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Differential regulation of IRESs in the Aurora A mRNA by bFGF through the mTOR complex TORC2 modulates Aurora A kinase expression

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Differential regulation of IRESs in the Aurora A mRNA by bFGF through the mTOR complex TORC2 modulates Aurora A kinase expression

A

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By

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Supervisory Professor: Leslie A. Krushel, Ph.D.

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ABSTRACT

Differential regulation of IRESs in the Aurora A mRNA by bFGF through the mTOR complex TORC2 modulates Aurora A kinase expression

Roy L. Voice III, B.S. MLS(ASCP)CM

Supervisory Professor: Leslie Krushel, Ph.D.

Identifying the mechanisms that contribute to tumorigenesis is a major area of focus in our fight against cancer. Epithelial malignant tumors, such as breast, colon, ovarian and pancreatic cancers have been shown to overexpress proteins that control cell mitosis, growth, and proliferation. One of those proteins is the Aurora A kinase. Aurora A kinase is a member of a small family of kinases that contribute to mitotic events such as centrosome duplication, separation, and maturation. Aurora A overexpression leads to genomic instability, which can contribute to tumorigenesis, on the other hand, inhibiting Aurora A expression leads to apoptosis, making it a target for anti-cancer drugs.

Recent studies have shown that protein synthesis can play a significant role in the regulation protein expression. The major mechanism by which translation is regulated is at the step of initiation. The canonical pathway utilizes the m7G cap structure at the 5' end of the mRNA to recruit the translational machinery including the 40S ribosome which scans the mRNA for the AUG start codon. Alternatively, the 40S robsome is recruited downstream of the cap to internal ribosomal entry site (IRES). IRESs are found in certain classes of viruses and a subset of eukaryotic mRNAs. IRES dependent

translation is thought to occur during times when the cap is down regulated such as during stress or cell proliferation.

Previously we found that the Aurora A mRNA contains an IRES and that IRES dependent translation appears to be a “major contributor” in the over-expression of Aurora A in cell lines that overexpress the protein. The elevation of Aurora A is due mainly to increased IRES translation. Further study demonstrates that the alternatively spliced aurora A 5’ UTR contains three independent IRESs localized in three different exons (exon 1b, exon 2, exon 2a). I examined whether growth factors which stimulate Aurora A expression, differentially stimulate different Aurora A IRESs. I found that addition of bFGF enhances Aurora A protein expression within 2 hours. Moreover, bFGF led to an increase in exon 1b and exon 2a but not exon 2 IRES activity. Further study showed that the translational response was mediated through the mTOR pathway. Cap-dependent translation is known to be mediated through one of the two mTOR complexes (mTORC1). Surprisingly my data reveal that the second complex, mTORC2, mediates bFGF induced IRES activity. This study is the first demonstration that growth factors differentially regulate IRESs in the same mRNA and that this is regulated through mTORC2. Importantly, this study identifies mTORC2 as a novel target for inhibition of Aurora A expression in cancer cells.

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CHAPTER I:

INTRODUCTION AND BACKGROUND

Causes

One of the leading causes of death is cancer. One out of four people will die by some form of cancer, placing it as the second highest cause of death in the U.S. Our research is directed against understanding the mechanisms that contribute to cancer and identifying targets to specifically kill cancer cells. Cancer occurs when cells lose the ability to inhibit their growth, proliferation, or cannot remain in senescence. Hyper-proliferative cells or cells that continue to growth in size can form tissue masses called tumors. Cancer originated from DNA mutations that result in numerous alteration in cellular processes, including intracellular signaling, protein synthesis and protein stability. Protein over-expression is often seen in a variety of tumors such as, breast, colorectal, pancreatic, and gastric, ovarian, and esophageal cancers (Bischoff, Anderson et al. 1998, Tanner, Grenman et al. 2000, Li, Zhu et al. 2003, Tong, Zhong et al. 2004, Jiang, Katayama et al. 2010, Zabaleta 2012). One protein that is often over-expressed in cancer is the Aurora A kinase and understanding the mechanisms underlying its over-expression may yield novel insight to cellular immortalization and tumorigenesis (Marumoto, Zhang et al. 2005, Mehra, Serebriiskii et al. 2013)

Aurora A kinase and Cancer

Organismal growth or survival relies heavily on the process of cellular division. Mitosis is a cellular process whereby replicated chromosomes are equally segregated to two daughter cells. Several kinases have been implicated in the exquisite regulation required for mitotic fidelity. The first Aurora kinase, dubbed Ip11, was discovered in *Saccharomyces cerevisiae* in a genetic screen for mutations causing abnormal

chromosomal segregation (Chan and Botstein 1993). Shortly thereafter, mutations in *Drosophila melanogaster* Ip11 homolog were found to result in chromosome segregation failure and monopolar spindle formation (Glover, Leibowitz et al. 1995). The name "Aurora" was given because these mutant monopolar spindles resembled the *aurora borealis*. In humans, there are three Aurora homologs. Aurora A, B, and C. Together these make up a family of serine/threonine kinases that are essential for mitotic events. More explicitly, Aurora A (as known as AIK; ARK1; AURA; BTAK; STK6; STK7; STK15; AURORA2; PPP1R47) was found to regulate mitotic centrosome separation, spindle assembly, and the G2/M transition (Barr and Gergely 2007, Fu, Bian et al. 2007). Regulation of Aurora A kinase expression, localization, and activation is crucial to maintain genetic stability. The cell cycle consists of four phases: G1, S, G2, and M. The Aurora A protein is barely detectable in G1, but Aurora A mRNA and protein levels rise during S phase and peak during G2/M. The APC/C (Anaphase Promoting Complex/Cyclosome)/Cdh1complex targets the Aurora A protein for degradation in late mitosis, just prior cytokinesis (Floyd, Pines et al. 2008).

Beginning in G2, Aurora A localizes to the pericentriolar material (PCM) of the newly duplicated centrosomes. Each centrosome is comprised of PCM and two centrioles, and function as the microtubule organizing centers of the mitotic spindle. Aurora A is recruited to the centrosomes upon the CDK11 (cyclin dependent kinase 11)-dependent localization of Plk1 (polo-like kinase 1) to the centrosome (Sunkel and Glover 1988, Lane and Nigg 1996). Aurora A also plays a role in spindle assembly with TPX2 (*Xenopus* kinesin-like protein 2) as a cofactor and activator. After nuclear envelope breakdown, Aurora A is localized to the pole proximal minus ends of microtubules (Fu,

Bian et al. 2007). TPX2 stimulates a conformational change to allow Aurora A auto phosphorylation, while at the same time preventing deactivation by protein phosphatase 1. In addition to enhancing the kinase activity of Aurora A, TPX2 localizes it to the spindle (figure 1.1). Aurora A then phosphorylates and recruits TACC (Transforming Acid Coiled Coil) which functions to stabilize spindle microtubules (Lee, Gergely et al. 2001, Kinoshita, Noetzel et al. 2005, Fu, Bian et al. 2007).

Evidence suggests centrosome separation is also dependent upon Aurora A as well. EXTAH is a complex that is required for bipolar assembly(Sauer, Korner et al. 2005, Koffa, Casanova et al. 2006). It consists of the microtubule MT crosslinking proteins (BimC-like kinesin Eg5, Aurora A, or TPX2), MT stabilizers (XMAP215), and MT bundling proteins (HURD) (Marumoto, Zhang et al. 2005, Dobson 2012). Eg5 is phosphorylated by Aurora A (Giet, Uzbekov et al. 1999) which promotes formation of the EXTAH complex (Barr and Gergely 2007). Consequently, inhibiting Aurora A or Eg5 leads to the formation of monopolar spindles and apoptosis.

Concomitant with separation, centrosomes undergo maturation, which refers to the recruitment of γ-tubulin along with PCM and coiled-coil proteins that facilitate microtubule nucleation (Fu, Bian et al. 2007). LATS2 (large tumor suppressor homolog 2) is another substrate of Aurora A. It is required for the γ-tubulin accumulation and spindle formation. When LATS2 was depleted from mammalian cells, the centrosomes failed to mature (Toji, Yabuta et al. 2004).

Aurora A kinase activity is required for entry into mitosis (figure 1.1). Once localized, Aurora A is activated by the LIM protein Ajuba (Fu, Bian et al. 2007). Ajuba

allows auto phosphorylation of Aurora A in late G2 promoting its progression into M phase. When Ajuba was silenced in HeLa cells with siRNA, phosphorylation of Aurora A greatly decreased and the cells were unable to enter mitosis (Hirota, Kunitoku et al. 2003). Additionally, Aurora A phosphorylates CDC25B (figure 1.1) which activates CDK1-clyclin B1 complex in late G2 (Cazales, Schmitt et al. 2005, Fu, Bian et al. 2007) stimulating entry into mitosis. Knocking down Aurora A expression leads to cell cycle arrest in G2/M and eventually cell death (Du and Hannon 2004, Fu, Bian et al. 2007).

Aurora A over-expression is observed in various types of cancers, including breast, pancreatic, gastric, esophageal, and ovarian (Li, Zhu et al. 2003, Tong, Zhong et al. 2004, Zabaleta 2012). Although Aurora A kinase contributes to multiple processes during G2/M as noted above, the most prominent result of Aurora A over-expression is centrosome amplification and cytokinesis failure, which can result in genetic instability. This observation was first seen in rat mammary models and cell cultures, centrosome amplification was demonstrated upon up-regulation of Aurora A (Goepfert, Adigun et al. 2002, Meraldi, Honda et al. 2002). Increased Aurora A expression also disrupts cell checkpoints. Paclitaxel (Taxol) is a chemotherapeutic agent that activates the spindle assembly checkpoint (Murata-Hori and Wang 2002, Marumoto, Zhang et al. 2005). Over-expression of Aurora A allows the cell to bypass this checkpoint increasing resistance to the drug. DNA damage has been reported to reduce Aurora A kinase activity, however Aurora A over-expression appears to impair the G2 DNA damage checkpoint (Marumoto, Zhang et al. 2005).

In vitro and in vivo studies confirmed that Aurora A expression induces tumors in a variety of different models. For instance, transgenic mice formed tumors when Aurora A was over-expressed (Wang, Zhou et al. 2006, Mountzios, Terpos et al. 2008). Furthermore, elevated Aurora A kinase activity can transform NIH 3T3 cells in vitro and stimulate tumor formation when they are injected into nude mice (Tong, Zhong et al. 2004, Wang, Zhou et al. 2006). Alternatively, down regulation or inhibition of Aurora A kinase causes defects in centrosome separation that lead to the formation of monopolar mitotic spindles (Cowley, Rivera-Perez et al. 2009). Aurora A depleted cells are arrested in mitosis by the spindle assembly checkpoint and ultimately succumb to apoptosis. It is of great interest to understand the mechanisms that regulate Aurora A expression as it could identify how Aurora A expression is elevated and contributes to tumorigenesis. Ultimately, targeted inhibition of these mechanisms may lead to more effective ways of treating cancer.

