Metastasis and the Impact of Translation in the Tumor and Host: a phospho-eIF4E Story

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ABSTRACT

Most cancer related deaths are due to metastatic progression. While deregulated protein synthesis in cancer cells is recognized as playing a major role in tumorigenesis, the association between the translational machinery and cancer dissemination have remained largely unexplored. To begin addressing this situation, the present thesis focuses on a single regulatory event: the phosphorylation of the cap-binding protein eIF4E. eIF4E is an oncogene that is overexpressed in human cancers, and its levels and activity correlate with poor survival. Phosphorylation on serine 209 is required for its oncogenic activity, and mice bearing a non phosphorylatable alanine mutant of eIF4E (S209A) are resistant to the development prostate cancer. Utilizing these mice and cells isolated therefrom, the current work establishes that cancer cells unable to phosphorylate eIF4E display reduced epithelial-to-mesenchymal transition (EMT) and invasive capacities. Furthermore, mammary tumors bearing the S209A mutation are less metastatic than their wild-type counterparts due to reduced translation of mRNAs encoding MMP3 and SNAIL. Surprisingly, eIF4E phosphorylation in non-transformed cells of the host also plays a role in metastatic progression of mammary tumors, as its detection in the tumor microenvironment correlates with poor survival in breast cancer patients. Consistently, S209A mice are resistant to metastasis, even when bearing tumors derived from 66cl4 cells that express wild-type eIF4E. Neutrophils are, at least in part, responsible for this phenotype: their aberrant accumulation is critical for lung colonization from 66cl4 tumors, however, the translation of anti-apoptotic mRNAs necessary for neutrophils to survive and accumulate is dependent on eIF4E phosphorylation. Interestingly, the small molecule inhibitor merestinib inhibits eIF4E phosphorylation in 66cl4 cells and in neutrophils *in vivo*, resulting in reduced neutrophil accumulation and the prevention of metastasis. Thus, the experiments outlined herein highlight the importance of translation in the metastatic process, uncover its important role in cells of the tumor microenvironment, and identify a therapeutic target whose inhibition in both the tumor and immune system contributes to the prevention of metastasis.

RÉSUMÉ

La mortalité chez les patients cancéreux est principalement associée à la formation de métastases. Alors que l'importance de la synthèse protéique dans la formation des tumeurs est bien établie, son rôle dans leur dissémination demeure inexploré. Pour étudier cet aspect du cancer, la présente thèse porte sur un phénomène régulatoire important dans la traduction des ARNm en protéine : la phosphorylation d'eIF4E. Ce facteur d'initiation est un oncogène qui est fréquemment surexprimés dans divers cancers, et son hyperactivité est associée à un mauvais pronostic. La phosphorylation sur la serine 209 est requise pour son activité : ainsi, il a été démontré que les souris porteuses d'une mutation à ce site (S209A) sont résistantes au développement du cancer de la prostate. À l'aide de ce modèle animal, cet œuvre permet d'établir que les cellules cancéreuses incapables de phosphoryler eIF4E sont moins aptes à procéder à la transition épithéliale-mésenchymateuse et à envahir la matrice extracellulaire. De plus, les tumeurs mammaires S209A sont moins métastatiques que les tumeurs de type sauvage, ce qui est causé par leur capacité réduite à traduire les ARNm encodant MMP3 et SNAIL. Surprenamment, la phosphorylation d'eIF4E dans les cellules non-transformées de l'hôte joue aussi un rôle dans le processus métastatique, puisqu'il y a une corrélation entre son expression dans les cellules du microenvironnement tumoral et la survie des patientes atteintes du cancer du sein. De plus, les souris S209A sont résistantes à la formation de métastases, même lorsque celles-ci proviennent de tumeurs 66cl4 exprimant la version sauvage d'eIF4E. Les neutrophiles sont responsables de ce phénomène, puisque leur accumulation anormale est requise pour la colonisation des poumons par les cellules 66cl4, alors que la traduction d'ARNm anti-apoptotiques nécessaires à leur survie et leur accumulation dépend de la phosphorylation d'eIF4E. De plus, un inhibiteur de plusieurs kinases, merestinib, bloque la phosphorylation d'eIF4E dans les cellules 66cl4 et les neutrophiles *in vivo*, permettant de prévenir leur accumulation et la formation de métastases. Ainsi, cette étude souligne l'importance de la traduction dans la dissémination métastatique, a permis de découvrir son rôle dans les cellules du microenvironnement tumoral, et identifie une cible thérapeutique dont l'inhibition à la fois dans la tumeur et le système immunitaire permet de combattre les métastases.

PREFACE AND CONTRIBUTION OF AUTHORS

The present thesis consists in large part of manuscripts published or submitted to peer-reviewed journals, of which I am the first or co-first author. Their citations are included here, with permission where required, and are distributed in the text as follows:

Chapter 1:

Robichaud N, Ruggero D, Schneider RJ, Sonenberg S. Translational Control of Cancer. In: Mathews MB, Sonenberg N, & Hershey JWB, editors. *Translation Mechanisms & Regulation*. Cold Spring Harbor: Perspectives; (expected publication 2018).

Robichaud N, Sonenberg N. eIF4E and Its Binding Proteins. In: Parsyan A, editor. *Translation and Its Regulation in Cancer Biology and Medicine*. Dordrecht: Springer Netherlands; 2014. p. 73-113.

Bhat M, Robichaud N, Hulea L, Sonenberg N, Pelletier J, Topisirovic I. Targeting the translation machinery in cancer. *Nat Rev Drug Discov*. 2015 Apr;14(4):261-78.

Robichaud N, Sonenberg N. Translational control and the cancer cell response to stress. *Curr Opin Cell Biol*. 2017 Apr;45:102-109.

Chapter 2:

Robichaud N, del Rincon SV, Huor B, Alain T, Petruccelli LA, Hearnden J, Goncalves C, Grotegut S, Spruck CH, Furic L, Larsson O, Muller WJ, Miller WH, Sonenberg N. Phosphorylation of eIF4E promotes EMT and metastasis via translational control of SNAIL and MMP-3. *Oncogene*. 2015 Apr 16;34(16):2032-42.

Chapter 3:

Robichaud N, Hsu BE, Istomine R, Alvarez F, Blagih J, Ma EH, Morales SV, Dai DC, Souleimanova M, Guo Q, del Rincon SV, Miller WH, Jones R, Piccirillo C, Siegel PM, Park M,

Sonenberg N. Translational Control in the Tumor Microenvironment Promotes Lung Metastasis via Phosphorylation of eIF4E in Neutrophils. (submitted to Cancer Cell 2017)

Contribution of authors:

The manuscripts forming the major part of this thesis included work from several co-authors to whom I owe many thanks. My contributions and those of my co-authors are delineated here.

Chapter 1 is a literature review consisting mainly of a manuscript entitled “Translational Control of Cancer”, which will constitute a chapter of the upcoming book “Translation Mechanisms & Regulation” for Cold Spring Harbor Perspectives. The original draft and all figures were my work, whereas all authors contributed extensive editorial work on numerous subsequent drafts. Chapter 1 also includes excerpts from other published reviews. For “Translational control and the cancer cell response to stress” and “eIF4E and its binding proteins”, I wrote the original draft and produced the figures, N.S. provided editorial support. For “Targeting the translation machinery in cancer”, I wrote parts of the original draft, revised the manuscript and produced the figures. L.H. wrote most of the first draft. I.T., J.P. and N.S. provided insight, support and revisions. M.B. also participated.

Chapter 2 was published in the journal Oncogene with the title “Phosphorylation of eIF4E promotes EMT and metastasis via translational control of SNAIL and MMP-3” Of note, S.V.d.R. and I contributed equally to the work. I contributed little to figures relating to TGFβ-induced EMT, where my only input was to provide reagents (constructs and small molecule inhibitors). I planned, performed and analyzed the experiments presented in all other figures, with guidance from N.S. I drafted the manuscript and compiled the figures, with editorial input from all authors. Some replication experiments were performed by co-authors, and O.L. performed most of the bioinformatics analysis presented in figure 2.2A. S.V.d.R. planned, performed and analyzed experiments on the induction of EMT *in vitro*, contributed to the writing and editing of the manuscript and the design of the figures. B.H.,

L.A.P., C.G., T.A., and S.G. participated in experiments on EMT. J.H. participated in rescue experiments on invasion. L.F. and W.J.M. were involved in mouse model development. C.H.S., W.H.M. and N.S. secured funding and supervised the research.

Chapter 3 has been submitted to Cancer Cell with the title "Translational Control in the Tumor Microenvironment Promotes Lung Metastasis via Phosphorylation of eIF4E in Neutrophils". While the list of authors is extensive, the work presented in the main figures and text was planned, performed, analyzed and drafted by N.R., except for the clinical data associated with the tissue microarray, which was collected by M.P.'s group and curated by M.S., and merestinib treatments of 66cl4 cells *in vitro*, which were performed by S.V.M. and D.C.D. Other contributions from co-authors were included in the supplemental figures or were provided in the form of sample preparation and preliminary experiments. B.E.H., R.I., F.A., J.B. and E.M. were involved in flow cytometry and neutrophil isolation experiments. Q.G. and S.V.d.R. were involved in mouse colony maintenance. W.H.M., R.J., C.P., P.M.S., M.P. and N.S. secured funding, recommended experiments, supervised the research and edited the manuscript.

Contributions to knowledge and elements of original scholarship:

This document has been eight years in the making. It is therefore not surprising that elements that were considered original at the time have since been described by other groups as well. This fact may lead to some unfortunate confusion when reading the literature review introducing my thesis, as it reviews work published as recently as August 2017. It therefore cites publications with experiments that would seem to take away from the novelty of my own investigations, but is merely a consequence of their release at a time near, or subsequent to, that of my own data. To avoid any misunderstandings, the elements of this thesis which were considered original scholarship at the time the research was conducted are delineated below. A more explicit description of publications preceding and following the experiments described here are included as the introduction of each chapter.

Elements that are not as original but nonetheless constitute distinct contributions to knowledge are also described here.

Elements of original scholarship:

1. Phosphorylation of eIF4E promotes migration and invasion in several cell models.
2. eIF4E phosphorylation promotes the translation of the mRNA encoding SNAIL.
3. eIF4E is phosphorylated downstream of TGFβ
4. Phosphorylation of eIF4E promotes EMT independently of the activity of the SMAD family of transcription factors.
5. Phospho-eIF4E promotes mammary tumor formation in the MMTV-PyMT mouse model
6. eIF4E phosphorylation promotes metastatic progression *in vivo*.
7. Phospho-eIF4E is detectable in the stromal cells of human breast cancer samples, and its presence correlates with poor survival.
8. Translational control in the TME plays a significant role in tumor biology
9. Phospho-eIF4E in the TME promotes metastatic progression
10. Phospho-eIF4E promotes the survival of neutrophils in response to G-CSF
11. Merestinib inhibits metastasis in a mouse mammary tumor model

Distinct contributions to knowledge

Other aspects were not entirely original but served to reinforce or dispute previously published observations:

1. The importance of translational control in cancer is well established; the current work contributes to the broader acceptance of this concept, in particular as it pertains to the phosphorylation of eIF4E.
2. Many of the known activities of eIF4E have been shown to require its phosphorylation for maximal effect, notably cellular transformation and cytokine production. Now, EMT, invasion and metastasis can be included in this list.

3. Activation of the MNKs has been linked to EMT in glioblastoma, but the mechanism invoked differed entirely and involved SMAD2, which we tested but could not observe.
4. Translation in cells of the TME has been largely ignored, despite reports indirectly supporting its potential role. Thus, eIF4F complex formation *in vitro* has been suggested to be affected by crosstalk between multiple myeloma cells and mesenchymal stem cells, and mTOR activity is important in both myeloid-derived suppressor cells and T cells in several disease models. The experiments herein explicitly establish a role for translation in the TME and demonstrate its effect on metastatic progression, solidifying previous findings and providing a solid rational for future investigation of this concept.

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I am also grateful for the financial support I have received in the form of grants and scholarships from government agencies, CIHR and FRQ-S, as well as private foundations, Susan G. Komen for the Cure and Terry Fox Research Institute. Even more so for the amazing fundraising in the Montreal community, led by the Goodman family and the wonderful people at Canderel, that allow for the creation of scholarships and travel awards that have been so important for my PhD research and that of my peers. Therefore, I extend my thanks

to the donors, organizers and armies of students and staff members involved in these efforts.

I would also like to acknowledge everyone that was involved in the experiments described herein. This includes all my co-authors in manuscripts included in this thesis, whose contributions are detailed in the previous section, as well as several other people who taught me how to perform critical experiments. I would therefore like to thank Chadi Zakaria for taking over the midi-prep I for some reason could never get to work, and for putting up with my sense of humor and general shenanigans; Colin Ratcliffe, Dongmei Zuo and the good people at the imaging facility for their help, training and advice on microscopy; Nadeem Siddiqui and Edna Matta Camacho for their advice on recombinant protein production and assays using them (too bad that project never led to anything…); Isabelle Harvey and Meena Vipparti for all the work they do for everyone in the lab; Annie Sylvestre, Annik Lafrance and everyone at McGill's Comparative Medicine and Animal Resources Centre, for all their help and training on experiments using mice; Julien Leconte and Vinicius Motta for flow cytometry training and cell sorting; and everyone at the histology facility for processing of mouse samples for immunohistochemistry. Many thanks to the following people for editorial help on various published works: Valerie Henderson, Dana Pearl, Soroush Tahmasebi, Tommy Alain and Chadi Zakaria. If I've forgotten anyone, please forgive me and accept these anonymous acknowledgements instead: Thank you everyone I've worked with over the last 8 years.

Finally, I would like to thank my family for all the support I've been lucky to have. My wife, Jida, for constant motivation and for just being awesome at everything. My daughter Naïad, for being a permanent joy in my life. And, also: happy hour.

CHAPTER 1

GENERAL INTRODUCTION

RATIONAL AND OBJECTIVES

Translational control plays a critical role in the regulation of gene expression, and its role in tumorigenesis is well established. Among other effects, deregulated translation in cancer promotes the unchecked proliferation, enhanced survival and aberrant vascularization associated with the development of primary tumors. However, in many cancer types including breast cancer, primary tumors are not lethal; rather, the eventual metastatic dissemination of cancer cells is responsible for disease-related mortality. Yet the importance of translation in this process has remained largely unexplored. Furthermore, many non-transformed cell types are of critical importance to metastasis. While these cells must translate mRNAs into proteins for their function, there is a severe dearth of information on how they regulate translation in the cancer setting. These gaps in our knowledge constitute an important problem to address, as there is a long list of inhibitors of translation in clinical trials, yet we know almost nothing regarding how they will affect metastasis, or the function of the immune system. The work presented in this thesis is based on the hypothesis that ***translation plays an important, as of yet unappreciated, role in the metastatic process by regulating the ability of cancer cells to disseminate and the ability of immune cells to respond to cancer***. As a first step in correcting this deficiency, the work presented in this thesis proposes to focus on a single regulatory event for which we possess an ideal mouse model, the phosphorylation of the eukaryotic initiation factor 4E, in order to achieve the following objectives:

1. Investigate the role of translation in the metastatic dissemination of cancer cells;
2. Investigate the role of translation in cells of the tumor microenvironment;
3. Determine the impact of targeting translation in both the tumor and host.

To fully appreciate the significance of this endeavor, one must be familiar with the current state of knowledge in the field of translational control of cancer. Therefore, a review of the relevant literature is presented here.

1. LITERATURE REVIEW

1.1 On the importance of translational control in cancer

1896: the modern Olympics were created, Wilhelm Röntgen described X-rays for the first time, Henri Becquerel discovered radioactivity and Giuseppe Pianese associated nucleolus size with cancer malignancy. While Pianese's discovery never reached the kind of fame and impact associated with these other achievements, it was the first indication, only to be understood a century late, that the translation of mRNAs to proteins is critical for cancer development and progression. Of course, Pianese could not have known the mechanism underlying the association between nucleoli and cancer in the 19th century, or even the term "nucleolus". Nonetheless, the importance of his discovery is demonstrated by the fact that today, in the modern era of molecular pathology, the number and size of nucleoli are still used as prognostic biomarkers for clinical outcome and tumor recurrence (*1*).

We now know that the nucleolus is the site of ribosome biogenesis, and cancer cells display larger nucleoli to support the increased ribosomal biosynthesis required for hyperactive proliferation. In fact, increased ribosome content is critical during cell cycle progression to ensure adequate doubling of protein content that signals cell division. Ribosomes themselves are enormously abundant, must be duplicated during cell growth to promote proliferation, and are thought to coordinate the two processes. One hypothesis proposes that mRNAs encoding ribosomal proteins are so abundant that they act as sponges, sequestering ribosomes from the low-abundance mRNAs necessary for cell cycle progression, until sufficient mature ribosomes are available for the translation of other transcripts (*2*).

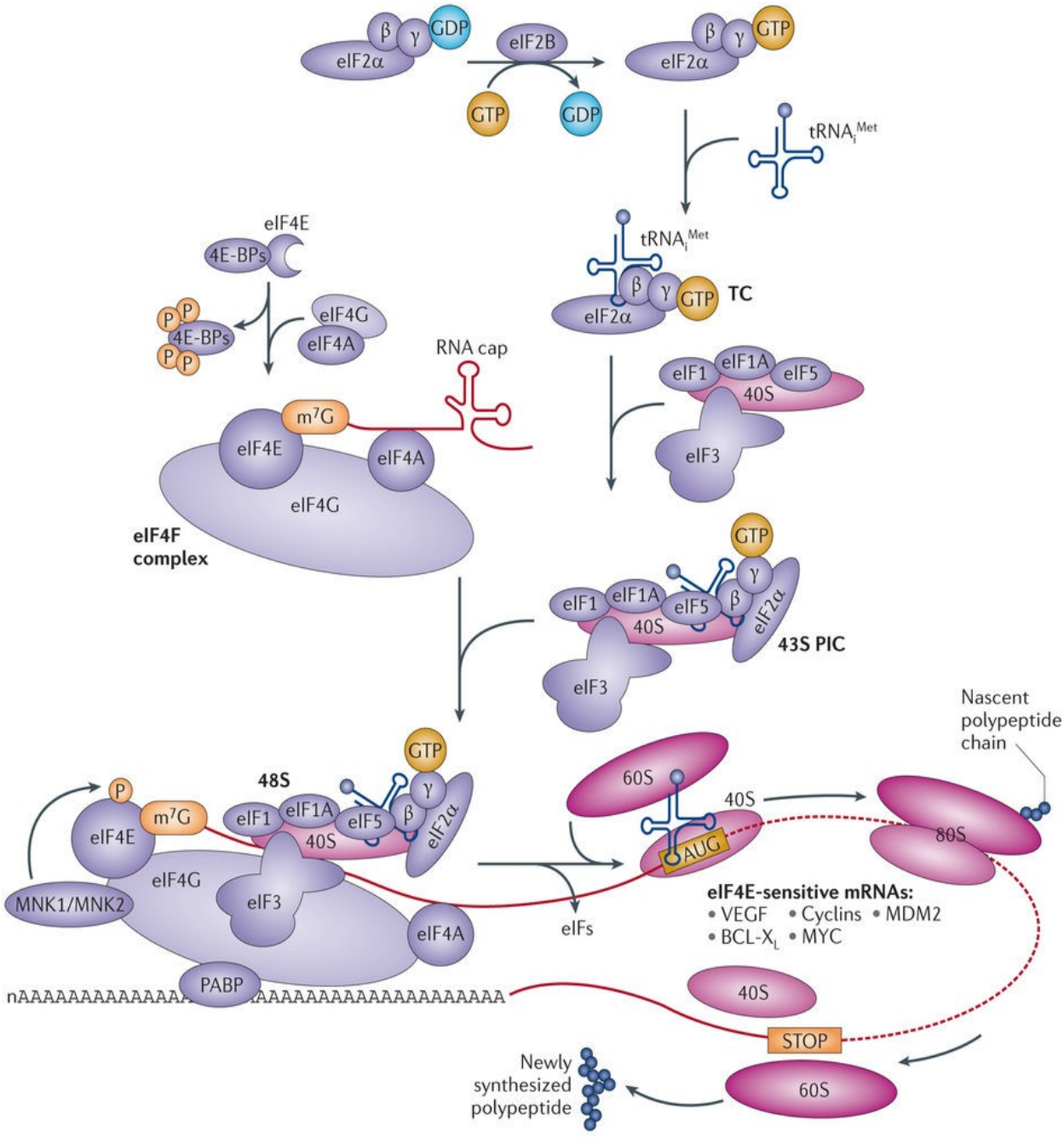
While this hypothesis has a certain elegant appeal, other mechanisms are also known to be at play, which are the subject of some excellent reviews (*3, 4*). Nonetheless, the abundance of ribosomes serves to remind us the extent of the resources dedicated to support mRNA translation. Indeed, in proliferating cells, up to 20% of cellular energy is used for translation, compared to 15% for transcription and replication, and 20% for various cation pumps (*5*). As most of transcription is directed to the synthesis of ribosomal RNA (rRNA) and mRNAs encoding

ribosomal proteins, it is clear that translation is easily the most energy demanding cellular process, which has been corroborated *in vivo* (*6*).

Considering the expense of dedicating so much energy to mRNA translation, this crucial step in gene expression is heavily regulated and has a profound impact on variations in protein levels. There have been several attempts to dissect the relative contributions of gene transcription, mRNA stability, mRNA translation and protein degradation on the steady-state levels of cellular proteins (*7-10*). These studies have highlighted the important differences between the transcriptome and proteome, with variations in mRNA levels accounting for 30-80% of variations in protein levels in cells and tissues. This moderate correlation is mostly attributed to translational regulation, as there is more than a 1000 fold difference in the translation rates of various mRNAs (*7, 10*). It is worth noting that there is some concern that experimental caveats may have affected the conclusions reached from some of these studies (*11*). However, the overwhelming consensus is that translation plays a major role in regulating gene expression, particularly in specific conditions such as cellular stress and cell growth (*9, 12*), two very important aspects of tumor biology.

1.2 Brief overview of translational control

Translation occurs in three steps: initiation, which includes all the steps leading up the recognition of the start codon; elongation, consisting of the sequential addition of amino acids corresponding to the mRNA sequence; and termination at the stop codon allowing ribosomes to be released or enter further rounds of translation. Our current understanding is that the key regulatory events are the formation of two trimeric complexes: eIF4F and the Ternary Complex (TC) (Figure 1.1). The TC's monopoly of the initiator methionine tRNA (Met-tRNAi) ensures that this complex is crucial for start codon recognition and translation initiation. eIF4F is responsible for mRNA recruitment and is required for the unwinding of secondary structures present in the 5'UTR of most cellular mRNAs (*13*).



Nature Reviews | Drug Discovery

Figure 1.1. Translation initiation. The rate-limiting phases of initiation involve controlling the assembly of a functional 40S subunit with its associated factors (43S pre-initiation complex (43S PIC)) and the access of PICs to the mRNA template (*14, 15*). The 43S PIC is a large multifactorial complex formed by the association of the 40S ribosomal subunit with eukaryotic translation initiation factors (eIFs) eIF1, eIF1A, eIF3, eIF5 and the ternary complex (TC) (*16, 17*). The TC consists of a trimeric complex involving eIF2 (containing α-, β- and γ-subunits), initiator methionyl tRNA (tRNAiMet) and GTP (*18*). The recruitment of the 43S PIC to the mRNA template is facilitated by eIF4F, a complex consisting of the mRNA 5′-cap-binding subunit (eIF4E), a large scaffolding protein (eIF4G) and the DEAD box RNA helicase (eIF4A) (*19, 20*). eIF4F recruits ribosomes to mRNA through eIF4E–mRNA cap and eIF4G–eIF3 interactions, resulting in the formation of a 48S initiation complex (*21*). eIF4G also interacts with the poly(A)-binding protein (PABP), which associates with the mRNA 3′ poly(A) tail, to cause mRNA circularization to stabilize mRNAs and bolster translation (*22*). The eIF4A helicase participates in the initial interactions of eIF4F with the mRNA 5′ end and may also facilitate scanning of the 40S ribosomal subunit towards the initiation codon by resolving the secondary structure in the 5′ untranslated region (UTR) (*23*). Recognition of the initiation codon by the 43S PIC leads to the release of eIFs and joining of the 60S ribosomal subunit (*23*). The formation of a translation-competent 80S ribosome marks the end of initiation and the beginning of elongation. BCL-XL, B-cell lymphoma extra large; 4E-BP, eIF4E-binding protein; m7G, 7-methylguanosine 5′-cap; MNK, MAPK-interacting kinase; VEGF, vascular endothelial growth factor.

In addition to these complexes, other initiation factors play important roles. These include eIF3, the bridge between eIF4F and the pre-initiation complex (PIC) (*24*); eIF5A, which appears to be involved in initiation, elongation, as well as other steps in mRNA metabolism (*25*); and eIF6, which is involved in ribosome biogenesis in the nucleolus and prevents premature joining of the 40S and 60S ribosomal subunits in the cytoplasm (*26*). As this thesis revolves around eIF4E and its phosphorylation, these topics are expanded upon here.

1.3 eIF4E Binds the Cap

Nuclear transcribed mRNAs possess a cap structure (m^7GpppN, where m^7G is 7-methylguanosine and N is any nucleotide) at their 5' end, which is critical for mRNA splicing and polyadenylation, stability and translation. eIF4E was first discovered as a 24 kDa protein that could be chemically crosslinked to the cap (*27, 28*). The structure of eIF4E resembles that of a cupped hand pinching the cap between finger and thumb (*29-31*). The molecular basis for the specificity of eIF4E for capped mRNAs is described in Figure 1.2. The most important interaction is the stacking of the guanine ring between two aromatic tryptophans, which is strengthened by delocalized positive charge arising from the methyl group of the cap structure. Further interactions occur between the cap-binding pocket and the guanine, the methyl group, the triphosphate bridge and the second nucleotide (Figure 1.2A-D). Each of these interactions is important for cap recognition, as demonstrated by the hierarchy of eIF4E's affinity to various cap analogs (*32, 33*) (summarized in Table 1.1). Impairing eIF4E's cap-binding activity by mutating W56 abrogates its oncogenic effects in mouse models and human cell lines (*28*).

Table 1.1 Relative Affinity of eIF4E for Various Cap Analogs

Interaction	Relative binding
Second nucleotide	m7GpppA>m7Gppp
Triphosphate bridge	m7GpppA>m7GppA m7Gppp>m7Gpp>>>m7Gp
Methyl group	m7Gppp>>>Gppp
m7: methyl group on position N7 of the guanine; G: guanosine; A: adenosine; p: phosphate group	
Ref: (*32, 33*)	

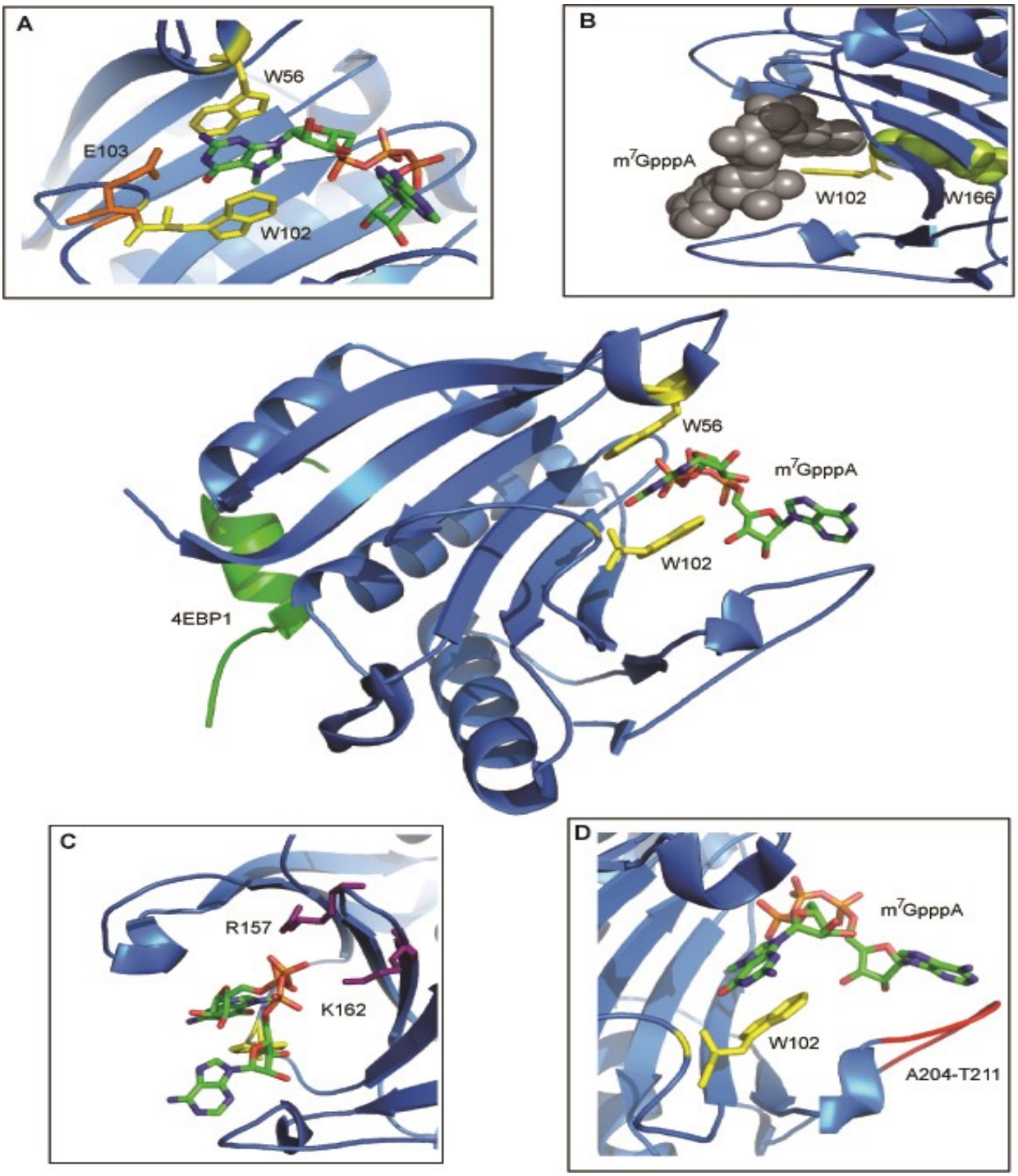

A
W56
E103
W102
B
m7GpppA
W102
W166
W56
m7GpppA
W102
4EBP1
C
R157
K162
D
m7GpppA
W102
A204-T211

Figure 1.2. 3D structure of cap-bound eIF4E and 4E-BP1 peptide. A, aromatic stacking of the purine ring between Trp 56 and Trp 102 and electrostatic interactions between N1/N2 and Glu 103; **B**, van der Waals interactions between the methyl group and Trp 166; **C**, electrostatic interactions of the triphosphate bridge with Arg 157 and Lys 162; **D**, interactions between the second nucleotide and residues of the flexible C-terminal loop of eIF4E. Structure from PDB 1WKW (*30*).

1.4 Regulation of eIF4E

1.4.1 eIF4E interacting proteins: eIF4G

The first protein that was described as a binding partner of eIF4E was eIF4G (*20, 34, 35*). The human genome encodes two functionally redundant eIF4G family members: eIF4GI and eIF4GII (*36*). These proteins bind to eIF4E, eIF3 and eIF4A, and can restore cap-dependent translation in rabbit reticulocyte lysates treated with rhinovirus $2A^{pro}$ (*20, 36*). A third family member, DAP5, cannot bind eIF4E and is involved in cap-independent translation. eIF4GI and II are ubiquitously expressed, though their relative expression varies in different tissues (*36*). eIF4GI was the first identified homolog and is generally used in studies on eIF4E-eIF4G interactions. Therefore, it is referred to as eIF4G in this thesis and the numbering of important residues relate to this homolog. Two conserved residues in eIF4E are critical for its interaction with eIF4G, Val69 and Trp73 (*37*). eIF4G interacts with eIF4E via the sequence YDREFLL, which conforms to the canonical eIF4E-binding motif, YXXXXLϕ (where X is any amino acid and ϕ is hydrophobic). RNA binding motifs in mammalian eIF4G stabilize the interaction of the eIF4F complex with the cap by maintaining eIF4E in the vicinity of the cap, increasing its local concentration and favoring the bound state (*38*). The RNA binding motifs on eIF4G likely do not affect the on/off binding rates of eIF4E to the cap structure itself (*39*).

1.4.2 eIF4E interacting proteins: 4E-BPs

The 4E-BPs are small (~15-20 kDa) proteins that interact with eIF4E (*40*). There are three known isoforms in mammals (4E-BP 1, 2, 3). Relatively little is known regarding 4E-BP3, especially in cancer. Most studies focus on 4E-BP1, and to a lesser extent 4E-BP2. Both isoforms 1 and 2 are ubiquitously expressed, although the predominant species varies across different tissues (*41, 42*). As these isoforms are functionally redundant in the context of cell growth and cancer (*43*), they are jointly referred to as the 4E-BPs. The 4E-BPs are regulated by the mammalian target of rapamycin complex 1 (mTORC1), which phosphorylates several residues in a hierarchical manner. First, Thr37/Thr46 are phosphorylated by the mTOR kinase, followed by Thr70 and finally Ser65 (*44*). Thr70 and Ser65 are responsive to extracellular cues such as serum

stimulation (*44*). Phosphorylation of all of these sites (the hyper-phosphorylated form) inhibits 4E-BPs' binding to eIF4E. In this manner, mTORC1 regulates eIF4E and translation.

The 4E-BPs act as inhibitors of eIF4E function by competing with eIF4G for binding to the dorsal side of eIF4E (*45, 46*), preventing the formation of the eIF4F complex for subsequent translation initiation (*40*). eIF4G and the 4E-BPs bind to eIF4E via their conserved eIF4E-binding motif (YXXXXLϕ) using a similar disorder-to-order transition mechanism, and possess similar affinities for eIF4E (*37, 47-49*). Despite this, the kinetics of binding differ widely, as the 4E-BPs display rates of binding and dissociation two to three orders of magnitude faster (*50*). Conceptually, this difference is consistent with the inhibitory role of the 4E-BPs, requiring rapid control of eIF4E binding, whereas eIF4G requires longer-lived interactions with eIF4E to promote translation initiation. Recent studies have discovered the molecular basis for this important distinction by uncovering a second eIF4E-binding site that differs between eIF4G and the 4E-BPs (*50, 51*). A structural study using full-length 4E-BP2 has confirmed the importance of the second binding site: while 4E-BP2 is intrinsically disordered, both eIF4E-binding sites possess significant transient secondary structure and contribute to 4E-BP2's affinity for eIF4E (*49*). The dynamic nature of the eIF4E/4E-BPs interaction is attributed to the second binding site (*49*).

1.4.3 eIF4E phosphorylation

eIF4E is phosphorylated on a single site, Ser209, by the map kinase integrating kinases (MNK1/2), which are activated in response to cellular stress and survival signals from the MEK/ERK and p38 MAPK pathways (*52, 53*). Phosphorylation requires prior binding of the MNKs to eIF4G, indicating that phosphorylation of eIF4E occurs after the formation of the eIF4F complex (*54*). eIF4E phosphorylation is not required for global translation as mutation of Ser209 to alanine or genetic abrogation of the MNKs has no deleterious effects in *in vivo* models (*55, 56*). Rather, eIF4E phosphorylation controls the translation of a specific subset of mRNAs, although the mechanism by which this occurs is uncertain. Originally, phosphorylation on S209 was predicted to increase eIF4E's affinity for the cap by establishing a salt bridge with lysine 159, clamping down on bound mRNAs (*29, 57*); however, the distance between the two

residues appears to be too great for such an interaction (*58*). Biophysical studies including surface plasmon resonance, stopped-flow kinetics and fluorescence titration experiments indicate that eIF4E phosphorylation decreases the affinity for the cap (*59-61*). This is due to electrostatic repulsion between the phosphorylated S209 and the intrinsic negative charges within the cap, a hypothesis supported by the fact that increasing the number of phosphate groups present in cap analogs exacerbates the effect of eIF4E phosphorylation (*61*). Phosphorylation by the MNKs requires eIF4G, and thus occurs after eIF4F complex formation (*54*). It has also been reported to require eIF3, suggesting that phosphorylation occurs after PIC recruitment (*62*). Thus, eIF4E phosphorylation promotes dissociation of eIF4E from the cap once scanning of the 5'UTR has begun. This may facilitate the dissociation of eIF4E from the mRNA and its recycling for further rounds of translation (*63*). Rapid recycling of eIF4E would be approximately equivalent to punctual increases in available eIF4E, which may explain why 5'UTR elements conferring sensitivity to eIF4E levels also confer sensitivity to eIF4E phosphorylation (*64*), and why many of the identified targets responding to eIF4E phosphorylation are targets of total eIF4E. However, this hypothesis is still lacking direct evidence.

1.5 Deregulated translation in cancer

Aberrant expression and phosphorylation of eIF4E have been linked to cancer, as have the other components of the eIF4F complex, regulators of TC formation, and many other initiation factors, as listed in Supplemental Table 1.2 (Annexe A). How do cancer cells highjack the translation machinery for their growth, survival and expansion to form tumors? And importantly, how do these changes in the activity of distinct translation factors, assumed to be important for the translation of all cellular mRNAs, confer specificity to the translation of selective mRNAs?

