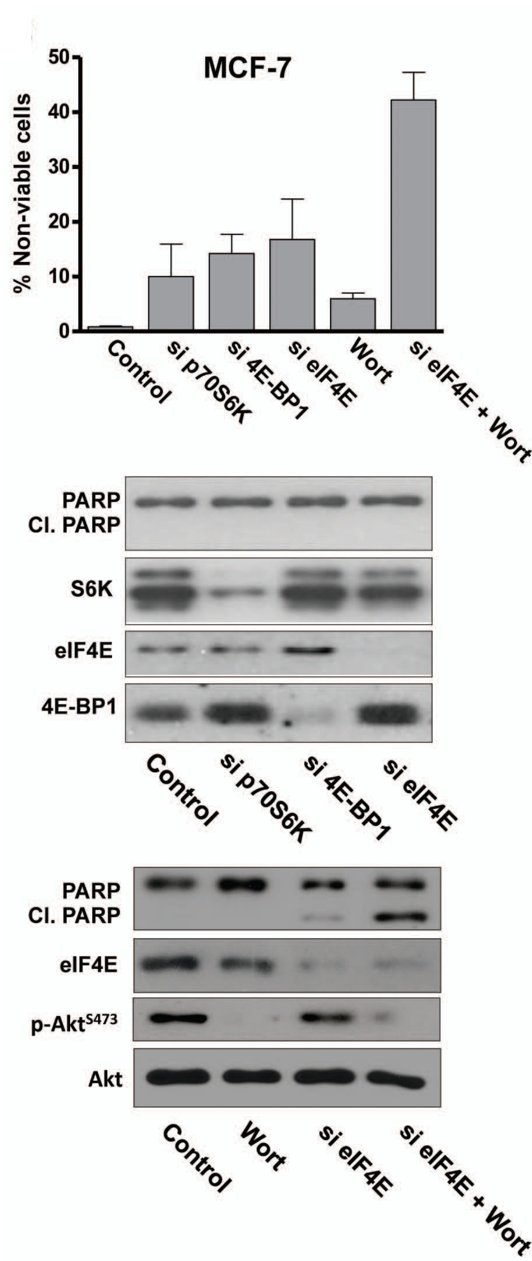
MDA-MB-231 cells were transfected at 50% confluence with negative control siRNA or siRNA targeted for S6 kinase, 4E-BP1 or eIF4E. 48 hours later, cells were observed by microscopy.

MDA-MB-231



3.9 Inhibition of eIF4E expression causes apoptosis in MCF-7 cells upon wortmannin treatment.

We also examined the effect of suppressing eIF4E in MCF-7 cells. In contrast to MDA-MB-231 cells, suppression of eIF4E does not significantly increase PARP cleavage or reduce cell viability **(Figure 3.9a A, B)**. However, treatment with wortmannin sensitizes the cells to eIF4E knockdown as a substantial increase in cleaved PARP and loss of cell viability is observed **(Figure 3.9a A, C)**. This correlates with microscopy images **(Figure 3.9b)**. Wortmannin causes an observed change in cell morphology but the cells remain viable. However, knockdown of eIF4E plus wortmannin treatment causes loss of epithelial phenotype as the cells round and lose adherence to the plate (indication of cell death) **(Figure 3.9b B)**. These data support the observation that MCF-7 cells are resistant to the suppression of 4E-BP1 phosphorylation by rapamycin **(Figure 3.5a)** and that this resistance is due at least in part to a feedback activation of Akt (Sun et al., 2005, O'Reilly et al., Wang et al., 2008).

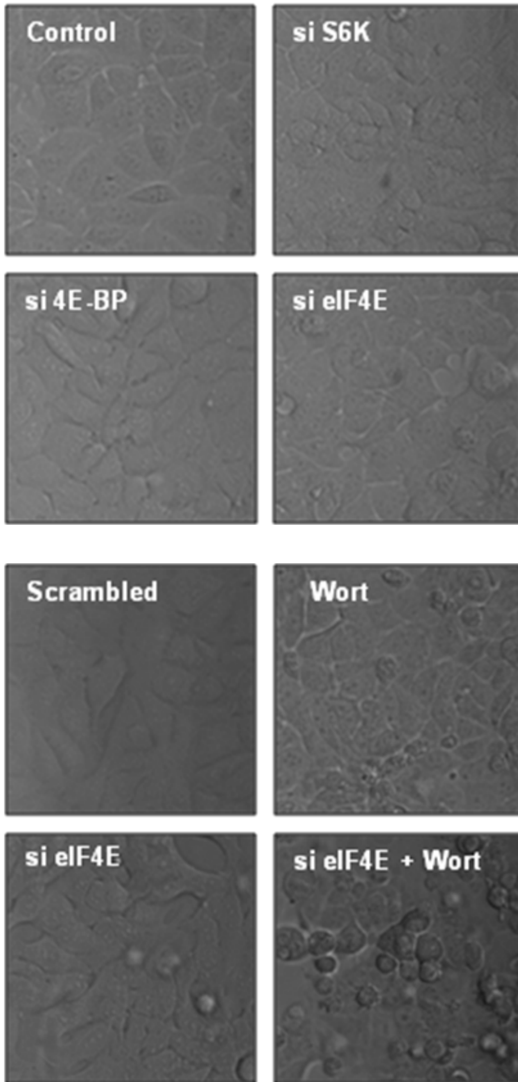


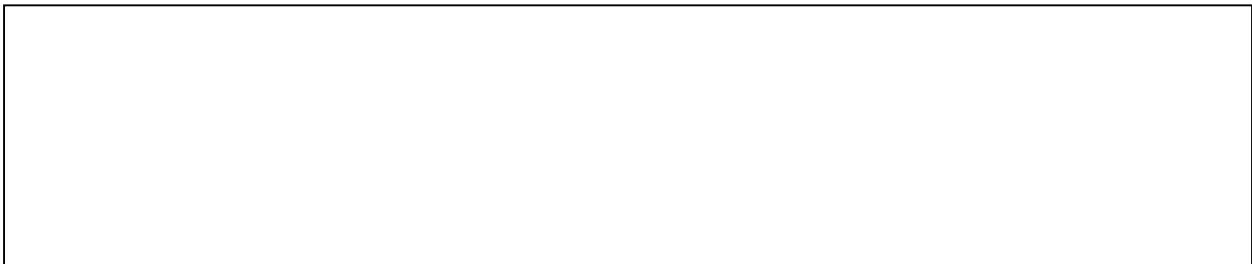
igure 3.9a Wortmannin sensitizes MCF-7 cells to eIF4E knockdown.

