Translational Control of Cancer:
An Exploration of eIF4E and its Role in Cellular Oncogenic Transformation

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the research sciences. As a graduate student, I have been fortunate to work as an LSSURP coordinator for two summers. The opportunity to interact with the talented students (and future leading scientists) was amazing. I will be a life-long supporter of LSSURP and similar programs.

Former fellow graduate student, Dr. Josephine Martin, Ph.D., and I decided to assemble a student group aimed at improving the lack of diversity within the University of Minnesota biomedical science community. We formed the Association of Multi-Cultural Scientists (AMS) with the intention of improving recruitment, retention and support for underrepresented minority scientists. Since the initiative's formation, AMS has taken off and its success has surpassed everyone's expectations. AMS now operates with a University funded budget and substantial student membership. I am grateful for the efforts of all those who were involved in the early development and current success of AMS (officers, faculty, staff, students, administration, etc.). I would like to especially acknowledge Dr. Robert Jones, Ph.D.; Dr. Jon Gottesman, Ph.D.; and Dr. Yoji Shimizu, Ph.D. Each of these individuals served a central role in AMS development, growth and operations.

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imaginable over the last several years, all of which have guided me to where I am today. Colin has not only offered his ear, but has always respected my opinions. I know I can count on him for an honest, caring assessment – which is often difficult to find. We have worked together in many different capacities over the last few years and his advice has been essential in my personal development. I will definitely miss our insightful conversations over drinks.

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Dedication

Over the last 5 years I have faced the greatest challenges of my life, thus far. Were it not for my strong belief in God as the source of my strength, and my foundation which is family- I simply “never would have made it”. My family has always held high expectations for me. Your belief in my abilities has continuously served to drive my ambition. I am inspired by all of you for various reasons – purity of character, achievements, talents, faith, perseverance, etc. With such an amazing group of individuals, possessing all of these traits, I never had to look beyond my own relatives for a role model. I am very grateful for everything that has been invested in me. I hope to continue making you all proud. For those younger than I and those still to come – I hope my accomplishments open doors and my experiences offer insight. However, do not simply retrace those footsteps lying before you. Find your *own* way. Discover your *own* passions. Pursue excellence within.

Therefore, I dedicate this dissertation to you all: Mom (Chandra), Dad (Jon), Chad, Blake, Gram (Callie), Ree (Marie), Granny (Evelyn), Grandad (Charles), Junie (James), Auntie (Charla), Kisha, Keewin, Barry, Lynn, Charles, Alan Sr., Briana, Kynton, Kyson, Kyla, Aaron, Tasha, Deion, Alan Jr., Jonathan, Kyle, Troy, Parrish, Jessica, Rochelle, Ryan, etc, etc, etc. (I have a lot of family:)

-mike

Abstract

Cancer is currently the 2nd leading cause of death in the U.S., responsible for approximately one quarter of the annual death rate. While cancer is genetically diverse, we suspect there may be common components of the cellular machinery where oncogenic signals converge, and that these critical regulatory nodes may represent molecular targets for new therapies. My approach derives from recent work on human breast and lung carcinoma, and murine models of lymphoid malignancies; which identify the cap-dependent protein synthesis machinery as a critical point of convergence and amplification of oncogenic signals emanating from the Ras/PI3K/Akt cascade. The rate limiting step in protein synthesis is initiation, mediated by the trimeric protein eukaryotic initiation factor 4F (eIF4F). The limiting component of eIF4F is the mRNA cap-binding protein, eukaryotic initiation factor 4E (eIF4E). Over expression of eIF4E transforms immortalized rodent fibroblasts and confers primary human cells with several cancer-related functions including decreased growth factor requirement for proliferation and survival, colony formation and anchorage independence. However, until very recently all experiments examining the oncogenic potential of eIF4E have involved constitutive over expression in stably transfected cell lines; leaving uncertain precisely which oncogenic functions could be ascribed directly to eIF4E, and which relate to its ability to suppress apoptosis – thus creating a permissive environment for subsequent oncogenic mutations. I have developed a high fidelity eIF4E inducible system which allows me to abruptly activate eIF4E over expression. With this system I am able to establish a direct connection

between eIF4E induction and autonomous cell proliferation. I have also been able to specify the cell cycle kinetics which occurs after eIF4E induction, including the ability of eIF4E to bypass growth factor initiation on the canonical proliferative pathway. The growth factor bypass properties of eIF4E are due to its ability to translationally activate cyclin D1, an occurrence I report for the first time. I also demonstrate the potential of pharmacological intervention targeting hyperactive translational activity. With the use of small molecular compounds which antagonize translation initiation, I demonstrate the ability to reverse eIF4E triggered proliferation in the eIF4E inducible system and the selective elimination of cancer cells with minimal toxicity in normal cells. This study has lead to a better understanding of how cap-dependent translation regulates cell proliferation and advanced the novel concept for cancer biology and therapeutics focused on translational control.

Table of Contents

List of Tables

List of Figures

Chapter 1.

Introduction

Cancer Epidemiology

Cancer is a group of diseases in which cells divide, move and survive autonomously; and is currently the 2nd leading cause of death among the U.S. general population (cdc.gov). Over the last 50 years cancer death rates have remained constant, while the median age of death has increased. Progress in cancer research and disease treatment is largely attributed for the increased life expectancy of cancer survivors. Recently the International Agency for Research on Cancer (IARC) released the December 2008 edition of the *World Cancer Report*, which shows that cancer will be the leading global cause of death by 2010. The American Cancer Society has acknowledged increasing cancer rates in developing countries as the next major challenge in the global fight against cancer.

The majority of high incidence cancers including breast, colorectal, prostate and lung are either chemo-resistant at the outset, or acquire this property when they recur (cdc.gov). Cancer is a genetic disease caused by multiple mutations leading to the acquisition of essential capabilities that define the malignant phenotype. Currently, there are approximately 300 genes causally implicated in carcinogenesis [(18)]. These genes, implied in carcinogenesis, have been observed to converge upon a small set of cellular pathways [(71)]. One approach to

developing effective cancer treatment is to identify regulatory hubs in the cell where signals from many different oncogenic pathways converge - for example a component of the cellular machinery that is regularly usurped by many cancers.

Difficulty in Cancer Treatment

Cancer chemotherapy is the treatment of cancer with chemical agents, and has long been plagued by cellular toxicity [39] and the onset of developed resistance to anti-cancer agents [15]. Most anti-cancer drugs target actively dividing cancer cells by preventing their progression through the cell cycle. These agents also target normal cells ranging from hair follicles, bone marrow, gastrointestinal lining, ovaries, and testicles- leading to adverse effects ranging from hair loss, diarrhea, mouth sores, low blood counts and death. Scientists have long attempted to avoid adverse effects by identifying cancer-specific characteristics for targeting. Identification of such targets, or regulatory hubs of cancer pathway convergence, would allow the selective elimination of cancer cells with a minimal effect on normal cells.

Translational Control: *Bona Fide Target Candidate*

A prime candidate for such a point of convergence is the cap-dependent translation initiation machinery. Traditionally, scientists have focused on genomic control, transcriptional regulation and post-translational modifications when studying cancer onset. Only in the last 15 years have scientists focused on translational control (protein synthesis) for cancer treatment.

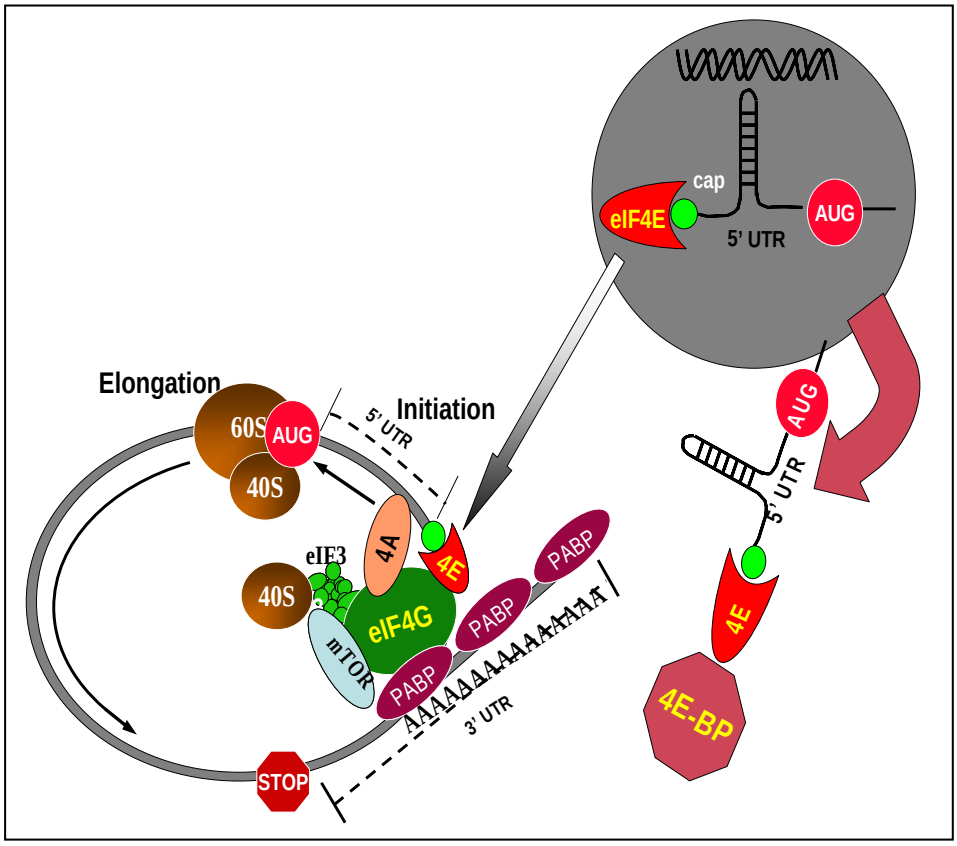
Translation and Protein Synthesis

Translation is the production of proteins by decoding mRNA produced by transcription. Translation occurs at the ribosomes, which are located in the cell's cytoplasm. Ribosomes are made up of a small and large subunit which surrounds the mRNA. In translation, messenger RNA (mRNA) is decoded to produce a specific polypeptide according its genetic code. The mRNA sequence serves as a template to guide the synthesis of a chain of amino acids that form a protein. Translation proceeds in four phases: activation, initiation, elongation and termination. Initiation is the rate limiting step of translation, mediated by the association of eukaryotic initiation factor 4F (eIF4F; a trimer consisting of the cap binding protein eIF4E, the eIF4G scaffold and the helicase eIF4A) with the 7-methyl guanosine cap at the transcript 5' terminus.

Binding of eukaryotic initiation factor 4E (eIF4E) to a 5', 7-methyl Guanosine mRNA cap facilities the vast majority of protein synthesis, and is known as Cap-dependent translation. Assembly of the eIF4F initiation complex is tightly regulated in normal conditions. The rate limiting factor, eIF4E, is often targeted for disruption to regulate the pathway. eIF4E Binding Proteins (4E-BPs) bind to eIF4E, preventing its association with scaffolding protein, eukaryotic initiation factor 4G (eIF4G). Once assembled, the initiation complex begins ribosome scanning (Figure 1).

Figure 1. Cap-Dependent Translation Pathway

Binding of eIF4E to the 5' mRNA cap structure facilitates nuclear export of selected transcripts, and primes mRNA for translation initiation when eIF4E associates with the docking protein eIF4G. The N-terminal part of eIF4G contains binding sites for eIF4E and the poly (A)-binding protein PABP. The phylogenetically conserved central third of eIF4G binds the helicase eIF4A, the multi-molecular complex eIF3 and RNA. The C-terminal portion of eIF4G has additional sites for binding eIF4A and the eIF4E-kinase Mnk-1 (not shown). The eIF3 complex associates in a growth-factor dependent manner with the kinase mTOR, its substrate the 40S ribosomal protein S6 and a component of the helicase system eIF4B. Thus, the eIF3 complex functions as a scaffolding platform to integrate the ribosome and several key initiation factors, and to coordinate their activity in response to regulatory signals. The resulting pre-initiation ribosome complex scans the 5' UTR to locate the initiation codon (AUG) where the 60S ribosome subunit joins to form an elongation competent 80S ribosome. The eIF4E/eIF4G interaction is negatively regulated by members of the 4E-BP family of translational repressor proteins. They compete with eIF4G for binding to eIF4E thereby sequestering capped transcripts into translationally inactive complexes.


cap
eIF4E
AUG
5' UTR
Elongation
60S
AUG
40S
5' UTR
Initiation
eIF3
40S
4A
4E
eIF4G
mTOR
PABP
PABP
PABP
AAAAAAAAAAAAAAA
3' UTR
STOP
AUG
5' UTR
4E
4E-BP

The eIF4E/eIF4G interaction is negatively regulated by members of the 4E-BP family of translational repressor proteins. Unphosphorylated 4E-BPs and eIF4G have similar binding affinity for, and share a common binding site on the eIF4E molecule [23]. 4E-BPs compete with eIF4G for binding to eIF4E, thereby sequestering capped transcripts into translationally inactive complexes. 4E-BPs are regulated by phosphorylation. The 4E-BP molecule contains 6 separate locations which may be phosphorylated, and 4E-BP phosphorylation is stimulated by growth factors and/or hormones. Once phosphorylated, the 4E-BP binding affinity for eIF4E is decreased. This leads to an increase in eIF4E binding to eIF4G, and progression of the translation initiation complex [53].

While most translation is cap-dependent, some mRNAs rely on internal ribosome entry sites (IRES) for initiation. In this mechanism of translation initiation, the 40S ribosomal subunit binds directly to a specific ternary structure within the 5' UTR of the mRNA, entirely bypassing the 5' end cap [52]. Ribosome shunting is another method in which initiation does not follow the mechanism seen in the cap-dependent scanning mechanism. In shunting translation initiation factors and the 40S ribosomal subunit assemble at the 5' end of the mRNA, begin to scan in the 3' direction and is given a signal enabling the complex to bypass a portion of the 5' UTR and continue scanning downstream closer to the initiating AUG [16]. Cap independent initiation of translation, mediated by IRES or ribosome shunting presents possible compensatory mechanisms in the case of interference with the eIF4F initiation complex.

Translational Control and Cancer

The positive and negative regulators of cap dependent translation control cell development, growth, and influence neuronal events controlling memory [53]. Pathological over expression of eIF4E has been shown to cause malignant conversion of rodent fibroblasts [35], human mammary epithelial cells [1] and promotes tumor formation in transgenic mice [59, 75]. Tumors isolated from humans express abnormally high levels of eIF4E [60]. Considering the malignant phenotypes caused by aberrant activation of eIF4E – it is logical that 4E-BP over expression would reverse its action. In this regard, not only does ectopically expressed 4E-BP negatively regulate the eIF4E transformed phenotype [57], but also hyper phosphorylated 4E-BPs are observed in human breast carcinoma cell lines [1].

Inequality in Translation

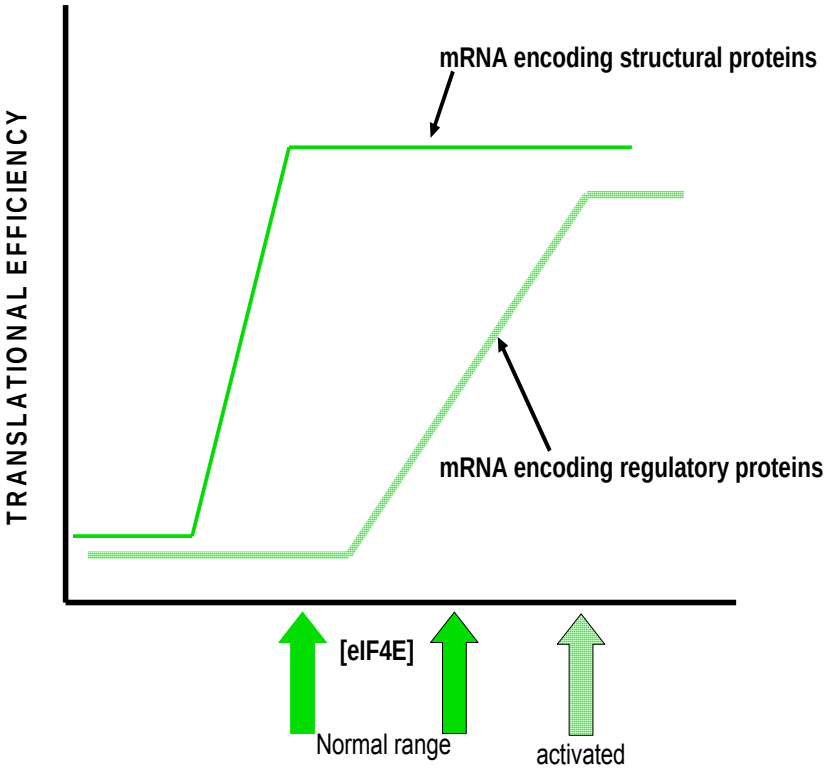
When the 4E-BPs prevent formation of the eIF4F complex by sequestering eIF4E [23] - there is a selective decrease in translation of certain mRNA's that require high levels of available eIF4E [20]. As a result, increased eIF4E activity does not globally elevate translation; instead it results in increased translation of a subset of mRNA [53]. The increased translation typically affects regulatory proteins which may be involved in cell cycle progression and other oncogenic functions. The shift in translation, however, does not appear to effect structural or "house-keeping" protein expression. Thus, activation of eIF4F by ectopically expressing eIF4E or eIF4G transforms rodent fibroblasts and promotes development of

lymphoma in the Eμ-Myc model of mouse B cell lymphoma, and down regulation of eIF4F by increasing 4E-BP reverts the malignant phenotype of breast carcinoma cells [1].

The theory of inequality in translation suggests eIF4E over expression facilitates the translation of regulatory mRNAs, which typically contain extensive secondary structures [31] (Figure 2).Therefore suppression of hyperactive eIF4E activity will selectively target mRNA responsible for cell cycle regulation. This theory provides an opportunity to selectively target mRNA involved in positive regulation of the cell cycle without harming uninvolved mRNA which are vital for systemic function.

Figure 2. Inequality in Translation

Hyperactive eIF4F selectively increases recruitment of ribosomes to regulatory mRNA transcripts. Increases in eIF4E, the limiting component of eIF4F, causes a selective increase in translation of mRNA encoding positive regulators of the cell cycle, survival and angiogenesis; without significantly changing translation of most structural proteins. This effect is mediated by physical-chemical characteristics of transcripts and by “user codes”, specific nucleotide sequences in the 5’ or 3’ UTR that regulate initiation and ribosomal scanning.


mRNA encoding structural proteins
TRANSLATIONAL EFFICIENCY
mRNA encoding regulatory proteins
[eIF4E]
Normal range
activated

The translation initiation machinery is tightly regulated during cell cycle progression

Cell cycle progression is controlled by a complex network of positive and negative regulatory proteins. Mutations in cell cycle regulatory proteins occur frequently in tumor cells and cell cycle deregulation is a universal feature of malignant transformation. eIF4E activity varies during entry and transit through the cell cycle [(65)]. 4E-BP1 (the most abundant member of the 4E-BP family) is bound to eIF4E during the S and G_2 phases as the cell prepares for mitosis, then dissociates from eIF4E during mitosis - only to bind eIF4E again after mitosis is complete. The bind and release action between 4E-BP1 and eIF4E is in coordination with the phosphorylation of 4E-BP1 at its Thr-70 and Ser-65 sites [(26)]. The mRNAs that preferentially associate with eIF4E during physiological cell cycle transit have not been directly identified, but the identity of such mRNAs have been studied in cells transformed by constitutive over expression of eIF4E [(65)].

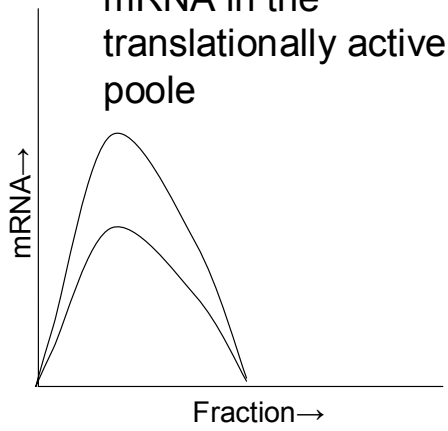
Polysome analysis provides insight in mRNA abundance, as well as its binding efficiency to ribosomes. Quantification with PCR (QTPCR) analysis enables us to detect and quantify relative mRNA levels. Fractionation separates mRNA samples by mass, which varies based on the average number of ribosomes bound per mRNA transcript, and allows us to detect translationally active mRNA based on its presence in heavy fractions. When plotting mRNA data in a graph, the x-axis represents increasing fraction mass, and the y-axis indicates mRNA

concentration (Figure 3). A vertical shift (peak movement along the y-axis) in mRNA analysis indicates an increase in total mRNA abundance, and is referred to as an “absolute” shift, while a horizontal shift (peak movement along the x-axis) in mRNA analysis indicates a change in ribosomal binding, and is referred to as a “relative” shift [32].

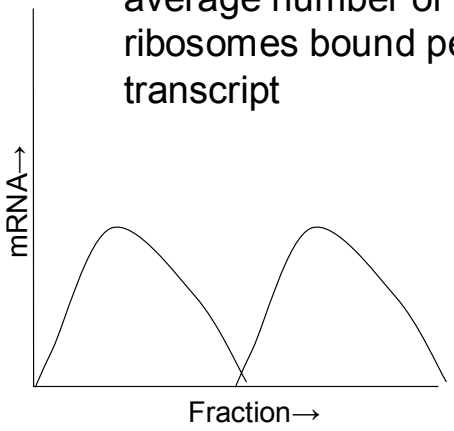
Figure 3. Polysomal Profile Analysis

The Absolute vs. Relative shift model describes possible changes in mRNA availability and translation. An absolute shift (change in polysomal profile along y-axis) indicates a change in the amount of mRNA available in the translationally active pool. A relative shift (change in the polysomal profile along the x-axis) indicates a change in the average number of ribosomes bound to the designated transcript. Most observations will contain both a relative and absolute shift, with a bias towards one.