1.5.1 Specific effects of general translation initiation factors

Curiously, the mechanism underlying the mRNA-specific effect of changes in general initiation factors was worked out before many of the factors involved were even identified. Indeed, a

mathematical model was derived demonstrating that, given constant elongation rates and limited PIC concentrations, mRNAs for which ribosome recruitment is inefficient will be disproportionately affected by any changes in translation initiation capacities (*65*). While based on rudimentary knowledge of ribosomal function, this model was sufficient to predict the response of α- and β-globin synthesis to inhibitors of translation. It can even be used to explain translational deregulation in cancer cells with our current understanding of this process, if we assume elongation rates are approximately constant and that PIC concentrations are low enough to allow for this disproportionate effect to occur, possibly due to oncogenic stress signals leading to eIF2α phosphorylation. In this context, any strategy taken by cancer cells to favour general ribosomal subunit joining at start codons should disproportionately improve the translation of mRNAs with poor initiation efficiencies. This is crucial for cancer cells, as it turns out that transcripts encoding oncoproteins tend to be poorly translated, purportedly to maintain tight control of their expression in normal cells, thus preventing transformation. Examples of such transcripts include those encoding the vascular endothelial growth factor VEGFA, the anti-apoptotic proteins BCL2 and MCL1, the oncogene MYC, as well as several cyclins and cyclin-dependent kinases. Thus, the model predicts that cancer cells should be particularly well equipped to maintain angiogenesis, survival and proliferation, even in times of stress, simply by increasing the availability of general initiation factors, or by removing the brakes on particularly poorly translated mRNAs. Some of the many ways by which this can be achieved are delineated here and summarized in Figure 1.3.

1.5.2 Mechanisms of deregulated translation in cancer (cancer inputs)

1.5.2.1 Changes in cis*: 5' and 3'UTR length and composition*

Sequence and structural motifs present in mRNAs determine their intrinsic translational efficiency and their ability to be regulated by trans-acting factors such as miRNAs, RNA-binding proteins and initiation factors. These motifs tend to be overrepresented in oncogenic transcripts, thus rendering these transcripts intrinsically poorly translated and subject to the kind of disproportionate regulation proposed by Lodish in 1974.

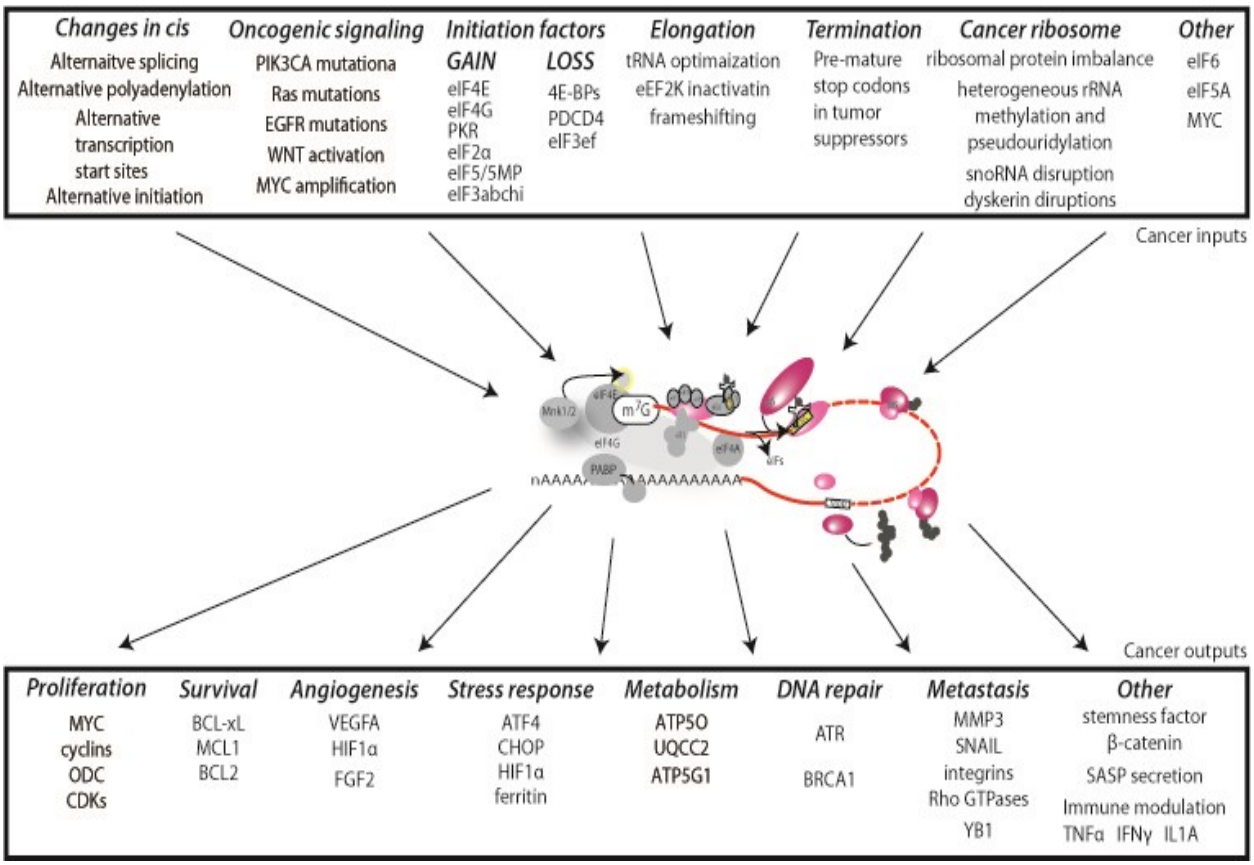

Changes in cis
Alternaitve splicing
Alternative polyadenylation
Alternative transcription start sites
Alternative initiation
Oncogenic signaling
PIK3CA mutationa
Ras mutations
EGFR mutations
WNT activation
MYC amplification
Initiation factors
GAIN
eIF4E
eIF4G
PKR
eIF2α
eIF5/5MP
eIF3abchi
LOSS
4E-BPs
PDCD4
eIF3ef
Elongation
tRNA optimaization
eEF2K inactivatin
frameshifting
Termination
Pre-mature stop codons in tumor suppressors
Cancer ribosome
ribosomal protein imbalance
heterogeneous rRNA methylation and pseudouridylation
snoRNA disruption
dyskerin diruptions
Other
eIF6
eIF5A
MYC
Cancer inputs
Mnk1/2
eIF4E
m7G
eIF4G
eIF4A
eIF5
PABP
nAAAAA
AAAAAAAAAAA
Cancer outputs
Proliferation
MYC
cyclins
ODC
CDKs
Survival
BCL-xL
MCL1
BCL2
Angiogenesis
VEGFA
HIF1α
FGF2
Stress response
ATF4
CHOP
HIF1α
ferritin
Metabolism
ATP5O
UQCC2
ATP5G1
DNA repair
ATR
BRCA1
Metastasis
MMP3
SNAIL
integrins
Rho GTPases
YB1
Other
stemness factor
β-catenin
SASP secretion
Immune modulation
TNFα IFNγ IL1A

Figure 1.3: Cancer inputs and outputs. Summarized view of the oncogenic lesions feeding into the translational machinery (cancer inputs) and of the resulting advantages conferred by aberrant translation, with examples of regulated mRNAs (cancer outputs).

Increased secondary structure (stability) of the 5' untranslated region (5'UTR) was one of the first *cis*-acting elements to be identified that affect the rate or efficiency of cap-dependent translation initiation. This was demonstrated by artificially enhancing base-pairing in reovirus mRNA by substitution of uridines for bromouridines, leading to increased dependence on eIF4E for translation; inosine substitution had the converse effect on secondary structure and translation efficiency (*66*). Oncogenic transcripts possessing stable 5'UTRs, and thus dependent on eIF4E, include those encoding MYC, ODC, and several cyclins and cyclin dependent kinases. Many other mRNA sequence elements can regulate the efficiency of translation. For example, exquisite dependence of eIF4E, but not eIF4A, has been demonstrated for the Translation Initiator of Short 5'UTR (TISU) element (*67*), whereas mRNAs containing internal ribosome entry sites (IRESs) are cap- and eIF4E-independent, but dependent on eIF4G and eIF4A (*68*). IRES-mediated translation allows for direct recruitment of ribosomes to start codons without the requirement for 5'UTR scanning (*68*). This mechanism is particularly important when scanning is impaired due to limiting eIF4E, such as in stress conditions (*68*). Furthermore, alternative initiation codons and inhibitory open reading frames upstream of the canonical AUG can severely hamper its identification by the 43S PIC; however, strategic placement of uORFs can lead to selectively increased translation in conditions of eIF2α phosphorylation (*13*). Additionally, structural or sequence motifs can recruit RNA-binding proteins to modulate mRNA translation, such as the transforming growth factor-β (TGFβ)-activated translation (BAT) element, an asymmetrical bulge-containing stem-loop that regulates the translation of mRNAs involved in epithelial-to-mesenchymal transition (*69*). Finally, binding sites for miRNAs are particularly common motifs that affect translation, in addition to mRNA stability, as are AU-rich motifs. All of these elements have been thoroughly reviewed elsewhere (*13, 68, 70-72*).

A particularly interesting example relates to terminal oligopyrimidine (TOP) motifs, consisting of a C and a series of 4-15 pyrimidines immediately following the cap structure. TOP motifs provide a unique "all-or-nothing" translational regulation to mRNAs encoding components of the translational machinery, though the underlying mechanism remains elusive (*73*). This was sequentially attributed to phosphorylation of RPS6 by the S6 kinases (*74*) and to the activity of

eIF4E downstream of 4E-BP phosphorylation (*75, 76*). However, cells lacking S6Ks or 4E-BPs retain the ability to modulate TOP mRNA translation downstream of mTOR (*73, 77, 78*). Many new candidates have emerged as possible TOP regulators, such as miR-10a/miR-10b, and several RNA-binding proteins: TIA-1/TIAR, ZNF9, La protein and the La-related proteins LARP1 and LARP7 (*79-84*). LARP1 in particular has garnered much interest, and has been proposed to regulate TOP mRNA stability, to bind to PABP, to bind to the cap, and to either increase or decrease TOP mRNA translation (*83, 85-87*). An interesting recent finding proposes that LARP1 interacts with the 40S ribosomal subunit to stabilize TOP mRNAs and involves this factor in the pathogenesis of ribosomaopathies (*88*). Further research into this field will hopefully resolve the controversy and confusion.

The majority of identified *cis-acting* elements described here reduce mRNA translation efficiency, and can be combined in individual mRNAs for efficient suppression. Since such combinations are commonly observed in oncogenic mRNAs to avoid abnormal expression and transformation, cancer cells can evolve a variety of mechanisms to bypass this control. The simplest involve shortening 5' and/or 3'UTRs to eliminate suppressive elements. For example, cancer cells can use alternative transcription start sites downstream of a regulatory element. This has been observed for the transcription of *Mdm2* which can skip two inhibitory uORFs (*89*). Similarly, inhibitory elements can be avoided by using alternative polyadenylation, leading to shorter 3'UTRs lacking miRNA binding sites, AU-rich elements, and other regulatory elements (*90*). These mechanisms appear to be wide-spread in cancer cells, whose transcriptomes are globally shorter, thus providing net advantages by removing the brakes on oncogenic mRNAs (*91*).

1.5.2.2 Oncogenic signaling

The most common mechanism for deregulated translation in cancer is probably through the activation of upstream signaling pathways. Indeed, the translational machinery is a major regulatory hub integrating signals from oncogenic signaling cascades to regulate the translation of important target mRNAs. mTOR in particular plays a crucial role by phosphorylating the 4E-

BPs to allow eIF4F complex formation. S6K is also important, as it phosphorylates several substrates such as programmed cell death 4 (PDCD4) to release eIF4A, eEF2K to alleviate inhibition of elongation through eEF2, as well as eIF4B to promote the helicase activity of eIF4F (*92-94*). In fact, signals from mTOR, MAPK and WNT all funnel to the activation of translation, which has been shown to be required for the transforming potential of these pathways (*95*). Many of the most commonly mutated genes across cancer types encode proteins in these pathways, including *PIK3CA*, *KRAS*, *PTEN*, *APC* and *EGFR* (*96*). Other commonly mutated pathways, such as NOTCH and SMAD, do not themselves regulate translation but require its concurrent activation to promote the expression of their target genes (*95, 97*). Particularly interesting is the case of the MYC oncogene, which promotes almost all aspects of translation (see below). Many of these signaling pathways present complex patterns of crosstalk and rewiring in cancer cells, as has been well documented between the mTOR and MAPK pathways (142) and been recently described for mTOR and GCN2 (143). The eventual connection at the translational machinery is a crucial aspect of this circuitry and presents an interesting opportunity for therapeutic targeting, as described at length elsewhere (144). Certainly, translation is far from the only effector of these pathways, yet its importance is demonstrated by the requirement for its activity in the transformed phenotype that they induce, as well as in the speed at which it is modulated (115). For example, following inhibition of upstream kinases such as AKT and KRAS, decreased translation efficiency occurs prior to any transcriptional changes in models of glioblastoma (145). Furthermore, direct deregulation of the translation machinery can occur independently of upstream signaling, or arise as a resistance mechanism to its inhibition, underscoring its significance in cancer (146-151).

1.5.2.3 Gain/loss of initiation factors

Aberrant expression of eIFs was the first mechanism identified that allows cancer cells to deregulate translation to their benefit, when eIF4E was found to transform NIH 3T3 cells *in vitro* (*98*) and to promote the development of tumors in transgenic mice (*99*). eIF4E was subsequently found to be overexpressed in a wide variety of human cancers, as were several other initiation factors, all of which have been comprehensively tabulated elsewhere (*100-102*).

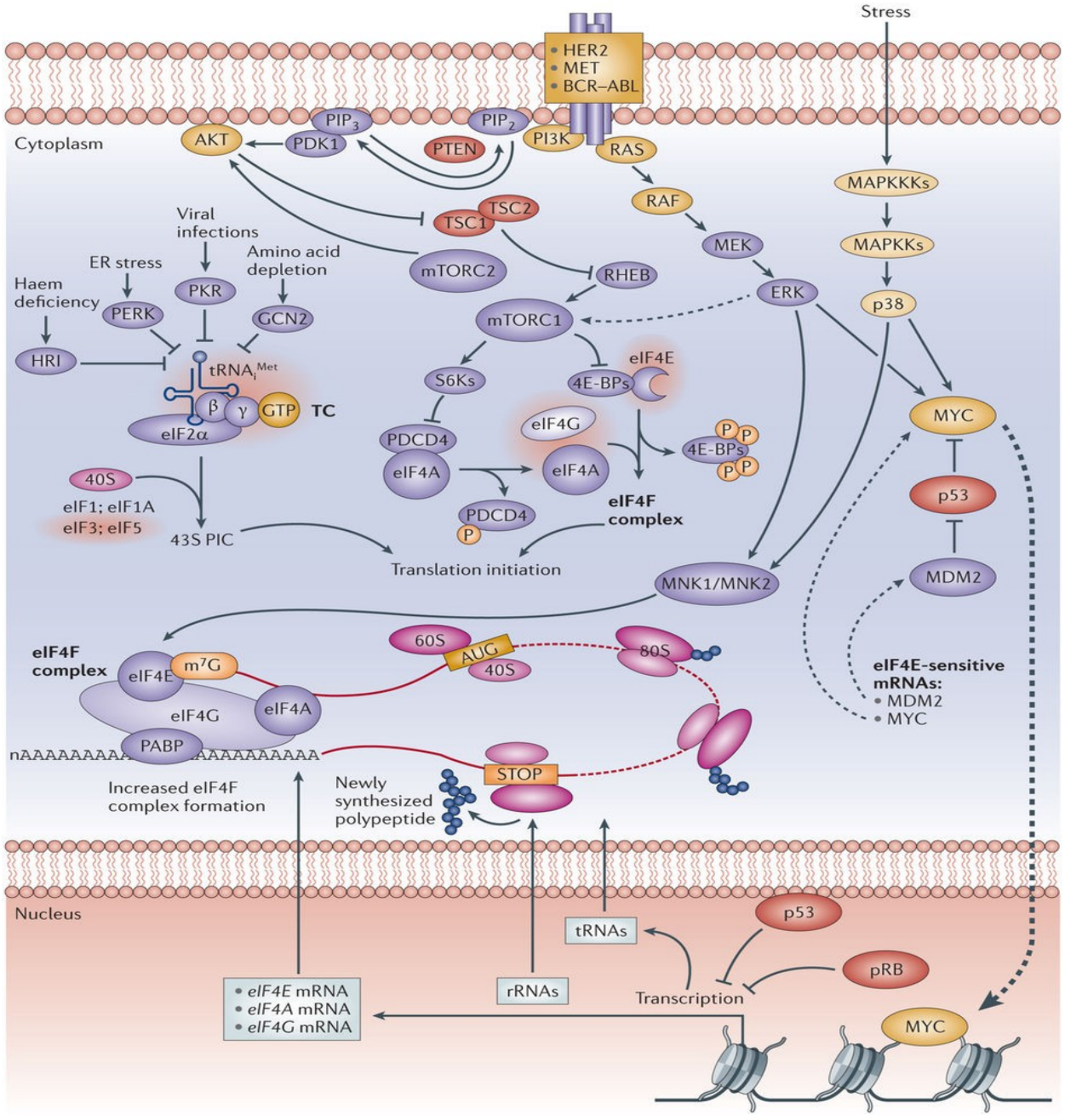


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Figure 1.4. The translation apparatus plays a pivotal role in mediating the effects of commonly dysregulated oncogenic signaling pathways in cancer. The signaling cascades by which hyperactive phosphoinositide 3-kinase (PI3K)–mammalian target of rapamycin (mTOR) and mitogen-activated protein kinase (MAPK) pathways induce formation of the eukaryotic translation initiation factor 4F (eIF4F) complex are indicated. Proteins encoded by commonly mutated oncogenes and tumour suppressors in these pathways are shown in yellow and red, respectively. On the left are the stresses leading to eIF2α phosphorylation, which inhibits translation by stabilizing the interaction between eIF2B and GDP–eIF2 (Ref. 235). Also shown is the transcriptional regulation of the translation machinery by the oncoprotein MYC that activates eIF4E, eIF4A and eIF4G and bolsters tRNAs and rRNA synthesis286, whereas inactivation of the p53 and pRB tumour suppressors releases their repression of tRNA and rRNA synthesis287. The net outcome of these alterations differs among tumour cells but is invariably linked to a disproportionate increase in the translation of eIF4E-sensitive transcripts such as MYC. Bar-headed lines indicate inhibition, and arrows indicate activation. The thick dashed arrow indicates nuclear translocation of MYC. Components of the translation machinery leading to neoplastic transformation when ectopically overexpressed are circled with red shading. 4E-BP, 4E-binding protein; ER, endoplasmic reticulum; ERK, extracellular signal-regulated kinase; GCN2, general control non-derepressible 2; HER2, human epidermal growth factor receptor 2; HRI, haem-regulated inhibitor; m7G, 7-methylguanosine 5′-cap; MAPKKK, mitogen-activated protein kinase kinase kinase; MEK, MAPK/ERK kinase (also known as MAPKK); MET, mesenchymal epithelial transition factor; MNK, MAPK-interacting kinase; mTORC, mTOR complex; PABP, poly(A)-binding protein; PDCD4, programmed cell death 4; PDK1, 3-phosphoinositide-dependent protein kinase 1; PERK, PKR-like ER kinase; PIC, pre-initiation complex; PIP2, phosphatidylinositol-4,5-bisphosphate; PIP3, phosphatidylinositol-3,4,5-trisphosphate; PKR, double-stranded RNA-dependent protein kinase; RHEB, RAS homologue enriched in brain; PTEN, phosphatase and tensin homologue; S6K, ribosomal S6 kinase; tRNAiMet, initiator methionyl tRNA; TSC, tuberous sclerosis.

1.5.2.3.1 *eIF4F complex formation*

Ribosomes are recruited to the 5' ends of mRNAs via the eIF4F complex, which consists of eIF4E, eIF4G and eIF4A (Figure 1.1). All three subunits can be deregulated in cancer cells: their genomic loci have all been shown to be amplified in human tumors, and they are all targets of the MYC oncoprotein (see below) (*100-102*). Of note, the relative importance of eIF4A expression levels is questionable. Of the two homologs with 90% identity that exist in humans, eIF4A1 is the most widely expressed and best studied, but genetic lesions in the *Eif4a1* gene are rare in cancer; in contrast, *Eif4a2* is amplified in 45% of lung squamous cell carcinomas, though this may be a side-effect of its location on a frequently amplified region of chromosome 3q (*103*). Indeed, eIF4A is expressed at much higher levels than other initiation factors, and is thus unlikely to be limiting, even in the cancer setting (*104*). Nonetheless, eIF4A activity is crucial for the translation of mRNAs with stable 5'UTR secondary structures, such as those encoding anti-apoptotic factors (*105*), and its inhibition leads to potent tumor suppression (*106*). In contrast, eIF4E and eIF4G act as classical oncogenes, their overexpression resulting in transformation *in vitro* and *in vivo* (*98, 99, 107, 108*). Importantly, it was recently demonstrated that eIF4E expression is normally maintained in excess for cap-dependent translation, and that loss of eIF4E by 50% is not limiting for general protein synthesis and embryonic development. Indeed, $eIF4E^{+/-}$ mice are viable and do not display any obvious phenotypes. On the contrary, $eIF4E^{+/-}$ cells and mice are remarkably resistant to cellular transformation, even when driven by a powerful oncogene such as *Hras-V12* (*109*).

As eIF4E has been the best studied initiation factor in cancer, other modes of deregulation have also been reported, for which similar mechanisms could potentially affect eIF4A and eIF4G. Thus, deregulated eIF4E can be achieved by activation of transcription factors other than MYC, by mRNA stabilization via loss of HuR or *Eif4e*-targeting miRNAs, as well as by increased phosphorylation (*110-112*). eIF4E phosphorylation by the MNKs promotes tumor development and dissemination (*55, 97, 113, 114*), and is elevated in human lung, breast and prostate cancers, among others (*115-117*). While the MNKs do not appear to be frequently amplified or

mutated, their expression can be increased by YB1 (*118*), though activation of upstream MAPK signaling is likely the most common means of promoting eIF4E phosphorylation in cancer cells.

Alternatively, eIF4F complex formation can be promoted by loss or inactivation of proteins responsible for the sequestration of eIF4A or eIF4E. Thus, despite the uncertain role of eIF4A amplification in cancer, loss of its inhibitor, PDCD4, appears to provide a significant advantage, as it acts as a tumor suppressor whose absence is associated with cancer cell invasion and poor survival in some cancers (*119-123*). Decreased PDCD4 expression is linked to post-transcriptional regulation by miR-21 and degradation following phosphorylation by S6Ks (*93*). While it is tempting to attribute PDCD4's tumor-suppressive properties to eIF4A inhibition, PDCD4 appears to be a multifunctional protein that has also been reported to regulate AKT and JNK signaling, and the transcription factors NF-κB, c-JUN and AP-1, through mechanisms that remain unclear (*124-126*). As one report attributes most of these functions to downstream effects of MYC regulation (*127*), the exact role of PDCD4 in cancer remains to be established.

In contrast, the eIF4E-binding proteins (4E-BPs), which compete with eIF4G for binding to eIF4E, clearly act as tumor suppressors via inhibition of cap-dependent translation. The 4E-BPs can either be lost, as occurs in pancreatic cancer but rarely in other cancer types, or functionally impaired by inhibitory phosphorylation via mTOR. Consequently, any activation of the mTOR pathway results in increased eIF4E availability for translation. While several studies linked elevated 4E-BP1 expression to cancer progression, suggesting a role as an oncogene rather than a tumor suppressor (*128, 129*), it appears that even when overexpressed in the metastatic setting, 4E-BPs are mostly hyper-phosphorylated in human cancers and therefore inactive, negating their overexpression as a cause of transformation. Confirming these data, our own unpublished observations indicate that genetic deletion of 4E-BP1/2 has no impact on tumor progression in a mouse model of PTEN-loss driven prostate cancer, as mTOR is highly active in this model. Of note, it is impossible to completely exclude the possibility that 4E-BP overexpression plays other, non-translational roles in cancer, which has been previously reported (*130*).

1.5.2.3.2 Pre-initiation complex formation

As delineated in Figure 1.1, translation begins with the formation of the 43S PIC formation, which is controlled by phosphorylation of eIF2α. Deregulated PIC formation in cancer cells is a complex issue that has led to some seemingly contradictory (*131*). On the one hand, it is generally thought that increased eIF2α phosphorylation would grant cancer cells a heightened ability to respond to the various stress conditions encountered along the path to malignancy by promoting the translation of uORF-containing stress-response transcripts such as ATF4 (*132*). Thus, overexpression of eIF2α or its kinase PKR have been shown to promote transformation in some contexts (*133-137*). On the other hand, long-term eIF2α phosphorylation engenders apoptosis, and has even prompted research into the development of cancer therapies promoting the activity of eIF2α kinases or the inhibition of their phosphatases (*138-140*). These results suggest that the outcome of eIF2α phosphorylation in cancer cells is highly context specific, perhaps related to disease site, tumor grade or underlying driver mutations. Likely, the ability of cancer cells to reap the benefits of eIF2α phosphorylation while avoiding its eventual anti-proliferative and apoptotic consequences relies on decoupling of the two processes. This could occur at the molecular level by enhancing translation dependent on alternative mechanisms such as IRESs, mRNA methylation and eIF2A (*132, 141-143*). Alternatively, chronology may be important, with sequential phosphorylation and quiescence in times of severe stress (e.g. hypoxia at early disease stages), followed by dephosphorylation and proliferation after adaptation (e.g. neo-vascularization) (*101*).

PIC formation can also be regulated by overexpression of eIF5 or its mimic proteins 5MP1/2. When present in excess, these proteins can bind to eIF2 and sequester it from the 40S ribosome (*144, 145*). Similarly to eIF2α phosphorylation, binding of eIF5 or the 5MPs reduces global translation but enhances that of uORF containing transcripts including ATF4 (*146*). This mechanism appears to be important for the malignant properties of some cancer types such as fibrosarcoma and salivary mucoepidermoid carcinoma (*147*).

1.5.2.3.3 eIF3, connecting the eIF4F and pre-initiation complexes

eIF3 is a multi-subunit complex that binds to eIF4G and the PIC, thus bridging mRNAs and the 40S ribosomal subunit and allowing scanning to occur (*13*). Theoretically, increased eIF3 levels should help promote this bridging and therefore the efficiency of translation initiation. However, several studies on eIF3 paints a more complex picture. Indeed, when overexpressed or knocked down in immortalized cells, individual subunits display oncogenic properties, while others behave more like tumor suppressors (*24*). Similarly, levels of eIF3a, b, c, h, i and m are increased in a variety of cancer types, while reduced expression of eIF3e and f have been reported. These associations should be interpreted with caution, as many rely on measurements of mRNA concentrations in patient samples, which may not reflect protein levels, or on RNAi-mediated depletion of eIF3 subunits to levels much lower than the minimum required to support translation, as discussed in depth elsewhere (*24*). Further complicating matters is the possibility of non-translational roles of certain subunits, such as eIF3a, which has been reported to bind to components of the cytoskeleton (*148, 149*), or eIF3f and i, which have been proposed to regulate signal transduction pathways (*150, 151*). Perhaps a better understanding of the different cancer-related roles of eIF3 subunits can be gleaned from recent studies highlighting their translation-specific roles. eIF3 can bind to mRNA structures in the 5'UTR of such cancer-relevant mRNAs as *c-Jun* and *Btg1*, resulting in translational activation of *c-Jun* and repression of *Btg1*; these opposing effects could be due to different eIF3 subunits (*152*). In fact, the same group has proposed that eIF3d specifically binds to the cap of the *c-Jun* mRNA and described an entirely novel model of specialized cap-dependent translation (*153*). In addition, loss of eIF3d and eIF3e has been linked to the translation of a metabolic program promoting the so-called Warburg effect. eIF3e has also been proposed to promote the phosphorylation of eIF4E (*62*). In this last case, it is difficult to reconcile the supposed tumor suppressive properties of eIF3e with the oncogenic aspects of eIF4E phosphorylation.

1.5.2.3.4 Alternative initiation

In stress conditions leading to eIF2α phosphorylation, translation is impaired but not abrogated. Recent publications have shed light on an alternative mechanism of recruiting initiator tRNAs to

ribosomes and the initiation of translation, which is dependent on eIF2A (*154*). This protein promotes initiation at non-canonical CUG and UUG codons, which encode leucine, leading to questions as to which tRNA species is used to initiate translation. While some groups have reported initiation with leucyl-tRNA, others have indicated that the eIF2A-initiator-tRNA interaction is much stronger (*155-158*). Notwithstanding this issue, eIF2A appears to play a crucial role in the translation of oncogenic mRNAs including c-Src, BiP, N-Ras, CD44, Ki67, Nrp1 and Rac1 (*155, 156, 159*). However, this role is limited to stress conditions and appears particularly important to cancer cells, as depletion of eIF2A has no effect on the proliferation of normal cells, but severely impedes tumorigenesis and the proliferation of cells with phosphorylated eIF2α (*155, 156*). This characteristic argues for therapeutic targeting of eIF2A, though no such therapy has yet been developed.

1.5.2.5 Translation elongation and termination

While much of the scientific focus has been on translation initiation, oncogenic changes in elongation and termination are emerging as important players in cancer. For example, a dominant role for the inhibition of elongation via the mTOR-S6K-eEF2K pathway has been demonstrated for intestinal tumor formation (*92*). Furthermore, the availability specific tRNA isoacceptors appears to play a role in tumorigenesis. Indeed, the speed of amino acid incorporation during the elongation phase is dependent on the availability of the corresponding charged tRNA (*160*). Several studies have reported distinct translational programs, where proliferating/cancer cells express an array of tRNAs optimized to correspond to the codon usage of pro-proliferative mRNAs, which is different than the codon usage and corresponding tRNAs of differentiated cells (*161-163*). Hence, in cancer cells the repertoire of available tRNAs is thought to be rewired such that the species required for the translation of oncogenic mRNAs are present at sufficient levels. In addition, elongation can also be deregulated in cancer via programmed -1 ribosomal frameshifting (-1 RPF), a process by which sequence elements force elongating ribosomes back by one base, leading to frameshifts, pre-mature stop codons and nonsense-mediated mRNA decay. Indeed, oncogenic pathways such as the JAK-STAT signaling pathway may be disproportionately regulated by -1 RPF, suggesting that members of this

pathway are normally translationally repressed at the elongation step, which could theoretically be relieved in cancer cells (*164*). This may explain the oncogenic role of silent mutations inducing frameshifting in tumor suppressors (*164*).

Aberrant termination in the cancer setting has garnered limited attention. However, termination at premature stop codons occurring due to somatic mutations in tumor-suppressor genes is also a cancer driver (*165*). This process promotes mRNA decay, known as nonsense-mediated mRNA decay (NMD). mRNA degradation by NMD prevents the expression of truncated proteins resulting from premature stop codons, as these may have dominant negative properties or gain-of-function activities that would be detrimental to cells (*165*). However, when occurring in tumor suppressors, reduced expression due to NMD is itself detrimental and can result in transformation (*165*). NMD can be prevented using aminoglycosides or small molecule drugs that promote readthrough of premature stop codons. Clinical introduction of a small molecule inhibitor for the treatment of Duchene Muscular Dystrophy, known as Translarna (Ataluren) by PTC Therapeutics is approved in a number of countries (*166, 167*).

1.5.2.5 Factors regulating multiple steps of translation

1.5.2.5.1 eIF6 and eIF5A

Two initiation factors also play definite, but confusing, roles at multiple steps of translation. The first is eIF6, an anti-association factor preventing aberrant interactions between the 40S and 60S ribosomal subunits. eIF6 must be displaced for the final step of ribosome formation in the nucleolus, a role emphasized by the finding that eIF6 is a key player in ribosomopathies, cancer syndromes characterized by aberrant ribosome synthesis (see below and (*164*)). In addition, eIF6 can theoretically promote 80S ribosome disassembly by preventing the re-association of post-termination 60S ribosomes, thereby shifting the equilibrium towards termination and preventing further rounds of initiation with prolonged sequestration (*26*). Thus, eIF6 may play multiple roles in deregulating translation. In addition to its role in ribosomopathies, aberrant eIF6 expression has been observed in colorectal and head and neck cancers, where it

accumulates in the nucleolus (*168, 169*). In contrast, reduced eIF6 levels have been shown to prevent oncogene-induced transformation, and delay lymphomagenesis (*170, 171*).

The second is eIF5A, which was originally described as an initiation factor important for the formation of the first peptide bond, but has since been found to play a role in the elongation of poorly translated tripeptides containing prolines, glycines and/or basic residues (*25, 172-174*). A role in translation termination has also been proposed based on the accumulation of stalled ribosomes at stop codons in cells lacking eIF5A (*174, 175*). There is much interest in this initiation factor, as both of its isoforms are overexpressed in a variety of cancers including pancreatic, hepatic, colon, lung and ovarian, and have been linked to the metastatic capacity of cancer cells (reviewed in (*25*)). As the only known mammalian protein containing a hypusine modification, which is a modified lysine, eIF5A is an attractive target, as its activity can be abrogated by inhibiting the enzymes catalysing the hypusination (*176*).

1.5.2.5.2 The MYC oncoprotein

Simultaneous deregulation of several aspects of translation can also occur via amplification of *Myc*. MYC is a promiscuous transcription factor that can target up to 15% of the genome, including the translation machinery (*177*). MYC promotes the synthesis of rRNAs and tRNAs by stimulating the activity of RNA polymerases I and III (*178, 179*), and can activate the transcription of *Eif2*, *Eif4e*, *Eif4a* and *Eif4g* (*180, 181*) (*182*). As the *Myc* mRNA was one of the earliest identified eIF4E-sensitive targets (*183*), there exists an interesting feed-forward mechanism between the MYC transcription factor and the translational apparatus. In addition, MYC-dependent protein synthesis increases eIF2α phosphorylation and activates autophagy as a survival pathway important for tumor development. Thus, MYC comprehensively deregulates translation to promote its own expression, and likely, that of survival factors required to escape MYC-induced apoptosis, to the great benefit of the transformed cells in which it is overexpressed. As a result, MYC-dependent tumors are addicted to activated translation, which provides an therapeutic opportunity to target protein synthesis in patients characterized by the classically untargetable MYC (*184*).

1.5.2.5.3 The cancer ribosome

Encompassing all of these possible oncogenic changes in translation initiation, elongation and termination are modifications in ribosomes themselves. A major boon to research on the importance of translation in cancer has been the discovery in the first years of the 21st century that inherited mutations in genes encoding ribosomal components or regulators thereof cause ribosomopathies, a family of syndromes leading to distinct pathological features including increased cancer susceptibility. The identity of the various mutated genes with their respective outcomes have been thoroughly reviewed elsewhere (*164*). Of note, most of these mutations are rarely observed in sporadic tumor cases, which present a different array of ribosomal defects. The basis for these discrepancies is unclear, as is the mechanism underlying the oncogenic effects of these mutations.

One hypothesis relies on observations that the stoichiometry of ribosomal components and rRNA modifications, such as methylation and pseudouridylation, varies in cancer cells. This suggests that individual ribosomes may possess unique modifications altering their ability to translate certain mRNAs with respect to others. “Cancer ribosomes” would therefore promote the translation of oncogenic mRNAs and/or restrict the translation of tumor suppressors. In support of this concept is the finding that disruption of dyskerin, the enzyme catalysing pseudouridylation, or of snoRNAs guiding dyskerin to rRNA sites, is common in cancer and can impair the translation of mRNAs encoding critical tumor suppressors such as p53 and p27 (*71*). Most recently, state of the art mass spectrometry to measure the absolute abundance of core ribosomal proteins, including those found mutated in ribosomopathies, has provided direct evidence of ribosome heterogeneity. Moreover, genome-wide ribosomal profiling of such heterogeneous ribosomes reveals that they preferentially translate distinct subsets of genes, including those critical for cellular proliferation, metabolism, and embryonic development (*185*). These findings support the notion that changes in ribosome composition may lead to specific pathologic features such as cancer, as a consequence of perturbations in the translation of specific networks of mRNAs.

Alternatively, the link between ribosomal components, ribosomopathies and cancer has been attributed to non-translational roles, mainly p53 stabilization (*3, 186*). These appear to play important roles in responding to nucleolar stress and regulating cell cycle progression. Thus, a sub-ribosomal complex composed of the 5S rRNA and ribosomal proteins RPL5 and RPL11, binds to and sequesters HDM2, resulting in p53 stabilization and cell cycle arrest. This complex forms when deregulated ribosome biogenesis leads to the accumulation of imbalanced ribosomal components, allowing the excess to inhibit proliferation until the proper balance is restored (*3*). Somatic loss of p53 signaling abrogates this effect, and is thought to be the cause of the cancer predisposition associated with ribosomopathies. A similar example relates to the ability of subsets of ribosomal proteins to modulate MYC expression, which in turn regulates nearly all the components of the translation machinery (*187*).

