Estrogen Receptor Alpha Translation: Mechanistic Insights and Therapeutic Implications

by

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A Dissertation

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To my family: James, Patricia, Zachary and Rachel

“Look closely. The beautiful may be small.” – Immanuel Kant

Abstract

The majority of human breast cancers are dependent on Estrogen Receptor Alpha (ER). These tumors are sensitive to inhibitors of ER, which serve to block ER activity or expression. Resistance to these therapies invariably develops and restores estrogen signaling in the tumor. ER+ breast cancers are also commonly dependent on the PI3K pathway which controls global cap-dependent protein translation through mTOR complex I (mTORC1). Inhibitors of PI3K/mTOR are effective in this setting when given with anti-estrogens.

Here we show that despite reducing global cap-dependent translation, PI3K and mTOR inhibitors do not reduce ER translation or expression. Translation of ER depends on RNA helicase, and translation initiation factor, EIF4A. Using small molecule inhibitors of EIF4A, we show that blocking ER translation impairs ER function and can block the growth of breast cancer models. The utility of EIF4A inhibition can be enhanced when combined with Selective Estrogen Receptor Degraders. This combination provides a deep and durable blockage of ER expression and a strong anti-tumor response. Finally, EIF4A inhibition can be exploited to block the translation of estrogen independent ER containing fusion proteins, whose incidence is being increasingly observed in anti-estrogen refractory patients.

BIOGRAPHICAL SKETCH

Jacob (Jake) Andrew Boyer was born on July 27th, 1992 in State College, Pennsylvania. Here he spent the next 21 years, attending both High School and College. Interested in science from a young age, Jake chose to study Biochemistry and Molecular Biology at Pennsylvania State University. While completing his studies, he worked in the laboratory of Dr. Gary Perdew in the department of Molecular Toxicology and Carcinogenesis. His work focused on a ligand activated transcription factor called the Aryl Hydrocarbon Receptor (AhR). Specifically, he studied the role of AhR in the regulation of chemokine gene expression. It was during his time at Penn State that Jake became interested in Signal Transduction, and particularly its dysregulation in human cancer.

Jake decided to pursue a PhD at the Gerstner Sloan Kettering School of the biomedical sciences at the Sloan Kettering institute in New York City. Even before his arrival in New York, Jacob was drawn to, and recruited by Dr. Neal Rosen (A circumstance for which I am forever grateful). Jacob worked on many projects in the Rosen lab, most of them involving the proto-oncogene mTOR kinase. Eventually, a serendipitous finding led Jake to investigate the mechanism of Estrogen Receptor protein translation. At the same time, he also worked in collaboration with Revolution Medicine in San Francisco, CA to characterize and develop selective inhibitors of mTOR Complex I.

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In order of appearance

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To my siblings: Thank you so much for your support and for the reminders of things most important.

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To my GSK classmates: There was, rather, a tensile excitement in that air which made one think—made me think for many years—that time spent asleep in New York was somehow time wasted. Whether this thought has lengthened or shortened my life I shall never know, but it has certainly colored it." – Christopher Hitchens.

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List of Abbreviations

4EBP: EIF4E Binding Protein

ADP: Adenosine diphosphate

AF1: Activation Function 1

AF2: Activation Function 2

AMP: Adenosine monophosphate

AMPK: AMP Kinase

AR: Androgen Receptor

ATP: Adenosine triphosphate

CASTOR: Cytoplasmic arginine sensor of TOR

DMSO: Dimethyl sulfoxide

E2: Estradiol

EdU: ethynyldeoxyuridine

eEF: Eukaryotic elongation factor

eIF: Eukaryotic Initiation Factor

ER: Estrogen Receptor (alpha)

ERE: Estrogen Response Elements

eRF: Eukaryotic Release Factor

FSH: follicle stimulating hormone

GAP: GTPase-activating Protein

GDP: Guanosine diphosphate

GEF: GTPase exchange Factor

GnRH: gonadotropin-releasing hormone

GTP: Guanosine triphosphate

hCG: chorionic gonadotropin

ICI: Imperial Clinical Industries

IRES: Internal Ribosome entry site

ITAF: IRES trans activating factors

LBD: Ligand Binding domain

LH: luteinizing hormone

M6A: 6-methyl adenosine

mRNA: messenger RNA

mTOR: mammalian target of rapamycin

mTORC: mTOR complex

NSG: NOD SCID gamma chain knockout mouse strain

PH: Pleckstrin homology

PI3K: Phosphatidylinositol-4,5-bisphosphate 3-kinase

PIC: Pre Initiation Complex

PIP3: Phosphatidylinositol (3,4,5)-triphosphate

PR: Progesterone Receptor

Raptor: Regulatory associated protein of mTOR

Rictor: Rapamycin-insensitive companion of mTOR

rRNA: Ribosomal RNA

SERD: Selective ER Degrader

SERM: Selective ER Modulator

S6K: S6 Kinase

TISU: Translation initiator of short 5’ UTR

tRNA: transfer RNA

uORF: upstream open reading frame

UPR: Unfolded protein response

UTR: Untranslated region

Chapter 1: Introduction

The central dogma of molecular biology posits that, with notable exceptions, information flow is generally from DNA to RNA to protein. DNA is replicated during cell division, RNA is generated from DNA via transcription, and proteins are synthesized by translating the information in mRNA to the language of amino acids. All three processes of information transfer: Replication, Transcription, and Translation are subject to tight and intricate regulation. In the case of transcription, lineage specific, and environmentally regulated transcription factors enhance or dampen select gene expression. Working in combination, these factors generate enormous diversity in the RNA expression profile of different cell types; it is precisely this control of gene expression that allows, for example, ductal mammary cells and neurons to be so different despite having identical DNA.

Conversely, it has long been thought that compared to transcription, translation is relatively straight forward; subject to quantitative but not qualitative regulation, and proceeds by only a few obligate mechanisms(Dever, 2012; Sonneveld et al., 2020) Elegant work by numerous investigators has shown this to be an oversimplification. Translation is subject to its own rich suite of combinatorial regulation, and is enjoying a period of investigative renaissance (Song et al., 2020; Sonneveld et al., 2020).

Understanding the ways in which various proto-oncogenes are translated is uncovering new targets for anti-cancer drugs (Malina et al., 2012; Pestova and Hellen, 2006; Song et al., 2021(Ernst et al., 2020). A major regulator of canonical

translation, mTOR kinase, has been a popular anti-cancer target for more than a decade, but as a single agent, its inhibition has yielded disappointing clinical responses(Lee et al., 2021; Sun, 2021). Among the many factors contributing to this outcome may be the many pro-survival genes whose translation can evidently proceed cap-independently during mTOR inhibition.(Chandarlapaty et al., 2011; Muranen et al., 2012; Rodrik-Outmezguine et al., 2011a) Understanding these mechanisms of translation informs the development of new therapies targeting select protein synthesis.

Eukaryotic Translation

Translation is the final step in the generation of functional protein decoded from information in DNA. Messenger RNA, generated via transcription, is used by the ribosomal machinery to synthesize polypeptides from constituent amino acids. Translation is traditionally divided into four phases: (1) Initiation (2) Elongation, (3) Termination and (4) ribosome recycling (Pelletier and Sonenberg, 2019). Translation is among the most energy intensive processes in the cell, and its estimated that 20% of ATP is used for this purpose(Xu and Ruggero, 2019). To protect against unnecessary, and potentially harmful, misuse of energy, translation is primarily regulated at the level of initiation, which is rate limiting for the process overall(Aitken et al., 2020; Pelletier and Sonenberg, 2019; Sonenberg and Hinnebusch, 2009)

In prokaryotes, base pairing between the mRNA and cognate 16S rRNA of the ribosome is sufficient to correctly situate the machinery atop the start codon

and begin translation. In contrast, and consistent with a greater control over gene regulation, eukaryotic initiation requires a coordinated sequence of events mediated by eukaryotic initiation factors (eIFs), which aid in the situation of the small ribosome atop the mRNA, facilitate the “scanning” of the small ribosomal machinery toward the situationally determined start codon, and final assembly of the complete and functionally competent ribosome to begin elongation(Kozak, 1999).

Canonical cap-dependent translation

Canonical translation begins with the recognition of the 5’ most element of the mRNA, the 7-methyl-guanosine triphosphate generated in RNA processing during transcription(Pelletier and Sonenberg, 2019; Sonenberg and Hinnebusch, 2009). This structure is frequently referred to as the “m7G”, “5’ cap” or simply “the cap.” Eukaryotic initiation factor 4E (EIF4E) is the major cytoplasmic cap-binding protein and nucleates the translation initiation complex. In addition to binding the m7G cap, eIF4E binds: eIF4G, eIF4A and eIF4B, this complex is referred to as the eIF4F complex. In parallel, a pre-initiation complex (PIC) consisting of: the small 40S ribosomal subunit, eIF1, eIF1A, eIF2, the eIF3 complex, and eIF5, is preassembled in preparation for EIF4F binding. The initiation factor, eIF2, is itself a member of the so-called “ternary complex”, which is comprised of: eIF2$\alpha/\beta/\gamma$, Met-tRNA$_i^{Met}$ (Methionine primed methionine-tRNA) and eIF2 bound GTP. The scaffold protein eIF4G is responsible for bridging EIF4F with the PIC, by way of eIF3 binding(Jaud et al., 2020; Pelletier and Sonenberg, 2019; Polunovsky et al.,

2013) eIF4B also contains an eIF3 binding domain and likely increases the avidity of PIC binding in conjunction with eIF4G. Upon EIF4F binding, eIF2 hydrolyzes GTP but remains associated with GDP.

Situated in the five-prime untranslated region 5' (UTR), the PIC, fully equipped with the required initiation factors, begins the process of "scanning" downstream toward the most situationally preferred AUG start codon. The preferred start codon is determined by a number of factors, including the regulated availability of initiation factors, surroundings sequences etc (Hinnebusch, 2011). Scanning is mechanistically facilitated via proteins eIF4A and eIF1/eIF1A. DEAD-box RNA helicase, EIF4A, utilizes ATP in the process of unwinding secondary structures in the mRNA UTR. The presence of 2 and 3 dimensional structures are common in 5' untranslated sequences and while they often have regulatory roles(discussed at length later), they must be denatured by EIF4A (and other helicases) in the process of scanning (Bourgeois et al., 2016; Hinnebusch, 2014; Parsyan et al., 2011). ATP independent factors EIF1 and EIF1A are thought to stabilize AUG binding in the P-site while destabilizing the binding of other codons(Holland et al., 2004). At this stage, eIF5-eIF2-GDP is released followed rapidly by the binding of eIF5B-GTP which facilitates the joining of the large 60S ribosome subunit(Holland et al., 2004; Pelletier and Sonenberg, 2019). The 80S ribosome is now competent to begin protein synthesis. This process overall is schematized in Figure 1.

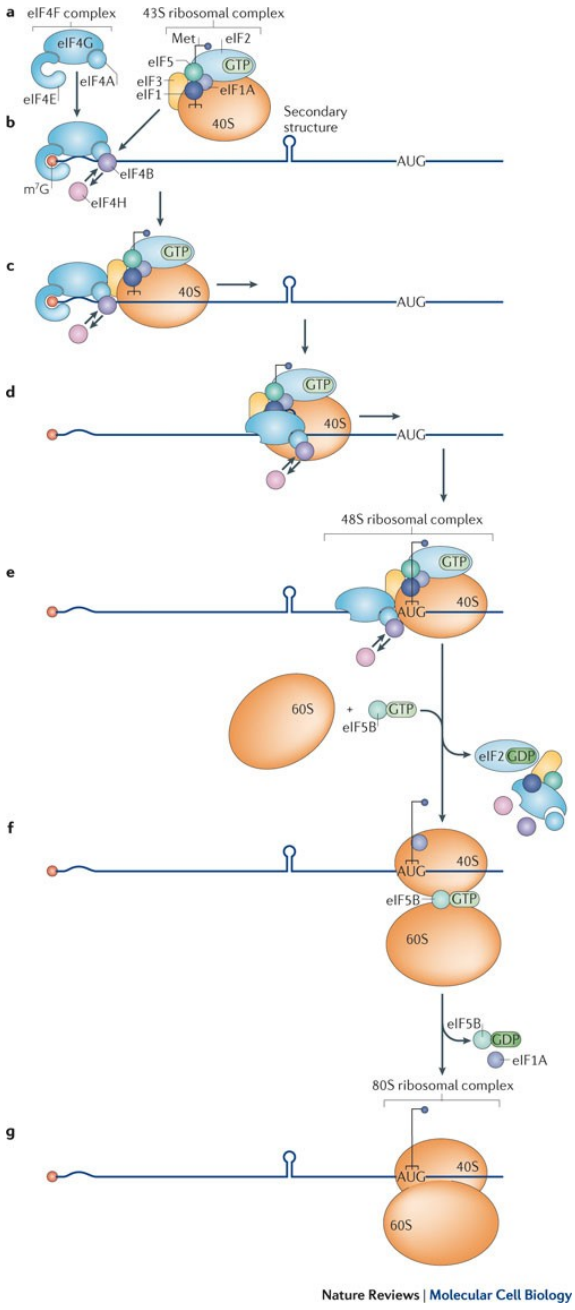


Figure 1.1: Translation Initiation in Eukaryotes

Parsyan et. al *Nat Rev Mol Cell Bio* (2011)

The second phase of translation is elongation, and proceeds independently of the initiation machinery. Here, a new suite of proteins, eukaryotic elongation factors (eEFs) are used. The 80S ribosome contains three compartments, “A” (Aminoacyl tRNA binding site), “P” (Peptidyl Transferase) and “E” (exit). During initiation, methionine-tRNA is placed in the “P” site as the nucleator for peptide synthesis. Subsequent charged tRNAs are carried to the ribosome “A” site by GTP bound eEF1A. The correct tRNA is retained in the A site via complementary binding between the codon in the mRNA and the anticodon in the tRNA. Peptide bond formation is catalyzed by highly conserved rRNA residues in the large ribosomal subunit, which as a group, form the peptidyl transferase center. The free amino group on the A site amino acid performs a nucleophilic attack on the carbonyl carbon of the P-site charged amino acid. This briefly transfers the elongating peptide to the “A” site tRNA, until ribosome translocation, which begins the process of elongation again, by putting the “A” site tRNA (now holding the elongating peptide) into the “P” site. This process is dependent on the GTPase activity of eEF1A, which is loaded with GTP each cycle with the aid of guanine exchange factor (GEF), eEF1B (Dever et al., 2018). This process repeats for each codon, with the growing polypeptide transferred in each step from the P-site to the A site, such that the latest amino acid added, is closest to the ribosome, and most C-terminal in the peptide chain.

Translation termination occurs when the ribosome complex reaches a stop codon: UAG, UAA, UGA. The proteins which participate in this final process, are termed: eukaryotic release factors (eRFs). In eukaryotes, these codons are

universally bound by, eRF1(Baierlein and Krebber, 2010). This protein mimics a tRNA in its structure, but lacking an attached amino acid, instead causes hydrolysis of the peptide bond upon its binding to the ribosome A site. This process is greatly catalyzed by the second release factor in eukaryotes, eRF3, which acts in a GTP dependent manner. The newly synthesized protein is thus released, and the ribosome must be recycled for another round of synthesis to occur.

Ribosome recycling involves the return of initiation factors, eIF1, eIF1A, and eIF3, which bind the small ribosomal subunit as before, as part of the nascent preinitiation complex. The protein Rli1, or ABCE1 in mammals, is also recruited by eRF1 and is required for the ATP dependent dissociation of the large and small ribosomal subunits, unprimed tRNA, and mRNA(Hellen, 2018; Zhou et al., 2020).

Signal Transduction Pathways Regulating Translation Initiation

Cell growth requires an increase in both global and select protein synthesis. Consequently, the translation machinery is a common convergence point for many signal transduction pathways which are frequently mutated in human cancer. These pathways and their interactions are schematized in Figure 1.2 below.

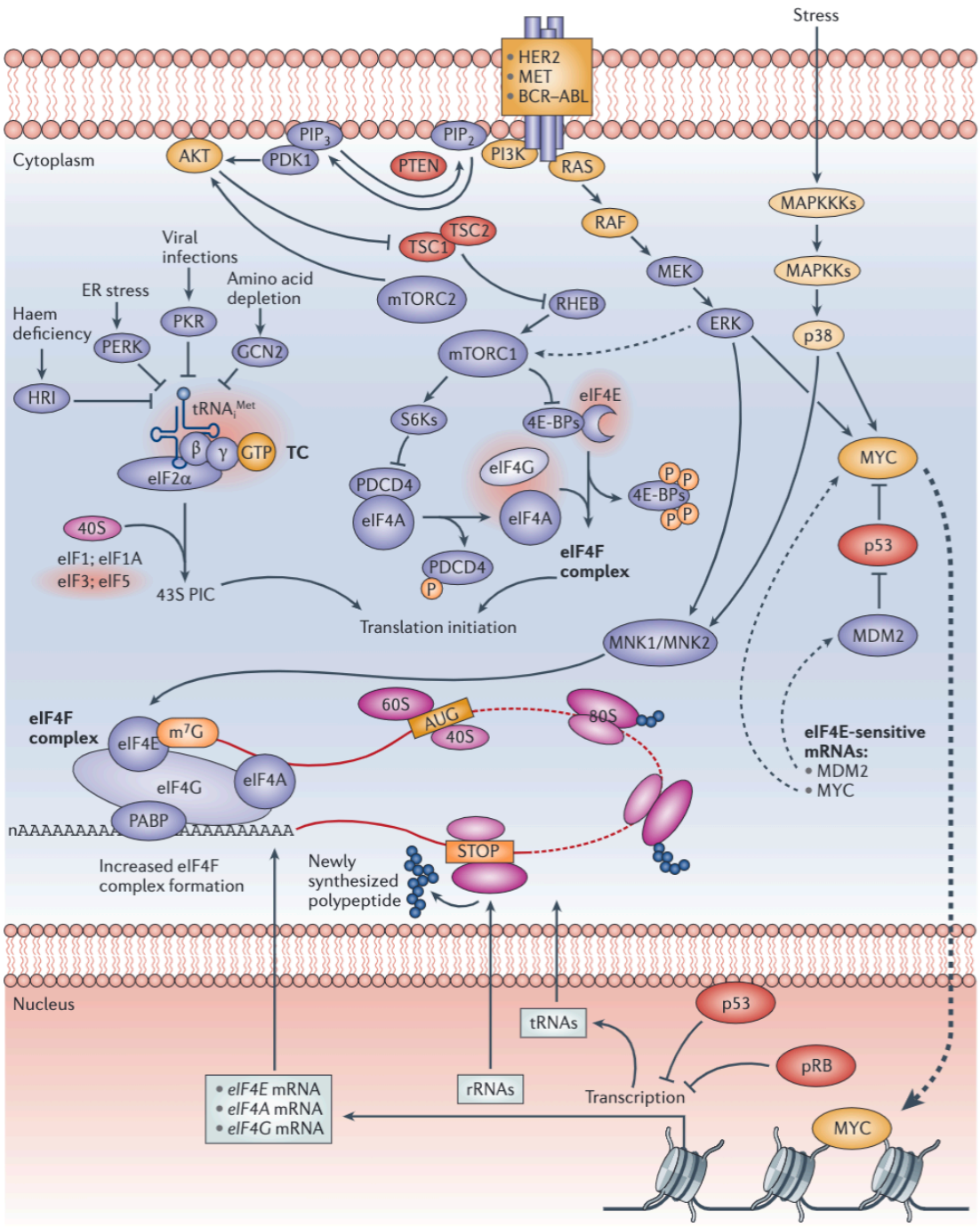


Figure 1.2: Signal Transduction pathways regulating translation

Bhat et. al Nature Reviews Drug Discovery *(2015)*

mTOR pathway

mTOR kinase is a serine/threonine protein kinase and member of the PI3K-related, PIKK family(Yang et al., 2013). The pathway is schematized in figure 1.3 below. The In mammalian cells, mTOR nucleates two distinct protein complexes, mTOR complex I (mTORC1) and mTOR Complex 2 (mTORC2). While the composition of mTOR containing complexes varies across organisms, mTOR kinase is a highly conserved regulator of anabolic metabolism in eukaryotes(Saxton and Sabatini, 2017). In mammals, mTORC1 is a regulator of nearly every metabolic network, and its functions can be broadly separated into suppression of catabolism and promotion of anabolism. mTORC1 exerts control over these functions by acting on a number of substrates, whose phosphorylation by mTOR kinase is facilitated by substrate binding protein, and mTORC1 member, Raptor(Hara et al., 2002; Kim et al., 2002).

Among the many functions ascribed to mTORC1 is the control of cap-dependent translation. mTORC1 phospho-inactivates 4E binding protein, (4EBP1), which otherwise binds and sequesters EIF4E away from the other initiation factors. mTORC1 phosphorylates 4EBP1 at multiple sites: T37/46, T70 and S65. These first two sites are high quality substrates and are thought to mediate priming of 4EBP1 for S65 phosphorylation, which regulates EIF4E binding directly (Roux and Topisirovic, 2018). When mTORC1 is inactive, 4EBP1 binds eIF4E at the expense EIF4G. In the absence of EIF4G binding, the required EIF4F complex cannot form and cap-dependent translation is halted. In addition,

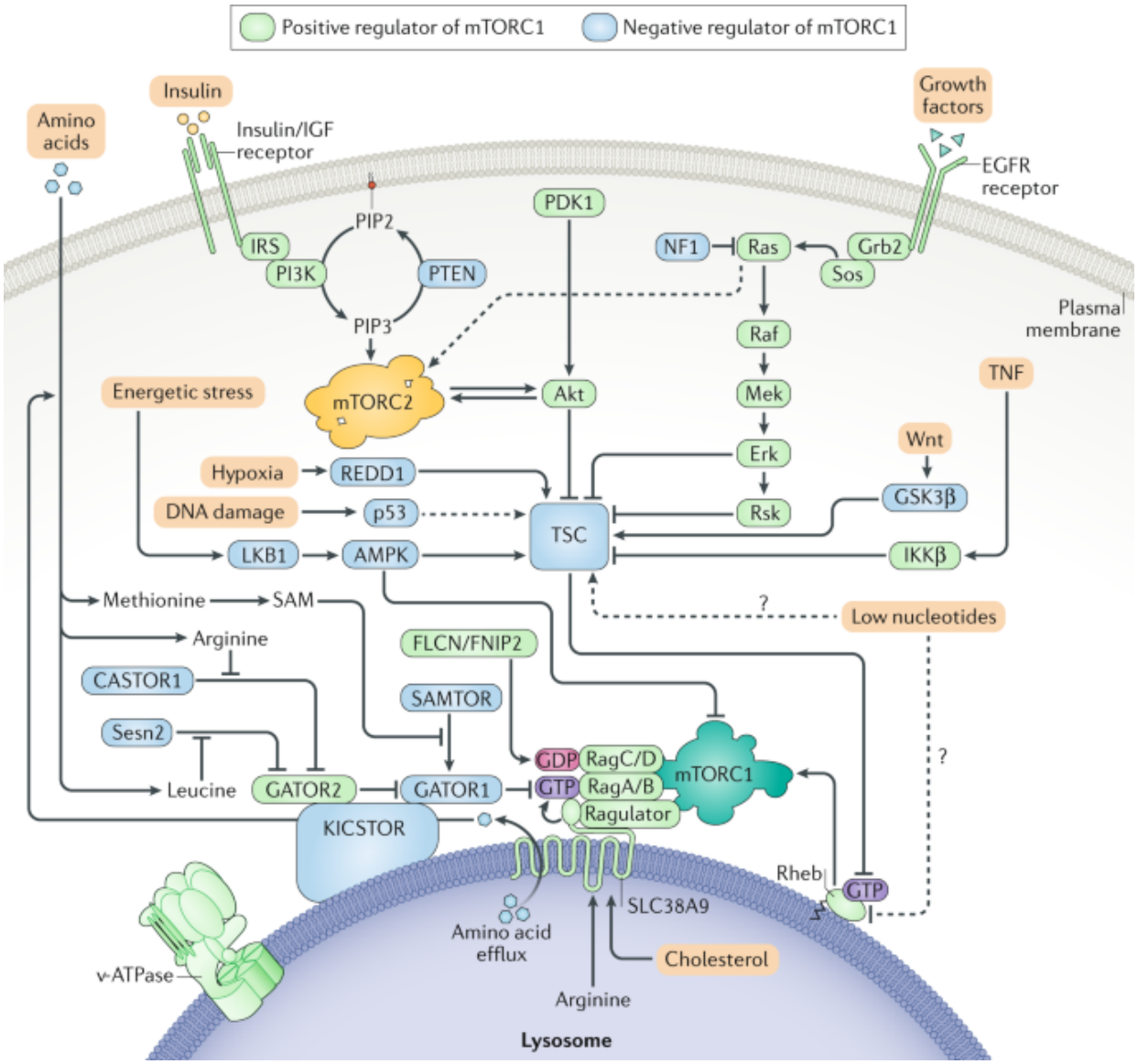


Figure 1.3: mTOR Signaling Network

Liu et. al *Nature Reviews Molecular Biology* (2020)

mTORC1 phospho-activates S6 Kinase (S6K). S6K1 then in turn phosphorylates and activates eIF4F complex member, eIF4B(Holz et al., 2005). in addition, S6K inactivates and degrades PDCD4, an important negative regulator of EIF4A(Moustafa-Kamal et al., 2020).

In addition to stimulating cap-dependent translation, mTORC1 also stimulates lipid and cholesterol biosynthesis directly via phosphorylation of transcription factor, Lipin-1, and indirectly through S6K mediated phosphorylation of SREBP transcription factors(Peterson et al., 2011).

mTORC1 is also an increasingly appreciated regulator of nucleotide biosynthesis. S6K phospho-activates the tri-enzymatic CAD complex, which is the rate limiting step in pyrimidine anabolism(Ben-Sahra et al., 2013). mTORC1 promotes purine biosynthesis by activating the ATF4 dependent transcription of multiple purine biosynthetic enzymes, including MTHFD2(Ben-Sahra et al., 2016).