\, B) MCF-7 cells were transfected with indicated siRNAs. Cells were evaluated for ıdicated proteins and cell viability at 48 hours. **(A, C)** Wortmannin (1uM) was added where ıdicated 24 hours prior to evaluating cell viability and 4 hours prior to evaluating protein :vels and PARP cleavage.

ckdown MCF-7 cells

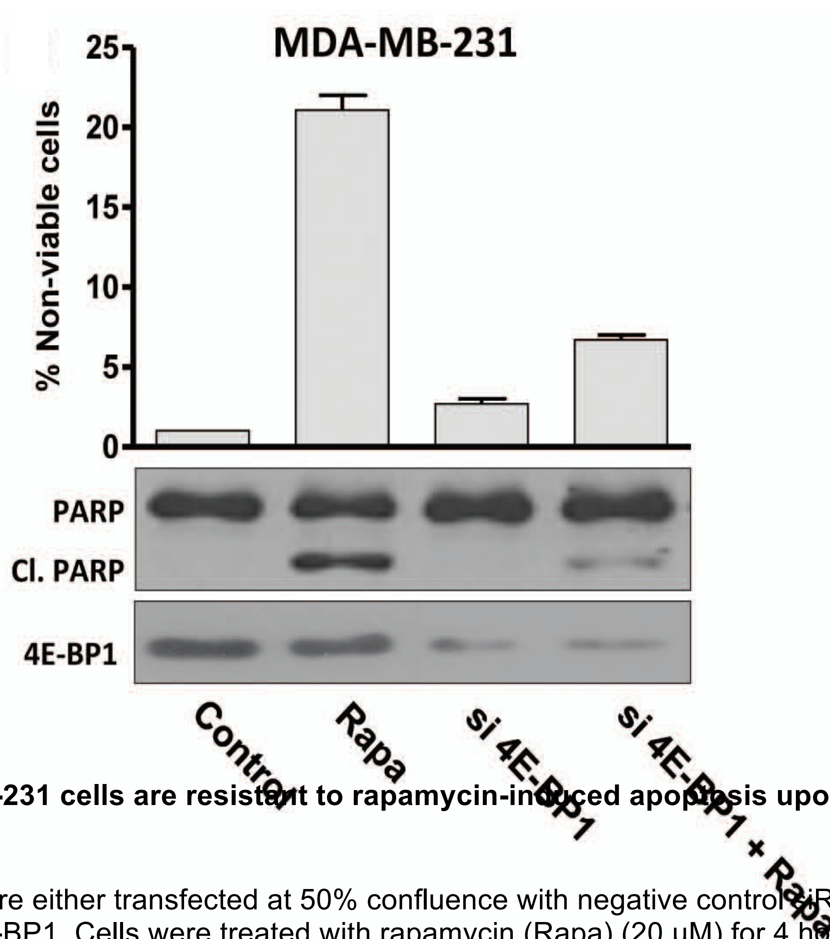
MCF-7 cells were transfected at 50% confluence with negative control siRNA or siRNA
jeted for S6 kinase, 4E-BP1 or eIF4E. 48 hours later, cells were observed by microscopy.
MCF-7 cells were transfected with negative control siRNA or eIF4E. Cells were treated
ı wortmannin (1 μM) as indicated. 48 hours later, cells were observed by microscopy.





3.10 High-dose rapamycin maintains specificity.

Since, it appeared that the apoptotic effect of high-dose rapamycin treatment is due to sequestration eIF4E by un-phosphorylated 4E-BP1, we reasoned that suppressing 4E-BP1 expression would de-sensitize the cells to high-dose rapamycin because there would be no 4E-BP1 to bind eIF4E. Therefore, we examined the effect of 20 µM rapamycin on cell viability on MDA-MB-231 cells treated with siRNA targeted for 4E-BP1. As shown in **Figure 3.10**, MDA-MB-231 cells treated with a control siRNA were sensitive to 20 µM rapamycin. However, cells treated with siRNA against 4E-BP1 were resistant to 20 µM rapamycin. These data further support the hypothesis that the effects of high-dose rapamycin are due to the direct suppression of 4E-BP1 phosphorylation and the indirect suppression of eIF4E. This is supported by the Dowling work discussed in **1.5**: raptor knockdown (Dowling) has the same effects as high-dose rapamycin (our study) upon loss of 4E-BP1 (Dowling) or knockdown of 4E-BP1 (our study). eIF4E is “free” and no cell death is observed. Importantly, this result also establishes that high-dose rapamycin treatment does not cause a global cytotoxic effect but rather a specific one, exerting a mechanistic repression of mTORC1.



ure 3.10 MDA-MB-231 cells are resistant to rapamycin-induced apoptosis upon 4E-1 knockdown.

A-MB-231 cells were either transfected at 50% confluence with negative control siRNA or NA targeted for 4E-BP1. Cells were treated with rapamycin (Rapa) (20 µM) for 4 hours to serve PARP cleavage and 24 hours for cell viability. All results are representative of periments repeated at least two times.

Chapter 4

Inhibition of S6 Kinase confers resistance to apoptosis induced by lack of eIF4E

4.1 Differential effects of eIF4E ablation and rapamycin treatment on MDA-MB-231 cells in the presence of serum

We had reported previously that high doses of rapamycin (20 μM) induce apoptosis in MDA-MB-231 **(Figure 4.1a A, B)** as well as in other cancer cell lines in the absence of serum (Chen et al., 2005; Chen et al., 2003; Gadir et al., 2008). The content in **Chapter 3** demonstrates the cytotoxic properties of rapamycin in MDA-MB-231 cells, implicating the importance of de-stabilizing the 4E-BP1/eIF4E complex. Here, we demonstrate that the apoptotic effect is due to suppression of 4E-BP1 phosphorylation by mTORC1, which prevents liberation of eIF4E. Suppression of 4E-BP1 phosphorylation leads to the sequestration and inhibition of eIF4E (Bjornsti and Houghton, 2004; Richter and Sonenberg, 2005). Consistent with this, we observe that suppression of eIF4E expression induces apoptosis **(Figure 4.1b A)**.

Prior work in the lab also demonstrated a cytostatic effect of high-dose rapamycin in the presence of serum **(elaborated on in Figure 3.1) (Figure 4.1a A, B)**. Thus, we were surprised to observe a cytotoxic effect upon knockdown of eIF4E in the presence of serum **(Figure 4.1b A, B)**. The loss of cell viability was accompanied by cleaved PARP, indicating apoptotic cell death **Figure 4.1b, B**.

B

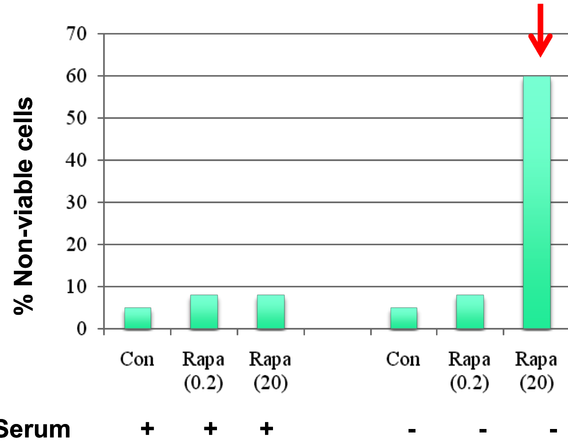
PARP
Cl. PARP

gure 4.1a MDA-MB-231 cells are resistant to high-dose rapamycin treatment in the esence of serum.