1.5.2.6 A variety of initiation factors with a variety of effects.

There are many ways in which cancer cells can regulate translation, all with different potential effects that are incompletely understood. The eIF4F complex alone provides multiple options. The simplest model for eIF4F complex activity in cancer would predict that modulating the levels or availability of any of its subunits would lead to changes in eIF4F complex formation, with similar results. In support of this, eIF4E, eIF4G and eIF4A are all critical for the translation of some initiation factors such as cyclin D1 (*101, 188-190*). However, functional parity does not always appear to be the case. Thus, eIF4E and eIF4G overexpression are found in different cancer types, and only eIF4E levels appear to affect metastatic progression (*101, 191*). Furthermore, inhibiting or depleting different subunits yields different results: whereas eIF4E inhibition tends to be cytostatic, eIF4A inhibition is potently cytotoxic (*106*). These discrepancies can be attributed to the varying roles of individual eIF4F subunits: the eIF4E-eIF4G interaction is required for all cap-dependent translation, with eIF4A only being required to unwind stable 5'UTR structures; in contrast, eIF4G and eIF4A, but not eIF4E, are required for cap-independent translation mediated by an internal ribosome entry site (IRES). Hence, specificity can be achieved by modulating specific eIF4F subunits. Examples of this phenomenon

include the upregulation of eIF4G seen in locally advanced breast cancer to promote IRES-mediated translation of VEGFA (*191*), or observed in response to genotoxic stress to promote the translation of factors involved in DDR (*192*). Another example is that of mitochondrial-induced apoptosis downstream of eIF4A inhibition, but not eIF4E (see section on energetic stress). In light of this, the array of possible ways for cancer cells to deregulate translation must provide a wide range of benefits, as well as possible therapeutic strategies, which remain to be fully understood.

1.5.3 Selective advantages of deregulated translation (cancer outputs)

1.5.3.1 Proliferation and apoptosis

Subsequent to the finding that eIF4E transforms fibroblasts, much research demonstrated the crucial role of translation in the regulation of proliferation and survival. Thus, several anti-apoptotic factors, cyclins and cyclin-dependent kinases have been shown to be regulated by eIF4E and many other initiation factors, a fact that has been extensively reviewed (*100, 101, 193-195*). While this has historically monopolized much of the research in the field, other aspects of cancer are also regulated at the level of translation (Figure 1.3). This is particularly striking when considering that at least one regulatory event, the phosphorylation of eIF4E, can promote neoplastic transformation and metastatic progression without affecting proliferation or survival (*55, 97*).

1.5.3.2 Angiogenesis

Angiogenesis can be promoted by a variety of translational mechanisms. Thus, FGF2 and VEGFA levels have long been associated with eIF4E overexpression in human tumors (*196, 197*). But VEGFA also possesses an IRES allowing for regulation by eIF4G (*191*) and uORFs that confer dependency on eIF2α phosphorylation (*198*), among the many layers of post-transcriptional regulation of VEGFA (*199*). This renders cancer cells particularly adaptable to oxygen deprivation, as seemingly all translational roads lead to neo-angiogenesis. Similarly, the major factor responsible for the response to hypoxia, HIF1α, can be translationally regulated by cap-dependent and independent mechanisms. Thus, in the context of hypoxia-induced mTOR

inhibition, 4E-BPs sequester eIF4E, promoting IRES-dependent expression of VEGFA and HIF1α (*191, 200*), in a process that may involve YB1 (*201*). However, in cells overexpressing eIF4E, or displaying hypoxia-resistant mTOR activation, sufficient eIF4E is available to promote cap-dependent translation of the mRNAs encoding these factors (*202-204*). Interestingly, HIF1α binds to the *Eif4e* promoter to promote its transcription, suggesting the possibility that the response to hypoxia could switch from an initial cap-independent mechanism, to a cap-dependent one (*203*). This model is supported by the fact that eIF4E promotes the translation of mRNAs encoding key hypoxia-response factors including the major hypoxia-related transcription factor, HIF1α, and growth factors promoting vascularization such as FGF2 and VEGFA (*196, 197, 202*). A more controversial report proposes that eIF4E2, also known as 4EHP, serves as an alternative cap-binding protein and recruits ribosomes via an alternative initiation complex during hypoxia (*205*). Of note, 4EHP is expressed at lower levels and possesses much poorer affinity for the cap than eIF4E (*206, 207*); therefore, how 4EHP could compete with eIF4E for cap-binding remains to be demonstrated. Specialized translation by non-canonical cap-binding proteins has also been proposed for eIF3d and the c-Jun mRNA (*153*).

1.5.3.3 Stress response

These mechanisms allow for VEGFA translation in a variety of stress conditions, particularly hypoxia, resulting in angiogenesis to increase tumor vascularization and overcome the oxygen deprivation. Many other stresses are encountered by cancer cells on the road to malignancy, including oncogenic stress, nutrient deprivation, and oxidative stress, with translational deregulation of the response providing cancer cells with the means to thrive in these highly stressed conditions (*132, 208*). The most striking example is that of eIF2α phosphorylation, which allows for the increased translation of uORF-containing stress-response mRNAs, including prototypical ATF4, CHOP, and many others. In fact, up to 49% of the transcriptome, and essentially all mRNAs translated in stress conditions, have been reported to include uORFs, and disproportionately encode proteins involved in pathways allowing cancer cells to adapt to their environment (*208-210*). Though beneficial in response to acute stress, the associated inhibition of general translation, if persistent, eventually causes cell death (*132, 208*). Cancer cells must

therefore develop mechanisms to dampen this global effect, either by fine tuning the amount and duration of eIF2α phosphorylation, or by dissociating the pro-apoptotic effects from the stress response. For example, eIF2α phosphorylation promotes the translation of its own phosphatase CreP, resulting in a feedback inhibitory loop (*210*). Furthermore, IRESs, mRNA methylation and eIF2A can help maintain protein synthesis when cap-dependent translation is inhibited, allowing cancer cells to survive the pro-apoptotic consequences of eIF2α phosphorylation (*132, 141-143*). However, further research is needed to better understand how cancer cells adapt translational control during prolonged stress. Recent advances in how translation modulates the cancer-specific stress response are delineated below.

1.5.3.4 Oncogene Induced Senescence (OIS)

A critical barrier to neoplastic transformation is the coupling of oncogenic stress with the pro-proliferative properties of oncogenes, resulting in senescence or apoptosis. This is related to accumulating DNA damage due to hyperactive proliferative signaling, and activation of the p53/p21 and p16INK4A tumor-suppressor pathways and growth arrest (*211-213*). Overexpression or hyperactivation of eIF4E was previously reported to act as a classical oncogene by inducing senescence in mouse embryonic fibroblasts and in B cells (*99, 214*). In contrast, recent studies using breast cancer models describe eIF4E as an exception to this rule, as its overexpression promotes sustained proliferation without inducing OIS (*215, 216*). While DNA damage occurs in cells overexpressing eIF4E (*215*), they benefit from the concomitantly increased translation of DNA repair factors such as BRCA1 (*217*), in addition to factors promoting survival and cell cycle progression (*216, 218*). As a result, they are able to overcome the anti-proliferative and pro-apoptotic signals associated with DNA damage and, thus, uncouple OIS from their oncogenic signaling (*215*). Beyond this apparent cell-type specificity of eIF4E-related OIS, it is well established that aberrant eIF4E expression can counteract RAS-mediated OIS and apoptosis induced by c-MYC, cooperating with these oncogenes to promote neoplastic transformation (*99, 219, 220*). Alternatively, cells undergoing transformation can evade OIS and oxidative stress via the eIF2α-ATF4 axis (*131, 221, 222*). Mechanistically, *eIF2*α phosphorylation promotes ATF4 mRNA translation, which in turn suppresses the transcription

of the major senescence inducing gene *Cdkn2a* (*131, 222*). Consequently, levels of p16INK4 and p19ARF decrease, preventing senescence. The implication of both eIF4E and eIF2 in the escape from OIS is demonstrated by a proteome-wide study of ubiquitinylation by Bengsch *et al.*, where translational control was identified as critically important in this process (*223*). Thus, cancer cells, even in pre-neoplastic stages, deregulate translation to evade senescence and achieve transformation.

1.5.3.5 Metabolism

Low levels of oxygen and/or nutrients can prevent proper energy production by cancer cells, as can mitochondrial damage and chemical inhibitors of oxidative phosphorylation (*224*). The link between translation and energy status is mediated by AMPK, which is activated by an increase in the AMP/ATP ratio, leading to phosphorylation of TSC2 and inhibition of translation via the mTOR/4E-BP axis. Phosphorylation of eIF2α downstream of AMPK has also been implicated, specifically in the context of drugs targeting NAD+ synthesis (*225*). Thus, energy deficits lead to the inhibition of translation and energy conservation. In turn, many mitochondrial proteins are regulated at the level of translation (*226, 227*). Interestingly, Gandin et al. demonstrated that translational co-regulation of these mitochondrial proteins and anti-apoptotic proteins constitutes a critical survival mechanism. As synthesis of proteins maintaining the integrity of the outer mitochondrial membrane (e.g. BCL2, MCL1, BIRC5) is decreased due to mTOR inhibition, mitochondrial activity is coordinately downregulated by preventing the synthesis of key electron transport chain components (e.g. ATP5O, ATP5G1, NDUFS6, UQCC2) (*105, 226*). Such co-regulation allows cancer cells to rapidly adapt to changing energetic states, but may also confer a targetable weakness, due to differences in the 5'UTRs of the two groups of mRNAs. Oxidative phosphorylation-related mRNAs possess short 5'UTRs enriched for translation initiator of short 5' UTR (TISU) elements, whereas survival-related mRNAs possess long, structured 5'UTRs (*105*). Thus, only the latter group requires the helicase activity of eIF4A to unwind 5'UTR secondary structures. This key difference may explain why mTOR inhibitors are cytostatic, whereas eIF4A inhibitors are cytotoxic, as mitochondrial activity continues despite decreased ability to maintain membrane integrity (*105*). Another important mechanism

linking energy status to translation has been reported, whereby energetic stress leads to alternative transcription start site selection, altering the 5'UTRs of a wide array of transcripts and therefore their potential for translational control (*228*). Many regulated mRNAs are themselves involved in translation, thus adding to the complexity of the interplay between energy status and translation. For example, under glucose starvation, the gene encoding poly-A binding protein (PABP) is transcribed from an alternative promoter, resulting in a transcript with a shorter 5'UTR lacking autoinhibitory sequences and displaying increased translation (*228*). Thus, there are several mechanisms that we are only beginning to uncover by which cancer cells can adapt translation to their energetic requirements. Whether this can be targeted in cancer patients remains to be seen, but it is an area of active investigation (*102*).

1.5.3.6 Drug Resistance

One of the most studied and clinically dire attributes of translational deregulation is the ability to confer drug resistance on cancer cells. There are numerous reports of resistance to chemotherapy, radiation and targeted therapies being mediated by alterations in the translational machinery (*95, 229-231*), which has been called a "nexus of resistance" (*231*). Some of the most recent observations include phosphorylated eIF4E-mediated resistance to radiation, DNA damage and alkylating agents (*232-234*), as well as resensitization of ovarian cancers to carboplatin by mTOR kinase inhibitors (*217*). Similar findings have been observed for eIF2α phosphorylation, in that use of salubrinal, a phosphatase inhibitor that prevents eIF2α dephosphorylation, enhances the efficacy of doxorubicin (*235*) and the proteasome inhibitor bortezomib (*139*). These effects are thought to be mediated by increased translation of mRNAs encoding DNA repair and survival factors in the case of deregulated eIF4F complex formation (*95, 217, 229-231*), and to the pro-apoptotic properties of long-term maintenance of eIF2α phosphorylation in the case of salubrinal (*139, 235*).

1.5.3.7 Emerging advantages of deregulated translation

While the impact of translation on proliferation, survival and the stress response has been known for decades, its involvement in other aspects of tumor biology are only now being

uncovered. Thus, a variety of translation initiation factors, most notably eIF4E and eIF4G, have been shown to promote the metastatic potential of cancer cells by promoting the translation of pro-metastatic factors such as MMPs, integrins, transcription factors involved in epithelial-to-mesenchymal transition, small GTPases involved in migration, survival factors and DNA damage and repair (DDR) factors (*97, 108, 191, 192, 236-242*). In addition, eIF4E hyperactivation in cancer cells increases the translation of key anti-oxidant proteins such as ferritin and members of the glutathione pathway to sustain cancer cell survival by reducing reactivate oxygen species (ROS) levels (*109*). Interestingly, a cytosine-rich motif known as CERT is present in the 5'UTRs of these anti-oxidant mRNAs and is functionally important to sensitize these mRNAs to eIF4E expression levels (*109*). Translational regulation of the response to ROS may be particularly important to maintain translation in conditions of oxidative stress, as cysteine oxidation of components of the translation machinery is particularly sensitive to ROS (*243*). Protein synthesis thus provides a crucial means for cancer cells to disrupt a variety of processes important for all steps of tumor biology.

1.5.4 Inhibitors of translation

Considering the importance of translation for many aspects of cancer cell biology, it is not surprising that many inhibitors of the translation machinery have been developed to treat cancer patients (Figure 1.5). The following section describes their mechanisms of action and clinical use.

1.5.4.1 mTOR inhibitors - Rapamycin and its analogs (Rapalogs)

Rapamycin (sirolimus) is a naturally occurring macrolide produced by *Streptomyces hygroscopicus*, which was first described as an antifungal agent in 1975 (*244*). Yeast TOR kinases, and subsequently mTOR, were identified as the sole targets of rapamycin (*245, 246*). Rapamycin binds to the FKBP-rapamycin-binding (FRB) domain of mTOR in a complex with the immunophilin FKBP12 (FK506-binding protein) (*246*). This is thought to induce conformational changes that weaken the Raptor:mTOR interaction (*247, 248*), thereby inhibiting mTORC1 (*249-252*). In addition, rapamycin:FKBP12 appears to induce steric changes in the FRB domain that

restrict substrate access to the catalytic site of mTOR (*249, 252*). The effects of rapamycin also seem to depend on the nature of the residues surrounding substrate phospho-acceptor sites, which partly reconciles why this drug inhibits phosphorylation of some, but not all mTORC1 phosphorylation sites (*253, 254*). Whereas mTORC2 is insensitive to acute rapamycin treatment (*250, 251*), prolonged rapamycin treatment suppresses mTORC2 levels in a subset of cell lines and in the liver, possibly as a consequence of newly synthesized mTOR molecules being sequestered by rapamycin:FKBP12 complexes (*255, 256*).

Several rapamycin analogs (rapalogs) were generated (everolimus, temsirolimus and ridaforolimus) to improve upon its pharmacodynamics properties, which exert anti-neoplastic activity in cancer cell lines and mouse models (*257*). All are in use in the clinic or in clinical trials (*258*). However, the efficacy of rapalogs in the treatment of human cancers has been less than expected (*259*). This has been attributed in part to incomplete inhibition of the 4E-BPs as well as to activation of AKT via disruption of mTORC1-S6K-PI3K and mTORC1-Grb10-PI3K negative feedback loops (*260-263*). In addition, rapalogs activate MAPKs in a PI3K-dependent manner (*264*). Thus, there are several mechanisms of resistance to this class of mTOR inhibitors.

1.5.4.2 Second-generation mTOR inhibitors

More recent therapeutic strategies aimed at mTOR have addressed some of the shortcomings associated with rapamycin. The activation of AKT can be avoided by using dual specificity inhibitors that target both mTOR and PI3K, which is the main AKT-activating kinase (*258, 265*). The clinical applicability of these dual inhibitors in cancer is unclear, and the results of current ongoing clinical trials of PI-103, NVPBEZ235 and other PI3K/mTOR inhibitors are not available (*258*). Active-site mTOR inhibitors (asTORi, also called TORCi and TORKinibs) have also been synthesized (*259*). These potently inhibit both mTORC1 and mTORC2 (*266-268*). Since mTORC2 can activate AKT by phosphorylating it on S473 (*269, 270*), asTORi suppress AKT signaling (*259*) and exhibit stronger anti-neoplastic effects as compared to rapalogs (*259*). However, it has been shown that loss of mTORC2 activity does not reduce the anti-proliferative activity of the asTORi PP242 or Torin1 (*266, 267*), therefore demonstrating that the inhibition of mTORC1 is

responsible for the anti-proliferative effects of asTORi. Considering that rapamycin strongly and sustainably inhibits phosphorylation of some (e.g. S6Ks), but not all (e.g. 4E-BPs) mTORC1 substrates (*271*), the superiority of the anti-neoplastic effects of asTORi relative to rapamycin might be a consequence of asTORi drastically inhibiting "rapamycin-resistant" mTORC1 outputs, including 4E-BP phosphorylation (*259, 266, 267*).

In preclinical models, asTORi have shown promising results, including bioavailability, anti-metastatic properties, increased potency compared to rapamycin *in vivo*, while displaying specificity for cancer over normal cells (*76, 272-274*). However, asTORi cannot completely escape the resistance mechanisms inherent to targeting of upstream signaling molecules. Several studies indicate that eIF4E and/or 4E-BP expression levels affect the efficacy of PI3K and mTOR inhibitors (*275-280*). These studies demonstrate that mTOR-targeted therapies would likely be ineffective in tumors that exhibit an elevated eIF4E/4E-BP ratio (reviewed in (*281*)). Mechanistically, this is explained by the inability of mTOR inhibitors to effectively suppress eIF4F assembly; with translation of "eIF4E-sensitive" mRNAs proceeding unabated (*275*). These findings suggest that eIF4E/4E-BP ratio in a tumor could be used as a stratification marker for patients receiving mTOR-targeted therapies (*275*), since expression of these markers varies widely – a situation that has been documented in several cancers, including prostate and breast (*116, 282*). Additional mechanisms of resistance to mTOR inhibition include a switch from cap-dependent to cap-independent translation (*283*) and compensation of reduced mTOR signaling by activation of MAPKs (*264*).

Finally, the biguanide metformin has been shown to significantly reduce the risk of cancer in diabetic patients (*284-287*). Metformin suppresses mTOR (*288*) and represses translation of cancer-related mRNAs, though the subsets of mRNAs targeted by metformin and other mTOR inhibitors are only partially overlapping (*227*). Currently, many clinical trials are repurposing metformin for cancer therapy, though initial results are disappointing (*289*).

1.5.4.3 MNK inhibitors

Several small molecule inhibitors targeting MNKs have been described, including CGP57380 and cercosporamide (*290-292*). These reduce eIF4E phosphorylation and repress neoplastic growth in cell culture (*290*). While CGP57380 is ineffective *in vivo*, cercosporamide was shown to drastically decrease the growth of lung colonies in an experimental metastasis assay, suggesting a possible use for MNK inhibitors in the treatment of metastatic cancers (*290*). However, both compounds exhibit significant off-target effects (*290, 293*). To address this problem, novel strategies for targeting MNKs have been developed. Several 5-(2-(Phenylamino)pyrimidin-4-yl)thiazol-2(3H)-one derivatives have been shown to take advantage of features unique to the catalytic domain of the MNKs: the presence in the magnesium binding site of a DFD- rather than the typical DFG-motif seen in other kinases, three atypical insertions in the kinase domain and a propensity to crystallize in the inactive conformation. This latter characteristic, unusual for a kinase, results in the exposure of an additional hydrophobic pocket which can be targeted for increased specificity (*294, 295*). Other strategies are based on resorcylic acid lactone analogues that chemically react with cysteines present in the catalytic domain (*296*), and retinoic acid metabolism blocking agents (RAMBA) which indirectly lead to MNK1 degradation and decreased eIF4E phosphorylation in breast cancer cell lines (*297*).

While inhibiting MNKs could serve as a means to prevent resistance to other chemotherapeutic drugs including rapamycin, gemcitabine and herceptin (*118, 298, 299*), resistance to MNK inhibitors remains unexplored; indeed, determinants of sensitivity to anti-MNK therapy are unknown. However, the position of MNKs downstream of MAPKs suggests that at least some of the mechanisms of resistance to MEK inhibitors (reviewed in (*300*)) may be relevant to MNKs.

1.5.4.4 Inhibitors of eIF4E-cap interaction

The ability of eIF4E to cause neoplastic transformation requires its cap-binding activity, since overexpression of an eIF4E mutant defective in cap-binding is not tumorigenic (*113*). Cap analogs have long been used in *in vitro* studies of eIF4E function, however they suffer from poor permeability and stability *in vivo* (*301*). To circumvent this problem, the design of pro-drugs with desirable pharmacokinetic properties has been undertaken. The N-7 benzyl

guanosine monophosphate tryptamine phosphoramidate pronucleotide 4Ei-1 was reported to inhibit cap-dependent translation and EMT in zebrafish (*302*), and cause chemosensitization of lung cancer cells to treatment with gemcitabine (*303*). High-throughput screening of chemical libraries for effective, bioavailable cap mimetics is ongoing (*304, 305*). More recently, delivery of traditional cap analogs has been achieved *in vivo* using virus-like particles, opening new possibilities for eIF4E-targeting in cancer (*306*).

Ribavirin was reported to be an eIF4E-cap inhibitor (*307*), but this has been disputed (*308, 309*). A clinical trial reported benefits in acute myeloid leukemia patients (*310*), but the mechanism of action is unknown and can be attributed to translation-independent biological activities inherent to ribavirin (*311*).

1.5.4.5 Inhibitors of eIF4E-eIF4G interaction

Another promising strategy to target the translational machinery is to interfere with the assembly of the eIF4F complex. High-throughput screening of chemical libraries identified 4EGI-1, 4E1RCat, and 4E2RCat as inhibitors of the eIF4E-eIF4G interaction (*312, 313*). These molecules effectively downregulate the translation of eIF4E-sensitive mRNAs and have shown promise in pre-clinical models (*312, 314*). Intriguingly, although 4EGI-1 abrogates binding of eIF4E to eIF4G, it does not prevent binding of 4E-BPs to eIF4E (*312*). Recently, structural studies of 4EGI-1 in complex with eIF4E have revealed that this compound binds to a hydrophobic pocket distal to the eIF4G-binding site, causing localized conformational changes that result in allosteric inhibition of eIF4E:eIF4G interaction (*315*). 4EGI-1 also inhibits eIF4E-independent translation, possibly due to activation of stress response pathways (*316, 317*). An alternative means of disrupting the eIF4E:eIF4G interaction is through the fusion of 4E-BPs to the ligand of a cancer-specific cell surface receptor. For example, 4E-BP1 fused to an analog of gonadotropin-releasing hormone prevented eIF4F complex formation and inhibited tumor growth in a mouse model of ovarian cancer (*318*). Taken together, these results demonstrate the potential clinical value of targeting the eIF4E:eIF4G interaction directly.

1.5.4.6 Inhibitors of eIF4A

Hippuristanol, Pateamine A, and silvestrol (rocaglamides) are eIF4A inhibitors which suppress translation (*319*). Hippuristanol belongs to a family of polyoxygenated steroids (*320*), which bind to the carboxy-terminal domain of eIF4A. It allosterically prevents eIF4A from interacting with RNA and blocks its helicase activity, both in its free form and as part of eIF4F (*320*). Pateamine A and the rocaglamide silvestrol increase the ATPase, RNA-binding, and helicase activities of eIF4A (*321, 322*). Both compounds appear to act as chemical inducers of dimerization, and increase the RNA-binding affinity of eIF4A to RNA in a non-sequence dependent manner, resulting in its depletion from the eIF4F complex (*321*). Pateamine A is an irreversible inhibitor of protein synthesis, likely the consequence of covalent inhibition of eIF4A, and is therefore very toxic *in vivo* (*321, 323*), though better tolerated derivatives have been developed (*324, 325*).

All three eIF4A inhibitors show pre-clinical efficacy in various cell and mouse models (*190, 322, 324-327*), with silvestrol demonstrating the highest potency *in vivo* (*190*). That inhibition of eIF4A is the mechanism by which silvestrol exerts its anti-proliferative properties on cells has been demonstrated by the identification of silvestrol-resistant eIF4A mutants that can rescue this effect (*328*). As expected for an eIF4F inhibitor, the translation of mRNAs harboring increased secondary structure in their 5' UTR are more sensitive to inhibition by silvestrol (*190, 329-331*).

The demonstration that the eIF4A inhibitors can re-sensitize lymphomas to DNA-damaging agents in tumors overexpressing eIF4E suggests that directly targeting the eIF4F complex can overcome resistance mechanisms described previously that lead to increased eIF4E availability or expression. Interestingly, one of the barriers to development of silvestrol *per se* as an anti-neoplastic agent is that resistance can be mediated by overexpression of ABCB1/P-glycoprotein; structure-activity relationship studies are underway to overcome this limitation (*329, 332, 333*).

1.5.4.7 Inhibitors of Ternary complex formation

Depending on the stimulus, intensity and duration, eIF2α phosphorylation can promote cell survival or have a deleterious effect on cell fate (*334*). A mutant of eIF2α which cannot be phosphorylated transforms NIH 3T3 cells (*335*), whereas sustained phosphorylation induces apoptosis (*336*). Therefore, increasing eIF2α phosphorylation is an attractive strategy to treat cancer (*337*). One way to achieve this is to activate the kinases residing upstream of eIF2α. BTdCPU, and related N,N'-diarylureas, promote eIF2α phosphorylation via the intracellular kinase HRI and show promising effects *in vitro* and *in vivo* (*138, 338*). Another strategy is to inhibit the dephosphorylation of eIF2α using phosphatase inhibitors, such as salubrinal (*339*). There is *in vitro* data suggesting a synthetic lethal relationship between salubrinal and the proteasome inhibitor bortezomib (*139*) and this is the basis of a clinical trial combining salubrinal with the proteasome inhibitor carfilzomib (NCT01775553). However, an important consideration in strategies aimed at increasing eIF2α phosphorylation is its pro-survival function in response to stressors, such as hypoxia and nutrient deprivation (*340, 341*). Dual effects of stimulators of eIF2α phosphorylation are exemplified by guanabenz, a compound that binds to protein phosphatase 1 and inhibits stress-mediated eIF2α dephosphorylation. Guanabenz was shown to promote the survival of HeLa cells under conditions of toxic ER stress (*342*), but inhibited tumor growth in a breast cancer mouse model (*140*). One possible means of avoiding the potentially detrimental effects of compounds like guanabenz would be to directly target TC formation, the feasibility of which has been demonstrated using a brominated derivative of fluorescein, NSC119889. This inhibitor prevents binding of $tRNA^{Met}$ to eIF2 *in vitro* (*343*), however, *in vivo* efficacy of direct TC inhibitors has yet to be established.

1.5.4.8 Other inhibitors of translation

A few other inhibitors of translation show promise in the treatment of cancer. A bacterial enzyme, asparaginase, catalyzes the hydrolysis of L-asparagine and at a lower rate L-glutamine (5% of asparagine rate) (*344*), and is used for the treatment of acute lymphoblastic leukemia and pediatric acute myeloid leukemia (*345, 346*). Asparaginase leads to depletion of L-asparagine and L-glutamine, which is accompanied by perturbations in amino-acid pools,

increased eIF2α phosphorylation via GCN2 and inactivation of mTORC1 (*347*). Another clinically-approved natural product that inhibits protein synthesis is homoharringtonine, which prevents the formation of the first peptide bond (*348, 349*) and is approved for the treatment of chronic myeloid leukemia (*350*). Transcription of ribosomal DNA by RNA polymerase I is often increased in cancer (*351*). The small molecule CX-5461 effectively inhibits RNA polymerase I (*352*). This causes accumulation of free ribosomal proteins, leading to disruption of nucleolar function and induction of p53-dependent apoptosis (*353*). CX-5461 has so far demonstrated antitumor activity in murine xenograft models (*352*). Another noteworthy inhibitor is GC7, which blocks the hypusination of eIF5A (*354*). This is particularly interesting because eIF5A is the only known protein to contain the amino acid hypusine (*355*). GC7-mediated inhibition of hypusination leads to apoptotic cell death and impaired tumor growth in a mouse model of melanoma (*354*).

1.5.5 Context specificity

It is convenient to picture deregulated translation as a continuous increase in translation of tumor-promoting mRNAs, and those favouring characteristics that contribute to overall cancer progression, such as proliferation, survival, angiogenesis, migration, etc. This view is supported by the observations that some aspects of deregulated translation appear to be uniformly distributed across entire tumors and predictive of outcome (*117, 356*), as well as by a wealth of studies utilizing carefully controlled environments that focus on very specific processes, some of which have been cited throughout this review. While the former publications validate translation as an important therapeutic target, and the latter have been absolutely crucial in developing our understanding of the translational control of cancer, they do not accurately represent the incredible plasticity, nor the dynamic nature, of this process. Cancer cells are not perpetually proliferating, and migrating, and promoting angiogenesis; rather, they respond to programmed and microenvironmental cues that steer the translational response in specific, advantageous directions.

To complicate matters further, different cell types within a tumor are responsible for different aspects of tumor biology. This has been highlighted by the ever-widening understanding of

intra-tumor heterogeneity, as tumors are comprised of many sub-populations of cells of varying types and functions. These include immune cells, stromal fibroblasts, vascular endothelial cells, stem-like tumor cells known as tumor initiating or cancer stem cells that are rare but able to repopulate entire tumors and likely underlie metastasis, among others. Moreover, different regions of tumors and their micro-environment are exposed to varying concentrations of nutrients and growth factors and respond across a gradient of stresses and in different ways that are cell type dependent. Thus, translational control may vary enormously across tumor regions and different cell populations within individual tumors, and we are only beginning to understand what these differences are, how they affect cancer and how best to strategize their therapeutic targeting.

1.5.5.1 Cancer cell stemness

The importance of translation in stem cells has long been appreciated, especially given the low transcriptional activity of these cells in embryonic and hematopoietic systems. More recently, translational regulation has been shown to be a major determinant in differentiation programs. Thus, the 4E-BPs are required to limit translation and ensure the maintenance of hematopoietic and embryonic stem cells (*357, 358*), whereas their loss promotes reprogramming to induced pluripotent stem (iPS) cells (*359*). Moreover, eIF2α phosphorylation promotes the maintenance of muscle stem cells (*360*), and ribosome biogenesis regulates neural stem cell self-renewal (*361*). Interestingly, DAP5 expression promotes developmental cell differentiation in normal tissues, purportedly by enhancing cap-independent translation that would circumvent inhibition by 4E-BPs and eIF2α phosphorylation (*362, 363*). While cancer cells are not stem cells *per se*, they have been shown to similarly rely on translational control. Thus, tumor initiating cells in a mouse skin cancer model display reduced protein synthesis, which is linked to aberrant uORF translation, and thus likely dependent of eIF2α phosphorylation (*364*). Phosphorylation of eIF4E has also been implicated in the stem-like phenotype of glioma stem cells, and in acute and chronic myeloid leukemia by promoting the translation of stemness factors such as β-catenin (*365-367*). These studies indicate that cancer stem-like cells are characterized by low protein synthesis compared with most cancer cells, and may be associated

with hypophosphorylation of 4E-BPs, or hyperphosphorylation of eIF4E or eIF2α. The spectrum of inhibitors of translation to which they respond may therefore be different than that of bulk tumor cells, which must be taken into consideration for potential cancer therapies.

1.5.5.2 Fibroblasts

Among the non-transformed cell types present in tumors, fibroblasts are often the most prevalent. Their important roles at all stages of tumor development, growth and metastasis have been extensively studied and reviewed elsewhere (*368*). Of interest to the current work is the finding that cancer associated fibroblasts displaying a senescence-associated secretory phenotype (SASP) are among the most highly translationally active cells. These cells secrete a wide variety of cytokines and chemokines, growth factors and matrix proteins which require an enormous amount of metabolic activity for their production (*368*). Interestingly, defects in ribosome biogenesis reminiscent of ribosomopathies have been reported to induce SASP in fibroblasts (*369*). Specifically, overexpression of TIF-IA, resulting in increased rRNA transcription, and depletion of rRNA processing enzymes, results in sequestration of MDM2 in the nucleolus by ribosomal components and the activation of p53 (*369*). Whether this plays any role in the pathogenesis of ribosomopathies, or the switch from a hypo- to hyper-proliferative disease, remains to be investigated. Furthermore, as the senescence appears to be induced through p53 activation, it is unclear how protein synthesis is regulated in this context. In other models, mTOR has been shown to promote the translation of the SASP-related immune modulator IL1A, and the MK-2 kinase (*370, 371*). It is unclear whether mTOR also affects the general biosynthetic capacity of senescent fibroblasts, though translational activation of TOP mRNAs is possible.

1.5.5.3 Immune cells

The importance of translation for immune cell populations is evident from the immune-suppressive effects of inhibitors such as rapamycin (*372, 373*). The role of mTOR in T cells, among others, is the subject of much ongoing investigation, in particular with respect to its importance in translation, metabolic reprogramming, differentiation and activation (*374-376*).

Furthermore, in a several disease states such as allografts, cancer and injury, mTOR appears to be important for the biology of myeloid derived suppressor cells (MDSCs); however, it is not always clear whether this reflects mTOR in the cells recruiting MDSCs, in the MDSCs themselves, or both (*377-379*). Despite this, little is known regarding translation regulatory events specific to immune cell populations overall, and specifically in the cancer context, although hints can be gleaned from research in other models. For example, decreased eIF4E levels have been reported to be important in the differentiation of T regulatory cells (*380*). eIF4E phosphorylation in a variety of immune cell populations also appears to be important for the synthesis of cancer relevant cytokines and chemokines such as TNFα and IFNγ (*381-384*), and for the development of experimental autoimmune encephalomyelitis (*385*). In neutrophils, our unpublished results demonstrate that eIF4E phosphorylation promotes survival via increased translation of anti-apoptotic proteins, thus promoting metastatic dissemination in a mouse model of breast cancer. Similarly, eFFECTOR Therapeutics has reported that a novel MNK inhibitor, eFT508, possesses immune-modulatory properties and are pursuing its development in Phase II clinical trials. This aspect is particularly intriguing, as it suggests that targeting eIF4E phosphorylation in both the cancer and immune compartments should improve patient outcome. Whether other means of inhibiting translation may be similarly useful or more potent is an intriguing direction that remains to be investigated.

1.5.6 Concluding remarks

As described herein, the translation of mRNAs into proteins is a critical aspect of the regulation of gene expression and plays a major in cancer. Every month brings new discoveries into the extent of its involvement, with previously unknown factors regulating novel aspects of tumorigenesis, in unsuspected cell types. Much of the information summarized here was unknown at the start of my studies in 2010. While it demonstrates the dynamic nature of research on translation and sharpens my interest in the field, it may lead to confusion for the reader as to the pertinence of certain experiments performed. Thus, the contextual setting of the research described in Chapters 2 and 3 is delineated at the start of each chapter.

CHAPTER 2

PHOSPHORYLATION OF EIF4E PROMOTES EMT AND METASTASIS VIA TRANSLATIONAL CONTROL OF SNAIL AND MMP-3

2.1 CONTEXTUAL SETTING

The field of translational control of cancer has enjoyed an extraordinary expansion in the last decade, as our understanding of its importance has broadened from promoting proliferation and survival to encompass every aspect of the hallmarks of cancer. The importance of the current work can therefore be best understood as part of this overarching theme of applying the known regulatory mechanisms of translation to new aspects of tumor biology. Considering this, a timeline of the relevant discoveries leading up to the experiments described in Chapter 2, and the following studies validating these findings, is presented here.

By the early 2000's, the importance of translation in cancer was well established. Much of the focus was on eIF4E, which was considered an oncogene promoting survival, proliferation and angiogenesis, and leading to transformation *in vitro* and *in vivo* (*194, 236*). In 2004, the phosphorylation of eIF4E was demonstrated to be required for its ability to transform NIH 3T3 cells *in vitro* (*114*), which was rapidly confirmed in an *in vivo* model of lymphoma (*113*). To validate these findings, the Sonenberg lab developed a mouse model in which the phosphorylation site of eIF4E is mutated to alanine at the endogenous locus (*55*). The resulting mice (termed eIF4E^{S209A} hereafter) were developmentally normal, born at Mendelian ratios and presented no overt phenotypes; however, they displayed a clear resistance to prostate cancer induced by loss of the PTEN tumor suppressor (*55*). Furthermore, mouse embryonic fibroblasts (MEFs) isolated from these mice were resistant to neoplastic transformation (*55*). Around the same time - in one case, in the same issue of *PNAS* - other studies validated these findings by demonstrating that inhibiting MNKs impaired xenograft and allograft growth, and that mice lacking MNKs were resistant to cancer development in the Lck-Pten lymphoma model (*56, 290*). Surprisingly, in many cases, abrogating eIF4E phosphorylation had little impact on proliferation or apoptosis, despite severely impeding focus formation, anchorage independent growth and tumor formation in mice (*55, 56, 290*). This suggested that other factors translationally regulated by eIF4E phosphorylation played important roles in tumorigenesis.

Clues as to what aspects of tumor biology, independent of proliferation and apoptosis, responded to translational control by eIF4E phosphorylation were provided by us and other groups. Thus, elevated phospho-eIF4E in patient samples was associated with more aggressive disease stages in prostate cancer, penile squamous cell carcinoma, nasopharyngeal carcinoma and non-small cell lung cancer (*55, 116, 386-388*). Metastatic lesions are usually the cause of death in these cancers, suggesting that the MNK/eIF4E axis may regulate the metastatic process. Furthermore, the role of eIF4E and the 4E-BPs in metastasis and patient survival was being established at this time (*116, 237, 282, 389*); it was thus plausible that phosphorylation be important for this activity of eIF4E. In line with this idea, we identified certain pro-metastatic factors as being controlled by phospho-eIF4E, notably the matrix metalloproteinases MMP-3 and MMP-9 (*55*). MMPs were known to cleave several component proteins of the extracellular matrix (ECM) to promote migration and invasion (*390*) and to induce epithelial-to-mesenchymal transition (EMT) (*391*). Furthermore, Grzmil et al. reported that MNK1 regulated the expression of SMAD2, thus affecting EMT in glioblastoma cells; however, this effect required 72h of MNK inhibition to be detectable, suggestive of secondary effects, and the involvement of eIF4E phosphorylation was unclear (*392*). An MNK inhibitor, cercosporamide, was also shown to inhibit the growth of experimental metastases derived from B16 melanoma cells; however, as the cells were first seeded in the lungs, followed by treatment, the impact of eIF4E on the metastatic process could not be assessed (*290*). These observations pointed to a role for eIF4E phosphorylation in metastasis, but direct experimental evidence for this link was lacking.