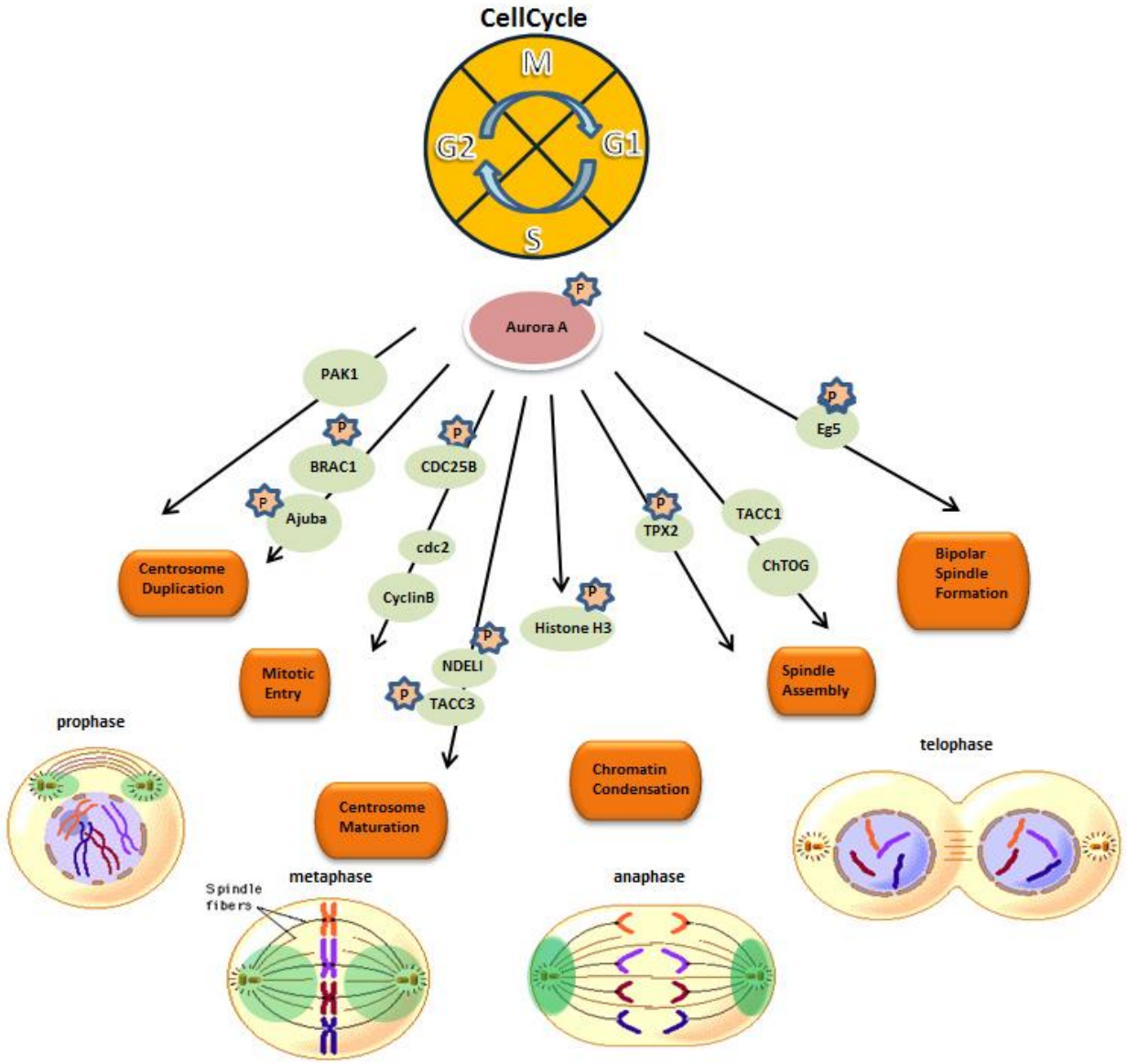


Figure 1.1 **Aurora A expression, localization and function during cell cycle.** Aurora A mRNA and protein (green) levels are detected during late S/ early G2 phase of the cell cycle and expression peaks during G2/M. Aurora A is targeted for destruction by APC/C through its D-box and A-box and is reduced to trace amounts following cytokinesis. During G2 phase, Aurora A (green) localizes to pericentriolar material and persists throughout mitosis. Additionally, it spreads to the minus ends of the mitotic spindle microtubules during mitosis. Aurora A is a key regulator of G2/M events, such as, centrosome duplication, mitotic entry, centrosome maturation, chromatin condensation, spindle assembly, and bipolar spindle formation, through activation of several substrates (Dobson 2012).

Regulation of Translation Initiation

A protein can be over-expressed by many mechanisms. The central dogma of gene expression is that DNA is transcribed to RNA which is translated into protein. Firstly, enhancing gene expression or gene duplication will lead to increased amounts of mRNA that potentially can be translated. Second, increased translation or stability of the mRNA and finally, protein stability could be increased. All three mechanisms could lead to steady state protein expression. All of these mechanisms have been proposed to contribute to Aurora A over-expression (Tanner, Tirkkonen et al. 1994, Tanner, Tirkkonen et al. 1995, Bischoff, Anderson et al. 1998, Zhou, Kuang et al. 1998, Tanner, Grenman et al. 2000, Dobson 2012). Little is known about differential protein stability between normal and Aurora A over-expressing cells. Finally, we have found in a group of breast cell lines that over express Aurora A, enhanced protein expression was due to increased protein synthesis (Dobson, Chen et al. 2013). Accordingly, my goal was to identify how Aurora A translation was regulated in these cells.

Protein synthesis is a tightly regulated process, primarily at the step of initiation. It has to be strictly regulated because during translation the cell utilizes 40% of its total energy (Meisenberg and Simmons 1998). The structure of the messenger RNA consists of: 1) a cap (m7GpppN, where N is any nucleotide) located at the 5' end, 2) a 5' untranslated region (UTR) or leader, 3) the open reading frame, 4) a 3' UTR, and 5) a poly(A) tail at the 3' end which plays a key role in mRNA stability and translational efficiency (Caponigro and Parker 1996, Gingras, Raught et al. 1999). Proteins that function to initiate, stabilize, and enhance translation are referred to as eukaryotic

initiation factors (eIF). The mechanism by which the general translation of eukaryotic mRNAs is initiated is referred to as cap-dependent translation. The cap, which is situated at the 5'end of the mRNA, is bound by eIF-4E. 4E in turn binds the scaffolding protein 4G which in turn recruits the remainder of the translational machinery including the 40S ribosome (Shatkin 1976).

Eukaryotic Initiation Factor 4 is a complex consisting of three polypeptides: eIF4A, eIF4E, and eIF4G (Gingras, Raught et al. 1999). eIF4E is the actual cap-binding factor and eIF4G is a scaffolding protein which has binding sites for 4E, PIC, PABP, and 4A and recruits the eIF3-ribosome (Gingras, Raught et al. 1999). eIF4A/4B binds to 4G and acts as a helicase to unwind the secondary structure of the mRNA allowing ribosomal scanning (Lorsch and Herschlag 1998, Gingras, Raught et al. 1999, Pestova and Kolupaeva 2002). The PIC (pre-initiation complex) forms when eIF2, GTP, and Met-tRNA bind to the 40S ribosome through a connection with eIF3. The 43S preinitiation complex needs a set of factors to guide and stabilize its position on the mRNA. Once the 40S ribosome is recruited, it then scans the mRNA for the in frame AUG start codon (figure 1.2 A).

There are several events that must occur for the canonical initiation factors to function properly. Formation of the translational complex can be regulated in multiple ways and subsequently alters the rate of protein synthesis and function. In addition, these events are affected by changes in the cellular environment, stress, oxidation, and mitosis (Pyronnet, Dostie et al. 2001). For example, eIF2 before it becomes part of the preinitiation complex, exists in a binary complex with GDP (Hinnebusch 1994). eIF2B is

a guanine nucleotide exchange factor that replaces GDP with GTP and the new pair (eIF2-GTP) becomes part of the preinitiation complex. eIF2 has an alpha subunit and in response to cellular stress is phosphorylated at serine 51 impairing eIF2B (Hinnebusch 1994, Veo 2003). This negatively affects protein synthesis by inhibiting formation of the preinitiation complex. Another example involves eukaryotic initiation factor 4E, whereby its activity is regulated by a family of proteins called 4E-BPs. 4E binding proteins compete for the site that eIF4G uses to bind 4E, thus preventing completion of the eIF4F complex (Pyronnet, Dostie et al. 2001). Mammalian target of rapamycin (mTOR) phosphorylates 4E-BP1 releasing it from eIF4E allowing translation to proceed. During stress mTOR activity is decreased causing hypophosphorylation of 4E-BP1 (Arsham, Howell et al. 2003). 4E-BP1 remains bound to eIF4E preventing formation of eIF4F (figure 1.3). Finally, inhibition of cap-dependent translation does not always result in a total loss of protein synthesis. For example, protein synthesis during G2/M occurs even though 4E-BP1 is hypophosphorylated (Pyronnet, Dostie et al. 2001). This result indicates translation can be initiated in a cap-independent manner.

IRES Mediated Translation

Many viral mRNA translate their mRNA even though viruses shut down host cell cap-dependent translation. Moreover, they recruit the translational machinery downstream of any cap structure. The sites to which the translational complex bind are termed internal ribosomal entry sitse or IRESs (figure 1.2 B). Many viruses are translated via an IRES and include: picornavirus, encephalomyocarditis virus (EMCV),

and the poliovirus (Pelletier and Sonenberg 1985, Jang, Krausslich et al. 1988, Pelletier and Sonenberg 1988). One of the initial assays to identify IRES activity was the creation of a dicistronic construct (Pelletier and Sonenberg 1988). Messenger RNA was generated that consisted of two reporter genes with the poliovirus leader inserted in between. The first reporter gene measured cap-dependent translation while the second could only be translated if the inserted poliovirus lead could recruit the ribosome independent of a cap structure (Pelletier and Sonenberg 1988, Komar and Hatzoglou 2005). What we learned from this work is that most viral IRESs thrive during times when cap-dependent translation is impaired and they have a "reduced requirement for canonical translation initiation factors" (Gradi, Imataka et al. 1998, Komar and Hatzoglou 2005).

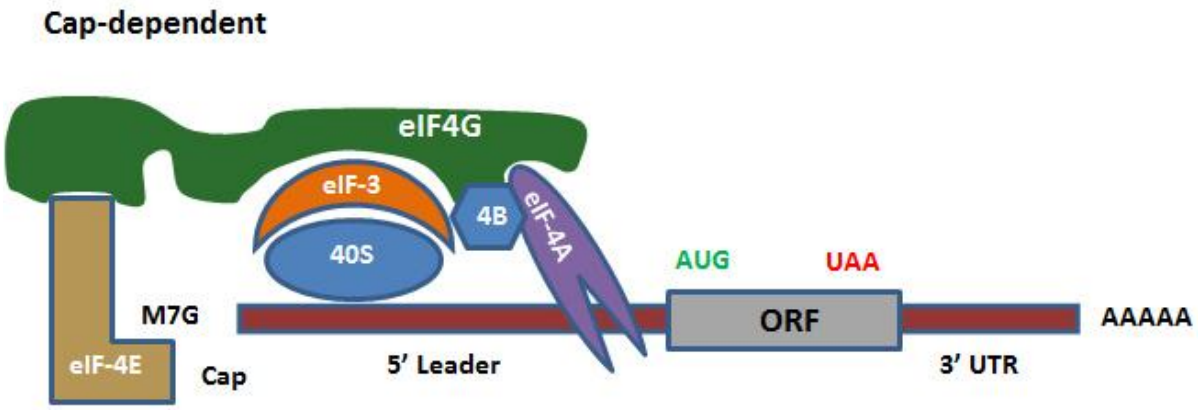
After viral IRESs were discovered, research focused on whether eukaryotic mRNA contain IRESs as well. In 1991, by making a circular mRNA with no cap, an IRES was discovered in the immunoglobulin heavy-chain binding protein (BiP) gene (Macejak and Sarnow 1991, Komar and Hatzoglou 2005). To identify a population of IRES contained in eukaryotic mRNA, cells were transfected with poliovirus. Polioviruses express a protease (2A) that cleaves the N-terminus of the scaffolding protein 4G. The N-terminus contains the 4E binding site. Consequently, expression of the 2A protease shuts down cap-dependent translation, however since, IRESs do not require 4E, translation of eukaryotic IRES should not be inhibited. A screen for eukaryotic IRESs was developed by measuring the number of ribosomes bound to the mRNA. What was found was that 3-5% of eukaryotic mRNA in fact have IRES elements (Johannes and Sarnow 1998, Qin and Sarnow 2004, Stoneley and Willis 2004). For

example, mRNAs encoding cysteine-rich angiogenic inducer 61 (Cyr61) and Proto-oncogene serine/threonine-protein kinase (Pim-1) were found to contain IRES elements (Johannes, Carter et al. 1999, Komar and Hatzoglou 2005). Cyr61 is a secreted factor that involved in cellular proliferation, differentiation, angiogenesis and tumor growth (Johannes, Carter et al. 1999). While Pim-1 is over-expressed in cancers such as prostate and bladder cancer (Dhanasekaran, Barrette et al. 2001, Valdman, Fang et al. 2004, Cibull, Jones et al. 2006, He, Bi et al. 2009, Guo, Mao et al. 2010, Merkel, Meggers et al. 2012)

Viral IRESs have been thoroughly characterized and key factors required for their proper functioning have been identified. However, between secondary structural differences, length of the 5' leader, and differential selection of cellular IRESs, general characterizations are difficult. For instance, platelet-derived growth factor-2 (PDGF2) and vascular endothelial growth factor mRNA can translate efficiently under conditions where eIF2-alpha is phosphorylated (Gerlitz, Jagus et al. 2002, Komar and Hatzoglou 2005). As mentioned earlier, phosphorylation of the alpha subunit of eIF2 inhibits cap-dependent translation. Cationic amino acid transporter (cat-1) mRNA has an IRES element that seems to require eIF2-alpha phosphorylation before it can activate translation (Fernandez, Yaman et al. 2002, Komar and Hatzoglou 2005). Some cellular mRNA can properly recruit the 40s small ribosomal subunit with or without different canonical initiation factors. Characteristics such as high G-C content, the length, and secondary structure of the 5' leader are hallmarks of some viral IRESs. Cellular mRNAs such as Apaf-1 (apoptotic protease activating factor) and XIAP-1 (x-linked inhibitor of apoptosis) are similar to viral IRESs with respect to their defined secondary structure and

length of the UTR (Veo 2003). However, eukaryotic IRESs are not always like viral IRESs. For example, the FMR2 5' UTR does not have IRES activity but is 480 bp long and 82% G-C rich (unpublished data). Additionally, the APP (amyloid precursor protein) IRES is relatively short (50 nucleotides) and exhibits full IRES activity (Veo 2003, Beaudoin, Poirel et al. 2008). Every mRNA has different characteristics and thus, for now, we must study them individually. The IRES elements of the Aurora A kinase and its regulation is what I focused on in this study.