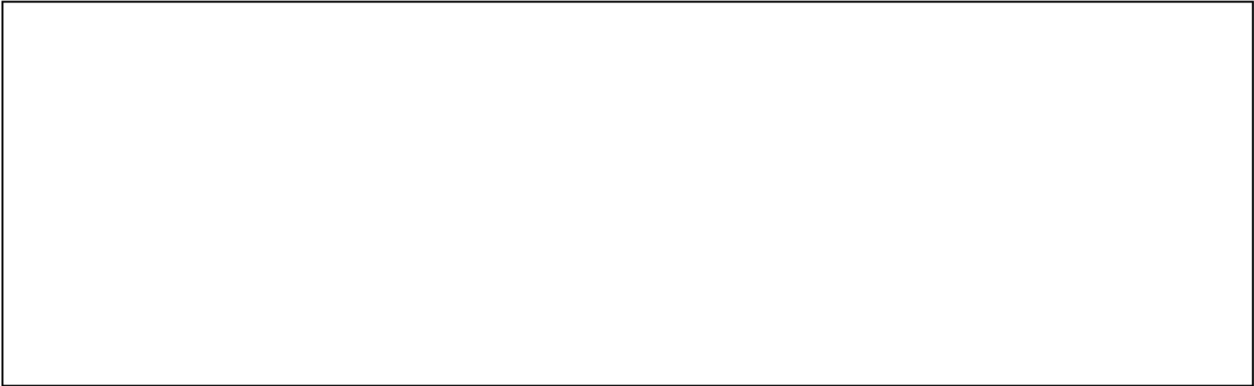
) Cells were d and treated at 90% confluence in 10% or 0% serum with DMSO or dicated conc tions of rapamycin (rapa) for 4 hours. Cell lysates were collected and munoblotted to indicate cleaved PARP.

) Cells were plated as in **(A)** and treated in 10% or 0% serum as indicated for 24 hours. e percentage of non-viable cells was determined at 48 hours as described in Materials and ethods.

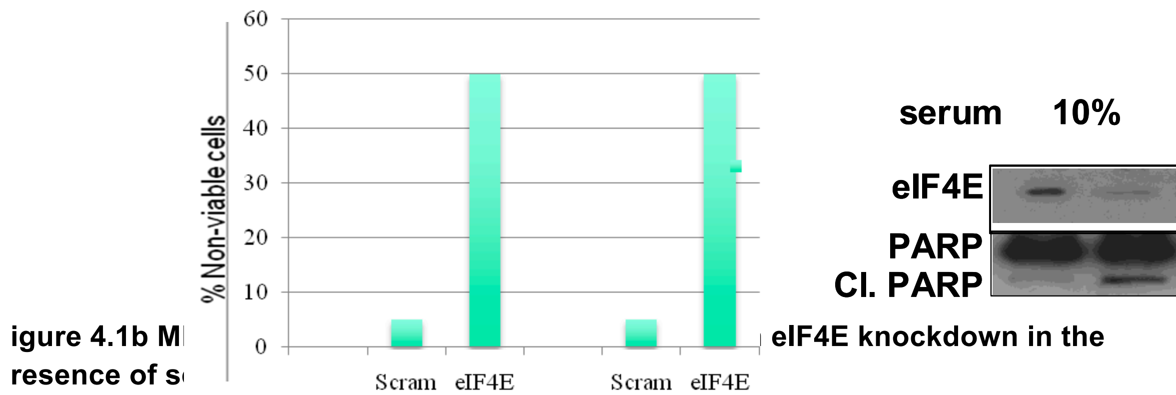
Control DMSO 2 μM rapa. 20μM rapa. Control DMSO 2 μM rapa. 20μM rapa.



Serum + + + - - -



B



igure 4.1b M **eIF4E knockdown in the resence of s**

Serum + + - -

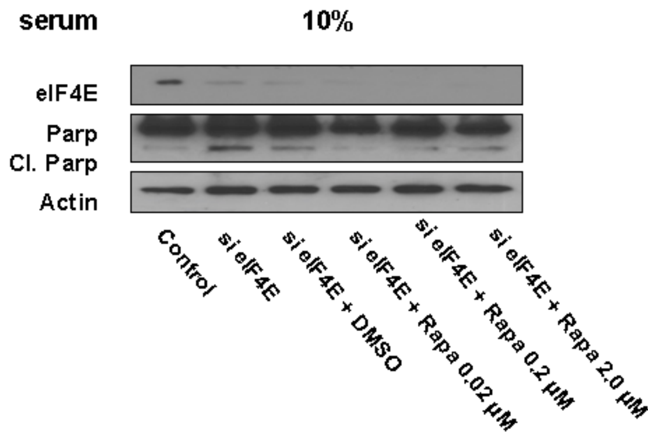
A) MDA-MB-231 cells were transfected at 50% confluence with negative control siRNA or iRNA targeted against eIF4E. The percentage of non-viable cells was determined at 48 ours as described in Materials and Methods.

B) Cell lysates were collected and immunoblotted to indicate cleaved PARP.

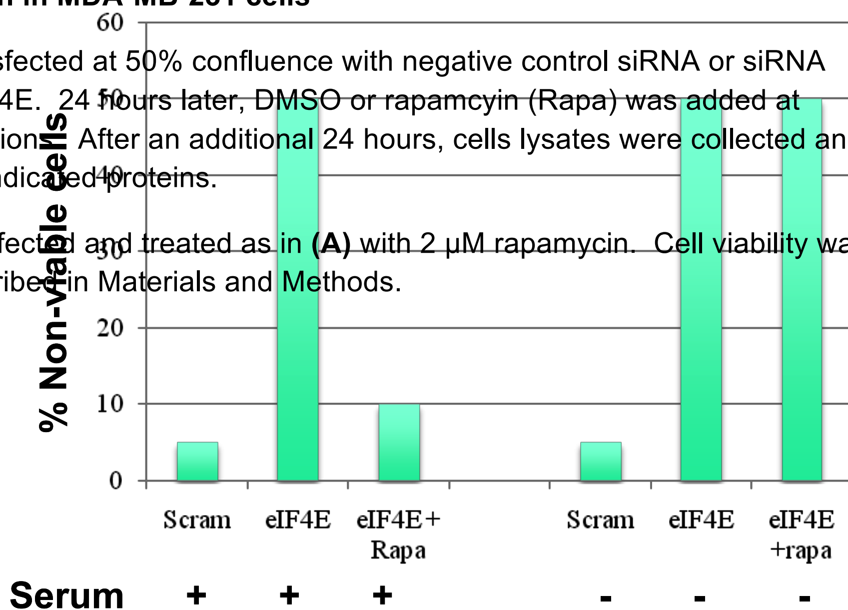
We reason that our observations are related to prior work in the lab (Gadir et al., 2008). In the presence of serum, there is a TGF-β-dependent G1 cell cycle arrest that prevents apoptosis (Gadir et al., 2008). Ongoing investigation is predicated on those findings.