- Absolute Shift
  - Increasing amount of mRNA in the translationally active poole



- Relative Shift
  - Increase in the average number of ribosomes bound per transcript



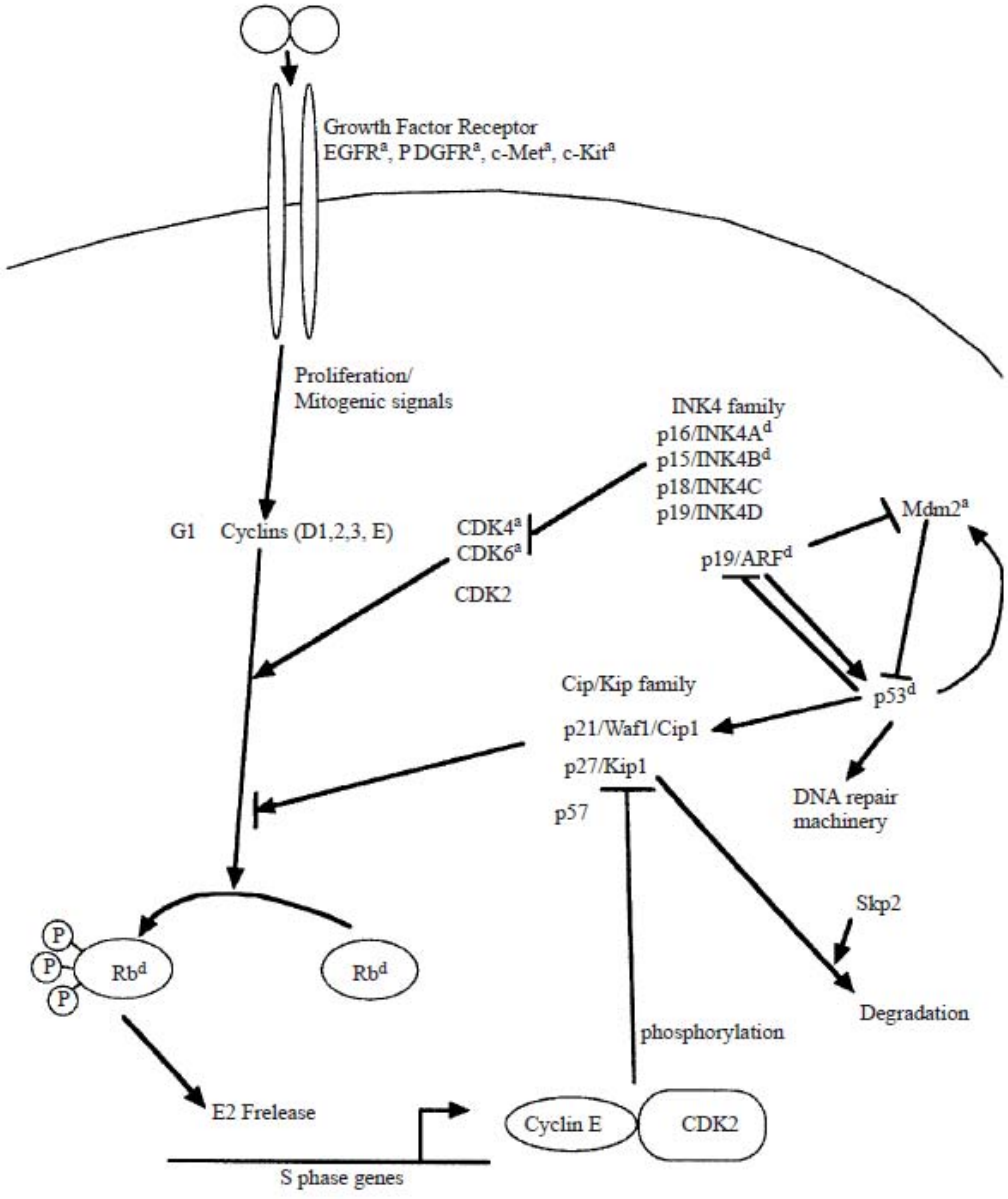
Transcripts showing enhanced translational efficiency as reflected by a shift in the average number of ribosomes bound per transcript include the cell cycle associated transcription factor, c-Myc, and the polyamine synthetic enzyme ornithine decarboxylase (ODC) [62]. Changes in translational rates for the critical regulator of the G_1-S transition, Cyclin D1, have not been reported. However, based on observing an absolute shift, previous research concludes increased cyclin D1 protein occurs through eIF4E facilitated nuclear-cytoplasmic mRNA transport rather than increased recruitment of ribosomes to its transcript [57].

Growth Factors Initiate Cell Cycle Progression

G_1 phase ushering of growth stimuli to cell cycle progression (Figure 4) is mediated by the mitogenic signaling induced transcription and translation of D-type cyclins, allosteric regulators of cdk4/6 [64]. The active cyclin D1/ cdk subunit, assembled in the cytoplasm, is translocated into the nucleus where it phosphorylates the retinoblastoma protein (Rb). Rb phosphorylation disenables its regulation of E2F-dependent transcription of genes required for S-phase entry, such as cyclin E [63].

Figure 4. The Canonical Growth Factor Activated Pathway

The well defined growth factor proliferative pathway is initiated by the binding of growth factors to a surface ligand. Growth factor binding activates cyclin D1 to bind cdk4/cdk6, then the active cyclin D1/cdk complex phosphorylates (and releases) Rb from the pleiotropic transcription factor, E2F. Freed from Rb inhibition, E2F carries out proliferative effects. The cdk complexes are antagonized by a complex of Ink and Kip inhibitors, which bind the kinase and prevent its ability to phosphorylate Rb protein.


Growth Factor Receptor
EGFR[a], PDGFR[a], c-Met[a], c-Kit[a]
Proliferation/
Mitogenic signals
INK4 family
p16/INK4A[d]
p15/INK4B[d]
p18/INK4C
p19/INK4D
G1 Cyclins (D1,2,3, E)
CDK4[a]
CDK6[a]
CDK2
Mdm2[a]
p19/ARF[d]
Cip/Kip family
p21/Waf1/Cip1
p27/Kip1
p57
p53[d]
DNA repair
machinery
Skp2
Degradation
P
P
P
Rb[d]
Rb[d]
phosphorylation
E2 Frelease
Cyclin E
CDK2
S phase genes

After G_1-S phase transition, cyclin D1 is rapidly phosphorylated by GSK3β on Thr-286. This phosphorylation of cyclin D1 causes immediate CRM1-dependent nuclear export [14]. Fbx4 recognizes the Thr-286 phosphorylated cyclin D1 which is subsequently poly-ubiquitylated and degraded by 26S proteasome [38].

Cell Cycle Regulation

The Rb & p53 pathways are the two primary regulators for cell cycle progression. The Rb pathway is composed of positive regulators cyclin D1, cdk4/6, and negative regulators p16/INK4A and Rb. Disruption of the normal function of a single component of the pathway (i.e. loss of p16/INK4A or Rb, or amplification of cdk4/6) is sufficient to lead to deregulated cell cycle progression and growth [74]. The p53 pathway is composed of p14/ARF (the human homologue of the mouse p19/ARF), the product of an alternative reading frame of the INK4a gene also encoding p16/INK4A , Mdm2, and p53. p14/ARF can activate p53 and promote the degradation of Mdm2, the latter sequesters and promotes p53 degradation, while p53 promotes Mdm2 expression and inhibits p14/ARF expression [4]. My studies will focus on the effects of deregulation within the Rb regulated pathway for proliferation.

Statement of Thesis

Over expression of eIF4E transforms immortalized rodent fibroblasts and confers primary human cells with several cancer-related functions; including decreased growth factor dependence for proliferation and survival, colony formation and anchorage independence. However, until very recently all experimental examination of the oncogenic potential of eIF4E had involved constitutive over expression in stably transected cell lines; leaving uncertain precisely which oncogenic functions could be ascribed directly to eIF4E over expression, and which relate to its ability to suppress apoptosis. This technological limitation has created a permissive environment for subsequent oncogenic mutations. While an eIF4E over expression model is clearly necessary to draw differences in comparison to cells which express endogenous levels, the continued over expression of eIF4E likely alters cellular characteristics and permits multiple mutations to accumulate. These pleiotropic alterations render the cell no longer comparable to normal cells expressing eIF4E at endogenous levels. To remedy this problem, I have developed an inducible cell system which has the ability to abruptly activate eIF4E over expression with mifepristone (progesterone antagonist) activation. This tool allows us to evaluate the kinetic effects of eIF4E over expression. Under these conditions I have the ability to examine detailed cellular mechanistic activity post eIF4E over expression.

Prior to my studies, the relationship between eIF4E activation and cell cycle machinery had not been systematically studied. In addition, it remained unclear if

there is a true cause and effect relationship between the activation state of eIF4E and cell proliferation, or if eIF4E is simply permissive by ensuring adequate levels of global protein synthesis required for the cell mass doubling required for cell division. In this dissertation, I have directly addressed the cause and effect question with the use of genetic and pharmacologic manipulation.

I utilized two independent approaches to regulate the function of the cap-dependent translation initiation apparatus. The first is genetic; such as ectopically expressing eIF4E or using RNAi to knock down components. The second is pharmacological. I have utilized compounds that: i) inhibit 4E-BP phosphorylation by targeting the regulatory mTOR kinase [78], ii) mimic 4E-BP activity (which bind/occupy the eIF4G binding site thus interrupting eIF4E/4G association) [41], iii) target the 7-methyl guanosine cap by binding eIF4E in place of the cap [19], thus preventing eIF4F complex assembly and translation initiation. I have used these tools to dissect mechanisms linking eIF4E to cell cycle regulation. With the use of the eIF4E inducible system, I have been able to examine the capability of these drugs to interfere with a fundamental oncogenic characteristic; autonomous cellular proliferation.

Completion of these studies have led to a better understanding of how cap-dependent translation regulates cell proliferation, and advanced a novel concept for cancer biology and therapeutics focused on translational control.

Chapter II.

Materials and Methods

Cell culture. NIH3T3 cells (designated “parental cells”) were maintained at 37^{o}C, 10% CO_2 in regular growth medium (Dulbecco's modified Eagle's medium [DMEM, Sigma] supplemented with 10% fetal bovine serum [FBS, Sigma], 25 mM Hepes, 100 units/ml penicillin, 100 units/ml streptomycin, and 125 ng/ml amphotericin [GIBCO]).

Inducible ectopic eIF4E system. Geneswitch NIH3T3 cells were purchased from Invitrogen and maintained in regular growth medium (previously described) supplemented with hygromycin (50ug/ml) at 37^{o}C, 10% CO_2. Plasmids encoding ***hemagglutinin (HA)***-tagged wild-type eIF4E [pKAS8-3] were linearized by digesting with SspI and transfected into log phase Geneswitch NIH3T3 cells. Two days post-transfection, cells were subcultivated and zeocin selection begun (500ug/ml zeocin). Transfected cells were incubated at 37^{o}C, 10% CO_2 under zeocin selection for 2-3 weeks to allow foci to form. Approximately 100 individual foci were isolated using 8mm cloning rings and expanded in growth medium under hygromycin and zeocin selection. For each clone, cells were added to 10 cm dishes, cultures continued overnight and the next morning were shifted to fresh medium with or without the inducer mifepristone (625pm final concentration). Cultures were continued for up to 48h and lysates were prepared.

Clones were screened for background expression of ectopic eIF4E without induction, and mifepristone-induced expression of HA-eIF4E by immunoblot as described in *methods* (Immunoblot analysis). Forty clones of Geneswitch NIH3T3 cells with mifepristone inducible eIF4E expression (designated “4E-inducible cells”) were expanded further and frozen under liquid nitrogen. To induce ectopic eIF4E, cells were incubated in serum free F-12 medium (serum starved) for 24 h, washed once with PBS and cultured for the indicated amount of time in serum free F-12 media with or without mifepristone (625nM).

Immunoblot analysis. To analyze protein abundance, cells were lysed with NP-40 lysis buffer, quantified using a BCA protein assay kit (Pierce). Equal amounts of cell extract protein per lane were subjected to SDS-PAGE, transferred onto nitrocellulose membranes, and identified using antibodies directed against; eIF4E (mouse monoclonal antibody, 1:500, Transduction Laboratories), cyclin E (mouse monoclonal antibody, 1:500, Santa Cruz Biotechnology), cyclin D1 (mouse polyclonal antibody, 1:500, BD Pharmingen), p27 (mouse polyclonal antibody, 1:2500, BD Pharmingen), p16 (mouse polyclonal antibody, 1:1000, BD Pharmingen), RB (mouse polyclonal antibody, 1:1000, BDPharmigen), 4E-BP1 (rabbit polyclonal antibody, 1:2000, BD Pharmingen), CDK 4 (mouse polyclonal antibody 1:1000, Cell Signaling), Lamin (mouse polyclonal antibody 1:1000, Cell Signaling) and Phosphorylated Rb Ser 780 (mouse polyclonal antibody 1:1000,

Cell Signaling). Actin (rabbit polyclonal antibody, 1:500, Sigma) served as a loading control.

Immunoprecipitation. To define the time course of cyclin D1/cdk4 complex formation after eIF4E induction, we followed established procedures to conduct a cyclin D1 pull-down assay [66, 77]. Lysates were collected and proteins quantified by immunoblot analysis (see above). Blots were probed first with mouse cdk4 (Cell Signaling, 1:500); stripped and re-probed for cyclin D1 (BDPharmingen, 1: 500).

Cell transfection. For stable transfection, cells were seeded into 35 mm wells of 6-well clusters. After 24 h, cells were transfected with 4 μg DNA using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. Cells were incubated in the presence of Lipofectamine 2000 for 6h, switched to regular growth (DMEM) medium containing 5% FBS for 24 h and cultures were continued in regular growth (DMEM) medium containing 10% FBS until use.

Cell based bicistronic assay. To quantify the global level of cap-dependent translation, we utilized a bicistronic reporter system (pcDNA-rLuc-polIRES-fLuc).

Cells were transfected with 1 μg of pcDNA3-rLuc-polio-fLuc. Cells were rinsed with PBS 24 h after transfection and induced to express ectopic eIF4E with mifepristone (625pM). After 24 h, cells were incubated with passive lysis buffer (Promega) for 15 min, cell debris was pelleted by centrifugation, and triplicate supernatant samples were assayed for Renilla and firefly luciferase activities in a Lumat LB 9507 luminometer (BG&G, Berthold, Germany) using the Promega dual luciferase reporter system.

Flow cytometry. For quantification of DNA content, cultured cells were detached with trypsin, washed with PBS, fixed with ice-cold 70% ethanol, and re-suspended in propidium iodide (PI) staining mixture as previously described [37, 48]. The percentages of cells with sub G1, G1, S and G2+M DNA content were determined on a FACSCalibur flow cytometer (Becton Dickinson) with the CellQuest program.

RNA preparation, fractionation and quantification. Polyribosome stratified RNA was fractionated and prepared as previously described [34]. Ten fractions of 0.5 ml were collected into tubes containing 50 μl of 10% SDS. RNA in the fractions was purified using Tri-reagent (Sigma), and reverse transcribed to cDNA using the TaqMan reverse transcriptase kit (Roche). To quantify RNA, Quantitative PCR (Q-PCR) was performed using the Roche Light-Cycler with SYBR Green dye (Roche). The primers were cyclin D1: 5′-GCGTACCCTGACACCAATCT and 5′-ATCTCCTTCTGCACGCACTT; and eIF4E: 5′-ACGTTCCAGATTACGCTGCT

and 5′-AGAGTGCCCACCTGTTCTGT. Before a primer pair was deemed acceptable for use in quantitative analysis, reaction products were subjected to gel electrophoresis to confirm the presence of a single PCR product of the appropriate length.

shRNA Viral Transduction. Lentiviral constructs expressing small hairpin RNA against mouse cyclin D1 (–NM_007631) were purchased from Open Biosystems (Huntsville, AL) for viral transduction of NIH 3T3 cells harboring the mifepristone inducible eIF4E construct. The target sequences of the three constructs used were:

Clone TRCN0000055233 – TCTAAGATGAAGGAGACCATT

Clone TRCN0000026883 - CCACGATTTCATCGAACACTT

Clone TRCN0000026948 - CCACAGATGTGAAGTTCATTT

Viral production and transduction was performed as follows. Each shRNA construct was transfected along with viral helper plasmids (pMDG & pCMVΔ8.91) into HEK 293 cells using the Fugene6 (Roche, Indianapolis, IN) transfection reagent at 1:3 DNA to Fugene6 ratio (w:v). 48 hours post transfection, media containing the virus was collected and filtered through a 0.45 μm filter. Media containing the virus was mixed with an equal volume of fresh growth medium containing 8 μg/ml polybrene (Sigma, St Louis, MO), and applied to confluent cells maintained in 6-well dishes which were centrifuged at 2000 rpm for 1 hour at room temperature and incubated at 37°C for 24 hours. After incubation, medium was removed, fresh growth medium was added and cultures were

continued 24 hours. Cells were induced with mifepristone and lysates were collected for immunoblot analysis of cyclin D1.

Sub-cellular fractionation. Protein analysis of cytoplasmic and nuclear compartments was conducted as previously described [58]. Following subcellular fractionation, lysates were analyzed for eIF4E (ectopic & endogenous), cyclin D1, cdk4 and actin by immunoblot. Subcellular fractionation for Rb was conducted with the NER-PER® Nuclear and Cytoplasmic Kit (ThermoScientific; Rockford, IL) as described in the product's protocol. Nuclear fractions were analyzed by immunoblot for phosphorylated Rb (ser 780) and nuclear Lamin.

RNA analysis. Parallel experiments proceeded identically for RNA isolation, substituting superase RNAse inhibitor (Applied Biosystems, 100 units/mL) for ROCHE complete tablets in all buffers. RNA from each fraction was purified using tri-reagent, reverse transcribed and subjected to Q-PCR.

Chapter III.

Inducible eIF4E System

Limitations in Prior Studies

A limitation of prior approaches to directly link eIF4E abundance with the acquisition of cancer related properties was the use of constitutive expression models *in vitro* and *in vivo,* which confounds the direct and indirect effects of eIF4E. Typically eIF4E was over expressed in 3T3 mouse fibroblasts, and then compared to naïve cells which expressed normal endogenous eIF4E levels. The differences observed in protein expression, mRNA abundance, proliferation and cell cycle activity, etc. were then attributed to eIF4E over expression. In order to define the direct cause and effect relationship between eIF4E and the acquisition of cancer-related functions, we realized the need to for a system in which we could abruptly activate eIF4E over expression, controlling the translation activation pathway.

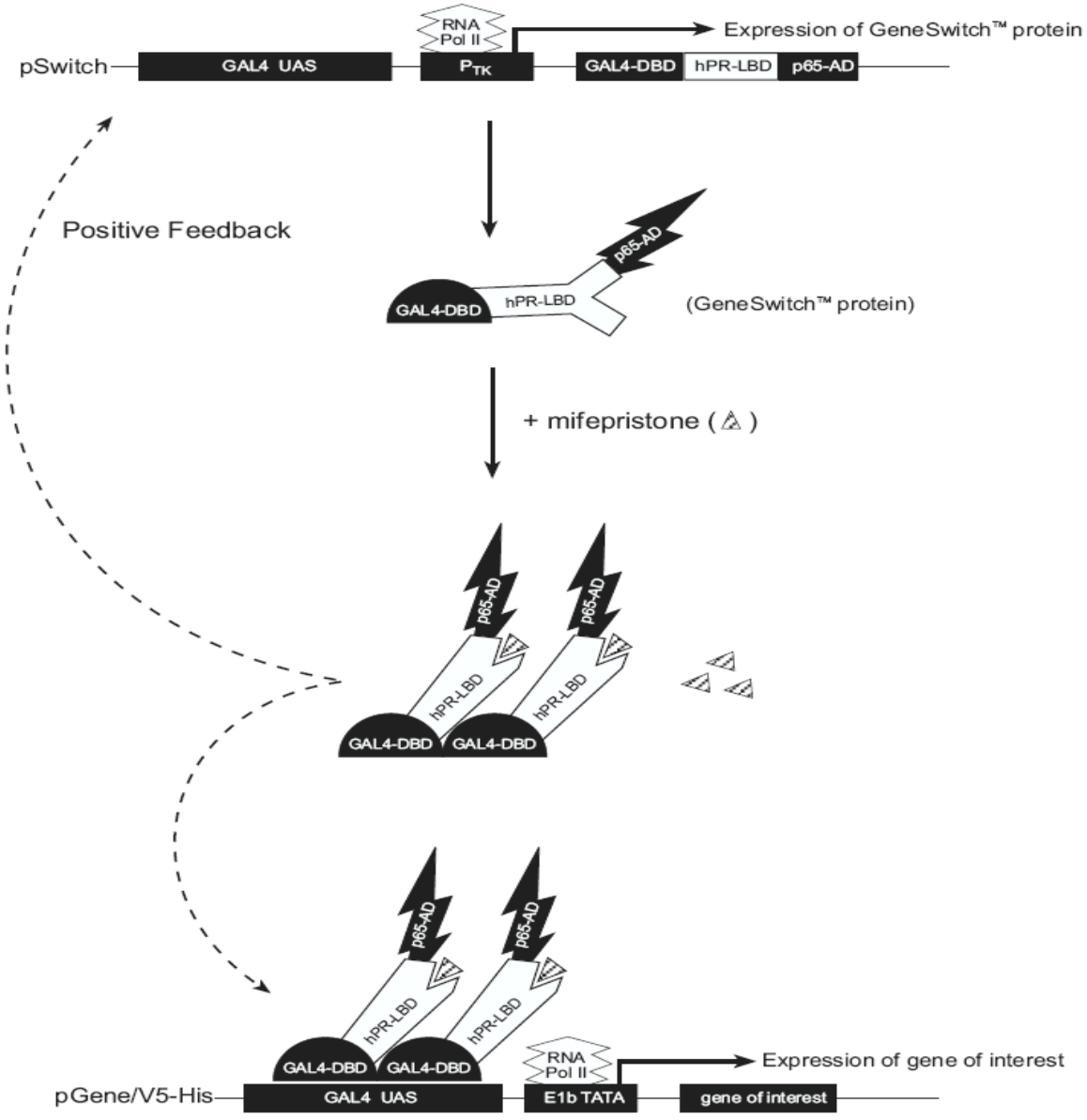
Mifepristone Activated eIF4E Induction

A previous study has utilized a tetracycline activated eIF4E inducible system to study 4E binding proteins, and its regulation through phosphorylation [(30)]. While the system offered promising potential- difficulty with leakage (ectopic eIF4E expression without activation) and varying eIF4E induction limited its use. We decided to develop a high-fidelity novel mifepristone activated eIF4E inducible

system free of leakage, while ensuring rapid and abundant eIF4E expression. We utilized the Invitrogen Geneswitch™ System (Figure 5) which includes cells conditioned to express the Geneswitch™ protein. The Geneswitch™ protein is activated by mifepristone; once bound by mifepristone, the Geneswitch™ protein-mifepristone complex binds Gal4 UAS to activate the expression of a particular gene of interest, in this case eIF4E.