Consistent with its role in biosynthetic metabolism, mTORC1 actively suppresses catabolic processes used to generate energy and metabolic intermediates during nutrient limitation. mTORC1 suppresses macroautophagy (autophagy) by phoso-inactivation of the ULK1/2 kinases(Kim et al., 2011). In the fed state, mTORC1 suppresses the use of extracellular nutrients obtained via macropinocytosis(Palm and Thompson, 2017; Palm et al., 2015, 2017). Further downstream, mTORC1 mediated phospho-inactivation of transcription factor, TFEB, has recently been shown to suppress the expression of genes involved in lysosome biogenesis, further limiting the availability of autophagy machinery (Martina et al., 2012).

mTORC2 is the second complex nucleated by mTOR, and is a major regulator of insulin signaling. In addition to mTOR kinase itself, mTORC2 also contains substrate binding protein, Rictor (analgous to Raptor in mTORC1), and mSin1, which contains the PH domain responsible for PIP3 binding and subsequent activation(Liu et al., 2015). The substrates of mTORC2 are primarily AGC kinases, and therefore mTORC2 cooperates with PDK1 to fully activate these proteins. For example, AKT is phosphorylated by mTORC2 at S473 and PDK1 at T308 (Manning and Toker, 2017; Rodrik-Outmezguine et al., 2011b)

AKT is the most well characterized mTORC2 substrate, and it responsible for amplifying the signal to many substrates in the AKT/PKB network(Manning and Toker, 2017). Among the most well-defined functions of AKT is insulin dependent glucose uptake, which depends heavily on mTORC2(Hagiwara et al., 2012; Yuan et al., 2012). While all AKT isoforms have been implicated in glucose uptake, this function has been most strongly linked to AKT2, which via AS160 phosphorylation, mobilizes GLUT4 containing vesicles to the membrane(Manning and Toker, 2017) mTORC2 has also been implicated in cell motility by virtue of its phosphorylation of PKC isoforms and attendant activation of cAMP production and actin remodeling(Liu et al., 2010). mTORC2 also phospho-activates AGC kinase, SGK, among whose activities include the phosphorylation of various anti-apoptotic proteins(Brunet et al., 2001; Mikosz et al., 2001)

Understanding the activation of each mTOR-containing complex is a rich and active field of investigation. mTORC1 is activated by both growth factors and nutrients, while mTORC2 is activated by growth factors alone. Lineage specific

receptor tyrosine kinases, of which insulin receptor is a special type, become activated upon cognate ligand binding. Subsequent binding of class I PI3K regulatory subunit, p85, activates the catalytic subunit of class I PI3K, p110. Activated PI3K catalyzes the phosphorylation of phosphoinositide 4,5 phosphate to phosphoinositide 3,4,5 triphosphate, which as stated above, acts as a binding site for PH domain containing proteins such as mTORC2 and AKT(Dibble and Cantley, 2015; Liu et al., 2015)

mTORC1 activation is much more complex, and is thought to require a two step mechanism, whereby amino acids first prime activation, followed by maximal induction by growth factors (Dibble and Cantley, 2015; Menon et al., 2014; Saxton and Sabatini, 2017). mTORC1 senses both glucose and amino acids, both of which must be in abundance for full activation. The mechanism by which glucose activates mTORC1 remains elusive, but is explained partly by the RAG GTPases, and AMPK kinase(Peng et al., 2014). Amino acid dependent mTORC1 activation is better understood, and involves both lysosomal and cytoplasmic signaling pathways. The two most important amino acids sensed by mTORC1 are leucine and arginine, both of which have a direct cytoplasmic sensor, Sestrin and CASTOR, respectively (Chantranupong et al., 2016; Dyachok et al., 2016; Hara et al., 1998; Wolfson et al., 2016). Through a series of controversial intermediate steps, amino acid bound forms of these sensors, relieve GAP suppression of RAGA/B GTP loading(Peng et al., 2014; Wolfson et al., 2016). At the same time, amino acids present in the lysosome, stimulate the activity of the RAGULATOR complex, which is a RAGA/B GEF(Bar-Peled et al., 2013; Sancak et al., 2010).

Finally, RAGA/B-GTP promote translocation of mTORC1 from the cytoplasm to the lysosome via RAPTOR. It is on the surface of the lysosome where mTORC1 encounters the growth factor activated, Rheb GTPase required for maximal activation.

mTORC1 senses growth factors downstream of mTORC2 and AKT. Among the many AKT substrates are TSC2, which is a member of the larger TSC complex, containing TSC1 and TSC2. TSC2 is a GAP for the Rheb GTPase, and it only known regulator, as it has no known GEF(Saxton and Sabatini, 2017). AKT phosphorylation of TSC2 on Thr 1462 inactivates its GAP activity and hence facilitates Rheb-GTP dependent mTORC1 activation(Manning et al., 2002). In addition, AKT phosphorylates and inhibits PRAS40, an inhibitory subunit of mTORC1, whose activity normally impairs raptor substrate binding(Kovacina et al., 2003). The molecular logic of mTORC1 and its regulation of cap-dependent translation is such that both nutrients and growth factors must be in abundance before translation can be initiated. In accordance with the extraordinary energy demand of translation, mTORC1 serves as the link between energy sufficiency and initiation of protein synthesis, specifically by sensing the cellular concentration of essential amino acids, arginine and leucine, needed for peptide elongation.

MAPK pathway

The RAS-MAPK pathway relays signals from various membrane bound receptors through the RAS family of GTPases. RAS isoforms (HRAS, KRAS, NRAS) activate a network of effectors, many of which control cell division(Stephen

et al., 2014) The RAF isoforms (ARAF, BRAF, CRAF) are well characterized RAS effectors and MAP kinase kinase kinases. RAF activation initiates a kinase cascade by phosphorylating MEK1/2, which in turn phosphorylates ERK1/2. ERK serves as a major amplifier of the RAS signal and activates a multitude of transcription factors and functional mediators, many of which, such as Myc and Cyclin D1 are involved in cell division (Matallanas et al., 2011; Stephen et al., 2014).

ERK is thought to promote protein translation in a number of ways, many through intermediate substrates. ERK phosphorylates and activates MNK kinase, whose binding to EIF4G normally localizes this protein to the EIF4F complex(Waskiewicz et al., 1997; Xu et al., 2021). Activated MNK phosphorylates EIF4E at S204 and enhances both global and oncogenic translation(Xu et al., 2021). ERK phosphorylates and activates the RSK family of kinases, which among other functions, phospho-activate, eIF4B, and phosphor-inhibit PDCD4(Pin et al., 2020). In addition, ERK and/or RSK are capable of phosphorylating many components of the mTOR pathway, all of which enhance mTOR output and attendant cap-dependent translation. These ERK and RSK substrates in this pathway include: Raptor, TSC2, LKB1, DEPTOR and S6(Roux and Topisirovic, 2012, 2018)

Negative regulation of Translation Initiation

Numerous pathways restrain translation during cellular stress. A major convergence point for negative regulators is the phosphorylation of eIF2α, which

severely restricts global translation. Serine 51 phosphorylation of eIF2α prevents the eIF2B dependent recycling of GDP for GTP, required for successive rounds of translational initiation (Jaud et al., 2020). One major eIF2α kinase is GCN2, which binds uncharged tRNAs, and acts as a general sensor of amino acid starvation(Dong et al., 2000). Another EIF2α kinase is the endoplasmic reticulum stress response (sometimes called the unfolded protein response) protein, PERK. The unfolded protein response is a general mechanism by which incorrectly folded proteins in the endoplasmic reticulum signal to halt translation in an attempt to restore homeostasis. The upstream most sensor of the UPR is the chaperone BiP(Bertolotti et al., 2000) Under homeostatic conditions, BiP binds the endoplasmic reticulum luminal domains of three major UPR effectors: PERK, IRE1 and ATF6. BiP binding to each protein inhibits their function as a UPR effector. In the case of the kinases, PERK and IRE1, BiP prevents their dimerization dependent activation. During the UPR, BiP binding to unfolded proteins leaves the dimerization interface unoccluded. PERK and IRE1 homodimerize and undergo trans-autophosphorylation; each kinase pair then activates a parallel pathway to restore protein homeostasis(Hetz, 2012) PERK phosphorylates eIF2α directly, which reduces the efficiency of global translation, as outlined above. IRE1 activation promotes its intrinsic RNAase activity, which in turn eliminates an inhibitory intron from the mRNA encoding transcription factor, XBP1. Functional XBP1 promotes the expression of various chaperones, ER quality control enzymes, and machinery involved in the degradation of non-functional proteins in the ER lumen. In the case of the third UPR mediator, ATF6, BiP normally occludes

a Golgi localization sequence. Once trafficked to the Golgi Apparatus, ATF6 is processed on both sides of the membrane to release the ATF6 cytoplasmic domain, a competent transcription factor with targets similar to XBP1(Thuerauf et al., 2007).

A final negative regulator worth mentioning is ATF4. While eIF2α phosphorylation reduces global translation efficiency, the translation of mRNA encoding ATF4 is elegantly activated under such circumstances. *ATF4* contains two upstream open reading frames (uORF), termed uORF1 and uORF2. During normal conditions of high eIF2α-GTP, initiation factors are in sufficient abundance to facilitate translation re-initiation at the more 3' uORF, uORF2. This element acts as an inhibitory cassette that normally suppresses ATF4 translation. During stress, eIF2α-GTP becomes limiting, and initiation at uORF2 occurs with reduced probability. Hence, translation occurring at uORF1 has the opportunity to proceed through the inhibitory element and translate a functional ATF4 protein(Vattem and Wek, 2004)ATF4 is a transcription factor, and acts as a major integrator of the stress response; promoting the transcription of amino acid transporters, apoptosis and autophagy genes (Wortel et al., 2017)

Cap-independent Translation

In addition to the canonical cap-dependent translation outlined above, it is becoming increasingly appreciated that select mRNAs can be translated by a variety of mechanisms which circumvent the cap-dependent/EIF4E requirement. Transcripts capable of such alternative initiation are enriched for stress response

proteins and proto-oncogenes, whose expression has evolved robustness to fluctuating nutrients and energy levels.

The information mediating cap-independent translation is often present in the 5' Untranslated region (UTR) of select mRNAs. Specific sequences, nucleobase modifications, as well as three dimensional structures are capable of initiating translation without eIF4E dependent assembly of the preinitiation complex. The complex array of regulatory information contained in the 5' UTR of select mRNAs can be appreciated in Figure 1.4A.

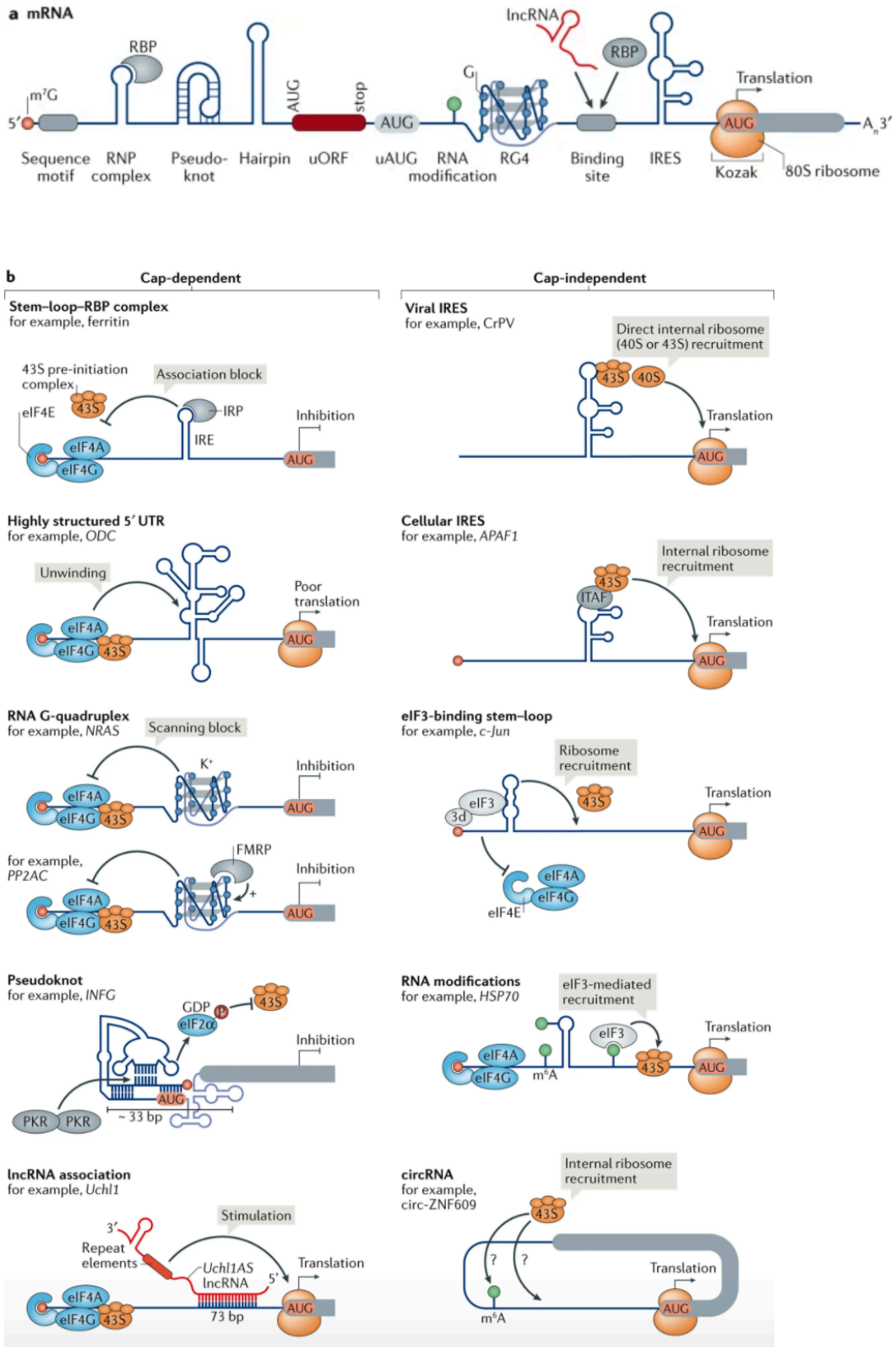

a mRNA
RBP
m7G
5′
AUG
stop
AUG
lncRNA
RBP
G
Translation
AUG
An 3′
Sequence motif
RNP complex
Pseudo-knot
Hairpin
uORF
uAUG
RNA modification
RG4
Binding site
IRES
Kozak
80S ribosome
b
Cap-dependent
Cap-independent
Stem–loop–RBP complex
for example, ferritin
43S pre-initiation complex
Association block
eIF4E
43S
IRP
IRE
Inhibition
eIF4A
eIF4G
AUG
Viral IRES
for example, CrPV
Direct internal ribosome (40S or 43S) recruitment
43S
40S
Translation
AUG
Highly structured 5′ UTR
for example, ODC
Unwinding
Poor translation
eIF4A
eIF4G
43S
AUG
Cellular IRES
for example, APAF1
Internal ribosome recruitment
43S
ITAF
Translation
AUG
RNA G-quadruplex
for example, NRAS
Scanning block
K+
Inhibition
for example, PP2AC
FMRP
+
eIF3-binding stem–loop
for example, c-Jun
Ribosome recruitment
eIF3
3d
43S
Translation
AUG
eIF4A
eIF4G
eIF4E
Pseudoknot
for example, INFG
GDP
P
eIF2α
43S
Inhibition
AUG
PKR
PKR
~ 33 bp
RNA modifications
for example, HSP70
eIF3-mediated recruitment
eIF3
eIF4A
eIF4G
m6A
43S
Translation
AUG
lncRNA association
for example, Uchl1
3′
Repeat elements
Stimulation
Uchl1AS lncRNA
5′
Translation
73 bp
circRNA
for example, circ-ZNF609
Internal ribosome recruitment
43S
?
?
Translation
m6A
AUG

Figure 1.4: Eukaryotic Translation Mechanisms

(A) Cartoon of an 5' untranslated region depicting regulatory elements

(B) Comparison between mechanisms of cap-dependent vs cap-independent mechanisms of translation.

The median length of UTR length in humans is 218 nucleotides, and while UTR length does not necessarily correlate with cap-independence, it provides a greater probability of forming cap-independent promoting structures(Leppek et al., 2018). While thermodynamic folding simulations can accurately predict formation of stable RNA structures, we currently have limited ability to predict which such structures, modifications and sequences mediate cap-independent translation. For these reasons, a candidate mRNA 5' UTR must be empirically tested with respect to its cap-independent activity(Leppek et al., 2018)

Internal ribosome entry sites (IRES), are those secondary mRNA structures in the 5'UTR capable of initiating translation independently of eIF4E. The first IRESs were discovered in viral genomes in late 1980s(Pelletier and Sonenberg, 1988). The field has since vastly expanded to include a multitude of IRES elements present in both viruses and eukaryotes. Viral IRES elements are traditionally stratified into 4 families, broadly characterized by the initiation factors on which they depend. Class I which are typified by Poliovirus IRES, utilize EIF4E, EIF4G, EIF3, EIF4A for the first round of translation, at which point viral protease 2A cleaves the EIF4E binding site of EIF4G. Further rounds utilize EIF4A, EIF3 and cleaved EIF4G, which proceeds much more efficiently, having lowered the

competition with eukaryotic EIF4E dependent RNAs(Pelletier and Sonenberg, 2019). Structurally quite different than type I and contain a tetraloop secondary structure. Functionally, class II IRESs initiate translation similarly to type I, but dispense entirely with the EIF4E dependence. Although type II IRESs require EIF4A dependent remodeling, scanning is not required for initiation. Class III IRESs, typified by Hepatitis C virus (HCV) are independent of all EIF4F members, and contain a loop structure which substitutes for EIF3 in binding the small subunit of the ribosome. Finally, type IV are the most versatile IRESs known and are exemplified by cricket insect paralysis virus (CrPV). These IRESs contain a 3D structure called a pseudoknot which mimics the initiator methionine tRNA and places it in the A site directly. Such IRESs therefore require no eukaryotic initiation factors(Pelletier and Sonenberg, 2019). Soon after the discovery of viral IRESs, the first cellular mRNAs were also identified. The first to be unearthed was the immunoglobulin heavy chain binding protein, BiP, and not coincidentally, in a cell with poliovirus infection, whose cap-dependent translation has been shut down (Komar and Hatzoglou, 2014). Recent work has suggested that as many as 10% of cellular mRNAs contain an IRES (Weingarten-Gabbay and Segal, 2016) The regulation of IRES utilization is controlled by so called IRES trans activating factors (ITAFs). These are RNA binding proteins, whose action being akin to transcription factors, bind specific IRES sequences or multi-dimensional structures and in poorly understood ways, recruit translation initiation machinery and or the 40S ribosomal subunit(Komar and Hatzoglou, 2014). La autoantigen/SSB is one such example and binds to the 5’ UTR of many IRESs, including: poliovirus IRES, XIAP and

Laminin Beta(Costa-Mattioli et al., 2004; Godet et al., 2019). Such ITAF molecules are themselves tightly regulated to ensure context specific expression of their target mRNAs. For example, in response to TGF-β, La/SSB was shown to re-localize from the nucleus to the cytoplasm and promote the IRES dependent translation of LamB1 (Petz et al., 2011, 2012)Similar to viruses, cellular IRES elements depend on a subset of initiation factors compared to the full suite used in conventional cap-dependent translation. While cap-independently translated mRNAs do not require EIF4E by definition, many other factors such as EIF4A and EIF4G may still be required. The exact initiation factors implicated in IRES mediated translation must at present be empirically examined for each mRNA, and depends on the exact IRES structure and sequence, as well as the participating ITAFs. More complicated still, alternative isoforms of the conventional cap-dependent machinery may be used in place of their canonical counterparts in IRES mediated initiation. For example, under stress conditions, EIF4G2 can be employed, which unlike the larger EIF4G1, lacks a binding site for EIF4E, while retaining those for EIF3 and EIF4A. Similarly, eIF3d, a member of the larger EIF3 complex can serve as an EIF4E substitute by binding the m7 cap under certain circumstances(Godet et al., 2019; Lee et al., 2016; Parra et al., 2018). A number of Cap-independent conferring mechanisms are outlined in Figure 1.4B

More recently, non-IRES mechanisms of cap-independent translation have been identified. One of the most exciting and evidently most general is mediated through 6-adenosine methylation (m6A) in the 5'UTR of various genes. This mechanism appears especially important for maintaining global translation when

mTOR and EIF4E activity is low (Coots et al., 2017). It was determined that the presence of a single m6A in the 5'UTR was sufficient to facilitate IRES independent and cap-independent translation(Wang et al., 2015). Evidently, multiple methylases can participate in this process, including but not limited to METTL3(Coots et al., 2017). Adenosine methylation provides a binding motif for ITAFs such as ABCF1 to recruit ribosome machinery. Based on a global approach, these authors identified estimate that as high as 30% of mRNAs are capable of utilizing this method of translational regulation.

Estrogen Receptor Alpha

The first estrogenic hormone was discovered in the early 1920s when Allen and Doisy induced the growth of female rodent reproductive tissue using injected ovarian follicle fluid. Subsequent work revealed multiple estrogen species produced in mammals. Allen and Doisy isolated the first, estrone (E1), from urine in 1929. Estriol (E3) and estradiol (E2) were discovered in 1930 and 1933 respectively. The mechanism of estrogen action would remain unknown until 1961, when Elwood Jensen used radiolabeled estradiol to identify a nuclear localized E2 binding protein, now known as ER alpha(Jensen, 1962; Tata, 2005). Estrogen Receptor became the founding member of a large superfamily of ligand activated transcription factors known as nuclear receptors, which includes: Androgen Receptor, Progesterone Receptor, Retinoic Acid Receptor and Glucocorticoid Receptor, among others. Such nuclear receptors come in two primary variates: Type I and Type II. In the absence of hormone, Type I receptors reside in the cytoplasm. Ligand binding induces nuclear entry, response element binding, and

transactivation of target genes. Type II nuclear receptors, conversely remain nuclear localized and response element bound, and associated with co-repressors. Ligand binding facilitates a change in conformation that switches to co-activator binding and target gene induction (Klinge et al., 1997)

ER is a typical type I nuclear receptor, which in the absence of ligand is cytoplasmically localized, and bound by HSP90. Upon Estradiol (E2) binding, the receptor is stabilized, sheds HSP90, dimerizes, enters the nucleus and binds DNA at sequences termed estrogen response elements or EREs. (Siersbæk et al., 2018). The ERE is a spaced palindromic sequence of the form: 5’ CAGGTCA nnn TGACCTG 3’, comprised of two half sites, to which each member of the dimer binds. The ER signaling cascade is schematized in figure 1.5.

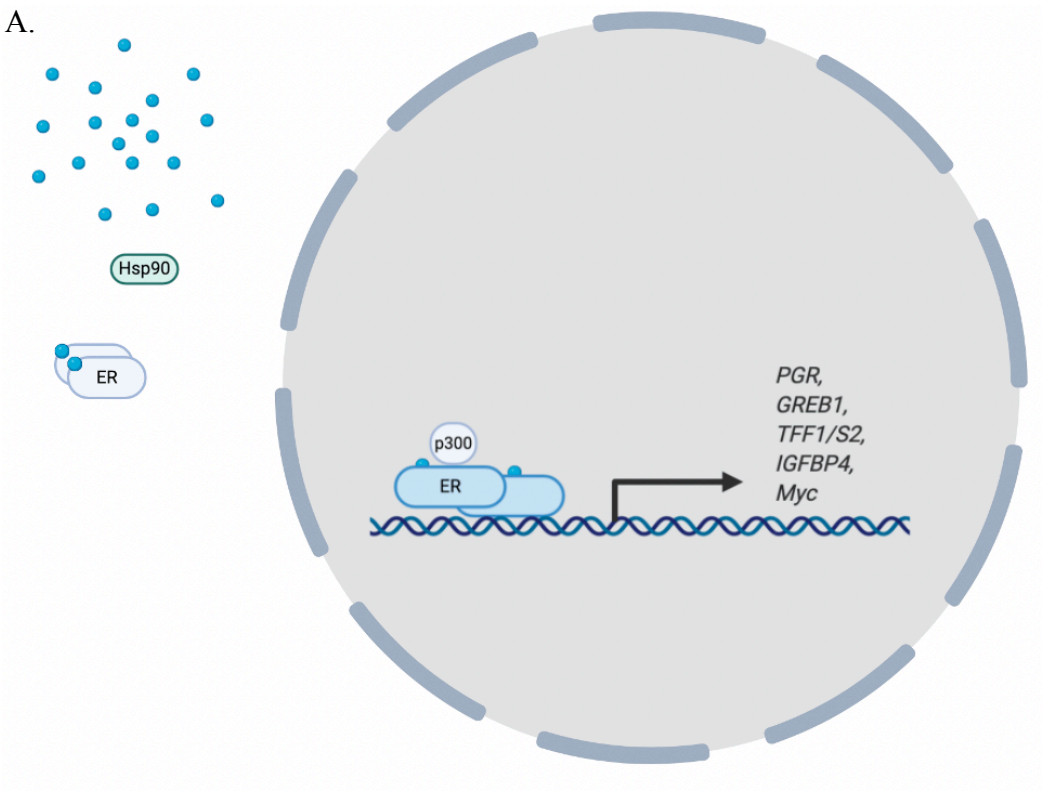


Figure 1.5: Schematic of ER signaling

(A) Estradiol is depicted as blue spheres, which bind ER and induce: dimzerization, nuclear entry and attendant gene expression.

ER alpha and other nuclear receptors have a conserved structure comprised of five domains: A/B, C, D, E and F. the N-terminal “A/B domain” contains the activation function 1 (AF1), which mediates ligand independent, but relatively weak transactivation of target genes. AF1 also contains phosphorylateable residues, which can be ERK and CDK2-cyclinA targets. The “C” domain is dual zinc finger DNA binding domain through which ER binds its response element. The hinge region “D” is important for coordinating the N and C terminal activation functions, and for mediating receptor turnover as the site of ubiquitination (Berry et al., 2008). “E” refers to the ligand binding domain, which contains the activation function 2 (AF-2) in which co-activators and repressors bind. Domain E is also responsible for the dimerization of receptor. Domain F is the C-terminal most domain and contributes to ligand specific activation and dimerization (Kumar et al., 2011).

Estrogen Receptor was later discovered to be expressed as two isoforms, ER alpha and ER beta. (Mosselman et al., 1996). These proteins are relatively well conserved, being most similar in their DNA binding domain, and differing most in their far N and C termini. The ligand binding domains of ER alpha and beta differ enough to generate quantitative differences in ligand preference. Estradiol binds with similar affinity well to each receptor, but activates ER alpha at least 10 fold more potently than ER beta (Zhu et al., 2006; Mosselman et al., 1996). Consequently, ER alpha and beta share some convergent functions but are not redundant, as ER alpha knockout produces infertility in both males and females. ER alpha also exhibits higher expression in most tissues and is the dominant driver

of hormone dependent breast cancer growth (Böttner et al., 2014). The two isoforms exhibit differential tissue expression and some non-overlapping target genes. In the ER literature, “ER” alpha is the isoform to which the acronym ER refers.

ER binds a diverse array of hormone ligands. The discrimination of ligand type is achieved by Helix-12 in the ligand binding domain. Agonists and antagonists both stabilize the receptor such that HSP90 can be shed, and both ligand types induce nuclear entry. However, agonists such as estradiol induce a conformation of Helix-12 whose inward movement uncovers a hydrophobic pocket on the surface of the ligand binding domain (LBD), which facilitates nuclear coactivator binding. Conversely, antagonists such as 4-hydroxytamoxifen induce an outward confirmation of Helix-12 whose orientation prevents co-activator binding(Bai and Gust, 2009; Katzenellenbogen et al., 2018).