In the work presented in this chapter, we investigated the role of eIF4E phosphorylation in metastasis. We demonstrated that phosphorylation of eIF4E promotes invasion in transformed MEFs, as well as TGFβ-induced EMT in normal epithelial cells. We identified mRNAs regulated by eIF4E phosphorylation, which could mediate its pro-metastatic effects, including *Snail* and *Mmp-3*. Importantly, we validated our findings *in vivo* using a metastatic mouse mammary tumor model. Taken together, these results demonstrated that eIF4E phosphorylation is a key event in the metastatic process.

2.2 RESULTS

2.2.1 eIF4E phosphorylation promotes migration and invasion

To explore whether eIF4E phosphorylation plays a role in metastasis, we utilized a cell model that we had previously developed (*55*). MEFs were derived from mice bearing the eIF4E^{S209A} mutant and their WT counterparts, and transformed with c-MYC and H-RAS^{V12}. These WT and eIF4E^{S209A} transformed MEFs display similar proliferation, cell cycle progression and levels of apoptosis, yet eIF4E^{S209A} MEFs possess reduced tumorigenic potential (*55*). We chose to use this model as it is not dependent on overexpression, and avoids targeting the MNKs, which phosphorylate additional proteins such as Sprouty2 (*393*), cPLA2 (*394*) and hnRNPA1 (*395, 396*). To investigate the role of phospho-eIF4E in metastasis, we studied WT and eIF4E^{S209A} transformed MEFs in assays that measure metastatic potential: anchorage independent growth (*397*), random migration (*398*), transwell invasion (*399*) and colony outgrowth in matrigel (*400*). As previously reported (*55*), eIF4E^{S209A} MEFs formed 2-fold fewer colonies when plated in agarose (Figure 2.1A). This effect was specific to eIF4E phosphorylation as treatment with the MNK inhibitor CGP57380 reduced colony formation in the WT MEFs in a dose-dependent manner, whereas eIF4E^{S209A} MEFs remained insensitive to this treatment (Figure 2.1A). Phosphorylation of eIF4E also promotes migration, as eIF4E^{S209A} MEFs displayed ~20% reduction in random migration speeds, as seen by time-lapse microscopy (Figure 2.1B). Accordingly, these cells traveled shorter distances than their WT counterparts (Figure 2.1C). Strikingly, invasion was severely impaired in eIF4E^{S209A} MEFs, as their invasion index was reduced 5-fold in a transwell invasion assay (Figure 2.1D). Corroborating these findings, in a matrigel colony outgrowth assay, colonies of WT MEFs exhibited a branched morphology, indicative of their invasion into the basement membrane matrix, while eIF4E^{S209A} colonies remained spherical (Figure 2.1E). Thus, eIF4E phosphorylation promotes *in vitro* characteristics that correlate with metastatic potential, prompting further investigation into its role in metastasis.

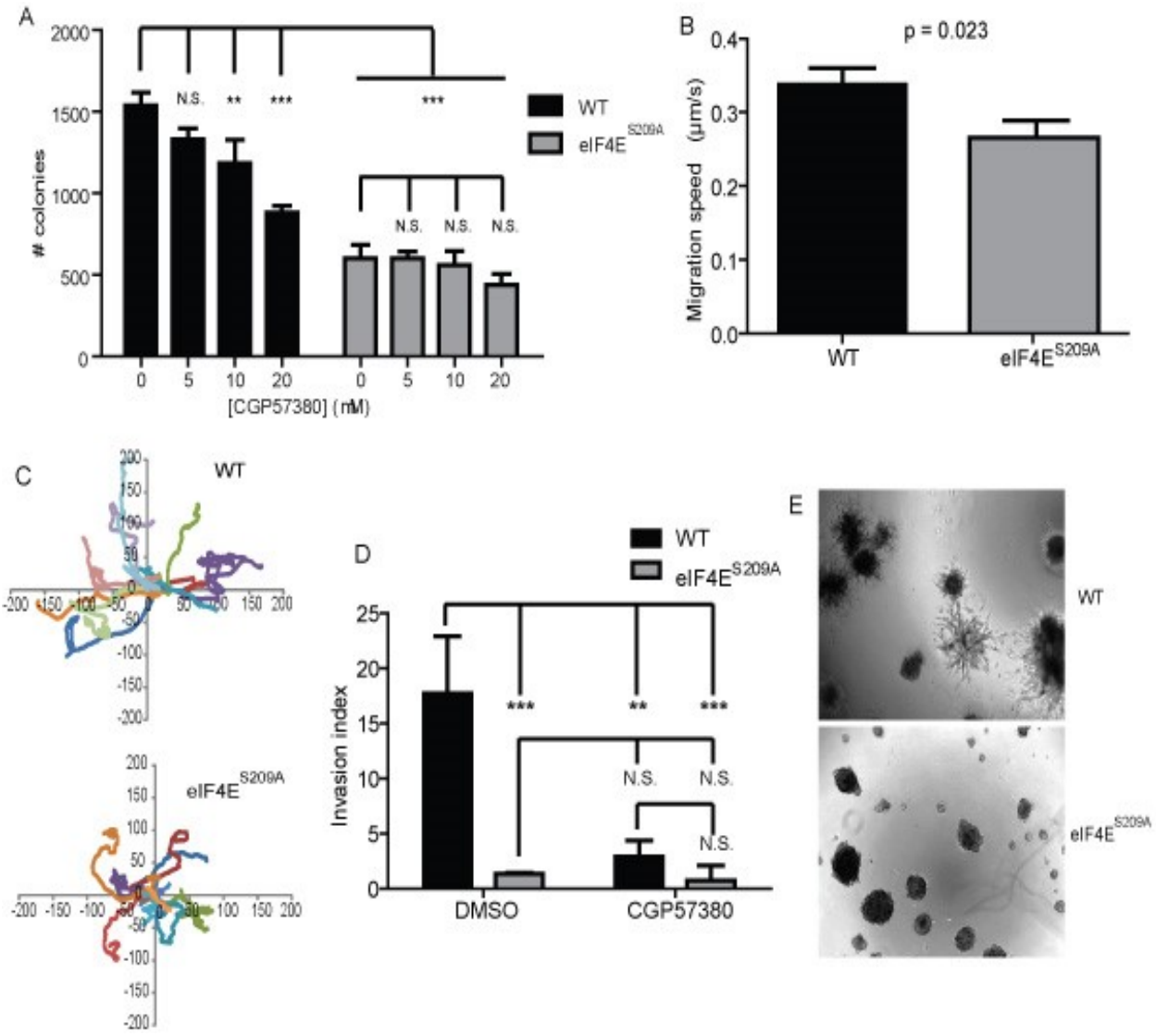
A
colonies
[CGP57380] (nM)
N.S.
**
***
WT
eIF4E S209A
B
p = 0.023
Migration speed (μm/s)
WT
eIF4E S209A
C
WT
eIF4E S209A
D
Invasion index
DMSO
CGP57380
E
WT
eIF4E S209A

Figure 2.1. eIF4E phosphorylation enhances *in vitro* metastatic properties. (A), Anchorage independent growth: WT and eIF4E^{S209A} MYC/RAS-transformed MEFs were plated in agarose and incubated for 2 weeks with the indicated concentration of CGP57380, and colonies of 8 or more cells were counted. **(B-C),** Random migration as monitored by time-lapse microscopy: migration speed **(B)** and representative migration paths **(C)** were determined using the Metamorph software. **(D)** Transwell invasion: invasion index is given as the percentage of cells having crossed the porous membrane of a Boyden chamber in the presence of a layer of matrigel versus in its absence. **(E)** Colony outgrowth: representative images of colony morphology for cells seeded in matrigel and incubated for 8 days. Error bars represent standard deviations (**A**, **D**) or standard errors (**B**). All results are representative of at least 3 independent experiments. Statistical significance determined by one-way ANOVA followed by Bonferroni's Multiple Comparison Test (**A**, **D**) or Student T-test (**B**); * indicates $p<0.05$; ** indicates $p<0.01$; *** indicates $p<0.001$; N.S. = non-significant.

2.2.2 Phosphorylated eIF4E promotes the translation of metastasis-related mRNAs.

To study the mechanism by which eIF4E phosphorylation favors pro-metastatic characteristics, the expression levels of the Y-box-binding protein 1 (YB1) were assessed in WT and eIF4E^{S209A} MEFs. This protein had previously been identified as playing an important role in eIF4E-mediated invasion (*76*). However, YB1 levels did not vary between the two cell lines (Figure S2.1), suggesting that this protein does not mediate the effect of phospho-eIF4E on invasion. To identify candidate factors that promote the pro-metastatic phenotype of WT MEFs, mRNAs translationally regulated by eIF4E phosphorylation were analyzed. To achieve this, pathway analysis was performed on a previously generated dataset of mRNAs translationally regulated by eIF4E phosphorylation (*55*). Pathways significantly increased in WT cells were identified and clustered using the DAVID functional annotation database (*401, 402*). Enriched clusters included potentially metastasis-related functions such as chemotaxis, proteases and immune signaling, as well as a general cancer cluster and one corresponding to glutathione metabolism (Figure 2.2A). Clusters corresponding to plasma membrane proteins, carbohydrate-binding proteins, leucine-rich repeat-containing proteins and zinc-binding proteins were also identified. The full list of clusters generated by DAVID and the mRNAs grouped in each cluster are presented in supplemental material (Figure S2.2, S2.3). To validate these findings and further investigate pro-metastatic mRNAs regulated by eIF4E phosphorylation, polysome profile analysis was performed on transformed WT and eIF4E^{S209A} MEFs serum-stimulated for 2h. qRT-PCR was used to monitor the distribution across a sucrose density gradient of mRNAs chosen for their relevance to invasion and metastasis, or because they were identified in the clustering analysis. There was no significant difference in global translation between WT and eIF4E^{S209A} MEFs, as the polysome profiles were nearly identical (Figure 2.2B). Accordingly, the distributions of housekeeping mRNAs *β-actin* and *Gapdh* across the sucrose density gradient were similar (Figure 2.2C, D). In contrast, the mRNA encoding MMP-3, which had been previously identified as being sensitive to eIF4E phosphorylation (*55*), was significantly shifted toward light polysomes in eIF4E^{S209A} MEFs (Figure 2.2E). This is indicative of regulation of translation initiation by eIF4E phosphorylation. Similarly, the polysomal distribution of *Snail* mRNA, an important transcription factor in the induction of EMT (*403*), was also significantly

shifted toward light polysomes in eIF4E^{S209A} MEFs (Figure 2.2F). However, no differences were detected for other factors tested including the EMT marker vimentin, the EMT-related transcription factor TWIST, the metalloproteinase MMP-14 and the vascular endothelial growth factor member VEGFC (Figure S2.4). Our results indicate that eIF4E phosphorylation promotes the translation of mRNAs encoding regulators of EMT and invasion such as SNAIL and MMP-3.

In keeping with the translational repression of *Snail* and *Mmp-3* in eIF4E^{S209A} MEFs, the expression of SNAIL and MMP-3 protein was also repressed in these cells (Figure S2.1 and Figure 2.3A). Next, it was pertinent to demonstrate that SNAIL and MMP-3 are responsible for phospho-eIF4E's pro-metastatic properties. To this end, we overexpressed eIF4E^{WT} or eIF4E^{S209A} in eIF4E^{S209A} MEFs; only eIF4E^{WT} restored SNAIL and MMP-3 levels and rescued the invasion defect of eIF4E^{S209A} MEFs (Figure 2.3A-B). Similarly, overexpressing either *Snail* or *Mmp-3* mRNAs lacking their regulatory 5' and 3'UTRs led to an increase in the expression levels of their respective proteins, in fact, to levels higher than those seen in WT MEFs (Figure 2.3A), and increased the invasion of eIF4E^{S209A} MEFs (Figure 2.3B). These experiments demonstrate that SNAIL and MMP-3 mediate the pro-invasive properties of phosphorylated eIF4E.

A

phospho-eIF4E regulated	Enrichment score
Glutathione metabolism	1.83
Chemotaxis	1.24
Cancer	1.18
Plasma membrane	1.16
Carbohydrate binding	1.15
Protease	1.12
Leucine-rich repeat	1.11
Immune response	1.05
Zinc-binding	1.03

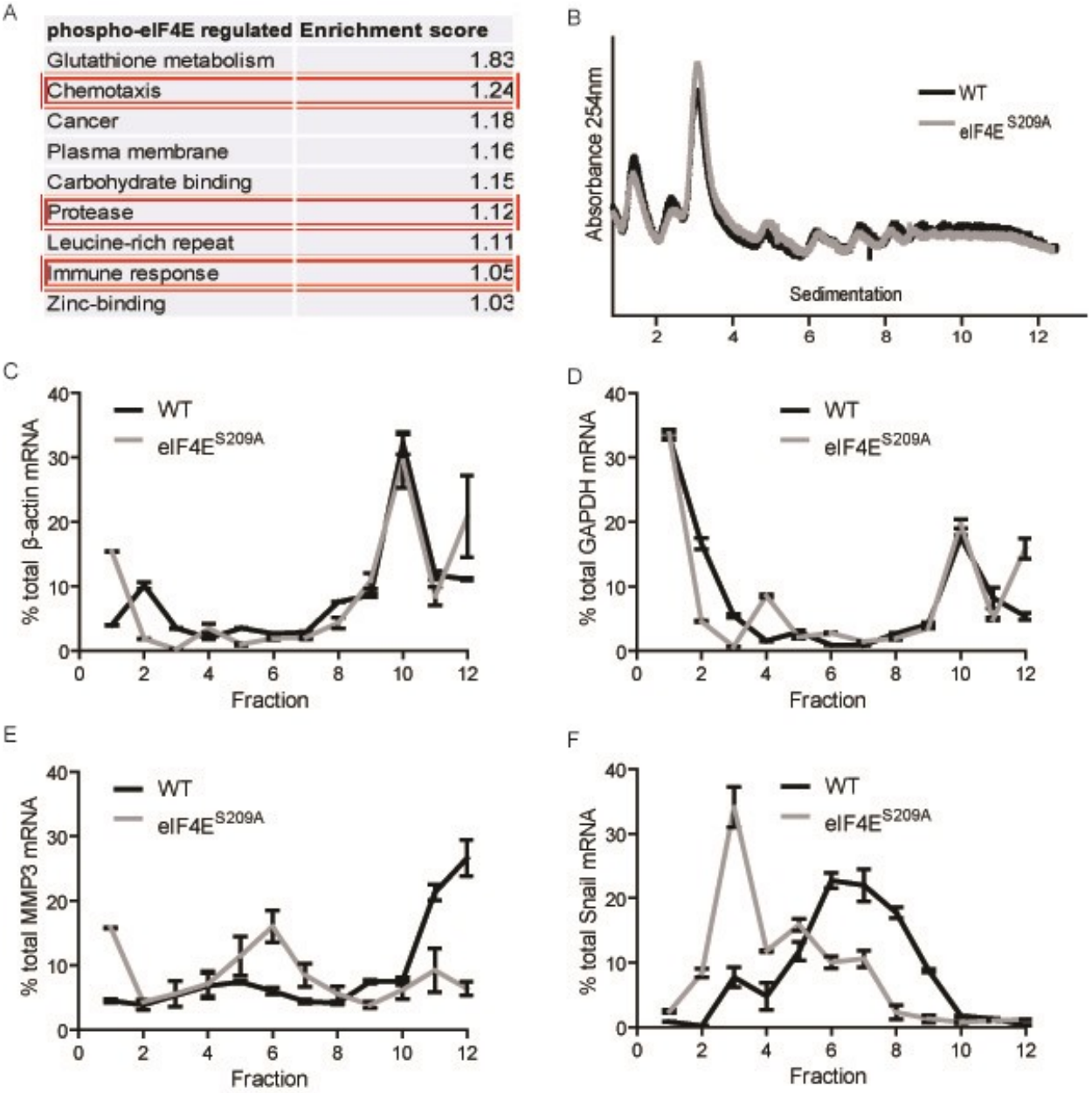

Figure 2.2. eIF4E phosphorylation promotes the translation of mRNAs involved in EMT/invasion and metastasis. (A), DAVID clustering of significantly enriched pathways from a previously generated list of phospho-eIF4E translationally-regulated mRNAs. Metastasis-related terms are in red boxes. **(B),** Polysome profile and qRT-PCR analysis of **(C),** β-*actin*, **(D),** *GAPDH*, **(E),** *Mmp-3* and **(F),** *Snail* mRNA in polysomal fractions of Myc/Ras-transformed WT and eIF4E^{S209A} MEFs. Levels of mRNA in each fraction are given as a percentage of the total. Error bars represent standard deviations. Results are representative of at least 3 experiments.

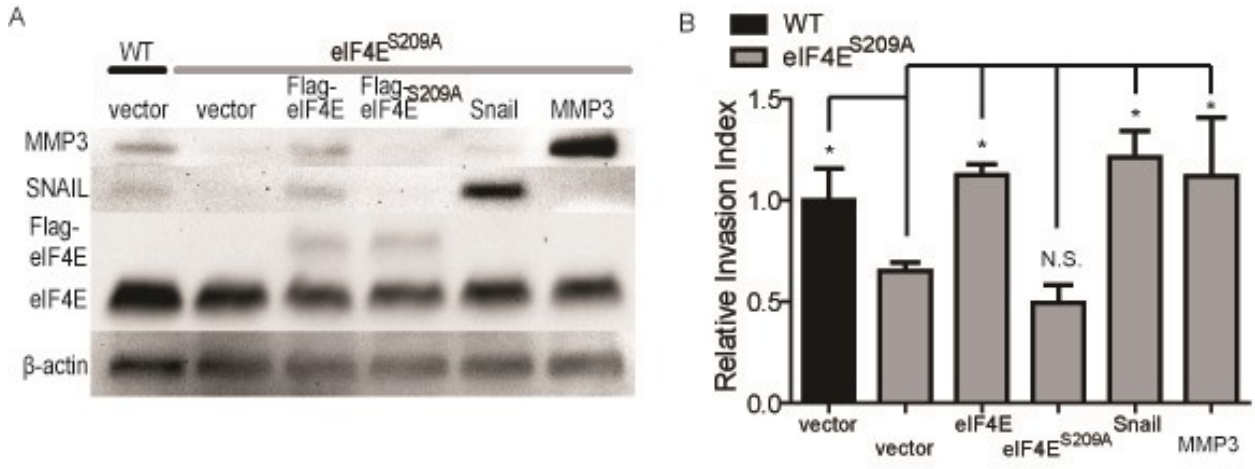
A
WT
eIF4E S209A
vector
vector
Flag-eIF4E
Flag-eIF4E S209A
Snail
MMP3
MMP3
SNAIL
Flag-eIF4E
eIF4E
β-actin
B
WT
eIF4E S209A
Relative Invasion Index
1.5
1.0
0.5
0.0
*
*
N.S.
*
*
vector
vector
eIF4E
eIF4E S209A
Snail
MMP3

Figure 2.3. Overexpression of eIF4E or phospho-eIF4E targets rescues invasion. (A) Western blot analysis and **(B)** transwell invasion of Myc/Ras-transformed WT and eIF4E^{S209A} MEFs overexpressing the indicated proteins relative to WT. Error bars represent standard deviations. Results are representative of at least 3 experiments. Statistical significance determined by one-way ANOVA followed by Bonferroni's Multiple Comparison Test; *p* values are given relative to eIF4E^{S209A} cells + vector; * indicates $p<0.05$; N.S. = non-significant.

2.2.3 eIF4E is phosphorylated during TGFβ-induced EMT

SNAIL and MMP-3 are important factors promoting cell invasiveness, metastasis and EMT (*391, 403*), therefore we investigated whether eIF4E phosphorylation could play a role in EMT. Since transformed WT and eIF4E^{S209A} MEFs are inadequate for studying EMT, we employed a well-studied cell model of EMT, in which normal murine mammary epithelial cells (NMuMG) are treated with TGFβ. This results in an easily observable transition from an epithelial morphology to a mesenchymal morphology, and is accompanied by quantifiable changes in the expression of EMT markers (*404*). Treatment with TGFβ engendered a strong increase in eIF4E phosphorylation in a time dependent manner (Figure 2.4A). Increased phospho-eIF4E levels were accompanied by an increase in the mesenchymal markers N-cadherin, fibronectin and vimentin, as well as a decrease in the epithelial marker, E-cadherin (for a review on EMT markers, see (*405*)). Ostensibly, TGFβ controls the phosphorylation of eIF4E via non-canonical signaling leading to p38 and MAPK activation (*406*). Indeed, the phosphorylation and activation of MNK1 was preceded by that of its upstream kinases, p38 and ERK (Figure 2.4B). In addition, chemical inhibition of ERK or p38 activation, using U0126 or SB203580, respectively, reduced TGFβ-stimulated phosphorylation of eIF4E (Figure 2.4C), which was abrogated by a combination of both inhibitors (Figure 2.4C). NMuMG cells engineered to express a dominant-negative mutant of p38 (p38AGF) or MKK3 (MKK3AL) are defective in undergoing a TGFβ-induced EMT (*407*). TGFβ failed to stimulate phosphorylation of eIF4E when p38 activation was blocked in p38AGF- and MKK3AL-NMuMG cell lines, consistent with non-canonical TGF-beta signaling impinging on eIF4E (Figure 2.4D). TGFβ-induced eIF4E phosphorylation was dependent on the TGFβ receptor kinase activity because SB431542, an inhibitor of the TGFβ receptor kinase (*408*), blocked eIF4E phosphorylation (Figure 2.4E). Taken together, these results demonstrate that activation of TGFβ signaling, which is frequently dysregulated in breast cancer (*409*), induces eIF4E phosphorylation, and that elevated phospho-eIF4E correlates with TGFβ-induced EMT.

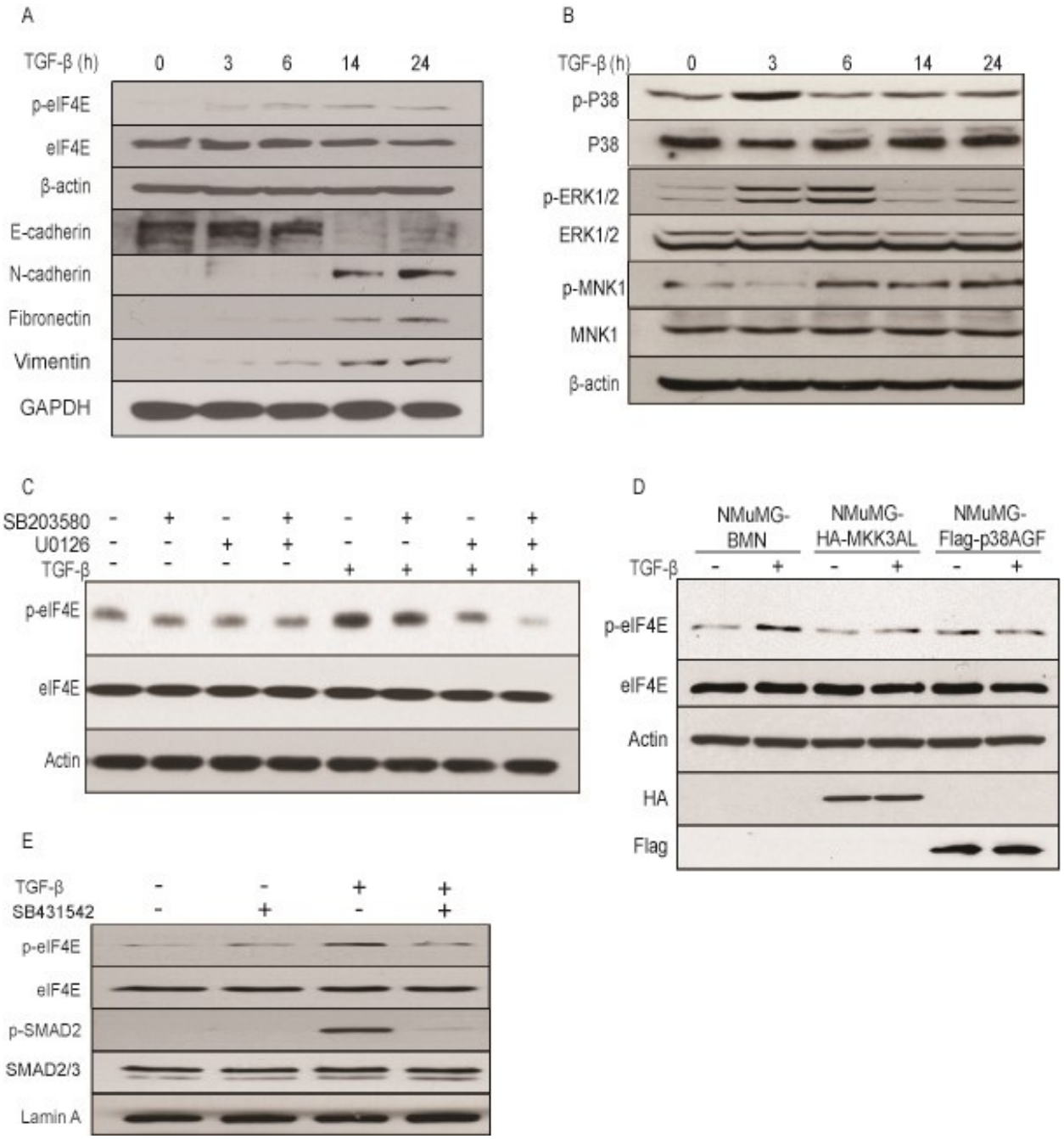
A
TGF-β (h) 0 3 6 14 24
p-eIF4E
eIF4E
β-actin
E-cadherin
N-cadherin
Fibronectin
Vimentin
GAPDH
B
TGF-β (h) 0 3 6 14 24
p-P38
P38
p-ERK1/2
ERK1/2
p-MNK1
MNK1
β-actin
C
SB203580 - + - + - + - +
U0126 - - + + - - + +
TGF-β - - - - + + + +
p-eIF4E
eIF4E
Actin
D
NMuMG-BMN
NMuMG-HA-MKK3AL
NMuMG-Flag-p38AGF
TGF-β - + - + - +
p-eIF4E
eIF4E
Actin
HA
Flag
E
TGF-β - - + +
SB431542 - + - +
p-eIF4E
eIF4E
p-SMAD2
SMAD2/3
Lamin A

Figure 2.4. eIF4E is phosphorylated downstream of MAPK during TGFβ-induced EMT. Western blot analysis of **(A)** EMT markers and **(B)** MAPK signaling in a time-course experiment of NMuMG cells treated with 5ng/mL TGFβ. Western blot analysis of the effect of ERK and p38 MAPK inhibitors **(C)**, expression of dominant-negative MAPK signal transducers **(D),** and TGFβ receptor inhibition **(E)** on TGFβ-induced eIF4E phosphorylation in NMuMG cells. Results are representative of at least independent experiments.

2.2.4 eIF4E phosphorylation promotes TGFβ-induced EMT

To determine whether eIF4E phosphorylation is a driver or a side-effect of EMT induction, we used a chemical inhibitor of the MNKs, CGP57380 (*292*). Treatment with this inhibitor blocked TGFβ-stimulated eIF4E phosphorylation and SNAIL expression (Figure 2.5A), without affecting *Snail* mRNA levels (Figure S2.5). Importantly, CGP57380 abrogated the molecular and morphological changes associated with EMT. Specifically, this inhibitor blocked the upregulation of the mesenchymal markers vimentin, fibronectin and N-cadherin by TGFβ (Figure 2.5A, upper panel), and favoured the maintenance of tight junctions, as seen by ZO-1 expression, characteristic of an epithelial phenotype (Figure 2.5A, lower panel and Figure S2.6). The effect of pharmacologically targeting MNK activity on TGFβ-stimulated EMT was not due to inhibition of the Smad2 transcriptional pathway, since TGFβ treatment resulted in comparable phosphorylation of Smad2 in the presence or absence CGP57380 (Figure 2.5A). To provide further evidence for the role of eIF4E phosphorylation in EMT, we knocked down MNK1 by siRNA. MNK1 was targeted because it is the isoform that responds to MAPK signaling, while MNK2 is relatively insensitive to upstream signaling and maintains a basal level of activation (*410*). Knock down of MNK1 prevented TGFβ-stimulated eIF4E phosphorylation as well as the TGFβ-induced expression of SNAIL and vimentin (Figure 2.5B, upper panel) and maintained the expression of ZO-1 (Figure 2.5B, lower panel and Figure S2.6), further demonstrating that MNK1-mediated eIF4E phosphorylation promotes EMT. As seen with the MNK inhibitor treatment, the effects of MNK1 knock down in NMuMG cells were not due to changes in SMAD2 activation (Figure 2.5B), and *Snail* mRNA levels were unaffected (Figure S2.5). Consistent with experiments performed using MEFs, YB1 levels were unaffected by eIF4E phosphorylation in NMuMG cells (Figure 2.5A, B).

Considering that the MNKs have targets other than eIF4E, notably Sprouty2 (*393*), cPLA2 (*394*) and hnRNPA1 (*395*), we performed a knockdown/add-back experiment to investigate the impact of phospho-eIF4E on EMT in a MNK-independent model. We stably expressed either $eIF4E^{WT}$ or $eIF4E^{S209A}$ in MCF10A cells, followed by shRNA silencing of the endogenous eIF4E. The shRNA was specific to the human eIF4E and therefore did not inhibit expression of the

exogenous murine eIF4E (Figure 2.5C). Knockdown of eIF4E impaired SNAIL expression and blocked TGFβ-mediated EMT, as indicated by reduced induction of vimentin and fibronectin (Figure 2.5C). Strikingly, when endogenous eIF4E was knocked down, only $eIF4E^{WT}$ but not $eIF4E^{S209A}$ rescued SNAIL, vimentin and fibronectin expression (Figure 2.5C). Importantly, only cells expressing $eIF4E^{WT}$, either the endogenous or exogenous form, expressed appreciable levels of SNAIL, vimentin and fibronectin (Figure 2.5C). In contrast, cells expressing the S209A mutant form of eIF4E had low amounts of the latter EMT markers, similarly to cells expressing low levels of eIF4E (Figure 2.5C). The effects we observe in the MCF10A model system indicate that defects in the MNK/eIF4E axis do not impinge upon canonical TGFβ signaling, since TGFβ-induced phosphorylation of Smad2 remained unchanged under all experimental conditions (Figure 2.5C). Thus, these experiments demonstrate a critical role for translational control by phosphorylated eIF4E in TGFβ-induced EMT.

2.2.5 eIF4E phosphorylation correlates with EMT and invasion *in vivo*

We validated our findings *in vivo* in a widely used model to study EMT and metastasis. This model consists of a series of isogenic mammary cancer cell lines (67NR, 168FARN, and 4T07) that are progressively more aggressive and present an increasingly mesenchymal phenotype (*411*). Specifically, when inoculated into the mouse mammary fat pad, the 67NR cell line forms only non-invasive primary tumors, 168FARN cells are locally invasive and detectable in the lymph node, while the 4T07 cells metastasize to the lung. In mammary tumors formed by these cell lines, increased MNK1 activation and eIF4E phosphorylation coincided with the acquisition of invasive properties and with the expression of SNAIL, as this protein was low in 67NR tumors, but increased in locally invasive 168FARN and metastatic 4T07 tumors (Figure S2.7). The correlation between SNAIL expression, eIF4E phosphorylation and the acquisition of invasive properties of these cells, *in vivo*, provides further evidence for the importance of eIF4E phosphorylation in the metastatic process.

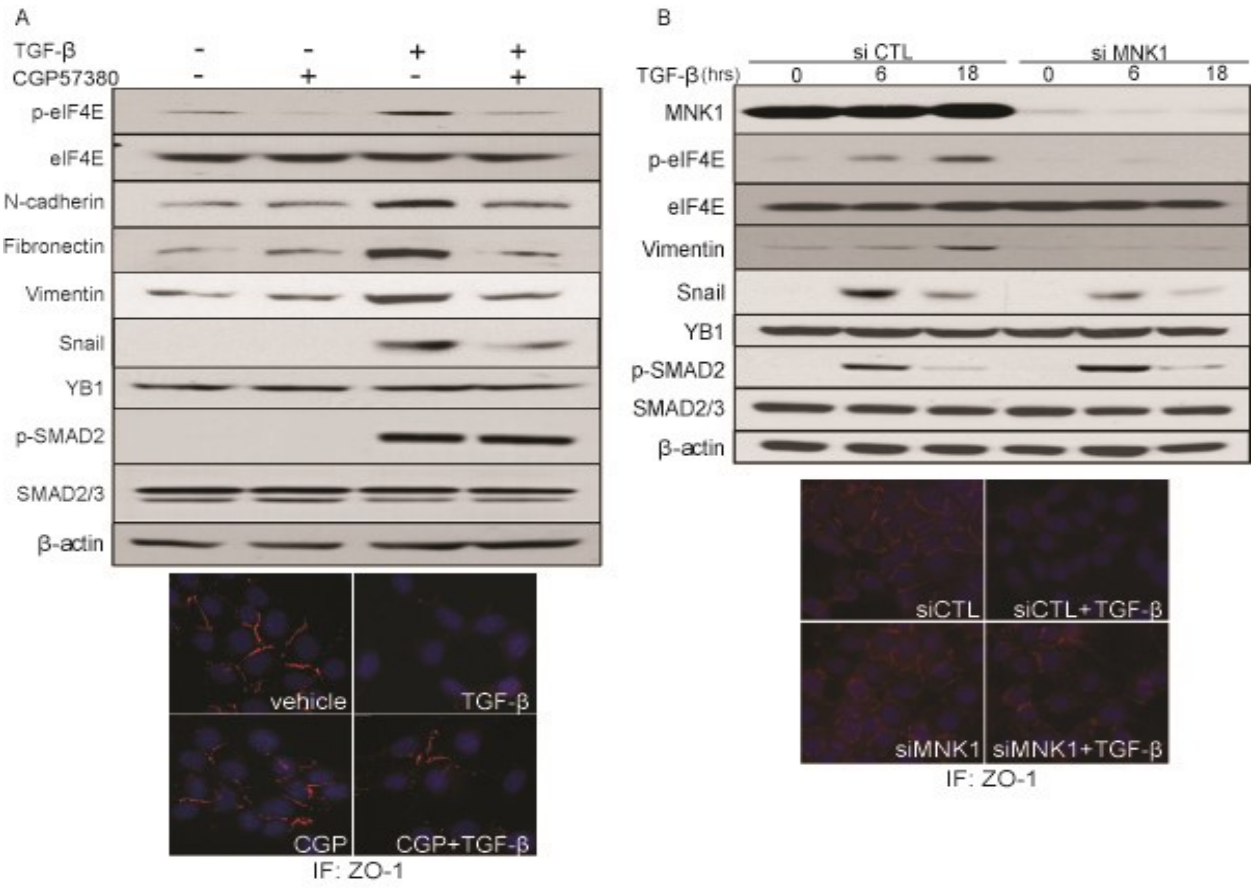
A
TGF-β - - + +
CGP57380 - + - +
p-eIF4E
eIF4E
N-cadherin
Fibronectin
Vimentin
Snail
YB1
p-SMAD2
SMAD2/3
β-actin
vehicle
TGF-β
CGP
CGP+TGF-β
IF: ZO-1
B
si CTL
si MNK1
TGF-β (hrs) 0 6 18 0 6 18
MNK1
p-eIF4E
eIF4E
Vimentin
Snail
YB1
p-SMAD2
SMAD2/3
β-actin
siCTL
siCTL+TGF-β
siMNK1
siMNK1+TGF-β
IF: ZO-1
C
CTL shRNA
eIF4E shRNA
HA-eIF4E
WT
S209A
TGF-β - + - + - + - +
HA-eIF4E
eIF4E
Snail
Fibronectin
Vimentin
p-SMAD2
SMAD2/3
GAPDH

Figure 2.5. eIF4E phosphorylation is required for TGFβ-induced EMT. **(A)** Western blot analysis (upper panel) and immunofluorescence (lower panel) of EMT markers in NMuMG cells treated with 5ng/mL TGFβ, with or without 20μM CGP57380. **(B)** Western blot analysis (upper panel) and immunofluorescence (lower panel) of EMT markers in NMuMG cells treated with 5ng/mL TGFβ in the presence of scrambled siRNA or siMNK1. **(C)** Western blot analysis of EMT markers in MCF10A cells expressing the indicated shRNAs and eIF4E variants.

2.2.6 eIF4E phosphorylation promotes tumor onset and metastasis in PyMT mammary tumor model

To assess the role of eIF4E phosphorylation in metastatic progression using a genetic model, eIF4E^{S209A} mice were crossed with MMTV-PyMT mice. In this mammary tumor model, tumors metastasize to the lungs with 100% penetrance (*412*). eIF4E^{S209A}-PyMT mice exhibited a significant delay in tumor onset, developing palpable tumors on average 2 weeks later than their WT counterparts (Figure 2.6A), confirming the importance of eIF4E phosphorylation in tumorigenesis (*55*). The penetrance of mammary tumor development was also reduced in eIF4E^{S209A}-PyMT mice, as approximately 10% percent remained tumor-free. Nonetheless, once established, both WT and eIF4E^{S209A} tumors grew at similar rates (Figure 2.6B). End-point tumors displayed similar proliferation, as evaluated by ki67 staining of tumor sections (Figure 2.6C), and similar apoptosis, as measured by caspase 3 activation (Figure 2.6D). Importantly, the metastatic potential of eIF4E^{S209A} tumors was reduced, as eIF4E^{S209A}-PyMT mice displayed a two-fold reduction in lung metastases as compared to WT-PyMT mice (Figure 2.6E, F). Metastatic burden was assessed at equivalent tumor burden rather than at a defined age; it therefore does not reflect the late onset of eIF4E^{S209A} tumors. Thus, eIF4E phosphorylation promotes the metastatic properties of eIF4E, in addition to its tumorigenic activity.