Figure 5. Geneswitch™ Inducible System

The inducible regulatory system, purchased from Invitrogen, is transfected into Mouse 3T3 fibroblasts. The Geneswitch protein is activated by exposure to 625pM mifepristone. The Geneswitch regulatory protein is expressed from pswitch. After binding to mifepristone the geneswitch protein changes confirmation. The geneswitch protein-mifepristone complex binds GAL 4 UAS to activate expression of the gene of interest, in this case- ectopic HA-tagged eIF4E.


RNA Pol II
Expression of GeneSwitch™ protein
pSwitch
GAL4 UAS
P TK
GAL4-DBD
hPR-LBD
p65-AD
Positive Feedback
(GeneSwitch™ protein)
+ mifepristone ( )
Expression of gene of interest
pGene/V5-His
E1b TATA
gene of interest

Geneswitch NIH3T3 cells were purchased from Invitrogen and maintained in DMEM with 10% fetal bovine serum, penicillin G (1,000 units/ml), streptomycin (1,000ug/ml), amphotericin (2.5 ug/ml) and hygromycin (50ug/ml) at 37^{o}C, 10% $CO_{2.}$ pKAS8-3 was linearized by digesting with *SspI* and transfected into four-10 cm plates of Geneswitch NIH3T3 cells (~ 70-80% confluent) as described above. Two days post-transfection, the pKAS80-3 transfected Geneswitch NIH3T3 cells were split 1:4 into 15 cm plates and zeocin selection begun (medium as above with addition of 500ug/ml zeocin). The transfected plates were incubated at 37^{o}C, 10% CO_2 under zeocin selection for 2-3 weeks to allow foci formation and medium changed periodically as required. Approximately 100 individual foci were isolated using 8mm cloning rings and expanded under hygromycin and zeocin selection. For each clone, two 10 cm plates were plated and cells were incubated to adhere to the plate overnight; cells were then incubated or induced with mifepristone (625pM final concentration) for 48h and freeze-thaw lysates prepared. Clones were screened for background expression and mifepristone-induced expression of HA-eIF4E by immunoblot.

Defined Media to Study Proliferation

Very specific media conditions must be maintained in order to ensure cell viability without activating proliferation. To accomplish these conditions, I utilized a defined media recipe which is conducive to serum starvation and mifepristone administration. F12 media enhanced with 25mM Hepes, 0.1mg/mL BSA, 10ug Transferin, 10^{-8}M Selenium is prepared and stored @ 4°C. Before use, the

defined media is warmed to 37°C and 40ng/mL IGF & 5ng/mL EGF are added to the media. When activating eIF4E induction, 625nM Mifepristone is added to the media (1:1000 mif to media), reaching a final concentration of 625pM. The Defined Media is used for serum starvation, mifepristone induction, drug treatment and any other experimental conditions applied to the inducible cells.

Experimental Protocol for Inducible System

One challenge we faced is how to achieve quiescence while retaining cell viability, since it is well known that when serum growth factors are withdrawn from NIH-3T3 cells at low cell density, apoptosis is triggered within 16h; well before quiescence [48]. Therefore, to detect trophic signals in our system, we followed a protocol developed 3 decades ago [46] which takes advantage of NIH-3T3 cells ability to undergo one round of cell division in response to trophic signals after they have reached "density arrest". Clone 45 cells were plated at a density of 3500 cells/cm^2. Once cells reached a confluence of approximately 50% (~36 hrs after plating), cells were re-fed with growth media (DMEM + 10%FBS). Cells were then incubated and allowed to grow until 90-95% confluence (~36 additional hrs). Cells were monitored daily to avoid overgrowth. Overgrowth of the cells causes rapid apoptosis, rendering cells useless for experimentation. When cells reach total confluence (90-95% confluent) they were shifted to defined media (F12 + IGF + EGF) which lacks growth factors for 24 hrs. Treatment with serum free defined media synchronizes cells, allowing entrance into quiescence (G_1 cell arrest) without causing cell death. After the 24h

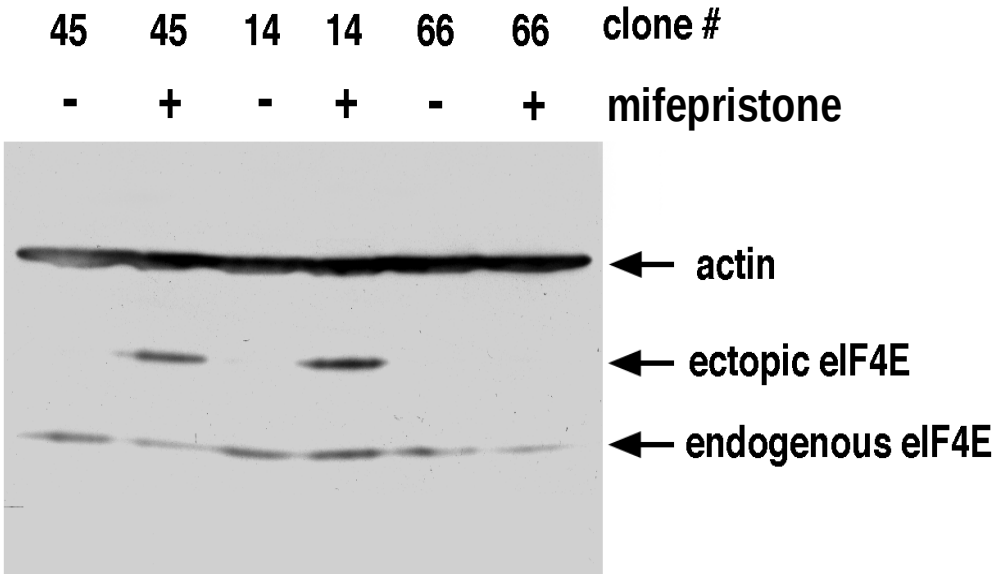
serum starvation, cells were prepared for mifepristone induction and subsequent experimentation. To induce eIF4E over expression, serum free media is replaced with fresh F12 media (+IGF, +EGF) containing mifepristone (625pM final concentration) for a minimum of 1h to induce ectopic eIF4E expression.

Validation of eIF4E Induction

Forty clones of Geneswitch NIH3T3 cells with mifepristone inducible eIF4E expression were expanded and frozen in liquid nitrogen. Of the forty clones detected to express mifepristone induced eIF4E, three of the clones (designated as clone 14, 45 & 66) were characterized under experimental conditions. Each of the clones was divided into two separate plates, grown to confluence and maintained as previously described. Once confluent and quiescent; one plate was treated with 625pM mifepristone for 24h, while the other remained in serum free media for the same duration. Cells were then harvested and lyates prepared. I observed maximal ectopic eIF4E expression in mif-induced clone 14, and a lesser (but substantial 2-fold) expression of ectopic eIF4E in clone 45 (Figure 6). Clone 66 had no ectopic eIF4E expression, and the endogenous eIF4E was similar in each clone. I also observed no leakage, or ectopic eIF4E expression without mifepristone induction, in each of the clones. This finding was significant as it proved the system was effective in over expressing eIF4E free of un-induced expression (leakage).

Figure 6. Protein Detection in eIF4E-Inducible Clones

Clones 14, 45 & 66 are maintained in regular growth medium (DMEM supplemented with 10% FBS), with or without mifepristone (625pM) for 24 h. Western blot is then conducted (see *methods*) to detect eIF4E (endogenous & ectopic). Analysis revealed ectopic (HA-tagged) eIF4E in clones 14 & 45. Shown is a representative immunoblot with actin shown as a loading control (5 independent replications gave similar results).


45 45 14 14 66 66 clone #
- + - + - + mifepristone
actin
ectopic eIF4E
endogenous eIF4E

I wanted to begin with three clones which each expressed ectopic eIF4E at high, medium and low levels. Clones 14, 45 and 66 met the requirements. Clone 14 was observed to express the highest amounts of ectopic eIF4E after mifepristone activation, in comparison to other clones. However, after continued cell culture and passages, clone 14 was observed to express eIF4E without mifepristone induction, also know as leakage. Clone 66 expressed virtually no ectopic eIF4E after mifepristone induction. My intentions were to utilize this clone as a negative control and compare its activity to cells which have a robust expression of ectopic eIF4E. Unfortunately, clone 66 induction was inconsistent in its inducible eIF4E expression, therefore causing me to discontinue its use. Clone 45 cells expressed roughly a 2-3 fold increase in eIF4E expression after induction. Most importantly, clone 45 cells were consistently activated to express ectopic eIF4E within 4hrs of mifepristone exposure. After continued and regular cell culturing, clone 45 cells also expressed no sign of leakage, which is vital to ensure abrupt activation of eIF4E. I decided to continue studies using clone 45 as the cell line to form the mifepristone inducible eIF4E cell system. Clone 45 proved ideal during pilot studies because it displayed 3 key properties: no leak, rapid expression of eIF4E after exposure to mifepristone, and a 2-fold increase in eIF4E levels closely mimicking the levels in cancer.

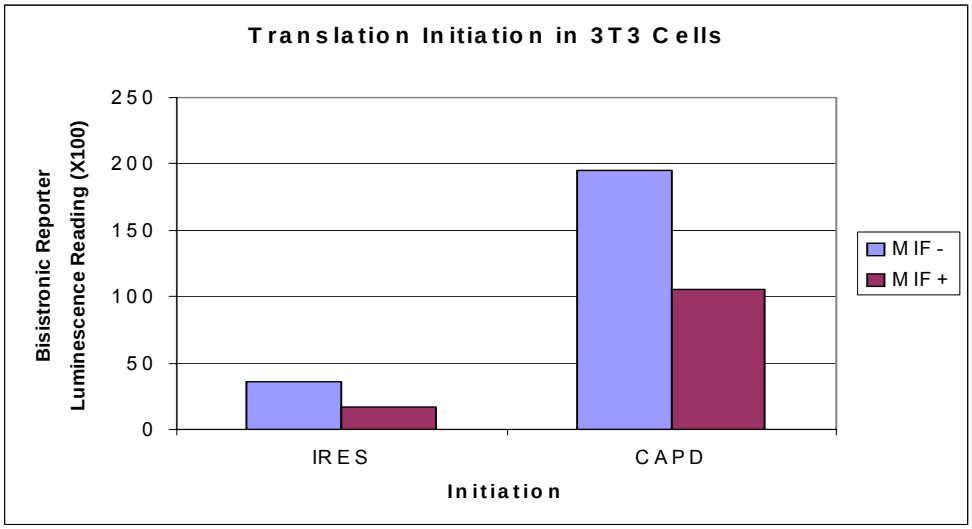
Validation of Translational Activation

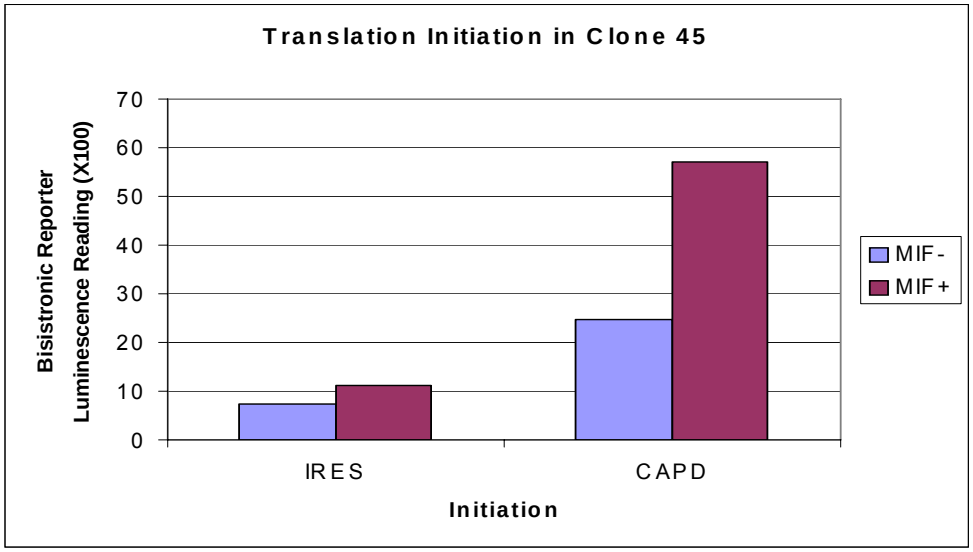
After demonstrating the ability to induce ectopic eIF4E in clone 45, I observed translational rates in order to verify activation of cap-dependent translation initiation with eIF4E over expression. To quantify the global level of cap-dependent translation, I utilized a bicistronic reporter system (pcDNA-rLuc-polIRES-fLuc). Cells were co-transfected with 1 µg of pcDNA3-rLuc-polio-fLuc and either wild-type or mutant pACTAG/HA-4E-BP1 (1 µg) or with 1 µg of pACTAG/HA vector. Cells were then rinsed with PBS 24 h after transfection and induced to express ectopic eIF4E with mifepristone (625pM). After 24 h, cells were incubated with passive lysis buffer (Promega) for 15 min, cell debris was pelleted by centrifugation, and triplicate supernatant samples were assayed for *Renilla* and firefly luciferase activities in a Lumat LB 9507 luminometer (BG&G, Berthold, Germany) using the Promega dual luciferase reporter system. Over expression of rate limiting factor, eIF4E, implies increased translational activity. Quantification of cap-dependent and internal ribosomal entry site (IRES)-mediated translation was conducted to establish the translational impact of mifepristone induced eIF4E over expression in Clone 45 cells. Translational activity was measured with the use of a bicistronic reporter system and using procedures established in our laboratory [(1)]. 3T3 fibroblasts and 4E inducible cells were maintained in identical conditions to measure both cap dependent and IRES mediated translation. Cells were either induced with mifepristone for 24 hrs or maintained in serum-free defined media. 3T3 cells exhibit a decrease in both IRES and cap dependent translation in response to mifepristone. However when

Clone 45 cells are treated with mifepristone, there is a slight increase in IRES and a 3-fold increase in cap dependent translation (Figure 7). Together these data confirm inducible cells respond to mifepristone induction with up regulation of ectopic eIF4E and increased translational activity, specifically cap dependent translation. Continued culture of serum-starved parental 3T3 cells was accompanied by an overall decreasing trend in both cap-dependent and cap–independent translation.

Figure 7. eIF4E Induction Stimulates Translational Activity

3T3 and WT cells were plated at a density of 250K/ well in a 6-well plate and transiently transfected with pcDNA3-rLuc-polio-fLuc for 24 hours. Cells were then treated with/ without 625pM mifepristone for 24 hrs, rinsed, collected and lysed. Renilla luciferase activity (cap-dependent translation) and firefly luciferase activity (IRES-mediated translation) were measured in a Lumat luminometer with the Promega dual luciferase reporter system. Data is shown for translational rates in 4E-inducible cells, and parental 3T3 cells as a reference.


Translation Initiation in 3T3 Cells
Bisistronic Reporter
Luminescence Reading (X100)
250
200
150
100
50
0
IRES
CAPD
Initiation
MIF-
MIF+

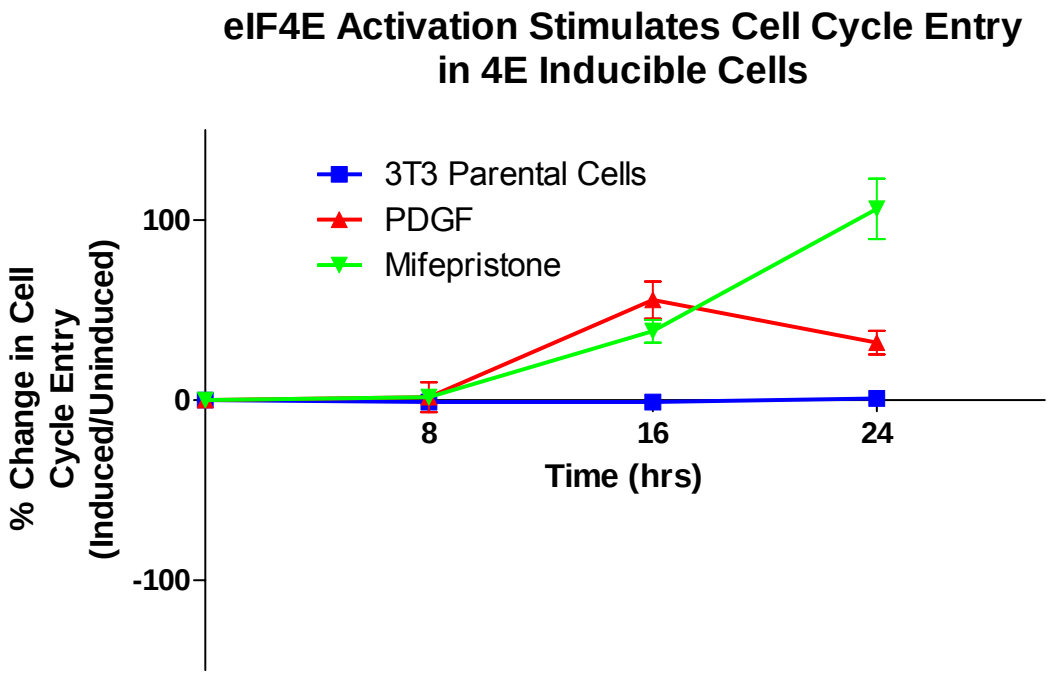

Translation Initiation in Clone 45
Bisistronic Reporter
Luminescence Reading (X100)
70
60
50
40
30
20
10
0
IRES
CAPD
Initiation
MIF-
MIF+

eIF4E Induction Triggers Cell Proliferation

As a prototype for a cancer related function, I chose autonomous proliferation. Cells were grown to confluence and quiesced (or synchronized in G0 arrest) by "density arrest" in growth medium (DMEM plus 10% FBS). Once quiesced, cultures were continued for 0, 8, 16, and 24 hours in serum free defined medium: i) lacking PDGF (negative control), ii) supplemented with PDGF (50pM; positive control) or iii) supplemented with the inducer Mifepristone (625pM). At the indicated time cells were harvested, fixed with ethanol and stained with propidium iodide. I measured the percentage of actively dividing cells, as % Cell Cycle Entry (CCE) with flow cytometry. Sixteen hours post treatment; I observed ~50% increase in CCE (%S phase +% G2/M phase) in cells incubated with PDGF. I observed a slightly lower percentage of mif-induced cells in CCE at the same time point (~40%). By 24h post treatment, we observed a 100% increase in CCE in mif-induced cells, while the % CCE in cells treated with PDGF actually decreased from 16 h (Figure 8). I attribute this finding to the pleiotropic effects of mif-induced eIF4E on proliferation, in comparison to the lesser effect of a single agent growth factor, PDGF. Cells with eIF4E over expression continue to divide while the efficacy of PDGF reaches a maximal peak, which is eventually exhausted.

Figure 8. eIF4E Induction Triggers Cell Cycle Entry

Cells are plated at a density of 3500/cm², incubated @ 37° C for 7 days in complete medium (DMEM plus 10% FBS). Resultant cells are confluent with no mitotic cells visible by phase contrast microscopy. Medium is removed and replaced with defined media (F12, containing Hepes, BSA, Transferin, selenite, linoleic acid; supplemented with IGF 40ng/mL ($5.3x10^{-8}$M) and EGF 5ng/mL ($7.5x10^{-10}$M) for 24h which are needed to preserve viability but not sufficient to trigger cell cycle entry. Quiescent cells are incubated for 24h in defined medium: i) lacking PDGF (negative control- black line), ii) supplemented with PDGF (50pM; positive control- red line), or supplemented with the inducer Mifepristone (625pM, experimental variable- red line)). DNA content is quantified by flow cytometry to calculate the proportion of cells in each phase of the cell cycle progression. Shown is the % of cells in S+G_2/M in each experimental condition. 3T3 Parental cells undergo identical conditions. At 16h, cells enriched with PDGF display maximum amount of CCE- but by 24h cells activated with mifepristone display a 2-fold increase CCE, exceeding that of cells treated with PDGF.


eIF4E Activation Stimulates Cell Cycle Entry
in 4E Inducible Cells
3T3 Parental Cells
PDGF
Mifepristone
% Change in Cell
Cycle Entry
(Induced/Uninduced)
100
0
-100
8
16
24
Time (hrs)

This finding is also significant as it is in accord with prior reports which demonstrate that even after prolonged serum starvation, 3T3 cells continue to enter S phase of the cell cycle [44]. This experiment shows that eIF4E stimulates cell cycle entry and ultimately proliferation. 3T3 Parental cells were used to control for of off-site target effects of mifepristone induction, and displayed no significant change in proliferation.

Chapter IV.

Mechanism Paper

This manuscript documents my work with the inducible eIF4E system, and describes the cell cycle kinetics which occur after mifepristone induction. This chapter was submitted for Publication in *Molecular and Cellular Biology*, March 2009.

eIF4E Mediated Cell Cycle Entry Requires Translational Activation of Cyclin D1

Jon M Underwood, Yong Y Kim, Jose R Gomez, Karen A Smith, Mark S Peterson, Vitaly A Polunovsky, Peter B Bitterman

Abstract

The cap-dependent translation initiation apparatus, eukaryotic initiation factor 4E (eIF4E) functions as a regulatory hub in the pathway to cancer; however, the pleiotropic effects of eIF4E on cell biology have complicated studies to elucidate the mechanism of eIF4E-mediated oncogenesis. We exploited an inducible system to abruptly increase eIF4E expression and determine if there was a direct connection between eIF4E abundance and a defining property of cancer, autonomous cell proliferation. By ectopically expressing eIF4E under control of a mifepristone inducible promoter, here we show that eIF4E triggers cell cycle entry in quiescent cells by increasing the translation of cyclin D1 mRNA through 2 mechanisms: i) increased ribosome recruitment; and ii) stimulation of nuclear export leading to increased cyclin D1 mRNA in the translationally active pool. Using shRNA targeting cyclin D1, we found that eIF4E triggered cell cycle entry was strictly dependent on translational activation of cyclin D1, and subsequently followed the canonical growth factor pathway to phosphorylation of the retinoblastoma protein. These data establish the molecular mechanisms linking increased levels of cellular eIF4E to proliferative autonomy – one defining property of malignancy.