A.



B.

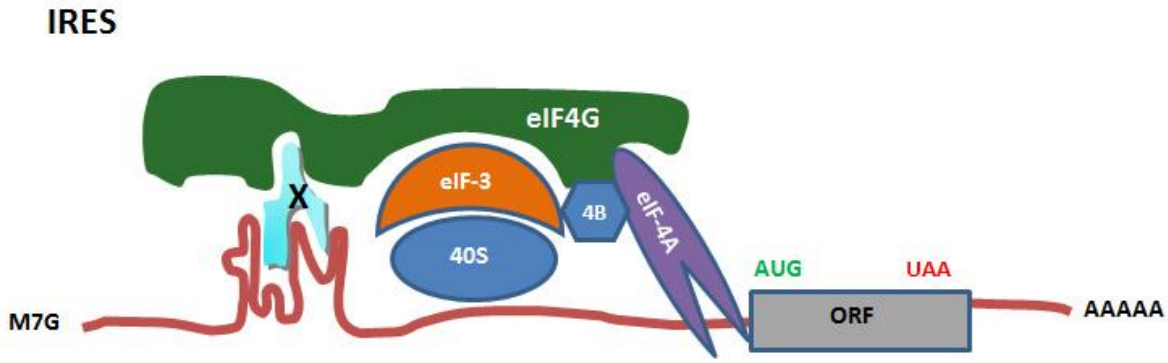


Figure 1.2 (A): **Primary mechanism of translation initiation: Cap-dependent** eIF4E binding to the m7G cap structure of the 5' UTR of a eukaryotic mRNA is the main regulatory step of cap-dependent translation initiation. eIF4E along with eIF4G and eIF4A form the eIF4F complex. eIF4B, eIF3, the 40S ribosomal subunit and the ternary complex (eIF2, the initiator tRNA and GTP - orange triangle) completes the formation of the preinitiation complex (Dobson 2012). According to the scanning hypothesis (Kozak and Shatkin 1978, Kozak 2001), this complex scans the 5' leader until it reaches the initiation codon where the 40S subunit joins the 60S ribosomal subunit to form the 80S ribosome. (B): **Alternative cap-independent mode of translation initiation:** Messenger RNA possesses a unique sequence in their 5' UTRs which allows recruitment of the 40S ribosome completely independent and downstream of its cap structure. IRES transacting factors (ITAFs) are suggested to enhancing translation through facilitating a bridge between the mRNA and some canonical initiation factors. Each IRES structure varies in length and structure and scanning is thought to not be possible (Hellen and Sarnow 2001, Stoneley and Willis 2004, Komar and Hatzoglou 2005).

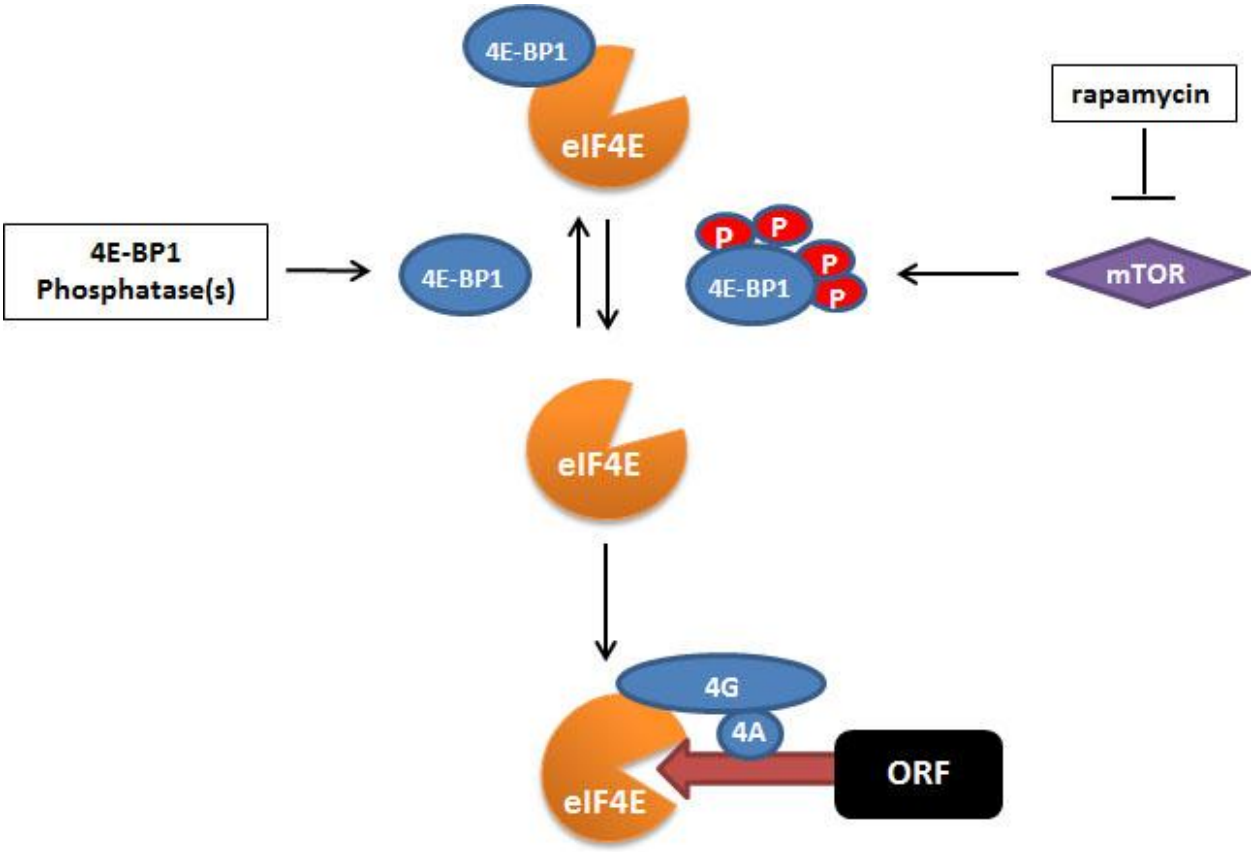


Figure 1.3: **4E binding protein mechanism of cap-dependent translation regulation** 4E-BP1 competes for the same binding site eIF4G uses to associate with eIF4E, thereby inhibiting successful recruitment of 4E to the cap structure. In response to activated PI3K/Akt/mTOR signaling, 4E-BP1 is hyperphosphorylated, formation of eIF4F complex occurs (4E, 4G, 4A), and cap-dependent translation is initiated. Drugs such as rapamycin can block mTOR phosphorylation of 4E-BP1 resulting in inhibition of cap-dependent translation.

Translation of the Aurora A kinase mRNA

In order to examine the mechanisms behind Aurora A over expression, we chose to study several different mammary epithelial cell lines (MCF10A, MCF12A, MCF-7, and HMECs). These cells were put in to two groups based on Aurora A protein expression. 10A and 12A over-expressed Aurora A while HMECs and MCF-7 had normal levels when compared to primary HMECs and WI38 (a primary fibroblast line) (Dobson, Chen et al. 2013). To find the cause of over expression, we considered three possibilities: increased transcription, increased translation, or protein stability. A measurement of Aurora A mRNA levels showed that all the cell lines transcribed this locus at about the same rate, except MCF-7 which was slightly more (Dobson, Chen et al. 2013). When protein levels were compared to transcript levels, we could see that MCF10A and MCF12A had increased protein to mRNA levels which suggested an increase in translation or protein stability. MCF-7 and HMECs did not demonstrate an increase in protein to mRNA (Dobson, Chen et al. 2013). Aurora A protein half-life was measured and revealed that protein stability is not the cause of increased levels of Aurora A that we observed (Dobson, Chen et al. 2013). Thus we hypothesized that increased protein synthesis contributed to Aurora A over-expression. However, there was no evidence that cap-dependent translation was differentially regulated. Instead we found an IRES and it was upregulated in Aurora A over-expressing cells. Moreover, it appeared to be the primary mechanism utilized to initiate translation this mRNA(Dobson, Chen et al. 2013). This analysis utilized an Aurora A 5' leader which was the longest reported in GenBank. Subsequently once the gene structure was defined and confirmed by PCR

(figure 1.4), multiple Aurora A 5' leaders were found. Essentially, IRESs were found in three distinct exons.

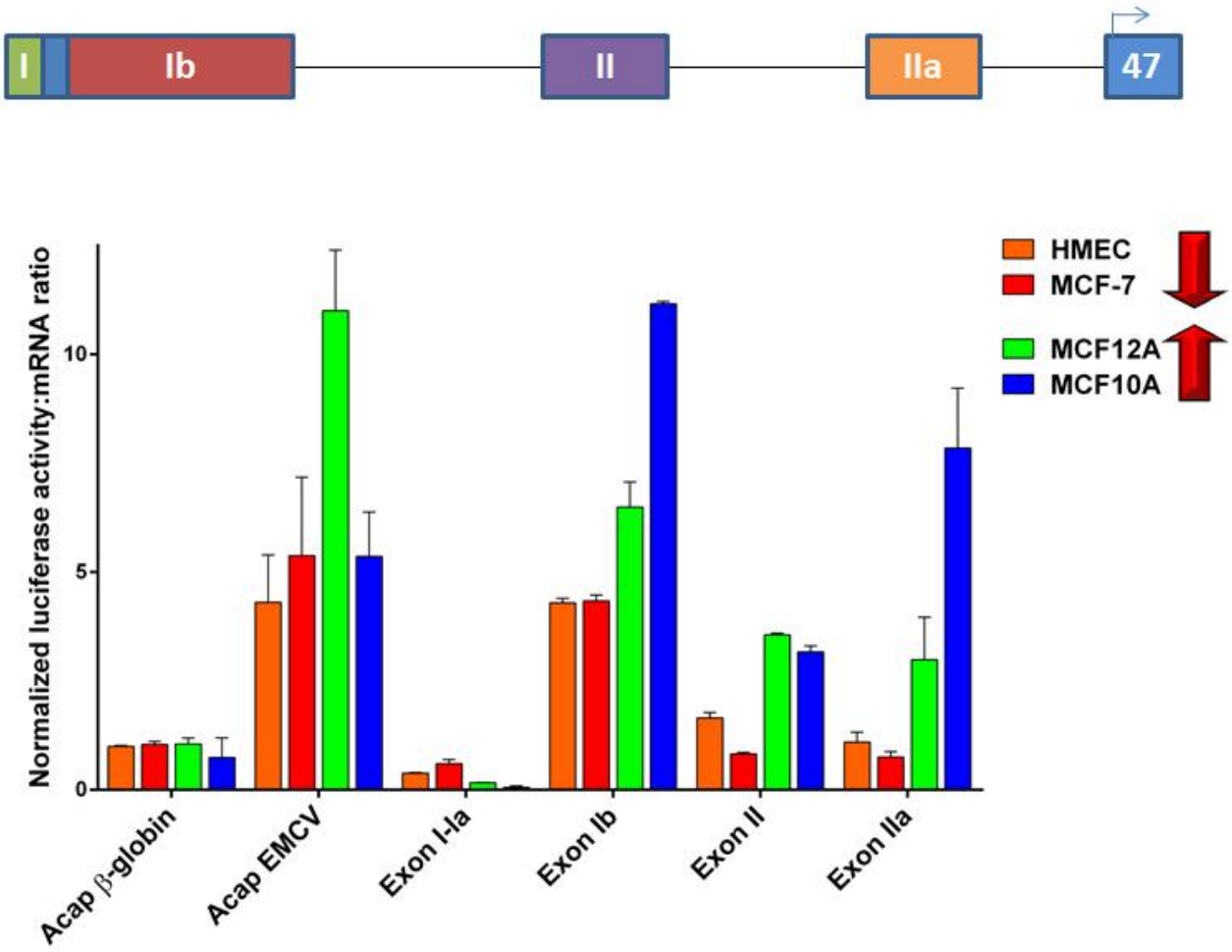


Figure 1.4 **Schematic diagram of the 5' UTR of the *Aurora A* gene and the IRES activity of each exon**. The 5' untranslated region of the *Aurora A* gene contains 5 exons (I, Ia, Ib, II, and IIA). IRES activity of each exon was measured in cells that express normal levels of Aurora A (HMEC and MCF7) and cells that over-express Aurora A (MCF12A and MCF10A). A ratio of luciferase to mRNA was made for each sample and compared to β-globin. n=3± standard deviation (SD).