4.2 Low-dose rapamycin or inhibition of S6 kinase expression prevents the apoptotic effect induced by inhibition of eIF4E expression in the presence of serum.

The data in **Figure 4.1a A, B** suggests that there is an additional effect of rapamycin that blunts the apoptotic effect of eIF4E ablation. Since high-dose rapamycin also suppresses S6 kinase phosphorylation, it is possible that suppression of S6 kinase phosphorylation confers resistance to high-dose rapamycin. Therefore, we examined the effect of low-dose rapamycin on MDA-MB-231 upon eIF4E knockdown. As shown in **Figure 4.2a**, cell death is suppressed or bypassed by the addition of rapamycin despite loss of eIF4E expression.



B



igure 4.2a Low-dose r cin prevents the apoptotic effect induced by inhibition f eIF4E expression in MDA-MB-231 cells

A) Cells were transfected at 50% confluence with negative control siRNA or siRNA argeted against eIF4E. 24 hours later, DMSO or rapamcyin (Rapa) was added at ndicated concentrations. After an additional 24 hours, cells lysates were collected and mmunoblotted for indicated proteins.

B) Cells were transfected and treated as in **(A)** with 2 μM rapamycin. Cell viability was letermined as described in Materials and Methods.

The surprising cytostatic effect of low-dose rapamycin treatment suggested that suppression of S6K triggers a protective mechanism. Thus, we inhibited both S6K and eIF4E expression **(Figure 4.2b)**. Such dual inhibition does not cause cell death, indicating that inhibition of S6K expression is sufficient to prevent death otherwise induced by singular knockdown of eIF4E.

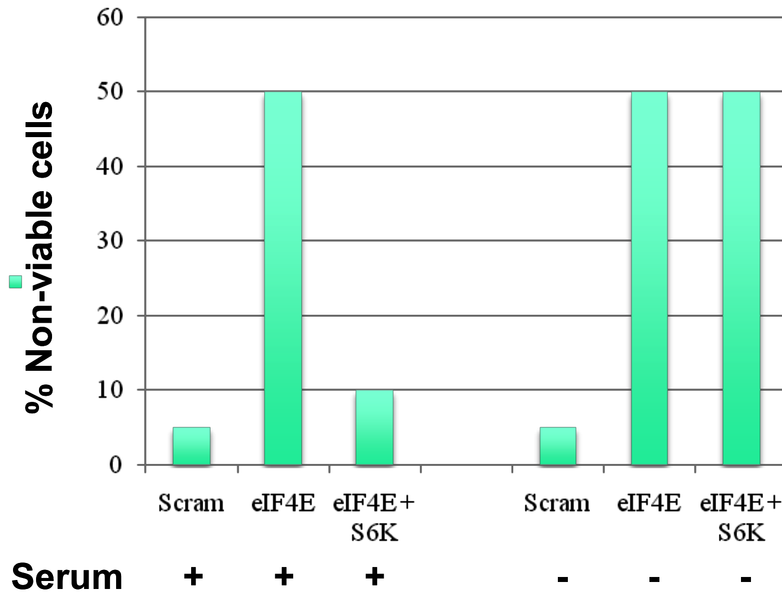


Figure 4.2b S6 kinase knockdown prevents the apoptotic effect induced by inhibition of eIF4E expression in MDA-MB-231 cells

Cells were transfected at 50% confluence with negative control siRNA or siRNA targeted against eIF4E. 24 hours later, DMSO or rapamycin (Rapa) was added at indicated concentrations. After an additional 24 hours, cell viability was determined as described in Materials and Methods.

Chapter 5

Discussion

5.1 Project Summary: Chapter 3

To date, we have revealed rapamycin as a site-specific, cytotoxic anticancer drug in the absence of serum. Our studies demonstrate that high-dose rapamcyin induces apoptosis because it effectively inhibits all phosphorylation sites on 4E-BP1, subsequently inactivating eIF4E. Importantly, we demonstrate that cancer cells are resistant to high-dose rapamycin upon 4E-BP1 knockdown, confirming that the drug retains its site-specific property regardless of dose. Furthermore, we show that high-dose rapamycin irreversibly compromises the integrity of the mTORC1 complex, as it pertains to mTOR-raptor association. We acknowledge the inefficacy of rapamycin in cancer cells that activate Akt upon mTORC1 inhibition. Under these circumstances, an inhibitor of both mTORC1 and mTORC2 complexes is necessary and sufficient to induce apoptosis.

5.2 Hierarchical 4E-BP1 phosphorylation correlates with rapamycin-induced apoptosis.

Our data clearly indicate that all four phosphorylation sites on 4E-BP1 must be inhibited to induce apoptosis in MDA-MB-231 breast cancer cells **(Figures 3.2a, 3.3)**. Complete inhibition requires high-dose rapamycin, with sites S65 and T37/46 retaining phosphorylation until such treatment. Specifically, phosphorylation at S65 is completely inhibited between 2 and 20 μM. However, inhibiting phosphorylation at T37 and T46 appears to be most resistant to rapamycin. Although not completely inhibited, phosphorylation is drastically reduced between 2 and 20 μM treatment. From **Figures 3.3, 3.8a** we attribute cell death to inhibition of translation (eIF4E knockdown or de-

phosphorylated 4E-BP1). Thus, it can be inferred that 4E-BP1 does not bind eIF4E unless T37 and T46 are de-phosphorylated. Our observation is supported by Dr. Gingra's work demonstrating hierarchical 4E-BP1 phosphorylation **(1.3)**. Phosphorylation of T70 and Ser 65 is not sufficient to disengage 4E-BP1 from eIF4E (Gingras et al., 2001a). Therefore, the stability of the 4E-EBP1/eIF4E complex is dependent on phosphorylation of sites T37/46. Ultimately, rapamycin's indirect inactivation of eIF4E, stipulated by direct inhibition of phosphorylation on 4E-BP1 sites T37 and T46, induces apoptosis.

5.3 Rapamycin-induced apoptosis is due to indirect inactivation of eIF4E in the absence of serum.

Figure 3.8a demonstrates that knockdown of eIF4E, not 4E-BP1, causes apoptosis. The result also suggests a requirement of eIF4E expression to maintain cell viability (in the absence of serum) and as discussed in **1.5**, is consistent with reports implicating it in cell proliferation and oncogenic transformation (Dowling et al., 2010; Lazaris-Karatzas et al., 1990). Increased oncogenic potential by elevated eIF4E expression and/or activity can also be attributed to phosphorylated 4E-BP1 (indicative of dysregulated mTORC1) which is associated with poor patient survival in melanoma (O'Reilly et al., 2009). Altogether, our data is consistent with a paradigm that mTOR survival signals are mediated by the phosphorylation of 4E-BP1 and the subsequent release of eIF4E.