Introduction

Cancer results from a series of somatic mutations that override or relax physiological controls on proliferation, survival and other critical life cycle and morphogenic events. As molecular mechanisms have been elucidated, most cancers show surprising genetic diversity despite remarkably uniform biology. The prototypical autonomous properties of a cancer cell are thought to emerge from several hundred cancer-associated genes that function in only a few, well defined cancer pathways [73]. One component of the cellular machinery that resides at a point of convergence of many oncoproteins – and is regularly usurped in cancer - is the cap-dependent translation initiation apparatus, eukaryotic initiation factor 4F (eIF4F). Initiation is the rate limiting step of translation, mediated by the association of eIF4F (a trimer consisting of the cap binding protein eIF4E, the eIF4G scaffold and the helicase eIF4A) with the 7-methyl guanosine cap at the transcript 5' terminus. Positive and negative regulators of cap dependent translation control cell development, growth and survival [53]. However, pathological over expression of eIF4E, the rate limiting component of eIF4F, causes malignant conversion of rodent fibroblasts [35] and human mammary epithelial cells [3] ; and promotes tumor formation in transgenic mice [59, 75]. Tumors isolated from humans express abnormally high levels of eIF4E [60] along with inactivated, hyper-phosphorylated forms of the eIF4E antagonists, the 4E-BP family of translational repressors. Pertinent to cancer biology, when the 4E-BPs prevent formation of the eIF4F complex by sequestering eIF4E [23], there is striking selectivity - with only a small proportion

of cellular mRNAs showing a substantial change in translation [20]. This translationally sensitive subset is highly enriched with genes assigned oncogenic functions [53].

The pleiotropic effects of eIF4E on the full range of cancer-related functions have made elucidating mechanism a challenge. In studies focused on individual genes of interest governing proliferation, activation of eIF4E was found to increase ribosome recruitment to c-Myc and ornithine decarboxylase [62]; and enhance nuclear-cytoplasmic transport of the cyclin D1 transcript [57]. In genome-wide translational profiling studies utilizing combined polyribosome-microarray analysis, functions assigned to eIF4E-sensitive genes are strongly skewed towards survival, apoptosis, morphogenesis and proliferation [33, 34]. However, all of these studies have been limited by reliance on established cell lines constitutively over expressing eIF4E, which precludes assignment of a specific cell function to a direct translational effect on a gene or group of genes.

A recent study exploited a tetracycline-inducible system to increase eIF4E expression and examine translational control of genes linked to the acquisition of apoptosis resistance [30]. We adopted a similar approach to determine if there was a direct connection between eIF4E abundance and a defining property of cancer, autonomous cell proliferation, and to define the mechanism. By ectopically expressing eIF4E under control of a mifepristone inducible promoter, here we show that eIF4E triggers cell cycle entry by increasing the translation of

cyclin D1 mRNA through 2 mechanisms: i) stimulation of nuclear export leading to increased cyclin D1 mRNA in the translationally active pool; and ii) increased ribosome recruitment. These data establish the mechanisms linking increased levels of cellular eIF4E and proliferative autonomy – a hallmark of malignancy.

Results

Induction of eIF4E triggers cell cycle entry.

To elucidate the mechanisms by which increased eIF4E abundance confers cells with cancer-related properties, we developed a switchable system in which ectopic, hemagglutinin tagged eIF4E (HA-eIF4E) could be induced with the progesterone antagonist, mifepristone. As a prototype cancer related function, we studied autonomous cell cycle entry. One challenge we faced was how to achieve quiescence while retaining cell viability. Prior reports indicate that when serum growth factors are withdrawn from NIH3T3 cells at low cell density, apoptosis is triggered within 16h; well before quiescence [48]. Therefore, to detect a positive effect of eIF4E on proliferation in our system if it existed, we followed a validated protocol [46] which takes advantage of the ability of serum-starved NIH3T3 cells to enter the cell cycle in response to trophic signals after “density arrest”. We screened more than 50 clonal cell lines and selected one line (clone 45) displaying no detectable baseline expression of ectopic eIF4E and a rapid 2-fold increase in eIF4E after exposure to mifepristone (approximating the levels observed in many cancers) [60] (Figure 9A, B). To determine whether the ectopic

HA-eIF4E was functional, we examined both cap-dependent and cap-independent translation using a bicistronic reporter mRNA in which translation of Renilla luciferase is cap-dependent and translation of Firefly luciferase is mediated by an internal ribosomal entry site. After addition of mifepristone to serum-starved parental NIH3T3 cells lacking the inducible eIF4E construct, both cap-dependent and cap–independent translation trended downward ($p<0.09$, both compared to the pre-mifepristone baseline; note negative numbers, Figure 9C). In contrast, in cells harboring the inducible eIF4E construct, addition of mifepristone led to an increase in both cap-dependent and cap-independent translation (reflecting the pleiotropic effects of eIF4E); with a statistically significant increase in cap-dependent translation compared to the pre-mifepristone baseline ($p<0.05$).

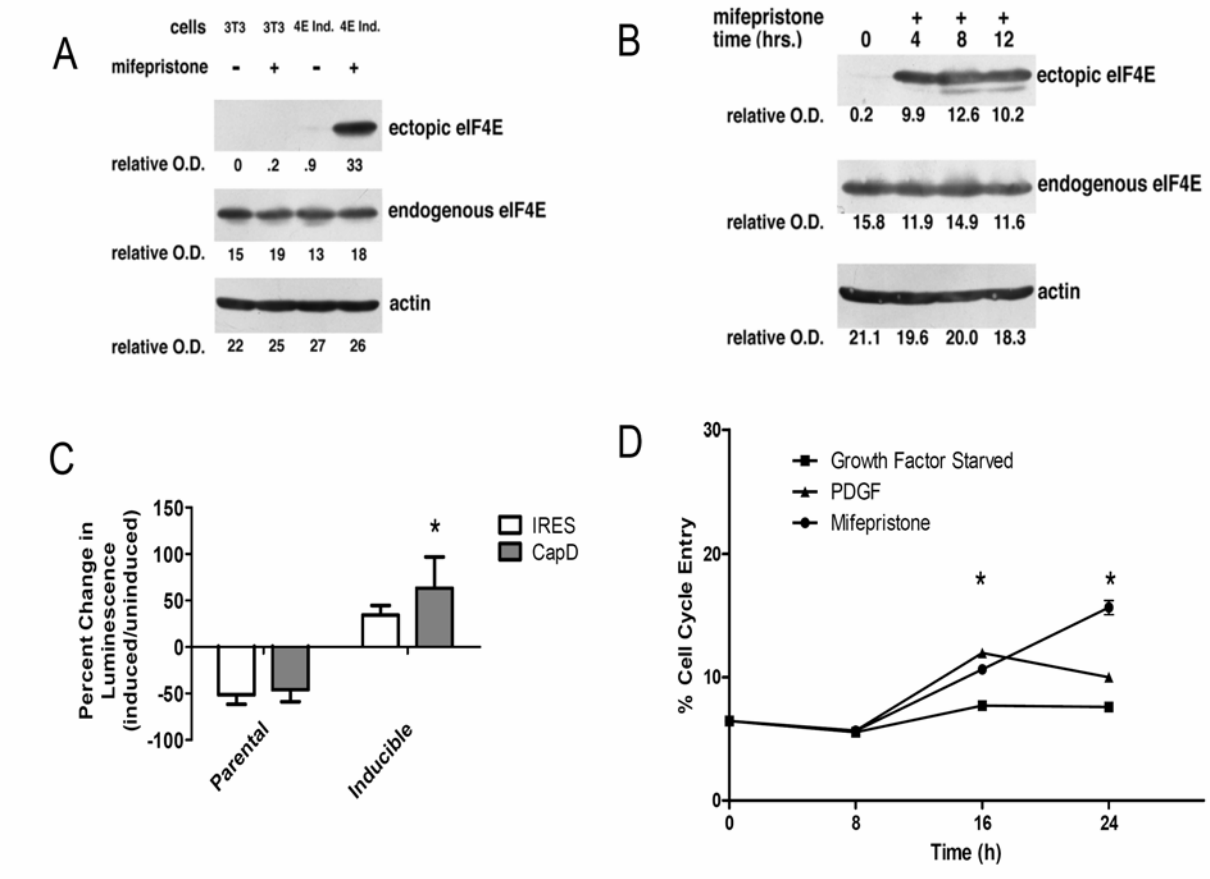
To examine the effects of eIF4E induction on cell cycle entry, cells were cultured in complete medium (DMEM plus 10% FBS) until confluent, continued for 24 hours in "defined medium" (F12 supplemented with IGF and EGF to preserve viability) and switched to defined medium: i) lacking PDGF (negative control), ii) supplemented with PDGF (50pM; positive control) or iii) supplemented with the inducer mifepristone (625pM). We quantified cell cycle entry by flow cytometry. In accord with prior reports, we observed a small, but persistent proportion of cells in S-phase, even at high density after serum starvation [(44)]. Sixteen hours after mifepristone addition, 11% of 4E-inducible cells were in S phase reaching a maximum of 15% after 24 h (Figure 9D). In contrast, un-induced cells never

exceeded 7% S phase entry during the period of observation. To exclude a clone-specific effect, we examined 3 additional clones and observed similar results. Mifepristone treatment of parental NIH3T3 cells had no impact on cell cycle distribution (not shown). These data establish a direct relationship between eIF4E abundance, translational activation and cell cycle entry.

Figure 9. Validation of Translational and Proliferative effects after eIF4E Induction

A. Mifepristone induction of eIF4E. Quiescent cells were incubated with mifepristone (625pM, 24h), lysed and both ectopic (HA-tagged) and endogenous eIF4E was quantified by immunoblot. Shown is a representative immunoblot with actin as a loading control (6 independent replications gave similar results). For all blots shown, numbers below each lane represent relative abundance determined by densitometry. **B. Kinetics of eIF4E induction.** Quiescent cells were incubated with mifepristone (625pM) for the time interval indicated. Cells were lysed and both ectopic (HA-tagged) and endogenous eIF4E was quantified by immunoblot. Shown is a representative immunoblot with actin as a loading control (4 independent replications gave similar results). **C. Quantification of translational activity.** Parental NIH3T3 and eIF4E inducible cells were transiently transfected with pcDNA3-rLuc-polio-fLuc, rendered quiescent and incubated with or without mifepristone for 24h. Both Renilla luciferase activity (cap-dependent translation) and firefly luciferase activity (IRES-mediated translation) were measured in a Lumat luminometer with the Promega dual luciferase reporter system. Results are shown as percent change (+/- SEM) in translation after induction compared to un-induced. *, $p<0.05$ (student's t test). **D. eIF4E induction triggers cell cycle entry.** Quiescent cells were incubated for 24h in defined medium: i) lacking PDGF (negative control; square symbol), ii) supplemented with PDGF (50pM; positive control; triangle symbol), or

supplemented with the inducer mifepristone (625pM; circle symbol). Cells were fixed and DNA content was quantified by flow cytometry. Shown is the percent of cells in S+G2/M as a function of time. Each data point is the average of triplicates from a single experiment; and the data shown are representative of 6 independent experiments that produced similar results. *, $p<0.01$ (student's t test). NIH3T3 parental cells showed no change in proliferation after mifepristone addition (data not shown).

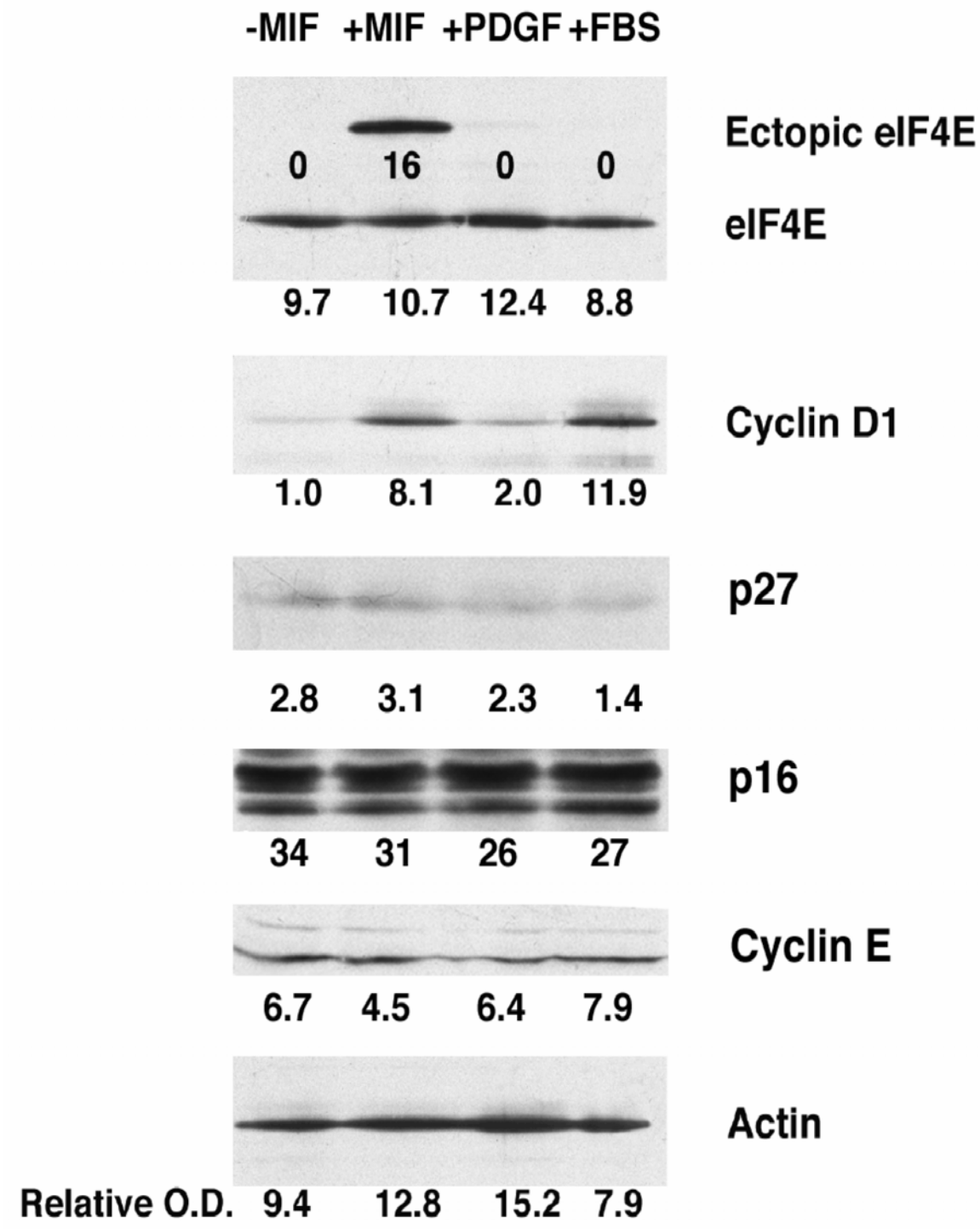

A
cells 3T3 3T3 4E Ind. 4E Ind.
mifepristone - + - +
ectopic eIF4E
relative O.D. 0 .2 .9 33
endogenous eIF4E
relative O.D. 15 19 13 18
actin
relative O.D. 22 25 27 26
B
mifepristone + + +
time (hrs.) 0 4 8 12
ectopic eIF4E
relative O.D. 0.2 9.9 12.6 10.2
endogenous eIF4E
relative O.D. 15.8 11.9 14.9 11.6
actin
relative O.D. 21.1 19.6 20.0 18.3
C
Percent Change in Luminescence (induced/uninduced)
150
100
50
0
-50
-100
*
IRES
CapD
Parental
Inducible
D
% Cell Cycle Entry
30
20
10
0
Growth Factor Starved
PDGF
Mifepristone
*
*
0
8
16
24
Time (h)

Increased cyclin D1 is required for eIF4E-triggered cell cycle entry.

Cyclins, their partner kinases, inhibitors and targets are known to regulate cell cycle entry and progression into S phase [25]. To examine those that were altered after eIF4E induction - and therefore might be implicated in the mechanism of its positive proliferative effects, we induced cells with mifepristone and surveyed key cell cycle proteins governing the G1/S checkpoint. To calibrate the response range, we utilized PDGF as a single trophic agent and serum as a pleiotropic stimulus. Among the cell cycle regulators surveyed, only cyclin D1 changed in an appropriate time frame prior to the onset of DNA synthesis (16h) – increasing 8-fold in abundance (Figure 10).

Figure 10. Analysis of G1/S checkpoint Regulators after eIF4E Induction

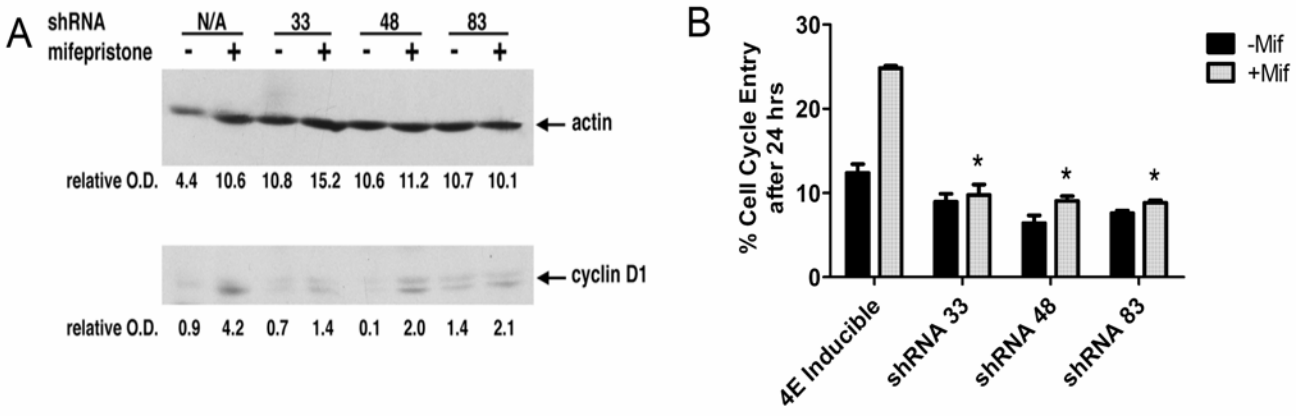
Quiescent cells were cultured in defined medium with or without 625pM mifepristone for 24h, lysed and subjected to immunoblot analysis for a panel of G1/S checkpoint regulators. Fetal bovine serum (FBS) and platelet derived growth factor B (PDGF) served as positive controls. Shown is a representative immunoblot with actin as a loading control (6 independent replications gave similar results).


-MIF
+MIF
+PDGF
+FBS
Ectopic eIF4E
0
16
0
0
eIF4E
9.7
10.7
12.4
8.8
Cyclin D1
1.0
8.1
2.0
11.9
p27
2.8
3.1
2.3
1.4
p16
34
31
26
27
Cyclin E
6.7
4.5
6.4
7.9
Actin
Relative O.D.
9.4
12.8
15.2
7.9

Prior studies document that introduction of cyclin D1 into quiescent primary hepatocytes is sufficient to trigger cell cycle entry [42, 43]. Therefore, it was in principle possible that eIF4E triggered cell cycle entry might be mediated solely by the observed increase in cyclin D1, or that the ability of eIF4E to promote cell cycle entry was pleiotropic. To address this issue, we used an RNAi approach to knockdown cyclin D1 after induction of eIF4E. We utilized 3 different shRNA for this purpose, each targeting a different region of the cyclin D1 transcript. We achieved knockdown of cyclin D1 ranging from 64% to 85% (Figure 11A); with a concomitant abrogation of eIF4E-triggered cell cycle entry in each case, including modest but reproducible suppression of basal S phase entry in the un-induced state (Figure 11B). Thus, eIF4E triggered cell cycle entry cannot be rescued by the pleiotropic effects of eIF4E on global translation – and depends upon the ability of eIF4E to increase cyclin D1.

Figure 11. Increased Cyclin D1 is Required for eIF4E-triggered Cell Cycle Entry

shRNA mediated knockdown of cyclin D1. A. NIH3T3 cells harboring the inducible eIF4E construct were virally transduced with one of three lentiviral shRNA constructs, each targeting a different region of the cyclin D1 mRNA (designated shRNA 33, 48 or 83). Empty lentiviral vector is used as a control. Quiescent cells were cultured in defined medium with or without 625pM mifepristone for 24h, lysed and subjected to immunoblot analysis for cyclin D1. Shown is a representative immunoblot with actin as a loading control (3 independent replications gave similar results). **B. Cyclin D1 knockdown attenuates eIF4E-mediated cell cycle entry.** Inducible cells expressing one of the 3 shRNA constructs or empty vector (control) were rendered quiescent, and incubated for 24h in defined medium with or without 625pM mifepristone, fixed and DNA content was quantified by flow cytometry. Shown is the percent of cells in S+G2/M +/- SD. *, p=0.001 (student's t test).