Based on our findings that eIF4E phosphorylation promotes the translation of mRNAs encoding SNAIL and MMP-3, we analyzed the expression of these factors by IHC in tumor samples from PyMT-WT and PyMT-eIF4E^{S209A} mice. Phosphorylation of eIF4E was elevated in WT tumors, as expected due to activation of the MAPK pathway by the PyMT oncogene (*413*), but remained undetectable in eIF4E^{S209A} tumors (Figure 2.7A). SNAIL and MMP-3 exhibited localized expression patterns in both WT and eIF4E^{S209A} tumors (Figure 2.7A). Indeed, as previously reported, these proteins were mainly expressed at tumor/stroma boundaries (*414, 415*). Importantly, WT tumors displayed increased SNAIL and MMP-3 expression (Figure 2.7A), resulting in a higher percentage of SNAIL and MMP-3 positive cells (Figure 2.7B,C). Taken together, these data support an important role for the phosphorylation of eIF4E in promoting invasion and metastasis through translational upregulation of its targets such as SNAIL and MMP-3.

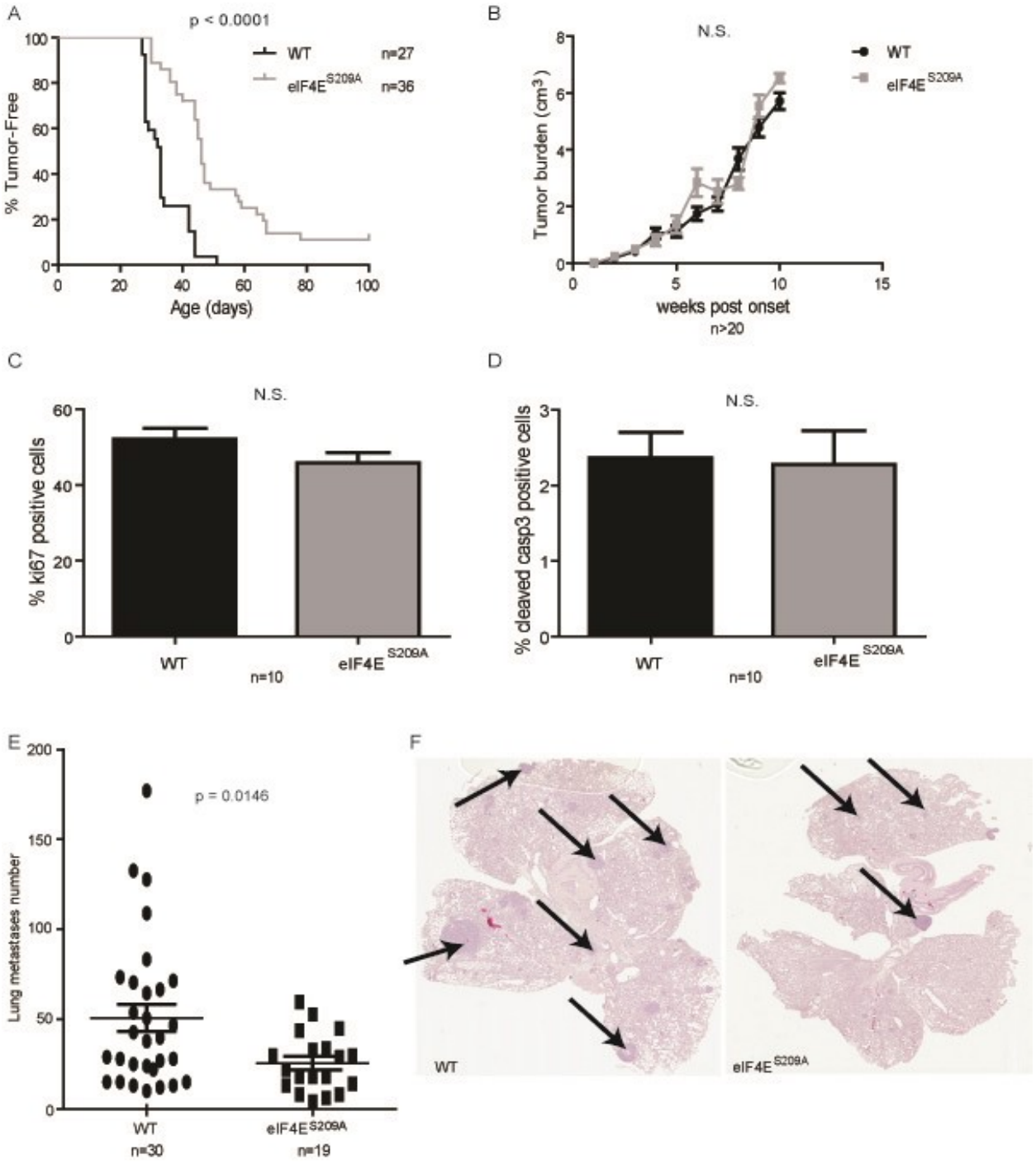
A
p < 0.0001
WT n=27
eIF4E S209A n=36
% Tumor-Free
Age (days)
B
N.S.
WT
eIF4E S209A
Tumor burden (cm3)
weeks post onset
n>20
C
N.S.
% ki67 positive cells
WT
eIF4E S209A
n=10
D
N.S.
% cleaved casp3 positive cells
WT
eIF4E S209A
n=10
E
p = 0.0146
Lung metastases number
WT
n=30
eIF4E S209A
n=19
F
WT
eIF4E S209A

Figure 2.6. eIF4E^{S209A} mice are resistant to mammary tumor development and metastasis. (A) Onset and **(B)** growth of mammary tumors in WT and eIF4E^{S209A} mice expressing the PyMT transgene. Quantification of **(C)** Ki67 and **(D)** cleaved caspase 3 immunohistochemical staining in tumor sections from WT and eIF4E^{S209A} mice, displayed as percentage of positive cells using ImageScope's nuclear stain algorithm. **(E)** Metastasis count and **(F)** representative lung images of WT and eIF4E^{S209A} mice at experimental end point. Arrows point to lung metastases. Statistical significance determined by Student T-test; *p*-values are indicated, N.S.=non-significant. The number of mice used is indicated in the figure.

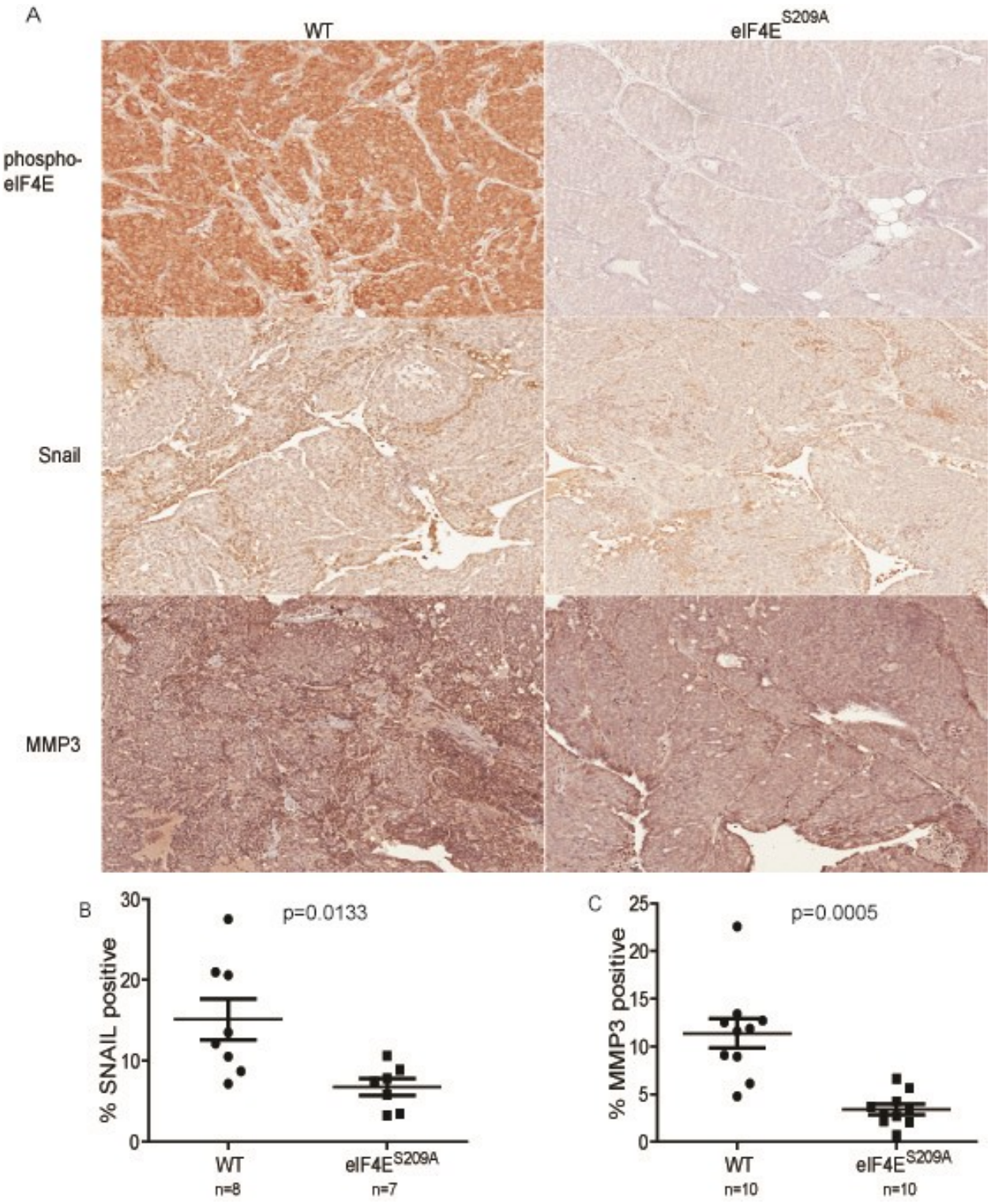
A
WT
eIF4E S209A
phospho-eIF4E
Snail
MMP3
B
p=0.0133
% SNAIL positive
30
20
10
0
WT
n=8
eIF4E S209A
n=7
C
p=0.0005
% MMP3 positive
25
20
15
10
5
0
WT
n=10
eIF4E S209A
n=10

Figure 2.7. eIF4E phosphorylation correlates with expression of SNAIL and MMP-3 in MMTV-PyMT tumors. **(A)** Representative images of IHC analysis of eIF4E phosphorylation, SNAIL and MMP-3 expression in primary MMTV-PyMT tumors. **(B)** SNAIL and **(C)** MMP-3 quantification of IHC analysis of primary MMTV-PyMT tumors: indicated are percentages of positive pixels using ImageScope's positive pixel count algorithm. Statistical significance determined by Student T-test; *p*-values are indicated. The number of mice used is indicated in the figure.

2.3 DISCUSSION

In Chapter 2, we showed that eIF4E phosphorylation promotes metastatic progression, in addition to its role in tumor development. We provided mechanistic insight into the role of eIF4E phosphorylation in metastasis, by showing that phospho-eIF4E was required for the translation of *Snail* and *Mmp-3* mRNAs, two factors that regulate EMT and invasion. Importantly, our investigation into the role of the MNK/eIF4E axis as an essential step during metastasis and TGFβ-induced EMT uncovered an unanticipated intersection of canonical and non-canonical TGFβ signaling to cooperatively promote EMT. We propose that a subset of mRNAs, transcriptionally induced by canonical TGFβ signaling, is better translated when eIF4E becomes phosphorylated via MNK1, which lies immediately downstream of the non-canonical TGFβ signaling pathway. Such co-regulation of transcription and translation has been evoked in other studies as well (*416-418*). For example, both ERK and β-catenin lead to transcriptional increases in c-Myc mRNA, and promote its translation by simultaneously activating cap-dependent translation; c-Myc in turn promotes the transcription of components of the translation machinery (*419-421*). A similar mechanism has been proposed for targets of NF-κB and Notch signaling (*421*).

Since our findings were published, they have been validated by other reports demonstrating similar EMT and invasion-promoting effects of MNKs and eIF4E phosphorylation in a variety of cell models (*297, 422, 423*). In addition, eIF4E phosphorylation has been correlated with poor outcome in melanoma and astrocytomas (*356, 424*). MNK1 had previously been linked to TGFβ signaling and was reported to promote the translation of SMAD2 (*392*). However, no differences in the level of SMAD2 expression or activation were observed in our experiments. Differences in cell type aside, the decreased SMAD2 levels upon MNK1 silencing observed by Grzmil et al. occurred after 72h and may be a result of secondary effects. All of the experiments here were performed after MNK1 siRNA silencing for 24h or less. These differences may explain the discrepancies between the two studies.

Our data demonstrated that eIF4E phosphorylation is important for EMT and invasion, which are early steps in the metastatic progression. This raised the interesting possibility of using eIF4E phosphorylation as a biomarker for tumors at high risk of metastasizing. However, it would be important to consider the levels of eIF4E phosphorylation in the context of total eIF4E expression, as well as expression and phosphorylation status of the 4E-BPs, as each of these markers can affect patient outcome, as seen in prostate cancer (*116*). Furthermore, based on results, we argued for the development of MNK inhibitors to prevent metastatic progression. At the time, the only bioavailable compound was cercosporamide, which had been shown to inhibit eIF4E phosphorylation and the growth of metastatic lung colonies in a B16 experimental metastasis assay (*290*). In this study, treatment with cercosporamide was initiated 24h after injection of the B16 cells, after the initial colonization of the lungs; these experiments could not have assessed the potential of preventing invasion by blocking eIF4E phosphorylation. Therefore, MNK inhibitors may possess important unexplored properties as metastasis preventive agents, which we will touch upon in Chapter 3. This issue will likely be addressed with the development of novel MNK inhibitors by Bayer and eFFECTOR, which are currently in clinical trials.

There are currently several inhibitors in clinical trials targeting eIF4E expression via antisense oligonucleotides, its availability through mTOR inactivation or its recruitment to the eIF4F complex by blocking the eIF4E-eIF4G interaction (*319*). However, the application of some of these inhibitors, as well as currently used therapeutic agents such as rapamycin (*299*), gemcitabine (*298*), cytarabine (*425*) and herceptin (*118*), leads to increased eIF4E phosphorylation and concomitant resistance to treatment. Therefore, inhibitors of eIF4E phosphorylation may be useful in preventing resistance to some anti-cancer drugs, in addition to blocking metastatic progression. Confirming this idea, in the years since the experiments in Chapter 2 were published, several reports highlighted the propensity of MNK inhibition to sensitize cancer cells to chemotherapy, radiotherapy and targeted therapy (*232-234, 426*). The promise of MNK inhibitors to provide clinical benefits is growing, and will be further discussed in Chapters 3 and 4.

2.4 MATERIALS AND METHODS

Reagents: TGFβ was purchased from PeproTech (USA). Insulin was from Sigma. Scrambled control siRNA and MNK1-specific siRNAs were from IDT (USA).

Mice and tissue preparation: eIF4E^{S209A} mice (*55*) were bred with MMTV-PyMT (*412*). Tumor onset in the mammary glands of control and experimental virgin mice was determined by palpation 3 times per week. Tumor growth was monitored by caliper measurements once per week or every 2 days near the experimental end point (single tumor above 2 cm^3 or total burden above 6 cm^3), at which point lung and tumor samples were collected. Step sections covering the entire lung were obtained and metastases were counted on all slides. The slide with the highest number of metastases was considered representative and used in further analyses. Lung and tumor samples were fixed in 10% formalin for paraffin imbedding and sectioning at 6 μm thickness. Pulmonary metastases were counted on H&E-stained 50 μm step-sections. Serial sections of tumor samples were obtained for immunohistochemistry. Immunohistochemistry was performed using the Vectastain ABC peroxidase system (Vector Labs, USA). Stained sections were quantified using Aperio ImageScope software using the positive pixel count algorithm. All experiments involving animals were conducted in accordance with McGill University (Montreal, QC, Canada) animal care guidelines.

Cell lines: 67NR, 168FARN and 4T07 cells were obtained from Fred Miller (Karmanos Institute, Detroit, USA). Cells ($5x10^5$) were injected into each 2nd mammary fat pad of BALB/c mice. Thirty days later, mice were sacrificed and the primary tumor harvested. Tumors obtained were immediately snap-frozen and pulverized under liquid nitrogen and stored at -80^oC until further use. Extracts were prepared by suspending the specimens in RIPA lysis buffer (*427*) and used for immunoblotting. Wild-type and eIF4E^{S209A} transformed MEFs were obtained as described (*55*). For rescue experiments, MEFs were transfected with pcDNA3.1 constructs using lipofectamine 2000 and selected with G418 sulfate. Normal Murine Mammary Gland (NMuMG) epithelial cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10%

FBS, insulin, 2mM glutamine, 100 U/ml penicillin, and streptomycin. MCF10A cells were cultured in DMEM supplemented with 5% horse serum, hydrocortisone (0.5 µg/ml), insulin (10 µg/ml), epidermal growth factor (20 ng/ml), and penicillin-streptomycin (100 µg/ml each). NMuMG-BMN, NMuMG-HA-MKK3AL, and NMuMG-Flag-p38AGF cells were kindly provided by A. Bakin (Roswell Park Cancer Institute) and cultured in the same media as parental NMuMG cells. MCF10A stable cells expressing eIF4E or eIF4E^{S209A} were infected with retrovirus produced in phoenix 293T cells, and subsequently selected with G418 sulfate and propagated. Cells were subsequently infected with lentiviral particles containing non-target control shRNA or human eIF4E shRNA (Sigma, USA). siRNAs were introduced into NMuMG using RNAiMAX (Life Technologies, USA) in accordance with the manufacturer's protocol.

Western blot analysis: Cell monolayers were washed with PBS and harvested with trypsin. Washed cells were pelleted and lysed in RIPA lysis buffer containing protease and phosphatase inhibitors (Roche). The lysate was cleared by centrifugation and protein concentration was determined with the Bio-Rad protein concentration assay solution (Bio-Rad, Mississauga, ON). 25 to 150 micrograms of protein were separated by 10-12% SDS-PAGE and electroblotted onto a nitrocellulose membrane. Membranes were incubated overnight at 4°C with the indicated primary antibodies, and the following day incubated with the appropriate secondary anti-rabbit or anti-mouse antibodies for 1-3h at room temperature. Membranes were developed with the enhanced chemiluminescence Western Blot Detection Kit. All Western blots shown are representative of at least 3 independent experiments.

Fluorescence microscopy: Cells were cultured on glass coverslips in 12-well plates for 24h, then fixed with ethanol:acetic acid at -20^{o}C, incubated with antibodies against the epithelial marker ZO-1 and then incubated with Alexa fluor 488 conjugated goat anti-rabbit IgG (Life Technologies, USA). Nuclei were stained with Hoechst. The mounted samples were scanned with a Leica DM LB2 microscope. Differences in ZO1 immunofluorescence were quantified by selecting a defined area corresponding to edges between two cells and by calculating the imageJ parameter “RawIntDen”, which is the sum of the pixel values in the selected area.

Migration and invasion: Random migration was monitored by time-lapse microscopy and analysed using MetaMorph Automation & Image Analysis Software (Molecular Devices, USA); 30,000 cells were plated in matrigel-coated 6 well plates and stimulated with 10% serum. Transwell migration assays were performed using 24-well cell culture inserts (BD Biosciences, USA) as described (*428*). Briefly, 30,000 cells were plated in the inserts in DMEM with 0.5% serum, which were placed in wells containing DMEM with 10% serum. After 16 hours, cells were counted on the bottom side of the inserts. Invasion index was obtained as the percentage of transwell migration with/without coating of the inserts with 50µL 5% growth factor-reduced (GFR) matrigel (BD Biosciences, USA). For colony outgrowth assays, 2000 cells were plated into 50µL GFR matrigel in 96-well plates and incubated for 7 days.

Plasmids and constructs: eIF4E and eIF4E^{S209A} constructs were described (*55, 98*). pcDNA3.1-FLAG-eIF4E^{WT} was described (*275*) and used to obtain pcDNA3.1-FLAG-eIF4E^{S209A} by quick-change PCR mutagenesis with the following primers:
5'-GACACAGCTACTAAGGCAGGCTCCACCACTAAAAATAGGTTTGTTGTTTAAGAAG-3',
5'-GTGGTGGAGCCTGCCTTAGTAGCTGTGTCTGCGTGGGACTGATAACC-3'.
MMP-3 cDNA was obtained from Origene (USA) in pCMV6-XL4 and cloned into pcDNA3.1 using the NotI restriction sites. pCDNA3.1-HA-SNAIL was obtained from Addgene (USA).

Polysome profile analysis, RNA isolation and RT-qPCR: Polysome profile analysis was carried out as described (*275*). RNA from each fraction was isolated using easy-BLUE kit (FroggaBio, Canada) and treated with DNaseTurbo (Ambion, USA) according to the manufacturer's instructions. Reverse transcription PCR (RT-PCR) of 100ng RNA of each fraction was carried out using SuperScript III First-Strand Synthesis System (Invitrogen). qPCRs were carried out in a Mastercycler Realplex^2 (Eppendorf) system using iQ Sybr green Supermix (Bio-Rad) according to the manufacturer's instructions.

Bioinformatics analysis: To identify genes whose translation is sensitive to eIF4E-phosphorylation we reanalyzed a previously generated microarray data set, obtained by polysome profile analysis of WT and eIF4E^{S209A} MEFs (*55*). To enable analysis of differential translation using the anota algorithm (*429*), we simulated a third replicate as described (*430*). This analysis results in an increase in false positives, but provides a ranking of genes that can be used for robust analysis, such as analysis of overrepresentation of genes belonging to specific pathways. To identify differential translation we used anota with the following settings: maxSlope=1.5, minSlope=(-0.5), maxRvmPAdj=0.15, selDeltaPT=log2(1.5) and minEff=log2(1.5). Identified differentially translated genes were further analyzed using DAVID (*431*).

2.5 ACKNOWLEDGMENTS

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CHAPTER 3

TRANSLATIONAL CONTROL IN THE TUMOR MICROENVIRONMENT PROMOTES LUNG METASTASIS VIA PHOSPHORYLATION OF EIF4E IN NEUTROPHILS

3.1 CONTEXTUAL SETTING

While our understanding of how translation is deregulated in cancer cells is growing, we know very little about translational control in cells that comprise the tumor microenvironment (TME). Yet, tumors are composed of many heterogeneous cell types, including endothelial cells, fibroblasts, adaptive and innate immune cells, which comprise the bulk of some tumors. Stromal cells play important roles in all steps of tumor development, growth, vascularization and metastasis (*432*). They also must respond to similar stresses and challenges as cancer cells (*433*), and purportedly can achieve this by regulating protein synthesis, though data explicitly demonstrating this is lacking. It is thus imperative to understand whether modulating translation in cells of the TME affects cancer progression, especially considering the numerous anti-cancer agents targeting translation that are currently in clinical trials (*102*).

When initiating this project, the possibility that translation in cells of the TME could play important roles in cancer had been indirectly suggested by a variety of studies demonstrating the importance of translation in non-transformed cell types. For example, translation plays important roles in the nervous system, where both eIF4F and eIF2 regulate aspects of behavior, including learning and memory, depression, autistic-like social and obsessive behaviors, as well as pain sensing (*434-438*). Translational control, particularly by the MNKs, of cytokine production in a variety of immune cell types had also been described in models such as Natural Killer T cell-mediated liver damage, Toll-like receptor activation in macrophages, neutrophil activation by LPS, interferon production and cellular responses to interferon during viral infections (*381-385, 439-441*). In T cells, phosphorylation of eIF4E had been linked to autoimmune encephalomyelitis, T helper type 17 differentiation in multiple sclerosis, whereas limiting eIF4E expression controls the differentiation of T regulatory cells (*385, 442*). Thus, the function of many cell types can be regulated by translation; however, whether this could have a significant impact on any aspect of cancer had never been demonstrated.

Here, we demonstrate that regulatory events affecting translation in cells of the TME impact cancer progression by investigating the importance of eIF4E phosphorylation in the TME of breast cancer patients, as well as by utilizing the eIF4E^{S209A} mouse model. By orthotopically

injecting a syngeneic mammary tumor cell line, 66cl4, in wild-type (WT) and eIF4E^{S209A} mice, we show that translational control in the TME plays a crucial role in metastatic progression, at least in part by modulating the survival of pro-metastatic neutrophils, which can be targeted using the small molecule merestinib.

3.2 RESULTS

3.2.1 Phosphorylation of eIF4E in breast cancer patients

We previously demonstrated that eIF4E phosphorylation in cancer cells promotes metastatic dissemination of mouse mammary tumors (*97*). To assess its importance in patients, we performed immunohistochemical analysis of eIF4E phosphorylation in human breast cancer samples in a tissue microarray representing tumors from 56 patients with associated normal tissue. Of the 56, 40 samples were of sufficient quality for analysis, and the expression of eIF4E phosphorylation was scored as high or low (see scoring examples in Figure S3.1). Interestingly, phosphorylated eIF4E was readily detectable in the stroma of patient samples, in addition to the neoplastic cells (Figure 3.1A); samples were therefore also separated based on the presence or absence of phospho-eIF4E in the TME. Phosphorylation of eIF4E was more common in both the cancer cells and TME of tumor tissue than in associated normal samples (Figure 3.1B-C). No differences were observed in the survival of patients when stratified according to eIF4E phosphorylation in the cancer cells (Figure 3.1D). Previous reports have indicated that eIF4E phosphorylation may be associated with survival in some cancer types but not others (*115, 356, 387, 424, 443*), which could be explained by the dominant role of deregulated mTOR/4E-BP/eIF4E axis (*116, 282*). In striking contrast, none of the patients in which eIF4E phosphorylation was absent in the TME succumbed to their disease (Figure 3.1E). This group also tended to present tumors of lower grade, possess a negative lymph node status, express at least one targetable hormone receptor, and be present in older patients (Figure S3.1). This may reflect a more prominent role of the MNKs in non-transformed cells. However, the sample size available for this experiment was too small to be conclusive, despite the statistical significance of the result (Figure 3.1E). Notwithstanding the limitations, our data suggest that eIF4E phosphorylation in the TME is common in breast cancer patients and is associated with poor survival, prompting us to investigate this phenomenon in a mouse model.

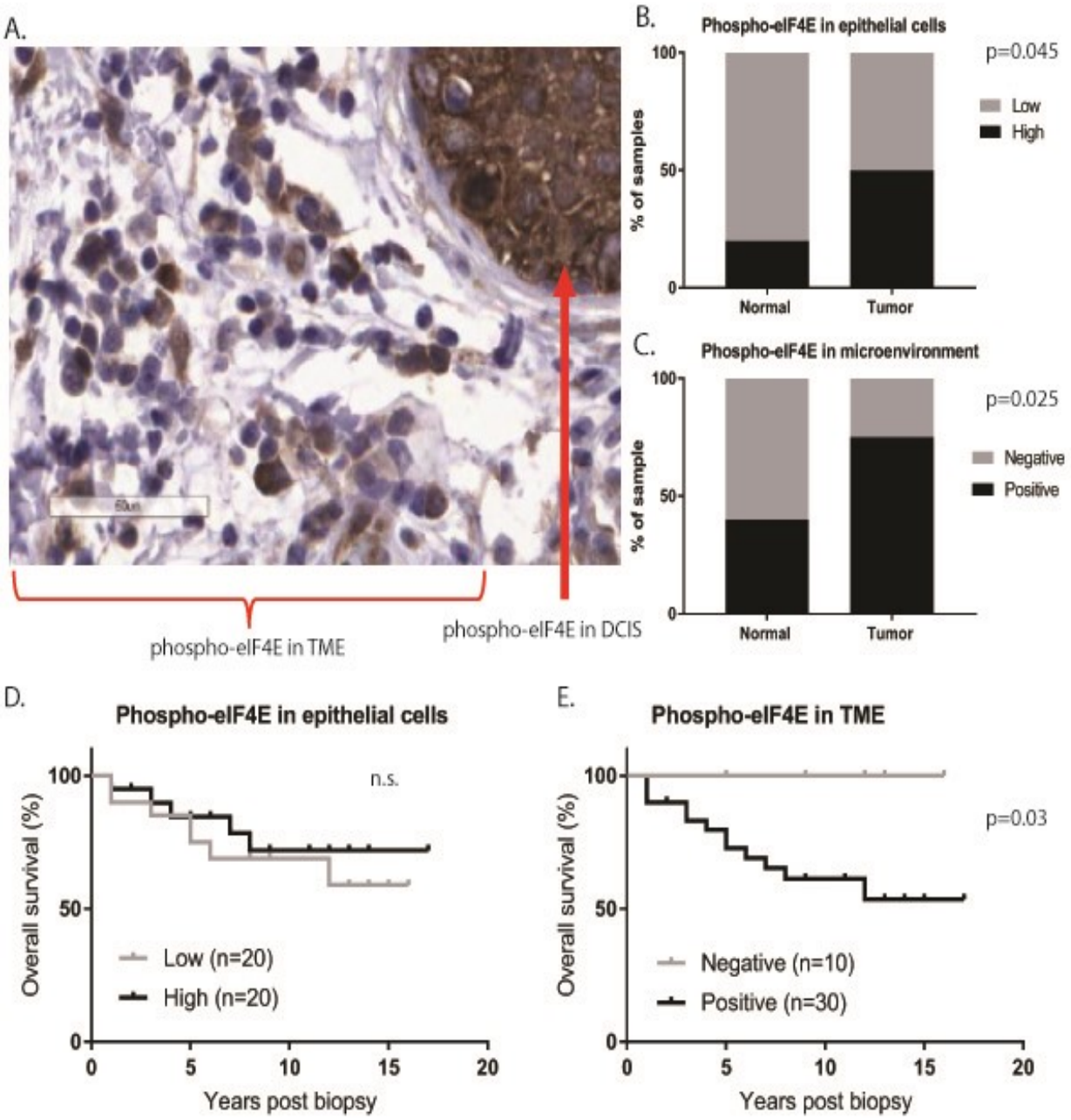
A.
phospho-eIF4E in TME
phospho-eIF4E in DCIS
B.
Phospho-eIF4E in epithelial cells
p=0.045
Low
High
% of samples
100
50
0
Normal
Tumor
C.
Phospho-eIF4E in microenvironment
p=0.025
Negative
Positive
% of sample
100
50
0
Normal
Tumor
D.
Phospho-eIF4E in epithelial cells
n.s.
Overall survival (%)
100
50
0
Low (n=20)
High (n=20)
0
5
10
15
20
Years post biopsy
E.
Phospho-eIF4E in TME
p=0.03
Overall survival (%)
100
50
0
Negative (n=10)
Positive (n=30)
0
5
10
15
20
Years post biopsy

Figure 3.1. Phosphorylated eIF4E in the TME of breast cancer patients correlates with poor survival. (A) Representative image of a TMA core displaying immunohistochemical detection of phospho-eIF4E in cancer cells (red arrow) and cells of the surrounding stroma (red brace) in a DCIS sample from a patient with triple negative breast cancer. (B) Distribution of samples displaying high or low eIF4E phosphorylation in the epithelial cells of normal and malignant mammary tissue. (C) presence or absence of phospho-eIF4E in the TME of normal and malignant mammary tissue. (D-E) Overall survival of breast cancer patients stratified according to eIF4E phosphorylation in the epithelial cells (D) or TME (E).

3.2.2 Phosphorylation of eIF4E in the TME promotes metastasis *in vivo*

To explore the role of translational control in the TME, we utilized the 66cl4 syngeneic mouse mammary tumor model. This cell line was chosen based on an interesting paradox in its metastatic properties: while it is well known to be incapable of invading blood vessels in a chorioallantoic membrane (CAM) assay, it metastasizes to the lungs when implanted in the fat pad of a syngeneic (BALB/c) host (*411*). As the metastases form in the lung parenchyma rather than within capillaries, the tumor microenvironment enables extravasation and metastasis in this model. When WT and eIF4E^{S209A} mice were subjected to orthotopic injections with 66cl4 cells, primary tumors developed with similar onset and growth rates in both mouse strains (Figure 3.2A-B). Consistent with these observations, all tumors were histologically indistinguishable, and immunohistochemical analysis revealed no differences in proliferation, apoptosis or vascularization (Figure 3.2C, Figure S3.2A). However, the absence of eIF4E phosphorylation in the TME of eIF4E^{S209A} mice was associated with a nearly complete resistance to the development of lung metastases (Figure 3.2D-F). In stark contrast, WT animals averaged 25 metastases/lung and displayed metastatic burdens of up to 50% of the total lung area. Interestingly, the few metastases present in eIF4E^{S209A} mice tended to be intravascular, suggesting a possible barrier to extravasation in these mice (Figure 3.2E-F). Similar results were obtained using a different model, in which EO771 cells were injected in C57/BL6 mice bearing the eIF4E^{S209A} mutation or lacking the MNKs (Figure S3.2B). Thus, the ability of cells within the TME to phosphorylate eIF4E has a significant impact on metastasis.

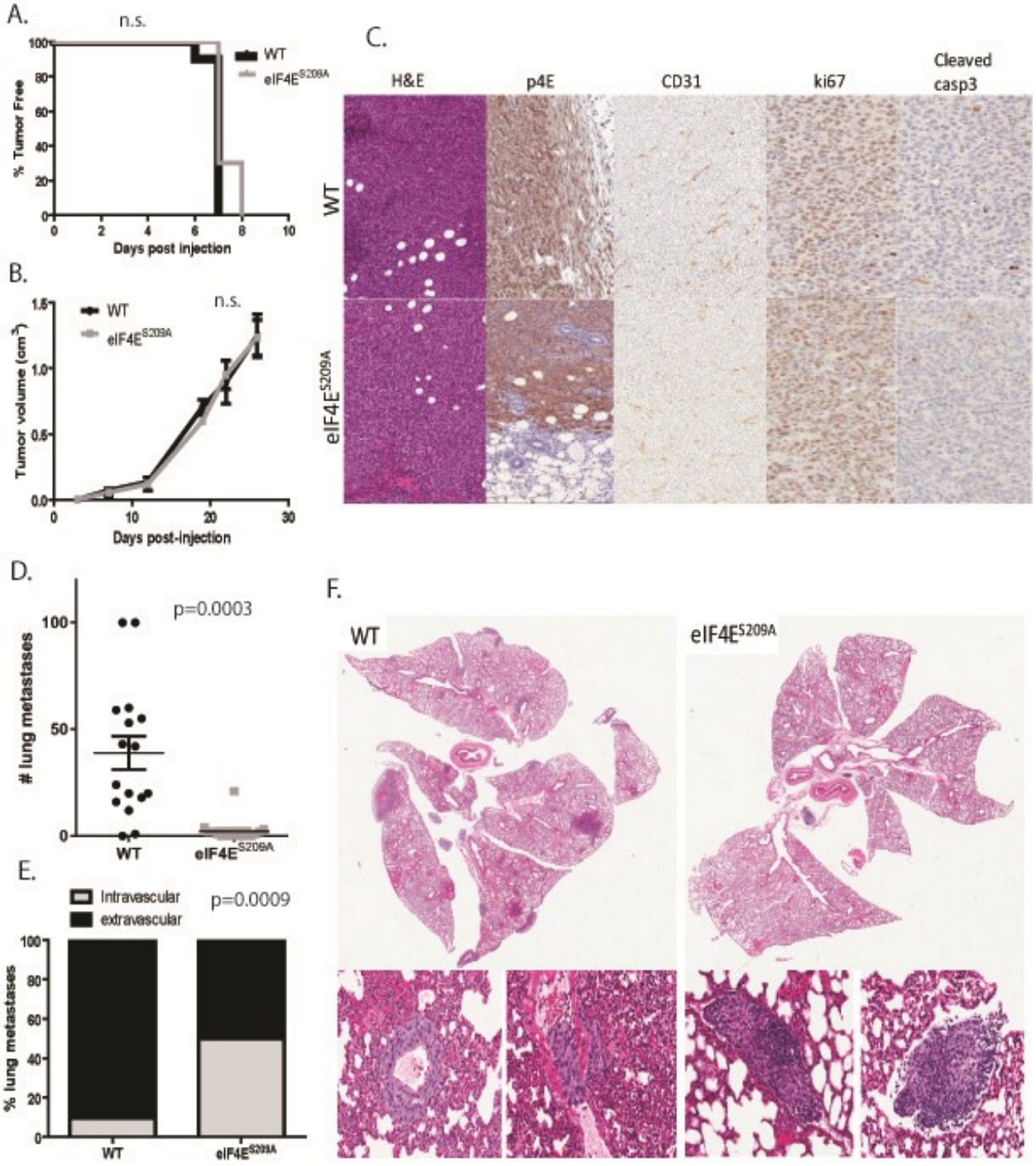
A.
n.s.
WT
eIF4E^S209A
% Tumor Free
Days post injection
B.
n.s.
WT
eIF4E^S209A
Tumor volume (cm^3)
Days post-injection
C.
H&E
p4E
CD31
ki67
Cleaved casp3
WT
eIF4E^S209A
D.
p=0.0003
# lung metastases
WT
eIF4E^S209A
E.
Intravascular
extravascular
p=0.0009
% lung metastases
WT
eIF4E^S209A
F.
WT
eIF4E^S209A

Figure 3.2. Phosphorylation of eIF4E in the TME promotes lung metastasis but has no effect on primary tumor growth. Onset (A) and growth (B) of tumors derived from 66cl4 cells injected into the mammary fat pad of WT and eIF4E^{S209A} mice (n=10). Tumors were assessed for differences in histology (H&E) and in the expression of the indicated markers by immunohistochemistry (C). Quantification (D) and representative image (F) of the number of lung metastases in tumor-bearing mice (n=16). Quantification (E) and representative examples (F, lower panel) of the proportion of intravascular lung metastases.