Regulating the Aurora A IRES

Little is known about the intracellular pathways that regulate IRESs. Through a kinase/phosphatase siRNA screen and a phospho-kinase array screen we were able to look at the sensitivity of Aurora A expression and IRES activity to different kinases. We found that the MAPK and PI3/Akt/mTOR pathways regulated IRES activity of the original Aurora A leader tested (exon 1-1a-1b-2). Differential mTOR phosphorylation between high Aurora A expressing and low expressing cells were demonstrated (Dobson 2012). mTOR is thought to play a role in IRES mediated translation in addition to cap-dependent translation (Ramirez-Valle, Badura et al. 2010). Mammalian target of rapamycin (mTOR), is a regulator of cellular growth and proliferation (Weber and Gutmann 2012). In yeast (*Saccharomyces cerevisiae*), deletion of the Tor1 gene led to a decrease in proliferation, and in *Drosophila melanogaster*, dTOR knockdowns showed a decrease in cell size and proliferative capability (Oldham, Montagne et al. 2000, Zhang, Stallock et al. 2000, Weber and Gutmann 2012). mTOR consists of two functional complexes: mTORC1 and mTORC2 (figure 1.5). The fundamental difference between the two complexes is the presence of raptor (complex 1) or rictor (complex 2) (Weber and Gutmann 2012). Downstream targets of mTORC1 include 4EBP and S6K-1, illuminating the regulation of protein synthesis as an important function. mTORC2, on the other hand, targets PKCα and Akt (S473) for phosphorylation (Weber and Gutmann 2012). EGF, which stimulates these pathways, was shown to increase Aurora A IRES activity but only through exon 2 (Lai, Tseng et al. 2010, Dobson, Chen et al. 2013). The IRES in exon 1b and 2a were not affected even though they were present. However, the question remained as to how the exon 1b and 2a IRESs are regulated.

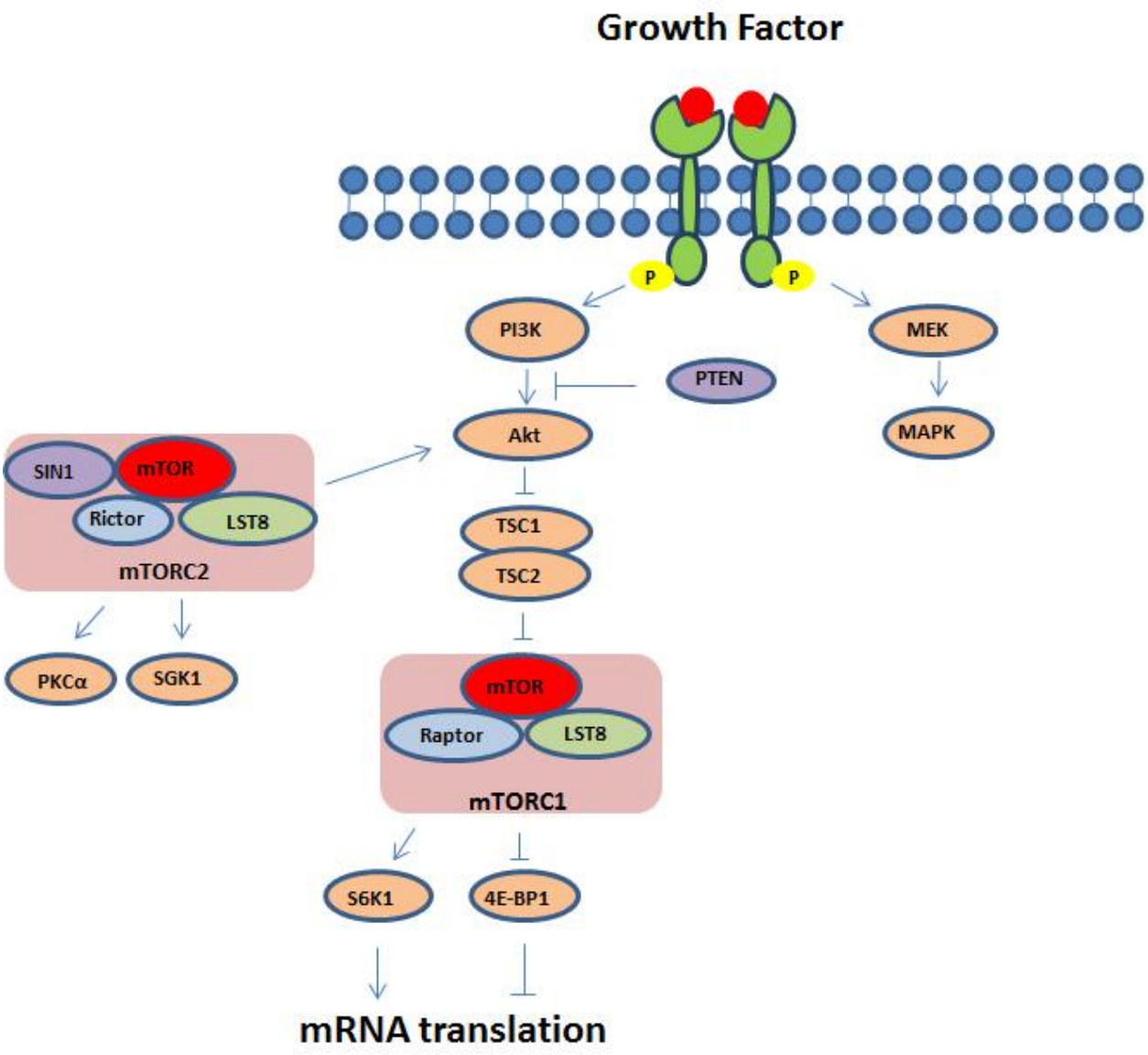


Figure 1.5 **Schematic of bFGF signaling pathway through mTOR.**

Growth factors (e.g. insulin, insulin-like growth factor 1, epidermal growth factor, and basic fibroblast growth factor) promote mRNA translation along with other cellular functions, primarily through two pathways: PI3K/Akt and MEK/MAPK. Mammalian target of rapamycin (mTOR) is the primary component of two functionally distinct complexes, mTORC1 and mTORC2. PTEN: Phosphatase and tensin homolog deleted from chromosome 10; TSC: Tuberous sclerosis complex; Raptor: Regulatory-associated protein of mTOR; Rictor: Rapamycin-insensitive companion of mTOR; MEK: Mitogen-activated protein kinase kinase; RSK: Ribosomal s6 kinase; PKCα: Protein kinase C α; SGK1: Serine/threonine-protein kinase 1; MAPK: Mitogen-activated protein kinase; PI3K: Phosphatidylinositol 3,4,5-kinase; P: Phosphate (modified from (Itamochi 2010).

CHAPTER II:

MATERIALS AND METHODS

Constructs and Cloning

The Aurora A 5' leaders (GenBank accession numbers: Aurora A, XM_114165) were PCR amplified from a human brain cDNA library (Clontech) and inserted into the mono luciferase vector - pRF (Stoneley, Paulin . 1998; Stoneley, Subkhankulova . 2000) (a generous gift from Dr. Anne Willis, University of Leicester) with EcoRI and NcoI restriction endonuclease sites. The monocistronic construct for *in vitro* transcription was created by digesting the *Photinus* Vector (I refer to the construct as *Photinus* instead of Firefly Luciferase) with EcoRV and BamHI releasing the *Photinus* gene and the SV40 3'untranslated region (3'-UTR). The single luciferase gene was inserted into the multiple cloning site of the SK+ Bluescript vector (Stratagene) downstream of the T7 promoter.

The Aurora A 5' UTR splice variants, containing exons 1b, 2, 2a, were identified by PCR amplification (with the 5' end primer targeting the downstream transcription start site and the 3' primer targeting the ORF just downstream of the start codon) from cDNA libraries created from WI-38, HMEC, MCF10A, MCF12A ,MCF-7, and HeLa S3 cell lines. PCR products were cloned into sequencing vectors using TOPO® TA Cloning® Kit (Invitrogen). These vectors were transformed in competent cells created with Z-Competent TM *E. coli* Transformation Kit & Buffer Set (Zymo Research) for amplification. Plasmid DNA was isolated using PureLink® Quick Plasmid Miniprep Kit (Invitrogen) or Nucleospin miniprep kit (Clontech) and submitted for sequencing. Verified leaders were PCR amplified from the Topo TA vectors with 5' and 3' primers containing EcoRI and NcoI endonuclease restriction sites and ligated into the monocistronic construct for *in vitro* transcription.

In Vitro Transcription

The monocistronic SK+ Bluescript vector was linearized with BamHI and used as templates for *in vitro* transcription. The linearized plasmid was purified by running digests on a 1% agarose gel, gel excising the linearized plasmid, and gel extracting using Zymoclean™ Gel DNA Recovery Kit (Zymo Research). Linearized plasmid DNA (1μg) was transcribed at 37°C for 3hrs. For the *in vitro* translation assay, monocistronic templates were transcribed using mMessage mMachine® T7 Ultra (Ambion) producing capped mRNA. For the RNA transfection assays, dicistronic and monocistronic templates were transcribed using MEGAScript® T7 (Ambion) producing either ApppG capped (New England Biolabs) or uncapped RNA. Cap priming of messages with an ApppG cap was done by the addition of 3μL 40mM G(5')ppp(5')A RNA cap structure analog and 2μL 15mM GTP to transcriptions. Transcriptions were then treated with 1μL DNase (2U/μL) for 15 mins at 37°C. mRNA was phenol/chloroform purified, isopropanol precipitated, and resuspended in nuclease free water. M7GpppG caps were added using Script Cap™ m7G capping system (Cellscript Inc.) and transcripts were poly (A) tailed using Poly (A) Polymerase tailing kit (Epicentre) per manufacturer's instructions. mRNA was phenol/chloroform purified, isopropanol precipitated, and resuspended in nuclease free water. mRNA quality was verified by running 1μg of mRNA on a Reliant® Precast RNA gel (LONZA) for 1.5hrs and stained with SYBR® gold (Invitrogen). RNA was stored at -80°C.

Cell Culture Maintenance

MCF10A cells were generously provided by Heide Ford (University of Colorado Anschutz Medical Center) and cultured as previously described (Ford 1998). Cells were cultured in

DMEM F-12 50/50 plus 10% FBS, 5% Pen/Strep, 50µl of Cholera Toxin, 250µl of Hydrocortisone, 500µl of insulin, and 300µl of EGF at 37°C, 5% CO_2.

RNA Luciferase Assays

Cells were transfected with 0.5µg of mRNA using the TransMessenger RNA transfection reagent (Qiagen) according to the manufacturer's directions. After 2 hours (RNA transfections), the cells were lysed with 250µl of lysis buffer (Promega). Forty microliters of the supernatant were used for the luciferase assays using the Single-Luciferase Reporter Assay (SLA) System and analyzed in a Luminoskan luminometer.