A
shRNA
N/A
33
48
83
mifepristone
- + - + - + - +
actin
relative O.D. 4.4 10.6 10.8 15.2 10.6 11.2 10.7 10.1
cyclin D1
relative O.D. 0.9 4.2 0.7 1.4 0.1 2.0 1.4 2.1
B
% Cell Cycle Entry after 24 hrs
30
20
10
0
-Mif
+Mif
*
*
*
4E Inducible
shRNA 33
shRNA 48
shRNA 83

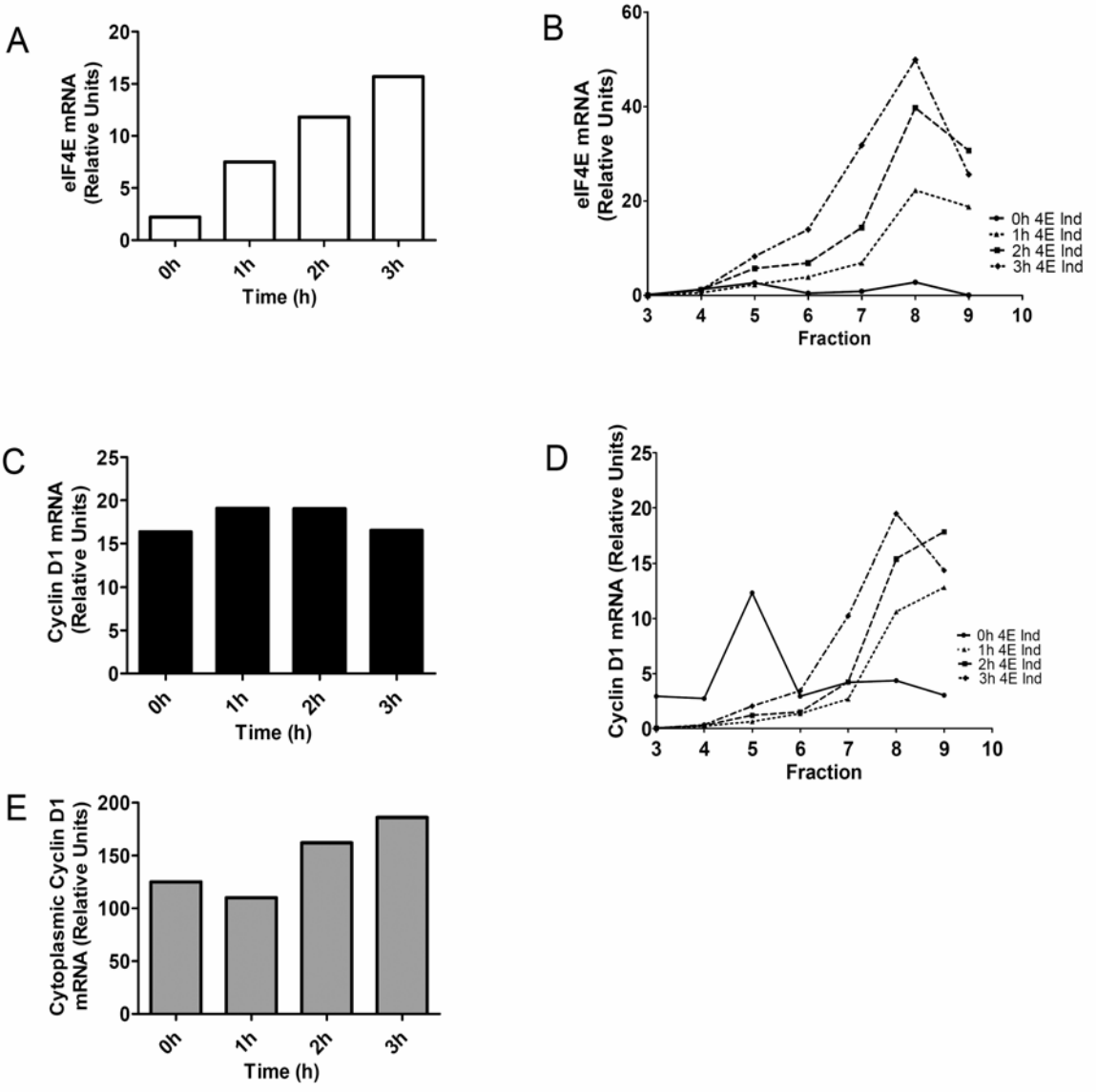
eIF4E increases ribosome recruitment to cyclin D1 mRNA.

Prior studies indicate that when established NIH3T3 cell lines constitutively express eIF4E, cyclin D1 abundance is increased through eIF4E facilitated nuclear export of the cyclin D1 mRNA, rather than through increased recruitment of ribosomes [(57)(56)]. To examine the mechanisms that operate when a cell with physiological eIF4E regulation is suddenly confronted with an enforced doubling of its eIF4E content, we monitored eIF4E and cyclin D1 mRNA abundance, association with ribosomes and sub-cellular distribution after addition of mifepristone. As expected, there was a rapid increase in ectopic eIF4E mRNA assessed by Q-PCR using primers specific for ectopically expressed eIF4E, containing the HA tag. (Figure 12A). This was accompanied by a progressive increase in the abundance of eIF4E mRNA in the polyribosome bound pool, with no change in the average number of ribosomes bound per transcript (note the progressive increased amplitude of the Q-PCR polyribosome profile with no shift in the position of its peak, Figure 12B). Cyclin D1 mRNA abundance did not change after eIF4E induction (Figure 12C). Instead we observed a rapid and striking shift of the cyclin D1 transcript from lighter into heavier polyribosome fractions within 1h, with no increase in cytoplasmic cyclin D1 abundance, indicating increased ribosome recruitment (Figure 12D, E). This was followed by a progressive increase in the amount of cyclin D1 mRNA in the polyribosome bound pool over the ensuing 3 h (Figure 12D), with a concomitant shift in the subcellular distribution of cyclin D1 mRNA into the cytoplasm, accounting for the progressive increased amplitude of the Q-PCR polyribosome profile with no

further shift in the position of its peak (Figure 12E). These results indicate that eIF4E powerfully activates cyclin D1 translation in 2 ways: i) by increasing ribosome recruitment; and ii) by facilitating its export from the nucleus into the cytoplasm.

Figure 12. Increased Ribosome Recruitment to the Cyclin D1 Transcript after eIF4E Induction

Quiescent cells were incubated with mifepristone (625pM) for up to 3h. Shown is the relative abundance of: **A.** Total ectopic eIF4E mRNA. **B.** Polyribosome fractionated ectopic eIF4E RNA. **C.** Total cyclin D1 mRNA. **D.** Polyribosome fractionated cyclin D1 RNA. **E.** Cyclin D1 mRNA in the cytoplasmic subcellular fraction. RNA was quantified by Q-PCR as a function of time after induction. Each data point/bar is the result of a single experiment; and the data shown is representative of 4 independent experiments that produced similar results.

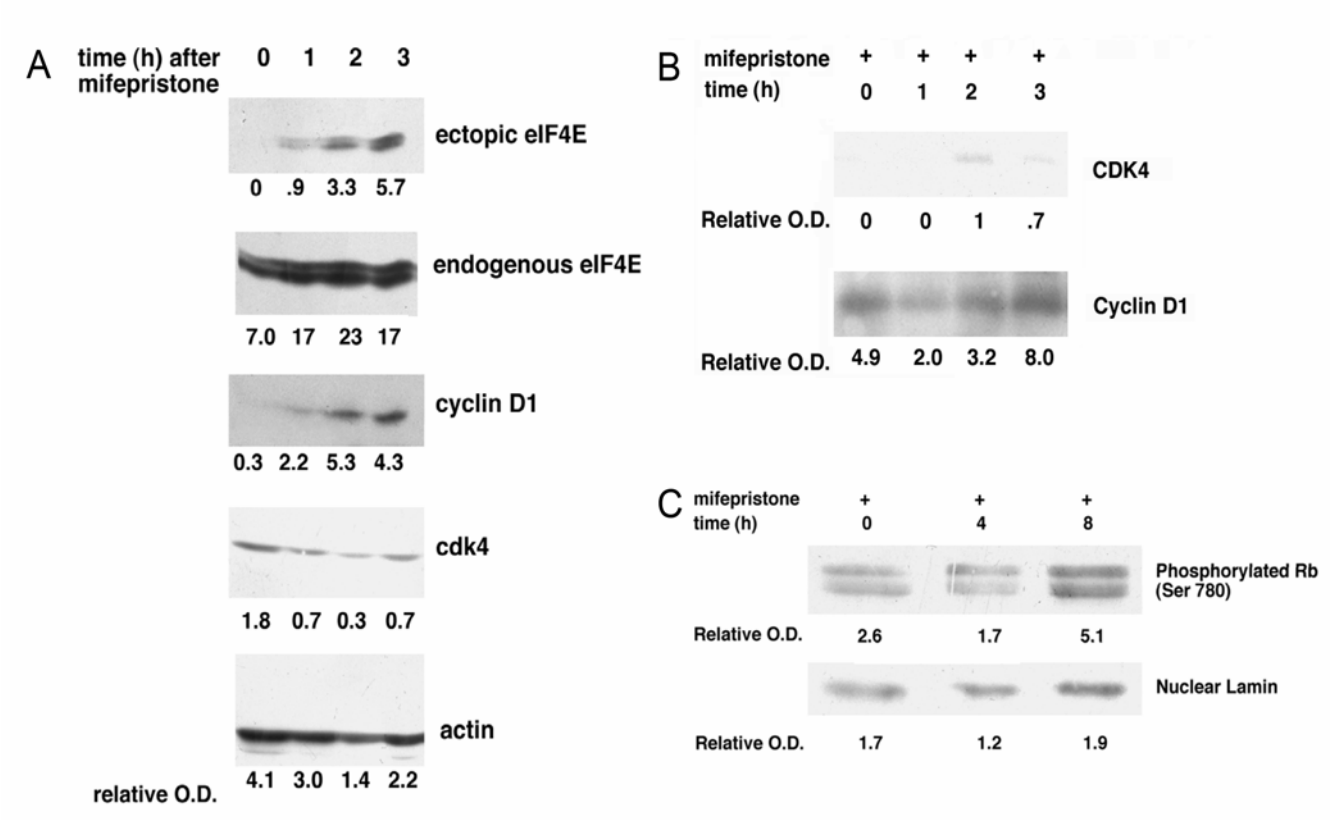

A
eIF4E mRNA (Relative Units)
20
15
10
5
0
0h
1h
2h
3h
Time (h)
B
eIF4E mRNA (Relative Units)
60
40
20
0
3
4
5
6
7
8
9
10
Fraction
0h 4E Ind
1h 4E Ind
2h 4E Ind
3h 4E Ind
C
Cyclin D1 mRNA (Relative Units)
25
20
15
10
5
0
0h
1h
2h
3h
Time (h)
D
Cyclin D1 mRNA (Relative Units)
25
20
15
10
5
0
3
4
5
6
7
8
9
10
Fraction
0h 4E Ind
1h 4E Ind
2h 4E Ind
3h 4E Ind
E
Cytoplasmic Cyclin D1 mRNA (Relative Units)
200
150
100
50
0
0h
1h
2h
3h
Time (h)

eIF4E-mediated proliferation follows the canonical growth factor pathway.

When quiescent cells are exposed to growth factors, cyclin D1 abundance in the cytoplasm increases as it associates with its partner kinases, cdk4 and cdk6, resulting in their activation. The resultant cyclin-cdk complexes enter the nucleus where they phosphorylate the retinoblastoma protein (Rb). Cyclin D1 in turn is rapidly phosphorylated by glycogen synthase kinase-3 beta (GSK3β) on Thr-286, triggering nuclear export and ubiquitin mediated degradation by the 26S proteasome [(49)]. To determine if eIF4E-mediated cell cycle entry followed this canonical growth factor initiated pathway, we traced the fate of cyclin D1 and its partner kinase cdk4 after eIF4E induction. Within 1h after mifepristone induction, the abundance of ectopic eIF4E and cyclin D1 in the cytoplasm increased sharply, with a concomitant decrease in cdk4 that was even more pronounced at 2h (Figure 13A). Within this time frame, we found cyclin D1 associated with cdk4 (Figure 13B), and by 8h post induction we observed a 2-fold increase in Rb phosphorylation (Figure 13C). These data indicate that eIF4E enables cells to bypass the early steps of the growth factor signaling cascade, initiating cell cycle entry by facilitating transit of the Rb-governed restriction point at the G1/S phase boundary.

Figure 13. eIF4E Bypasses Apical Steps in the Canonical Growth Factor-Initiated Proliferative Pathway

Quiescent cells were incubated with mifepristone (625pM) for the indicated time interval; lysed; and separated into subcellular fractions (cytoplasmic or nuclear). **A.** Immunoblot analysis for cytoplasmic cdk4, cyclin D1 and eIF4E. Actin is shown as a loading control (3 independent replications gave similar results). **B.** Association of cyclin D1 with cdk4. Nuclear lysates were immunoprecipitated with anti-cyclin D1 antibody to isolate cyclin D1 and its binding partners. Shown is a representative immunoblot for cdk4 and cyclin D1 (2 independent replications gave similar results). **C.** Phosphorylation of Rb after eIF4E induction**.** Nuclear lysates were subjected to immunoblot analysis for phosphorylated Rb using a reagent specific for phosphorylation on Ser 780. Nuclear lamin is used as a loading control (3 independent experiments gave similar results).


A
time (h) after mifepristone
0 1 2 3
ectopic eIF4E
0 .9 3.3 5.7
endogenous eIF4E
7.0 17 23 17
cyclin D1
0.3 2.2 5.3 4.3
cdk4
1.8 0.7 0.3 0.7
actin
4.1 3.0 1.4 2.2
relative O.D.
B
mifepristone + + + +
time (h) 0 1 2 3
CDK4
Relative O.D. 0 0 1 .7
Cyclin D1
Relative O.D. 4.9 2.0 3.2 8.0
C
mifepristone + + +
time (h) 0 4 8
Phosphorylated Rb (Ser 780)
Relative O.D. 2.6 1.7 5.1
Nuclear Lamin
Relative O.D. 1.7 1.2 1.9

Discussion

Sustained activation of eIF4F is on the causal pathway to cancer [47]. When its rate limiting component, eIF4E, is over expressed in rodent cells, it functions as an oncogene *in vitro* and *in vivo*; and when over expressed together with the catalytically active subunit of telomerase, eIF4E confers human cells with apoptosis resistance and the capacity for clonal and anchorage-independent growth [48]. However, in nearly all prior reports eIF4E has been over expressed in a constitutive manner, leaving uncertain whether newly acquired oncogenic properties arose directly from the resultant alterations in translational control – or indirectly from long term cellular adaptations that occur, and new mutations that persist (due to apoptosis resistance) when cap-dependent translation is durably activated *in vivo* or *in vitro*. To test whether increasing eIF4E abundance could confer cells with a hallmark property of cancer, autonomous proliferation; we engineered NIH3T3 cells with a mifepristone inducible eIF4E construct, and quantified cell cycle entry after increasing eIF4E abundance to levels typical of human cancer. Here we show that eIF4E triggers transit of the G1/S phase checkpoint within 16h, following the canonical growth factor pathway downstream of cyclin D1. Translation of cyclin D1 was rapidly activated after eIF4E induction by increased ribosome recruitment and increased flux of cyclin D1 mRNA into the translationally active cytoplasmic pool. These data establish that eIF4E directly confers cells with proliferative autonomy, and reveals the mechanism by which eIF4E liberates cells from the physiological requirement for growth factors to enter the cell cycle.

Prior reports firmly establish that activation of eIF4F is on the causal pathway to cancer [10, 13, 27, 47, 72, 79]. The evidence includes direct gain and loss of function experiments in animal models and in human and animal cell lines [3, 35]. In this line of investigation, most investigators have manipulated the levels and activity of the rate limiting component of the eIF4F complex, eIF4E. Over expression of eIF4E causes malignant conversion of rodent fibroblasts [35] and human mammary epithelial cells [3]; and promotes tumor formation in transgenic mice [59, 75]. Tumors isolated from humans express abnormally high levels of eIF4E [60]. While the prowess of eIF4E as an oncogene has been firmly established, its pleiotropism has complicated studies of mechanism.

Our approach to simplifying this problem was to focus on a single, but sentinel property of malignant transformation, proliferative autonomy; and develop a high fidelity inducible system that simulated the levels of eIF4E observed in human cancer. We began our studies with significant prior knowledge derived from more than a decade and a half of studies from many laboratories using constitutive over expression models *in vitro* and *in vivo.* These studies document that several pathways linked to cell cycle progression including growth factor production, polyamine metabolism and the cell cycle machinery itself are all subject to translational control [58][61].

In accord with prior reports in constitutive systems [57], our studies verify that enforced over expression of eIF4E increases cyclin D1 abundance. Cyclin D1 is

tightly regulated by several transcriptional and post-transcriptional mechanisms [21]. Prior to our study, there was no evidence that eIF4E could alter the efficiency of ribosome recruitment to the cyclin D1 transcript. Results in constitutive systems indicate that the sole mechanism was accelerated transit of the cyclin D1 mRNA from the nucleus to the cytoplasm where it entered the translationally active pool [11, 12, 68, 69]. In contrast, here we report a very rapid increase in the recruitment of ribosomes to the cyclin D1 transcript after eIF4E over expression, followed later by the increase in nuclear-cytoplasmic transport documented in previous reports.

Translational control includes a system of regulatory elements that generally reside in the 5' and 3' untranslated regions of a transcript permitting rapid and large changes in the translation of individual mRNA – or groups of mRNA sharing one or more regulatory elements (reviewed in [29]). These elements associate with sequence-specific RNA-binding moieties (proteins or miRNA) that orchestrate their splicing, nuclear export, localization, stability and ribosome recruitment. While our study does not directly reveal the molecular mechanisms mediating the increased ribosome recruitment to the cyclin D1 transcript in response to enforced over expression of eIF4E, curated databases of 5' and 3' RNA regulatory elements provide a clue. A survey of UTR elements conducted with UTRscan reveals 1 regulatory element in the 3' UTR of the cyclin D1 transcript; and TARGETscan identifies 14 putative miRNA targets (Table. 1). A recent report has implicated the 3' UTR elements HuR and AUF1 in translational

control of eIF4E expression by regulating eIF4E mRNA stability [70]. This same report suggests that HuR and eIF4E may control the translation of a common set of transcripts, including cyclin D1. Future studies focused on the interaction between eIF4E abundance and the functions of these elements have the potential to elucidate the full set of mechanisms controlling the translation of cyclin D1.

Table 1. Known Regulatory Elements in the UTR of the Cyclin D1 Transcript

Shown are the results of a search for regulatory elements in the untranslated regions (5' and 3' UTR) of the murine cyclin D1 transcript conducted with UTRscan (non-miRNA targets) and TARGETscan (miRNA targets).

RNA Regulatory Elements

miR Family	Gene Symbol	UTR start	UTR end
let-7/98	CCND1	2000	2006
miR-1/206	CCND1	911	917
miR-106/302	CCND1	924	930
miR-15/16/195/424/497	CCND1	1728	1734
miR-15/16/195/424/497	CCND1	1805	1811
miR-17-5p/20/93.mr/106/519.d	CCND1	925	931
miR-19	CCND1	1593	1599
miR-202/202-3p	CCND1	1999	2005
miR-203	CCND1	916	922
miR-23ab	CCND1	1945	1951
miR-34a/34b-5p/34c/34c-5p/449/449abc/699	CCND1	1853	1859
miR-503	CCND1	1727	1733
miR-503	CCND1	1804	1810
miR-590/590-3p	CCND1	2652	2658
miR-873	CCND1	1986	1992
3' UTR Regulatory Element			
15-LOX-DICE	CCND1	N/A	N/A

To dissect whether activation of cyclin D1 was required for cell cycle entry or was simply one of many trophic pathways activated by an increase in eIF4E abundance, we induced eIF4E expression in cells harboring cyclin D1 shRNA. By using 3 different shRNA targeting distinct regions of the cyclin D1 mRNA, we were able to achieve a range of cyclin D1 knockdown. Of note, each of the shRNA constructs blocked transit of the G1/S checkpoint upon eIF4E induction. These data are in accord with the idea that eIF4E triggered cell cycle entry follows the canonical growth factor pathway, skipping past the apical growth factor stimulated steps to join the cell division pathway at cyclin D1. To address this inference experimentally, we replicated prior experiments [77] tracing the fate of cyclin D1 and found an increase in cyclin D1/cdk4 complex assembly with subsequent phosphorylation of Rb. These results show that translational activation of cyclin D1 is required for eIF4E mediated cell cycle entry, and that eIF4E can substitute for growth factors to stimulate cell cycle entry, following the canonical growth factor pathway.

Available data begin to unveil those cancer-related functions that can be directly ascribed to activation of eIF4E. Experiments conducted using a tetracycline inducible system show that eIF4E confers cells with resistance to apoptosis by increasing translation of several survival genes [30]; and also document translational activation of ornithine decarboxylase (ODC), a positive regulator of cell proliferation. Although this study clearly illustrates the pleiotropic action of eIF4E; here we document that translational activation of cyclin D1 is necessary

for cell cycle entry, and that other growth promoters that are eIF4E-responsive cannot rescue cells from this requirement.

Our study provides novel insights into how the translational machinery functions in oncogenesis. Enforced over expression of eIF4E translationally activates cyclin D1, stimulates cell cycle entry, and thus is capable of short-circuiting the canonical growth factor pathway. As a point of convergence of many oncogenes, activation of cap-dependent translation may serve as a biomarker for early cancer detection and prevention. In addition, studies in model systems targeting eIF4E abundance with antisense oligonucleotides and the integrity of eIF4F with novel small molecules that mimic 4E-BP activity already show promise [22] [41]. These observations serve as proof of concept that cap-dependent translational control is a legitimate target for cancer therapy, and provide the foundation for future studies in which the regulation of the translational machinery is utilized for cancer therapy in humans.

Chapter V.

4E Binding Proteins as Potential Tumor Suppressors: A Study in Knockout Mice

Chapter V describes a collaborative study in which I assisted my colleague and lab mate, Yong Kim. The following is an excerpt for his 2009 publication in *Nature Medicine*. I have provided the Abstract, Introduction, Methods, Discussion and References sections from the publication; to provide the study motivation and novel findings, as well as a detailed description of my contributions to the study.

The 4E-BP Family of Translational Repressors Function in Tumor Defense

Yong Y Kim, Linda Von Weymarn, Ola Larsson, Danhau Fan, Jon M Underwood, Stephen S Hecht, Peter B Bitterman

My contributions to this study are primarily based on tissue harvesting and protein detection. I assisted Yong Kim in collecting mouse lungs and processing samples by subcellular fraction (described below). I then analyzed the samples by western blot throughout the study.

Abstract

Activation of the cap-dependent translation initiation complex, eIF4F, is on the causal pathway to cancer. Although the three member 4E-BP family of translational repressors is known to mediate restraint of eIF4F-dependent ribosome recruitment, a definitive role for the 4E-BPs in tumor defense is not established. Here we show that *4ebp1*$^{-/-}$/*4ebp2*$^{-/-}$ mice manifest increased sensitivity to lung tumorogenesis induced by the tobacco carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), and develop tumors with markedly increased vascularity. At baseline in *4ebp1*$^{-/-}$/*4ebp2*$^{-/-}$ mice, we observed translational activation of genes promoting oncogenesis as well as translational activation of VEGF. Our data also unveiled a previously unrecognized, translationally controlled, feed-forward loop involving the cytochrome P450 enzyme system that increases the carcinogenic potency of NNK in the 4E-BP deficient state. Our data firmly establish a role for the 4E-BPs in tumor defense and elucidate a molecular mechanism for the non-linear cumulative risk of lung cancer in smokers.