3.2.3 Excluding potential mediators of the effect of phospho-eIF4E in the TME

To identify cell type(s) with phospho-eIF4E-dependent effects on metastasis, immune profiling was performed in the spleen, bone marrow and lymph nodes of non-tumor bearing WT and eIF4E^{S209A} mice. No differences were observed in the steady-state levels of any of the cell types analyzed, including monocytes, granulocytes, T cell subpopulations and B cells, or in cytokine production by T cells (Figure S3.2C-G), indicating that phosphorylation of eIF4E is dispensable for normal hematopoiesis. In the context of 66cl4 mammary tumors, previous studies have found neutrophils, natural killer (NK) cells and the ratio of CD8$^+$ T-cells to T-regulatory (CD8$^+$/T$_{reg}$) cells to be related to metastatic potential (*444-446*). However, analysis of dissociated primary mammary tumors indicated that the CD8$^+$/T$_{reg}$ ratio remained unchanged in WT and eIF4E^{S209A} mice (Figure S3.3A). To further investigate the role of T cells, we compared WT fully immune competent BALB/c mice to T-cell deficient BALB/SCID mice. Tumor growth and metastatic burden were similar in both strains (Figure S3.3B, C), indicating that T cells are functionally suppressed in this model. Taken together, these data indicate that T cells are unlikely to mediate the effect of eIF4E phosphorylation in the TME. To explore the role of NK cells in our model, we performed lung colonization experiments by injecting 66cl4 cells in the tail vein of WT and eIF4E^{S209A} mice following NK cell depletion. Interestingly, depletion of NK cells led to a three-fold increase in lung nodules, confirming the importance of this cell type as a first line of defense against metastatic colonization (Figure S3.3D). However, the observed increase was, if anything, greater in WT than eIF4E^{S209A} mice, indicating that NK cell-mediated protection from metastasis was at least as effective in WT mice and could therefore not be responsible for the observed decrease in lung metastasis in eIF4E^{S209A} mice. Strikingly, the difference in lung colonization between WT and eIF4E^{S209A} mice, which was dramatic in mice bearing primary tumors, was negligible in the experimental metastasis assay, where cells were injected in the tail vein in the absence of a primary tumor (Figure S3.3D). This observation suggests the requirement for a pre-metastatic niche, the formation of which would be dependent on eIF4E phosphorylation within cells that comprise the lung microenvironment.

3.2.4 Neutrophils promote metastasis

Neutrophils have been implicated in the formation of a pre-metastatic niche in the lungs of tumor bearing mice (*447*). We therefore wished to investigate the contribution of neutrophils to lung metastasis in eIF4E^{S209A} mice. Splenomegaly was apparent in tumor-bearing WT mice, in which the spleens weighed nearly seven times more than those of non-tumor bearing mice due to a more than 100-fold increase in neutrophils (Figure 3.3A-C). This was mirrored in the blood of tumor bearing WT mice (Figure 3.3D), where neutrophils accumulate as early as two weeks post injection of 66cl4 cells in the mammary fat pad and reach concentrations averaging $150*10^9$ cells/L by four weeks (Figure 3.3D); normal concentrations were in the range of 0.5-$3*10^9$ cells/L (Figure 3.3D). Importantly, at the metastatic site, neutrophils represented <20% of cells in naïve mice, which increased to an average of 60% in animals with lung metastases (Figure 3.3E-F). This accumulation correlated with metastatic burden (Figure 3.3E). In striking contrast, neutrophil accumulation in eIF4E^{S209A} tissues was minimal (Figure 3.3D-F). It is noteworthy that neutrophil infiltration in primary tumors was mostly absent, except in necrotic areas, in both backgrounds (Figure 3.3F), possibly explaining why phospho-eIF4E in the TME had little impact on primary tumor development (Figure 3.2A-B).

To establish a causative role for neutrophil accumulation in promoting metastasis in this model, we performed depletion experiments using antibodies specific to Ly6G (clone 1A8). This marker is expressed on myeloid cells in the bone marrow but is restricted to neutrophils in peripheral tissues, and its targeting with anti-Ly6G antibodies depletes neutrophils without affecting other white blood cells (Figure S3.3E-F). In our model, both circulating mature neutrophils and immature neutrophils (band cells) were successfully depleted in peripheral blood by anti-Ly6G treatment (Figure 3.4A-B). Neutrophil depletion conferred significant protection from lung metastasis, but had no effect on primary tumor growth (Figure 3.4C-D). Thus, neutrophil depletion recapitulated our findings in eIF4E phosphorylation deficient mice, supporting the conclusion that neutrophils mediate the different metastatic dispositions of WT and eIF4E^{S209A} mice.

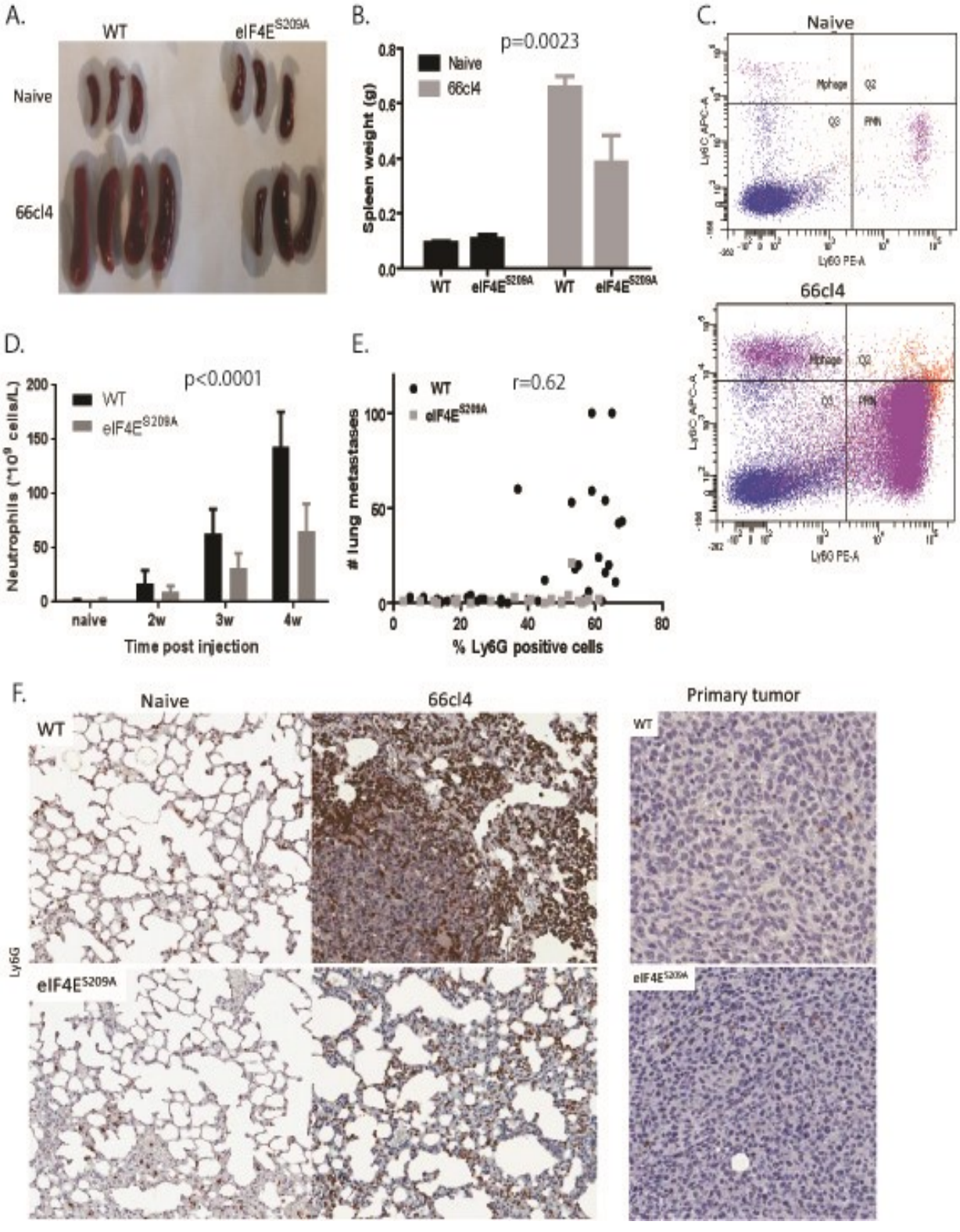
A.
WT
eIF4E S209A
Naive
66cl4
B.
p=0.0023
Naive
66cl4
Spleen weight (g)
WT
eIF4E S209A
WT
eIF4E S209A
C.
Naive
66cl4
Mphage
PMN
Ly6G APC-A
Ly6G PE-A
D.
p<0.0001
WT
eIF4E S209A
Neutrophils (*10^9 cells/L)
naive
2w
3w
4w
Time post injection
E.
r=0.62
WT
eIF4E S209A
lung metastases
% Ly6G positive cells
F.
Naive
66cl4
Primary tumor
WT
eIF4E S209A
Ly6G

Figure 3.3. Neutrophils accumulate in tissues of tumor-bearing WT and eIF4E^{S209A} mice. The spleens of naïve and tumor-bearing mice were isolated (A) to determine their weight (B) and cellular composition (C). Shown in purple are CD11b positive cells, identified as monocytes (top left quadrant) and granulocytes (bottom right quadrant) based on the expression of Ly6C and Ly6G. CD11b negative cells are shown in blue and are Ly6C/Ly6G negative (bottom left quadrant). (D) Circulating neutrophil concentrations in the blood of tumor bearing mice (n=4). (E-F) Quantification and representative images of neutrophils in lung and tumor sections based on immunohistochemical detection of Ly6G. (E) The percentage of Ly6G positive cells of Ly6G-stained lungs (F) was determined using an automated quantificiation algorithm in ImageScope and plotted against the number of lung metastases to determine their correlation (r=0.62 Spearman correlation). Ly6G was largely absent in primary tumors (F, right panel).

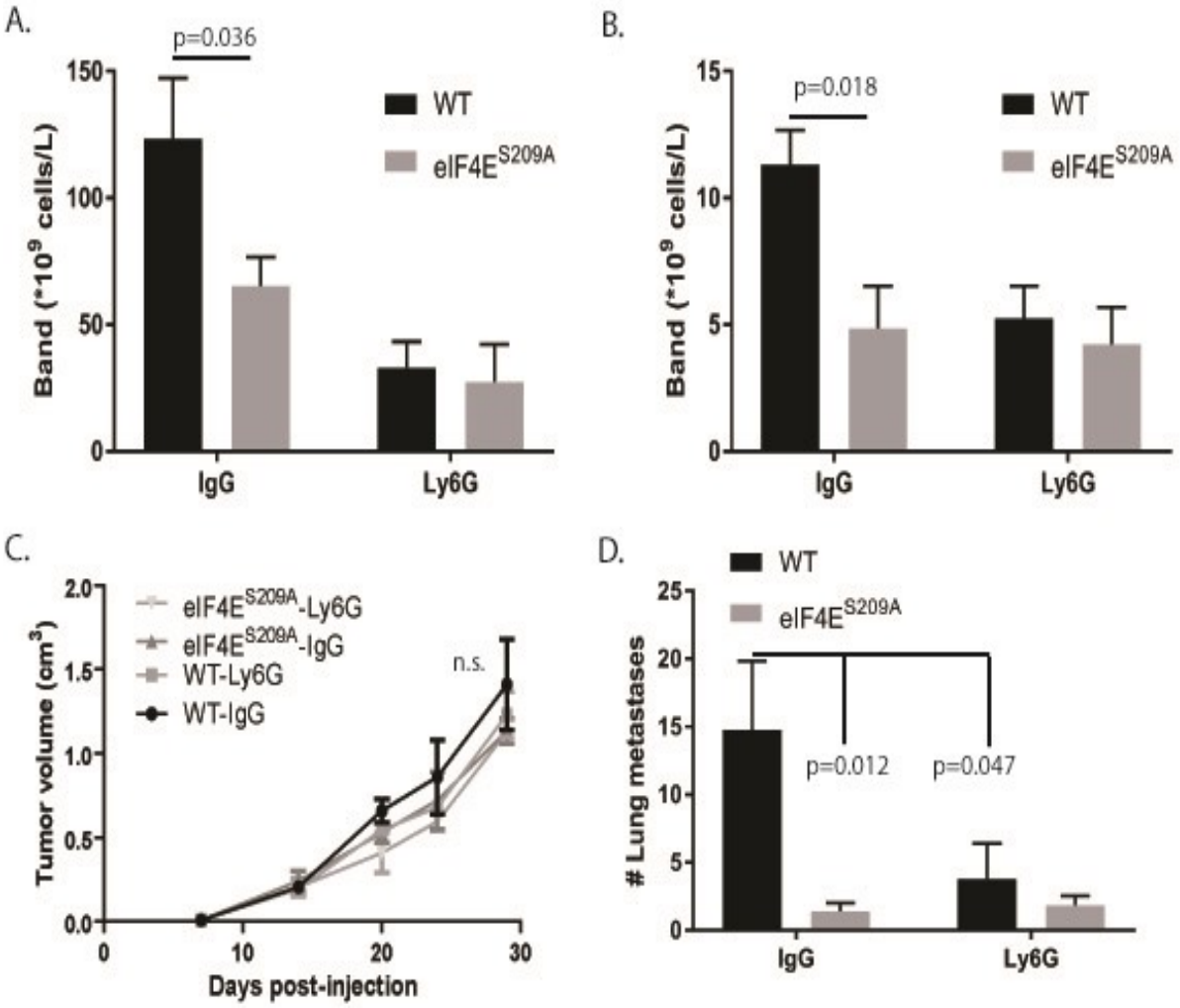
A.
p=0.036
Band (*10^9 cells/L)
150
100
50
0
WT
eIF4E^S209A
IgG
Ly6G
B.
p=0.018
Band (*10^9 cells/L)
15
10
5
0
WT
eIF4E^S209A
IgG
Ly6G
C.
eIF4E^S209A-Ly6G
eIF4E^S209A-IgG
WT-Ly6G
WT-IgG
n.s.
Tumor volume (cm^3)
2.0
1.5
1.0
0.5
0.0
0
10
20
30
Days post-injection
D.
WT
eIF4E^S209A
Lung metastases
25
20
15
10
5
0
p=0.012
p=0.047
IgG
Ly6G

Figure 3.4. Anti-Ly6G depletion of neutrophils impairs metastasis. Neutrophils were depleted from WT and eIF4E^{S209A} mice bearing palpable 66cl4 tumors (7 days post injection) by daily intra-peritoneal injections of an anti-Ly6G antibody (clone 1A8). Control mice received injections of a non-specific antibody of the same isotype (labelled "IgG"). Quantification of circulating neutrophils (A) and band cells (B) in naïve mice and in mice at four weeks post-injection of 66cl4 cells. Tumor growth was monitored throughout the experiment by caliper measurements (C). Lung metastases were detected by H&E staining and quantified in (D). N=4-5 mice per condition.

3.2.5 Phospho-eIF4E promotes neutrophil survival and accumulation

Many pro-metastatic roles have been described for neutrophils, including proteolytic remodelling of the extra-cellular matrix, increased angiogenesis, enhanced extravasation and immune suppression of multiple cell types including T cells and NK cells (reviewed in (*447*)). However, none of these activities, when assayed *ex vivo*, differed significantly in response to eIF4E phosphorylation (Figure S3.4A-C), highlighting the importance of eIF4E phosphorylation in neutrophil accumulation, rather than function, in our system. Furthermore, in tumor-bearing mice, circulating levels of cytokines and chemokines involved in neutrophil recruitment were similar in both genotypes (Figure 3.5A). In particular, G-CSF, which is tumor-derived in this model, increased 100-fold in tumor bearing WT and eIF4E^{S209A} mice equally (Figure 3.5A).

Next, we explored the possibility that neutrophil differentiation and/or survival were dependent on eIF4E phosphorylation. Myeloid progenitor cells defined as Lin$^-$ Sca1$^+$ cKit$^+$ (LSK) were isolated from the bone marrow of WT and eIF4E^{S209A} mice and differentiated to neutrophils *ex vivo* in the presence of G-CSF (Figure S3.4D). Both WT and eIF4E^{S209A} LSK cells were differentiated to neutrophils, but the numbers of eIF4E^{S209A} neutrophils obtained was reduced due to decreased viability (Figure S3.4E-F). Strikingly, blood smears revealed a 6-fold increase in the proportion of apoptotic neutrophils in eIF4E^{S209A} tumor-bearing mice, corresponding to fewer total circulating neutrophils (Figure 3.5B-C). Furthermore, neutrophils isolated from the blood of tumor-bearing eIF4E^{S209A} mice displayed reduced G-CSF-induced survival when cultured *ex vivo* (Figure 3.5D). Interestingly, no differences were observed between WT and eIF4E^{S209A} neutrophils when cultured in the absence of G-CSF (Figure 3.5D). This may explain why eIF4E phosphorylation didn't affect the circulating neutrophil numbers of naïve mice.

Previous studies have established the key role of translation in the regulation of expression of members of the BCL2 family of anti-apoptotic proteins (*113, 290, 448*). Accordingly, analysis of circulating neutrophils isolated from the blood of tumor bearing mice revealed that eIF4E^{S209A} neutrophils express lower amounts of the anti-apoptotic proteins BCL2 and MCL1

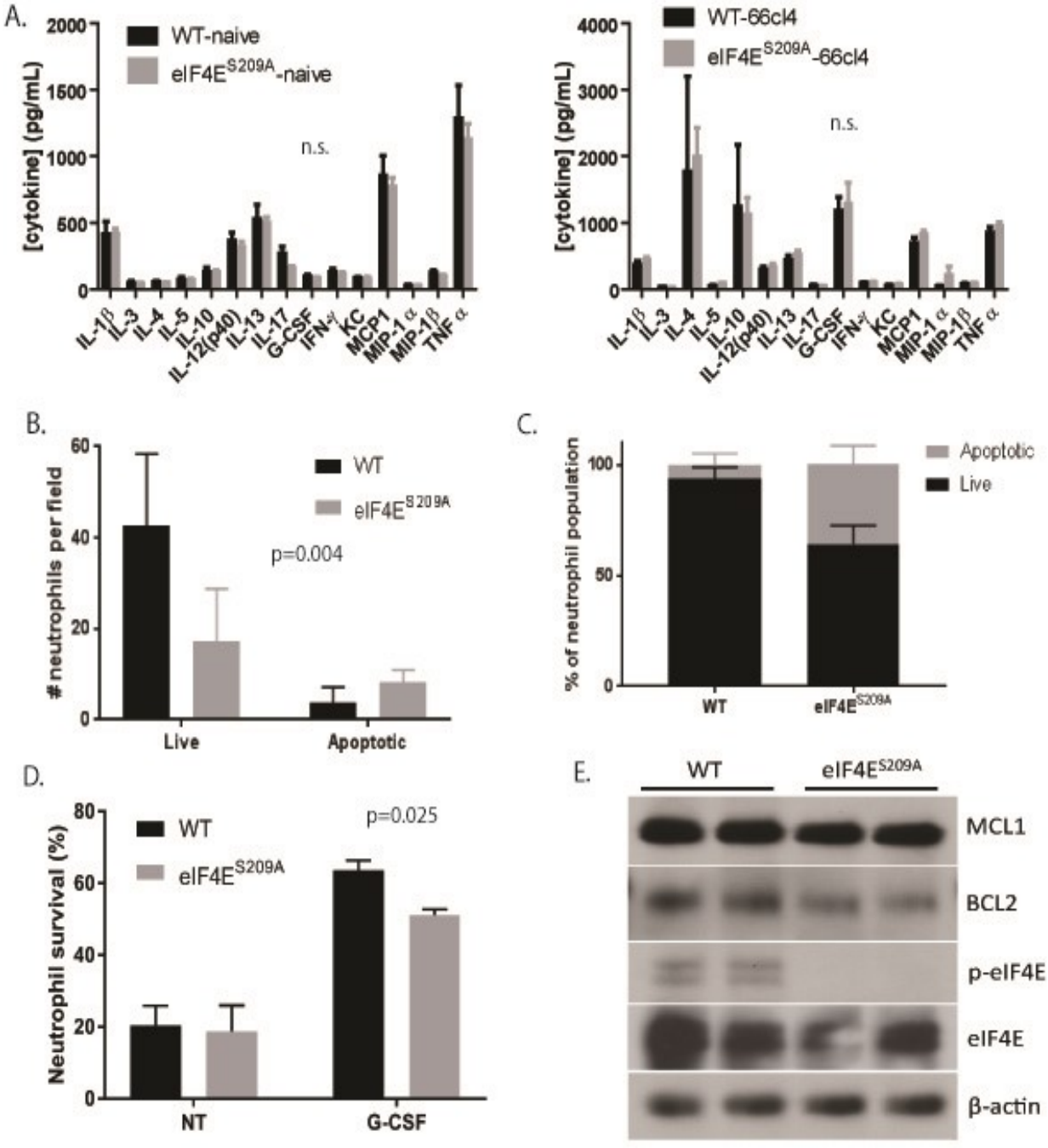
A.
WT-naive
eIF4E^S209A-naive
[cytokine] (pg/mL)
n.s.
IL-1β IL-3 IL-4 IL-5 IL-10 IL-12(p40) IL-13 IL-17 G-CSF IFN-γ KC MCP1 MIP-1α MIP-1β TNFα
WT-66cl4
eIF4E^S209A-66cl4
B.
#neutrophils per field
WT
eIF4E^S209A
p=0.004
Live
Apoptotic
C.
% of neutrophil population
Apoptotic
Live
WT
eIF4E^S209A
D.
Neutrophil survival (%)
p=0.025
NT
G-CSF
E.
WT
eIF4E^S209A
MCL1
BCL2
p-eIF4E
eIF4E
β-actin

Figure 3.5. Phosphorylation of eIF4E promotes neutrophil survival in response to G-CSF. (A) Multiplex ELISA results quantifying the indicated cytokines and chemokines from the serum of naïve (left) (n=5) or tumor bearing (right) (n=9) WT and eIF4E^{S209A} mice. (B-C) The survival of circulating WT and eIF4E^{S209A} neutrophils was assessed on Giemsa-stained blood smears of tumor-bearing mice. (B) Total numbers of live and apoptotic neutrophils counted in a microscope field at 40X magnification (n=5). (C) Proportion of live and apoptotic neutrophils counted in A. (D) Apoptosis in response to cytokine withdrawal was assessed in circulating neutrophils from tumor-bearing mice: neutrophils were isolated by density gradient centrifugation and incubated for 24h in the presence or absence of G-CSF; apoptotic neutrophils were identified by flow cytometry, shown is percentage of Ly6G positive cells stained positive for Annexin V (n=3). (E) Neutrophils were isolated from the blood of tumor bearing mice by density gradient centrifugation and lysed for western blot analysis of the indicated proteins.

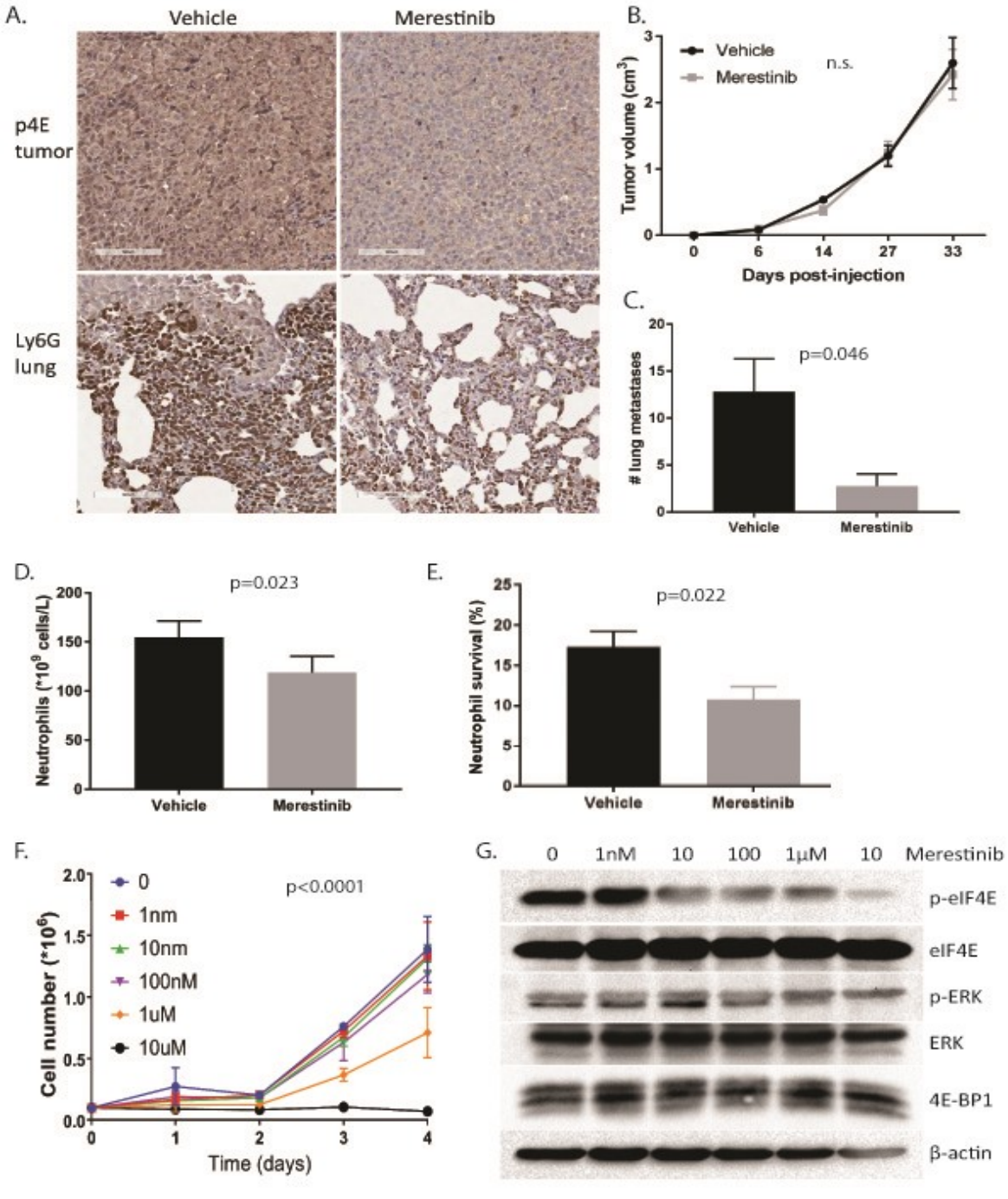
A.
Vehicle
Merestinib
p4E tumor
Ly6G lung
B.
Vehicle
Merestinib
n.s.
Tumor volume (cm3)
Days post-injection
C.
p=0.046
# lung metastases
Vehicle
Merestinib
D.
p=0.023
Neutrophils (*10^9 cells/L)
Vehicle
Merestinib
E.
p=0.022
Neutrophil survival (%)
Vehicle
Merestinib
F.
p<0.0001
0
1nm
10nm
100nM
1uM
10uM
Cell number (*10^6)
Time (days)
G.
0
1nM
10
100
1μM
10
Merestinib
p-eIF4E
eIF4E
p-ERK
ERK
4E-BP1
β-actin

Figure 3.6. Merestinib inhibits eIF4E phosphorylation, neutrophil survival and metastatic progression of 66cl4 tumors. (A-C) BALB/c mice bearing palpable 66cl4 tumors were treated with 12mg/kg merestinib daily for 14 days. (A) Immunohistochemical analysis of primary tumors and lungs; (B) tumor growth; and (C) metastatic burden of vehicle and merestinib treated animals. (D) Circulating neutrophil concentrations in the blood of vehicle and merestinib-treated animals 14 days post-injection. (A-D) n=4 mice per group. (E) Neutrophils were isolated from the blood of 8 tumor-bearing mice and treated *ex vivo* with or without merestinib (100nM) for 24 hours in the presence of G-CSF; their survival was monitored by flow cytometry. (F-G) 66cl4 cells were treated with merestinib *in vitro* for the indicated times and concentrations; proliferation was monitored by trypan blue exclusion, and Western blot analysis of the indicated proteins was performed on lysates of 66cl4 cells treated for 4 hours (G).

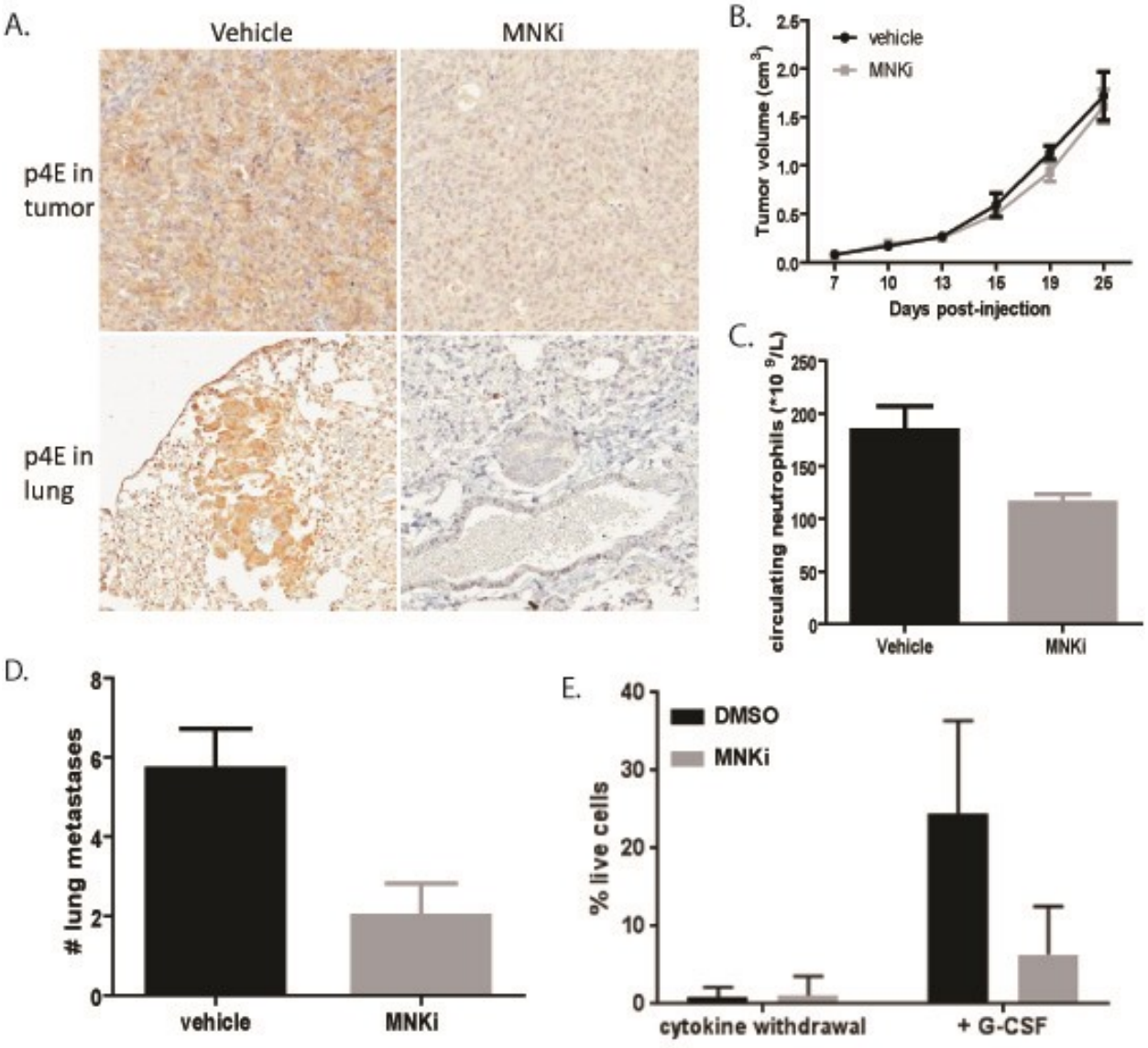
A.
Vehicle
MNKi
p4E in tumor
p4E in lung
B.
vehicle
MNKi
Tumor volume (cm3)
Days post-injection
C.
circulating neutrophils (*10 9/L)
Vehicle
MNKi
D.
lung metastases
vehicle
MNKi
E.
DMSO
MNKi
% live cells
cytokine withdrawal
+ G-CSF

Figure 3.7. MNK inhibition blocks eIF4E phosphorylation, neutrophil survival and metastatic progression of 66cl4 tumors. (A-C) BALB/c mice bearing palpable 66cl4 tumors were treated with a MNK inhibitor (MNKi) daily for 14 days. (A) Immunohistochemical analysis of primary tumors and lungs; (B) tumor growth; and (C) metastatic burden of vehicle and MNKi treated animals. (D) Circulating neutrophil concentrations in the blood of vehicle and MNKi-treated animals 28 days post-injection. (A-D) n=5 mice per group. (E) Neutrophils were isolated from the blood of 4 tumor-bearing mice and treated *ex vivo* with or without MNKi for 24 hours in the presence or absence of G-CSF; their survival was monitored by flow cytometry, shown is percentage of Ly6G positive cells positively stained with Annexin V.

(Figure 3.5E). This is consistent with a previous report linking neutrophil survival to regulation of MCL1 by MNKs (*449*). Thus, our results demonstrate that neutrophil accumulation is reduced in eIF4E^{S209A} mice due to impaired resistance to apoptotic cell death.

3.2.6 Targeting eIF4E phosphorylation in the tumor and TME prevents metastasis

Our results indicate that eIF4E phosphorylation in the TME promotes metastasis by increasing neutrophil survival. Thus, small molecule inhibitors of eIF4E phosphorylation would be expected to prevent metastatic progression, especially considering our previous work demonstrating that eIF4E phosphorylation in cancer cells promotes metastasis. To investigate this, eIF4E phosphorylation was blocked *in vivo* using merestinib, a multi-kinase inhibitor that was recently demonstrated to be a potent MNK inhibitor reducing the translation of *Bcl2* and *Mcl1* in mouse xenograft and allograft models (*365, 450-452*). In our model, merestinib inhibited eIF4E phosphorylation in the primary tumor and metastatic site (Figure 3.6A). This treatment had no effect on primary tumor growth, but prevented neutrophil accumulation and the development of lung metastases (Figure 3.6B-D). Furthermore, a 100nM concentration of merestinib was sufficient to inhibit the survival of neutrophils cultured *ex vivo* in the presence of G-CSF (Figure 3.6E). In contrast, there was no effect on the proliferation of 66cl4 cells treated with this dose of merestinib (Figure 3.6F), despite efficient inhibition of eIF4E phosphorylation starting at 10nM (Figure 3.6G), which is consistent with the finding that merestinib had no effect on the growth of primary tumors (Figure 3.6A). Importantly, merestinib did not inhibit mTOR signaling, as phosphorylation of 4E-BP1 and S6 were unaffected (Figure 3.6G), whereas MAPK signaling was only affected at higher doses that correlated with decreased proliferation, as shown by ERK dephosphorylation at 1μM and 10μM merestinib (Figure 3.6G). To validate these findings, a second MNK inhibitor was used. This inhibitor is reported to be highly specific for the MNKs, but Material Transfer Agreements prevent us from disclosing its identity. Despite this limitation, MNK inhibition confirmed our findings using merestinib, as eIF4E phosphorylation decreased in tumors and lungs (Figure 3.7A), which had no effect on primary tumor growth (Figure 3.7B) but was accompanied by a reduction in circulating neutrophils (Figure 3.7C) and lung metastasis (Figure 3.7D). Furthermore, MNK inhibition blocked

neutrophil survival in response to G-CSF (Figure 3.7E). Taken together, these results support our findings that eIF4E phosphorylation promotes metastatic progression via increased neutrophil survival, and argue for its chemical inhibition in cancer patients using MNK inhibitors such as merestinib.

3.3 DISCUSSION

The experiments documented herein establish for the first time an important role for translational control in cells of the TME. Using an orthotopic model wherein 66cl4 mammary tumor cells are injected into syngeneic BALB/c mice, we demonstrate that eIF4E phosphorylation in the non-transformed cells of the host promotes metastatic lung colonization. We validate this finding in an unrelated orthotopic model using EO771 cells and syngeneic C57/BL6 mice. We show that eIF4E phosphorylation promotes neutrophil survival, thus favoring metastasis to the lung. Interestingly, we demonstrate that eIF4E phosphorylation in the TME of breast cancer patients correlates with poor outcome, though validation studies with larger sample sizes are critical to confirm this effect.