ER is directed to proper response elements with the aid of partner transcription factors, most notably FoxA1 and GATA3 (Hurtado et al., 2011). FoxA1 is a pioneer factor thought to localize ER to its appropriate target genes, but also grant accessibility to otherwise heterochromatic regions(Carroll et al., 2005). GATA3 similarly directs ER activity and is thought to act upstream of both ER and FOXA1. It’s localization to ER target genes such as TFF1 in the absence of Estrogen suggests it acts as a potential licensing factor for ER binding to specific regions of the genome(Theodorou et al., 2013). In the agonist confirmation, ER transactivation is further aided by coactivator proteins such as p300, BRD4 etc, which enhance the likelihood of RNA polymerase II recruitment and attendant

target gene transcription(Farcas et al., 2020). In contrast, antagonist bound ER, binds co-repressor proteins such as NCoR, and can participate in gene silencing with the polycomb repressor complex (Farcas et al., 2020).

ER dependent gene activation is limited by turnover of the receptor itself. ER is subject to ligand dependent turnover, and displays a half-life proportional to the amount of exogenous estrogens. Receptor turnover is proteasome dependent, and likely involves polyubiquitination of K302 and K303(Berry et al., 2008). The mechanism of ER turnover appears ligand dependent. Turnover induced by estradiol is thought to take place in the cytoplasm, whereas ER degradation in response to SERDs such as fulvestrant appears to occur in the nucleus(Calligé and Richard-Foy, 2006; Guan et al., 2019).

Estrogen Receptor Alpha in Physiology

Estrogens mediate many function in animals, including: reproductive development, endocrine regulation of fat metabolism, behavioral changes, maintenance of bone density among others (Eyster, 2016). Estrogen is employed as an important reproductive hormone in all vertebrates, and estrogen receptor alpha is well conserved across vertebrate evolution. ER is expressed most highly in the ovary, mammary and endometrial tissues, but plays important roles in other tissues, including: brain, liver, beta cells, white adipose cells and T cells(Mauvais-Jarvis, 2011). Following puberty, estradiol is produced by the ovaries in a reaction involving the enzyme aromatase. Estrogen is responsible for development of female secondary sex characteristics, maturation toward sexual maturity, and in

some mammals mating behavior during estrus. Estrogen has unique importance in breast development in early puberty. E2 causes the formation of a nascent branching structure of developing luminal cells. Of particular note, are the terminal end buds, which form as a nucleating site for complex secondary branches that develop until adulthood(Watson and Khaled, 2020). Mammary ductal branching in puberty is driven by those 40% of luminal cells which express Estrogen receptor(Rusidzé et al., 2021; Watson and Khaled, 2020). Additional branching and involution occur in cyclical fashion during the estrus cycle. A general and well characterized ER target gene is progesterone receptor, which during the high progesterone setting of pregnancy promotes the development of milk producing alveoli in the mature luminal duct (Watson and Khaled, 2020)

In postpubescent mammals, the levels of circulating estrogen are controlled by the estrous/mensural cycle. The frequency of the estrous cycle is variable, and can occur on the order of a month, as in humans, or over the course of a year as in wolves. The estrous cycle may be accompanied by external uterine shedding, as in mensuration, or may involve internal involution. In humans, gonadotropin-releasing hormone (GnRH) is released monthly by the hypothalamus. This promotes the pituitary gland to release follicle stimulating hormone (FSH) and luteinizing hormone (LH). These hormones promote the development of egg stem cells into follicles, and promote estrogen production by aromatase expressing granulosa cells in the ovary. Rising estrogen levels, block pituitary release of FSH and LH, while simultaneously enhancing pituitary sensitivity to GnRH, and acting on endometrial cells to initiating thickening of the uterine lining. A dominant follicle

is selected via paracrine negative feedback, and completes meiosis. An egg from this follicle is released into the fallopian tubes, where it awaits fertilization. The ruptured follicle becomes the corpus luteum, comprised of epithelial cells secreting and responding to estrogen. ER in these cells stimulates PGR expression as a direct target gene. Progesterone prepares the uterus for embryo implantation, and prevents initiation of another estrus cycle. Over the next 2 weeks in humans, the corpus luteum becomes inactive unless fertilization occurs, in which case the early placenta of the implanted egg releases a hormone called human chorionic gonadotropin hCG. hCG promotes continued progesterone expression which maintains the uterine lining and prepares the body for pregnancy (Hill, 2021).

Estrogen Receptor in Cancer

Estrogen-ER signaling is implicated in many cancer types including: Lung, Colon, Prostate, Ovarian, Endometrial and Breast(Loibl et al., 2021; Maingi et al., 2020; Stabile and Siegfried, 2004). High levels of estrogens are associated with an increased risk of endometrial cancer, and women taking selective estrogen receptor modulators such as tamoxifen are at an increased risk of endometrial cancer(Liang and Shang, 2013). Many epithelial ovarian cancers also display high levels of ER alpha expression, although the clinical significance of this is unclear(Fernandez et al., 2020). Breast cancer is by far the tumor in which ER is most implicated, and the most common cancer type worldwide(Loibl et al., 2021). Breast cancer is traditionally stratified into 3 clinical subtypes: 1. ER+ (70%), Her2 amplified (20%) and triple negative or basal (10%)(Koboldt et al., 2012),. Age

remains the greatest risk factor for breast cancer, with the age adjusted mean diagnosis at 64 years old(Wörmann, 2017). Additional risk factors include BRCA1/2 mutation, and chest irradiation in patients younger than 30(Warner, 2011). The mechanisms underlying ER dependent carcinogenesis is a field of active study. However, many of the mediators of ER induced carcinogenesis are known and are implicated in both cancer and normal physiology. These include familiar oncogenes such as cyclin D1 and c-Myc, which mediate entry into S phase(Butt et al., 2005; Caldon et al., 2010; Liang and Shang, 2013; Musgrove et al., 2011) . ER also upregulates anti-apoptotic proteins such as bcl-2 and bcl-xl(Liang and Shang, 2013). ER also contributes to angiogenesis by promoting IL-8 and VEGF secretion(Liang and Shang, 2013)

It is currently unclear what promotes the transition of an ER+ luminal cell to ER+ cancer, especially considering the lack of mutations in genes coding for ER or its co-factors in primary tumors(Chi et al., 2019). A number of authors have postulated a mechanism whereby cancer associated epigenetic changes reveal or conceal ER binding regions in the genome. Cancer unique ER binding was found enriched and reduced at enhancers upstream of oncogenes and tumor suppressors respectively(Xiao et al., 2018).

Estrogen Receptor as a therapeutic target in breast cancer

ER antagonism as a breast cancer therapy was discovered serendipitously. Scientists at Imperial Clinical Industries (ICI) lead by Arthur L. Walpole were studying pharmacological interventions to improve fertility. In particular, they were studying synthetic non-steroidal triphenyl compounds known to mimic estrogen activity(Jordan, 2006). In 1968, Walpole and his team had identified a compound (ICI46-474) which was a partial estrogen antagonist/agonist (Harper and Walpole, 1967) and a potential postcoital contraceptive in rats. In 1971, Lars Terenius demonstrated an anti-tumor effect of two related synthetic triphenyl estrogens, MER-25 and nafoxidine, in a DMBA induced model of rat mammary cancer(Terenius, 1971). In that same year enough momentum had developed for a clinical trial of ICI46-474. The first results demonstrated a just under 25% partial response rate, with remarkably low side effects or toxicity(Cole et al., 1971). Subsequent studies for indication optimization showed that tamoxifen was most effective in early stage tumors and if given for multiple (now 5 to 10) years following tumor excision, and only in women whose tumors were ER positive(Gajdos and Jordan, 2002). Today, tamoxifen is the drug of choice for treating ER+ positive breast cancer.

Detailed studies went on to reveal tamoxifen and the larger class of selective ER modulators (SERMs) as tissue specific agonists/antagonists. Tamoxifen induces a similar confirmation of ER in both breast and endometrium, however the relative expression of coregulators in each tissue results in a divergent pharmacological response. The high expression of co-activator NCOA1,

and enhanced modulation of AF-1 in endometrial tissue converts tamoxifen to an agonist in that setting, thus explaining why a potential side effect of long term tamoxifen usage is an increased risk of endometrial cancer(Patel and Bihani, 2018).

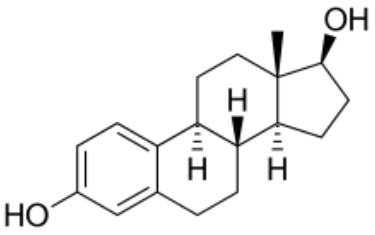
Structure function analysis of tamoxifen and its metabolites, informed the synthesis of new potent classes of SERMs: benzothiophene, indole and naphthalene(Wallace et al., 2004). Raloxifene is one such molecule and exhibits a more complete antagonist activity compared to tamoxifen, though with a far less favorable pharmacokinetic profile, exhibiting a bioavailability of only 2%(Patel and Bihani, 2018). By virtue of inhibiting ER dependent gene expression and attendant ER turnover, antagonists of the SERM class induce ER stabilization, the opposite effect of agonists such as estradiol.

The search for an anti-estrogen that would work in tamoxifen resistant tumors, informed the development of a second class of ER antagonist, termed Selective ER Degraders (SERDs). These compounds were also conceived by scientists at Imperial Clinical Industries (ICI). Compound libraries and subsequent structure function analysis demonstrated that steroidal compounds with long 7alpha aliphatic side chains of between 15 and 19 carbons were potent ER antagonists. These compounds impart a structure of helix 12 that prevents any association with the ligand binding domain. This unique binding mode, induces the proteosomal dependent degradation of ER, while acting as a complete anti-estrogen(Wakeling, 1989; Wijayaratne and McDonnell, 2001; Sharma et al., 2018). The first such compound was ICI 164,384, which upon refinement became the

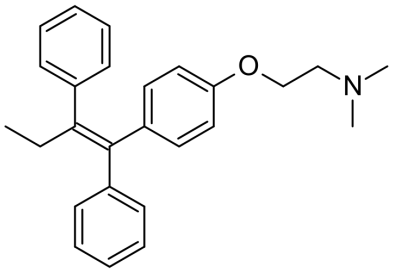
currently clinical compound fulvestrant (ICI 182,780)(Wakeling and Bowler, 1992). The first trials of fulvestrant were done in comparison to tamoxifen in post-menopausal women set to undergo surgery. Fulvestrant was significantly better than placebo or tamoxifen, at both causing tumor regressions and inhibiting ER dependent gene expression(Robertson, 2001). One drawback is that owing to its long hydrophobic tail, fulvestrant has poor pharmacokinetics and must be administered Intramuscularly (Wang and Sharma, 2020). Therefore, there has been much interest in developing orally bioavailable SERDs. The chemical diversity of new SERDs with better PK has provided insight into ER pharmacology. For example, a recent study comparing the mechanisms of structurally diverse SERDs with tamoxifen revealed that SERDs partly exert their efficacy by reducing ER nuclear mobility, and that antagonism precedes ER degradation(Guan et al., 2019). In addition, although E2 and fulvestrant both induce receptor turnover, recent data indicates that the mechanisms may differ for E2 and fulvestrant. For example, one study demonstrated that the nuclear localization of ER was necessary for fulvestrant but not E2 induced receptor turnover, fitting the aforementioned impaired nuclear mobility model(Casa et al., 2015). This implies also that E2 and fulvestrant may induce both overlapping and distinct E3 ligases, depending on the intracellular compartment.

The third and final class of ER targeting agents are the aromatase inhibitors. Aromatase (CYP19A1) is a member of the cytochrome p450 family of oxidoreductases, and converts testosterone into estrogen(Yoshio et al., 1987). Aromatase is found expressed in most tissues, but is comparatively most active in

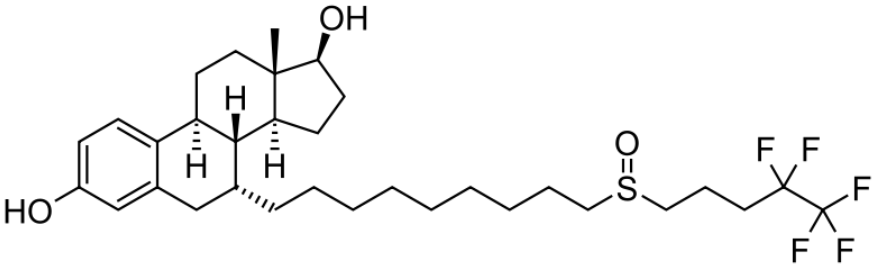
ovarian and placental cells(Blakemore and Naftolin, 2016) . Aromatase inhibitors (AI) were designed in the late 1970s as an attempt to treat metastatic patients, for whom surgical intervention was ineffective or only mildly effective(Santen et al., 1977, 1982). The first AI was the compound, aminoglutethimide, and although exhibiting in vitro and in vivo activity, was limited by toxicity(Blakemore and Naftolin, 2016). Current aromatase inhibitors are divided into two classes: Type I are steroidal in nature and act as irreversible inhibitors, while type II are non-steroidal and reversibly block the enzyme by interacting with the heme co-factor required for substrate oxidation(Buzdar et al., 2002). Currently employed examples include exemestane (type I) and letrozole (type II)(Blakemore and Naftolin, 2016; Buzdar et al., 2002). When compared to tamoxifen, aromatase inhibitors have demonstrated approx. 3 fold enhanced activity in breast cancer prevention and adjuvant treatment(Amir et al., 2011) . As of now, AI treatment is the first line therapy for advanced disease, although fulvestrant is comparable in certain head to head trials ((Ma et al., 2015)Roberson 2014)l and nearly 80% of patients initially respond(Ma et al., 2015). Figure 1.6 illustrates the structures of various antiestrogens currently employed in cancer therapy.



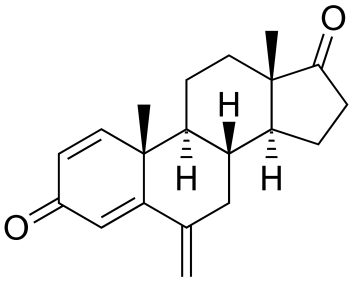
Estradiol



Tamoxifen (SERM)



Fulvestrant (SERD)



Exemestane (steroidal aromatase inhibitor)

Figure 1.6: Structure of Estradiol and Anti-Estrogens used as cancer therapies

In addition to anti-estrogens, other targeted therapies have enjoyed recent success in ER+ breast cancer. Cell cycle entry is a well appreciated aspect of Estrogen receptor's oncogenic function. Cyclin D1 is a both an ER target gene and an ER co-factor, and cyclin D1 protein overexpression may be present in up to 50% of cases (Casimiro et al., 2013; Murphy and Dickler, 2015; Musgrove et al., 2011, Arnold and Papanikolaou, 2005). A major development has been the advent of CDK4/6 inhibitors (Murphy and Dickler, 2015). Preclinical data showed especially high efficacy for selective CDK4/6 inhibitors in the setting of ER+ breast cancer, including those with acquired resistance to anti-estrogens(Murphy and Dickler, 2015). In vitro studies with the first CDK4/6 inhibitor, pablociclib, demonstrated an enriched sensitivity among hormone positive, RB proficient, luminal tumors(Finn et al., 2009). Additional studies demonstrated a combination synergy with CDK4/6 inhibitors and tamoxifen. This led to a clinical trial which culminated in the approval of palociclib in 2015(Spring et al., 2020). There are now 3 approved CDK4/6 inhibitors in clinic, and all approved with both fulvestrant and aromatase inhibitors(Spring et al., 2020).

Endocrine Resistance in Breast Cancer

Despite the profound success of anti-estrogens in treating breast cancer, resistance inevitably develops. Insensitivity can arise via adaptation, which is usually an early event involving the reorientation of signaling pathways, or overt genomic resistance, usually occurring after prolonged treatment. Signaling

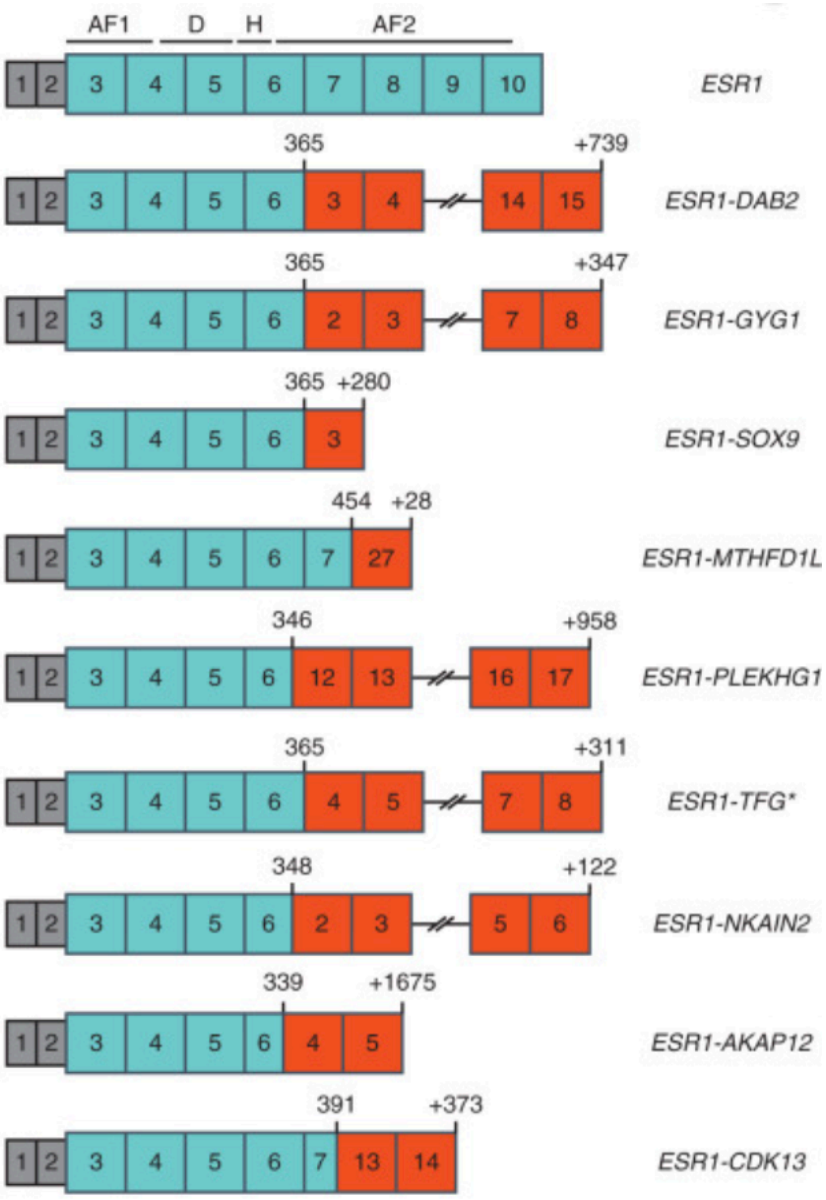
pathways mediating cell growth and division are normally limited by negative feedback loops which suppress upstream or parallel pathways. This ensures that the growth signal is properly received and self-terminating ((Chandarlapaty, 2012; Lito et al., 2012)). In cancer, these growth pathways remain active, and with them, extensive negative feedback. Upon inhibition of oncogenic drivers, the negative feedback is relieved and these upstream and parallel pathways are reactivated((Chandarlapaty et al., 2011; Rodrik-Outmezguine et al., 2011). This attenuates the activity of the targeted therapy in some cells, and can lead to the accumulation of persisters, which may eventually develop overt resistance mutations. This relief of negative feedback is a general phenomenon of targeted therapy and many such cases exist in ER positive breast cancer. For example, Xiao et. Al found an important negative feedback loop involving ER dependent expression of Src family member, CSK, which normally is induced by ER and restrains the activity of a pro-growth kinase, PAK2. Upon anti-estrogen treatment, CSK levels decline, and PAK2 can promote mitogenic growth in the absence of ER signaling(Xiao et al., 2018) Wu et. Al found an EZH2 dependent silencing of ER co-factor GREB1 in tamoxifen resistant tumors. This silencing shifted the balance of co-factors and converted tamoxifen into an agonist(Wu et al., 2017). Karthik et. al observed an activation of PI3K/mTOR and mTORC1 dependent ribogenesis following tamoxifen treatment in so called breast cancer stem-like cells(Karthik et al., 2015).

Genetic mechanisms of resistance to endocrine therapy also exist. For example, ESR1 mutations frequently occur following prolonged treatment with

anti-estrogens, and may be seen in up to 20% of ER+ endocrine resistant patients(Jeselsohn et al., 2015). The most common of these mutations, Y537 and D538, occur at the base of helix 12, and allow the ligand binding domain to adopt an agonist confirmation in the absence of ligand(Katzenellenbogen et al., 2018). In conjunction, these mutations reduce affinity of antagonists for the receptor, by virtue of stabilizing the agonist confirmation in the absence of ligand. These mutations have higher basal and inducible activity at many target gene sites(Jeselsohn et al., 2015; Katzenellenbogen et al., 2018; Reinert et al., 2018; Robinson et al., 2013; Toy et al., 2013). In addition to exhibiting reduced antagonist binding and conferring estrogen independent growth, these mutants may also display neomorphic functions, that localize mutant ER to new metastasis promoting loci(Jeselsohn et al., 2018). ESR1 amplification is an additional mechanism of acquired resistance, and is observed with varying frequency depending on the study in question, potentially up to 20% of AI inhibitor resistant patients(Adélaïde et al., 2008; Holst et al., 2007).

One recently implicated mechanism of acquired resistance is that of ESR1 fusions. These variants fuse the N-terminal 6 or 7 exons of ER to any number of C-terminal partners. The result is loss of the ER ligand binding domain and constitutive, hormone independent, receptor activation(Hartmaier et al., 2018; Lei et al., 2018; Li et al., 2013). Increasingly modern sequencing technology has also led to increased detection of such fusions, and up to 1% of anti-hormone refractory patients may express such ER variants (Kim and Han, 2021). This is reminiscent of glioma and prostate cancers in which EGFR or AR may be mutated in such a

way to lose binding binding capability, which also renders these receptors and ligand independent and constitutively active. Recurrent C-terminal partners include: CCDC170, YAP1, Sox9, AKAP12, etc(Hartmaier et al., 2018; Ma et al., 2015). Similar to ESR1 mutation, these alterations not only cause hormone independent gene expression and endocrine resistance, but these fusions also induce poorly understood neomorphic activity(Kim and Han, 2021). A representative group of ER fusions identified by Hartmaier et. al is depicted in figure 1.7



ESR1 fusions observed in endocrine resistant patients

Non ESR1 implicated pathways are also frequently observed as acquired resistance mechanisms. For example, amplification of various receptor tyrosine kinases can activate AKT and MAPK pathways, that enhance ER phosphorylation in the AF-1 domain, and thus induce some reduced ligand dependent co-factor interactions and target gene transactivation. By virtue of degrading ER itself, this is one potential area where SERDs such as fulvestrant may be more effective that AIs which only inhibit the available AF-2 activating estrogens(Robertson, 2002).

Epigenetic mechanisms of resistance to anti-estrogens have also been found. For example, mutations in the SWI/SNF complex are common in endocrine resistant ER+ breast cancer, and usually are found as mutations in ARID1A. Inactivation of ARID1A results in a relative loss of luminal signature in treated cells, and a transition to a less ER dependent basal like state(Xu et al., 2020).

Estrogen Receptor-PI3K cross talk

In breast cancer, the PI3K mTOR pathway is second in importance only to ER, and these pathways interact extensively with therapeutic implications. PI3Kα (*PIK3CA*) is mutated in up to 40% of all breast cancers, and significantly enriched in ER+ subtype (cbioportal). PI3K mutations activate the downstream components of the insulin network, and thus engender mutant cells with enhanced glucose uptake, enhanced migration, enhanced cap-dependent translation and other anabolic functions through mTOR(Fruman and Rommel, 2014). Additional alterations that activate the PI3K pathway such as ERBB2 and AKT are also frequently observed(Koboldt et al., 2012) Inhibiting PI3K or mTOR has been an

attractive strategy in ER+ breast cancer for some time. PI3K is expressed as four different isoforms, with PI3K alpha (*PIK3CA*) being the primary mutant isoform in ER+ breast cancer. Inhibitors which engage all PI3K isoforms have enhanced toxicity which limits their use in vivo(Baselga et al., 2017). These targeted therapies are often combined with anti-estrogens (Miller et al., 2010). One of the first combination trials used everolimus, an analog of mTORC1 inhibitor, rapamycin with Aromatase inhibitor, exemestane (Baselga et al., 2012). The hazard ratio for patients receiving exemestane plus everolimus vs exemestane alone was 0.43, a substantial result which provided proof of principle for successive PI3K ER dual targeting. Following the development of a better tolerated, PI3K alpha selective inhibitor, alpelisib, a clinical trial combing PI3K alpha inhibitors with fulvestrant, nearly doubled the time to progression in previously anti-estrogen treated patients, and motivated FDA approval of alpelisib for this indication(André et al., 2019).

Adaptation to PI3K inhibitors occurs by multiple mechanisms. Inhibition of AKT via PI3K/mTORC2 inhibition relieves the negative feedback AKT exerts on FOXO family members, this has the effect of resensitizing the cell to upstream signaling by inducing the transcription of growth factor receptors such as Her3 and Insulin Receptor(Chandarlapaty et al., 2011; Muranen et al., 2012; Rodrik-Outmezguine et al., 2011a). Similarly, residual PDK1 activity can reactivate mTORC1 and cell growth following PI3K inhibition by activating an SGK-TSC axis(Castel et al., 2016). In ER+ breast tumors, PI3K inhibition also activates Estrogen receptor activity itself. First, PI3K inhibition can activate transcription of

ESR1 by relieving negative feedback exerted on Foxo3a by AKT(Bosch et al., 2015). Secondly, PI3K inhibition also relieves AKT dependent negative feedback exerted on KMT2D, a lysine methyltransferase whose activity enhances ER gene expression, and whose activity reduces the efficacy of PI3K alpha inhibitors(Toska et al., 2017). These later most mechanisms are schematized in figure 1.8

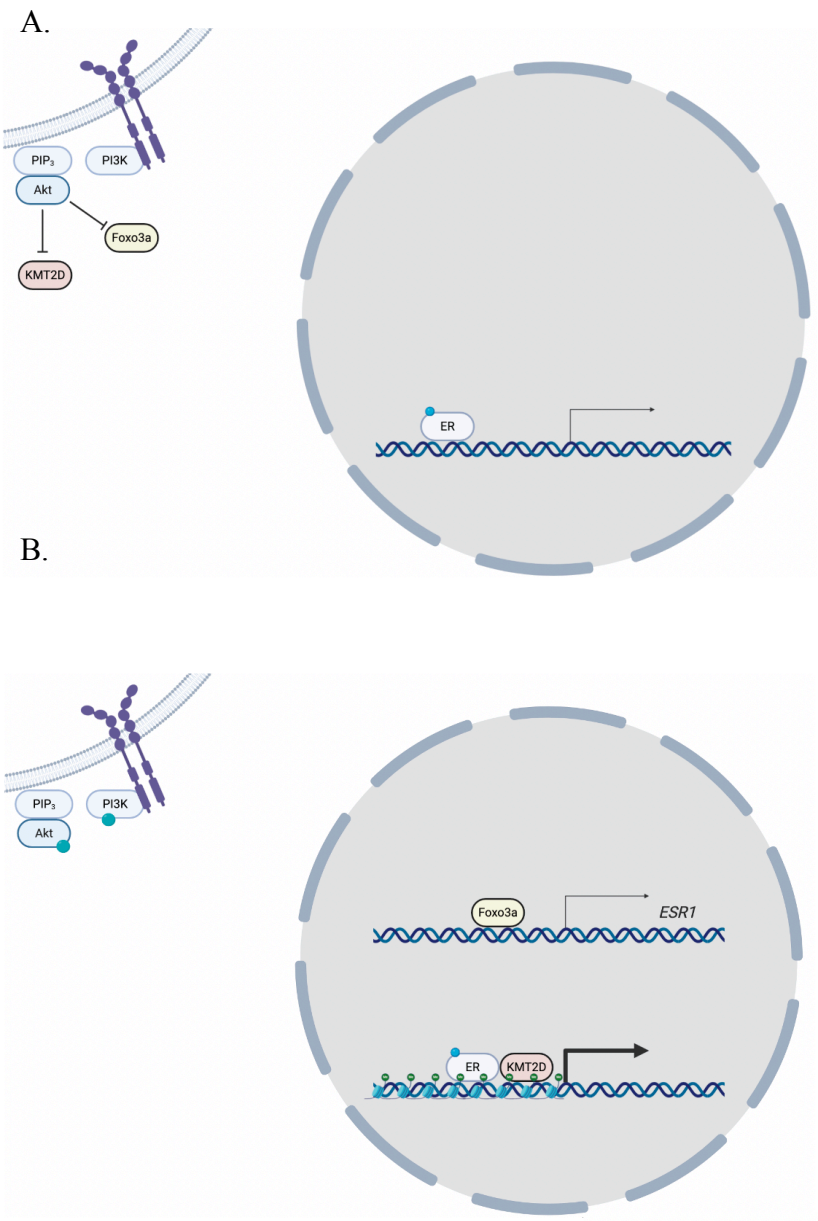


Figure 1.8 Mechanisms of enhanced ER activity following PI3K/AKT inhibition

(A) In cells with active PI3K/AKT/mTOR, FOXO family members and lysine methyltransferase, KMT2D are suppressed.