RNA Extraction/qRT-PCR

Total RNA was extracted using TRIzol® Reagent (Sigma) followed by PureLink™ RNA Mini Kit (Invitrogen). cDNA libraries were synthesized using iScript™ cDNA Synthesis Kit (Bio-Rad), including a (-)RT control. For primer pairs see table 3. qRT-PCR was performed using a Roche Lightcycler® 480 with either LightCycler® 480 SYBR Green I Master (Roche) or soAdvanced™ SYBR® Green Supermix (Bio-Rad) per manufacturer's instructions.

W**estern Blot Analysis**

Cells were harvested in cell lysis buffer (Promega) with protease (Roche) and phosphatase inhibitors (Pierce). Cells were centrifuged for 20 minutes at maximum speed 4°C to remove cell debris. The lysate was removed to a fresh tube and stored at -80□C. 5X SDS loading dye (100% glycerol, 20% SDS, 1M Tris pH 6.8, DTT, bromophenolblue) were added to aliquoted lysate samples. Samples are boiled for 5-10mins. Samples were loaded onto

a 10% SDS-PAGE gel, and run in 1X SDS Running Buffer (Tris Glycine) at 200 Volts for 35-45 mins. The transfer apparatus was assembled as follows: black side of sandwich, sponge, 3 filter paper squares, gel, nitrocellulose, 3 filter paper squares, sponge, clear side of sandwich. The transfer sandwich was placed in the apparatus with the black to black. 1X transfer buffer (Tris Glycine and 100% MeOH) was added up to the top of the apparatus. The gel was transferred for 35 mins at 400 mAMPS. The blot was blocked with 5% milk in 1X TBST (blocking solution) for 1hr at room temperature. The blot was washed 2 times with ddH_20. The blot was exposed to 1° antibody diluted in TBST (of PBS containing 0.1% Tween 20) for 1hr at RT or overnight at 4°C. The blot was washed with 1X TBST for 5 minutes and repeated 4X. The blot was exposed to 2° antibody diluted in or TBST for 1hr (anti-mouse) at RT. The blot was washed 4 times for 5 minutes in 1X TBST. The blot was rinsed in 2X ddH_20 for 3mins. Immunoreactive bands were detected using Amersham® ECL-plus chemiluminescent detection reagent (Promega-Aurora A study). The blot was detected with either x-ray film or STORM imager and quantified using Image Quant software.

Table 2.1 **Primary and secondary antibodies.**

Antibodies	Dilution	Source
Rictor	1:2000	(53A2)Cell Signaling
phosphorylated p70 S6	1:1000	(49D7) Cell Signaling
Gapdh anti-mouse	1:2500	(ab9485) Abcam-FMR1
Raptor anti-rabbit	1:2000	Milliepore
Aurora A anti-mouse	1µg/ml	(35C1) Calbiochem
HRP-conjugated anti-mouse	1:2500 – 1:10,000	Promega

Table 2.2 **RNA transfection with TransMessenger**

Transfection Agent	6.0µL of TransMessenger/1ug of RNA
Additional Reagent	3.2 µL of enhancer/1ug of RNA
RNA	0.5ug of RNA*
Transfection Buffer	100µL
Volume of medium w/o serum on	300µL
Final Volume per well	400µL

Table 2.3 **qRT-PCR Primer Sets**

Gapdh target	FORWARD: 5′-ACA GTC AGC CGC ATC TTC TT-3′ REVERSE: 5′ GTT AAA AGC AGCCC TGG TGA-3
Photinus target	FORWARD: 5′-AAA GCT CCC AAT CAT CCA AA-3′ REVERSE: 5′-GAG ATG TGA CGA ACG TGT-3′

Growth Factor Induction

On the first day 2.0 x10^5 cells/per well were plated in 12 well dish and incubated O/N in normal growth conditions. On day two, the growth medium was replaced with serum free media. Cells +/- 10 ng/ml TGF-β, 5ng/ml basic FGF, 50ng/ml of IGF-I and cells were incubated for two hours. At this time the cells were lysed with appropriate buffer (see western blotting). When performing RNA transfections the whole media was replaced with serum free media for 4hrs then RNA was transfected at the same time of bFGF induction. RNA transfection with cells harvested, luciferase activity, and transcript level were measured after two hours.

Drug Inhibition

On the first day 2.0 x10^5 cells/per well were plated in 12 well dish and incubated O/N in normal growth conditions. On day two, the growth medium was replaced with serum free media for 2 hours then incubated with rapamycin (0.01μM) or pp242 (0.1μM). After 2 hours, cells +/- bFGF (5ng/ml) were incubated for two hours. At this time the cells were lysed with appropriate buffer (see western blotting). When performing RNA transfections the whole media was replaced with serum free media for 4hrs then RNA was transfected at the same time of bFGF induction. RNA transfection with cells harvested, luciferase activity, and transcript level were measured after two hours. P-values were calculated using students t-test.

siRNA Transfection

Day one: 1.0 x10^5 cells/per well were plated in 12 well dishes and incubated O/N in whole media. Day two: whole media was replaced with 500μl of fresh, pre-warmed whole media; 20nM of siRNA (Invitrogen) was added to 100μl of serum free media. After which, 5ul of Transfection Reagent (Invitrogen) was added and the mixture was incubated at room temperature for 10-15 minutes. siRNA reagent mixture was added to each well and the cells were incubated for 24hrs. The next day, the growth factor induction experiment was performed as previously described.

CHAPTER III: RESULTS

Overview

In cells that over-express the Aurora A kinase, the Aurora A mRNA is primarily translated via an IRES. (Dobson 2012). Recently we found that the Aurora A 5' leader contains three independent IRES located in exons 1b, 2, and 2a (figure 1.4). We also found that EGF induces IRES activity via exon 2 but not exons 1b or 2a (Dobson, Chen, and Krushel unpublished observations). Thus, I examined the effect of other growth factors that may regulate the use of the exon 1b and exon 2a Aurora A IRESs.

bFGF Increases Endogenous Aurora A Protein Expression

To identify growth factors regulating high Aurora A expression, I first examined the effect of bFGF, TGF-β, and IGF-I, since their receptors are expressed by MCF10A cells. MCF10A cells were serum starved for 4 hours then bFGF (5ng/ml), TGF-β, (10ng/ml) or IGF-I (50ng/ml) was added for two hours. Addition of basic FGF led to a 210% increase in Aurora A expression, whereas IGF-I increased Aurora A by 30% and TGF-β had no effect on protein levels (figure 2.1). The increase observed after the addition of bFGF was similar to that stimulated by EGF (Dobson 2012). Since the greatest increase in Aurora A protein was elicited by bFGF, I examined the effect of bFGF on Aurora A IRES activity.

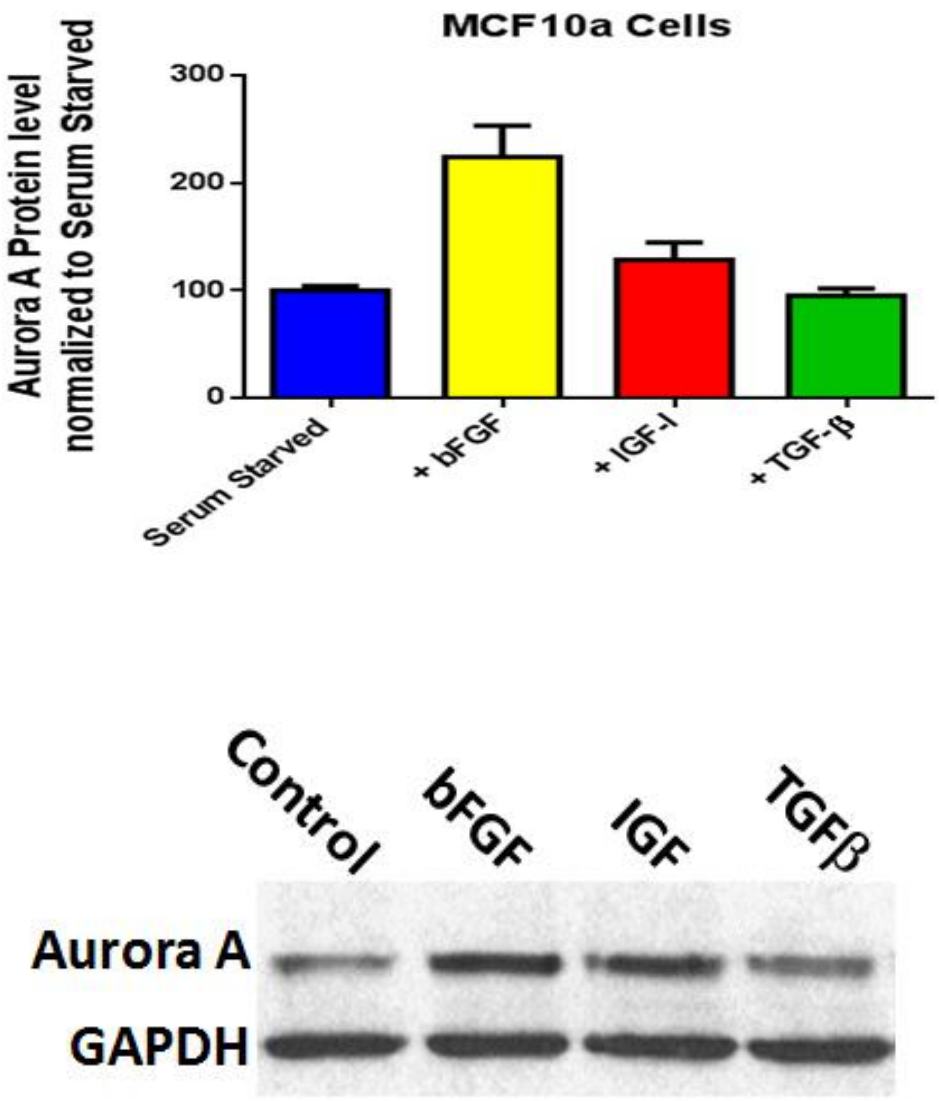


Figure 3.1 **Growth Factor induced Aurora A protein expression.** bFGF, IGF-I, and TGF-β were added to serum starved MCF10A cells. After 2hrs the cells were harvested. Aurora A protein expression was analyzed via western blot. Aurora A protein levels were determined using Image J and Gapdh was used as a loading control. The Aurora A to Gapdh ratio in serum starved cells were set to one and used to normalize Aurora A expression in the presence of individual growth factors. n=3± SD.

bFGF Increases IRES Activity of Aurora A

EGF and bFGF employ very similar signaling pathways (Schlessinger 2004, Hebert, Wu et al. 2009), suggesting the two growth factors possibly stimulate IRES activity through the same pathway. To determine if bFGF stimulated Aurora A IRES activity, exons 1b, 2, and 2a were inserted into a monocistronic construct containing the *Photinus* luciferase reporter gene. The DNA constructs were in-vitro transcribed, capped with ApppG (preventing cap-dependent translation and maintaining RNA stability) and poly (A) tailed for RNA stability. The MCF10A cells were serum starved for 4 hours followed by simultaneous addition of (5ng/ml) bFGF and A- cap luciferase mRNA containing the different exons (ex 1b, ex 2, and ex 2a). Two hours later the cells were harvested and RNA was reverse transcribed. Using real-time PCR the exon containing transcripts were measured. The *Photinus* luciferase to mRNA ratio was calculated and normalized to serum starved samples. The luciferase protein/RNA ratio from the mRNA containing exon 1b increased 60%, whereas exon 2a containing mRNA increased approximately 110%. Interestingly, exon 2 IRES activity only managed a 20% increase (figure 2.2). I conclude that the bFGF induced increase in Aurora A protein levels is mediated by the IRESs contained in exon 1b and 2a. Interestingly, addition of bFGF only exerted a minimal effect on exon 2 IRES activity, whereas it was the only IRES to be stimulated by EGF (Dobson, Chen, and Krushel unpublished observations).