5.4 Strength of mTOR-raptor association determines S6K and 4E-BP1 phosphorylation

We attribute the differential effects of low- and high-dose rapamycin treatment to the partial versus complete dissociation of raptor from mTOR. Rapamycin's direct action on mTOR disrupts the mTOR-raptor association **(1.6a)**. While low-dose rapamycin treatment weakens, but still allows for, the association between raptor and mTOR, high-dose rapamycin nearly obliterates it. This is show in **Figure 3.4a** (compare low-dose rapamycin treatment (2nd bar) and high-dose rapamycin treatment (4th bar) to control). This suggests that the interaction between raptor-S6K is weaker than the raptor-4E-BP1 interaction. The Blenis group supports this and additionally proposes that the affinity for each substrate changes the structure of mTOR to facilitate phosphorylation (Choo and Blenis, 2009).

mTOR-raptor association is completely regained with a cross-linker after low-dose rapamycin treatment, suggesting that loss of S6K phosphorylation can be restored. On the other hand, the cross-linker is incapable of restoring mTOR-raptor association after high-dose rapamycin treatment suggesting that the complex is irreversibly compromised.

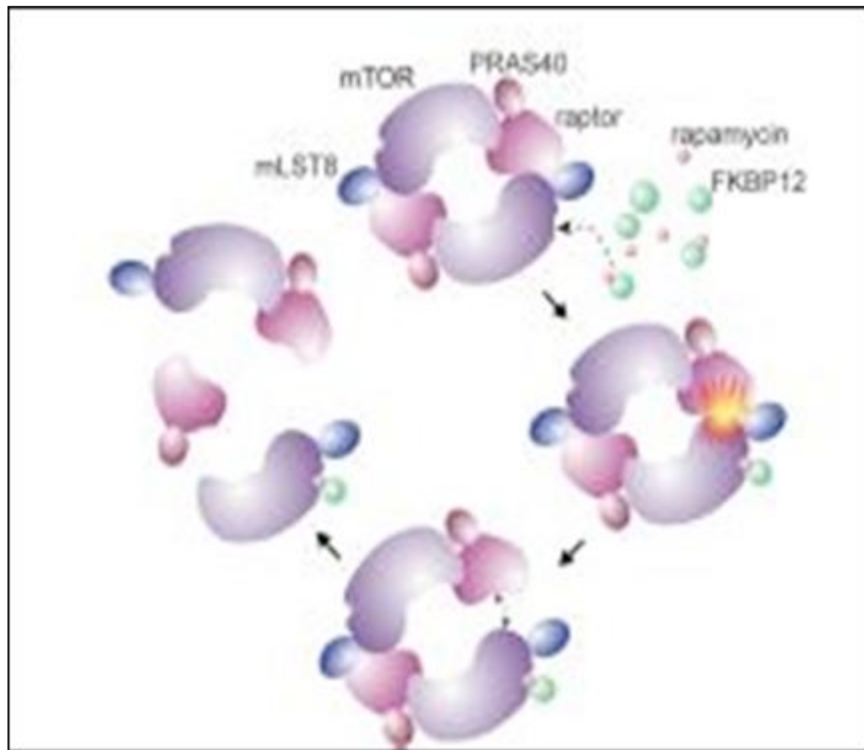
In support of our findings, a prior biochemical study indicated that binding of FKBP12-rapamycin to mTORC1 induces a conformational change that weakens the mTOR-raptor interaction (Kim et al., 2002).

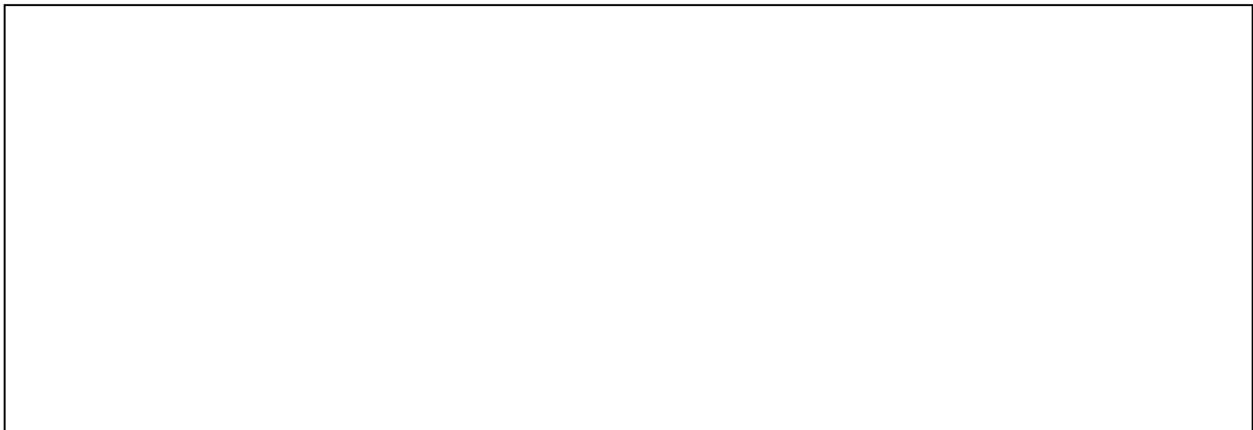
Another study by the Sabatini group depicts the alterations of mTORC1 with rapamycin treatment (Yip et al., 2010). Here, the investigators have determined the three-dimensional structure of mTORC1 by cryo-electron microscopy **(Figure 5.1)**.

The initial binding of one FKBP12-rapamycin to mTORC1 causes a subtle conformational change in mTOR that weakens the mTOR-raptor interaction, but does not suffice to disrupt the dimeric architecture. Moreover, the bound FKBP12-rapamycin likely occludes the binding of or blocks access to the active site for larger-sized substrates such as S6K1. Over time, either amplified structural strain caused by the first FKBP12-rapamycin or, perhaps, the binding of a second rapamycin complex leads to a fast disintegration of the already "weakened" mTORC1 and the complete abolishment of 4E-BP1 phosphorylation.

(**Yip et al., *Mol. Cell,* 2010)**

They treated purified intact human mTORC1 (by a method they developed) with 100 nM rapamycin. S6K phosphorylation was suppressed acutely (after 5 minutes of treatment) while long exposures (2 hours) suppressed the phosphorylation of 4E-BP1 at T37/46. Though the rapamycin dose and treatment time differs greatly than the parameters in our study, the net effect and conclusion is the same. Weakened complex assembly is associated with loss of S6K phosphorylation while an obliterated complex is associated with loss of 4E-BP1 phosphorylation.





These results place raptor at the forefront of substrate recognition and presume that it is indispensable for mTORC1 activity in as far as activation of S6K and 4E-BP1. While our results and those of other investigators (mentioned here: Choo and Blenis, 2009; Kim et al., 2002; Yip et al., 2010) indicate this is the case, mTORC1 has many other downstream functions independent of protein S6K and 4E-BP1, including but not limited to, roles in lipid synthesis and autophagy. In other words, raptor may be dispensable for other mTORC1 functions.