(B) When PI3K/mTOR is inhibited, this suppression is relieved and ER becomes activated.

Thesis Outline

In the following study, we have investigated the mechanism and clinical implications surrounding Estrogen Receptor translation. The impetus for this study arose from the previous finding that inhibition of PI3K/mTOR and cap-dependent protein translation did not reduce expression of short half-life protein, ER, but indeed enhanced its activity.

We hypothesized that ER is translated in a cap-independent/mTOR independent manner, which ensures its expression when mTOR is low. In chapter 3 we test this hypothesis in a variety of ways. We first confirm previous findings that ER expression is robust to mTOR inhibition, despite being a short half-life protein. We demonstrate that ER is translated in a cap-independent manner, and that this likely involves an authentic IRES element present in the 5' UTR of ER coding gene, *ESR1.*

In chapter 4, we further discuss the mechanism of ER translation. Specific features of the ESR1 5' UTR, motived our investigation of the RNA helicase and eukaryotic initiation factor, EIF4A. Using small molecule inhibitors and genetics, we show that ER translation is dependent on EIF4A. We demonstrate that reducing ER levels via EIF4A inhibition, blocks ER dependent gene expression and enhancer binding. EIF4A inhibition blocked the growth of ER dependent cell lines at the low nanomolar range as a single agent.

In chapter 5 we turn our attention to the clinical implications of targeting ER via EIF4A inhibition. To minimize ER expression, we combined EIF4A inhibitors

with Selective ER degrader, Fulvestrant. The combination of these inhibitors more effectively inhibited tumor growth than either compound alone, and this effect was seen both in vitro and in vivo. We finally investigated the effect of EIF4A inhibition on ER variants associated with endocrine resistance. Both the ER D538G and ER-Sox9 fusion remained dependent on EIF4A for their expression, and EIF4A inhibition blocked the growth of models harboring these ER variants.

In the final chapter we discuss important details, clinical implications, limitations and future directions as they relate to the current study.

Chapter 2: Materials and Methods

Mammalian Cell Culture

All cell lines were obtained from the American Type Culture Collection (ATCC). Cell lines were maintained in a 1:1 mixture of DMEM:F12 medium supplemented with 4 mM glutamine, 100 units ml^{-1} each of penicillin and streptomycin, and 10% serum (FBS) and incubated at 37 °C in 5% CO_2.

Immunoblotting

Cells were collected in ice cold PBS and lysed with RIPA lysis buffer (Pierce #89901) supplemented with Halt protease and phosphatase inhibitors (Pierce Chemical). Lysates were briefly sonicated before centrifugation at 20,000 × g for 5 minutes at 4°C. The supernatant was collected, and protein concentration was determined using the BCA kit (Pierce) per manufacturer's instructions. Equal amounts of protein (20µg) in cell lysates were separated by SDS–PAGE, transferred to nitrocellulose membranes (GE healthcare), immunoblotted with specific primary and secondary antibodies and detected by chemiluminescence with the ECL detection reagents from Thermo Fisher or Millipore.

Cell Titer Glo Determination of cell viability

Cell viability was determined using a CellTiter-Glo Luminescent Cell Viability Assay kit (Promega). Between 2000-5000 cells were plated in 96-well plates, and after 24hr treated with indicated compounds for various times. 100 µl of prepared

reagent was added to each well. The contents of the wells were mixed on a plate shaker for 15min, and then luminescence was measured by an Analyst AD (Molecular Devices). For GI50 curves, cells were treated for 72hr and day 0 values were subtracted from each group. The relative growth was normalized to the day 3 untreated samples. Sigmoidal growth inhibition curves were calculated using a four-parameter model in Graph Pad Prism 8.

mRNA extraction and RT-qPCR

mRNA was isolated using Trizol based phenol chloroform extraction. cDNA was synthesized using Quantitect Reverse Transcription kit (Qiagen). Transcript quantification was done using Applied Biosystems Taqman probes and ABI 7500 real-time quantitative PCR system. For data analysis, cycle numbers were normalized to housekeeping gene, Rplp0 and then to untreated control ($2^{-\Delta\Delta Ct}$).

ER Reporter Assay

T47D KBluc were plated in normal DMEM F12. Cells were then washed twice and media was changed to DMEM F12 lacking phenol red and containing charcoal stripped FBS (-E2) , with or without indicated drug (s) for an additional 24hr. Cells were then stimulated with estradiol for a final 24hr. Firefly luciferase activity was measured via dual luciferase assay reporter system (Promega) according to the manufacturer's instructions.

Dual Luciferase Assay for Cap-independent Translation

5' UTR elements or part of the GAPDH gene body(REF) were cloned into the dual luciferase assay construct. 1.5 million cells/6cm plate were transfected with 2μg of the construct using lipofectamine 2000 at a ratio of 3:1 lipofectamine to μg DNA. Firefly and Renilla activity luciferase activity was measured 24hr post transfection via dual luciferase assay reporter system (Promega) according to the manufacturer's instructions.

Chromatin Immunoprecipitation Assay (CHIP)

MCF7 were plated in normal DMEM F12. Cells were then washed twice and media was changed to DMEM F12 lacking phenol red and containing charcoal stripped FBS (-E2) , with or without indicated drug (s) for an additional 24hr. Cells were then stimulated with 10nM Estradiol for 1hr. ChIP was performed using the Simple ChIP Enzymatic Chromatin IP kit (Agarose Beads) from Cell signaling (#9002) according to the manufacurer's instructions. ER enhancer binding was measured via PCR amplification of the ER enhancer upstream of TFF1/pS2. Primers were from Cell Signaling (#9702)

AHA labeling and click chemistry

MCF7 were starved of methionine for 30min with simultaneous treatment of vehicle, or increasing doses of mTOR kinase inhibitor, then pulsed with 100uM AHA for 2hr. 200ug of protein was used for click chemistry and was performed using biotin-alkyne and protein reaction buffer kits from thermos fisher. AHA-

biotin-alkyne labeled proteins were pulled down with streptavidin beads and the indicated proteins analyzed by immunoblotting.

Bioinformatic analysis of 5' UTR features

Thermodynamic features and predicted 3D structures were obtained by entering NCBI (add all the gene names) into the RNAfold WebServer from University of Vienna Institute for theoretical chemistry. Predicted number of G-quadraplexes were determined via QGRS Mapper from Rampo College of New Jersey Bioinformatics.

Methionine Starvation and restimulation

Cells were plated in complete media, Cells were washed twice and changed to media lacking methionine for 24hr. Cells were then placed back into complete media with or without 20nM Silvestrol, 10nM RapaLink-1, or 50ug/ml Cycloheximide for indicated times.

Densitometry

Signal intensity of immunoblots was quantified using ImageJ (Fiji)

Edu Labeling and Cell Cycle Analysis

MCF-7 that had been treated for 48 with fulvestrant (concentration), silvestrol (concentration) or fulvestrant and silvestrol (concentrations) were incubated with EdU (10uM) for 1.5h at 37C. Cells were then processed with a Click-iT Plus EdU

Alexa Fluor 594 flow cytometry kit (Thermo Fisher, C10646) following the manufacturer recommendation. Cells were analyzed by flow cytometry on a LSRFortessa instrument (BD Biosciences) and data were analyzed using FlowJo (TreeStar).

Annexin V staining

Cells were then collected and stained with Annexin V-APC (Biolegend, 640941) and 4′,6-diamidino-2-phenylindole (DAPI; ...). Cells were analyzed by flow cytometry on a CytoFLEX LX (Beckman Coulter), and data were analyzed using FlowJo (TreeStar).

SiRNA knockdown of eukaryotic initiation factors

Cells were transfected for 72hr with Dharmacon SMARTpool nontargeting or siRNA designed against indicated eukaryotic initiation factors. Transfection was aided by preincubation of siRNA with lipofectamine RNAiMAX (Thermo Fisher Scientific) and used according to the manufacturer's instructions.

Puromycin incorporation assay

Cells were pulsed for the last 30min of indicated treatment times with 1uM puromycin, followed by cell lysis and detection of puromycin incorporation by immunoblotting with anti-puromycin antibody (Kerafast).

In vitro cap-binding affinity assay

Experiments were conducted using the protocol from (ref). 200ug of lysate was incubated with m7G conjugated agarose beads (Jena Biosciences) for 2hr at 4 degrees with rotation. Beads were washed 3 times with ice cold lysis buffer. Bound proteins were eluted with 1x loading buffer with heating at 95 degrees for 5 min. EIF4F complex composition was analyzed by immunoblotting using indicated antibodies.

Xenograft Experiments

Eight-week-old athymic nu/nu female mice (MCF7) (Harlan Laboratories), or NOD scid gamma mice (T47D) were injected subcutaneously with 10 million cells together with matrigel (BD Biosciences). 17β-Estradiol pellets (0.18 mg or 0.72mg/90 days release) (Innovative Research of America) were implanted subcutaneously 3 days before tumor cell inoculation. Once tumors reached an average volume of 100 mm^3, mice were randomized (n = 3-5 mice per group) to receive CR31B(+/-) in 10% Captisol 1mg/kg i.v. twice/week, Fulvestrant in 5% EtOH and 95% castor oil twice/week. Tumors were measured twice weekly using calipers, and tumor volume was calculated using the formula:
length × $width^2$ × 0.52. All tumors were collected 24hr following the final dose. Samples were lysed and processed as previously described(Will et al., 2014). For PD studies, tumors of average volume of 200-300mm^3 were collected 24hr following a single dose.

Table 2.1: Materials and Reagents

Reagent	Source	Identifier
Cell Culture		
DMEM Ham's F12 50/50 Mix	Corning	10-090-CV
L-Glutamine	MSKCC Media Core	N/A
DMEM F12 (Without Methionine)	MSKCC Media Core	N/A
Charcoal stripped Fetal Bovine Serum	Thermo Fisher	12676029
DMEM F12 (Phenol Red Free)	MSKCC Media Core	N/A
Opti-MEM	Thermo Fisher	31985070
DMEM F12 (Without Amino Acids)	MSKCC Media Core	N/A
Antibodies		
Anti-ER (N-Terminal)	Cell Signaling Technologies (CST)	13258S
Anti-ER (C-Terminal)	CST	8664S
Anti-Cyclin D1	Thermo Fisher	MA5-16356
Anti-p4EBP1 (S65)	CST	9451S
P4EBP1 (T37/46)	CST	2855S
Anti-Actin	CST	3700S
Anti-pAKT (S473)	CST	4060S
Anti-pS6 (S235/S236)	CST	4858S
Anti-EIF4G1	CST	2469S
Anti-EIF4E	CST	2067S
Anti-t4EBP1	CST	9644S
Anti-Progesterone Receptor	CST	8757S
Anti-GREB1	CST	65171S
Anti-cleaved PARP	CST	5625S
Anti-Myc	CST	9402
Anti-Androgen Receptor	CST	5153
Anti-LC3B	CST	3836
Anti-ATG5	CST	9980
Anti-eIF1	CST	12496
Anti-eIF1AX	Thermo Fisher	PA5-42809
Anti-eIF2 alpha	CST	5324
Anti-eIF3a	CST	3411
Anti-eIF3d	Abcam	Ab155419
Anti-eIF4A1	CST	2490
Anti-eIF4A2	Abcam	Ab31218
Anti-eIF4B	CST	3592
Anti-eIF4E3	Thermo Fisher	PA5-50954
Anti-eIF4G2	CST	5169
Anti-eIF5	CST	13894
Anti-eIF5B	Abcam	ab89016
Drugs/Chemicals		
Cycloheximide	Millipore Sigma	01810
RapaLink-1	Revolution Medicine	N/A
Puromycin	Thermo Fisher	A1113802
L-Azidohomoalanine hydrochloride (AHA)	Milipore Sigma	900892
Actinomycin D	Millipore Sigma	A9415
Fulvestrant	Selleckchem	1191
Estrogen pellets	Innovative Research of America	NE-121
Beta-Estradiol	Millipore Sigma	E2758
Lipofectamine 2000	Thermo Fisher	11668500
Lipofectamine RNAi Max	Thermo Fisher	13778075
Hygromycin B	Thermo Fisher	10687010

Silvestrol	Wendel Lab (MSKCC)	N/A
CR-31-B (+/-)	Wendel Lab (MSKCC)	N/A
Hippuristanol	Pelletier Lab (McGill)	N/A
Pateamine A	Pelletier Lab (McGill)	N/A
Oligonucleotides	IDT	
sgGFP-F	CACCGGGGCGAGGAGCTGTTCACCG	N/A
sgGFP-R	AAACCGGTGAACAGCTCCTCGCCCC	N/A
Sg4EBP1-F	CACCGGGAAATTCCTGATGGAGTGT	N/A
Sg4EBP1-R	AAACACACTCCATCAGGAATTTCCC	
ESR1 Intron 6 guide RNA	GCTCCTGAACGAATACACTG	N/A
Sox9 Intron 2 guide RNA	CGGGACGGAGATAGCTTGTC	N/A
Cell Lines		
MCF7	ATCC	HTB-22
T47D	ATCC	HTB-133
ZR75-1	ATCC	CRL-1500
BT474	ATCC	HTB-20
MCF7 ER D538G	Chandarlapaty Lab (MSKCC)	N/A
T47D ER-Sox9	This Study	N/A
T47D KBluc	ATCC	CRL-2865
MCF7-4EBP1-4A	This Study	N/A/
SiRNA		
ON-TARGETplus siRNA hEIF1	Dharmacon (Horizon)	L-015804-02-0005
ON-TARGETplus siRNA hEIF2a	Dharmacon (Horizon)	L-014766-01-0005
ON-TARGETplus siRNA hEIF3a	Dharmacon (Horizon)	L-019534-00-0005
ON-TARGETplus siRNA hEIF3d	Dharmacon (Horizon)	L-017556-00-0005
ON-TARGETplus siRNA hEIF4A1	Dharmacon (Horizon)	L-020178-00-0005
ON-TARGETplus siRNA hEIF4A2	Dharmacon (Horizon)	L-013758-01-0005
ON-TARGETplus siRNA hEIF4E	Dharmacon (Horizon)	L-003884-00-0005
ON-TARGETplus siRNA hEIF4E3	Dharmacon (Horizon)	L-032845-01-0005
ON-TARGETplus siRNA hEIF4G1	Dharmacon (Horizon)	L-019474-00-0005
ON-TARGETplus siRNA hEIF4G2	Dharmacon (Horizon)	L-011263-00-0005
ON-TARGETplus siRNA hEIF4G3	Dharmacon (Horizon)	L-019530-00-0005
ON-TARGETplus siRNA hEIF5	Dharmacon (Horizon)	L-021336-00-0005
ON-TARGETplus siRNA hEIF5B	Dharmacon (Horizon)	L-013331-01-0005
Plasmids		
pcDNA RLUC POLIRES FLUC	Addgene	Plasmid #45642
pCW57.1-4EBP1_4xAla	Addgene	Plasmid #38240
V2 Lentiviral CRISPR	Addgene	Plasmid #52961
qPCR		
TaqMan Universal PCR Master Mix	Thermo Fisher	4305719

PGR taqman primer	Thermo Fisher	Hs01556702_m1
GREB1 taqman primer	Thermo Fisher	Hs00536409_m1
TFF1 taqman primer	Thermo Fisher	Hs00907239_m1
IGFBP4 taqman primer	Thermo Fisher	Hs01057900_m1
Serpina1 taqman primer	Thermo Fisher	Hs00165475_m1
TP53INP1 taqman primer	Thermo Fisher	Hs01003820_m1
Misc		
CellTiter-Glo	Promega	G9241
SimpleChIP Plus Enzymatic ChromatinIP Kit (Magnetic Beads)	CST	9005
Annexin v staining kit	Biolegend	640919
Edu staining kit	Thermo Fisher	C10646
Dual Luciferase Reporter Assay System	Promega	E1910
SimpleChIP Tff1 promoter primers	CST	9702S
Click-iT Protein Reaction Buffer Kit	Thermo Fisher	C10276
Streptavidin-Agarose	Sigma	S1638
Immobilized aminophenyl-m7 GTP	Jena Biosciences	AC-155S
Software		
Imagej		N/A
FlowJo 10	FlowJo LLC	
Graphpad Prism 8	Graph Pad	

Chapter 3: Cap-Independent Translation of Estrogen Receptor

Introduction

Hormone dependent cancers such as breast and prostate often have concurrent alterations in the PI3K/mTOR pathway, and extensive crosstalk occurs between these pathways. High PI3K output has been shown to exert negative feedback on both androgen and estrogen receptors, albeit by different mechanisms(Bosch et al., 2015; Carver et al., 2011; Toska et al., 2017). In the case of estrogen receptor alpha, elegant studies revealed different means by which ER becomes activated following PI3K inhibition. First, it is well appreciated that AKT exerts negative feedback on the insulin pathway by inhibiting the activity of members of the FOXO family(Chandarlapaty et al., 2011). Bosch et. al found that in addition to controlling insulin sensitivity, FOXO3a also positively regulates ER (*ESR1*) transcription. Consequently, when AKT is inhibited, *ESR1* and total protein expression increases(Bosch et al., 2015). Even more striking is the effect that PI3K inhibition exerts on ER activity. Toska et. al demonstrated that AKT suppresses the activity of the lysine methyltransferase, KMT2D, which normally augments ER dependent gene expression. PI3K inhibition increased ER enhancer binding and ER dependent gene expression. This came with the attendant resistance to anti-hormonal therapies in vitro and in vivo(Toska et al., 2017).

Conventional wisdom asserts that the majority of eukaryotic translation is initiated by the cap-binding protein and initiation factor EIF4E. This type of translation is referred to cap-dependent, and is under the control of mTOR complex

I (mTORC1). It should be noted that EIF4E is not the only cap-binding protein capable of initiating translation. The proteins, cbp20 and cbp80 form a nuclear resident cap-binding protein complex that couples nascent mRNA to translation, non-sense mediated decay and other functions(Ishigaki et al., 2001). However, the degree of contribution to global translation played by this complex is unknown, and typically EIF4E initiated translation is referred to cap-dependent. In this study, we interchangeably use the terms, “cap-dependent”, “mTOR dependent”, and “EIF4E dependent”.

ATP competitive mTOR inhibitors have shed light on mTOR control of protein synthesis(Hsieh et al., 2012; Thoreen et al., 2012) It has been appreciated that while mTOR inhibition greatly reduces global translation, residual translation remains, and by definition must occur using very low levels of mTOR activity, or use a qualitatively different mechanism of translation initiation. Such mechanisms are of special importance for proteins exhibiting short half-lives which depend on continual synthesis for expression.

In light of the previous work demonstrating activation of ER during PI3K inhibition we hypothesized that ER may be one such protein whose translation might proceed cap-independently, especially when mTOR is inhibited. Our resolve at testing this hypothesis was enhanced when we observed that ER has a short protein half-life.

In this chapter, we demonstrate that ER translation can proceed in an EIF4E (cap-independent) manner and that this cap-independent translation is initiated by elements in the ESR1 5’ UTR .

Results

Estrogen Receptor Expression is robust to mTOR/EIF4E Inhibition

mTOR is a major regulator of protein translation, primarily as the regulator of cap-binding protein EIF4E. We treated ER+ breast carcinoma cell line MCF7 with dual mTORC1/2 inhibitor, RapaLink-1(Rodrik-Outmezguine et al., 2016) or global translation inhibitor, cycloheximide as a function of time. In the presence of cycloheximide, ER protein expression declined as a function of time with a half-life of between 4 and 8hr (Figure 3.1A).

It was previously reported that inhibition of AKT/mTOR kinase relieves feedback inhibition of Estrogen Receptor Alpha (ER), both at the level of protein expression and receptor activity((Bosch 2015,Toska 2017)). Indeed, when we treated cells with mTOR inhibitor, RapaLink-1, ER target protein, Progesterone Receptor expression was enhanced 4.5 fold by 48hr (Figure 3.1B.I). Global protein translation assayed via puromycin incorporation was reduced by nearly 90% at 24hr (Figure 3.1B.I). Cyclin D1 expression tracked very closely with global translation during mTOR inhibition, and is consistent with cyclin D1 being translated in a canonical cap dependent manner((Averous et al., 2008; Benedetti and Graff, 2004; Konicek et al., 2008; Tan et al., 2000)). In contrast, ER levels increased slightly and reached a constant maximum by 16hr. mTOR controls cap dependent translation primarily through inhibitory phosphorylation of substrate, 4EBP((Lee et al., 2021)). When dephosphorylated under conditions of mTOR inhibition, 4EBP1 binds EIF4E at the expense of EIF4G and cap dependent translation is antagonized. mTOR inhibition with RapaLink-1 durably abolished

phosphorylation of both mTORC1 and mTORC2 targets: S6 (S235/236), 4EBP1 (T37/46) and AKT (S473) by 4hr (Figure 3.1B.II). Dephosphorylation of 4EBP1 was coincident with enhanced binding of 4EBP1 to the EIF4E-m7G cap complex, with concurrent displacement of EIF4G. These results are quantified via densitometry in Figure 3.1B.III.

These results indicate that while both global and cap-dependent translation are reduced by mTOR inhibition, ER and target expression is either unaffected or enhanced. Since mTOR controls cap dependent translation primarily through substrate 4EBP1, we expressed a doxycycline inducible 4EBP1 mutant termed 4EBP1 “4A” whose mTOR sites have all been mutated to alanine (T37A/T46A/S65A/T70A)(Thoreen et al., 2012). This renders 4EBP1 constitutively active and uncoupled from the inhibitory action of mTORC1. Expression of this mutant ablated cap-dependent translation as a function of doxycycline dose. Using m7-guanosine cap pulldown assays, we observed that 100ng/ml doxycycline resulted in saturation binding of 4EBP1 to EIF4E and at the expense of EIF4G. Cyclin D1 expression anti-correlated with increasing 4EBP1-4A expression, whereas estrogen receptor expression was unaffected even at 1000ng/ml doxycycline (Figure 3.1C). In reciprocal fashion, we ablated 4EBP1 expression in MCF7 using CRISPR Cas9 (Figure 3.1D). Upon mTOR inhibition with RapaLink-1, only the control sgGFP, but not sg4EBP1 expressing cells showed a significant decrease in cyclin D1 expression—indicating EIF4E dependent translation. Estrogen receptor expression however was similarly unaffected by these treatments in either the sgGFP or sg4EBP1 expressing MCF7. Estrogen receptor

and Cyclin D1 were both inhibited by global translation inhibitor cycloheximide, irrespective of 4EBP1status. These results strongly imply the EIF4E independent expression of ER.

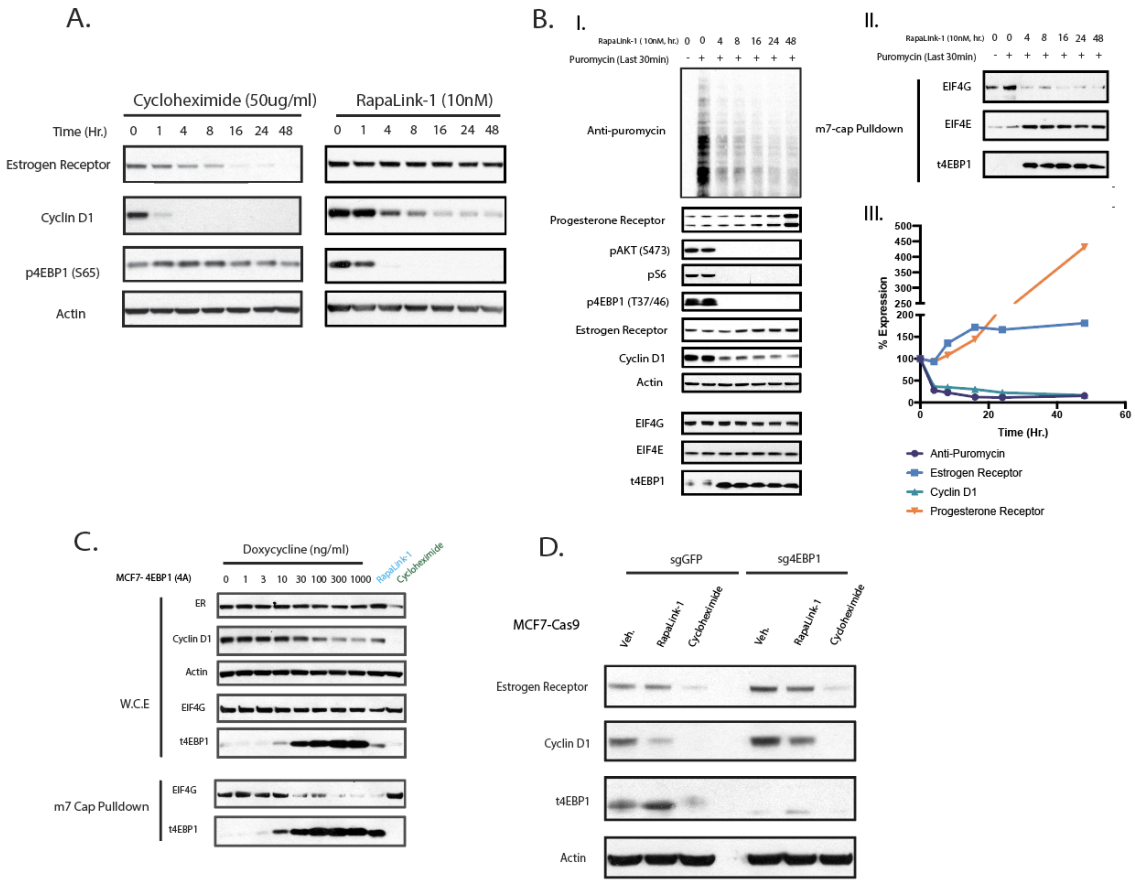


Figure 3.1: ER expression is robust to mTOR (EIF4E) inhibition.