Our data clearly implicate neutrophils in the development of lung metastases from 66cl4 tumors. Emerging evidence suggests that neutrophils represent a phenotypically diverse cell population, with subsets possessing pro-metastatic versus anti-metastatic functions (*445, 447, 453-455*). In our model, pro-metastatic functions clearly dominate. Neutrophil depletion reduces lung colonization in WT mice, and fails to enhance colonization in eIF4E^{S209A} mice, which would be expected if anti-metastatic neutrophils were playing a major role. This contrasts the conclusions of Granot et al., who report that neutrophil depletion increases lung metastasis (*454*). However, most of the experiments in the latter publication use the related 4T1 cells, which elicit differential neutrophil responses. In one experiment, circulating neutrophil numbers in mice bearing 66cl4 tumors were much lower than in mice bearing 4T1 tumors, and in fact similar to those seen in naïve mice, in contrast to our results (*454*). This distinction might arise simply from a difference in the time of analysis: Granot et al. measure neutrophil levels 7 days after engraftment. At this time point, 66cl4 tumors are barely palpable,

circulating G-CSF levels are low and neutrophils have yet to accumulate in the circulation, whereas 4T1 tumors grow much faster and produce more G-CSF than 66cl4 tumors. In our experiments with 66cl4-derived tumors, neutrophils accumulate by day 14 and reach peak concentrations around $150*10^9$/L after 3-4 weeks (Figure 3.4). Thus, our results are consistent with previous findings showing that neutrophils favor the metastatic process (*445, 455*).

Our experiments did not identify any specific neutrophil functions affected by eIF4E phosphorylation, as none of those tested changed significantly between WT and S209A neutrophils. As these experiments were by no means exhaustive, there may be additional layers of phospho-eIF4E mediated regulation of neutrophils that we were unable to identify (*456*). It is also possible that metastatic cancer cells seeded in the lungs attract neutrophils. However, neutrophil infiltration to the lung occurs as early as 2 weeks following primary tumor injection, mirroring their accumulation in the blood; this is earlier than the appearance of macroscopic nodules (data not shown). In addition, neutrophil infiltration was limited in the primary tumors, but extensive in the blood, spleen and lungs (Fig. 3.3). It seems unlikely that such a tissue distribution would occur if cancer cells were facilitating neutrophil recruitment to the lungs, rather than the other way around. Our data therefore supports the notion that aberrant neutrophil accumulation is a cause, rather than an effect, of metastasis to the lungs.

Unexpectedly, our results demonstrate that apoptosis in neutrophils is regulated by the phosphorylation of eIF4E, while no such effect is seen in 66cl4 cells, MEFs or in primary tumors (Figure 3.5) (*55, 97*). In this regard, it is noteworthy that immature neutrophils express BCL2, whereas mature neutrophils depend primarily on MCL1 for survival, in contrast to other cell types that may express a battery of anti-apoptotic proteins (*457*). As they seem to rely on individual BCL2-family members at any given time, neutrophils may be particularly sensitive to translational regulation of BCL2 and MCL1. Several groups have reported the regulation of these factors by eIF4E levels and phosphorylation (*113, 448*). The impact of eIF4E phosphorylation on apoptosis is therefore highly context specific. This may explain why our previous studies using prostate and breast cancer models found no link between apoptosis and

eIF4E phosphorylation, in contrast to other groups studying models of lymphoma and melanoma (*113, 290*).

Several other cell types may be playing important roles that we did not identify in our mouse model. Possibilities include macrophages, in which eIF4E phosphorylation has been linked to cytokine production (*381, 458*), as well as regulatory T cells, where eIF4E levels have been reported to play important roles (*380*), and IFNγ production by Th1 cells, which has recently been linked to the p38-MNK-eIF4E axis (*383*). The case of T cells is interesting, as our data indicate that they are functionally suppressed by neutrophils in the WT setting (Figure S3.3A-C, 4B), but do not preclude a role for phospho-eIF4E in these cells when relieved from neutrophil suppression in the $eIF4E^{S209A}$ setting. In fact, the translational regulation of various cytokines by these cells reported in other contexts (*383, 458*), as well as data presented by the pharmaceutical company eFFECTOR regarding the effects of their own MNK inhibitors (*459*), support this notion. In contrast, work with T cells lacking MNKs has indicated that these kinases, and eIF4E phosphorylation, are dispensable for normal T cell function, but indirectly affect their differentiation and activity in a T cell extrinsic manner, using an *in vivo* model of autoimmune encephalomyelitis (*385, 458*), mirroring our own findings in the TME.

Merestinib had no effect on primary tumor growth in our model, despite inhibiting tumor growth in mouse models of non-small cell lung cancer, leukemia and glioblastoma (*365, 451, 452, 460*). As discussed above, this may be reflective of the importance of phospho-eIF4E sensitive anti-apoptotic factors in various cell types. However, it is also likely that these differences arise from the lack of specificity of merestinib. Indeed, this inhibitor targets an array of kinases including MET, for which it was originally developed (*452*). Any of these other targets may contribute to the effects of merestinib observed by us and others, and their relative expression and importance in these various models has not been compared. Interestingly, our *in vitro* assays demonstrated that merestinib inhibits eIF4E phosphorylation at doses much lower than those required to inhibit proliferation (Figure 3.6F-G). It is therefore likely that targets other than the MNKs mediate the anti-proliferative effects of merestinib, that their

inhibition in 66cl4 cells require higher doses of the drug, and that such high doses were not reached in our *in vivo* model. Whether anti-proliferative doses of merestinib can be achieved in patients will need to be determined. Of note, the similar effects obtained when using merestinib or a more specific MNK inhibitor were similar, arguing for primarily MNK-specific effects of merestinib in our model. Irrespective of these issues, we and others show that merestinib treatment has clear beneficial properties in cancer cells and in the TME, supporting its clinical development.

Possible clinical implications of our findings are noteworthy. Approximately 10% of cancer patients present with pathologically high levels of neutrophils (>20-50*10^9 cells/L of blood) (*461*). This can be caused by high levels of G-CSF expression from primary tumors and is linked to lung colonization and poor prognosis (*461*). However, the interplay between cancer and neutrophils is complex, as the main concern for most patients is neutropenia resulting from chemotherapy, where insufficient neutrophils lead to an inability to efficiently combat bacterial infections. These patients are generally treated with G-CSF to stimulate neutrophil production by the bone marrow (*461*). Thus, depleting neutrophils with monoclonal antibodies in the manner used in this and other publications is not practical in the clinic. However, inhibiting eIF4E phosphorylation, which prevents pathological accumulation of neutrophils in our mouse model, but not normal neutrophil homeostasis, may be beneficial to cancer patients. This is an especially attractive approach, as eIF4E phosphorylation appears to be dispensable for normal immune cell development (Figure S3.2). In addition, our previous findings indicate that eIF4E phosphorylation in cancer cells also promotes metastatic progression, further arguing for the development of MNK inhibitors for the prevention of metastasis (*97, 290*). However, as eIF4E phosphorylation has no effect on primary tumor growth (Figure 3.2A-B), such a phospho-eIF4E targeting therapy would most likely be useful in combination with other treatments.

Immunotherapeutic drugs, including inhibitors of CTLA4 and PD1/PDL1, are particularly promising candidates to combine with MNK inhibitors. These compounds disrupt signals that suppress T cell mobilization, proliferation and function, leading to an increase in activated

tumor reactive T cells. The introduction of checkpoint inhibitors has led to a major revolution in the treatment of some cancer types such as melanoma (*462*). We and others have shown that neutrophils can inhibit T cell proliferation and function (Figure S3.4) (*447*), raising the possibility that preventing aberrant neutrophil accumulation with MNK inhibitors may improve the efficacy of immunotherapy. This is especially interesting considering the requirement for eIF4E phosphorylation in both the cancer and stromal compartments for efficient metastasis. In this regard, NF1 mutant melanomas may be particularly sensitive: they are expected to be responsive to immunotherapy due to their high mutational load (*463*), and sensitive to the effects of MNK inhibition similarly to other NF1 mutant tumors (*464*). This is a striking contrast to the immunosuppressive effects of many chemotherapeutic regimens and some targeted therapies such as rapamycin. Research in this direction is therefore highly warranted, and is promisingly expected to enter clinical trials later this year.

3.4 MATERIALS AND METHODS

Experimental model and subject details

Human samples. Human breast cancer samples were collected from needle biopsies of primary tumors from female patients between 30 and 90 years of age (see Figure S3.1) undergoing treatment at the McGill University Health Centre (MUHC) between 1999 and 2004 who provided written, informed consent (MUHC REB protocols SDR-99-780). Information regarding clinical variables was obtained through review of medical records. Haematoxylin and eosin (H&E)-stained sections from each sample were evaluated by an attending clinical pathologist with expertise in breast tissue to identify areas of normal tissue, DCIS and invasive carcinoma. Sections from biopsy cores were arranged as a tissue microarray on a single microscope slide used for immunohistochemistry. The TMA included samples from 56 patients, 40 of which were used for analysis. Reasons for excluding 16 patient samples were absence, or insufficient amount, of material on slide (12 patients), or tissue originating from a recurrence rather than a primary tumor (4 patients).

Animal models. Immunocompetent WT BALB/c and eIF4E^{S209A} mice (*55*) were bred in house after 12 backcrosses. SCID mice were from Jackson Laboratories. All experiments

involving animals were conducted in accordance with McGill University (Montreal, QC, Canada) animal care guidelines. Mice were maintained at 22^{o}C with a continuous water supply, standard solid food and bedding, and a 12-hour light/dark cycle (light 6:30am-6:30pm). Treatment naïve were injected with 66cl4 cells between the ages of 8-12 weeks and weighed between 19 and 22 grams. Mice were examined twice weekly during tumor growth for caliper measurements and to monitor health and assess ethical endpoints (tumor size greater than 2cm^3, dehydration, hunched back, weight loss), or daily for experiments with drug treatments or immune cell depletion. Tumor volume was estimated at the indicated time points using the formula V=(4/3*(3.14159)*(Length/2)*(Width/2)^2) and caliper measurements of the longest and shortest diameters of the tumor. Age-matched WT and eIF4E^{S209A} mice were distributed in blocks of 2-3 mice of each genotype in cages of 4-5 mice. This blocking strategy was also used for merestinib treatments and neutrophil depletion. Experiments were repeated up to include the numbers of mice indicated in each figure legend (4-10 mice per group). Merestinib was provided by Eli Lilly and injected orally at 12mg/kg daily, formulated in 20% PEG 400/80% (20% Captisol in H_2O). For depletions, antibodies were injected intra-peritoneally with 50µL of anti-asialo-GM1 (eBioscience) for NK cells or 5.5mg/kg of anti-Ly6G (BioXCell) in saline for neutrophils. At endpoint, mice were anaesthetized with isoflurane, and then euthanized with CO_2 and cervical dislocation. For experiments requiring blood collection, heart punctures were performed following isoflurane anaesthesia, followed by cervical dislocation. Tissues were collected and processed immediately using 48-hour formalin fixation of tissues for immunohistochemistry. For experiments on neutrophil accumulation in circulation, 50µL of blood was collected weekly from the saphenous vein into EDTA coated capillary tubes.

Cell culture. 66cl4 cells were obtained from W.H.M. These cells were originally isolated from a primary mammary tumor from a female BALB/c mouse due to a Mouse Mammary Tumor Virus infection. They were subsequently selected for resistance to thioguanine and ouabain. 66cl4 cells were cultured in DMEM (Wisent) with 10% fetal bovine serum (FBS) (Wisent) and 1% penicillin/streptomycin (P/S) (Sigma), at 37^{o}C with 5% CO_2. Neutrophils were isolated from the blood of tumor bearing mice by diluting 200µL in 5mL of PBS, which was loaded onto a Histopaque gradient consisting of a dense fraction of 3mL Histopaque 1119 and a

light fraction of Histopaque 1077. Columns were centrifuged at 800g for 30 minutes at room temperature and neutrophils were collected from the interface between the two Histopaque solutions, diluted in 10mL PBS and pelleted by centrifugation for 5 minutes at 800g. Residual RBCs were lysed by resuspending pellet in 5mL ACK buffer and incubating 5 minutes at room temperature. Neutrophils were then washed in PBS and resuspended in RPMI with 2% FBS and 1% P/S for ex vivo apoptosis experiments, or in PBS with 2% FBS for flow cytometry. To obtain high density and low density neutrophils, cells from the Histopaque interface and between the Histopaque 1077 and PBS layers were collected and further purified by flow cytometry using a BD Aria Fusion sorter.

Method details

Immunohistochemistry. Formalin fixed and paraffin embedded samples from breast cancer patients and mouse organs were rehydrated by progressive washes for 5 minutes each in xylene (3x), 100% ethanol (2x), 70% ethanol, water. Antigens were retrieved using a citrate buffer pH 6 and heated for 15 minutes in a pressure cooker. Sections were washed in PBS, blocked in 2.5% normal horse serum for 10 minutes, followed by incubation with primary antibodies overnight. After PBS washes, endogenous peroxidases were quenched with 3% H_2O_2 in PBS for 5 minutes, followed by further PBS washes and incubation with HRP-conjugated ImmPRESS secondary antibodies. DAB was used as a peroxidase substrate for sample staining, Hematoxylin QS as a couterstain. All reagents except primary antibodies were from Vector Labs, primary antibodies used were anti-CD31 (Dianova), anti-phospho-eIF4E clone EP2151Y (Abcam), anti-ki67 (BD Pharmingen), anti-cleaved caspase 3 clone 5A1E (Cell Signaling), anti-Ly6G clone 1A8 (BD Pharmingen). In mouse lungs and tumors, percentages of cells positively stained with anti-Ly6G, ki67, CD31 or cleaved caspase 3 were determined using Aperio ImageScope software. Scoring of TMA for eIF4E phosphorylation in the epithelial cells and TME was performed independently by 2 blinded scorers. Phospho-eIF4E expression in cancer cells was categorized as “low” if undetectable or if expression was low and sparse; it was categorized as “high” if expression was uniformly detectable or if expression was sparse but very high. Phospho-eIF4E expression in the TME was categorized as “positive” if at least 5 cells outside the

tumor bed expressed phospho-eIF4E, otherwise it was categorized as “negative”. This number was chosen because it was small enough to allow analysis of sections with limited areas of stroma, but large enough to reduce the odds of a single randomly stained area affecting the analysis. The 2x2 scoring method was chosen to limit groups, due to the small sample number.

Histology. Primary tumor histology as analysed in H&E stained sections by a pathologist with experience in breast cancer. Lung metastases were counted in 5 H&E stained step sections with 50µm spacing, metastases occurring in the same position of sequential sections were only counted once. Metastases were considered intravascular if entirely enclosed by an endothelial layer. All analyses were performed blind.

Flow cytometery. For flow cytometric analysis, spleens were dissociated by manual crushing with a syringe plunger and cell strainer, bone marrow cells were washed out of mouse femurs with PBS using a 26-gauge needle after cutting off the extremities, tumors were dissociated by incubating minced pieces in collagenase B for 30 minutes at 37^{o}C. RBCs were lysed by incubating in ACK buffer for 5 minutes. The resulting cell populations were washed in PBS, and 10^6 cells were resuspended in 100µL PBS with 2% FBS and various combinations of the following antibodies from BioLegend: APC-Annexin V, PE-anti-Ly6G, PerCP/Cy5.5-anti-Ly6C, APC/Cy7-anti-CD11b, AF488-anti-Sca1, PE-anti-cKit, AF700-anti-mouse lineage cocktail.
Multiplex ELISA. Circulating cytokines were measured in the serum using Bio-Plex Pro Mouse Cytokine 23-plex Assay #M60009RDPD (Bio-Rad) following the manufacturer’s instructions. Serum was diluted 1:4 in sample diluent, and standards were prepared as recommended. Samples and standards were incubated in a 96 well plate with magnetic antibody-conjugated beads for 30 minutes at room temperature, followed by incubation for 30 minutes with the detection antibody and incubation with streptavidin-PE for 10 minutes at room temperature. Wash steps were performed between all incubations. Finally, samples were resuspended in Assay buffer and inserted into a Luminex 200 plate reader system. Cytokines below the detection limit of the assay were removed from further analysis.

Cytokine withdrawal. Neutrophils isolated as described above were incubated for 24 hours in the presence or absence of 25ng/mL recombinant G-CSF (Peprotech) and 100nM merestinib at a density of 10^6 cells per well in a 12-well culture dish with 1mL culture medium.

They were then collected by centrifugation at 800g for 5 min, washed in Annexin V staining buffer and resuspended in 100µL Annexin V staining buffer containing Annexin V and anti-Ly6G-PE for flow cytometric analysis.

Proliferation. To monitor cell proliferation in response to merestinib, 10^6 66cl4 cells were plated in each well of 6 well plates. Individual plates were prepared for each of the 4 days included in the experiment, and the experiment was performed in triplicate. In all plates, every day following initial plating, media was replaced with fresh media containing vehicle (DMSO) or merestinib at the indicated concentrations. Every 24 hours of treatment, cells were trypsinized and counted in a set of 3 plates by trypan blue exclusion.

Western blots. For merestinib treatments of 66cl4 cells, $5*10^6$ cells were plated in each well of a 6-well plate, the following day, they were incubated with the indicated concentrations of merestinib for 4 hours and lysed in RIPA buffer. Protein concentration was determined using Bio-Rad Protein Assay and 20µg of protein was loaded on a 10% SDS-PA gel, then transferred to a nitrocellulose membrane for immunoblotting. For neutrophils, their intrinsic protease activity prevents quantification of protein content, as samples are significantly degraded in that time. Therefore, neutrophils were counted to obtain equal neutrophil, were flash frozen in liquid nitrogen and lysed directly in 1X Laemmli buffer for protein separation by SDS-PAGE. Antibodies used were anti-MCL1 (Cell Signaling), anti-BCL2 (Cell signaling), anti-eIF4E (BD Biosciences), anti-phospho-eIF4E clone EP2151Y (Abcam), anti-β-actin clone AC15 (Sigma), anti-ERK (Santa Cruz), anti-phospho-ERK (Cell Signaling), anti-4E-BP1 (Cell Signaling).

Quantification and statistical analysis

All statistical analysis was performed using GraphPad Prism software. All graphs, except those presenting fractions of entire populations such as Kaplan-Meier curves, are shown as means +/- SEM. Significance was defined using the standard p=0.05 cut-off. P-values, and the values and meanings of *n* can be found in the figures and legends. Statistical tests used are outlined below.

Unpaired t-test. For comparisons of only two groups without paired samples, the unpaired t-test was used, as was the case for Figure 3.6C-E, where there was no indication that populations deviated significantly from a normal distribution or had widely different variations.

2-way ANOVA. Several experiments were performed where a variety of measurements were taken in two groups, including figures 3.3B, 4ABD, 5ABD and 6F; these were analyzed by two-way ANOVA. Some p-values are indicated for pair-wise comparisons of interest and were computed using Sidak's multiple comparison test.

Repeated measures ANOVA. For experiments monitoring tumor growth and neutrophil accumulation in circulation, several measurements at different time points were performed on the same animals; thus, repeated measures ANOVA was used in figures 3.2B, 3D, 4C and 6B.

Fisher's exact test. For experiments in which data could be categorized as a 2X2 contingency table, Fisher's exact test was used. This is the case in the following figures: Figure 3.1B, where all samples contained either normal epithelial cells or neoplastic epithelial cells that expressed either high or low levels of eIF4E phosphorylation; Figure 3.1C, where all samples contained either normal epithelial cells or neoplastic epithelial cells, and were either positive or negative for eIF4E phosphorylation in cells of the surrounding stroma; Figure 3.2E, where metastases were either intra-vascular or extra-vascular and present in either WT or $eFI4E^{S209A}$ mice; Figure 3.5C, where neutrophils where either segmented or apoptotic and present in either WT or $eFI4E^{S209A}$ mice.

3.5 ACKNOWLEDGEMENTS

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CHAPTER 4

GENERAL DISCUSSION

4.1 How does eIF4E phosphorylation affect translation?

In the current thesis, the importance of eIF4E phosphorylation in cancer cells and neutrophils is established. However, it remains unclear how eIF4E phosphorylation affects the translation of specific mRNAs at the molecular level. Many models have been proposed, one of which has been introduced briefly in Chapter 1; these are discussed in greater detail here, in light of the data presented in Chapters 2 and 3.

4.1.1 Switch to alternate initiation mechanisms

The phosphorylation of eIF4E has been proposed to inhibit cap-dependent translation while promoting cap-independent translation via IRESs (*465*). This hypothesis is supported by the finding that eIF4E phosphorylation reduces its affinity for the cap, thus purportedly favoring cap-independent translation (*59-61*). Such a mechanism would be particularly useful in stress conditions that inhibit mTOR but activate p38 MAPK signaling, such as hypoxia (*466*). In line with this, eIF4G upregulation has been shown to promote IRES-mediated translation of *Vegfa* when 4E-BPs are hypophosphorylated in response to hypoxia (*191*). Furthermore, MNK activity promotes cap-independent translation by the *Myc* IRES in multiple myeloma cells treated with rapamycin (*465*). Similarly, MNKs promote the translation of oncolytic poliovirus mRNAs containing IRES sequences (*467*), although similar effects have also been attributed by the same group to other targets of the MNKs (*468*).

Unfortunately, this is a recurring theme in studies on the effects of eIF4E phosphorylation: as its modulation is most easily achieved by MNK inhibition or knockdown, most studies rely exclusively on these methods, and attribute the reported effects to the best characterized substrate, eIF4E. However, the design of these studies cannot distinguish between MNK targets including eIF4E (*469*), Sprouty2 (*393*), cPLA2 (*394*) and hnRNPA1 (*395, 396*). As a result, several effects ascribed to eIF4E phosphorylation have subsequently been attributed to other effectors, including translational regulation of TNFα and the afore-mentioned regulation of cap-independent translation in virus-infected cells (*396, 468*).

In contrast, the work presented here unambiguously attributes pro-metastatic functions to translational control via eIF4E phosphorylation. While we did not specifically investigate IRES-mediated translation, our results fail to uncover any evidence of increased cap-independent translation. Thus, polysome profiling of S209A MEFs did not detect any change in the translation of the *Myc* mRNA, or other commonly reported IRES-bearing mRNAs such as *Vegfa* or *Bcl2* (*55*). Of note, these experiments were performed in optimal growth conditions where cap-dependent translation is predominant, whereas previous reports were performed in the context of mTOR inhibition, perhaps explaining the discrepancy. While decreased expression of BCL2 in S209A neutrophils was detected in Chapter 3, we cannot attribute this effect to impaired IRES-mediated translation, as it could result from the blockade of either cap-dependent or -independent translation. Thus, while a phospho-eIF4E dependent switch to IRES-mediated translation is not required to explain our results, it cannot be wholly excluded.

Phosphorylation of eIF4E has also been reported to control the identity of the major cap-binding protein by promoting the translation of the *Eif4e* mRNA, with MNK inhibition resulting in the translation of the alternative cap-binding protein eIF4E3 (*470*). Thus, phospho-eIF4E was proposed to promote oncogenic cap-dependent translation, in opposition to the supposed tumor-suppressive program of eIF4E3-mediated cap-dependent translation (*471*). While eIF4E3 levels were not assessed in any of our experiments, eIF4E levels were routinely monitored in cells lacking MNKs, in cells bearing the S209A mutation, and in response to MNK inhibition; as a result, we are confident that eIF4E levels are not affected by its phosphorylation in any cells tested, including neutrophils, breast and prostate cancer cell lines, B16 melanoma cells, primary MEFs, transformed MEFs, and mouse mammary tumors. We cannot, however, exclude that such an effect exists specifically in DLBCL.

4.1.2 Modulating protein-protein interactions

One possible mechanism by which phosphorylation of eIF4E may affect translation is by altering its affinity for certain interacting proteins. This has been reported as the mechanism underlying increased migration and invasion downstream of eIF4E phosphorylation. Thus, eIF4E phosphorylation reduces its affinity for the repressor of translation cytoplasmic fragile-X protein-interacting protein 1 (CYFIP1), resulting in increased availability for cap-dependent translation (*422*). In contrast, phospho-eIF4E has been proposed to increase its affinity for the eIF4E transporter 4E-T, leading to its recruitment to mRNP granules, in response to a variety of stressors (*233*). As both of these reports were published subsequent to our own work presented in Chapter 2, neither mechanism was investigated in the experiments presented therein. Nonetheless, it is unclear how phosphorylation of eIF4E would affect its affinity for its known interacting proteins, as S209 is present on a flexible loop near the cap-binding channel, opposite the canonical binding site for eIF4G, the 4E-BPs, CYFIP1 and 4E-T. While secondary binding sites nearer S209 have been reported for eIF4G and the 4E-BPs (*50, 51*), the effect of phosphorylation on this region remains unclear. Indeed, there is as of yet no structural information on the phosphorylated form of eIF4E.

In contrast, eIF4E-binding partners may influence eIF4E phosphorylation by the MNKs. Indeed, MNK1 must be recruited by eIF4G to phosphorylate eIF4E (*54*). Thus, binding of any other interactor would prevent MNK1 recruitment and eIF4E phosphorylation, which has been demonstrated in the case of 4E-BPs binding to eIF4E as a result of mTOR inhibition (*472*). However, it remains to be established whether other eIF4E interacting proteins can recruit MNKs in certain conditions. It is also unclear whether any intermediates are required for phosphorylation of eIF4E by MNK2; while MNK2 can bind eIF4G, studies establishing the requirement for eIF4G have focused on MNK1 (*54, 410, 473*). MNK2 also displays higher basal activity and is relatively independent of activation from upstream signaling (*410, 474*). There is as-of-yet unpublished data supporting the hypothesis of eIF4G-independent phosphorylation of eIF4E by MNK2 (Graff JR, personal communication). As a result, there may be unsuspected mechanisms of eIF4E phosphorylation that are independent of eIF4G

and its proposed role in promoting initiation factor recycling after 43S PIC recruitment. If correct, this may partially explain why some phenotypes associated with eIF4E phosphorylation appear to be MNK1 dependent while others appear to be MNK1 dependent, as observed in studies distinguishing between individual eIF4E kinases (for example: (*97, 298, 392, 474*)).

One interesting possibility that has yet to be investigated is that eIF4E phosphorylation may play a role in substituting cap-binding proteins in mRNP granules. Indeed, phospho-eIF4E has been reported to play a role in the stress response and to be elevated in P-bodies and/or stress granules (*233, 475*). In parallel, it has been suggested that eIF4E may be displaced from the cap in conditions of miRNA-mediated silencing in favor of the alternative cap-binding protein 4EHP (*476*). While there is currently no data to support this idea, it may be worth speculating that phosphorylation of eIF4E could reduce its affinity for the cap, thus promoting its replacement by 4EHP. Of course, this depends on the possibility that eIF4E can be phosphorylated independently of the eIF4F complex for processes other than translation initiation. Testing this hypothesis should be straightforward, for example by repeating the experiments contained in reference (*476*), but in cells and lysates lacking the MNKs or with recombinant eIF4E bearing the S209A mutation.

4.1.3 Promoting initiation factor recycling after 43S PIC recruitment

As described in section 1.4.3, one model proposes that eIF4E phosphorylation decreases its affinity for the cap and occurs after scanning has begun to promote initiation factor recycling (*63*). Thus, phosphorylation of eIF4E is hypothesized to have similar effects to increased eIF4E availability or expression, an idea supported by the fact that all three of these contexts increase the translation of mRNAs containing stable hairpins in their 5'UTRs (*13, 64*). While not explicitly tested, several reports support this model. When ribosomes and their interacting proteins are separated from other cytoplasmic proteins in rabbit reticulocyte lysates by centrifugation or chromatography, the fraction of eIF4E bound to ribosomes is phosphorylated at higher proportion (~60%) than "free" eIF4E (~30%) (*477*). Furthermore,

the majority of eIF4E is unbound. These findings support the idea that phosphorylation occurs following ribosome recruitment, and results in eIF4E release, consistent with early reports that eIF4E binds to 48S initiation complexes but not 80S ribosomes (*478*). Conceptually, this would allow for eIF4E to participate in the formation of new 48S complexes during AUG recognition and 60S subunit joining, a particularly slow process, as shown by the accumulation of 40S and 80S ribosomes at start codons (*479*).

In addition, dephosphorylation of eIF4E must be relatively rapid to prevent accumulation of phosphorylated eIF4E in the cytoplasm. While phosphatase activity is generally assumed to be faster than that of kinases, the identity of the eIF4E phosphatase remains uncertain; it thus remains to be demonstrated whether the kinetics of its dephosphorylation are sufficient to support the proposed model of increased initiation factor recycling. Encouragingly, several phosphatases in the MAPK pathway display high catalytic efficiencies, in the range of 10^6 $M^{-1}s^{-1}$, 1000-fold greater than that of their respective kinases (*480*). While eIF4E dephosphorylation has not been thoroughly investigated, protein phosphatase 2A (PP2A) has been reported to be responsible for dephosphorylation of both MNK1 and eIF4E (*481*). However, it has also been reported to dephosphorylate 4E-BP1, thus, eIF4E may be a direct PP2A target or be indirectly regulated by controlling eIF4F complex formation and MNK activity (*482*). Interestingly, the B56 regulatory subunit of PP2A has been identified as a possible interactor with eIF4G (*483*). If correct, this finding would implicate the presence of an eIF4E phosphatase in close proximity with its substrate, allowing for rapid and dynamic phosphorylation and dephosphorylation, such as would be required for the initiation factor recycling model.

Although our results cannot answer this issue, they highlight important differences in the kinetics of phosphorylation and dephosphorylation of signaling intermediates upstream of eIF4E. Thus, MAPK signaling increases by 3 hours of TGFβ stimulation and returns to baseline levels by 14 hours; in contrast MNK1 and eIF4E phosphorylation remain elevated for at least 24 hours. This suggests that the phosphatases responsible for the dephosphorylation of

MNKs are distinct from those responsible for upstream kinases. For the MNKs, the phosphatase likely displays only a low level of expression and/or activity, as its phosphorylation remains high despite reduced activity of its upstream kinase. The activity of the eIF4E phosphatase likely remains constant, as eIF4E phosphorylation remains at a constant, increased level, mirroring that of its activated kinase. We believe that the phosphorylation of eIF4E is unlikely to simply achieve saturation caused by hyperactive MNKs, as even their most potent activators result in only about 75% of eIF4E present in the phosphorylated form (*484*).

Of note, the finding that TGFβ stimulation results in the translational activation of mRNAs transcriptionally induced by TGFβ highlights another potential role of eIF4E phosphorylation: recruitment to new mRNAs. Indeed, it is possible that, in unstimulated cells, eIF4E is mostly bound to mRNAs required to maintain their homeostasis. Upon stimulation, eIF4E must be redirected to new mRNAs needing to be translated to respond to the stimulus. Phosphorylation may be a means to release eIF4E from mRNAs previously undergoing translation to promote the translation of newer ones. The basis for this idea was proposed as part of the initiation recycling hypothesis, and has yet to be thoroughly tested (*63*).

In fact, all aspects of how eIF4E phosphorylation affects translation may need to be revisited. While there have been many studies on this topic, the design and conclusions of the experiments may have been biased by unrealistic expectations skewed by an unfortunate historical error. Indeed, the phosphorylation site of eIF4E was initially misidentified as serine 53 (*485*). Eventually, the correct site was identified by the same group and independently confirmed (*486, 487*), but in the meantime, various studies using an S53A mutant had concluded that eIF4E phosphorylation was an absolute requirement for translation initiation (*98, 488-491*). As a result, subsequent studies on S209 were characterized by statements that its phosphorylation was “not required” for a variety of processes, including functional replacement of yeast eIF4E, the reconstitution of eIF4E-depleted rabbit reticulocyte lysates, cell growth, organismal development, or *de novo* protein synthesis following hypertonic

stress (*474, 492, 493*). However, revisiting the data presented in these reports tells a different story. First, it is not surprising that phosphorylated S209 plays no role in *Saccharomyces cerevisiae*, as yeast do not possess an ortholog of MNK1 or residues equivalent to S209 in the eIF4E ortholog. In contrast, flies, which can phosphorylate deIF4E via their MNK1 ortholog, display growth defects in response to amino acid deficiency (*494*). Second, while the data presented in (*493*) shows that reconstitution of eIF4E-depleted rabbit reticulocyte lysate with high levels of eIF4E S209A is equivalent to reconstitution with WT eIF4E, it is clearly less effective at intermediate levels. Similar observations can be made regarding *de novo* protein synthesis, as small molecule MNK inhibition does not entirely prevent polysome formation following hypertonic stress, but clearly reduces its efficiency (*492*). Thus, it may be useful to further investigate the requirement of eIF4E phosphorylation for the initiation of translation by performing reconstitution experiments with increasing amounts of eIF4E mutants, or by testing de novo protein synthesis following various stresses in cells lacking MNKs or expressing eIF4E S209A. A role for eIF4E phosphorylation in directing initiation factors to newly synthesized mRNAs could also be tested by controlling the sequential addition of *in vitro* synthesized reporter mRNAs in rabbit reticulocyte lysates reconstituted with eIF4E mutants.

Clearly, more work is needed to dissect the molecular effects of eIF4E phosphorylation, its impact on translation, and potentially other cellular processes. Curiously, many of the competing hypotheses described above may contain some truth, as they are not mutually exclusive. Changes in affinity for the cap and binding partners may occur simultaneously, as can changes in recycling and subcellular localization. There may even be different contexts during which eIF4E phosphorylation may be dynamic and promote recycling, or continuously elevated and reduce cap binding and cap-dependent translation.

4.2 Revisiting the pro-metastatic effect of eIF4E phosphorylation in cancer cells

The experiments presented herein demonstrate that translational control plays an important role in the metastatic process, both in cancer cells and in the cells of the TME. Specifically,

we demonstrate that eIF4E phosphorylation promotes the translation of mRNAs in different cell types that promotes their pro-metastatic functions. In neutrophils, this favors their survival in response to G-CSF by promoting the translation of mRNAs encoding the anti-apoptotic factors BCL2 and MCL1. In cancer cells, invasion is favored by promoting the translation of mRNAs encoding SNAIL and MMP3, two factors that promote EMT. Furthermore, we validate some of these findings *in vivo*, by demonstrating that neutrophil survival in response to eIF4E phosphorylation leads to aberrant accumulation and favors lung colonization by cancer cells in an orthotopic model. We also used a genetic model that confirms that tumors expressing phospho-eIF4E are more metastatic and express more SNAIL and MMP3. However, the conclusions drawn from the genetic experiments in Chapter 2 should be tempered by our findings in Chapter 3. Indeed, in the MMTV-PyMT model, the S209A mutation is present in all cell types of the eIF4E^{S209A} mice, including neutrophils. Thus, we cannot exclude that the observed difference in metastatic potential between WT and S209A mice is due to differential phospho-eIF4E-dependent neutrophil survival, rather than phospho-eIF4E-dependent cancer cell invasion. Considering that neutrophils have been described to play an important role in the metastatic dissemination of MMTV-PyMT tumors (*495*), our inability to separate the contributions of the cancer cells from those of the neutrophils is an important caveat.

In an attempt to address this issue, we have devised several plans to use orthotopic models in which we can individually control eIF4E phosphorylation in the tumor and the host. Unfortunately, these have so far been hampered by significant technical limitations. In one case, WT and eIF4E^{S209A} tumors were isolated, dissociated and reinjected into naïve recipient mice of both genetic backgrounds. We tested the four possible combinations of phospho-eIF4E in the cancer cells only, the TME only, both compartments or neither, in an effort to dissect the relative contribution of each. While some of our other findings were validated, notably that eIF4E phosphorylation in cancer cells, but not the TME, promotes the onset of mammary tumors, none of the mice developed metastatic lung colonies, preventing any further analysis. This was surprising, considering the high metastatic burden observed in

almost all mice in the genetic MMTV-PyMT model. The reduced lung colonization is likely due to a reduced tumor load in the orthotopic model, where mice bear a single primary tumor, as opposed to 10 or more in the genetic model. Alternatively, we endeavored to abrogate eIF4E phosphorylation in 66cl4 cells to compare their metastatic potential with the parental line. First, we used shRNAs targeting MNK1/2, with the objective of isolating clones with stable knockdown of these kinases. However, MNK expression invariably was recovered within one week, preventing their use *in vivo*, where tumors form over 4-5 weeks. We are currently circumventing this problem by using CRISPR-CAS9 gene editing to knock out the MNKs, and are awaiting the identification of successfully mutated clones. While experiments using these cells will be crucial in confirming that eIF4E phosphorylation in cancer cells plays a role in metastasis *in vivo*, our previously obtained *in vitro* data indicating that phospho-eIF4E promotes EMT and invasion remains valid.

4.3 Other inhibitors of translation in immune space

In this thesis, emphasis was entirely placed on the phosphorylation of eIF4E as a model for translational control. However, many other regulatory events in protein synthesis may be important in the metastatic process. In fact, considering the relative innocuousness of the eIF4E^{S209A} mutation, other targets may provide even greater benefits. Thus, the Pelletier lab has demonstrated that eIF4F complex formation is critical in cancer cell dissemination, and that its pharmacological targeting using inhibitors of mTOR or eIF4A efficiently prevents metastasis (*237*). However, nothing is known regarding the effect of eIF4A inhibition in neutrophils or any other immune cell population, although it is potently cytotoxic in Jurkat cells and various lymphoma and leukemia models (*496, 497*).

More is known regarding how mTOR inhibitors affect the tumor microenvironment, as they are well documented to be immunosuppressive. Thus, mTOR inhibitors prevent T cell growth and proliferation, while promoting Treg development, resulting in impaired adaptive immunity (*373, 498*). Rapamycin, in particular, has been used as a clinical immunosuppressive for decades. Interestingly, one model demonstrates the importance of

mTOR in both cancer cells and TME: renal transplant recipients that develop Kaposi's sarcoma. Indeed, these tumors can appear in patients receiving cyclosporin to prevent graft rejection. Interestingly, substituting rapamycin for cyclosporin maintains immunosuppression by inhibiting mTOR in T cells, but also leads to tumor regression by inhibiting mTOR in cancer cells (*499, 500*). Other reports indicate that mTOR regulates the accumulation of myeloid-derived suppressor cells (MDSC) in transplant and allograft recipients, although it is unclear whether this is due to effects intrinsic to MDSCs, or whether this is secondary to effects in other cell types (*377-379*).