Nonetheless, additional experiments might strengthen our argument. We could knockdown raptor and mTOR in both the MDA-MB-231 and MCF-7 cells. We would expect that knockdown of raptor and mTOR would inhibit phosphorylation of both S6K and 4E-BP1. Cell death would only be observed in MDA-MB-231 cells as MCF-7 cells would survive by inhibition of the mTORC1 negative feedback loop **(1.6d, 5.4)**.

We also could measure restoration of S6 kinase phosphorylation after addition of the cross-linker upon low-dose rapamcyin treatment **(Figure 3.4a, 3rd bar)**.

A cell viability experiment in cells treated with high-dose rapamycin plus cross-linker would correlate complex integrity with viability: cells would not respond to the addition of the cross-linker and undergo apoptosis as they do by high-dose treatment alone. This would confirm that mTOR-raptor association is critical for cell viability in MDA-MB-231 cells, or further, in cells that do not inhibit the negative feedback loop. In fact, complex dissociation may stimulate Akt activation in the MCF-7 cells. We could compare Akt activation in MCF-7 cells treated with the cross-linker after low and high-dose rapamcyin. We might expect that the obliteration of the raptor-mTOR association in the latter treatment would also accompany hyper-phosphorylated Akt.

5.5 Activation of Akt by rapamcyin necessitates development of other mTOR inhibitors.

Despite de-phosphorylated 4E-BP1, MCF-7 cells survive high-dose rapamycin treatment. **Figures 3.2a and 3.5a** demonstrate this correlation as no cleaved PARP is observed at the high-dose treatment. Instead, high-dose rapamycin treatment results in a progressive increase in phosphorylation of Akt at S473, with a robust signal produced at 20 μM. This hyper-phosphorylation is a contrast to phosphorylation observed at nM concentrations reported previously (O'Reilly et al., 2006; Sun et al., 2005). The observation that the high-dose induces Akt activation is indicative of the negative feedback loop in the mTOR pathway **(1.6d)**.

Wortmannin sensitizes the cells to rapamycin-induced apoptosis. Correspondingly, stimulation of Akt phosphorylation in MDA-MB-231 cells with insulin results in rapamycin-resistance with no PARP cleavage observed. Together, these data indicate that Akt activation is necessary and sufficient to prevent death by rapamycin. As such, it would seem fitting that targeting mTORC2 alone would be the superior anti-cancer strategy. But as addressed in **6.4**, this is not a viable option.

As is evident in our study, the negative feedback loop is not observed in all cancer cell lines (compare MDA-MB-231 to MCF-7). Nonetheless, it may explain tumor recalcitrance to rapamycin and rapalog treatment in some patients. A clinical trial for the rapalog, everolimus revealed an increase in the phosphorylation of Akt at S473 observed in fifty percent of the tumors (Tabernero et al., 2008). While it was not apparent how this impacted on the overall effect of everolimus, there may be interesting differences in the effects of rapalogs themselves.

5.6 Rapamycin dose stipulates cell cycle arrest in the presence of serum.

Enhancement of the 4E-BP1/eIF4E interaction, through the expression of a constitutively active 4E-BP1 mutant (in which all phosphorylation sites are mutated to alanine) **(1.5)**, leads to a decrease in cell size and inhibition of cell cycle progression in a manner that mimics rapamycin treatment (Fingar et al., 2002). This 4E-BP1 mutant also slows G1 progression and prevents tumor growth (Lynch et al., 2004). These findings suggest that the ability of rapamycin to inhibit cancer cell growth is in part mediated through de-phosphorylation of 4E-BP1.

These studies, along with the hierarchy of 4E-BP1 phosphorylation, support data shown in **Figure 3.2a, A**. Here, we see a difference in the reduction in S-phase content between the MDA-MB-231 cells and the MCF-7 cells upon rapamycin treatment up to 2 μM. Generally, though, the low-dose rapamycin treatment only modestly slows progression through the cell cycle. This correlates with de-phosphorylation of S6K. However, at 20 μM, both cell lines exhibit drastic reduction in S-phase content relative to their controls. Possibly, the effect at 20 μM represents de-phosphorylated 4E-BP1 at T37 and T46 with the other sites de-phosphorylated upon lower dose treatment **(Figure 3.3)**. This coordinates S6K and 4E-BP1 phosphorylation status with progression through the cell cycle. It also suggests a chronological order of phosphorylation: S6 kinase at T389 then 4E-BP1 (hierarchically).

5.7 Dual inhibition of S6 kinase and eIF4E bypasses apoptosis.

Our current study **(Chapter 4)** is to further investigate the surprising observation that while high-dose rapamycin treatment (indirect inactivation of eIF4E) induces

apoptosis only in the absence of serum **(Figure 4.1b)**, knockdown of eIF4E (direct inactivation of eIF4E) induces apoptosis in both the presence and absence of serum **(Figure 4.1a)**. Thus, there is a mechanistic difference between the indirect and direct inactivation of eIF4E.

Since high-dose rapamycin treatment also inhibits phosphorylation of S6K and does not induce apoptosis in the presence of serum, we reasoned that protection from apoptosis induced by high-dose rapamycin could be due to suppression of S6K. Consistent with this hypothesis, concomitant knockdown of eIF4E plus either low-dose rapamycin treatment (up to and including 2 μM) **(Figure 4.2a)** or dual S6K and eIF4E knockdown **(Figure 4.2b)** prevents cell death otherwise induced by knockdown of eIF4E alone.

5.8 Neither S6K nor eIF4E alone is sufficient to cause cell cycle arrest

Currently, we are investigating the mechanism by which this occurs. Inhibition of S6K phosphorylation by low-dose rapamycin, in the presence of serum modestly reduces the number of cells in S-phase (Figure 3.1-MDA-MB-231 cells). Instead, drastic reduction of S-phase content is observed at high-dose rapamycin. Thus, it can be inferred that inhibition of S6K activity alone is not sufficient to induce cell cycle arrest. Dual inhibition of S6K and eIF4E activity is required to induce arrest. From the rapamycin experiments, it is not possible to isolate the effect on cell cycle arrest by eIF4E inhibition alone. The phenotype (dead cells) observed by knockdown of eIF4E is actually a fortuitous one as it makes it apparent that inactive eIF4E is also not sufficient

to induce arrest. Therefore, neither S6K nor eIF4E inhibition alone is sufficient to induce cell cycle arrest.

Importantly, we do note that knockdown of S6K in the absence of serum (and therefore, the absence of TGF-β signaling) does not cause cell death. We infer that the 4E-BP1/eIF4E complex is intact **(3.4a, b, 5.4)**. That this is sufficient for cell viability can may be supported from the information provided in **1.5** (loss of S6K is not critical for cell cycle progression).