(A) MCF7 were treated for time t with either 10nM RapaLink-1 (mTOR inhibitor) or cycloheximide (50µg/ml).

(B) MCF7 were treated time t with 10nM RapaLink-1. (I) To measure global translation, 1µM puromycin was pulsed for the last 30min, and analyzed by immunoblotting with an anti-puromycin antibody. (II) For cap pulldowns, cell lysates were incubated for 2hr with m7-Guanosine conjugated agarose beads, before washing, elution and running on a 4-12% poly acrylamide gel. (III) Results from 3.1B.I and 3.1BII quantified via densitometry.

(C) MCF7 were treated 24hr with increasing doses of doxycycline to induce 4EBP1 (T37A/T46A/S65A/T70A) "4EBP1 4A" expression. As a comparison, cells were also treated with 10nM RapaLink1 or 50ug/ml Cycloheximide for an equivalent time.

(D) MCF7 expressing Cas9 and guide RNAs targeted against GFP (control) or 4EBP1 were treated 24hr with RapaLink-1 or Cycloheximide.

ER is translated in a cap (EIF4E) Independent manner

As a way to more directly measure protein synthesis, as oppose to total expression, we set up a way to assay the synthesis of many proteins under various conditions. We starved ER+ Breast carcinoma cell line, T47D of methionine for 24hr to halt translation initiation. We then added back methionine as a function of time in the presence or absence of mTOR inhibitor, Rapalink-1 (Figure 3.2A). Estrogen receptor was resynthesized in both vehicle and mTORi containing conditions, and with similar kinetics. Expression began to rebound at 8hr. and continued to increase over the next 16hrs. (Figure 3.2B). While the kinetics of ER resynthesis was similar in both conditions, the absolute level of ER at 24hr. was lower in the mTOR inhibitor treated cells, potentially indicating that while ER can be translated cap-independently it may be less efficient. We examined Myc as a positive control, as its cap-independence is well established(Stoneley et al., 2000). The dynamics of Myc resynthesis was similar to that of ER, rebounding by 16 and 24hr in both conditions, although again, the absolute level reached over this interval was lower in the mTOR vs Veh. treated cells. In contrast to Myc and ER, cyclin D1 was only resynthesized in the veh treated cells, and consistent with its cap-dependent translation, could not be resynthesized under conditions of mTOR inhibition. Similar results for ER and Cyclin D1 resynthesis were obtained in MCF7 (Figure 3.2C). As a final method of validating cap-independent ER translation, we took advantage of the methionine derivative, L-azidohomoalanine (AHA)(Landgraf et al., 2014). In the absence of methionine this synthetic amino acid is used as an

initiator residue for translation. The azide moiety in place of the thiol ether of methionine allows click chemistry and subsequent biotin-Strepavidin affinity purification of de-novo synthesized proteins. We starved MCF7 cells of methionine for 30min in the context of increasing doses of fast acting, ATP competitive mTOR inhibitor, INK1028, followed by addition of AHA for 2hr. Translation of all 3 proteins: ER, Myc and Cyclin D1 was mildly inhibited with as low as 1nM INK0128. However Cyclin D1 was more deeply inhibited as the concentration of INK0128 was increased, reaching a saturable minimum at 30nM (Figure 3.2D). For ER and Myc however, increasing doses did not produce substantial effects beyond 1nM. This potentially suggests, as before, a preference for cap-dependent translation unless mTOR is inhibited. We also utilized the AHA assay to profile translation under control of the 4EBP1-4A mutant. Expression of Cyclin D1 was greatly reduced with increasing levels of doxycycline (4EBP1-4A expression). In contrast, translation of Myc was mildly inhibited with 100ng/ml dox, while ER was unaffected at even the highest 4EBP1-4A expression level (Figure 3.2E).

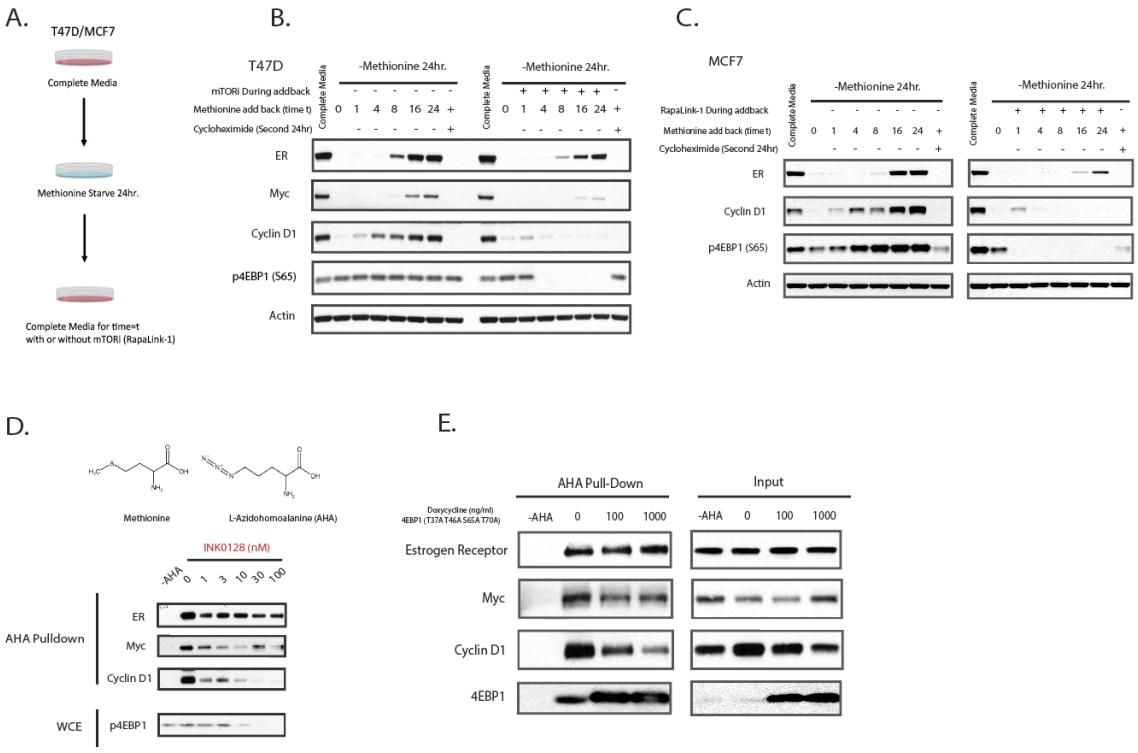


Figure 3.2: ER is translated in a cap (EIF4E) Independent manner

(A) Schematic for methionine starve and resynthesis assay.

(B) T47D were starved of methionine for 24hr to block global translation. Cells were then restimulaed with complete media as a function of time, with or without translation inhibitors, for up to an additional 24hr.

(C) MCF7 were starved of methionine for 24hr to block global translation. Cells were then restimulaed with complete media as a function of time, with or without translation inhibitors, for up to an additional 24hr.

(D) MCF7 cells were starved of methionine for 30min. At the same time, cells were treated with increasing doses of mTOR kinase inhibitor, INK0128. Cells were then pulsed with L-Azidohomoalanine (AHA) for 2hr. 200ug of lysate were subjected to a click chemistry reaction using biotin conjugated alkyne. AHA labeled proteins were then isolated via streptavidin-agarose assisted precipitation.

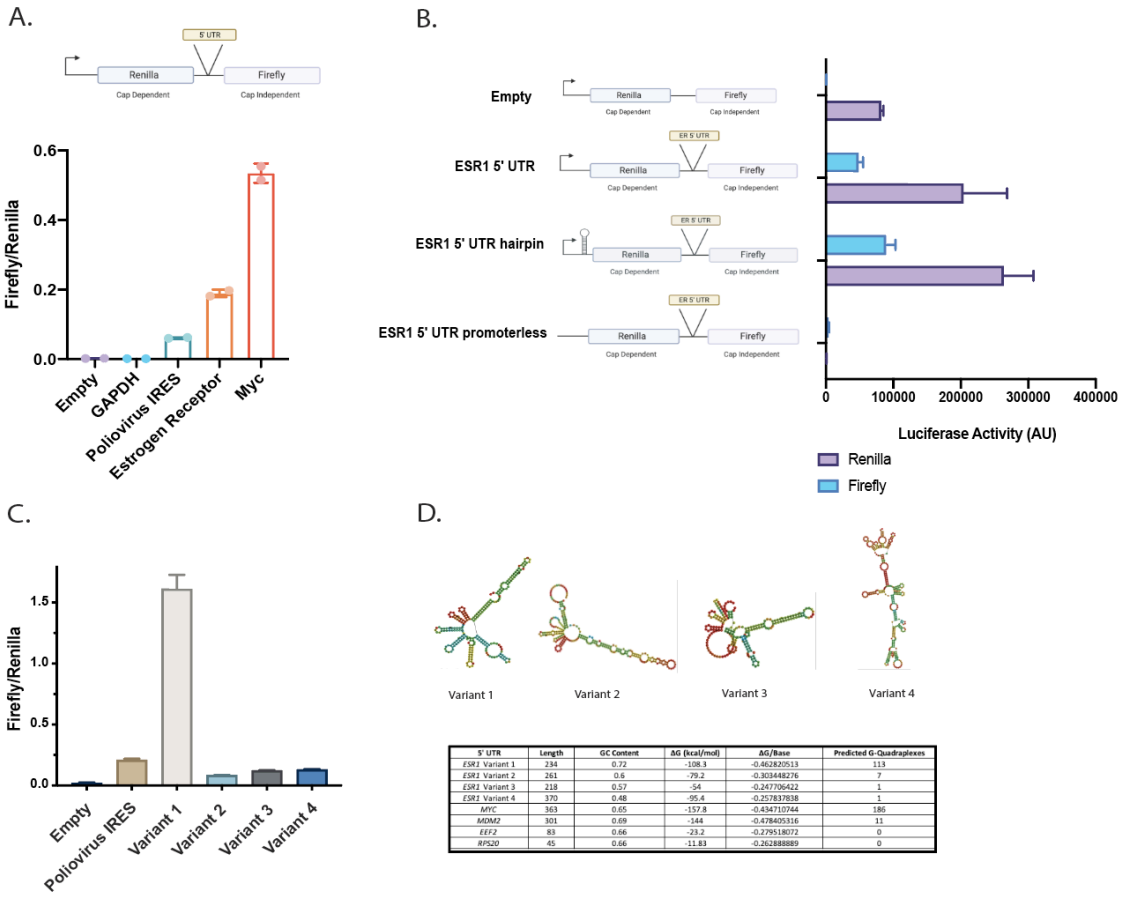
(E) MCF7 expressing doxycycline inducible 4EBP1 (T37A,T46A,S65A,T70A) were plated in 1μg/ml doxycycline for 24hr followed by methionine starvation for 30min. Cells were then pulsed with L-Azidohomoalanine (AHA) for 2hr. 200μg of lysate were subjected to a click chemistry reaction using biotin conjugated alkyne. AHA labeled proteins were then isolated via streptavidin-agarose assisted precipitation.

ESR1 5' UTR mediates cap-independent translation

Organisms from viruses to mammals have evolved a variety of methods to ensure cap independent translation of select mRNAs(Jackson et al., 2011; Leppek et al., 2018; Shatsky et al., 2010, 2018; Stoneley et al., 2000; Terenin et al., 2017) Often the elements facilitating cap-independent translation are contained in the mRNA 5' untranslated region (5' UTR). To test whether the 5' UTR of ER (*ESR1*) exhibits cap independent activity, we took advantage of a previously developed bicistronic luciferase vector. This vector contains a cap-dependently translated Renilla luciferase followed by a firefly luciferase whose cap independent expression is under control of a cloned insert (Figure 3.3A). Cap independent activity is measured as the ratio of Firefly/Renilla luciferase. The empty vector, as well as a coding region of GAPDH used as a negative control elsewhere(Zhang et al., 2020) exhibited no cap-dependent activity. The positive control Poliovirus IRES was capable of driving cap independent translation approx. 20fold higher than that of the empty insert. We observed that the 5' UTR of ESR1 was capable of driving cap-independent translation approx. 120fold higher than the empty insert and 5 fold higher than the poliovirus IRES control. As another positive control, we used an annotated IRES element from the Myc 5' UTR. This region was most efficient at driving cap independent translation and surpassed all other elements tested (Figure 3.3A). We confirmed that the cap independent activity of the *ESR1* 5' UTR was not an artifact of cryptic promoter activity or read through (Figure 3.3B).

To understand the mechanism by which ER is translated, we analyzed the four distinct transcript variants all encoding ER alpha (NCBI gene: *ESR1*). While

variant 1 is the most common in both healthy and cancerous tissue, we reasoned that the unique features of the other three variants could provide mechanistic clues as to the translation of ER. When we assessed the cap independence of these variants, we noted that only variant 1 was capable of driving significant cap-independent translation (Figure 3.3C). At the sequence and structural levels, the 5' UTR of variant 1 exhibited a number of unique features. Variant 1 was found to contain the highest GC content (72%), and was predicted to form the most stable 2 dimensional structure, both in its entirety and also per nucleobase (Figure 3.3D). In addition, variant 1 was predicted to form a large number of overlapping G-quadraplex structures. G-quadraplexes are a highly stable 3D arrangement of interacting guanosines coordinated by monovalent cations, most commonly potassium(Largy et al., 2016).These structures are known to play a dual role in translational regulation. The stable structures formed by these moieties may facilitate cap-independent translation or provide other regulatory information. However, by virtue of their stability, G-quadraplexes reduce translational efficiency, and their denaturation often requires the aid of RNA helicases during start codon scanning(Bugaut and Balasubramanian, 2012). For comparison, the 5' UTR of ESR1 transcript variant 1 exhibited features more similar to those highly structured 5'UTRs known to facilitate cap-independent translation such as MYC and MDM2. These later genes also exhibited highly negative predicted folding energy, both total and per nucleobase (Figure 3.3D).



5' UTR	Length	GC Content	ΔG (kcal/mol)	ΔG/Base	Predicted G-Quadraplexes
ESR1 Variant 1	234	0.72	-108.3	-0.462820513	113
ESR1 Variant 2	261	0.6	-79.2	-0.303448276	7
ESR1 Variant 3	218	0.57	-54	-0.247706422	1
ESR1 Variant 4	370	0.48	-95.4	-0.257837838	1
MYC	363	0.65	-157.8	-0.434710744	186
MDM2	301	0.69	-144	-0.478405316	11
EEF2	83	0.66	-23.2	-0.279518072	0
RPS20	45	0.66	-11.83	-0.262888889	0

Figure 3.3: 5' UTR of ESR1 mediates cap-independent translation

(A) 1.5 million MCF7 cells were plated in 6cm dishes, and transfected with 2ug of the indicated dual luciferase constructs for 24hr. Firefly and Renilla luciferase activity was measured via luminescence and, and "cap-independent activity" of the various 5' UTR elements were quantified as the ratio of firefly to renilla. Representative results for n=2 samples per group.

(B) MCF7 were transfected with dual luciferase constructs, containing an empty cassette or the ESR1variant1 5'UTR. The 5' UTR of ESR1 was subcloned into vectors containing a hairpin upstream of the renilla cassette, or lacking any upstream promoter. Representative results for n=2 samples per group.

(C) 1.5 million MCF7 in 6cm dishes were transfected for 24hr with 2ug of dual luciferase containing constructs containing the four 5' UTR transcript variants of ESR1. Luciferase expression was measured via luminescence and quantified as the ratio of Firefly/Renilla activity. Representative results for n=2 samples per group.

(D) Predicted RNA structures and thermodynamic features for ESR1 5' UTR variants.

Chapter 4: EIF4A Controls ER Expression and Function

Introduction

EIF4A is a well conserved RNA helicase and founding member of the "DEAD box" family of nucleic acid binding proteins (Kikuma et al., 2004). EIF4A was initially identified in the 1970s, and later cloned in the late 1980s by Nielsen et. al(Nielsen et al., 1985) (Rogers et al., 2002). DEAD Box helicases have a RecA like core domain which includes the necessary Asp-Glu-Ala-Asp residues required for RNA binding and helicase activity. EIF4A is unique among DEAD box containing proteins in that it consists solely of the helicase core(Andreou and Klostermeier, 2012). EIF4A is encoded by two isoforms in mammals, EIF4A1 and EIF42, which share 95% sequence and both associate with EIF4E and EIF4G. EIF4A1 appears more abundant and unlike EIF4A2 is required for cell viability(Steinberger et al., 2020). A third EIF4A isoform, EIF4A3 shares only 65% sequence homology and plays a role in the nucleus, and is essential for non-sense mediated mRNA decay(Bordeleau et al., 2005). The helicase activity of EIF4A is stimulated by partner protein, EIF4B, which is in turn activated by phosphorylation via S6K and ERK1/2 substrate, RSK at Ser422(Holz et al., 2005; Shahbazian et al., 2006). Conversely the activity of EIF4A is repressed by protein, PDCD4 which sequesters EIF4A away from both EIF4G and RNA(Suzuki et al., 2008). As alluded to above, the major role of the EIF4A1/2 proteins is to aid in the process of

scanning, whereby the 40S subunit must traverse the 5' UTR of the target RNA and bind to the preferred start codon. EIF4A unwinds thermodynamically stable structures in the 5' UTR. In the course of initiation, EIF4A binds to both EIF4E and EIF4G family members, with this trimer referred to as EIF4F complex. EIF4A also binds to EIF4GII/DAP5, a shortened version of EIF4G which lacks a binding site for EIF4E and is utilized under conditions of cap-independent (EIF4E) initiation(Godet et al., 2019). Indeed, EIF4A may be especially important for cap-independent translation, whereby complex 5'UTR structures may act as authentic IRES elements, whose structure recruits the ribosome in an EIF4E independent manner, but requires unwinding during scanning. The generality of EIF4A as an initiation factor is contested among experts, and predicting mRNAs uniquely dependent on EIF4A has been challenging. Numerous profiling studies have attempted to define the subset of mRNAs most sensitive to EIF4A inhibition. Investigators have identified numerous 5' UTR motifs as predictive of EIF4A dependence, but no one motif appears necessary to confer EIF4A dependence. Wolfe et. al demonstrated that many of clinically important oncogenes in leukemia and lymphoma are marked by 5' UTR G quadraplex elements, and that such elements are predictive of translational dependence on EIF4A(Wolfe et al., 2014). Such genes include: Myc, ADAM10, Bcl-2 and MDM2. Modelska et. al identified a number of 5' UTR elements correlating to EIF4A dependency. Among the least predictive was the overall length of the 5' UTR, followed by the predicted stability of the full length sequence. Most predictive was the percentage of G/C content. The logic here being that the density of difficult to traverse sequences is most

dependent on helicase activity, whereas long relatively unstable sequences can form stable structures by virtue of avidity, but lack requirement of helicase activity in comparison.

EIF4A has recently become a promising drug target. One class of EIF4A inhibitors are the natural products of the rocaglate family. These compounds are found in the roots of certain species of the genus Aglaia tree found in southeast Asia(Ebada et al., 2011). These compounds inhibit EIF4A in the low nanomolar range and include the natural products Rocaglamide and Silvestrol, and the synthetic derivatives such as CR-31-B (+/-)(Chu et al., 2019). These compounds share a core cyclopenta[b]benzofuran structure which inhibits EIF4A function by durably stabilizing the association of EIF4A with the substrate mRNA. Another class of EIF4A inhibitor, also a natural product is, Pateamine A. This inhibitor is structurally dissimilar to rocaglates, but binds in a similar mode (engaging mRNA and protein) to inhibit EIF4A. The final type of EIF4A inhibitor is the steroidal type compound, Hippuristanol. This compound engages EIF4A in the carboxy terminus of the protein and sequesters EIF4A away from substrate mRNA(Steinberger et al., 2020). Pharmacologically improved rocaglates have been developed for treatment of a variety of tumor types, and are now entering in the clinic (See discussion)(Ernst et al., 2020).

Having established that ER is translated in a cap-independent manner, we next turned our attention to potential proteins on which ER translation might depend. We were particularly intrigued by the predicted 5’ UTR features of ESR1 transcript

variant 1. This isoform is by far the most abundantly expressed in breast cancer and breast cancer cell lines (data not shown). As explained above, software predicted an abundance of G-quadraplexes in this 5' UTR element. Coupled with the previous results suggesting that G-quadraplexes predict EIF4A sensitivity, we analyzed the potential relationship between ER translation and EIF4A.

Results

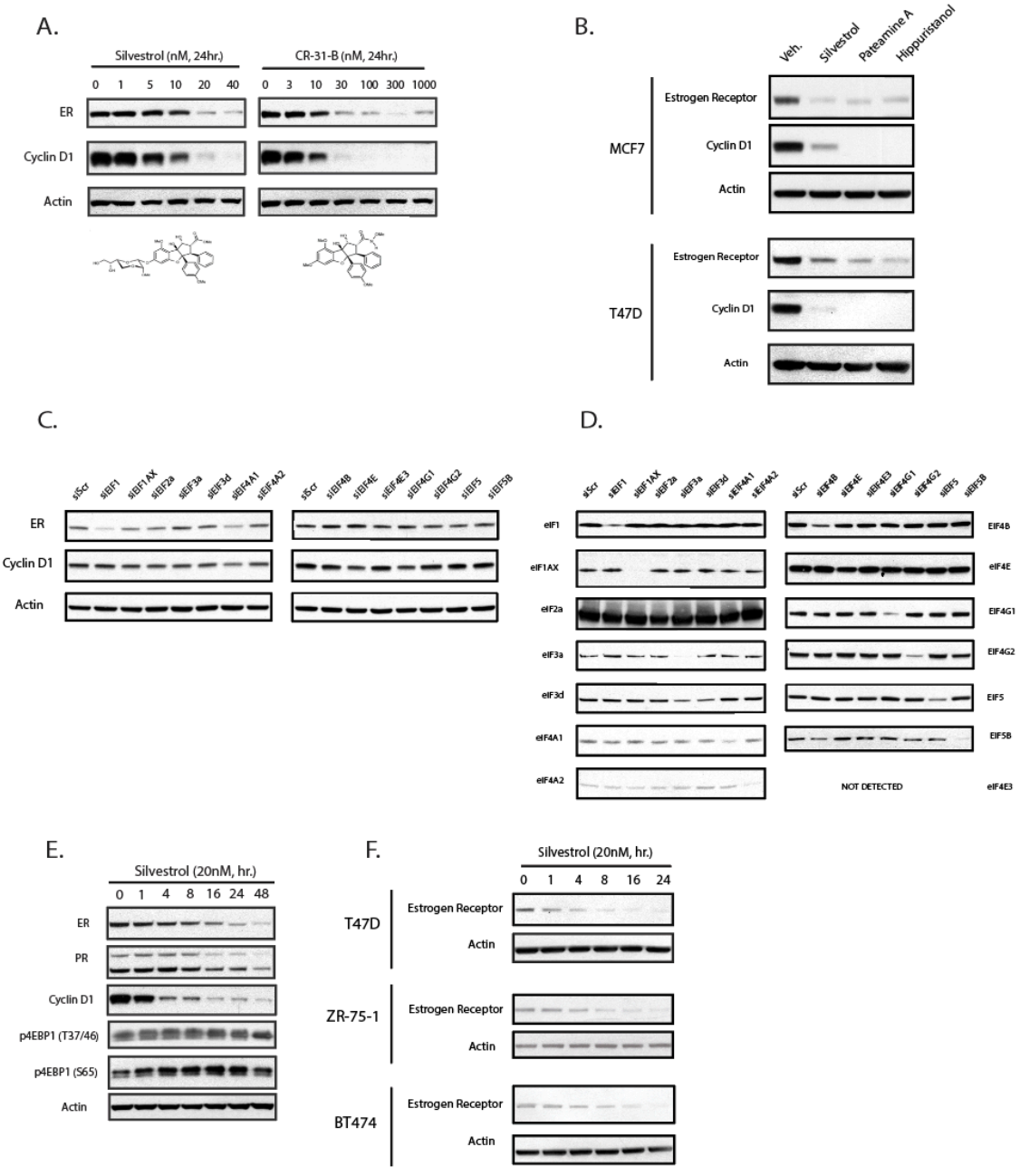
ER is translated in an EIF4A dependent manner

It has been reported by multiple groups that 5 ' UTR elements with longer than average length, stability or in particular containing G-quadraplexes, are dependent on RNA helicases to unwind the 5'UTR during translation initiation. One especially important helicase in this regard is the dead-box containing protein, EIF4A. To determine whether EIF4A controlled the expression of ER, we used a number of small molecules inhibitors of EIF4A. The rocaglate silvestrol and its synthetic derivative, CR31B, both saturably inhibited ER expression at between 20 and 30nM (Figure 4.1A). Structurally and mechanistically distinct EIF4A inhibitors, Hippuristanol and Pateamine A, showed qualitatively similar effects in ER+ cell lines MCF7 and T47D (Figure 4.1B). In parallel, we knocked down a number of the translation initiation components (Figure 4.1C and Figure 4.1D). The only factors whose knockdown reduced ER expression were EIF1 and EIF4A1, whereas cap-dependently translated Cyclin D1 was reduced by not only eif4A1 knockdown, but also by knockdown of the cap binding protein, EIF4E and partner scaffold protein EIF4G1. Silvestrol treatment decreased ER expression in a time dependent

manner, beginning as early as 4hr and continuing to decrease for up to 48hr (Figure 4.1E). These kinetics recapitulated the ER half-life determined earlier by cycloheximide treatment (Figure 3.1A). Similar effects on ER expression were observed in other ER+ cell lines, T47D, ZR75-1 and BT474 treated with silvestrol (Figure 4.1F). To determine whether EIF4A inhibition directly prevented ER translation, we again turned to our methionine deprivation and resynthesis assay. We starved cells of methionine for 24h followed by readdition of methionine for 4 or 24hr in the presence of mTOR inhibitor, RapaLink-1, Silvestrol, or cycloheximide. In the absence of any translation inhibitors, ER and Cyclin D1 were completely resynthesized when methionine was added back. Conversely, cycloheximide addition completely prevented the resynthesis of both ER and cyclin D1 when methionine was added back. Inhibition of mTOR however, prevented only the resynthesis of cap-dependently translated Cyclin D1, where as ER resynthesis was unaffected (Figure 4.1G). Silvestrol treatment prevented resynthesis of both ER and cyclin D1. This suggests a common requirement of EIF4A on these transcripts but a difference in their requirement for EIF4E as shown in figure 1. To determine if the 5' UTR of ESR1 conferred sensitivity to EIF4A inhibition, we again used the dual luciferase reporter from figure 3.3. The renilla cassette is under cap-dependent translation whereas the firefly translation is dependent on ESR1 5 'UTR IRES. Therefore, the Renilla expression serves as a normalization factor whereas firefly measures the activity of *ESR1* 5'UTR driven activity in the presence of silvestrol. Silvestrol inhibited expression of the *ESR1* IRES driven element in a dose dependent manner, with an IC50 of approx. 5nM (Figure 4.1H). This indicates

that the ESR1 5' UTR can drive cap independent expression but that this activity depends on EIF4A. To confirm that silvestrol affects ER expression via translation, we treated T47D and MCF7 with a fixed dose of silvestrol in the presence of increasing doses of cycloheximide for time t. We observed similar rates of ER expression decay between cells treated with silvestrol vs. cycloheximide and silvestrol, and that cycloheximide treatment was the dominant inhibitor of global translation whether or not silvestrol was added (Figure 4.1I, Figure 4.1J)**.** To determine the effects of EIF4A inhibition on *ESR1* mRNA, we subjected cells to a fixed dose of silvestrol with or without actinomycin D, which blocks global transcription. With silvestrol alone, we noticed an initial 2 fold spike in ESR1 levels by 8hr which gradually declined to half basal levels by 24hr (Figure 4.1K). The inclusion of actinomycin D blocked this initial spike in ESR1, suggesting a role for de novo transcription in this phase. The inclusion of Actinomycin D treatment also accelerated the decline in ESR1 levels suggesting that the reason for eventual ESR1 decline in the presence of silvestrol was likely due to mRNA degradation and not blockade of ESR1 synthesis by inhibiting an intermediate transcription factor. Additionally, on the protein level, Actinomycin D inclusion with silvestrol did not accelerate the decline in ER levels post treatment compared to silvestrol alone (Figure 4.1K). Furthermore the increase in mRNA levels at up to 8 hr with Silvestrol alone was not reflected at the protein level, further indicating that the decrease in ER levels as a function of time was due to blockage of translation and not the result of any changes in ESR1 levels.