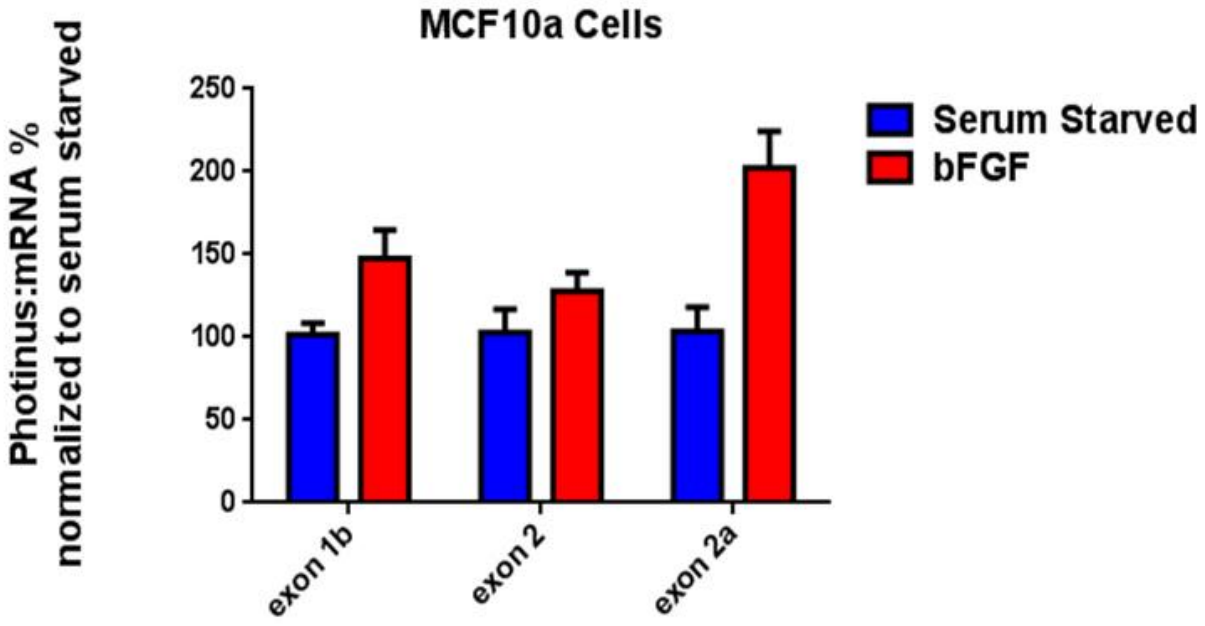


Figure 3.2 **bFGF induced Aurora A IRES activity.** ApppG capped monocistronic *Photinus* luciferase mRNA containing an exon from the 5' leader of Aurora A was transfected into serum starved MCF10A cells in the presence and absence of bFGF. *Photinus* luciferase protein/mRNA ratios were determined. The ratio for the serum starved untreated cells was normalized to 100. bFGF ratios were normalized to serum starved samples. Performed in triplicate, n=3 $\pm$ SD.

bFGF Stimulates an Increase in Aurora A IRES Activity Through the mTOR Pathway

bFGF is known to increase protein synthesis through mTOR. Moreover, preliminary evidence indicates EGF increases exon 2 IRES activity through mTOR. Therefore, I hypothesize that bFGF regulates IRES activity by a similar mechanism. To determine if mTOR is involved in IRES-dependent translation, I selected two mTOR inhibitors: rapamycin (mTORC1) and pp242 (mTORC1 and mTORC2). Rapamycin blocks 4EBP-1 and S6K phosphorylation. MCF10A cells were serum starved for 2 hours before mTOR inhibitors rapamycin (0.01nM) and pp242 (0.1nM) were added. Serum starvation continued for 2 more hours, bFGF was added, and (A) capped mRNA (containing the three exons) was transfected. The cells were harvested 2 hours later as noted above. bFGF increased exon 1b and exon 2a translation and was not blocked by rapamycin. Rapamycin did however reduce bFGF induced exon 2 IRES use by an average of 30%. pp242 exerted a minimal effect on bFGF induced exon 1b activity. On the other hand, pp242 blocked the bFGF increase in exon 2a IRES activity (figure 2.3A). To determine if Aurora A protein expression was regulated in a manner similar to what was observed from the IRES activity, a western assay was performed. bFGF induced a 70% increase in Aurora A expression which was only modestly inhibited by rapamycin but was completely blocked by pp242 (figure 2.3C). I conclude that the bFGF increase of exon 1b and particularly exon 2a IRES activity contributes to the increase in Aurora A expression through the mTOR pathway. Since pp242 was more effective than rapamycin in blocking IRES activity and protein expression, it suggests that either mTORC1 and mTORC2 together or mTORC2 alone is mediating this translational response.

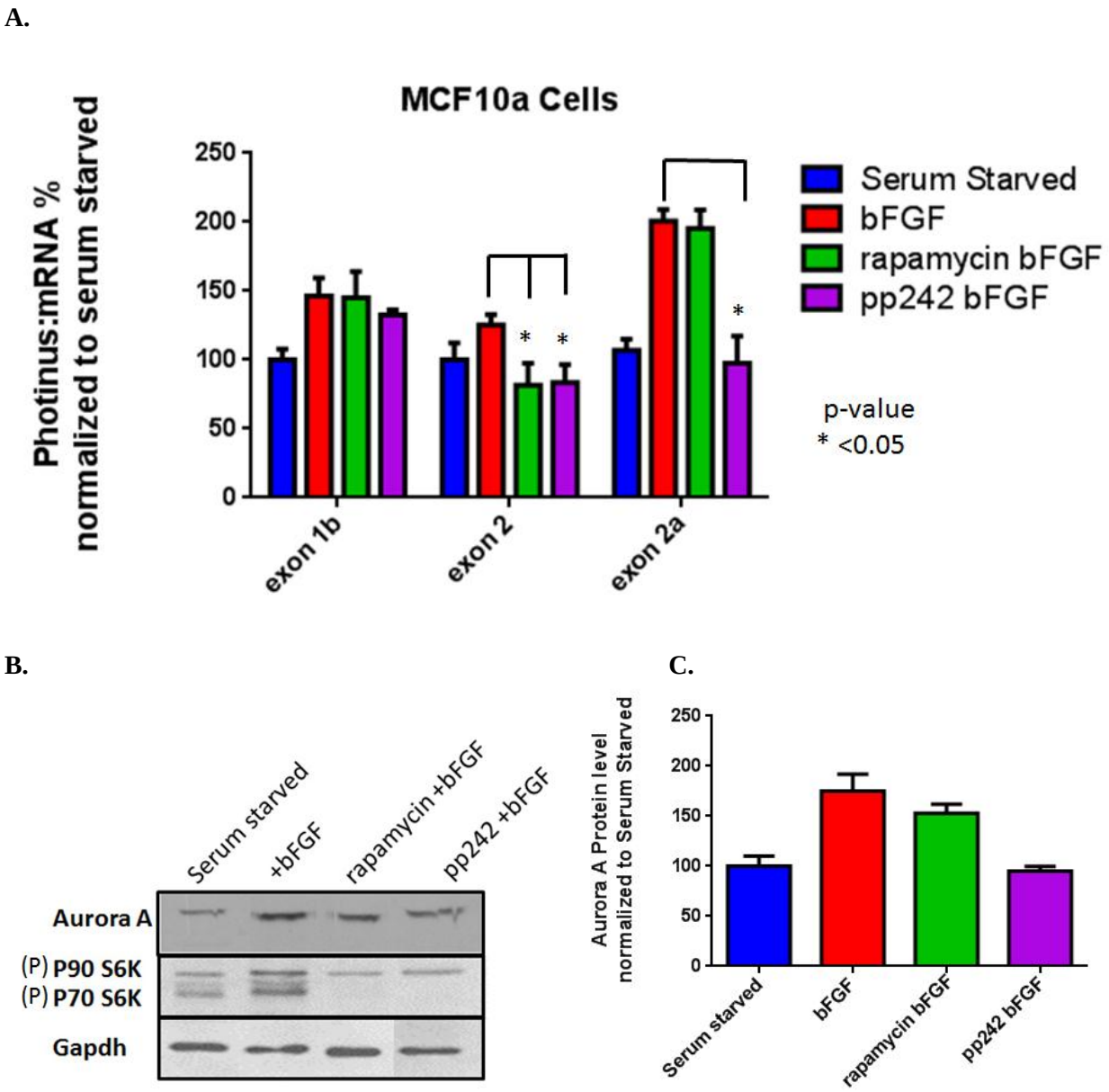
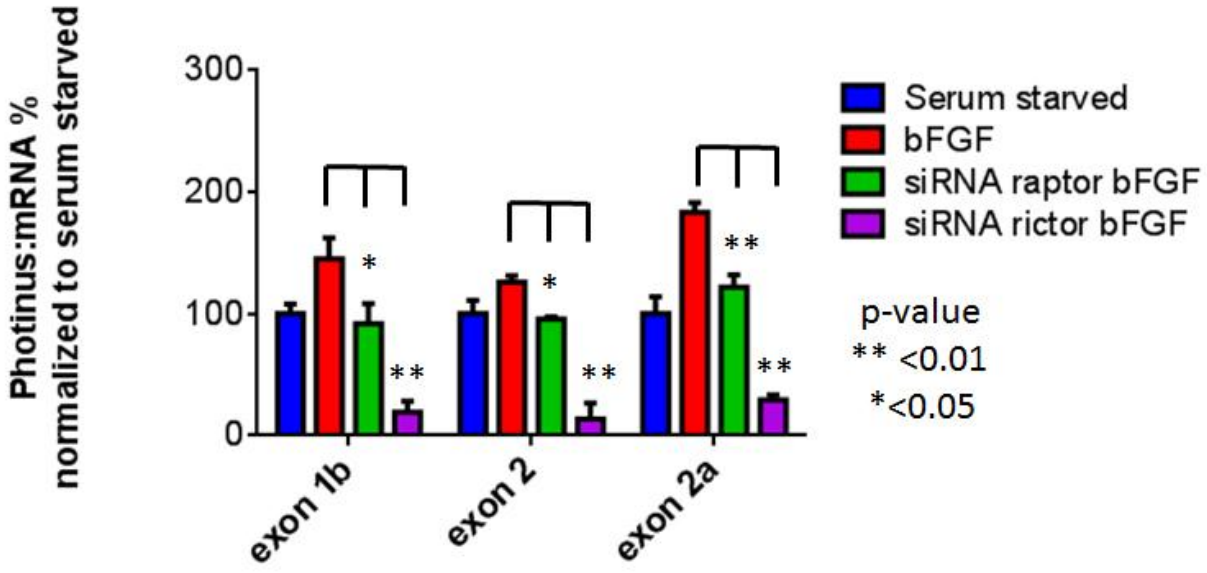


Figure 3.3 (A) **Aurora A protein expression and IRES activity after mTOR inhibition.** ApppG capped and poly A tailed monocistronic mRNA containing each exon was transfected into serum starved MCF10A cells. bFGF was added in the presence of rapamycin or pp242. *Photinus* to mRNA ratios of serum starved samples were set to one and each condition was normalized to serum starved. Performed in triplicate, n=3± standard deviation (SD). (B) Western blot analysis displays reduction of p70 S6K (a downstream target of mTORC1) with rapamycin and pp242. Represents effectiveness of the drug and the same dose was used for all inhibitor experiments. (C) Quantified Aurora A protein expression during treatment with Rapamycin (mTORC1) or pp242 (mTORC1 and mTORC2) in the presence of bFGF. N=3 ± SD. Western blots were quantified using image J software.

mTORC2 is a Major Regulator of IRES Activity

To determine the mTOR complex(s) mediating IRES activity and Aurora A protein expression, they were inhibited individually. I prevented the formation of both mTORC1 and mTORC2 using siRNA directed against raptor and rictor respectively. These proteins are required for stabilization of the mTOR complex (Zinzalla, Stracka et al. 2011, Weber and Gutmann 2012). Scrambled siRNA was used as a negative control. MCF10A cells were treated with siRNA directed against raptor and rictor for 24 hours, after which the cells were serum starved for 4 hours and then bFGF was added along with the exon containing monocistronic (coding *Photinus* luciferase) A-capped RNA. The cells were then incubated for 2 hours in the presence of the bFGF and harvested. Luciferase and RNA levels were quantified. A ratio of protein to mRNA was determined for each sample and normalized to the serum starved samples. A western assay confirmed that expression of raptor and rictor were successfully reduced using siRNA (figure 2.4A). Knocking down raptor led to a reduction in bFGF induced IRES activity of exon 2a by 40% and completely blocked the increase in exon 1b and exon 2 activity. Knocking down rictor greatly diminishes Aurora A IRES activity of exon 1b and exon 2 to 15% of basal levels and exon 2a to 30 % of basal levels. The western assay revealed a correlation with Aurora A endogenous protein expression. Taken together these results indicate bFGF increases Aurora A protein via mTOR2 which in turn increase exon 1b and exon 2A mediated IRES-dependent translation of Aurora A.