5.9 TGF-β-mediated cell cycle arrest requires coordinate inhibition of S6K and eIF4E.

The cell cycle arrest observed with high-dose rapamycin treatment in the presence of serum is dependent on TGF-β signaling which is suppressed by mTORC1 (Gadir et al., 2008; Gadir et al., 2007; Song et al., 2006). However, TGF-β signaling can be activated with nanomolar doses that suppress S6 kinase phosphorylation (Gadir et al., 2007).

Therefore, we hypothesize that inhibition of S6K initiates TGF-β signaling but that concomitant inhibition of eIF4E is required for complete cell cycle arrest. Future studies outlined in **Chapter 6** are designed to test this.

Chapter 6

Conclusions

and

Future Studies

6.1 Significance

The basic research perspective presented here addresses two related problems underlying the disappointment associated with rapamycin as an anti-cancer drug in clinical trials: complete de-phosphorylation of 4E-BP1 and inhibition of the mTORC1 negative feedback loop.

6.2 S6K activity is the readout for rapamycin efficacy

Evaluating the inhibition of mTORC1 should include not only the phosphorylation status of S6 kinase but of 4E-BP1 as well. But, measuring the phosphorylation of S6K and/or its substrate S6 is much technically much easier than measuring phosphorylation of 4E-BP1 with phosphor-specific antibodies. Thus, utilization of phospho-S6K as an in vivo readout assay for mTORC1 activity has become pervasive (Choo and Blenis, 2009). If measured in clinical trials, the effect on 4E-BP1 phosphorylation is observed at site T70 (Tabernero et al., 2008). Coincidentally, T70 is de-phosphorylated by rapamycin at 20nM, the same dose that de-phosphorylates S6K **(1.6c, Figure 3.3)**. Our work clearly indicates that cytotoxic effects are only observed upon inhibition of all 4E-BP1 phosphorylation sites.

Still, stalwart adherence to using S6K as a marker for rapamycin efficacy is not without reason. It reflects the principal requirement of successful Phase I clinical trials design: safety. The maximum tolerated dose (MTD), the maximum dose of a drug that does not harm the patient, of rapamycin is in the nanomolar range (Cloughesy et al., 2008; Tabernero et al., 2008, Hsieh et al., 2010). With emphasis on safety, the clinical

approach has been to administer drugs only at low doses, obeying a so-called "nanomolar rule." Consequently, potentially efficacious chemotherapeutic agents have been cast aside (Wong et al., 2012). This perspective supports a departure from the nanomolar rule. With regard to our study: more is better.

6.3 Maximum tolerated dose exceeds high-dose rapamycin: is there a solution?

Then, we do acknowledge that the level of rapamcyin required to suppress 4E-BP1 in cell culture would be equivalent to a dose higher than the MTD. To advocate the use of high-dose rapamcyin would necessitate a means of drug delivery capable of targeting tumors with high specificity. One possible strategy is to tag rapamycin with glucose, which is taken up preferentially by most cancer cells (the well-established Warburg effect (reviewed by DeBerardinis et al., 2008; Vander Heiden et al., 2009). This seems like a plausible method as glucose has been used to enhance the uptake of a photodynamic therapeutic agent into cancer cells (Chen et al., 2004). With such a strategy, delivery of high-dose rapamycin may be achieved. An aside, there may also be secondary, beneficial effects conferred by rapamycin treatments which have been implicated in prolonging lifespan (Blagosklonny, 2009, 2010; Foster, 2010).

Thus, while targeting 4E-BP1 phosphorylation and eIF4E-dependent protein synthesis, may present challenges, understanding the limits of rapamycin-based therapies suggests novel approaches that can exploit the critical mTORC1 signaling node that promotes cell cycle progression and survival in what may be virtually all human cancer cells.

6.4 Catalytic inhibitors

The experiments with MCF-7 cells illustrate the paradox of rapamycin/rapalog treatment: inhibiting mTORC1 activity stimulates pro-survival mTORC2 (Akt) activity. The initial solution was to develop catalytic inhibitors that effectively target both mTOR complexes (mTORC1 and mTORC2). In fact, we observe that in lieu of rapamycin, catalytic inhibitor AZD8055 causes apoptosis, sufficiently inhibiting the activation of mTORC1 substrate 4E-BP1 and that of mTORC2 substrate Akt in MCF-7 cells. The pitfall of the catalytic inhibitor is its probable toxicity to the patient. Currently, however, there is no pharmacological way to inhibit mTORC2 without also affecting mTORC1. The fact that both complexes share the same catalytic domain makes the prospect of developing an mTORC2-specific inhibitor daunting.

6.5 Current clinical trials: combining allosteric and catalytic inhibitors

The latest approach to induce maximal inhibition of mTOR in cancer cells is to combine allosteric and catalytic inhibitors at low doses. The rationale is that rapamycin/rapalogs change the conformation of mTORC1 such that the catalytic center is more accessible or has a higher affinity for the catalytic inhibitors. For example, investigators in one study used rapamycin and PI-1-3 (a dual PI3K/mTOR inhibitor) in ovarian and prostate cancer. The benefits were twofold:

1. At the molecular level, combined inhibition of mTOR prevented the rebound activation of Akt that is seen after treatment with rapamycin/rapalogs and caused more sustained inhibition of Akt phosphorylation. A synergistic response is observed, eliciting greater anti-tumorigenic potential than by singular treatment with either drug.

2. There was no increase in toxicity as doses administered were well below the maximum tolerated dose (Mazzoletti et al., 2011).

At a recent conference (Keystone Symposium: Cancer and Metabolism, February 2012) I attended, it was revealed that a Phase I clinical trial with rapalog everolimus (RAD-001) and catalytic inhibitor BEZ235 is underway. Lower doses of both drugs are being administered than would otherwise be administered alone. Importantly, it was emphasized that the study was designed to measure 4E-BP1 and eIF4E inhibition (Nyfeler, 2012).

6.6 Tumor progression may be predictive of cytotoxic or cytostatic effects of rapamycin.

Tumors adapt to stress conditions such as hypoxia and nutrient deprivation, developing and progressing despite these imperfect conditions. In fact, their responses contribute to development and progression (Wellen et al., 2010). We have established a cytostatic effect in the presence of serum and a cytotoxic effect of rapamycin in the absence of serum. As such, this may have implications in targeting tumors with high-dose rapamycin at different stages of tumorigenesis. Early stage tumors, developing in nutrient-replete conditions may respond to high-dose rapamycin by arresting while late stage tumors growing in harsher conditions may be susceptible to cell death.