The major regulator of ER turnover is estradiol, in that exposure to ligand induces nuclear receptor turnover. To determine whether silvestrol affected the rate of ER degradation, we cultured cells in phenol red free, charcoal stripped serum media to reduce the amount of exogenous estrogens. At baseline, this increases ER expression levels by approx. 2 fold. When given silvestrol, the rate of decrease in ER expression was the same, whether or not exogenous estrogens were present, albeit reaching a lower absolute level when in the presence of estrogens (Figure 4.1L). Taken all, these results indicate that silvestrol blocks ER expression by inhibiting translation and not by another indirect mechanism involving mRNA or effects on ER protein half-life.


A.
Silvestrol (nM, 24hr.)
0 1 5 10 20 40
CR-31-B (nM, 24hr.)
0 3 10 30 100 300 1000
ER
Cyclin D1
Actin
B.
Veh.
Silvestrol
Pateamine A
Hippuristanol
MCF7
Estrogen Receptor
Cyclin D1
Actin
T47D
Estrogen Receptor
Cyclin D1
Actin
C.
siScr siEIF1 siEIF1AX siEIF2a siEIF3a siEIF3d siEIF4A1 siEIF4A2
siScr siEIF4B siEIF4E siEIF4E3 siEIF4G1 siEIF4G2 siEIF5 siEIF5B
ER
Cyclin D1
Actin
D.
siScr siEIF1 siEIF1AX siEIF2a siEIF3a siEIF3d siEIF4A1 siEIF4A2
siScr siEIF4B siEIF4E siEIF4E3 siEIF4G1 siEIF4G2 siEIF5 siEIF5B
eIF1
eIF1AX
eIF2a
eIF3a
eIF3d
eIF4A1
eIF4A2
EIF4B
eIF4E
EIF4G1
EIF4G2
EIF5
EIF5B
NOT DETECTED
eIF4E3
E.
Silvestrol (20nM, hr.)
0 1 4 8 16 24 48
ER
PR
Cyclin D1
p4EBP1 (T37/46)
p4EBP1 (S65)
Actin
F.
Silvestrol (20nM, hr.)
0 1 4 8 16 24
T47D
Estrogen Receptor
Actin
ZR-75-1
Estrogen Receptor
Actin
BT474
Estrogen Receptor
Actin

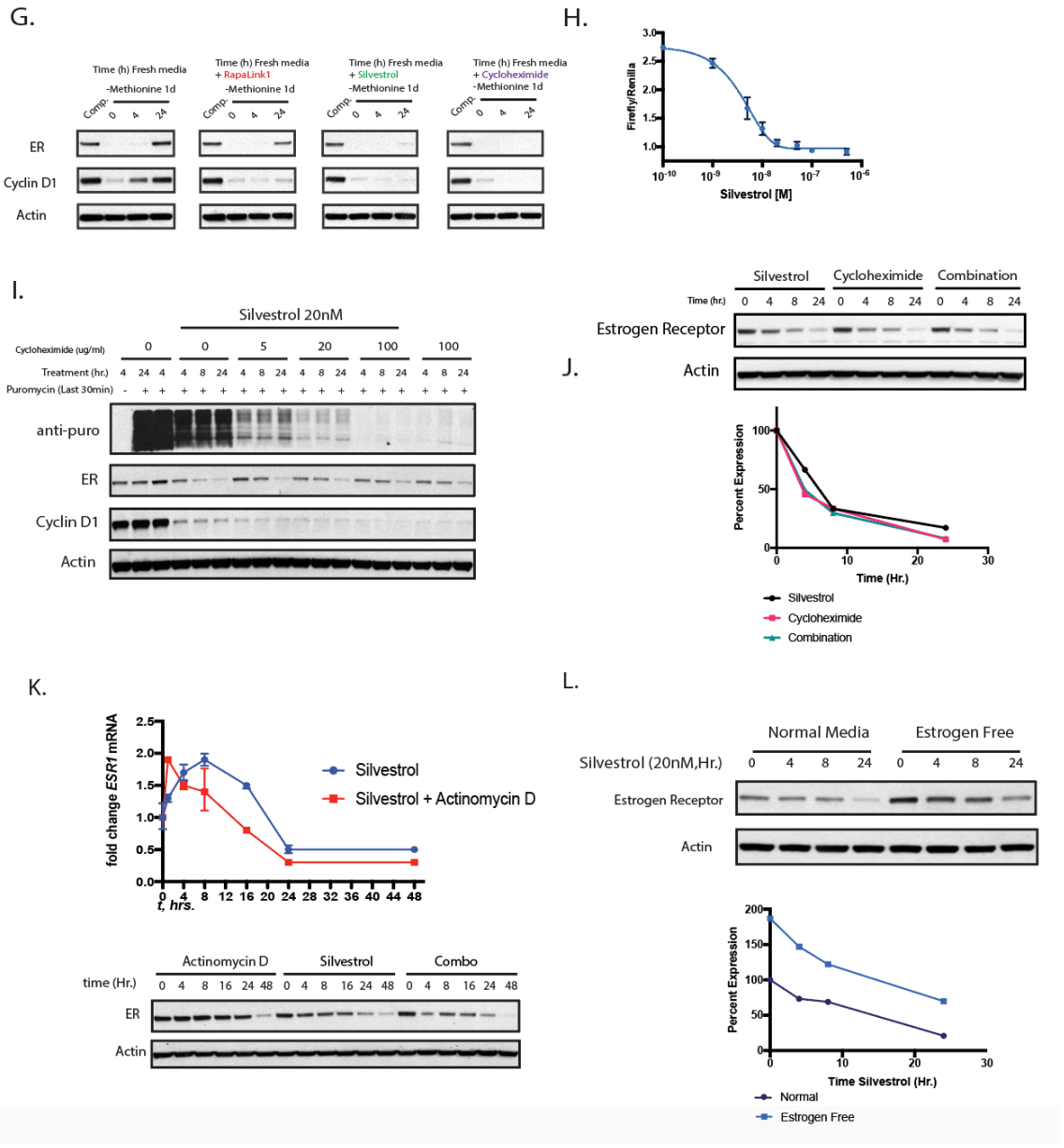

G.
Time (h) Fresh media
-Methionine 1d
Time (h) Fresh media
+ RapaLink1
-Methionine 1d
Time (h) Fresh media
+ Silvestrol
-Methionine 1d
Time (h) Fresh media
+ Cycloheximide
-Methionine 1d
Comp.
0 4 24
ER
Cyclin D1
Actin
H.
Firefly/Renilla
Silvestrol [M]
I.
Silvestrol 20nM
Cycloheximide (ug/ml)
0 0 5 20 100 100
Treatment (hr.)
Puromycin (Last 30min)
anti-puro
ER
Cyclin D1
Actin
J.
Silvestrol
Cycloheximide
Combination
Time (hr.)
0 4 8 24 0 4 8 24 0 4 8 24
Estrogen Receptor
Actin
Percent Expression
Time (Hr.)
Silvestrol
Cycloheximide
Combination
K.
fold change ESR1 mRNA
t, hrs.
Silvestrol
Silvestrol + Actinomycin D
Actinomycin D
Silvestrol
Combo
time (Hr.)
0 4 8 16 24 48 0 4 8 16 24 48 0 4 8 16 24 48
ER
Actin
L.
Normal Media
Estrogen Free
Silvestrol (20nM,Hr.)
0 4 8 24 0 4 8 24
Estrogen Receptor
Actin
Percent Expression
Time Silvestrol (Hr.)
Normal
Estrogen Free

Figure 4.1: ER expression is EIF4A dependent

(A) MCF7 were treated for 24hr with inc. doses of EIF4A inhibitors, Silvestrol or CR-31-B (+/-)

(B) MCF7 or T47D treated 24hr with EIF4A inhibitors, Silvestrol (20nM), Pateamine A (1uM) or Hippuristanol (1uM).

(C) MCF7 were transfected with siRNA targeted against the indicated eukaryotic initiation factors for 72hr.

(D) Western blot showing knockdown of proteins referred to in Figure 4.1C

(E) MCF7 treated for the indicated times with 20nM Silvestrol

(F) T47D, ZR-75-1 and BT474 treated for the indicated times with 20nM Silvestrol.

(G) MCF7 were starved of methionine for 24hr followed by restimulation with complete media with or without silvestrol 20nM, RapaLink-1 10nM or cycloheximide 50ug/ml.

(H) 1.5 million MCF7 plated in 6cm dishes were transfected for 24hr with dual luciferase plasmid containing the 5' UTR of ESR1. At the same time, cells were subjected to inc doses of silvestrol. Luciferase activity was measured via luminescence.

(I) T47D treated with 20nM Silvestrol for the indicated times, with or without increasing doses of cycloheximide (5-100ug/ml). Puromycin was pulsed for the last 30min of indicated times to track global protein translation. Estrogen Receptor levels were quantified via densitometry and were plotted as a function of time post silvestrol treatment.

(J) MCF7 treated with 20nM Silvestrol or 50ug/ml cycloheximide or the combination for 4, 8 or 24hr. Estrogen Receptor percent expression was measured via densitometry.

(K) MCF7 were treated for indicated times with Actinomycin D (3nM), Silvestrol (20nM) or the combination. Collected cells were used for RNA or total protein isolation and subsequent qRT-PCR or immunoblotting.

(L) MCF7 were washed and placed in complete or phenol red free charcoal stripped FBS containing media (Estrogen Free) for 24hr, followed by treatment with silvestrol 20nM for the indicated times.

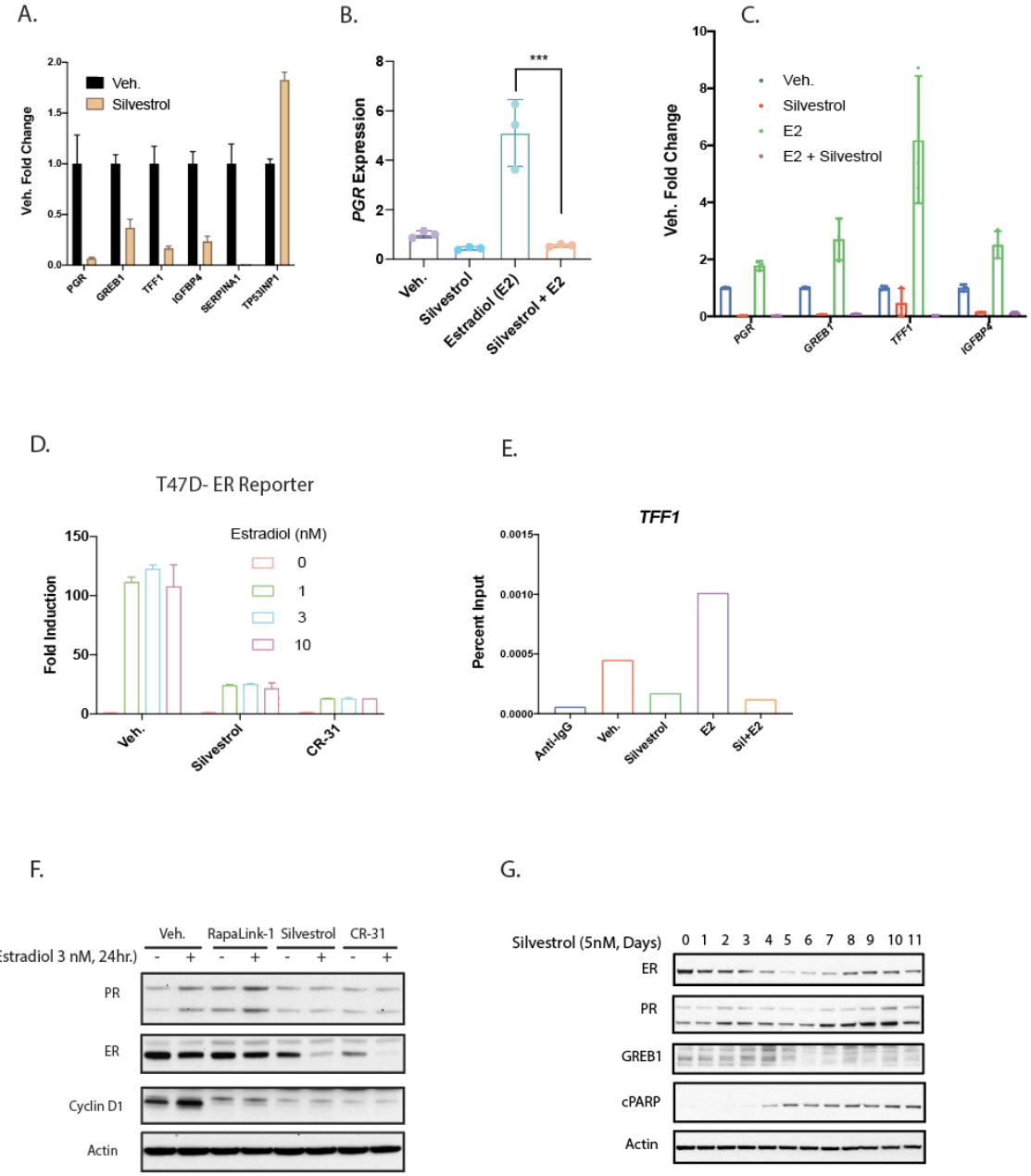
EIF4A Inhibition blocks cell growth and inhibits ER function

We next turned our attention to whether the decreased ER levels following EIF4A inhibition resulted in decreased receptor function. We treated cells for 24hr with Silvestrol and analyzed expression levels of canonical ER targets. All ER target genes with the exception of TP53INP1 were greatly reduced by silvestrol treatment. The least affected gene was GREB1 which was lowered by more than 2 fold, whereas the most affected gene, SERPINA1 was completely abolished. TP53INP1 is a gene normally repressed by ER. In this case, treatment with silvestrol induced its expression, consistent with inhibition of ER affecting gene expression in both directions (Figure 4.2A). We also determined whether silvestrol treatment could attenuate the ability of estradiol to activate canonical ER targets. We cultured cells in phenol red free media for one day with or without silvestrol. We then added 10nM estradiol for an additional day to stimulate ER. Estradiol treatment increased *PGR* expression by nearly 5 fold. Pretreatment with silvestrol prevented such induction, and even lowered expression below baseline (Figure 4.2B). We performed a similar experiment in related cell line, T47D. In this case cells were starved of estrogens for 3d in the presence of silvestrol, as in our experience this cell line requires a longer starvation period before appreciable *PGR* expression can be induced with estradiol. Stimulation with 10nM estradiol induced canonical target genes, *PGR*, *GREB1*, *TFF1* and *IGFBP4*. Pretreatment with silvestrol not only prevented induction of these targets, but again, lowered the basal expression (Figure 4.2C).

To avoid confounding effects of silvestrol on mRNA levels, we performed CHIP-qPCR to determine if the reduced ER levels resulting from EIF4A inhibition resulted in reduced presence of ER on its response elements. We again starved cells of estrogen for 1 day in the presence or absence of silvestrol, followed by 1hr stimulation with 10nM Estradiol. Estradiol treatment resulted in enrichment of ER on the promoter region of target, *TFF1,* and this was attenuated by silvestrol pretreatment (Figure 4.2E). Finally, we used a reporter cell line, stably expressing a firefly luciferase cassette driven by estrogen response elements. As low as 1nM estradiol produced saturable luciferase induction in these cells. If pretreated with either silvestrol or CR31B, the estrogen driven luciferase expression was severely attenuated, even at 10nM estradiol (Figure 4.2D). We also analyzed endogenous ER targets at the protein level in this cell line (Figure 4.2F). As previously demonstrated, mTOR inhibition with Rapalink-1 blocked cap-dependently translated cyclin D1 but did not effect ER expression. mTOR inhibition, likely through mTORC2, did evidently enhance its activity as measured by increased PR expression, and this effect was further accentuated by estradiol treatment, as was previously reported (Toska et al., 2017) . Both silvestrol and CR31B treatment reduced ER and cyclin d1 expression and prevented the estradiol dependent induction of PR on the protein level. We also analyzed the long term effects of EIF4A inhibition on ER and its targets (Figure 4.2G and 4.2H). At 5nM, the kinetics of ER and target inhibition was quite slow. ER levels declined more or less linearly

over the course of 5 days, before rebounding over the next 5 days. This was accompanied by a slight inhibition of ER target, GREB1 at 6d, which was followed by a rebound parallel to that of ER. Interestingly there was almost no inhibition of PR expression using the 5nM dose, suggesting that different doses of silvestrol can produce differing effects on ER targets. The 5nM dose also produced an increase in apoptotic marker, cleaved PARP, that began at 4 days and persisted the full 11 days. In contrast, the 20nM dose of silvestrol produced complete inhibition of ER by 4d and did not rebound up to day 7. The experiment was terminated at 7d due to inability to collect enough cells passed that time. In addition, *PGR* and *GREB1* expression was durably inhibited in by 3 days, and these substrates remained suppressed over the duration. The cleaved PARP induced by 20nM silvestrol peaked at 3d and declined over the next 4, likely due to the death and detachment of cells that were then not collected. The ability of silvestrol to durably inhibit ER and its targets, led us to investigate whether the compound could be washed out when cells were washed and exposed to fresh media. We treated cells with 20nM silvestrol for either 24 or 72hr. followed by washout for time t up to an additional 24hr. We observed that following 24hr silvestrol treatment, ER expression began to rebound at 4hr post washout, and completely returned to baseline at 24hr post washout. Conversely, when cells were treated with silvestrol for a full 72hr, ER expression only slightly returned post washout, but remained repressed at 24hr post washout. In addition, 3d treatment with silvestrol potently inhibited PR expression which was maintained for up to 24hr post washout (Figures 4.2I and 4.2J).

To determine the effect of EIF4A inhibition on cell growth, we treated cells with increasing doses of silvestrol for up to 7 days. Cell growth was partially inhibited at as low as 5nM and saturably inhibited at 20nM, with a linear decrease in cell viability below baseline as a function of time (Figure 4.2K). Silvestrol and CR-31-B produced comparable effects on cell growth, exhibiting 5.2, and 6.38nM GI50s respectively (Figure 4.2L). We obtained similar values in T47D, silvestrol was still slightly more potent than CR31B, GI50s at 3.53nM and 7.2nM respectively (Figure 4.2M).


A.
Veh.
Silvestrol
Veh. Fold Change
PGR
GREB1
TFF1
IGFBP4
SERPINA1
TP53INP1
B.
***
PGR Expression
Veh.
Silvestrol
Estradiol (E2)
Silvestrol + E2
C.
Veh.
Silvestrol
E2
E2 + Silvestrol
Veh. Fold Change
PGR
GREB1
TFF1
IGFBP4
D.
T47D- ER Reporter
Estradiol (nM)
0
1
3
10
Fold Induction
Veh.
Silvestrol
CR-31
E.
TFF1
Percent Input
Anti-IgG
Veh.
Silvestrol
E2
Sil+E2
F.
Veh.
RapaLink-1
Silvestrol
CR-31
(Estradiol 3 nM, 24hr.)
PR
ER
Cyclin D1
Actin
G.
Silvestrol (5nM, Days)
0 1 2 3 4 5 6 7 8 9 10 11
ER
PR
GREB1
cPARP
Actin

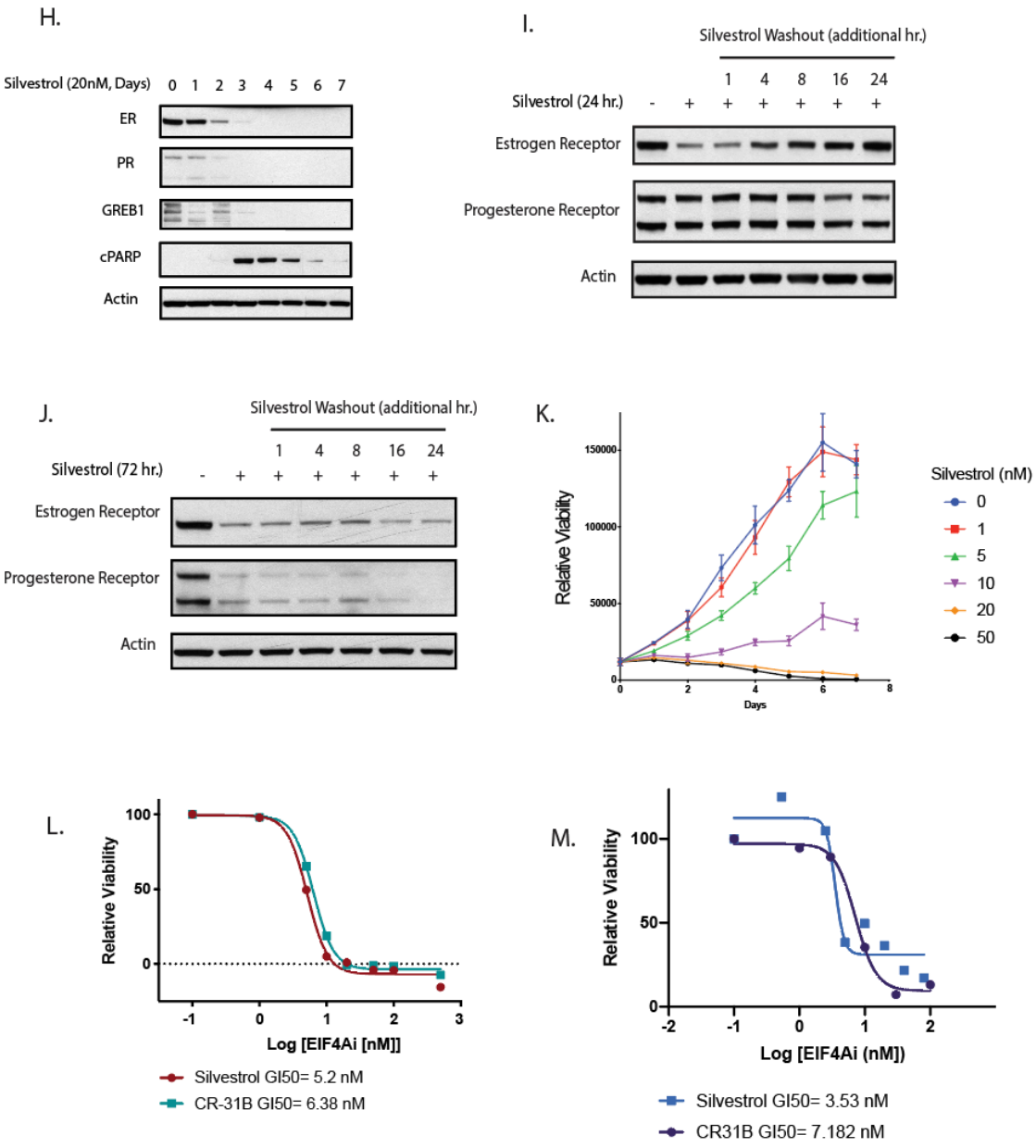

H.
Silvestrol (20nM, Days) 0 1 2 3 4 5 6 7
ER
PR
GREB1
cPARP
Actin
I.
Silvestrol Washout (additional hr.)
1 4 8 16 24
Silvestrol (24 hr.) - + + + + + +
Estrogen Receptor
Progesterone Receptor
Actin
J.
Silvestrol Washout (additional hr.)
1 4 8 16 24
Silvestrol (72 hr.) - + + + + + +
Estrogen Receptor
Progesterone Receptor
Actin
K.
Relative Viability
150000
100000
50000
0
0 2 4 6 8
Days
Silvestrol (nM)
0
1
5
10
20
50
L.
Relative Viability
100
50
0
-1 0 1 2 3
Log [EIF4Ai [nM]]
Silvestrol GI50= 5.2 nM
CR-31B GI50= 6.38 nM
M.
Relative Viability
100
50
0
-2 -1 0 1 2
Log [EIF4Ai (nM)]
Silvestrol GI50= 3.53 nM
CR31B GI50= 7.182 nM

Figure 4.2: EIF4A Inhibition blocks cell growth and inhibits ER function

(A) MCF7 treated for 24hr with 20nM Silvestrol followed by analysis of canonical ER target genes by RT-qPCR

(B) MCF7 were plated in DMEM F12 containing charcoal stripped FBS and lacking phenol red, followed by treatment with silvestrol (20nM) for 24hr. Cells were then stimulated with 10nM estradiol for an additional 24hr. PGR expression was analyzed via RT-qPCR.

(C) T47D were plated in DMEM F12 containing charcoal stripped FBS and lacking phenol red, followed by treatment with silvestrol (20nM) for 24hr. Cells were then stimulated with 10nM estradiol for an additional 24hr. ER target gene expression was analyzed via RT-qPCR.