A.



B.

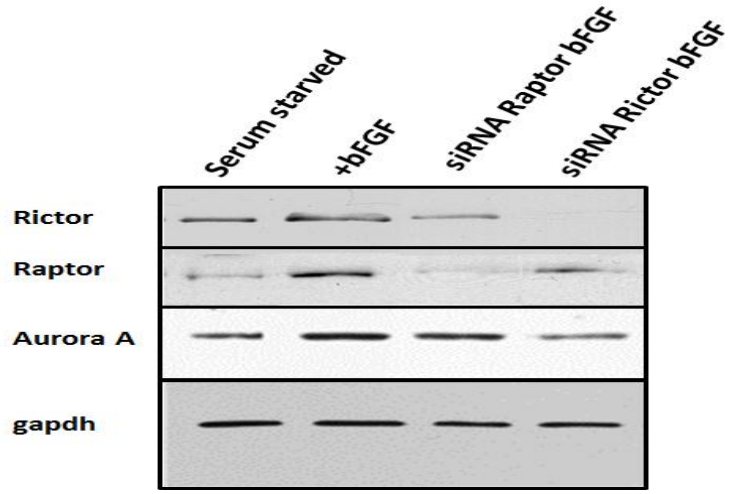


Figure 3.4 **Aurora A IRES activity after TORC1 and TORC2 inhibition. (**A) mTOR complex 1 and 2 were inhibited with siRNA against proteins raptor and rictor respectfully. Scrambled siRNA was used as a negative control. A capped monocistronic mRNA containing each exon was transfected into MCF10A cells in the presence of bFGF and siRNA. Performed in triplicate, n=3 $\pm$ SD (B)Western assay shows siRNA knock down of raptor and rictor along with effects on Aurora A protein expression +/- bFGF induction.

CHAPTER IV: DISCUSSION and

FUTURE DIRECTION

Overview

In my study, I show that growth factors can regulate protein expression by enhancing translation of mRNAs that contain specific IRESs. Specifically, I demonstrate that the bFGF induced increase in Aurora A expression was mediated by IRESs situated in exon 1b and exon 2a of the Aurora A 5' leader. Furthermore, my study indicates that the ERK and PI3K/Akt pathways converge on the two mTOR complexes, TORC1 and TORC2. Further experimentation indicates that of the two mTOR complexes, mTORC2 more so than mTORC1 mediates this translation mechanism. In summary, my results identify a novel pathway and translational mechanism contributing to over-expression of an oncoprotein. In turn this over-expression may contribute to tumorigenesis.

Intracellular pathways regulating IRES dependent translation are not well understood. Many cellular processes/stimuli are known to affect IRES activity, yet, the pathways mediating this affect have yet to be analyzed. For example, increasing intracellular Fe^{++} increases APP IRES activity and this effect is dependent on de novo transcription (Beaudoin, Poirel et al. 2008). However, it does not affect IRES dependent translation of the Tau mRNA (Krushel unpublished observations). Oxidative stress stimulates an increase in the translation of the HIV-1 IRES (Gendron, Ferbeyre et al. 2011). On the other hand, the nucleolar protein dyskerin, exhibits a negative effect on the IRES activity of VEGF (Rocchi, Pacilli et al. 2013). In addition some growth factors are positive stimuli for IRES activity.

Basic FGF can increase cap dependent translation (Pardo, Arcaro et al. 2002), however, little is known about its involvement in IRES dependent translation. bFGF promotes cellular proliferation and differentiation in a variety of cell types. Its major signaling pathways are

PI3K/Akt and MAPK. bFGF is often increased in cancers such as bladder and breast cancer (Hebert, Wu et al. 2009, Li, Gao et al. 2012). The protein has been detected in urine and nipple fluid in patients with bladder cancer at a level significantly higher than control patients and patients who were successfully treated (Li, Gao et al. 2012). I found that inducing epithelial breast cells with bFGF stimulates an increase in Aurora A kinase protein synthesis. Similar IRES studies have shown platelet derived growth factor to increase IRES dependent translation of Laminin (Petz, Them et al. 2012) and EGF to increase cap and IRES dependent translation of Aurora A (Lai, Tseng et al. 2010, Chen, Torcia et al. 2013).

bFGF is intimately linked to breast cancer (Li, Gao et al. 2012, Tiong, Mah et al. 2013) and therefore, the phenomena observed in my ex-vivo study may be a representation of what occurs in vivo. Aurora A protein expression is increased with bFGF signals through mTORC2 to increase IRES dependent translation. This regulation of Aurora A may be a major contributing factor as to why over-expression of bFGF is involved in cancer. We know inhibition of bFGF reduces the proliferative abilities of various cell types (Pratsinis and Kletsas 2007, Hebert, Wu et al. 2009), however, it has not been determined if bFGF is required for Aurora A over expression. It would be of interest to determine the dependency, of Aurora A expression in vivo, on bFGF. bFGF also has an IRES (Le and Maizel 1997) and whether it contributes to bFGF over-expression in certain breast cancers is not known. It would be of interest to determine whether bFGF and Aurora A IRESs and protein are regulated in a similar manner during cellular immortalization. For example, hnRNP A1 and possibly hnRNP E2 are ITAFs that regulate bFGF and Aurora A IRES respectively (Bonnal, Pileur et al. 2005). It is likely Aurora A IRESs in 'non-breast' cells are regulated in the same manner. HeLa cells, which are from the cervix,

also show increased Aurora A IRES activity (Dobson 2012), however, the effects of growth factors has not been investigated.

Another candidate pathway regulating IRES activity is though the MNK kinases. Reducing MNK activity blocks IRES dependent translation stimulated by rapamycin (Shi, Frost et al. 2013). It has been suggested that cellular stress is stimulating this pathway (Ueda, Watanabe-Fukunaga et al. 2004, Shi, Frost et al. 2013). My results indicated that rapamycin did not affect IRES activity, but inhibiting mTORC1, by knocking down raptor, did partially block bFGF increased IRES activity. In this paper, the authors did not examine any potential role of mTORC2. It is possible that MNK does contribute to IRES activity by interacting with IRES-transacting factor (ITAFs). For example, MNK kinases phosphorylate hnRNP A1 (Buxade, Parra et al. 2005, Shi, Frost et al. 2013), which is an ITAF, enhancing translation of myc and cyclin IRES (Shi, Frost et al. 2013).

Cap-dependent translation is regulated by intracellular pathways that converge on mTOR. Mammalian target of rapamycin is localized to two complexes TORC1 and TORC2. Of the two complexes, TORC1 was shown to be critical for this mode of translation. To regulate cap dependent translation through mTOR, growth factors signal through PI3K/Akt and MAPK pathways. Phosphorylation of Akt (T308 and S473) inhibits TSC 2 which is an inhibitor of mTOR (Weber and Gutmann 2012). TSC2 is also inhibited by ERK 1/2. One report from the Schneider lab indicated that mTORC1 also mediate IRES dependent translation of EMCV (Ramirez-Valle, Badura et al. 2010). I also found similar results in which knockdown of raptor, thus abolishing TORC1 activity, blocked the bFGF induced increase in EMCV IRES activity. Indeed IRES activity was below control levels (Figure 3.1). Originally, IRESs were proposed to occur or be up-regulated when cap dependent translation was inhibited (Pyronnet, Dostie et al.

2001), yet our evidence suggests that both cap and IRES modes of translation are regulated through mTOR. Preliminary data show silencing raptor (a unique mTORC1 stabilizer) reduces Tau IRES by 80% (Leslie Krushel, Niza Nemkul unpublished observations). Therefore, it was interesting that these same pathways also mediate IRES dependent translation. This observation is even more remarkable since it was initially proposed that IRES translation was up-regulated when cap-dependent translation was reduced.

My studies show that mTORC2 not mTORC1 regulates Aurora A IRES activity. This result is the first demonstration of the role of mTORC2 in IRES dependent translation. The drug pp242, which inhibits TORC1 and TORC2, dramatically reduced IRES activity, whereas rapamycin, which affects TORC1, had no effect. Moreover, abolishing the TORC2 complex by knocking down rictor dramatically reduced Aurora A IRES activity and also blocked the bFGF induced increase of the EMCV IRES (Figure 3.1).

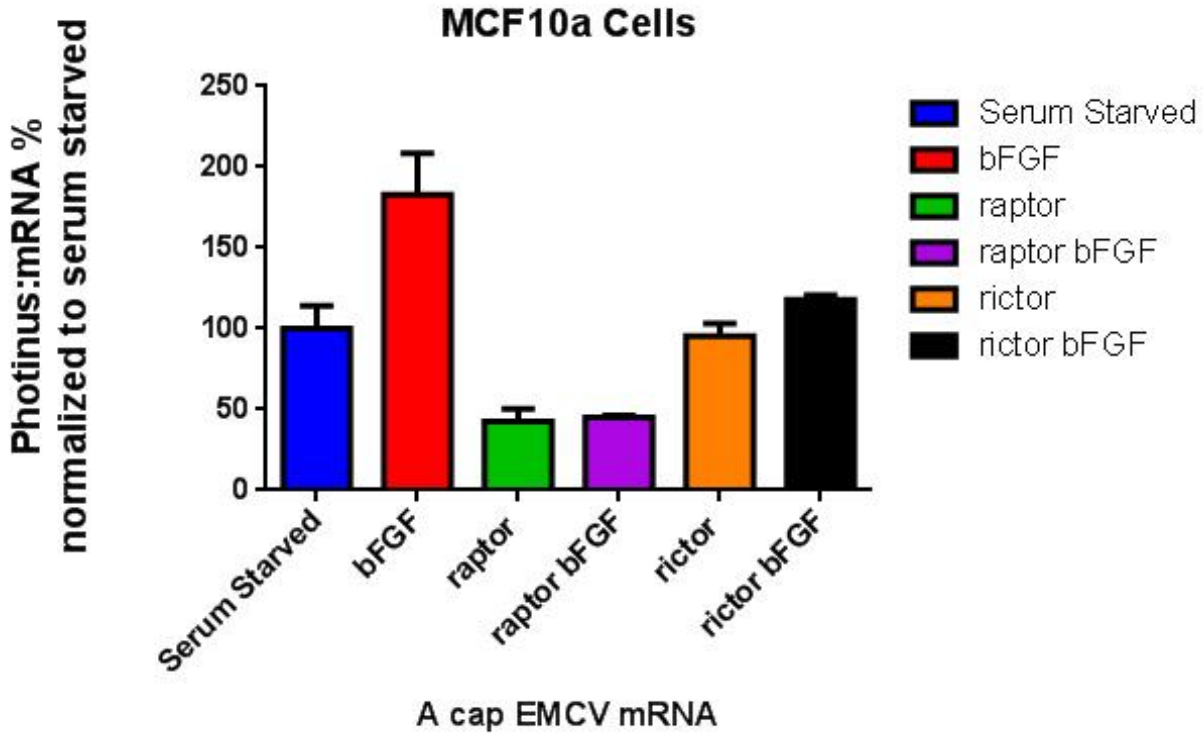


Figure 4.1 **EMCV IRES activity after TORC1 and TORC2 inhibition.** ApppG capped monocistronic *Photinus* luciferase mRNA containing EMCV 5' leader transfected into serum starved MCF10A cells in the presence and absence of bFGF and TORC inhibitors raptor and rictor. A serum starved condition served as both a control for transfection efficiency and representation of basal IRES activity. *Photinus* to mRNA ratios, from the serum starved sample, was normalized and set to 100. Performed in triplicate n=2 $\pm$ SD.