6.7 Summary

In summary, the effects of rapamycin on cancer cells have been difficult to evaluate because of differential effects observed in different cancer cells and the

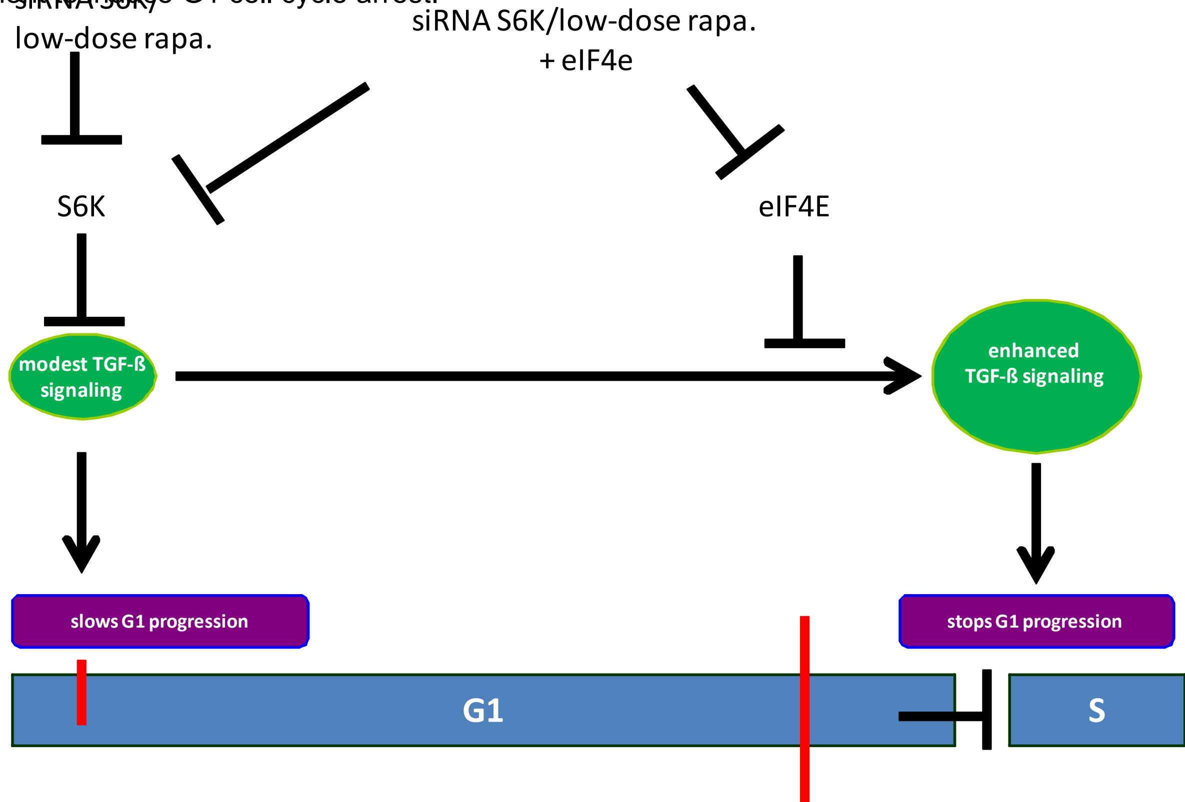
varying concentrations needed to suppress different downstream targets of mTOR. In this study, we have revealed that the cytotoxic effect of high-dose rapamycin treatment is due to the complete dissociation of mTOR from raptor, which results in the inhibition of 4E-BP1phosphorylation. We also show that rapamycin-resistance can be achieved by hyper-phosphorylation of Akt at the mTORC2 site at S473. These complex responses to rapamycin reveal both problems and opportunities for targeting mTOR, which has been implicated in the signals that promote cell cycle progression and survival in human cancers.

6.8 Future Studies-overview

Currently, we are investigating the mechanism by which inhibition of S6K expression confers resistant to apoptosis otherwise induced by knockdown of eIF4E. As discussed in **Chapter 1.8**, our hypothesis is that although TGF-β signaling is initiated by inhibition of S6K (phosphorylation or expression), its cytostatic effect is only achieved by concomitant inhibition of eIF4E. Based on **Chapter 4** and the aforementioned prior work in the lab, we propose a model **(Figure 6.8)**.

igure 6.8 Proposed model. Dual inhibition of S6K and eIF4E activity result in oordinative induction of TGF-β signaling to mediate G1 cell cycle arrest

hibition of S6K activity is necessary to initiate TGF-β signaling which slows G1 cell ycle progression. Concomitant knockdown of eIF4e enhances TGF-β signaling ecessary and sufficient to induce G1 cell cycle arrest.



6.8a G1 arrest is achieved with concomitant knockdown of S6K and eIF4E

As our model suggests **(Figure 6.8)**, singular knockdown of S6K modestly induces TGF-β signaling; only retarding cell cycle progression through G1. TGF-β signaling is enhanced only upon dual knockdown of S6K and eIF4e. At this point, cells arrest in late G1.

6.8b Suppression of apoptosis by S6 kinase ablation is dependent on TGF-β signaling.

We previously reported that the ability of serum to suppress the apoptotic effect of high-dose rapamycin treatment was due to the presence of TGF-β in serum, which induces a G1 cell cycle arrest. This arrest protects cells because progression into S-phase upon high-dose rapamycin results in apoptosis (Gadir et al., 2008). Currently, we are examining whether low-dose rapamycin or S6 kinase ablation induces TGF-β signaling that is sufficient to bypass apoptosis. MDA-MB-231 cells will be transfected with siRNA targeting eIF4E and S6 kinase and treated with a neutralizing anti-TGF-β antibody or SB431542, a compound that inhibits the TGF-β receptor. The hypothesis is that inhibition of TGF-β signaling will relieve the protective effect on the cells and the cells will undergo apoptosis. Again, we will observe cell viability by cleaved PARP and quantitative analysis by hemocytometer or Coulter counter. Furthermore, we are examining the downstream targets of TGF-β signaling such as Smad phosphorylation, p21 and p27.

Because we attribute such protective effects to TGF-β signaling , we are also investigation BT-549 breast cancer cells in this study because they do not express protein kinase Cδ (Jackson et al., 2005), which is required for TGF-β signaling (Perillan et al., 2002; Runyan et al., 2003). We reported previously that these cells could not be rescued from the apoptotic effects of high- dose rapamycin treatment in serum (Gadir et al., 2008). Therefore, our hypothesis is that BT-549 cells will undergo apoptosis despite the concomitant addition of low-dose rapamycin or S6K knockdown with knockdown of eIF4E. Since these cells lack intact TGF-β signaling, this outcome would support the

idea that inhibition of S6K expression or phosphorylation prevents apoptosis by lack of eIF4E activity by inducing TGF-β signaling.

6.8c Low dose rapamycin induces G1 arrest in cells with suppressed eIF4E expression

While low dose rapamycin induces TGF-β signaling, it only weakly suppresses G1 cell cycle progression in MDA-MB-231 cells. High dose (20 μM) is required to completely block synchronized cells from entering S-phase. We are examining if low-dose rapamycin (or knockdown of S6 kinase) is sufficient to induce full G1 cell cycle arrest upon eIF4E ablation. We are investigating G1, S and G2/M phase content. We predict a G1 content that is similar to high-dose rapamycin treatment. This would further support the idea that TGF-β-dependent suppression of G1 cell cycle progression requires suppression of both S6K and 4E-BP1 phosphorylation, or similarly, suppression of S6K phosphorylation and suppression of eIF4E expression.

Chapter 7

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