(D) T47D-kBluc cells were plated in DMEM F12 containing charcoal stripped FBS and lacking phenol red, followed by treatment with 20nM Silvestrol or 30nM CR31B for 24hr. Cells were then stimulated with the indicated concentrations of estradiol for an additional 24hr. ER dependent gene expression was measured as firefly luciferase luminescence

(E) MCF7 were placed in DMEM F12 containing charcoal stripped FBS and lacking phenol red, followed by treatment with 20nM Silvestrol for 24hr. Cells were then stimulated with 10nM estradiol for 1hr. ER binding to the TFF1 enhancer element was analyzed by CHIP and quantified by RT-qPCR.

(F) T47D kBluc were placed in DMEM F12 containing charcoal stripped FBS and lacking phenol red with or without 10nM RapaLink-1, 20nM Silvestrol, 30nM CR31B for 24hr. Cells were then stimulated with 3nM estradiol for an additional 24hr.

(G) MCF7 were treated for up to 11 days with 5nM Silvestrol

(H) MCF7 were treated for up to 7 days with 20nM Silvestrol

(I) MCF7 were treated for 24hr. with 20nM Silvestrol. Cells were then washed and placed in drug free media for time t, up to an additional 24hr. MCF7 Day 3 GI50 curves for Silvestrol and CR31B. GI50 values were determined via 4 parameter non linear model fitting in graph pad prism 8.

(J) MCF7 were treated for 72hr. with 20nM Silvestrol. Cells were then washed and placed in drug free media for time t, up to an additional 24hr.

(K) MCF7 was treated for up to 7 days with indicated concentrations of Silvestrol. Growth was quantified as a function of time (days) via Cell Titer Glo luminescence.

(L) MCF7 Day 3 GI50 curves for Silvestrol and CR31B. GI50 values were determined via 4 parameter non linear model fitting in graph pad prism 8.

(M) T47D Day 3 GI50 curves for Silvestrol and CR31B. GI50 values were determined via 4 parameter non linear model fitting in graph pad prism

Chapter 5: Therapeutic applications for EIF4A inhibitors in ER+ Breast Cancer

Introduction:

Fulvestrant is a clinically employed selective ER degrader. We wondered whether combining Fulvestrant with EIF4A inhibition could reduce ER levels even further than either compound alone. In this chapter we first test the combination of silvestrol and fulvestrant on ER levels, gene expression and tumor growth.

The majority of patients with ER+ breast cancer initially respond to endocrine therapy but resistance inevitably develops. Development of new strategies to target mechanisms mediating such endocrine resistance remains a principal focus of breast cancer oncology. One such mechanism of resistance is the expression of ER variants which have reduced or no sensitivity to ER antagonists. These include ER mutants such as those occurring at W537 or D538. As expressed in the introduction, these mutants occur near the base of helix 12 and impart the helix with enhanced flexibility. Such a confirmation allows the receptor to adopt a partial agonist confirmation in the absence of ligand. Another set of variants mediating endocrine resistance are the ER fusion proteins which lack a ligand binding domain entirely and can signal without any exoigenous ligand(Katzenellenbogen et al., 2018; Toy et al., 2017). In the following experiments, we test the ability of EIF4A inhibitors to block the expression of these ER variants.

Results

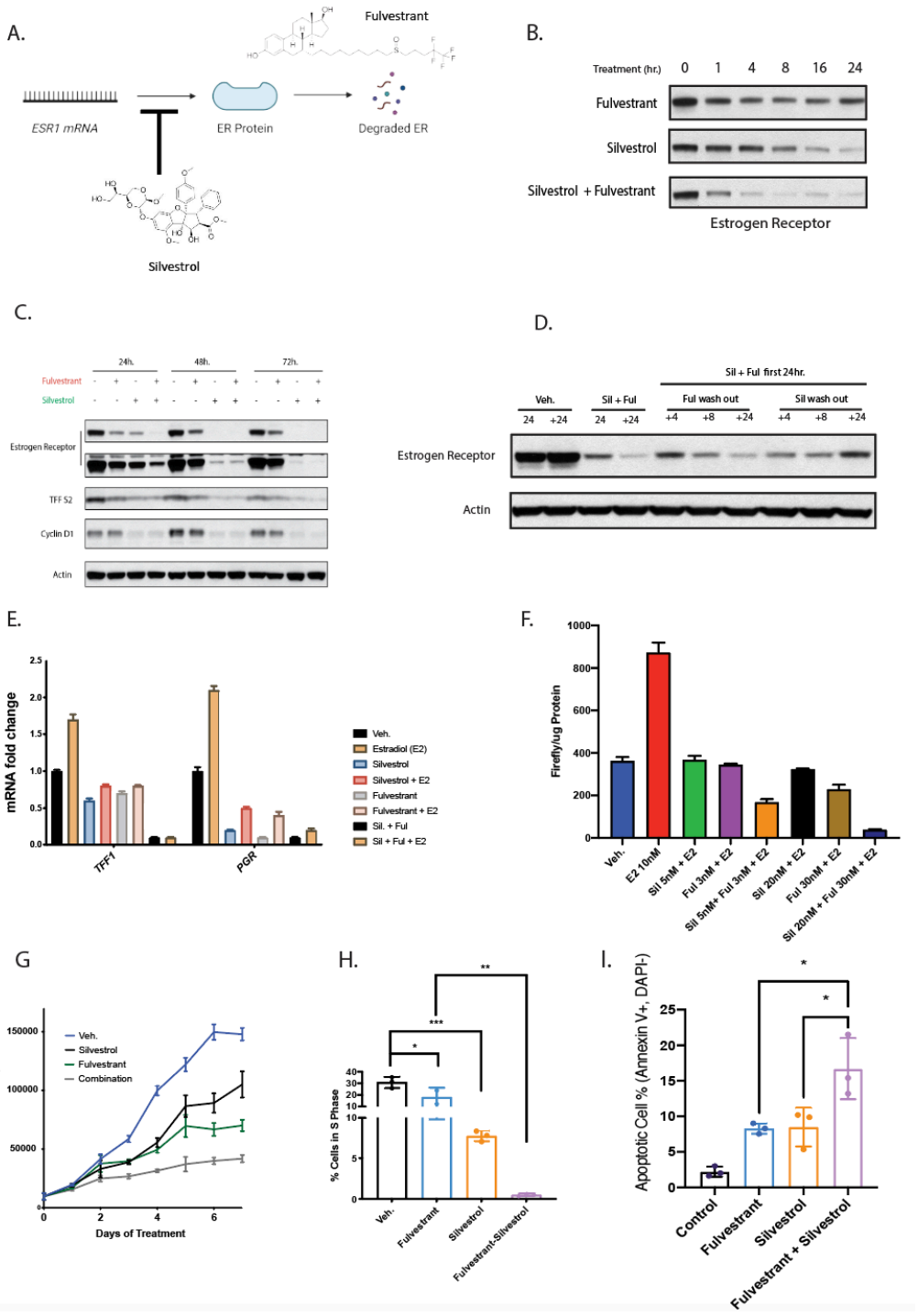
EIF4A inhibition combined with fulvestrant minimizes ER expression and blocks tumor growth

Protein expression is a balance between synthesis and degradation. Our data suggested that inhibition of EIF4A lowered ER expression to a greater degree when cells were in exogenous estrogen (Figure 5.1K). This is consistent with estrogens being the primary the determinant of receptor half-life. We therefore reasoned that a selective ER degrader such as fulvestrant, which induces receptor turnover without receptor activation, could work in concert with EIF4A inhibition to minimize ER levels (Figure 5.1A). We treated cells with fulvestrant, silvestrol or the combination for up to 24hr. Fulvestrant exerted its maximial inhibition on ER levels by 1hr (Figure 5.1B). Silvestrol suppressed ER expression more deeply, but required longer to do so. The combination of fulvestrant and silvestrol exhibited the kinetics of fulvestrant and the inhibitory capacity of silvestrol, thus lowering the cumulative ER expression quickly and to a minimum. Long term treatment with these compounds demonstrated the power of blocking ER synthesis via silvestrol. In the first 24hr, combination silvestrol/fulvestrant treatment blocked ER expression more than either compound alone. By 48hr however, silvestrol alone had become as effective as the combination. This is likely due to ER turnover and continued blockade of protein synthesis well passed the ER half life of 4-8hr. This is in contrast to fulvestrant, which by 48hr had lost some of its initial effectiveness. The addition of silvestrol attenuated this rebound (Figure 5.1C). In light of these results, we reasoned that a logical schedule for a silvestrol/fulvestrant combination

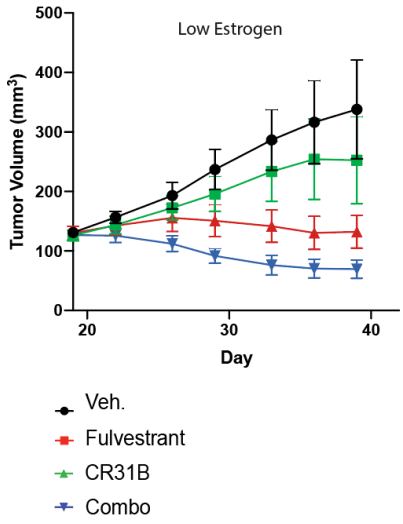
might involve an initial treatment with silvestrol and fulvestrant to cause receptor degradation, followed by silvestrol to block new receptor synthesis. To test this, we treated cells with the combination for 24hr, followed by washout and treatment with either fulvestrant or silvestrol alone for time t. We observed oppositely phased rebound dynamics for silvestrol and fulvestrant in this second 24hr time period. The cells receiving only silvestrol (Ful washout) had a rapid increase in ER expression followed by gradual decrease as silvestrol blocked ER synthesis. In contrast, those cells receiving only fulvestrant during the second window (Sil washout), exhibited a gradual increase in receptor expression as newly synthesized receptor reached the fulvestrant determined turnover rate (Figure 5.1D). To determine whether combination fulvestrant, silvestrol could attenuate estradiol induced gene expression, we starved cells of estrogen, followed by pretreatment with either compound alone or the combination, and stimulated with estradiol for 1d. Both fulvestrant and silvestrol reduced basal and inducible TFF1 and PGR mRNA levels. The combination of both compounds however was markedly better than either alone (Figure 5.1E). We obtained similar results using our ERE driven luciferase expressing T47D cell line (Figure 5.1F). As low as 5nM Silvestrol and 3nM fulvestrant completely suppressed the inducable activity of the reporter. The combination of both compounds however, not only reduced inducible activity, but reduced reporter expression to approx. half of baseline. Using these lower doses also produced combinatorial inhibition of cell growth in vitro. 5nM silvestrol and 3nM fulvestrant were each observed to block cell growth by around 50% at day 3. The combination inhibited cell growth approx. twice as well as either

compound alone and this effect persisted up to 7d (Figure 5.1G). As another measurement of proliferation, we examined whether silvestrol, fulvestrant or the combination reduced the number of cells in S phase, as assayed via Edu incorporation. At baseline, approx. 30% of MCF7 cells were in S phase, and at the chosen doses, only single agent silvestrol significantly lowered this fraction. The combination of fulvestrant with silvestrol was highly effective at reducing the fraction of S phase cells, lowering the percentages to 2.5 and 0.5 respectively (Figure 5.1H). To assess the levels of apoptotic cell death following silvestrol and fulvestrant treatment, we assayed annexin V positive cells following 72hr of treatment. The combination produced approx. twice as much apoptotic cell death as either compound alone (Figure 5.1I). Finally, we examined the effects of these compounds in vivo. As a way to examine the estrogen dependence of our findings, we used MCF7 xenografts in mice preimplanted with either a low (0.18) or high (0.72) estrogen pellets. In the low estrogen context, fulvestrant demonstrated strong efficacy and prevented tumor growth (Figure 5.1J). For in vivo studies we used the EIF4A inhibitor, CR31B, capable of being dosed in mice. In the low estrogen setting, CR31B produced only a mild effect as a single agent. The combination of CR31B with fulvestrant produced a mild regression. In contrast, the combinatorial effect was most strongly seen in the high estrogen setting (Figure 5.1K). In this case, neither fulvestrant nor CR31B produced any substantial effect as single agents. The combination however produced a profound regression, durable up to 45 days. Importantly, over the course of the experiment, there was no statisticsally significant change in mouse weights, indicating that both single

agents and the combination were well tolerated (Figure 5.2L). The combination treatment also produced a deeper inhibition of downstream ER targets compared to either treatment alone (Figure 5.1M).

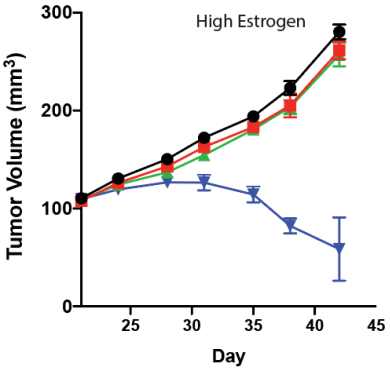

A.
Fulvestrant
ESR1 mRNA
ER Protein
Degraded ER
Silvestrol
B.
Treatment (hr.) 0 1 4 8 16 24
Fulvestrant
Silvestrol
Silvestrol + Fulvestrant
Estrogen Receptor
C.
24h.
48h.
72h.
Fulvestrant
Silvestrol
Estrogen Receptor
TFF S2
Cyclin D1
Actin
D.
Sil + Ful first 24hr.
Veh.
Sil + Ful
Ful wash out
Sil wash out
24 +24 24 +24 +4 +8 +24 +4 +8 +24
Estrogen Receptor
Actin
E.
mRNA fold change
TFF1
PGR
Veh.
Estradiol (E2)
Silvestrol
Silvestrol + E2
Fulvestrant
Fulvestrant + E2
Sil. + Ful
Sil + Ful + E2
F.
Firefly/ug Protein
Veh.
E2 10nM
Sil 5nM + E2
Ful 3nM + E2
Sil 5nM+ Ful 3nM + E2
Sil 20nM + E2
Ful 30nM + E2
Sil 20nM + Ful 30nM + E2
G
Cell Viability
Days of Treatment
Veh.
Silvestrol
Fulvestrant
Combination
H.
% Cells in S Phase
Veh.
Fulvestrant
Silvestrol
Fulvestrant-Silvestrol
I.
Apoptotic Cell % (Annexin V+, DAPI-)
Control
Fulvestrant
Silvestrol
Fulvestrant + Silvestrol

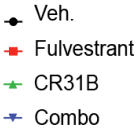
J.


Low Estrogen
Tumor Volume (mm3)
500
400
300
200
100
0
20
30
40
Day

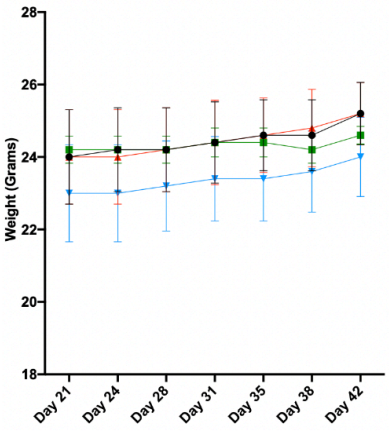
Veh.
Fulvestrant
CR31B
Combo

K.


High Estrogen
Tumor Volume (mm3)
300
200
100
0
25
30
35
40
45
Day


Veh.
Fulvestrant
CR31B
Combo

L.


Weight (Grams)
28
26
24
22
20
18
Day 21
Day 24
Day 28
Day 31
Day 35
Day 38
Day 42

M.

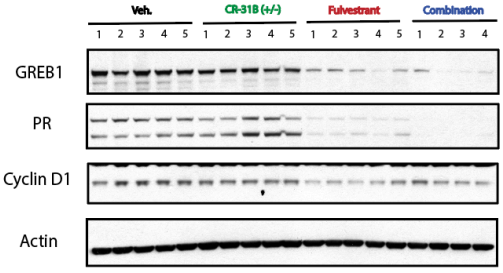

Veh.
CR-31B (+/-)
Fulvestrant
Combination
1 2 3 4 5 1 2 3 4 5 1 2 3 4 5 1 2 3 4
GREB1
PR
Cyclin D1
Actin

Figure 5.1: EIF4A inhibition combined with fulvestrant minimizes ER expression and blocks tumor growth

(A) Schematic Showing the rationale for dual EIF4A inhibitor/Fulvestrant Treatment.

(B) MCF7 were treated for indicated times with Silvestrol (20nM), Fulvestrant (30nM) or combination.

(C) MCF7 were treated for 24, 48 or 72 hr with Silvestrol (20nM), Fulvestrant (30nM) or the combination

(D) MCF7 were treated with Silvestrol (20nM) and Fulvestrant (100nM) for 24 hr (24) followed by washout of either fulvestrant or Silvestrol for 4, 8 or 24 (+4, +8 +24 notation).

(E) MCF7 were plated in phenol red free charcoal stripped FBS DMEM F12. Cells were then treated with Veh. or Silvestrol (5nM), Fulvestrant (3nM) or the combo for 24hr. Cells were finally stimulated with 10nM Estradiol for an additional 1 day before isolation of mRNA for RT-qPCR.

(F) T47D kBluc were placed in DMEM F12 containing charcoal stripped FBS and lacking phenol red with or without the indicated doses of silvestrol or fulvestrant for 24hr. Cells were then stimulated with 10nM estradiol for an additional 24hr. Firefly expression was quantified via luminescence and normalized to mg protein obtained via BCA protein quantification

(G) MCF7 were treated with either 5nM silvestrol, 3nM fulvestrant or the combination for up to 7d. Cell growth was measured daily via ATP-glo luminescence.

(H) Percentage of cells in S phase as measured by 1.5h EdU pulse incorporation of MCF-7 treated for 48h with fulvestrant (3nM), silvestrol (5nM) or the combination. Mean of three biological replicates is plotted.

(I) MCF7 were treated with either 5nM silvestrol, 3nM fulvestrant, or the combination for 72hr. Annexin V positive cells were quantified via flow cytometry.

(J) Nude mice were implanted with estrogen pellets (0.18 mg) for 3d before injection of MCF7 10million cells/mouse. Once tumors reached 100 mm^3 mice were treated twice weekly with 200mg/kg Fulvestrant SubQ, CR31B (1mg/kg i.v.) or combination.

(K) Nude mice were implanted with estrogen pellets (0.72 mg) for 3d before injection of MCF7 10million cells/mouse. Once tumors reached 100 mm^3 mice were treated twice weekly with 200mg/kg Fulvestrant SubQ, CR31B (1mg/kg i.v.) or combination.

(L) Kinetic measurement of mouse weights over the course of the “high estrogen experiment”

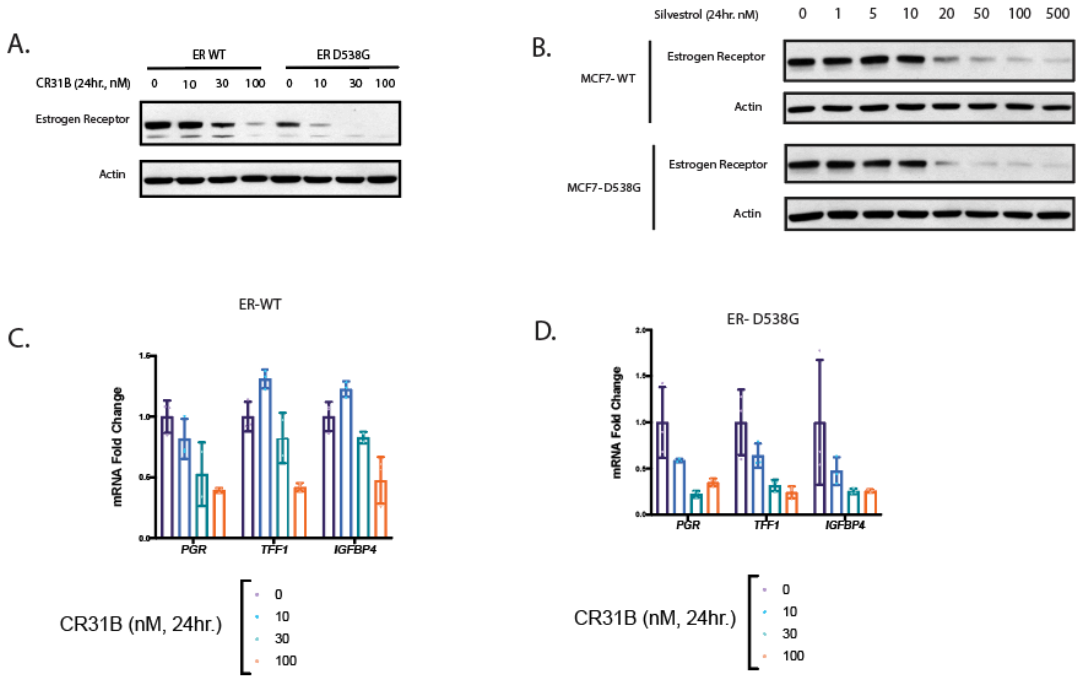
(M)Tumors were collected 24hr after the final dose, followed by lysis and preparation for immunoblotting Data corresponding with Figure 5.1K.

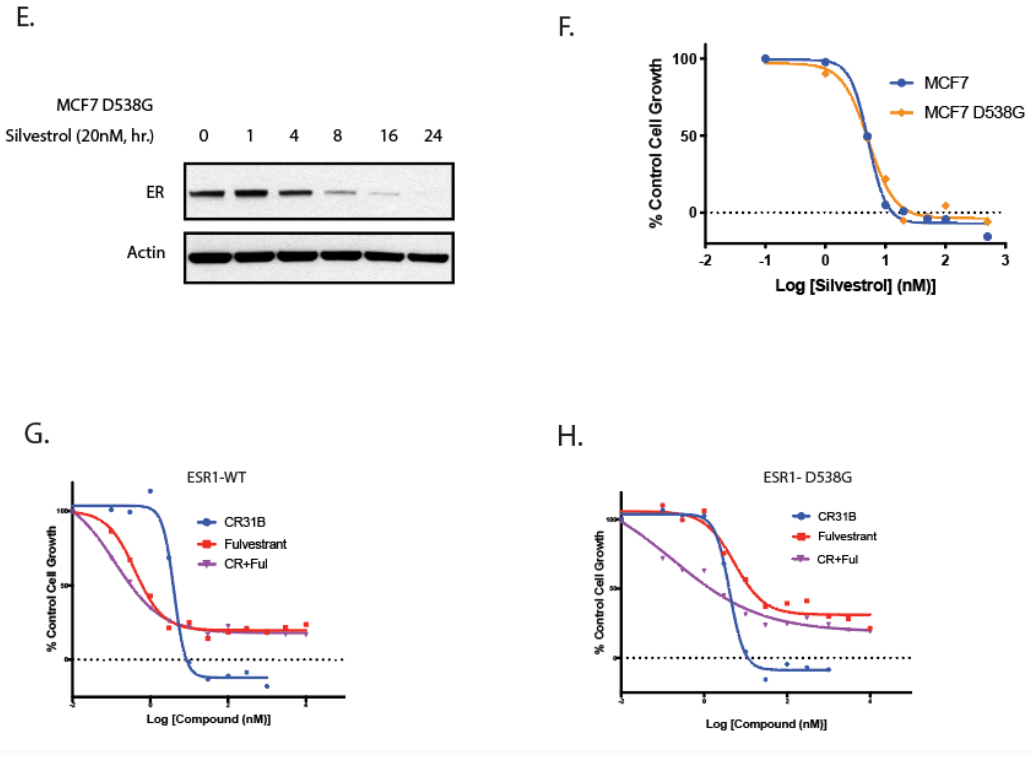
EIF4A inhibition blocks the expression of clinically significant ER variants

Having shown that ER is translated in an EIF4A dependent manner, we decided to test whether EIF4A inhibition could block the expression of clinically important ER variants. ESR1 mutants such as D538G allow a relaxed conformation of helix-12, thus mimicking an agonist bound receptor confirmation, which confers reduced hormone dependence, hyperactivity, and resistance to anti hormonal therapy. We used a previously characterized MCF7 derived cell line expressing a knocked in version of ESR1-D538G(Toy et al., 2013, 2017). Compared to wildtype ER, the D538G variant was expressed at a lower basal level, potentially owing to its higher its activity (Figure 5.2A). We compared the ability of CR31B to block expression of wt and D538G ER. The ER-D538G variant was reduced to approx.. 50% expression by 10nM CR31B, and saturably inhibited by 30nM. Consistent with higher baseline expression, the WT variant was approx. 3 fold less potently affected. Both wt and D538G variants demonstrated a similar response to silvestrol, and 20nM was a saturating dose for blocking ER expression (Figure 5.2B). EIF4A inhibition at the same doses of CR31B also attenuated ER target gene expression at 24hr (Figure 5.2 C and Figure 5.2D).CR31B at 30nM produced a slight inhibition of *PGR, TFF1* and *IGFBP4* in the WT ER expressing MCF7, whereas this dose produced a saturable inhibition of target gene expression in the D538G variant. The D538G variant also displayed a half life similar to wt ER, between 4 and 8hr in standard phenol red containing DMEM F12 (Figure 5.2E).

To determine the sensitivity of the ER D538G mutant in terms of cell growth, we treated either the WT or ER D538G expressing cells for 3d with increasing doses of CR31B. Both cell lines were equally sensitive to CR31B, exhibiting GI50 values of approx. 4nM (Figure 5.2F). In contrast, the ER D538G expressing cell line was at least 10 fold less sensitive to fulvestrant compared to wildtype, as previously demonstrated(Toy et al., 2017). We also tested whether the inclusion of a small amount of CR31B (3nM) could re-sensitize the D538 mutant cells to fulvestrant. When this small dose of CR31B was included, the dose of fulvestrant required to inhibit cell growth of the D538G cells was enhanced by 10x (Figure 5.2 G, H). The second ER variant we investigated was that of the recently characterized ESR1-fusions. At least 1% of hormone therapy relapsed patients are thought to harbor ESR1 fusion proteins(Kim and Han, 2021). These variants contain the N-terminus of ER fused to a variety of C-terminal partners. The ligand binding domain of ER is lost in the process and allows receptor activation in the absence of estrogen. Using CRISPR-Cas9 technology, we generated a T47D cell line harboring a previously isolated ESR1-Sox9 fusion (Figure 5.2 I). This fusion produces a protein slightly larger than wildtype estrogen receptor and can be detected at approx. 75kd via immunoblotting (Figure 5.3J). To select for high expression, we placed these cells in estrogen free media for at least 5 weeks (charcoal stripped serum, phenol red free). The ability of cells harboring the ESR1 fusion to grow in low estrogen conditions produced an enrichment of fusion expression. The ESR1-Sox9 fusion cells were substantially less sensitive to fulvestrant compared to the wildtype analog, requiring over 1μM to block growth

by 50% (Figure 5.2K). Expression of both the fusion and the WT ER variant were equally sensitive to CR31B, being saturably inhibited by 30nM 24hr post treatment. In addition, the ESR1-Sox9 fusion appeared to have a similar half-life compared to that of WT ER, approx. 4hr under normal media conditions (Figure 5.2L). 30nM CR31B also produced a saturable inhibition of ER expression and canonical ER target genes in both the WT and fusion expressing cells (Figure 5.2 M,N,O). In contrast to the differential sensitivity of the fusion expressing cells to fulvestrant, both the fusion and the WT expressing cells were equally sensitive to CR31B in vitro, exhibiting GI50 values of approx. 5nM (Figure 5.2P). Finally, we tested the effect of EIF4A inhibition on ESR1-Sox9 expressing xenografts in vivo. Bi weekly doses of 1mg/kg CR31B produced appreciable inhibition of tumor growth for up to 75 days after treatment (Figure 5.2Q). Such results suggest that these analyzed variants retain their dependence on EIF4A for expression, and that this can be therapeutically exploited.