The rate limiting step in IRES translation is proposed to be the non-canonical RNA binding proteins termed IRES transacting factors (ITAFs). For example, the cellular concentration of the La protein regulates IRES activity mediated by the amyloid precursor protein (APP) 5' UTR (e.g., reducing or over-expressing La decreases and increases APP IRES activity respectively) (Beaudoin, Krushel unpublished observations). This result indicates that the concentration of La regulated APP IRES activity and in turn the levels of APP in the cell. The mechanism by which TORC2 mediates Aurora A IRES activity is unknown. TORC2 has been associated with ribosomes (Oh, Wu et al. 2010) both large and small subunits. TORC2 leads to transcription via certain transcription factors. For example, the APP IRES up-regulated by Fe^{++} requires de novo transcription, however, bFGF increases IRES activity as early as two hours. We do not know if this is enough time to transcribe and translate. It is possible the TORC2 regulates 80S ribosome formation at the initiation start site or if it forms a unique complex with the IRES. For example, recent data (Juan Chen and Leslie Krushel unpublished observation) have indicated that PCBP2 (hnRNP E1) may be an Aurora A ITAF. It has been found that PCBP2 binds TORC2 (Ghosh, Srivastava et al. 2008). On the other hand, TORC2 could phosphorylate substrates that act as ITAFs. We have evidence that polypyrimidine tract binding protein, a RNA binding protein, is phosphorylated by different kinases influencing various regulatory functions of mRNA processing (Xie, Lee et al. 2003, Vavassori and Covey 2008). It is possible that post translational modifications other than phosphorylation of ITAFs could be the rate limiting step. For example, kinase pathways could regulate other 'protein modifiers' such as acetylases and methylases which could enhance or impair the affinity of RNA binding proteins for transcripts (Liu and Dreyfuss 1995, Rho, Choi et al. 2007, Blackwell and Ceman 2012).

Substrates that mediate IRES activity downstream of mTORC2 are not known. mTOR, as part of mTORC2, is known to phosphorylate Serum and glucocorticoid induced protein kinase 1 (SGK1),PKCα, and is best known for phosphorylating Akt (S473) (Garcia-Martinez and Alessi 2008, Zinzalla, Stracka et al. 2011, Weber and Gutmann 2012). SGK1 is activated by mTORC2 leading to potassium, sodium, and chloride channel activation. It also stimulates proteolysis and glycogenolysis. There are no reports of SGK1s involvement in IRES dependent translation. Protein Kinase C alpha (PKCα), when increased, has been shown to increase Hif-1α IRES activity via AMPK (Mizrachy-Schwartz, Cohen et al. 2011). Thus, it would be useful to determine if inhibition of PKCα reduces Aurora A basal or bFGF induced IRES activity. Akt (protein kinase B) has two phosphorylation sites, S473 and T308 (Yung, Charnock-Jones et al. 2011, Dobson 2012) . IRES activity may be affected by this differential phosphorylation. Downstream of growth factors and other cellular stimuli, Akt is a key player in various cellular processes such as translation, apoptosis and cell proliferation. Phosphoinositol-dependent kinase 1 (PDK1) phosphorylates Akt at T308, mTORC2 and PDK2 phosphorylate Akt S473 (Dobson 2012). Both are required for full activation of the kinase. Interestingly, evidence shows differential regulation of T308 and S473 results in a variation in Akt substrate phosphorylation (Jacinto, Facchinetti et al. 2006, Yung, Charnock-Jones et al. 2011, Dobson 2012). This differential phosphorylation could affect the Akt substrate TSC2 which would then have positive or negative effects on the mTOR pathway. We have demonstrated in MCF7 cells, that translate Aurora A primarily in a cap-dependent manner, phosphorylation of S473 was not detectable, however, T308 phosphorylation was (Dobson 2012). Moreover, in MCF10A cells, that primarily translate Aurora A by utilizing the IRES, S473 phosphorylation is elevated while T308 phosphorylation was lower than MCF7. We found a correlation between increased exon 2 IRES

activity and elevated Aurora A protein expression. Analyzing S473 and T308 levels of phosphorylation in bFGF induced cells may reveal possible differences which lead to selective increases of Aurora A IRESs.

It has yet to be determined if the bFGF increase in Aurora A IRES activity occurs in all cells or only those that over express Aurora A. We had shown that primary cells like HMECs translate Aurora A mRNA via the cap but still retain the ability to utilize the IRES. On the other hand, a transformed breast cell line (MCF7), appears to have lost the ability to utilize the Aurora A IRES and is solely dependent on cap dependent translation (Dobson 2012). Thus, it would be useful to determine the cells that express the bFGF receptor and examine how they respond.

I have shown that bFGF increases exon 1b and exon 2a IRES activity (predominantly exon 2a), but, EGF increases exon 2 IRES activity. Although the intracellular pathways from EGF and bFGF overlap, at some point there must be a divergence. It is highly likely that downstream pathways unique to bFGF and EGF receptors are critical for controlling the different IRESs, possibly by differential post translational modifications, translations of ITAF mRNA, and/or transcription of RNA binding proteins which could act as ITAFs. Proteins such as Crk and SHP2 are in the FGF signaling pathway and not in the EGF pathway (Schlessinger 2004, Tiong, Mah et al. 2013). Crk, also known as proto-oncogene c-Crk or p38, is an adaptor protein and mediator of signal transduction (Tiong, Mah et al. 2013) SHP2 is a protein tyrosine phosphatase that regulates growth factor induced cell growth and proliferation through the RAS/MAPK pathway (Chen, Sung et al. 2006). Additionally FGF receptor interacts with docking protein fibroblast growth receptor 2 (FRS2) forming a complex with Grb2 and SHP2 (Schlessinger 2004). This complex is not found in the EGF pathway and is one of many unique complexes between bFGF and EGF.

The differential use of IRES and cap dependent translation in the different cell types could be exploited to target cancer cells. We have shown that cap dependent translation is dominant in primary cells, at least one tumorigenic cell line, and the Aurora A over-expressing cells utilize IRES. Thus, targeting IRES mediated translation may spare normal cells and kill a subset of cancer cells. Creating small molecules that interfere with the binding of the putative ITAF or 40S ribosome to the Aurora A mRNA would be a useful approach. Indeed, small molecules have been created to bind to the 5' UTR of the theophylline mRNA preventing 80S ribosome assembly and consequently inhibit its translation (Pelletier and Sonenberg 1985, Harvey, Garneau et al. 2002).

Determining if increased IRES activity is responsible for the increase in Aurora A expression in vivo is difficult. It is critical to identify the ITAF(s) and determine if its level or post translational modifications change in breast cancer. If this is the rate limiting step, knocking down the ITAF or inhibiting its post translational modification in mouse models of breast cancer could be very enlightening. I have shown basic fibroblast growth factor (bFGF) increases IRES activity of specific exons in the Aurora A 5' leader primarily through the mTORC2 pathway. This novel finding is a step in the direction of identifying a method of selectively reducing Aurora A expression specifically in cancer cells and not in normal expressing cells. There is still much to learn about cellular regulation of Aurora A's IRES, more work need to be done to unveil mTORC2s mechanism of regulating IRES dependent translation.

Future Direction

My research has shown that bFGF stimulation through MAPK and Akt which in turn targets TORC2 leading to enhanced translation of Aurora A protein via the IRES located on exon

2a and to a lesser degree exon 1b. The next objective is to learn more about mTORC2, but our understanding of this process is incomplete. There are four questions/objectives I want to address 1: How dependent is Aurora A on bFGF? 2: What are roles of TORC2 substrates in IRES mediated translation? 3: What ITAFs associate with Aurora A mRNA? 4: Is Aurora A IRES activity sensitive to their expression levels or post translational modifications?

EGF and bFGF both exhibit and increase in Aurora A protein synthesis by stimulating IRES activity. Using siRNA to knock down bFGF, in cells that over-express Aurora A, we can then observe the effects on Aurora A synthesis. Furthermore, we could look at IRES activity and changes in phosphorylation of PI3K/Akt and ERK pathways. On the other hand, using FGFR inhibitors may prove more beneficial. There are four active FGF receptors (FGFRs) (Daniele, Corral et al. 2012) and bFGF binds all of them. Reports have shown FGF 1, 2, and 3 to be amplified in breast cancers as well as ovarian, bladder, and gastric cancers (Ishizuka, Tanabe et al. 2002, Kunii, Davis et al. 2008, Nord, Segersten et al. 2010, Weiss, Sos et al. 2010, Daniele, Corral et al. 2012, Tiong, Mah et al. 2013). We could identify which receptors are responsible for the increase in Aurora A by using small molecule receptor tyrosine kinase inhibitors to individually inhibit each receptor. If one or a combination of the FGFRs is responsible for the increase in Aurora A this information would allow us to use mouse models of breast cancer. Utilizing tumorigenic mice, we knockout those FGFRs to examine if Aurora A is reduced in vivo and if the tumors are reduced.

Downstream targets of mTORC2 include: PKCα, Akt(S473), and SGK1. To further explore the pathway leading to IRES usage, we can individually silence these proteins using siRNA and measure IRES activity with or without bFGF or EGF induction. All growth factors signal through the same pathways Hebert, Wu et al. (2009), however our studies suggests bFGF

and EGF regulate different IRESs. It would be beneficial to uncover the IRES dependent functional differences between these two growth factors. We have previously observed differential phosphorylation of Akt between cell lines that primarily use cap dependent translation and those that use IRES. Thus, to identifying Akt phosphorylation patterns in EGF and bFGF induced cells, we would use Aurora A over-expressing cells induced with bFGF and EGF and observe Akt phosphorylation at S473 and T308. This may illuminate the key differences between the growth factors which are responsible for specific IRES up- regulation.

Identifying ITAFs should be a priority. ITAFs have been identified such as hnRNPs, PTB, DAP5, and Unr (Komar and Hatzoglou 2005). Multiple techniques including, Mass spectrophotometry, IP pull down assays, and/or RNA-seq may provide a better understanding of which ITAFs bind to Aurora A mRNA. We could then use siRNA to manipulate those proteins and determine if they really are essential for IRES dependent translation. Maybe there are different ITAFs for different IRESs. Post transcriptional modification may play a role in ITAF association to the Aurora A mRNA as well. For example PTB is phosphorylated by protein kinase A leading to its localization to the CD40L mRNA resulting in increased stability (Xie, Lee et al. 2003, Vavassori and Covey 2008). Some ITAFs have been shown to double as splicing factors which may explain how they initially associate with the mRNA (Blaustein, Pelisch et al. 2007). Regulation of splicing may very well directly affect how IRES elements are regulated. This process shares similarity with pathways that increase expression of Aurora A. Well-known splicing regulatory factors are members of two protein families serine/arginine-rich (SR) proteins and heterogeneous nuclear ribonucleoproteins (hnRNPs) which include PCBP1 and PCBP2 (Blaustein, Pelisch et al. 2007). PCBP2 could regulate splicing of Aurora A and remain associated to the mRNA. PCBP2 also associates with mSIN1 (Ghosh, Srivastava et

al. 2008) which is a major component of the mTOR2 complex. This link between PCBP2 and mTORC2 could be the mechanism that leads to increased translation of the Aurora A mRNA. It would be of interest to knock this protein down and observe the effects on Aurora A IRES activity.

We classified the cells base on the level of Aurora A protein expression. Over-expressing cells tend to predominantly use IRES dependent translation, while normal Aurora A expressing cells utilize the cap. bFGF stimulates an increase in protein and IRES activity although this is only in vitro. I have shown mTORC2 to be critical for IRES mediated translation of Aurora A. The goal is to selectively target cancer cells and not normal cells. Thus, we may have gained insight into how we can achieve this by discovering mTORC2s role. Further investigation is needed to better understand the mechanism behind TORC2s involvement. Targeting IRES activity whether through mTORC2 or by selectively mutating exon 2 or exon 2a, as those IRESs were increased by bFGF and EGF, may very well lead to the reduction of IRES activity that is vital for cancerous cells. Moreover basal cap dependent translation of Aurora A will remain active sparing normal cells. Future studies that identify the fundamental mechanisms by which Aurora A protein is synthesized will lead to more efficient methods of targeting the protein for anti-cancer drug therapy.

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Vita

Roy Lee Voice III is the oldest of four, born January 5 1984 in Waco, TX. He graduated from Linden-Kildare High School, a member of the National Honors Society, and all-star baseball player. He then pursued a higher education from Texas Southern University in the discipline of Clinical Laboratory Science. Immediately after graduating he worked in Corpus Christi, TX as a Medical Technologist for one year before returning to Houston. After becoming employed by the Transfusion Medicine department at MD Anderson Cancer Center, Roy learned about the University of Texas Health Science Center Graduate School of Biomedical Sciences. While working full time, he took courses as a non-degree seeking student. After two semesters, he had completed the course requirements for the Master of Science degree. He then enrolled as a full time Masters student and joined the Krushel Lab to begin his research. He has goals of joining the F.B.I criminal laboratory as a DNA Analyst.

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