Introduction

Biomedical scientists have recognized for more than a decade that components of the cap-dependent translation initiation machinery are more abundant and active in cancer than in their non-malignant counterparts; an adjustment previously assumed to serve the increased metabolic needs of a cancer cell. It came as somewhat of a surprise when studies of hematological malignancies, breast cancer and lung cancer documented that cancer is subject to translational control; and that sustained hyper-activation of the cap-dependent translation initiation apparatus leads to spontaneous cancers in mice [(59)]. In fact, more than three quarters of the approximately 300 cancer-related genes recognized to date activate eIF4F directly or indirectly [(13)]. Many known oncogenic factors are either upstream modulators of eIF4F (e.g. HER-2, EGFR, c-MYC, Ras) or downstream effectors of its function (e.g. Cyclin D1, BCL-X_L) [(8)]. Thus, the cap-dependent initiation step of protein synthesis is a key regulatory hub in the gene expression pathway, and its sustained aberrant activation is a property of cancer.

The basic translation initiation apparatus is conserved from yeast to humans with only minor differences [(20)]. Cap-dependent translational control is exerted by regulation of the abundance and activity of eIF4E, the rate-limiting component of the eIF4F complex. A wide variety of extracellular regulatory cues stimulate eIF4F assembly and cap-dependent translation by signaling the phosphorylation of 4E-BPs on six serine/threonine sites through the Ras/PI3K/AKT/mTOR kinase cascade [(51)]. Hyperphosphorylation decreases the affinity of the 4E-BPs for

eIF4E which is free to participate in eIF4F assembly. In mammals, three proteins with a high degree of structural and functional identity - 4E-BP1, 4E-BP2 and 4E-BP3 - constitute the 4E-BP family of translational repressors. Why this redundancy? The answer is not clear, since mice engineered to be null for *Eif4ebp1* and *Eif4ebp2* develop and reproduce normally. So it seems that just one of the 4E-BPs is needed for physiological regulation of protein synthesis during development and most homeostatic functions in post-natal life. However, the most ubiquitous translational repressor, 4E-BP1, displays attributes of a tumor suppressor: (a) it is functionally inactivated in aggressive breast carcinomas by hyperphosphorylation [(1)]; and (b) when ectopically expressed in breast and lung carcinoma cells, 4E-BP1 suppresses tumorigenicity [(1)]. These findings support the idea that the 4E-BP family of translational repressors might not only function in their canonical role as global regulators of mRNA recruitment to the ribosome, but also in tumor surveillance.

To examine whether 4E-BP family members function in tumor defense, we sought a clinically relevant mouse cancer model and chose lung cancer triggered by the tobacco carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) – one of the most abundant and best studied of the tobacco carcinogens [(25)]. NNK produces neoplastic lung lesions in mice that closely resemble their human counterparts; progressing from adenomatous hyperplasia to pre-malignant adenomas to adenocarcinoma. Currently, adenocarcinoma is the most frequent non-small cell lung carcinoma in humans, representing 35 to 40% of all lung

cancers. Although NNK is non-carcinogenic; when taken up by the cell it is metabolized by the cytochrome P450 enzyme superfamily into carcinogens that create DNA adducts leading to oncogenic mutations. The most frequent of these is a gain–of-function point mutation at RAS codon 12[24]. Oncogenic Ras in turn activates translation through kinase cascades leading to phosphorylation of both eIF4E and the 4E-BPs [5]. Here we show that mice genetically engineered to lack *Eif4ebp1* and *Eif4ebp2* (*4ebp1*$^{-/-}$/*4ebp2*$^{-/-}$) manifest increased sensitivity to NNK-induced lung tumorigenicity. Our study definitively establishes a role for the 4E-BPs in tumor defense, and provides proof of concept for translational research efforts targeting the cap-dependent translation initiation apparatus for cancer therapy.

Western blot analysis

Lung tissue was flushed with PBS and homogenized before cells were lysed with RIPA buffer (150mM NaCl, 6mM $NaHPO_4$, 4mM NaH_2PO_4, 2mM EDTA, 1% NaDOC, 1% NP40, 0.1% SDS) supplemented with Protease Inhibitor Cocktail Tablets (Roche, Switzerland), phosphatase inhibitors Na3OV4 (1mM) and beta-glycerophosphate (50mM). The protein content of lysates was quantified and equal amounts were resolved by 15% SDS-PAGE. Proteins were transferred onto nitrocellulose and analyzed for eIF4E, 4E-BP1, 4E-BP2, and 4E-BP3. Antibodies for eIF4E (BD Biosciences, San Diego, CA) and 4E-BP1 (Abcam, Cambridge, MA) were purchased, and antibodies for 4E-BP2 and 4E-BP3 were provided by Dr. Nahum Sonenberg.

Sub-Cellular Fractionation

Mouse lung samples were collected & homogenized. Tissue was resuspended with 1mL PBS, and then transferred to a 1.5 ml microcentrifuge tube and pelleted by centrifugation at 500 × *g* for 2-3 minutes. A pipette was used to carefully remove and discard the supernatant, leaving the pellet as dry as possible. 200 µl ice-cold CER I (provided in NE-PER® kit, ThermoScientific) is added to the cell pellet, and cells are vortexed vigorously on the highest setting for 15 seconds to fully suspend the cell pellet. The tube is incubated on ice for 10 minutes. 11 µl ice-cold CER II (provided in NE-PER® kit, ThermoScientific) is added to the tube, and the tube is vortexed for 5 seconds on the highest setting- then incubated on ice for 1 minute. The tube is vortexed again for 5 seconds on the highest setting, then centrifuged for 5 minutes at maximum speed in a microcentrifuge (~16,000 × *g*). The supernatant (cytoplasmic extract) was immediately transferred to a clean pre-chilled tube. (Place this tube on ice until use or storage @ -80°C). The pellet,which contains nuclei, is suspended in 100 µl ice-cold NER (provided in NE-PER® kit, ThermoScientific), and the tube is then vortexed on the highest setting for 15 seconds. The sample is placed on ice and continuously vortexed for 15 seconds every 10 minutes, for a total of 40 minutes. The tube is then centrifuged at maximum speed (~16,000 × *g*) in a microcentrifuge for 10 minutes. The supernatant (nuclear extract) is immediately transferred to a clean pre-chilled tube, and stored @ -80°C until use.

DISCUSSION

The concept that activation of cap-dependent translation is on the causal pathway to cancer is now established [59]. However, the inverse concept - that the 4E-BP family of cap-dependent translational repressors function in tumor defense - could only be inferred from available experimental evidence [1]; providing us with strong motivation to perform a definitive *in vivo* study. Using mice genetically engineered to lack 2 of the 3 members of the 4E-BP family; and a high fidelity tobacco carcinogen mouse model of lung cancer that closely recapitulates the human disease, here we report that 4E-BP deficiency sensitizes mice to NNK-induced lung tumorigenesis, firmly establishing a role for the 4E-BPs in tumor defense. In addition, we report that lung tumors in 4E-BP deficient mice showed a marked increase in vascularity compared to their wild type counterparts, identifying angiogenesis as a critical translationally controlled step in the cancer pathway. Moreover, at baseline before NNK challenge, we show a significant skewing of the *4ebp1*$^{-/-}$/*4ebp2*$^{-/-}$ translational profile towards translational activation of genes promoting cancer-related properties. Finally, we unexpectedly discovered a potential feed-forward loop involving the cytochrome P450 enzyme system that increases the carcinogenic potency of NNK in the 4E-BP deficient state – providing a novel molecular mechanism for the potency of cigarette smoke as a human lung carcinogen.

Evidence for the role of eIF4F activation in cancer has been accumulating for more than a decade[1-4]. Since the 4E-BPs are the primary negative regulators of

eIF4F function, our hypothesis that *4ebp1*$^{-/-}$/*4ebp2*$^{-/-}$ mice would be sensitized to NNK-induced lung tumorigenesis had strong antecedent experimental grounding [(1)]. Indeed, as we predicted, our study shows that NNK increases the sensitivity of *4ebp1*$^{-/-}$/*4ebp2*$^{-/-}$ mice to NNK, establishing a role for the 4E-BP family of cap-dependent translation repressors in tumor defense.

Morphological analysis of tumors revealed a striking increase in tumor microvessel density in *4ebp1*$^{-/-}$/*4ebp2*$^{-/-}$ mice; a result identifying a role for the 4E-BPs in restraint of tumor neovascularization. Activation of eIF4F is known to selectively activate translation of the angiogenic protein VEGF [(9)]. We found increased recruitment of ribosomes to VEGF in the *4ebp1*$^{-/-}$/*4ebp2*$^{-/-}$ mice; creating a primed state for increased neovascularization of NNK-triggered tumors. However, we note that translational control of angiogenesis in not simply unidirectional. In breast carcinoma, high level over expression of 4E-BP1 promotes angiogenesis by triggering a switch from cap-dependent to IRES mediated translation [(40)]. This leads to translational activation of VEGF which can be translated in a cap-independent as well as a cap-dependent manner – giving it a selective advantage to recruit ribosomes when cap-dependent translation is repressed. These data highlight the importance of translational control in governing the propensity of tumors to progress by recruiting a circulation, and provide a molecular mechanism for the increased vascularity we observed in the lung tumors from *4ebp1*$^{-/-}$/*4ebp2*$^{-/-}$ mice.

To elucidate the molecular mechanism by which the 4E-BP deficiency sensitizes mice to NNK, we analyzed the baseline lung RNA ribosome recruitment pattern genome-wide. There was a marked skewing of the *4ebp1*$^{-/-}$/*4ebp2*$^{-/-}$ molecular landscape towards translational activation of genes enriched for cancer related functions in metabolism, proliferation, and transcription. Our finding is consistent with current understanding of translational deregulation as a causal pathway to cancer resulting in selective translation of proliferative factors leading to increases in metabolism. Also, it is tempting to speculate that a similar shift in the global translational profile towards cancer may be recapitulated in the smoker's lung; a possibility in accord with the clinical finding that hyperphosphorylated 4E-BP1 is a negative prognostic factor in breast, ovarian, and prostate cancer [(7)].

Our most exciting result was completely unexpected; the discovery of a feed-forward loop enabling NNK to become a progressively more potent carcinogen in the smoker. We found that *4ebp1*$^{-/-}$/*4ebp2*$^{-/-}$ mice were more efficient at catalyzing NNK to its carcinogenic metabolites, methane diazohydroxide and 4-(3-pyridyl)-4-oxobutane-1-diazohydroxide, and that this resulted in increased methylated DNA adducts. The mechanism involved selective translational activation of CYP2A5, the primary enzyme catalyzing NNK into its carcinogenic metabolites [(17)]. This finding points to a novel role for the 4E-BPs in regulating the cytochrome P450 enzyme system. Combining the published literature with our current findings, we have unveiled a pernicious feed-forward loop in smokers. At

the outset, physiological levels of CYP2A5 in the lung will catalyze NNK into methane diazohydroxide and 4-(3-pyridyl)-4-oxobutane-1-diazohydroxide which forms DNA adducts, some of which will preferentially create a codon 12 gain of function mutation in RAS [54]. The resultant production of oncogenic Ras activates the PI3K/Akt/mTOR pathway which in turn leads to hyperphosphorylation of the 4E-BP family members [5], i.e. a *4ebp1*$^{-/-}$/*4ebp2*$^{-/-}$ phenocopy. We show here that CYP2A5 is translationally activated in the 4E-BP deficient state, increasing the catalysis of subsequent exposure to NNK into carcinogenic metabolites resulting in accelerated accumulation of DNA adducts. These data help to explain the non-linear cumulative risk of developing lung cancer from cigarettes [24].

Although our study does not definitively establish a role for the 4E-BP family of proteins in defense against malignancies other than adenocarcinoma of the lung; together with the published literature it strongly suggests a more general role in other epithelial malignancies. Our findings, therefore, provide impetus for current efforts to develop new classes of therapeutics aimed at normalizing translational control in malignancy. These efforts include mTOR inhibitors designed to restore 4E-BP function and a number of compounds targeting the integrity or activity of the eIF4F complex. Our findings also provide guidance about the potential utility of array-based translational profiling to begin identifying and stratifying at-risk states for the development of lung and other malignancies.

Chapter VI.

Pharmacological Tools

Pharmacologic Rationale

Cancer treatment with chemotherapy, or pharmacological intervention, faces many barriers due to toxicity. The intended goal of chemotherapy is to terminate cancer growth- but very often normal cells are targeted as well, leading to severe toxic effects. An ideal treatment would include selective disease targeting which reverts oncogenic phenotype- without targeting non oncogenic protein involved in general physical function. While a “silver-bullet” in cancer treatment is very rare- the precedent has been set with Gleevec [(50)]. Researchers developed Gleevec (Imantinib), an inhibitor of tyrosine kinases, (targeting Bcr-Abl fusion protein) for the treatment of chronic myeloid leukemia (CML). Imantinib selectively inhibits activation of target proteins involved in cellular proliferation.

I aimed to apply similar principals in identifying an aberration specifically seen in oncogenic protein and target this feature for selective pharmacological interference. In this case the aberration is increased protein synthesis (translation), and we aim to target translation initiation by way of the eIF4F initiation complex responsible for cap-dependent translation. Based on previous research in the field; targeting of the cap-dependent machinery should negatively

regulate hyperactive translation, causing a decrease in oncogenic protein synthesis with little effect on house-keeping proteins.

The majority of my studies are rooted in my selected field of graduate study, Pharmacology. I have utilized drugs provided by collaborators in the eIF4E model system to gain more understanding of translational machinery and the potential for its antagonism in disease treatment. There are several factors to consider when utilizing pharmacologic tools to interrupt a system, including “ADME”. ADME is an acronym for absorption, distribution, metabolism and elimination; and refers to the variables involved in drug activity. The compounds explored in this study were all characterized and their “ADME” properties defined through the use of trials intended to maximize their efficiency. I will focus on drug potency and efficacy in terms of its ability to reverse oncogenic properties.

Background of eIF4F antagonists

Prior chapters in this report firmly establish the translational machinery as a point of convergence in cancer onset. There has also been a significant amount of research dedicated to developing pharmacological tools which interfere with eIF4F assembly to reverse oncogenic properties. There are various ways to target eIF4F assembly, and scientists have been successful in exploiting the well defined cap-dependent pathway to find targets for intervention.

Pharmacological Targets for Cap-Dependent Translation Interference

7-methyl guanosine capped mRNA binding to eIF4E is necessary to initiate eIF4F assembly. Once eIF4E binds the 7-methyl guanosine cap, the complex moves into the cytoplasm and continues along the translation initiation pathway. Our lab has produced a library of 7-methyl cap analogs which bind eIF4E and interferes with the eIF4E-cap mRNA complex [19]. In this study, cell permeable inhibitors of eIF4E bound to the 7-methyl guanosine cap mRNA- and thus regulated cap-dependent translation.

The 4E-BPs are the well defined natural antagonist for eIF4F assembly. 4E-BPs compete (at similar affinity) with eIF4G- for binding to the cap activated translation initiator molecule and limiting factor, eIF4E. In cancer onset, the 4E-BPs are increasingly phosphorylated which leads to decreased affinity for eIF4E, leaving the protein free to bind eIF4G and continue towards eIF4F assembly. Researchers have produced compounds which mimic 4E-BPs, and shown that these compounds have the ability to inhibit cellular expression of oncogenic proteins in multiple cancer cell lines and reverse tumor formation [41]. While this study was successful, many 4E-BP mimics have struggled with the difficulty of mimicking the protein-protein interaction necessary to sequester eIF4E. Previous studies also show antisense oligonucleotides designed to selectively target eIF4E have the ability to repress the expression of eIF4E regulated proteins (cyclin D1, VEGF, c-myc, Bcl-2), induce apoptosis, and preventing endothelial cells from forming vessel-like structures [22].

I have utilized compounds which mimic both the 7-methyl guanosine cap and 4E-BPs, as well as increase 4E-BP activity. These compounds will be used to revert oncogenic properties observed in eIF4E over expression- with use of the 4E-inducible system. With the following experiments, I have gained a better understand of the potential for translational machinery as novel molecular targets for cancer therapy, with the use of eIF4F antagonists. I have also provided more insight on the possibility of utilizing the cap-dependent translational machinery as a bio-marker for early cancer detection and prevention.

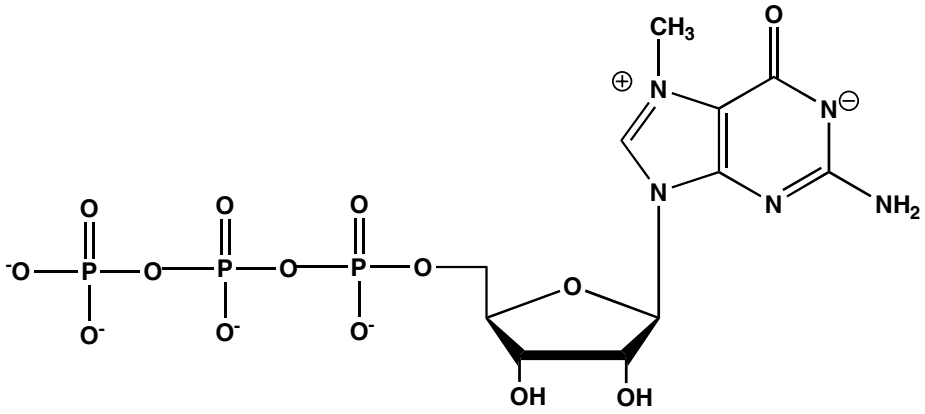
7 Methy Cap Analogs: Targeting the eIF4E-7methyl cap complex

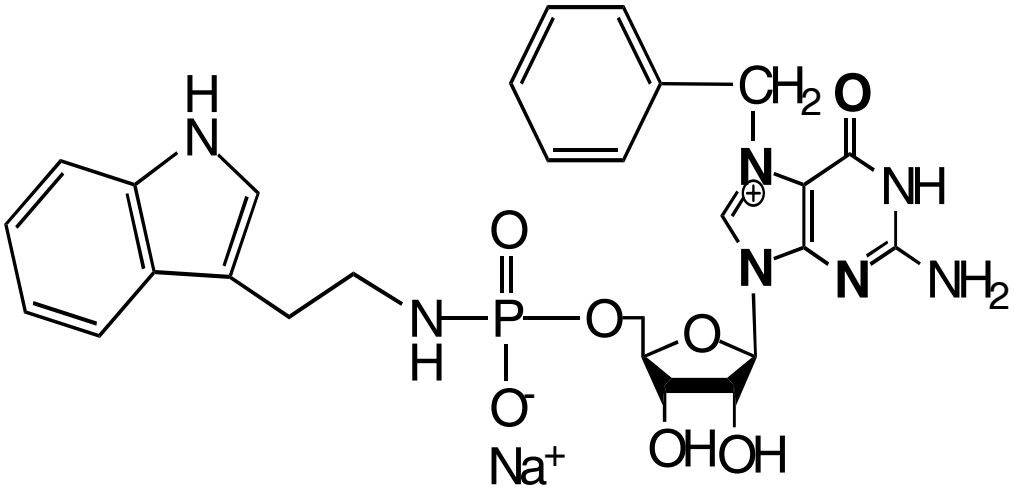
7-methyl cap analogs were prepared by lab collaborator, Phalgani Ghosh in the Wagner lab (Medicinal Chemistry). For my studies, I utilized *PG-175,* a 1st generation- cap analog mimic of 7-methy GTP. PG-175 acts by competing with the 7-methyl cap for binding to eIF4E (Figure 14, Figure 15). I utilized a bicistronic, cell-free assay; which measures the compound's ability to suppress cap dependent translation. PG-175 was administered to 231 breast cancer cellular lysate to measure its binding affinity to eIF4E in comparison to 7 methyl GTP (a portion of the natural 7 methyl guanosine substrate). PG-175 was shown to bind eIF4E with affinity similar to t-methyl GTP (Figure 16).

Figure 14. 7-methyl Cap Analogs

A. PG-175 is a 1st generation mimic of 7-methyl GTP (which forms the mRNA cap) cap analogs which acts by binding to and sequestering eIF4E, preventing its ability to initiate cap dependent translation initiation. The compound is produced by collaborators Phalgani Ghosh & Rick Wagner.

7 Methyl GTP





PG-175 (synthetic mimic of 7-methyl GTP)

Figure 15.Crystal Structure Analysis for eIF4E-*PG175* Binding

A nucleoside cap analog that antagonize mRNA cap binding to eIF4E. We have developed a “cap analog”, designated *PG-175* that antagonizes association of the 7-methyl cap with eIF4E. Shown is a model of eIF4E and *PG-175* docked at the putative cap analog binding site.