A.
ER WT
ER D538G
CR31B (24hr., nM)
0 10 30 100 0 10 30 100
Estrogen Receptor
Actin
B.
Silvestrol (24hr. nM)
0 1 5 10 20 50 100 500
MCF7- WT
Estrogen Receptor
Actin
MCF7- D538G
Estrogen Receptor
Actin
C.
ER-WT
mRNA Fold Change
PGR
TFF1
IGFBP4
CR31B (nM, 24hr.)
0
10
30
100
D.
ER- D538G
mRNA Fold Change
PGR
TFF1
IGFBP4
CR31B (nM, 24hr.)
0
10
30
100


E.
MCF7 D538G
Silvestrol (20nM, hr.)
0 1 4 8 16 24
ER
Actin
F.
MCF7
MCF7 D538G
% Control Cell Growth
Log [Silvestrol] (nM)]

G.
ESR1-WT
CR31B
Fulvestrant
CR+Ful
% Control Cell Growth
Log [Compound (nM)]
H.
ESR1- D538G
CR31B
Fulvestrant
CR+Ful
% Control Cell Growth
Log [Compound (nM)]

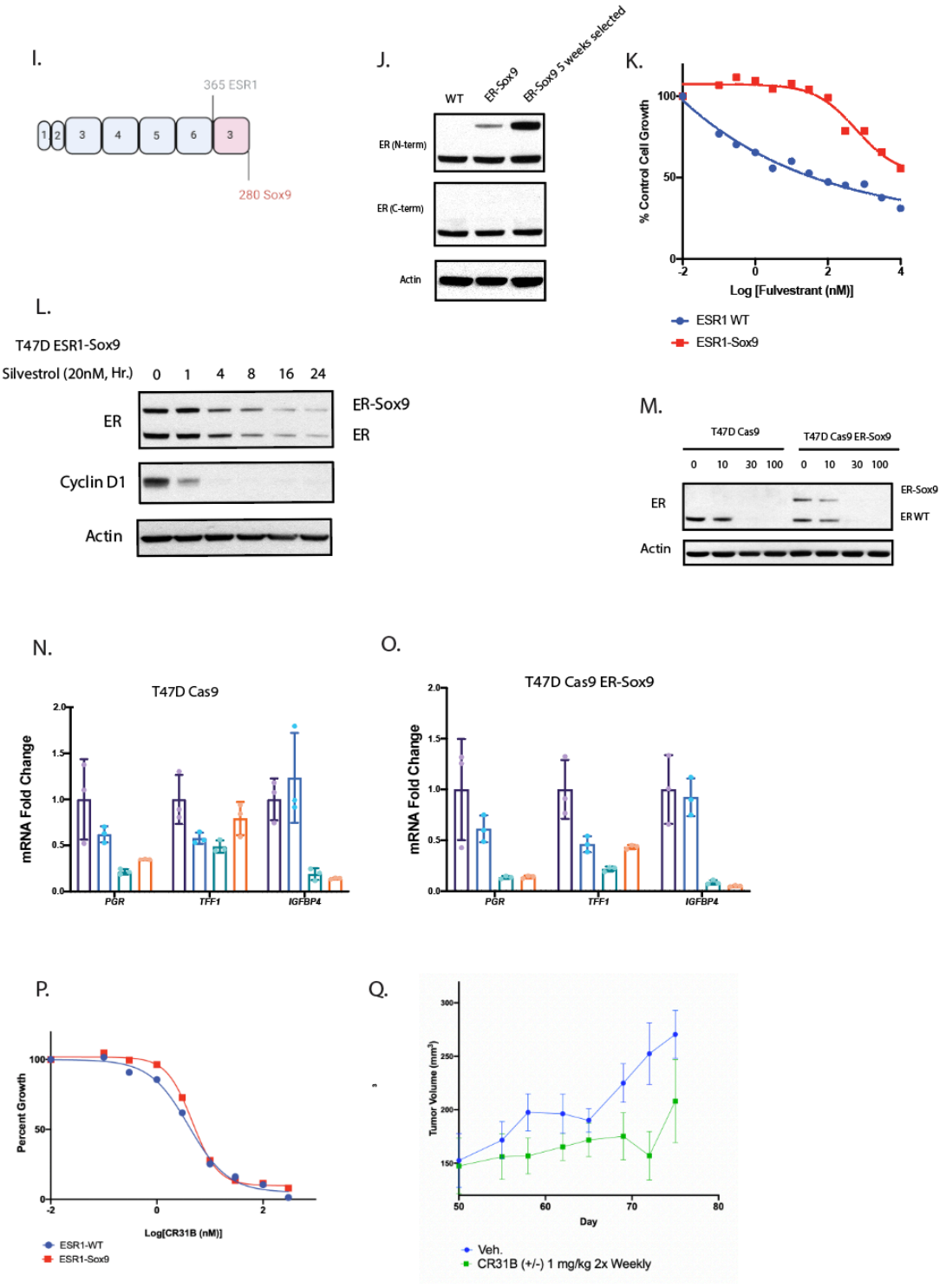

I.
365 ESR1
1
2
3
4
5
6
3
280 Sox9
J.
WT
ER-Sox9
ER-Sox9 5 weeks selected
ER (N-term)
ER (C-term)
Actin
K.
% Control Cell Growth
100
50
0
-2
0
2
4
Log [Fulvestrant (nM)]
ESR1 WT
ESR1-Sox9
L.
T47D ESR1-Sox9
Silvestrol (20nM, Hr.)
0
1
4
8
16
24
ER
ER-Sox9
ER
Cyclin D1
Actin
M.
T47D Cas9
T47D Cas9 ER-Sox9
0
10
30
100
0
10
30
100
ER
ER-Sox9
ER WT
Actin
N.
T47D Cas9
mRNA Fold Change
2.0
1.5
1.0
0.5
0.0
PGR
TFF1
IGFBP4
O.
T47D Cas9 ER-Sox9
mRNA Fold Change
2.0
1.5
1.0
0.5
0.0
PGR
TFF1
IGFBP4
P.
Percent Growth
100
50
0
-2
0
2
Log[CR31B (nM)]
ESR1-WT
ESR1-Sox9
Q.
Tumor Volume (mm³)
300
250
200
150
50
60
70
80
Day
Veh.
CR31B (+/-) 1 mg/kg 2x Weekly

Figure 5.2: EIF4A inhibition blocks expression of clinically significant ER variants

(A) MCF7 expressing either wild type ESR1 or ESR1 D538G were treated 24hr with increasing doses of CR31B. MCF7 were treated for indicated times with Silvestrol (20nM), Fulvestrant (30nM) or combination.

(B) MCF7 expressing either wild type ESR1 or ESR1 D538G were treated 24hr with increasing doses of Silvestrol.

(C) MCF7 ESR1 WT were treated 24hr with increasing doses of CR31B. mRNA levels of canonical ER targets were quantified via RT-qPCR

(D) MCF7 ESR1 D538G were treated 24hr with increasing doses of CR31B. mRNA levels of canonical ER targets were quantified via RT-qPCR

(E) MCF7 ESR1-D538G was treated for the indicated times with 20nM silvestrol.

(F) MCF7 or MCF7 ESR1 D538G were treated for 72hr with inc. doses of CR31B. Cell viability was quantified using ATPglo Luminescence.

(G) MCF7 ESR1 WT were treated for 72hr. with inc doses of CR31B, Fulvestrant, or increasing doses of Fulvestrant in the presence of fixed 3nM CR31B.

(H) MCF7 ESR1 D538G were treated for 72hr. with inc doses of CR31B, Fulvestrant, or increasing doses of Fulvestrant in the presence of fixed 3nM CR31B.

(I) Schematic showing CRISPR Cas9 constructed ESR1-Sox9 fusion. Numbers indicate the residues contributed by each protein (First 365 amino acids of ER and the last 280 of Sox9)

(J) Immunoblot showing expression of ER-Sox9 fusion protein in cells selected in estrogen free conditions for 5 weeks. Fusion protein can only be detected by probing with N-terminal targeted ER antibody.

(K) T47D Cas9 or T47D Cas9 ESR1-Sox9 were treated for 72hr with inc. doses of Fulvestrant. Cell viability was quantified using ATPglo Luminescence.

(L) T47D Cas9 ESR1-Sox9 treated for the indicated times with 20nM Silvestrol.

(M) Cas9 or T47D Cas9 ESR1-Sox9 were treated for 24Hr with increasing doses of CR31B .

(N) T47D Cas9 were treated 24hr with increasing doses of CR31B. mRNA levels of canonical ER targets were quantified via RT-qPCR.

(O) T47D Cas9 ESR1-Sox9 were treated 24hr with increasing doses of CR31B. mRNA levels of canonical ER targets were quantified via RT-qPCR.

(P) T47D Cas9 (H) or T47D Cas9 ESR1-Sox9 were treated for 72hr with inc. doses of CR31B. Cell viability was quantified using ATPglo Luminescence

(Q)NSG mice were implanted with estrogen pellets (0.18 mg) for 3d before injection of T47D Cas9 ESR1-Sox9 10million cells/mouse. Once tumors reached 100 mm^3 mice were treated twice weekly with CR31B (1mg/kg i.v.).

Chapter 6: Discussion

Conclusions

In the present study we have investigated the mechanisms of Estrogen Receptor Alpha translation and the potential therapeutic implications. We demonstrate that Estrogen Receptor Alpha can be translated in a cap/EIF4E independent manner. This explains why, despite being a short half-life protein, ER expression and activity can be maintained and even enhanced when PI3K/AKT/mTOR is inhibited. We show that the 5' UTR of ESR1, and especially that of transcript variant 1 is capable of driving a high degree of cap-independent translation. We hypothesize that this 5' UTR likely contains an authentic IRES element. Analysis of the 5' UTR predicted a highly favorable 2D confirmation, and the presence of abundant G-quadraplex elements. Consistent with these 5' UTR features, we find that ER translation depends on the RNA helicase and eukaryotic initiation factor, EIF4A. Small molecule inhibitors of EIF4A are capable of reducing ER expression and blocking ER dependent gene induction. EIF4A inhibitors block growth of ER+ breast cancer cell lines in the low nanomolar range. To reduce ER levels and tumor growth even further, we combined EIF4A inhibitors with selective ER degrader, Fulvestrant. This combination reduced ER levels to a minimum and exhibited powerful antitumor activity in breast cancer xenografts. Finally, we showed that EIF4A inhibition can block the expression of clinically significant ER variants which mediate resistance to endocrine therapies. We focused on the ER D538G mutant and an ER-Sox9 fusion. EIF4A inhibition blocked the expression of both variants with an attendant inhibition of tumor growth. Taken broadly, these

results suggest that ER is translated in a unique way, and that its continued expression depends on EIF4A, but not EIF4E. This EIF4A dependence is clinically exploitable using small molecule inhibitors of EIF4A.

Clinical Considerations

We have shown that targeting ER translation via EIF4A inhibition is a viable strategy to block growth of breast cancer models. This strategy is particularly effective when blocking ER synthesis is combined with inducers of ER degradation, such as Fulvestrant. EIF4A inhibitors were surprisingly well tolerated in vivo as indicated by the lack of weight loss in either the single agent or combined groups. EIF4A has been shown to regulate the translation of many important oncogenes, such as, Myc, Bcl2, MDM2 etc. As a result, EIF4A inhibitors have become an attractive target in many tumor types. Indeed at the time of this publication (Nov 2021), the company eFFECTOR therapeutics has initiated a multi-institutional dual phase I and II clinical trial using their eif4a inhibitor, eft226 (Zotatifin). The trial is currently recruiting patients with relapsed solid tumors, or tumors harboring activing variants of Her2, Her3, FGFR1/2 and KRAS. Their existing data suggested that these RTKs and KRAS are EIF4A dependently translated(Ernst et al., 2020). In addition to these inclusion criteria, we are gratified to have the opportunity to include a handful of endocrine resistant ER+ breast cancer patients in the MSKCC branch of the effector trial. We hope these patients respond, and will eagerly await the results of this study.

EIF4A inhibition has promise as a clinical target, but a number of factors likely inform the best setting for its use. Protein expression is a balance between synthesis and degradation. For long half-life proteins, blocking synthesis is likely an ineffective way of blocking protein expression. Therefore, an oncogene exhibiting a long turnover time is a comparatively poor target compared to a protein such as Myc, Cyclin D1, Bcl2 or ER. Targeting translation of a short half-life oncogene also affords flexibility in dosing schedule, as a short lived protein will be reduced in expression rapidly following drug administration. Conversely, a long half-life oncogene requires prolonged treatment before protein expression is decreased, which may require comparatively high/prolonged dosing. In the current study, we reasoned that combining an EIF4A inhibitor with a degrader of ER would further reduce the apparent receptor half-life and widen the therapeutic window. A treatment regimen of two compounds that work on opposite phases of ER expression may be a candidate for intermittent therapy. For example, based on our results, one might envision giving successive rounds of fulvestrant to reduce receptor levels, followed by an EIF4A inhibitor to block receptor synthesis. Less frequent, and lower effective doses of EIF4A inhibitors may mitigate potential toxicity associated with targeting a general eukaryotic translation factor. Most immediately, this implies that as we enroll patients harboring ER fusions, each protein must be considered empirically as far as half-life and translational dependence. Most fusions are likely to retain EIF4A dependent translation, as the 5' fusion partner is ER itself. However, the contribution of the 3' partner to protein half-life, and ultimately the response to EIF4A inhibition will have to individually

examined for each of the detected fusions. In the current study, it appears that coincidentally or otherwise, the ER-Sox9 fusion protein has a remarkably similar half-life to wild type ER, and this likely signifies this particular fusion as a potential EIF4A target going forward.

Analogies to Androgen Receptor

Following our discovery that ER is translated in an EIF4A dependent manner, we investigated a number of other nuclear receptors for the same requirement, most notably, androgen receptor (AR). AR is also a type I nuclear receptor and plays the analogous role of ER in driving prostate cancer. Similar also to ER, AR is a target of various anti-endocrine therapies which eventually lose effectiveness(Lu et al., 2020; Sobhani et al., 2018; Tan et al., 2015). One AR variant capable of producing endocrine resistance is the alternatively spliced product, AR variant 7 (AR-V7). Similar to ER fusions, this variant lacks the ligand binding domain (although in this case with no fusion partner), and signals independent of exogenous androgens. We tested the ability of EIF4A inhibitors to block AR expression. A simple but reproducible experiment demonstrated that indeed not only AR, but also AR-V7 were sensitive to EIF4A inhibition. Similar to ER, AR expression was not affected by selective mTORC1 inhibition, perhaps implying a degree of cap-independence. Many additional studies are required to put this finding into proper context, however preliminary data suggests a potential generality of EIF4A in regulating nuclear receptor translation.

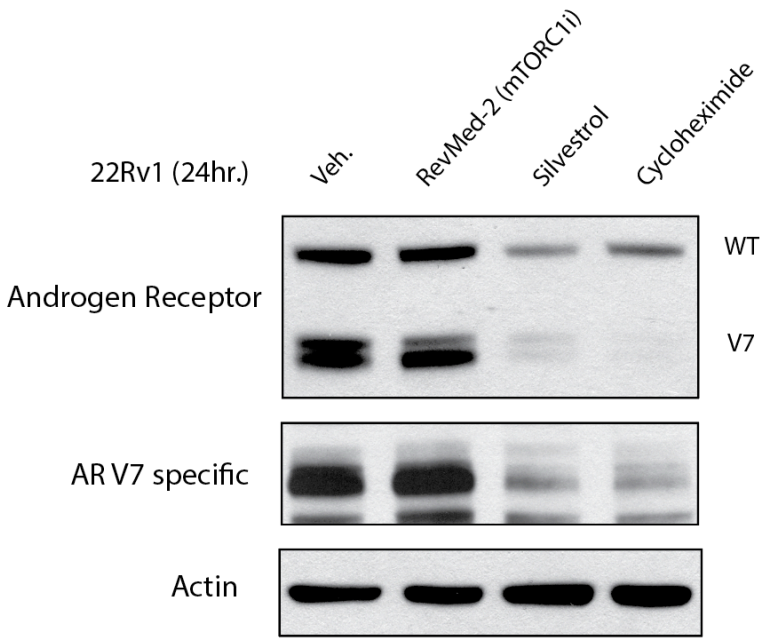


Figure 6.1: EIF4A inhibition blocks AR expression

(A) AR-V7 harboring cell line, 22rv1 was treated for 24hr with the RevMed-2 (100nM), Silvestrol (20nM) or cycloheximide (50ug/ml).

EIF4A vs EIF4E dependence

One consideration revealed by the current study is the complexity of characterizing proteins based on their EIF4E vs EIF4A dependence. These are not mutually exclusive categories. For example, Cyclin D1 depends on both EIF4E and EIF4A for its translation, whereas ER depends on EIF4A alone. Conversely, there are mRNAs which depend on EIF4E but not EIF4A. These include so called TISU elements, or translation initiator of short 5' UTRs, which are mRNAs with below average length 5'UTR elements, and can therefore initiate without abundant ATP required for scanning(Elfakess et al., 2011; Sinvani et al., 2015). There are mRNA that can initiate translation independent of both EIF4A and EIF4E. These include elements such as Hepatitis C Virus IRES (HCV), and some eukaryotic mRNAs as well(Leppek et al., 2018). Visually, this can be interpreted as concentric circles depicted below (Figure 6.2A). While each mRNA likely differs in the initiation factors used, the distinction of EIF4E vs EIF4A dependence is one axis along which mRNAs can be grouped, and this designation is likely particularly important when studying inhibitors of mTOR/EIF4E.

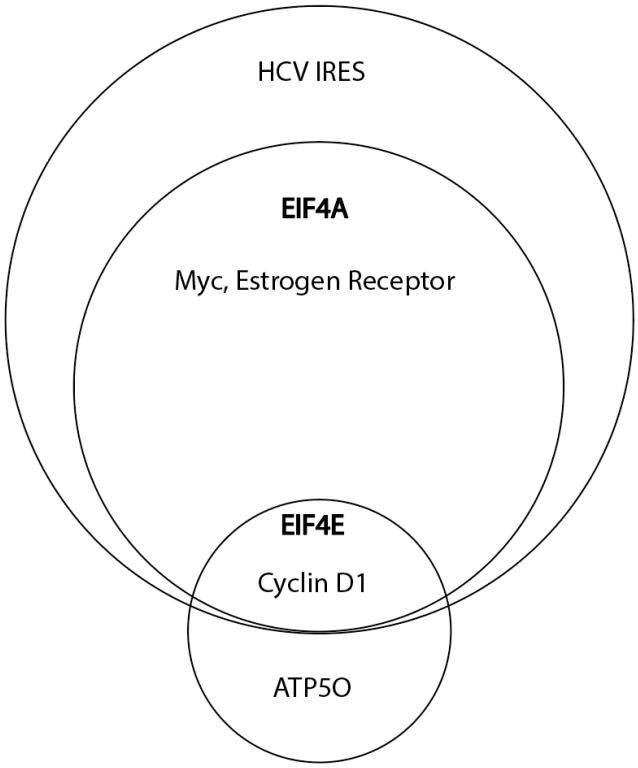


Figure 6.2: Concentric circles depicting EIF4E vs EIF4A dependently translated proteins

Limitations

Our study is subject to a number of important limitations, many of which can be addressed in the future. First, our study is cancer, and specifically breast cancer focused. Our understanding of EIF4A dependent ER translation is therefore limited to this case. We did test a number of endometrial and ovarian cell lines which have been said to express ER. In our hands, detection of ER in the endometrial models was a challenge that limited our ability to conclude anything about EIF4A dependent ER regulation in this context. One ER+ ovarian cell line, SKOV3, exhibited ER expression that was very low compared to ER+ breast cancer cell lines. Nonetheless, ER expression was similarly reduced by silvestrol treatment, and with the same kinetics as the breast cell lines (Data not shown). Whether this finding will extend to ER positive ovarian tumors in vivo is a question that requires further study. In addition, this study did not address whether ER is EIF4A dependently translated in normal tissue, breast or otherwise. These studies are currently ongoing, but must be undertaken using alternative detection methods for ER. Even in the tissues such as breast and ovary where ER plays an especially important role, only a minority of cells (up to 40%) express ER. Therefore, in the current approach, we are dosing healthy mice with EIF4A inhibitor and analyzing ER expression in various tissues via immunohistochemistry. In chapter 3.3 we discussed four ER transcript variants, which all include encode identical ER alpha protein. They are many additional transcript variants of ER alpha, and spliceoforms utilizing upstream promoters which may precede the coding region by multiple kb (NCBI Gene *ESR1*). The clinical significance of these additional ER variants is an

area of active investigation, but these isoforms are known to exhibit tissue specific expression, and likely context specific function(Flouriot et al., 2000; Kos et al., 2000; Reid et al., 2002). To what extent these variants are dependent on EIF4A vs EIF4E will need to be tested, especially in those variants using entirely distinct 5' UTR regions.

In this study, we demonstrated that ER can be translated in a cap-independent manner, and that the *ESR1* 5' UTR is sufficient to mediate such activity. Furthermore, we demonstrated that EIF4A is necessary for the functionality of this element to drive cap-independent translation. We did not however study the mechanism of cap-independence in detail. For example, throughout the written part of this work, we refer to this element as forming an IRES. This cannot be concluded with absolute certainty, however this was supported by abundant circumstantial evidence. First, the 5' UTR of *ESR1* is predicted to form a stable structure with similar parameters to that of other genes known to exhibit cap-independent translation. Second, we performed m6A immunoprecipitation and RNA sequencing to determine whether *ESR1* or other genes of interest were methylated in the 5' UTR. We did not observe ESR1 variant 1 to be adenosine methylated in the 5' UTR in either MCF7 or T47D (Data not shown). A substantial clarifier with respect to the mechanism of ER translation would arise if an ITAF with a known mechanism were found to participate. These ITAFs are in a sense analogous to transcription factors, and couple IRES elements to the ribosome and initiate translation using some subset of eukaryotic initiation factors. We have produced some very preliminary data to suggest that the ITAF,

ABCF1 might may a role in regulating ER translation. We performed siRNA knockdown of various general RNA binding proteins and ITAFs. Only ABCF1, and only in the context of mTOR inhibition seemed to reduce ER expression. More work will be needed to identify potential additional mediators of ER translation. A factor with more specificity to ESR1 translation may be identified and provide a better therapeutic target than EIF4A going forward.

Future Directions

In addition to clarifying the addressable limitations in our study, we will focus on one particularly interesting future direction. As outlined in the introduction, the major physiological regulator of mTOR is amino acid availability. Along side the presented data, we conducted many experiments examining the effect of amino acid starvation on ER expression. Similarly to direct mTOR inhibition, ER expression was robust to amino acid starvation (Figure 6.3A). This led us to ask two questions. The first, and the one addressed in this study, is: what is the mechanism of ER cap-independent translation? The second, and a source of ideas to be pursued in the future is: what is the source of amino acids for ER translation when exogenous amino acids are lacking? We hypothesized that macroautophagy (autophagy) derived amino acids might fuel translation under nutrient limiting conditions. To test this, we generated cell lines lacking ATG5, a protein required for autophagy. We then turned again to our methionine starvation and restimulation assay for translation. WT cells or cells lacking ATG5 were starved of methionine for 24hr followed by addition of media lacking all amino acids for time t. In response

to amino acid starvation, the ATG5 wt, but not knockout cells were able to undergo autophagy, as marked by productive LC3 lipidation. Important cap-independently translated oncoproteins such as ER, AR and Myc were capable of being resynthesized under conditions of amino acid starvation. In contrast, cyclin D1 could not be resynthesized during amino acid starvation. In the ATG5 KO cells, none of the proteins could be remade, likely indicating that autophagy derived amino acids were channeled to synthesize cap-independently translated proteins under nutrient limitation. It is my intention to continue this investigation during my postdoctoral studies. Specifically, my aim is to generalize our findings about estrogen receptor to other short half-life oncogenes whose expression requires continual translation. The source of amino acids fueling essential protein translation and the mechanism by which these essential proteins are translated during low nutrient conditions will be the focus of my future studies as a postdoctoral fellow.

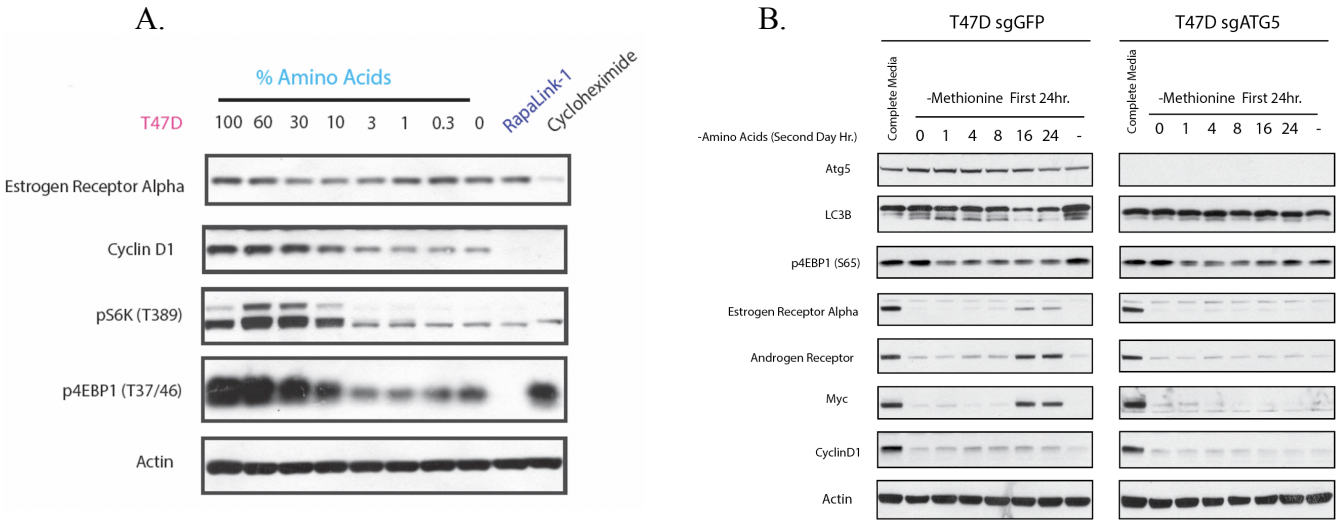


Figure 6.3: Autophagy fuels translation during amino acid limitation

(A) T47D were placed in DMEM F12 with the indicated percentage of amino acids relative to normal media. Cells were alternatively treated with RapaLink-1 (10nM) or cycloheximide (50ug/ml). All treatments performed for 24hr.

(B) T47D sgGFP or sgATG5 were starved of methionine for 24hr followed by replacement of media lacking all amino acids for time t.

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