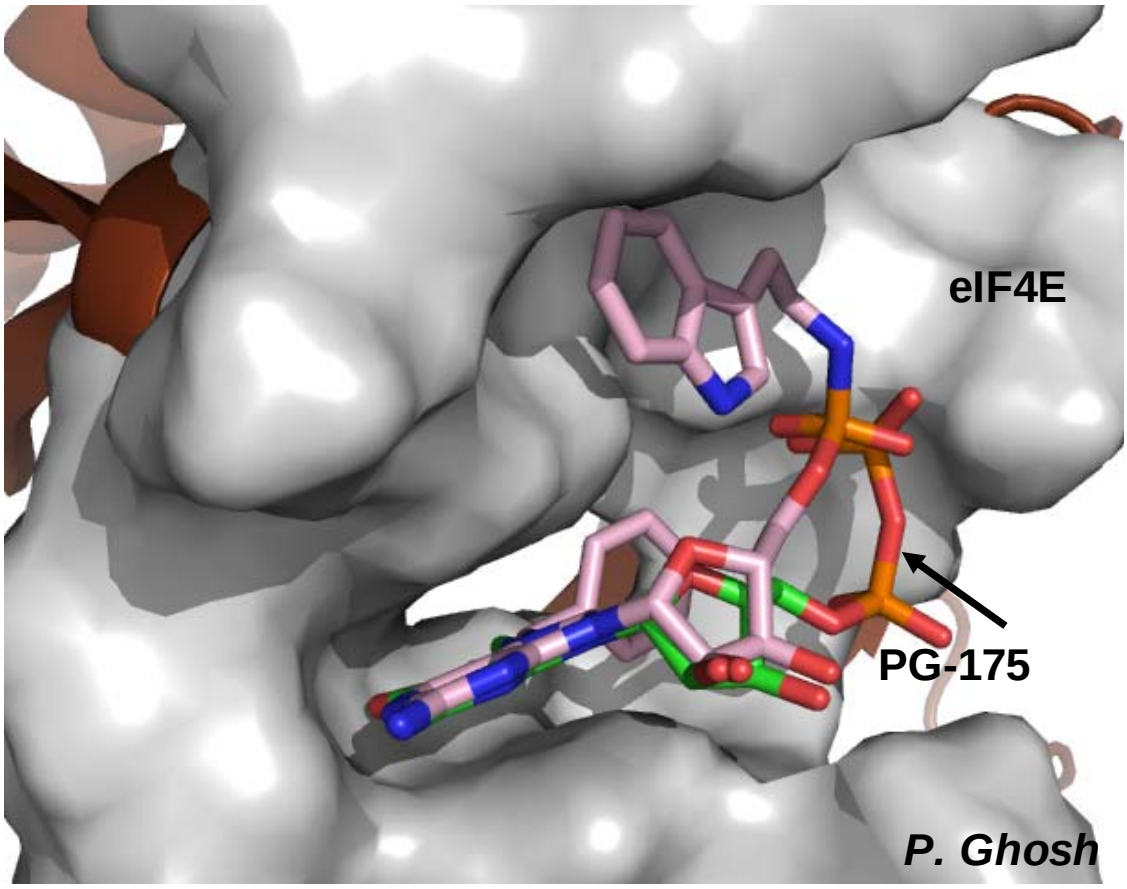
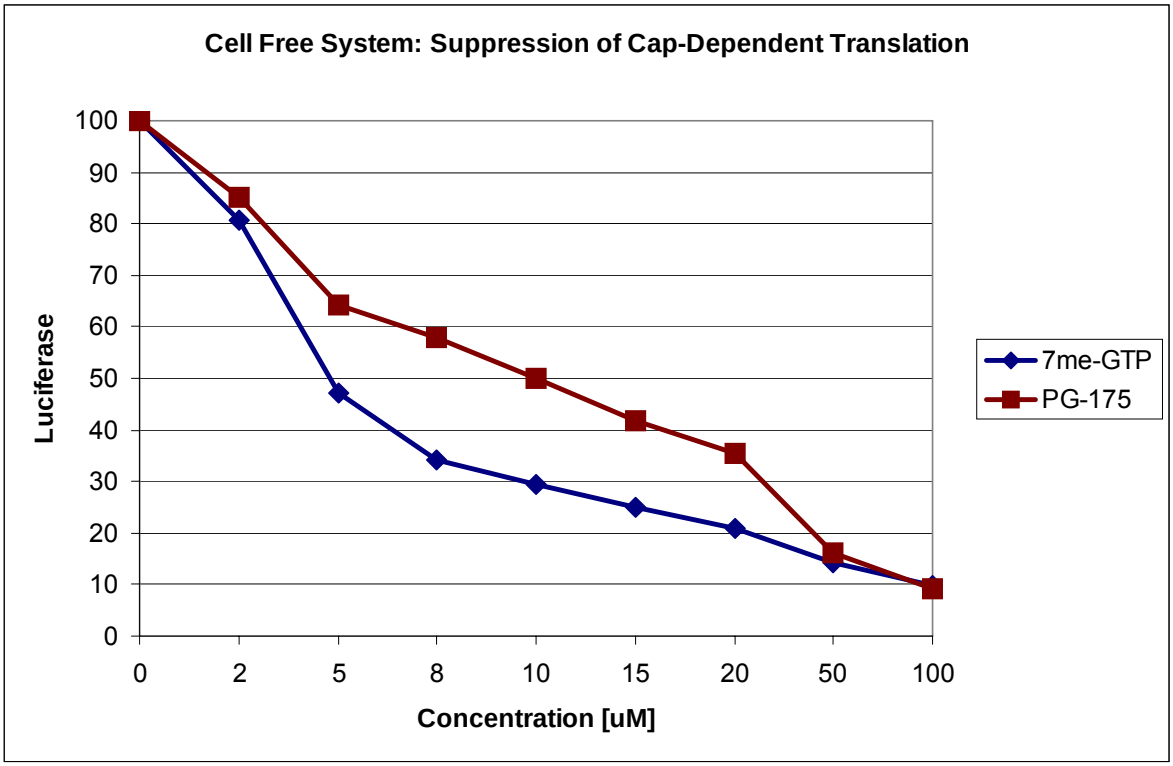

eIF4E
PG-175
P. Ghosh

Figure 16. 7-methyl GTP vs. *PG-175*: Affinity for eIF4E Molecular Binding

Bicistronic cell free assay reveals PG-175 is effective in inhibiting translation pathway by competing with the natural substrate for eIF4E binding. Cell lysates containing the bicistronic reporter are treated to increasing dosage of *PG-175.* Both Renilla luciferase activity (cap-dependent translation) and firefly luciferase activity (IRES-mediated translation) were measured in a Lumat luminometer with the Promega dual luciferase reporter system.

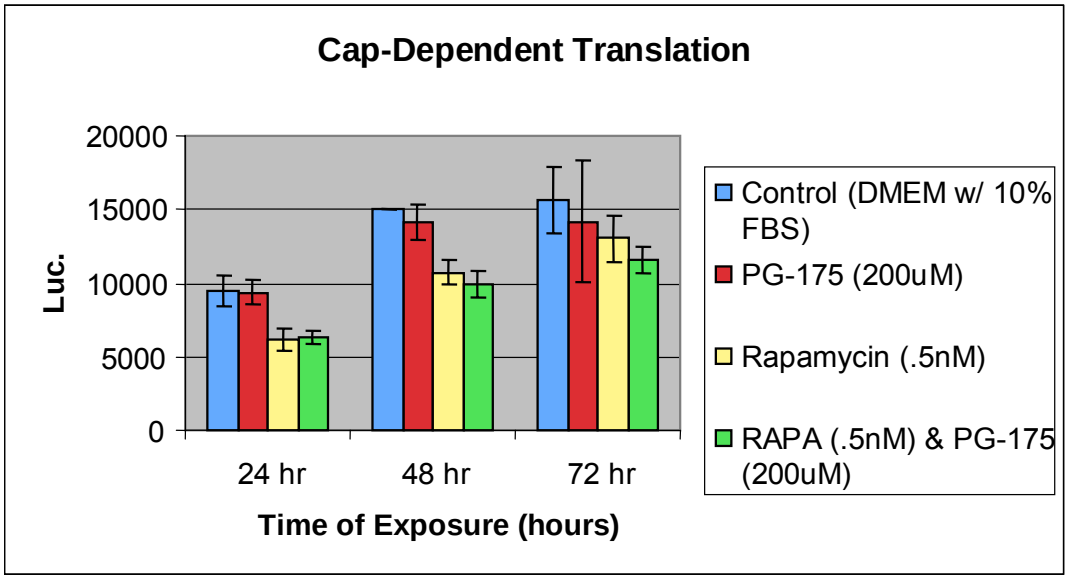

Cell Free System: Suppression of Cap-Dependent Translation
Luciferase
100
90
80
70
60
50
40
30
20
10
0
0
2
5
8
10
15
20
50
100
Concentration [uM]
7me-GTP
PG-175

The compound was administered to 231 Breast Cancer cells in a cell based, bicistronic assay which detects translational activity (described in *methods*). The mTOR inhibitor, Rapamycin (Sirolimus®), was used as a positive control due to its well established ability to suppress cap-dependent translation. mTOR is a kinase responsible for mitogen-induced cell proliferation/survival signaling and its activation positively regulates cap-dependent translation initiation [(28)].

While Rapamycin (0.5nM) displayed a strong and immediate suppression of cap-dependent translation, administration of *PG-175* (200uM) displayed very little suppression- reaching a maximum of ~5% by 72h (Figure 17). However when *PG-175* is co-administered with positive control Rapamycin I observed maximal efficacy in translational suppression. This finding indicates a synergistic effect between the cap analog and mTOR inhibitor by targeting different sites in the cap dependent translational pathway.

Figure 17. PG-175 Demonstrates Moderate Suppression of Cap Dependent Translation

The bicistronic system (see methods) is utilized to detect PG-175 efficacy in suppressing cap-dependent translation. 231 Breast cancer cells were transiently transfected with pcDNA3-rLuc-polio-fLuc, then rendered quiescent and incubated with or without mifepristone for 24h. Both Renilla luciferase activity (cap-dependent translation) and firefly luciferase activity (IRES-mediated translation) were measured in a Lumat luminometer with the Promega dual luciferase reporter system.


Cap-Dependent Translation
20000
15000
10000
5000
0
Luc.
24 hr
48 hr
72 hr
Time of Exposure (hours)
Control (DMEM w/ 10% FBS)
PG-175 (200uM)
Rapamycin (.5nM)
RAPA (.5nM) & PG-175 (200uM)

The 7-methyl cap analog compounds displayed very little effect when administered independently, and when co-administered with mTOR kinase inhibitor, Rapamycin. The mTOR kinase phosphorylates (and inactivates) 4E-BPs, therefore mTOR inhibition would lead to increased activity of the natural eIF4F antagonist. The synergic effect of the combination of compounds appears to surpass the cap-dependent suppression of Rapamycin or the cap analog independently. From this result, I conclude that while the 7-methyl cap analog compounds displayed low efficacy, targeting the 7 methyl cap-eIF4E complex is a feasible alternative for suppressing cap-dependent translation.

4E-BP Mimic: Targeting the association of eIF4E with eIF4G.

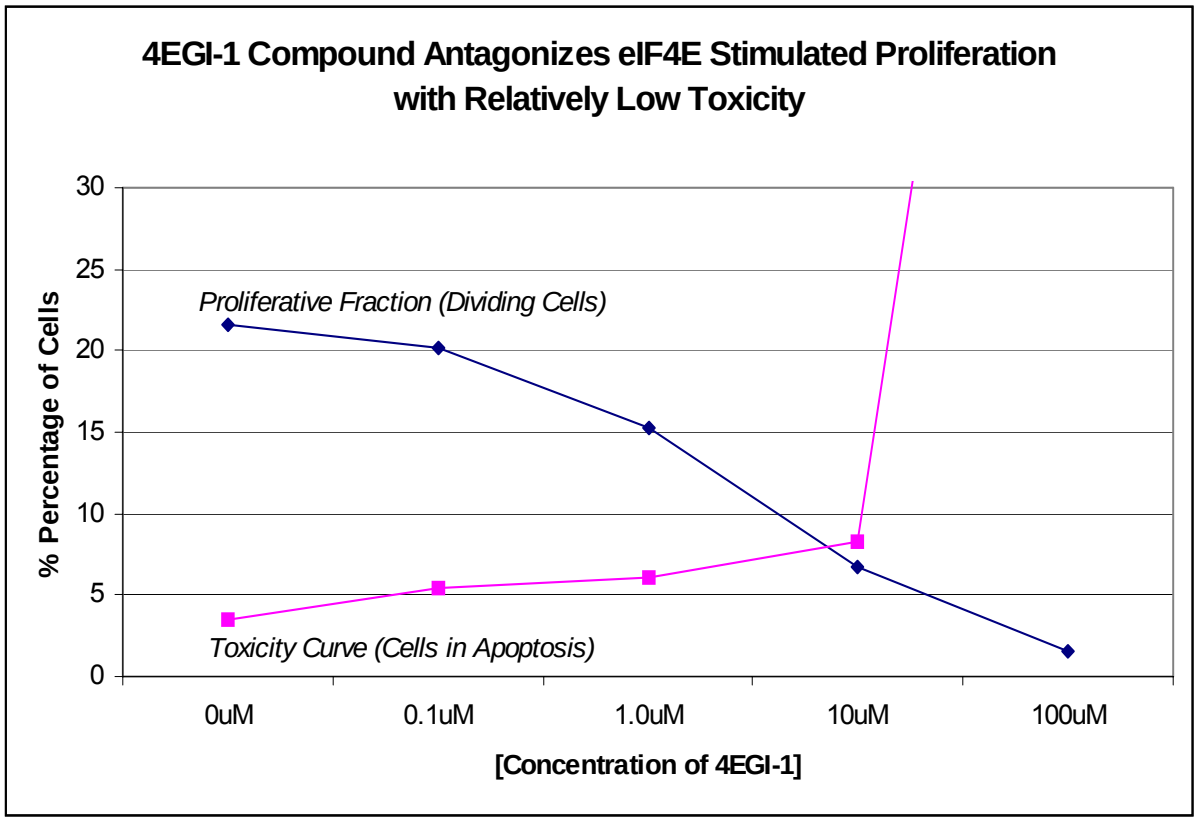
To mimic 4E-BP activity, I used a compound designated *4EGI-1* which inhibits eIF4F assembly by blocking association of eIF4E with eIF4G (generously provided by Dr. Gerhard Wagner) [41] . The 4E-inducible system was used in testing of this compound in an effort to reverse the previously described increase in proliferation triggered by mifepristone activation (*Chapter III*).

4E-Inducible cells were maintained, grown to confluence and then activated as previously reported. When mifepristone treated cells were co-administered 4EGI-1 for a period of 24 hours, there was a dose-dependent suppression of actively dividing cells, or % CCE. This finding is important as it was my first demonstration of an eIF4F antagonist reversing an eIF4E triggered oncogenic property. This experiment was followed by a toxicity analysis to determine if

4EGI-1 induces toxicity. Concentrations of compound which were effective in reversing proliferation (0.1uM-10uM) were non toxic as judged by the proportion of hypodiploid (apoptotic) cells (Figure 18).

Figure 18. 4EGI-1 Exhibits a Dose Response Reversal in Proliferation, with Minimal Toxicity in the 4E Inducible System

Inducible cells were rendered quiescent, and then incubated for 24h in defined medium with 625pM mifepristone and increasing dosages of 4EGI-1. Cells were then fixed and DNA content was quantified by flow cytometry. Shown is the proportion of cells with S + G_2/M (proliferative) and hypodiploid (apoptotic) DNA was measured to determine toxicity.

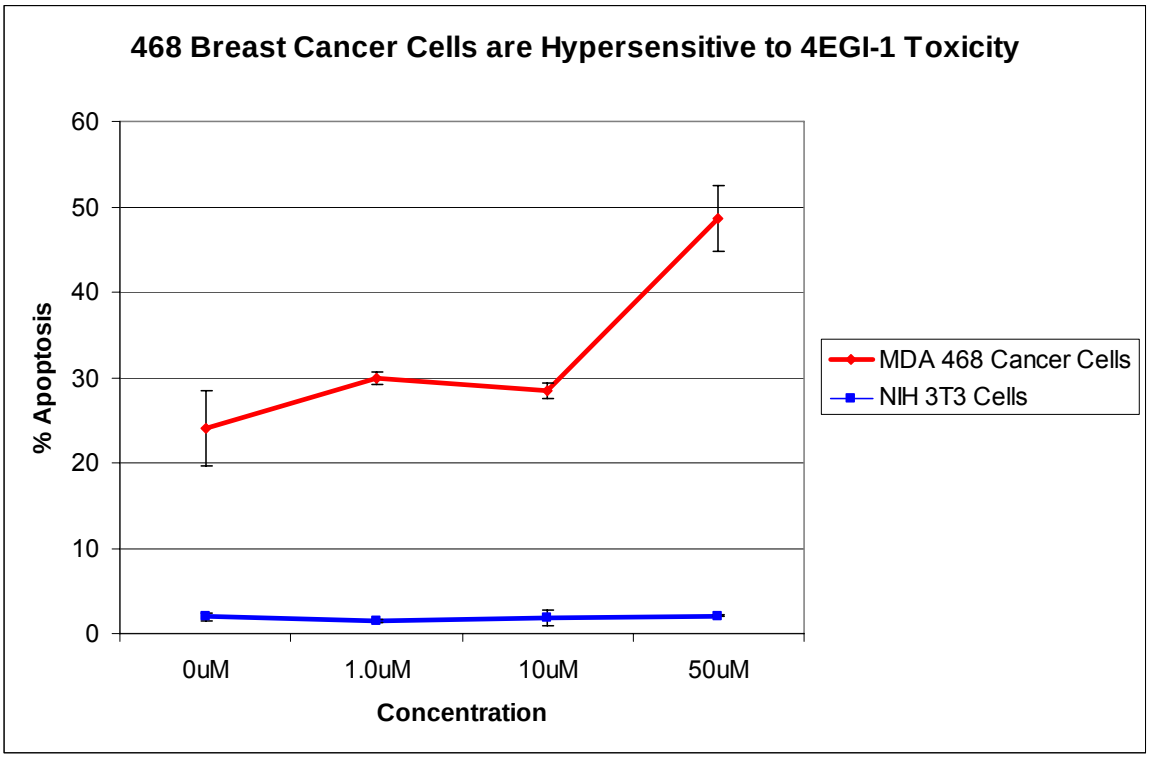

4EGI-1 Compound Antagonizes eIF4E Stimulated Proliferation with Relatively Low Toxicity
% Percentage of Cells
30
25
20
15
10
5
0
Proliferative Fraction (Dividing Cells)
Toxicity Curve (Cells in Apoptosis)
0uM
0.1uM
1.0uM
10uM
100uM
[Concentration of 4EGI-1]

4E-GI-1 Triggers Apoptosis in 468 Breast Cancer Cells

In the *statement of thesis*, I proposed a concept that increased eIF4E stimulates translation for a subset of mRNA. The increased translation typically affects regulatory proteins which may be involved in cell cycle progression and other oncogenic functions, without harming “house-keeping” proteins. Therefore a drug which targets cap-dependent translational machinery should act selectively to target cancer cells while showing little to no toxicity in normal cells. To test this, I used MDA 468 breast cancer cells which are known to over express eIF4E and non-transformed NIH-3T3 cells (Figure 19).

Figure 19. Breast Cancer Cells are Hypersensitive to 4EGI-1 Administration

468 Breast cancer cells are plated (300K/well) in a 6 well plate, Incubated for 48hrs in regular growth medium, then treated with increasing dosages of 4EGI-1 (also administered in regular growth medium). Cells were then fixed and DNA content was quantified by flow cytometry. Shown is the proportion of apoptotic cells (measured by hypodiploid DNA content). 468 cells are hypersensitive to 4EGI-1, while 3T3 fibroblasts (control condition) used in a parallel experiment, show no apoptosis in concentrations up to 50uM.


468 Breast Cancer Cells are Hypersensitive to 4EGI-1 Toxicity
% Apoptosis
60
50
40
30
20
10
0
0uM
1.0uM
10uM
50uM
Concentration
MDA 468 Cancer Cells
NIH 3T3 Cells

An increasing dosage of 4EGI-1 (which antagonizes association between eIF4E and eIF4G) triggers apoptosis in 468 cancer cells. I observed as much as 50% cell death when treated with 4EGI-1 (50uM). In comparison, 3T3 fibroblasts were able to withstand exposure to 4EGI-1 (50uM) with less then 3% cell death. This data supports my assumption of selective “translational targeting” as an approach to cancer therapy. We know 468 Breast cancer cells over express eIF4E, and therefore contain elevated amounts of complex mRNAs which make the cells susceptible to eIF4E suppression.

Research Potential

There is a strong potential for future studies analyzing translational machinery as novel targets in cancer therapeutics. I have demonstrated the ability to reverse oncogenic characteristics in a high fidelity system with the use of compounds which inhibit eIF4F assembly. While the results in 7 methyl cap analogs were modest, the experiments with *PG-175* provide proof of concept; targeting the 7 methyl cap-eIF4E complex formation is a feasible method in suppressing cap dependent translation. These studies also provide another use for the 4E-inducible system as a screening device for eIF4F antagonists. Use of novel compounds in the 4E inducible system will effectively test the compounds ability to reverse the most basic of oncogenic functions; proliferation.

Therapeutic Implications

The data collected with use of the pharmacological tool, *4EGI-1*, provides significant promise in therapeutic usage. The 4E-BP mimic was successful in reversing eIF4E triggered proliferation, and showed very little toxicity at the effective concentrations. The very aggressive 468 breast cancer cells appeared to be hypersensitive to treatment with *4EGI-1*. The LC50 concentration in 468 breast cancer cells induces apoptosis in less than 3% of naïve 3T3 mouse fibroblasts. These data imply *4EGI-1* is highly effective in causing apoptosis in cancer cells (which express high levels of eIF4E), without causing cell death (toxicity) in cells expressing normal levels of eIF4E. Following this logic also suggests the possibility to selectively target cancer cells by interrupting eIF4F, and eradicating the often seen toxicity associated with chemotherapy treatment.

Chapter VII.

Discussion

Translational Control Holds Major Implications in Cancer

My entrance into the Bitterman- Polunovsky lab was preceded by a massive compiling of information about Translational Control and its implications in Cancer. These findings were made by members of my lab, as well as numerous researchers and collaborators worldwide.

As expected- one of the most energy expensive processes in cellular function (protein synthesis) is under a tightly restricted regulatory complex. The translational machinery which orchestrates protein synthesis has been shown to regulate a wide variety of cellular processes, including cell development, growth and neuronal events including memory storage [53]. Arguably, the most significant impact within the Translational Control field has come from the many studies which have confirmed deregulation of the translational machinery is on the causal pathway to cancer [47]. Researchers identified eIF4E to be the limiting rate factor in the cap dependent translation process and an oncogene responsible for transformative characteristics in normal cells. With the use of human breast carcinoma and murine lymphoid malignancies, the cap-dependent protein synthesis machinery was shown to be a critical point of convergence and amplification of oncogenic signals emanating from the Ras/PI3K/Akt cascade.

Over expression of eIF4E was shown to cause malignant conversion of rodent fibroblasts [35], human mammary epithelial cells [2] and promotes tumor formation in transgenic mice [59, 75]. Consequently, tumors isolated from humans have been shown to express abnormally high levels of eIF4E [60]. Scientists have also demonstrated the ability of complex antagonist, 4E-BP1, to reverse phenotypes seen after eIF4E over expression. BPs have been shown to prevent assembly of the eIF4F complex by sequestering eIF4E [23] which results in a decrease in certain mRNAs that require high levels of available eIF4E [20]. Ectopically expressed BP's were shown to partially reverse the eIF4E transformed phenotype [57], and hyper phosphorylated BPs, which dissociate from eIF4E, were observed in transformed mammary cell lines [1].

Scientists had previously confirmed increased eIF4E activity does not result in elevated global translation. Instead it results in the increased translation of a subset of mRNA with extensive secondary structure in the 5' UTR and typically involved in the growth regulatory process [31, 53]. This feature is significant as it provides potential for the selective targeting of mRNA that has an elevated dependence upon eIF4F complex assembly [33]. As mentioned previously, decreased eIF4E activity causes a reversal of malignant phenotypes. Compounds that mimic the anti-translational activity of BP's may serve as useful agents to selectively treat malignancies where eIF4E is high [53]. Theoretically, there is potential for compounds to reverse/ suppress oncogenic properties without harming normally functioning cells.

Oncogenic transformation begins with alterations in cell cycle progression. While there were significant findings in the transformative properties of translational machinery deregulation, the mechanism of action leading to the phenotype was relatively unknown. The limitation of prior knowledge was largely due to the experimentation being limited to cells which constitutively over express eIF4E. Even with the existing experimental limitations, scientists were able to prove eIF4E activity is important for both entry and transit through the cell cycle [65]. Scientists also demonstrated the cell cycle progression is largely regulated by BP1 phosphorylation; phosphorylated (inactive) 4E-BPs dissociate from eIF4E, leaving the limiting factor free to drive mRNA translation. Scientists had shown that BP1 is bound to eIF4E during the S and G2 cell cycle phases as the cell prepares for mitosis, then dissociates from eIF4E during mitosis- only to bind eIF4E again after mitosis is complete. The bind and release action between BP1 and eIF4E is in coordination with the phosphorylation of BP1 at the Thr-70 and Ser-65 sites [26].

The most pertinent question of translational machinery activity is the detection of the target mRNA's whose translation is activated in 4E over expressing cells [65]. Cell cycle regulators, c-*myc* and RAS, have been shown to become activated in 4E transformed cells- which may account for its malignant phenotype [36]. When 4E is over expressed in NIH-3T3 cells there appears to be an increased expression of cyclin D [56] and ornithine decarboxylase (ODC) [62]. These proteins are required for regulating cell cycle progression and growth. Cyclin D1 is

required for cell cycle progression from G_1 to S phase [76] and ODC plays an essential role in cell growth and differentiation [45]. Prior results in constitutive systems indicated that the sole mechanism was accelerated transit of the cyclin D1 mRNA from the nucleus to the cytoplasm where it entered the translationally active pool [11, 12, 55, 58, 67-69]. There had been no reported evidence of eIF4E regulating cyclin D1 by stimulating its translation.

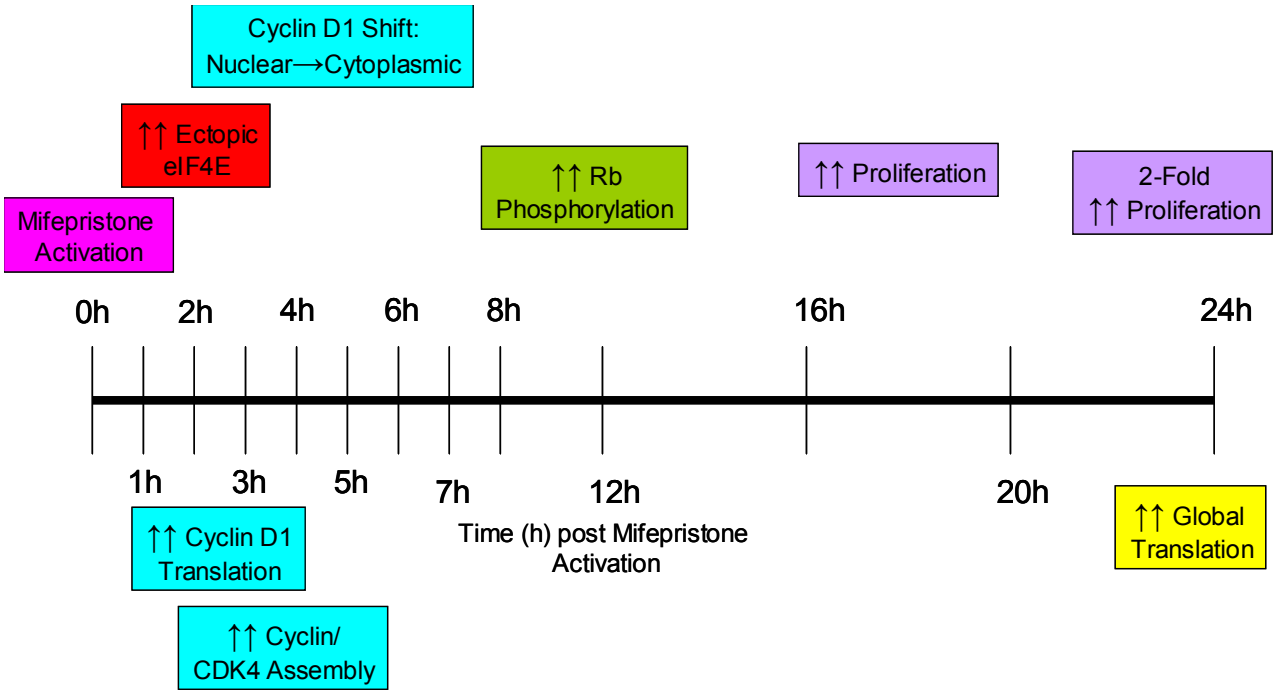
As I entered the field, there existed a well established connection between increased translational machinery, stimulated cell cycle progression and ultimately oncogenesis. While there were substantial examples of oncogenes which increased after eIF4E over expressing cells, there was a gap in knowledge when identifying the mechanism of eIF4E modification to cell cycle kinetics. I was able to examine the underlying molecular mechanisms and make novel contributions to the field. My contributions include: i. Development of a high-fidelity eIF4E inducible system, ii. Demonstrating that abrupt over expression of eIF4E causes a 2-fold increase in proliferation within 24h, iii. Proving eIF4E regulates the G1/S phase gatekeeper, cyclin D1, by increasing its translation, iv. Demonstrating eIF4E can bypass growth factor initiation of the canonical proliferative pathway by acting directly upon (and activating) cyclin D1, and v. Advancing pharmacological evidence for the potential of eIF4F antagonists to intervene (and reverse) eIF4E triggered oncogenic properties.

Potential of the eIF4E Inducible System

Prior to my work, discoveries in eIF4E were made with the use of cells which constitutively over express eIF4E. We now understand eIF4E to be an extremely potent and pleiotropic oncogene- and it's continued over expression likely mutates cells over time. Comparing constitutive over expressing eIF4E cells to normal cells may have created biased experimental conditions. With the use of the eIF4E inducible system- we now have the ability to abruptly activate eIF4E over expression, and trace it's morphology in comparison to inactivated cells which do not over express eIF4E. This study utilizes mifepristone (progesterone antagonist) to activate protein synthesis of eIF4E. We developed this system with the full intentions of exploiting eIF4E over expression to closely examine its trophic capabilities. In my studies eIF4E is typically activated in serum deprived conditions, lacking any growth factors which would initiate proliferation. Prolonged serum deprivation in the 4E-Inducible cells would typically cause quiescence and eventually death. Therefore my findings of increased proliferation after selective eIF4E activation in serum deprived conditions- are even more significant when considering these cells were completely inactive, and soon to be dead. This system holds great potential in revealing more actions initiated by eIF4E. I have thoroughly investigated manipulations in cell cycle progression after mifepristone activation, and developed a complete timeline which follows cell cycle kinetics culminating in stimulated proliferation (figure 20). By understanding the chronological sequence of eIF4E triggered mutations, we can better understand its transformative process.

Figure 20. eIF4E Inducible System: Cell Cycle Kinetics Timeline

Within 1h mifepristone activation, I observed a rapid and drastic increase ectopic eIF4E expression followed by increased cyclin D1 expression. At 2h I observe increasing association between cyclin D1 and cdk4, which is followed by increased nuclear export of cyclin D1 at 3h. Within 8h post mifepristone activation I observed increased phosphorylation (ser 780), and by 16h post activation I document increasing cell cycle entry. By 24h post activation I observed a 2-fold increase in proliferation, as well as increased global translation (cap dependent and IRES) in serum starved- eIF4E induced cells.


Cyclin D1 Shift:
Nuclear⟶Cytoplasmic
↑↑ Ectopic
eIF4E
Mifepristone
Activation
↑↑ Rb
Phosphorylation
↑↑ Proliferation
2-Fold
↑↑ Proliferation
0h
2h
4h
6h
8h
16h
24h
1h
3h
5h
7h
12h
20h
↑↑ Cyclin D1
Translation
↑↑ Cyclin/
CDK4 Assembly
Time (h) post Mifepristone
Activation
↑↑ Global
Translation

This system will also be useful for analyzing other biological effects of eIF4E over expression, immediately after activation. The eIF4E inducible system provides a promising model for drug experimentation. Because these cells function as normal fibroblasts prior to eIF4E over expression, we can utilize chemical compounds to interfere with eIF4F assembly in an attempt to reverse eIF4E triggered oncogenic characteristics. The ability to reverse an eIF4E triggered event, such as proliferation, in the 4E-Inducible cells will validate further examination of a potential therapeutic compound.

Novel Findings in eIF4E Kinetics

The trophic effects of eIF4E had been well documented prior to my entry into the field. However, my work has provided a better understanding of the cellular kinetics post eIF4E activation. Prior to my findings, cells over expressing eIF4E- were shown to exhibit increased proliferation in comparison to cells which express normal levels of eIF4E protein [(65)]. Therefore scientists have concluded eIF4E over expression leads to increased proliferation, without developing a cause and effect relationship.

I show an immediate response in proliferation after induction of eIF4E over expression. Within 16h of eIF4E activation, I begin to see increased proliferation reaching a maximum 2-fold increase by 24h. This finding provides insight on the potent and pleiotropic effects of eIF4E activity due to its immediate and drastic effects in proliferation. The eIF4E triggered stimulation of proliferation led me to

further examine it's manipulation of cell cycle kinetics, which are known to regulate proliferation.

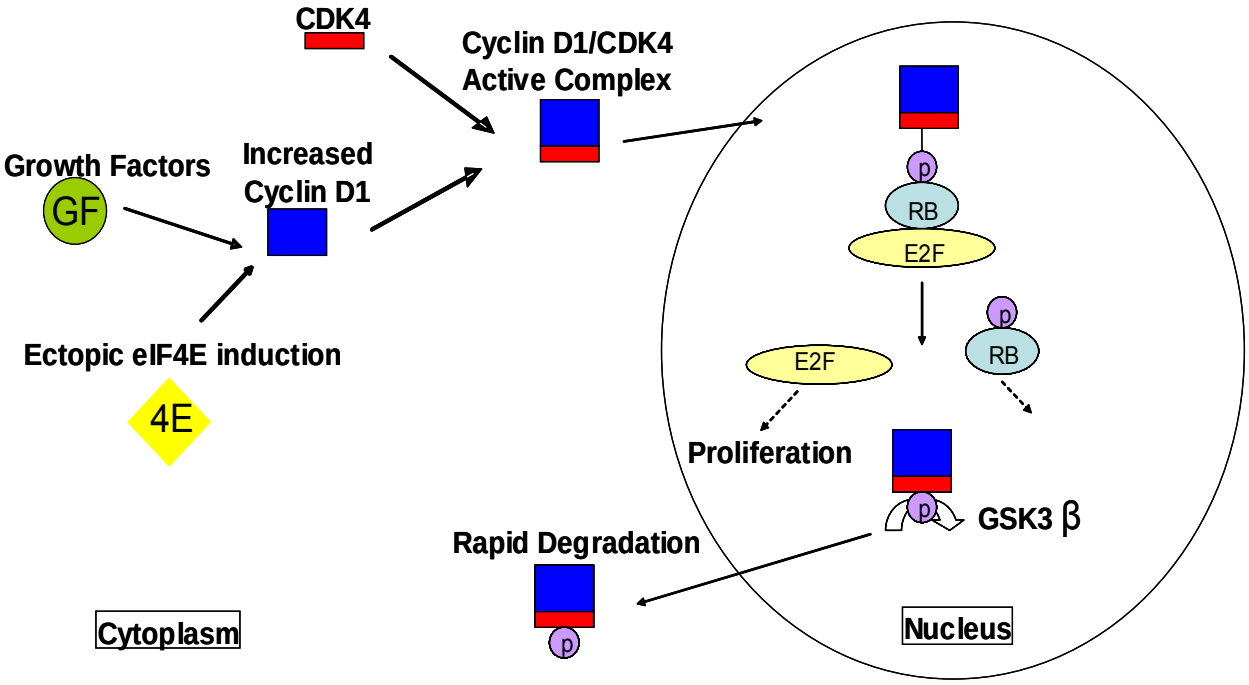
eIF4E Bypass of Growth Factors

Just as scientists had previously reported changes in proliferation after eIF4E over expression- the same was true for cell cycle kinetics which precedes proliferation. Scientists showed cells which over express eIF4E also expressed increases in positive cell cycle regulators, cyclin D1 & ornithine decarboxylase (ODC) [56, 62]. Scientists also demonstrated an increase in ODC translation, an effect which was not observed in cyclin D1. With use of the 4E-Inducible model, I was able to show increased cyclin D1 protein expression, and an increase in the average number of ribosomes bound to cyclin D1 within 1h of mifepristone activated eIF4E over expression. This increase in ribosome bound to cyclin D1 results in increased translation and ultimately protein synthesis. This finding is completely novel and implies eIF4E is capable of bypassing growth factor initiated proliferation by directly activating cyclin D1 translation, and stimulating its nuclear export into the cytoplasm. I also observed down stream effects of eIF4E over expression, on the canonical proliferative pathway. When quiescent cells are exposed to growth factors, the cytoplasmic cyclin D1 protein abundance is increased. Cyclin D1 then associates with its partner kinases, cdk4 and cdk6, resulting in an activated phosphorylation complex. The resultant cyclin/cdk complex enters the nucleus where it phosphorylates the retinoblastoma protein (Rb) [49]. I traced the fate of cyclin D1 with a pull down assay, and found an

increase of cyclin D1/cdk4 complex assembly 2h post mifepristone activated eIF4E over expression. I then observed increased Rb phosphorylation 8h post mifepristone activation. Each of these events takes place well before the observed increase in proliferation (16h), and combines to provide a detailed description of how eIF4E triggered increase in cell cycle kinetics leading to proliferation- independent of growth factors. I have developed a model which outlines the eIF4E bypass pathway (Figure 21).

Figure 21. The Cell Division Bypass: Deregulated eIF4E Substitutes for Growth Factors in the Canonical Proliferative Pathway

I propose a model which supports the established mechanism and introduces novel components to the canonical proliferative pathway. My research suggests that ectopically induced eIF4E translationally activates cyclin D1. Following cyclin D1 activation- cdk4 associates with cyclin D1 to form the active kinase unit, leaving the cytoplasmic storages of cdk4 temporarily depleted. The active cyclin D1/cdk4 complex is then translocated into the nucleus where it phosphorylates Rb. The cyclin D1 itself is then phosphorylated by GSK3β, exported from the nucleus and degraded.


CDK4
Cyclin D1/CDK4
Active Complex
Growth Factors
GF
Increased
Cyclin D1
Ectopic eIF4E induction
4E
p
RB
E2F
E2F
p
RB
Proliferation
p
GSK3 β
Rapid Degradation
p
Cytoplasm
Nucleus

Pharmacological Intervention of eIF4F Assembly

Binding of eIF4E to the 5' mRNA cap structure facilitates nuclear export of selected transcripts, and primes mRNA for translation initiation when eIF4E associates with the docking protein eIF4G. The N-terminal part of eIF4G contains binding sites for eIF4E and the poly (A)-binding protein PABP. The phylogenetically conserved central third of eIF4G binds the helicase eIF4A, the multimolecular complex eIF3 and RNA. The resulting pre-initiation ribosome complex scans the 5' UTR to locate the initiation codon (AUG) where the 60S ribosome subunit joins to form an elongation competent 80S ribosome. The eIF4E/eIF4G interaction is negatively regulated by members of the 4E-BP family of translational repressor proteins. They compete with eIF4G for binding to eIF4E thereby sequestering capped transcripts into translationally inactive complexes. 4E-BPs are regulated by mTOR mediated phosphorylation caused by hormones and growth factors [(47)].

The rate limiting step in protein synthesis is initiation, mediated by the trimeric protein initiation factor eIF4F; and the limiting component of eIF4F is the mRNA cap-binding protein, eIF4E. The cap-dependent translation initiation pathway relies upon assembly of the eIF4F complex. Potentially, compounds which can interfere with this process will prevent eIF4F complex assembly, and suppress translation initiation. I utilized compounds which target eIF4F assembly at various steps in its assembly within the 4E-Inducible system in an effort to reverse eIF4E

triggered proliferation. This includes compounds which mimic the 7-methyl cap to bind eIF4E, mimic 4E-BP activity and potentiate 4E-BP activity.

mTOR Suppression

The mTOR Kinase is capable of phosphorylating (therefore inactivating) 4E-BPs[78]. Phosphorylation by mTOR is initiated by hormones and growth factors, and leads to 4E-BP deregulation of eIF4F assembly. We have access to a compound, Rapamycin, which targets and suppress mTOR allowing a potentiated 4E-BP efficacy. This compound was successful in suppressing cap dependent translation and eIF4E triggered proliferation; therefore was used as a positive control in my early drug studies.

eIF4E Cap Analogs

The 7-methyl guanosine cap binds to eIF4E to initiate cap dependent translation. One strategy to decrease cap dependent translation is to antagonize the interaction between eIF4E and the 7-methyl guanosine cap. In our drug discovery program, we have developed a 7-methyl guanosine cap analogs to compete with the 7-methyl cap for the binding site on eIF4E[19]. After utilizing various cap analogs which exhibited limited efficacy, I focused on the use of the leading compound BG-I-77 for my drug selection studies. BG-I-77 showed slight efficacy in reversing eIF4E triggered proliferation in the 4E-Inducible system when used independently. However when the cap analog is co-administered with Rapamycin, I observed a suppression of eIF4E-triggered proliferation which

exceeded standard Rapamycin. This finding suggests the cap analog is most effective when used in combination with Rapamycin possibly due to increased permeability or target site action. Continued development of BG-I-77 to improve cell permeability, receptor binding and pro drug activation has been successful and will soon be document by lab mate Alexey Benyumov.

4E-Binding Protein Mimics

If the eIF4E-mediated proliferative response depends upon its function in cap-dependent translation initiation, then agents that directly antagonize eIF4F function should negatively regulate proliferation. To test this theory, I have used a 4E-BP mimicking compound designated 4EGI-1 that inhibits eIF4F assembly by blocking association of eIF4E with eIF4G [(41)]. This compound was generously provided by Dr. Gerhard Wagner, Harvard University. When mifepristone treated cells were incubated with 4EGI-1 for a period of 24 hours, there was a dose-dependent decrease in proliferating cells triggered by eIF4E over expression. I observed a significant proliferative suppression (EC50) at relatively low concentrations (<10uM). Reversal of eIF4E triggered proliferation at this concentration was significant due to the low dosage. In the same cells, I was able to demonstrate a very low potential for toxicity and hypersensitivity in cancer cells. These findings were very important for my study as they validate my hypothesis that selective targeting of translational machinery will reverse/ terminate cells reliant on increased translational activity (cancer cells)- while not harming cells which maintain normal endogenous translational rates (normal

cells). This finding has many clinical implications in selective targeting of cancer cells and decreased toxicity in normal cells.

Therapeutic Implications

My work has contributed to the field by providing a better understanding of how cap-dependent translation regulates cell proliferation, and therefore helped advance a novel concept for cancer biology and therapeutics focused on translational control. We now know that eIF4E acts to directly activate cyclin D1, bypassing the initiating effects of growth factors. We also know that the selective targeting of eIF4E reverses an oncogenic characteristic, autonomy for cellular division, without inducing cellular toxicity. This finding is imperative as it provides the possibility of drug specificity in a wide range of diseases in which hyperactive eIF4E is a hallmark feature. My research has demonstrated that abrupt activation of eIF4E causes cells to exhibit oncogenic transformative properties such as proliferation. Therefore drugs which target eIF4F assembly will target a point of convergence of multiple cancer pathways to have an early and potent effect in cancer intervention and prevention.

A drug which contains features such as selective targeting of a point of convergence and minimal toxicity will have enormous potential in clinical implications. The intervention potential of such drugs will be useful in situations where early detection of cancer progression has been established. Such a compound would be useful in treating pulmonary tumor lesions before they

metastasize leading to lung cancer. Preventive capabilities will also be useful, as they can be used in situations where individuals have increased risk of disease occurrence. For example, women treated for breast cancer have increased risk of disease recurrence- even after successful treatment. Such a drug which continually suppresses cancer transformation by acting at a point of oncogenic pathway convergence could potentially be useful as maintenance therapy after initial chemotherapy treatment.

A drug of this capacity has major implications in pharmacogenomics and pharmacogenetics. Pharmacogenetics is genetic variation that gives rise to differing response to drugs, while pharmacogenomics is the broader application of genomic technologies to new drug discovery and further characterization of older drugs. As the pharmacology field continues to develop and advance- individualized medicine will increasingly come to the forefront. Along with the potential for individualized medicine will come the need to analyze individual genetic make-up. Pharmacogenetic factors have also been strongly implicated in cancer treatment and chemotherapy[6]. Since we know that increased eIF4E and the translational machinery is on the causal pathway to cancer, genes involved in translation regulation will be leading candidates for examination in oncogenic potential. Individuals with a genetic propensity toward increased eIF4F assembly will likely also have an increased potential for cancer onset. These individuals will greatly benefit from maintainence therapy of a drug which antagonizes hyperactive eIF4E activity.

Toxicity is the poisoning of cellular functions, and has plagued chemotherapy treatment since its origination. Often chemotherapy has targeted healthy and cancer cells alike causing a wide range of adverse effects ranging from nausea to death. Chemotherapeutic toxicity has limited treatment options at times, due to the inability of patients to handle the harsh side effects associated with some drugs. Interference of a cellular process observed in all cells will lead to cell death in the targeted diseased cells and in healthy cells as well. However, if you can identify a feature specific to cancer cells and not observed in normal healthy cells, this feature may be exploited for the selected targeting of cancerous cells. Overactive translational machinery marked by the increased eIF4F assembly (and hyperactive eIF4E) is a characteristic explicitly seen in cancer when compared to normal cells. This makes increased eIF4F assembly a prime candidate for selective targeting in cancer. My research has demonstrated compounds which antagonize eIF4F assembly (either by mimicking 4E-BP activity or eIF4E sequestration) are effective in killing cancer cells, while having little to no toxic effects in normal cells. This finding suggests the clinical usage of drugs which target eIF4F assembly will eliminate cancer cells without harming normal cells, avoiding toxicity. The discovery of a cancer selective, non-toxic drug capable of intervention and prevention will be a major breakthrough in cancer treatment.

Chapter VIII.

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