Another targetable branch of translation is the eIF2α pathway, with small molecules being developed that can increase or reduce eIF2α phosphorylation, or inhibit TC recruitment to the PIC. These compounds show promise in treating leukemias and other cancer types by inhibiting translation initiation and causing apoptosis (*102, 138-140, 314, 338*), but their effects on non-transformed immune cell types remains to be determined. One study exceptionally investigated the effect of PERK inhibition on immune cells, in particular in the brain microenvironment, and found that normal immune function remained intact, while ER-stress induced inflammation was reduced (*501*). Another study has reported that guanabenz-induced increases in eIF2α phosphorylation inhibits the expression of GM-CSF, IL6 and COX2, which may be beneficial (*502*). The effect of such therapeutic strategies on both the tumor and its microenvironment remains to be investigated. Of note, eIF2α phosphorylation was shown to play an important role in separating T helper cell priming from activity by preventing the translation of mRNAs encoding inflammatory cytokines at the priming site and in circulation; their translation at the target site occurred following a second T cell activation event, resulting in eIF2α dephosphorylation (*503*). It is thus possible that targeting the eIF2 pathway in patients would affect T cell mobilization in the cancer setting, but how and to what extent remains to be seen.

The importance of translation in T cells is especially relevant to the cancer setting, as these are considered the major cell type in controlling tumor progression, and are the target of

immunotherapeutic drugs currently providing significant benefits to cancer patients that had previously lacked useful therapeutic options. Interestingly, the expression of initiation factors increases in activated T cells over that of quiescent T cells to support the increased protein synthesis associated with their proliferation (*504, 505*). In contrast, eIF4E levels and mTOR activity decrease in T regulatory cells (Tregs), and the resulting reduction in translation is required for their differentiation (*380, 498*). Considering these findings, great care must be taken when utilizing inhibitors of translation to determine their effects on the immune system. For example, if an inhibitor of mTOR causes apoptosis in cancer cells and prevents the accumulation of MDSCs, but simultaneously reduces $CD8^+$ T cell proliferation and activity while increasing Treg differentiation, the net outcome is difficult to predict. It may, in fact, be undesirable if the balance favors immunosuppression over cancer cell killing. Biomarkers should be identified that can predict which effect will be dominant to determine which patients would benefit most from mTOR inhibitors, for example, levels of circulating MDSCs, presence and activity of infiltrating T cells, etc. Alternatively, targets with beneficial effects in both cancer cells and the immune system could be prioritized. Such is the case for the phosphorylation of eIF4E, as presented here, but other, yet-to-be-identified targets likely exist.

Conclusion and perspectives

The work presented in this thesis highlights the importance of translation in cancer. First, it identifies the phosphorylation of eIF4E as a key event promoting the translation of pro-metastatic mRNAs in cancer cells. Second, it expands the importance of eIF4E phosphorylation to not only cancer cells, but also non-transformed cells of the tumor microenvironment, by demonstrating its role in promoting the survival of neutrophils in response to G-CSF. Overall, the findings described herein underscore a need to study the effects of inhibitors of translation currently being developed on both cancer cells and the TME.

As we ponder the sometimes overwhelming complexity of translational deregulation in cancer, we are left wondering which aspects will prove most vulnerable and amenable to therapeutic intervention. eIF2A certainly appears to be a promising target, though the knowledge of its potential is too recent for drug discovery to be in anything but its infancy. Certainly, there is much interest in the development of eIF4A inhibitors, as well as several other translation initiation factors. Beyond the potency of such drugs on cancer cells, several questions will need to be addressed. What will their effects be on other cell types of the TME? What are the possibilities for cancer cells to develop resistance to such inhibitors? While downstream effectors of oncogenic kinases can easily be identified, overexpressed, constitutively activated or phosphorylated by alternative pathways, it is unclear what equivalent process could circumvent the inhibition of translation. Can feedback inhibition limit the efficacy of targeting translation, as seen with kinase inhibitors? While several positive feedback loops have been described, most notably the interplay between MYC and the translational machinery, examples of negative feedback, while existent, have been largely ignored by the field. Certainly, it is an exciting time to study the translational control of cancer.

References

1. Ruggero, D. (2012) Revisiting the nucleolus: from marker to dynamic integrator of cancer signaling, *Science signaling 5*, pe38.
2. Thomas, G. (2000) An encore for ribosome biogenesis in the control of cell proliferation, *Nature cell biology 2*, E71-72.
3. Gentilella, A., Kozma, S. C., and Thomas, G. (2015) A liaison between mTOR signaling, ribosome biogenesis and cancer, *Biochimica et biophysica acta 1849*, 812-820.
4. Derenzini, M., Montanaro, L., and Trere, D. (2017) Ribosome biogenesis and cancer, *Acta histochemica 119*, 190-197.
5. Buttgereit, F., and Brand, M. D. (1995) A hierarchy of ATP-consuming processes in mammalian cells, *The Biochemical journal 312 (Pt 1)*, 163-167.
6. Rolfe, D. F., and Brown, G. C. (1997) Cellular energy utilization and molecular origin of standard metabolic rate in mammals, *Physiological reviews 77*, 731-758.
7. Schwanhausser, B., Busse, D., Li, N., Dittmar, G., Schuchhardt, J., Wolf, J., Chen, W., and Selbach, M. (2011) Global quantification of mammalian gene expression control, *Nature 473*, 337-342.
8. Vogel, C., and Marcotte, E. M. (2012) Insights into the regulation of protein abundance from proteomic and transcriptomic analyses, *Nature reviews. Genetics 13*, 227-232.
9. Aviner, R., Shenoy, A., Elroy-Stein, O., and Geiger, T. (2015) Uncovering Hidden Layers of Cell Cycle Regulation through Integrative Multi-omic Analysis, *PLoS genetics 11*, e1005554.
10. Edfors, F., Danielsson, F., Hallstrom, B. M., Kall, L., Lundberg, E., Ponten, F., Forsstrom, B., and Uhlen, M. (2016) Gene-specific correlation of RNA and protein levels in human cells and tissues, *Molecular systems biology 12*, 883.
11. Li, J. J., Bickel, P. J., and Biggin, M. D. (2014) System wide analyses have underestimated protein abundances and the importance of transcription in mammals, *PeerJ 2*, e270.
12. Cheng, Z., Teo, G., Krueger, S., Rock, T. M., Koh, H. W., Choi, H., and Vogel, C. (2016) Differential dynamics of the mammalian mRNA and protein expression response to misfolding stress, *Molecular systems biology 12*, 855.
13. Hinnebusch, A. G., Ivanov, I. P., and Sonenberg, N. (2016) Translational control by 5'-untranslated regions of eukaryotic mRNAs, *Science 352*, 1413-1416.
14. Sonenberg, N., and Hinnebusch, A. G. (2009) Regulation of translation initiation in eukaryotes: mechanisms and biological targets, *Cell 136*, 731-745.
15. Jackson, R. J., Hellen, C. U., and Pestova, T. V. (2010) The mechanism of eukaryotic translation initiation and principles of its regulation, *Nature reviews. Molecular cell biology 11*, 113-127.
16. Hinnebusch, A. G., and Lorsch, J. R. (2012) The mechanism of eukaryotic translation initiation: new insights and challenges, *Cold Spring Harbor perspectives in biology 4*.
17. Benne, R., and Hershey, J. W. (1978) The mechanism of action of protein synthesis initiation factors from rabbit reticulocytes, *The Journal of biological chemistry 253*, 3078-3087.
18. Safer, B., Anderson, W. F., and Merrick, W. C. (1975) Purification and physical properties of homogeneous initiation factor MP from rabbit reticulocytes, *The Journal of biological chemistry 250*, 9067-9075.
19. Gingras, A. C., Raught, B., and Sonenberg, N. (1999) eIF4 initiation factors: effectors of mRNA recruitment to ribosomes and regulators of translation, *Annual review of biochemistry 68*, 913-963.
20. Grifo, J. A., Tahara, S. M., Morgan, M. A., Shatkin, A. J., and Merrick, W. C. (1983) New initiation factor activity required for globin mRNA translation, *The Journal of biological chemistry 258*, 5804-5810.

21. Lamphear, B. J., and Panniers, R. (1991) Heat shock impairs the interaction of cap-binding protein complex with 5' mRNA cap, *The Journal of biological chemistry 266*, 2789-2794.
22. Kahvejian, A., Svitkin, Y. V., Sukarieh, R., M'Boutchou, M. N., and Sonenberg, N. (2005) Mammalian poly(A)-binding protein is a eukaryotic translation initiation factor, which acts via multiple mechanisms, *Genes & development 19*, 104-113.
23. Hinnebusch, A. G. (2014) The Scanning Mechanism of Eukaryotic Translation Initiation, *Annual review of biochemistry*.
24. Hershey, J. W. (2015) The role of eIF3 and its individual subunits in cancer, *Biochimica et biophysica acta 1849*, 792-800.
25. Mathews, M. B., and Hershey, J. W. (2015) The translation factor eIF5A and human cancer, *Biochimica et biophysica acta 1849*, 836-844.
26. Brina, D., Miluzio, A., Ricciardi, S., and Biffo, S. (2015) eIF6 anti-association activity is required for ribosome biogenesis, translational control and tumor progression, *Biochimica et biophysica acta 1849*, 830-835.
27. Sonenberg, N., Morgan, M. A., Merrick, W. C., and Shatkin, A. J. (1978) A polypeptide in eukaryotic initiation factors that crosslinks specifically to the 5'-terminal cap in mRNA, *Proceedings of the National Academy of Sciences of the United States of America 75*, 4843-4847.
28. Topisirovic, I., Svitkin, Y. V., Sonenberg, N., and Shatkin, A. J. (2011) Cap and cap-binding proteins in the control of gene expression, *Wiley interdisciplinary reviews. RNA 2*, 277-298.
29. Marcotrigiano, J., Gingras, A. C., Sonenberg, N., and Burley, S. K. (1997) Cocrystal structure of the messenger RNA 5' cap-binding protein (eIF4E) bound to 7-methyl-GDP, *Cell 89*, 951-961.
30. Tomoo, K., Matsushita, Y., Fujisaki, H., Abiko, F., Shen, X., Taniguchi, T., Miyagawa, H., Kitamura, K., Miura, K., and Ishida, T. (2005) Structural basis for mRNA Cap-Binding regulation of eukaryotic initiation factor 4E by 4E-binding protein, studied by spectroscopic, X-ray crystal structural, and molecular dynamics simulation methods, *Biochimica et biophysica acta 1753*, 191-208.
31. Matsuo, H., Li, H., McGuire, A. M., Fletcher, C. M., Gingras, A. C., Sonenberg, N., and Wagner, G. (1997) Structure of translation factor eIF4E bound to m7GDP and interaction with 4E-binding protein, *Nature structural biology 4*, 717-724.
32. Niedzwiecka, A., Marcotrigiano, J., Stepinski, J., Jankowska-Anyszka, M., Wyslouch-Cieszynska, A., Dadlez, M., Gingras, A. C., Mak, P., Darzynkiewicz, E., Sonenberg, N., Burley, S. K., and Stolarski, R. (2002) Biophysical studies of eIF4E cap-binding protein: recognition of mRNA 5' cap structure and synthetic fragments of eIF4G and 4E-BP1 proteins, *Journal of molecular biology 319*, 615-635.
33. Hu, G., Gershon, P. D., Hodel, A. E., and Quiocho, F. A. (1999) mRNA cap recognition: dominant role of enhanced stacking interactions between methylated bases and protein aromatic side chains, *Proceedings of the National Academy of Sciences of the United States of America 96*, 7149-7154.
34. Etchison, D., Milburn, S. C., Edery, I., Sonenberg, N., and Hershey, J. W. (1982) Inhibition of HeLa cell protein synthesis following poliovirus infection correlates with the proteolysis of a 220,000-dalton polypeptide associated with eucaryotic initiation factor 3 and a cap binding protein complex, *The Journal of biological chemistry 257*, 14806-14810.
35. Prevot, D., Darlix, J. L., and Ohlmann, T. (2003) Conducting the initiation of protein synthesis: the role of eIF4G, *Biology of the cell / under the auspices of the European Cell Biology Organization 95*, 141-156.
36. Gradi, A., Imataka, H., Svitkin, Y. V., Rom, E., Raught, B., Morino, S., and Sonenberg, N. (1998) A novel functional human eukaryotic translation initiation factor 4G, *Molecular and cellular biology 18*, 334-342.
37. Marcotrigiano, J., Gingras, A. C., Sonenberg, N., and Burley, S. K. (1999) Cap-dependent translation initiation in eukaryotes is regulated by a molecular mimic of eIF4G, *Molecular cell 3*, 707-716.

38. Yanagiya, A., Svitkin, Y. V., Shibata, S., Mikami, S., Imataka, H., and Sonenberg, N. (2009) Requirement of RNA binding of mammalian eukaryotic translation initiation factor 4GI (eIF4GI) for efficient interaction of eIF4E with the mRNA cap, *Molecular and cellular biology 29*, 1661-1669.
39. Slepenkov, S. V., Korneeva, N. L., and Rhoads, R. E. (2008) Kinetic mechanism for assembly of the m7GpppG.eIF4E.eIF4G complex, *The Journal of biological chemistry 283*, 25227-25237.
40. Pause, A., Belsham, G. J., Gingras, A. C., Donze, O., Lin, T. A., Lawrence, J. C., Jr., and Sonenberg, N. (1994) Insulin-dependent stimulation of protein synthesis by phosphorylation of a regulator of 5'-cap function, *Nature 371*, 762-767.
41. Tsukiyama-Kohara, K., Vidal, S. M., Gingras, A. C., Glover, T. W., Hanash, S. M., Heng, H., and Sonenberg, N. (1996) Tissue distribution, genomic structure, and chromosome mapping of mouse and human eukaryotic initiation factor 4E-binding proteins 1 and 2, *Genomics 38*, 353-363.
42. Lin, T. A., and Lawrence, J. C., Jr. (1996) Control of the translational regulators PHAS-I and PHAS-II by insulin and cAMP in 3T3-L1 adipocytes, *The Journal of biological chemistry 271*, 30199-30204.
43. Rousseau, D., Gingras, A. C., Pause, A., and Sonenberg, N. (1996) The eIF4E-binding proteins 1 and 2 are negative regulators of cell growth, *Oncogene 13*, 2415-2420.
44. Gingras, A. C., Raught, B., Gygi, S. P., Niedzwiecka, A., Miron, M., Burley, S. K., Polakiewicz, R. D., Wyslouch-Cieszynska, A., Aebersold, R., and Sonenberg, N. (2001) Hierarchical phosphorylation of the translation inhibitor 4E-BP1, *Genes & development 15*, 2852-2864.
45. Haghighat, A., Mader, S., Pause, A., and Sonenberg, N. (1995) Repression of cap-dependent translation by 4E-binding protein 1: competition with p220 for binding to eukaryotic initiation factor-4E, *The EMBO journal 14*, 5701-5709.
46. Mader, S., Lee, H., Pause, A., and Sonenberg, N. (1995) The translation initiation factor eIF-4E binds to a common motif shared by the translation factor eIF-4 gamma and the translational repressors 4E-binding proteins, *Molecular and cellular biology 15*, 4990-4997.
47. Gosselin, P., Oulhen, N., Jam, M., Ronzca, J., Cormier, P., Czjzek, M., and Cosson, B. (2011) The translational repressor 4E-BP called to order by eIF4E: new structural insights by SAXS, *Nucleic acids research 39*, 3496-3503.
48. Fletcher, C. M., and Wagner, G. (1998) The interaction of eIF4E with 4E-BP1 is an induced fit to a completely disordered protein, *Protein science : a publication of the Protein Society 7*, 1639-1642.
49. Lukhele, S., Bah, A., Lin, H., Sonenberg, N., and Forman-Kay, J. D. (2013) Interaction of the eukaryotic initiation factor 4E with 4E-BP2 at a dynamic bipartite interface, *Structure 21*, 2186-2196.
50. Umenaga, Y., Paku, K. S., In, Y., Ishida, T., and Tomoo, K. (2011) Identification and function of the second eIF4E-binding region in N-terminal domain of eIF4G: comparison with eIF4E-binding protein, *Biochemical and biophysical research communications 414*, 462-467.
51. Mizuno, A., In, Y., Fujita, Y., Abiko, F., Miyagawa, H., Kitamura, K., Tomoo, K., and Ishida, T. (2008) Importance of C-terminal flexible region of 4E-binding protein in binding with eukaryotic initiation factor 4E, *FEBS letters 582*, 3439-3444.
52. Buxade, M., Parra-Palau, J. L., and Proud, C. G. (2008) The Mnks: MAP kinase-interacting kinases (MAP kinase signal-integrating kinases), *Frontiers in bioscience : a journal and virtual library 13*, 5359-5373.
53. Waskiewicz, A. J., Johnson, J. C., Penn, B., Mahalingam, M., Kimball, S. R., and Cooper, J. A. (1999) Phosphorylation of the cap-binding protein eukaryotic translation initiation factor 4E by protein kinase Mnk1 in vivo, *Molecular and cellular biology 19*, 1871-1880.
54. Pyronnet, S., Imataka, H., Gingras, A. C., Fukunaga, R., Hunter, T., and Sonenberg, N. (1999) Human eukaryotic translation initiation factor 4G (eIF4G) recruits mnk1 to phosphorylate eIF4E, *The EMBO journal 18*, 270-279.

55. Furic, L., Rong, L., Larsson, O., Koumakpayi, I. H., Yoshida, K., Brueschke, A., Petroulakis, E., Robichaud, N., Pollak, M., Gaboury, L. A., Pandolfi, P. P., Saad, F., and Sonenberg, N. (2010) eIF4E phosphorylation promotes tumorigenesis and is associated with prostate cancer progression, *Proceedings of the National Academy of Sciences of the United States of America 107*, 14134-14139.
56. Ueda, T., Sasaki, M., Elia, A. J., Chio, II, Hamada, K., Fukunaga, R., and Mak, T. W. (2010) Combined deficiency for MAP kinase-interacting kinase 1 and 2 (Mnk1 and Mnk2) delays tumor development, *Proceedings of the National Academy of Sciences of the United States of America 107*, 13984-13990.
57. Shibata, S., Morino, S., Tomoo, K., In, Y., and Ishida, T. (1998) Effect of mRNA cap structure on eIF-4E phosphorylation and cap binding analyses using Ser209-mutated eIF-4Es, *Biochemical and biophysical research communications 247*, 213-216.
58. Tomoo, K., Shen, X., Okabe, K., Nozoe, Y., Fukuhara, S., Morino, S., Ishida, T., Taniguchi, T., Hasegawa, H., Terashima, A., Sasaki, M., Katsuya, Y., Kitamura, K., Miyoshi, H., Ishikawa, M., and Miura, K. (2002) Crystal structures of 7-methylguanosine 5'-triphosphate (m(7)GTP)- and P(1)-7-methylguanosine-P(3)-adenosine-5',5'-triphosphate (m(7)GpppA)-bound human full-length eukaryotic initiation factor 4E: biological importance of the C-terminal flexible region, *The Biochemical journal 362*, 539-544.
59. Scheper, G. C., van Kollenburg, B., Hu, J., Luo, Y., Goss, D. J., and Proud, C. G. (2002) Phosphorylation of eukaryotic initiation factor 4E markedly reduces its affinity for capped mRNA, *The Journal of biological chemistry 277*, 3303-3309.
60. Slepenkov, S. V., Darzynkiewicz, E., and Rhoads, R. E. (2006) Stopped-flow kinetic analysis of eIF4E and phosphorylated eIF4E binding to cap analogs and capped oligoribonucleotides: evidence for a one-step binding mechanism, *The Journal of biological chemistry 281*, 14927-14938.
61. Zuberek, J., Wyslouch-Cieszynska, A., Niedzwiecka, A., Dadlez, M., Stepinski, J., Augustyniak, W., Gingras, A. C., Zhang, Z., Burley, S. K., Sonenberg, N., Stolarski, R., and Darzynkiewicz, E. (2003) Phosphorylation of eIF4E attenuates its interaction with mRNA 5' cap analogs by electrostatic repulsion: intein-mediated protein ligation strategy to obtain phosphorylated protein, *RNA 9*, 52-61.
62. Walsh, D., and Mohr, I. (2014) Coupling 40S ribosome recruitment to modification of a cap-binding initiation factor by eIF3 subunit e, *Genes & development 28*, 835-840.
63. Scheper, G. C., and Proud, C. G. (2002) Does phosphorylation of the cap-binding protein eIF4E play a role in translation initiation?, *European journal of biochemistry / FEBS 269*, 5350-5359.
64. Korneeva, N. L., Song, A., Gram, H., Edens, M. A., and Rhoads, R. E. (2016) Inhibition of Mitogen-activated Protein Kinase (MAPK)-interacting Kinase (MNK) Preferentially Affects Translation of mRNAs Containing Both a 5'-Terminal Cap and Hairpin, *The Journal of biological chemistry 291*, 3455-3467.
65. Lodish, H. F. (1974) Model for the regulation of mRNA translation applied to haemoglobin synthesis, *Nature 251*, 385-388.
66. Sonenberg, N., Guertin, D., Cleveland, D., and Trachsel, H. (1981) Probing the function of the eucaryotic 5' cap structure by using a monoclonal antibody directed against cap-binding proteins, *Cell 27*, 563-572.
67. Elfakess, R., Sinvani, H., Haimov, O., Svitkin, Y., Sonenberg, N., and Dikstein, R. (2011) Unique translation initiation of mRNAs-containing TISU element, *Nucleic acids research 39*, 7598-7609.
68. Komar, A. A., and Hatzoglou, M. (2011) Cellular IRES-mediated translation: the war of ITAFs in pathophysiological states, *Cell Cycle 10*, 229-240.

69. Chaudhury, A., Hussey, G. S., Ray, P. S., Jin, G., Fox, P. L., and Howe, P. H. (2010) TGF-beta-mediated phosphorylation of hnRNP E1 induces EMT via transcript-selective translational induction of Dab2 and ILEI, *Nature cell biology 12*, 286-293.
70. Jonas, S., and Izaurralde, E. (2015) Towards a molecular understanding of microRNA-mediated gene silencing, *Nature reviews. Genetics 16*, 421-433.
71. Truitt, M. L., and Ruggero, D. (2016) New frontiers in translational control of the cancer genome, *Nature reviews. Cancer 16*, 288-304.
72. Wurth, L., and Gebauer, F. (2015) RNA-binding proteins, multifaceted translational regulators in cancer, *Biochimica et biophysica acta 1849*, 881-886.
73. Meyuhas, O., and Kahan, T. (2015) The race to decipher the top secrets of TOP mRNAs, *Biochimica et biophysica acta 1849*, 801-811.
74. Jefferies, H. B., Reinhard, C., Kozma, S. C., and Thomas, G. (1994) Rapamycin selectively represses translation of the "polypyrimidine tract" mRNA family, *Proceedings of the National Academy of Sciences of the United States of America 91*, 4441-4445.
75. Thoreen, C. C., Chantranupong, L., Keys, H. R., Wang, T., Gray, N. S., and Sabatini, D. M. (2012) A unifying model for mTORC1-mediated regulation of mRNA translation, *Nature 485*, 109-113.
76. Hsieh, A. C., Liu, Y., Edlind, M. P., Ingolia, N. T., Janes, M. R., Sher, A., Shi, E. Y., Stumpf, C. R., Christensen, C., Bonham, M. J., Wang, S., Ren, P., Martin, M., Jessen, K., Feldman, M. E., Weissman, J. S., Shokat, K. M., Rommel, C., and Ruggero, D. (2012) The translational landscape of mTOR signalling steers cancer initiation and metastasis, *Nature 485*, 55-61.
77. Tang, H., Hornstein, E., Stolovich, M., Levy, G., Livingstone, M., Templeton, D., Avruch, J., and Meyuhas, O. (2001) Amino acid-induced translation of TOP mRNAs is fully dependent on phosphatidylinositol 3-kinase-mediated signaling, is partially inhibited by rapamycin, and is independent of S6K1 and rpS6 phosphorylation, *Molecular and cellular biology 21*, 8671-8683.
78. Miloslavski, R., Cohen, E., Avraham, A., Iluz, Y., Hayouka, Z., Kasir, J., Mudhasani, R., Jones, S. N., Cybulski, N., Ruegg, M. A., Larsson, O., Gandin, V., Rajakumar, A., Topisirovic, I., and Meyuhas, O. (2014) Oxygen sufficiency controls TOP mRNA translation via the TSC-Rheb-mTOR pathway in a 4E-BP-independent manner, *Journal of molecular cell biology 6*, 255-266.
79. Pellizzoni, L., Cardinali, B., Lin-Marq, N., Mercanti, D., and Pierandrei-Amaldi, P. (1996) A Xenopus laevis homologue of the La autoantigen binds the pyrimidine tract of the 5' UTR of ribosomal protein mRNAs in vitro: implication of a protein factor in complex formation, *Journal of molecular biology 259*, 904-915.
80. Markert, A., Grimm, M., Martinez, J., Wiesner, J., Meyerhans, A., Meyuhas, O., Sickmann, A., and Fischer, U. (2008) The La-related protein LARP7 is a component of the 7SK ribonucleoprotein and affects transcription of cellular and viral polymerase II genes, *EMBO reports 9*, 569-575.
81. Huichalaf, C., Schoser, B., Schneider-Gold, C., Jin, B., Sarkar, P., and Timchenko, L. (2009) Reduction of the rate of protein translation in patients with myotonic dystrophy 2, *The Journal of neuroscience : the official journal of the Society for Neuroscience 29*, 9042-9049.
82. Damgaard, C. K., and Lykke-Andersen, J. (2011) Translational coregulation of 5'TOP mRNAs by TIA-1 and TIAR, *Genes & development 25*, 2057-2068.
83. Tcherkezian, J., Cargnello, M., Romeo, Y., Huttlin, E. L., Lavoie, G., Gygi, S. P., and Roux, P. P. (2014) Proteomic analysis of cap-dependent translation identifies LARP1 as a key regulator of 5'TOP mRNA translation, *Genes & development 28*, 357-371.
84. Orom, U. A., Nielsen, F. C., and Lund, A. H. (2008) MicroRNA-10a binds the 5'UTR of ribosomal protein mRNAs and enhances their translation, *Molecular cell 30*, 460-471.
85. Aoki, K., Adachi, S., Homoto, M., Kusano, H., Koike, K., and Natsume, T. (2013) LARP1 specifically recognizes the 3' terminus of poly(A) mRNA, *FEBS letters 587*, 2173-2178.

86. Fonseca, B. D., Zakaria, C., Jia, J. J., Graber, T. E., Svitkin, Y., Tahmasebi, S., Healy, D., Hoang, H. D., Jensen, J. M., Diao, I. T., Lussier, A., Dajadian, C., Padmanabhan, N., Wang, W., Matta-Camacho, E., Hearnden, J., Smith, E. M., Tsukumo, Y., Yanagiya, A., Morita, M., Petroulakis, E., Gonzalez, J. L., Hernandez, G., Alain, T., and Damgaard, C. K. (2015) La-related Protein 1 (LARP1) Represses Terminal Oligopyrimidine (TOP) mRNA Translation Downstream of mTOR Complex 1 (mTORC1), *The Journal of biological chemistry 290*, 15996-16020.
87. Lahr, R. M., Fonseca, B. D., Ciotti, G. E., Al-Ashtal, H. A., Jia, J. J., Niklaus, M. R., Blagden, S. P., Alain, T., and Berman, A. J. (2017) La-related protein 1 (LARP1) binds the mRNA cap, blocking eIF4F assembly on TOP mRNAs, *eLife 6*.
88. Gentilella, A., Moron-Duran, F. D., Fuentes, P., Zweig-Rocha, G., Riano-Canalias, F., Pelletier, J., Ruiz, M., Turon, G., Castano, J., Tauler, A., Bueno, C., Menendez, P., Kozma, S. C., and Thomas, G. (2017) Autogenous Control of 5'TOP mRNA Stability by 40S Ribosomes, *Molecular cell*.
89. Landers, J. E., Cassel, S. L., and George, D. L. (1997) Translational enhancement of mdm2 oncogene expression in human tumor cells containing a stabilized wild-type p53 protein, *Cancer research 57*, 3562-3568.
90. Mayr, C. (2016) Evolution and Biological Roles of Alternative 3'UTRs, *Trends in cell biology 26*, 227-237.
91. Mayr, C., and Bartel, D. P. (2009) Widespread shortening of 3'UTRs by alternative cleavage and polyadenylation activates oncogenes in cancer cells, *Cell 138*, 673-684.
92. Faller, W. J., Jackson, T. J., Knight, J. R., Ridgway, R. A., Jamieson, T., Karim, S. A., Jones, C., Radulescu, S., Huels, D. J., Myant, K. B., Dudek, K. M., Casey, H. A., Scopelliti, A., Cordero, J. B., Vidal, M., Pende, M., Ryazanov, A. G., Sonenberg, N., Meyuhas, O., Hall, M. N., Bushell, M., Willis, A. E., and Sansom, O. J. (2015) mTORC1-mediated translational elongation limits intestinal tumour initiation and growth, *Nature 517*, 497-500.
93. Dorrello, N. V., Peschiaroli, A., Guardavaccaro, D., Colburn, N. H., Sherman, N. E., and Pagano, M. (2006) S6K1- and betaTRCP-mediated degradation of PDCD4 promotes protein translation and cell growth, *Science 314*, 467-471.
94. Raught, B., Peiretti, F., Gingras, A. C., Livingstone, M., Shahbazian, D., Mayeur, G. L., Polakiewicz, R. D., Sonenberg, N., and Hershey, J. W. (2004) Phosphorylation of eucaryotic translation initiation factor 4B Ser422 is modulated by S6 kinases, *The EMBO journal 23*, 1761-1769.
95. Robichaud, N., and Sonenberg, N. (2014) eIF4E and Its Binding Proteins, In *Translation and Its Regulation in Cancer Biology and Medicine* (Parsyan, A., Ed.), pp 73-113, Springer Netherlands, Dordrecht.
96. Kandoth, C., McLellan, M. D., Vandin, F., Ye, K., Niu, B., Lu, C., Xie, M., Zhang, Q., McMichael, J. F., Wyczalkowski, M. A., Leiserson, M. D., Miller, C. A., Welch, J. S., Walter, M. J., Wendl, M. C., Ley, T. J., Wilson, R. K., Raphael, B. J., and Ding, L. (2013) Mutational landscape and significance across 12 major cancer types, *Nature 502*, 333-339.
97. Robichaud, N., del Rincon, S. V., Huor, B., Alain, T., Petruccelli, L. A., Hearnden, J., Goncalves, C., Grotegut, S., Spruck, C. H., Furic, L., Larsson, O., Muller, W. J., Miller, W. H., and Sonenberg, N. (2015) Phosphorylation of eIF4E promotes EMT and metastasis via translational control of SNAIL and MMP-3, *Oncogene 34*, 2032-2042.
98. Lazaris-Karatzas, A., Montine, K. S., and Sonenberg, N. (1990) Malignant transformation by a eukaryotic initiation factor subunit that binds to mRNA 5' cap, *Nature 345*, 544-547.
99. Ruggero, D., Montanaro, L., Ma, L., Xu, W., Londei, P., Cordon-Cardo, C., and Pandolfi, P. P. (2004) The translation factor eIF-4E promotes tumor formation and cooperates with c-Myc in lymphomagenesis, *Nature medicine 10*, 484-486.
100. Ruggero, D. (2013) Translational control in cancer etiology, *Cold Spring Harbor perspectives in biology 5*.

101. Silvera, D., Formenti, S. C., and Schneider, R. J. (2010) Translational control in cancer, *Nature reviews. Cancer 10*, 254-266.
102. Bhat, M., Robichaud, N., Hulea, L., Sonenberg, N., Pelletier, J., and Topisirovic, I. (2015) Targeting the translation machinery in cancer, *Nature reviews. Drug discovery 14*, 261-278.
103. TheCancerGenomeAtlasNetwork. (2012) Comprehensive genomic characterization of squamous cell lung cancers, *Nature 489*, 519-525.
104. Duncan, R., Milburn, S. C., and Hershey, J. W. (1987) Regulated phosphorylation and low abundance of HeLa cell initiation factor eIF-4F suggest a role in translational control. Heat shock effects on eIF-4F, *The Journal of biological chemistry 262*, 380-388.
105. Gandin, V., Masvidal, L., Hulea, L., Gravel, S. P., Cargnello, M., McLaughlan, S., Cai, Y., Balanathan, P., Morita, M., Rajakumar, A., Furic, L., Pollak, M., Porco, J. A., Jr., St-Pierre, J., Pelletier, J., Larsson, O., and Topisirovic, I. (2016) nanoCAGE reveals 5' UTR features that define specific modes of translation of functionally related MTOR-sensitive mRNAs, *Genome research 26*, 636-648.
106. Chu, J., and Pelletier, J. (2015) Targeting the eIF4A RNA helicase as an anti-neoplastic approach, *Biochimica et biophysica acta 1849*, 781-791.
107. Fukuchi-Shimogori, T., Ishii, I., Kashiwagi, K., Mashiba, H., Ekimoto, H., and Igarashi, K. (1997) Malignant transformation by overproduction of translation initiation factor eIF4G, *Cancer research 57*, 5041-5044.
108. Silvera, D., Arju, R., Darvishian, F., Levine, P. H., Zolfaghari, L., Goldberg, J., Hochman, T., Formenti, S. C., and Schneider, R. J. (2009) Essential role for eIF4GI overexpression in the pathogenesis of inflammatory breast cancer, *Nature cell biology 11*, 903-908.
109. Truitt, M. L., Conn, C. S., Shi, Z., Pang, X., Tokuyasu, T., Coady, A. M., Seo, Y., Barna, M., and Ruggero, D. (2015) Differential Requirements for eIF4E Dose in Normal Development and Cancer, *Cell 162*, 59-71.
110. Hariri, F., Arguello, M., Volpon, L., Culjkovic-Kraljacic, B., Nielsen, T. H., Hiscott, J., Mann, K. K., and Borden, K. L. (2013) The eukaryotic translation initiation factor eIF4E is a direct transcriptional target of NF-kappaB and is aberrantly regulated in acute myeloid leukemia, *Leukemia 27*, 2047-2055.
111. Topisirovic, I., Siddiqui, N., Orolicki, S., Skrabanek, L. A., Tremblay, M., Hoang, T., and Borden, K. L. (2009) Stability of eukaryotic translation initiation factor 4E mRNA is regulated by HuR, and this activity is dysregulated in cancer, *Molecular and cellular biology 29*, 1152-1162.
112. Jiang, C. C., Croft, A., Tseng, H. Y., Guo, S. T., Jin, L., Hersey, P., and Zhang, X. D. (2014) Repression of microRNA-768-3p by MEK/ERK signalling contributes to enhanced mRNA translation in human melanoma, *Oncogene 33*, 2577-2588.
113. Wendel, H. G., Silva, R. L., Malina, A., Mills, J. R., Zhu, H., Ueda, T., Watanabe-Fukunaga, R., Fukunaga, R., Teruya-Feldstein, J., Pelletier, J., and Lowe, S. W. (2007) Dissecting eIF4E action in tumorigenesis, *Genes & development 21*, 3232-3237.
114. Topisirovic, I., Ruiz-Gutierrez, M., and Borden, K. L. (2004) Phosphorylation of the eukaryotic translation initiation factor eIF4E contributes to its transformation and mRNA transport activities, *Cancer research 64*, 8639-8642.
115. Fan, S., Ramalingam, S. S., Kauh, J., Xu, Z., Khuri, F. R., and Sun, S. Y. (2009) Phosphorylated eukaryotic translation initiation factor 4 (eIF4E) is elevated in human cancer tissues, *Cancer biology & therapy 8*, 1463-1469.
116. Graff, J. R., Konicek, B. W., Lynch, R. L., Dumstorf, C. A., Dowless, M. S., McNulty, A. M., Parsons, S. H., Brail, L. H., Colligan, B. M., Koop, J. W., Hurst, B. M., Deddens, J. A., Neubauer, B. L., Stancato, L. F., Carter, H. W., Douglass, L. E., and Carter, J. H. (2009) eIF4E activation is commonly elevated in advanced human prostate cancers and significantly related to reduced patient survival, *Cancer research 69*, 3866-3873.

117. Ramon y Cajal, S., De Mattos-Arruda, L., Sonenberg, N., Cortes, J., and Peg, V. (2014) The intra-tumor heterogeneity of cell signaling factors in breast cancer: p4E-BP1 and peIF4E are diffusely expressed and are real potential targets, *Clinical & translational oncology : official publication of the Federation of Spanish Oncology Societies and of the National Cancer Institute of Mexico*.
118. Astanehe, A., Finkbeiner, M. R., Krzywinski, M., Fotovati, A., Dhillon, J., Berquin, I. M., Mills, G. B., Marra, M. A., and Dunn, S. E. (2012) MKNK1 is a YB-1 target gene responsible for imparting trastuzumab resistance and can be blocked by RSK inhibition, *Oncogene 31*, 4434-4446.
119. Yang, H. S., Jansen, A. P., Komar, A. A., Zheng, X., Merrick, W. C., Costes, S., Lockett, S. J., Sonenberg, N., and Colburn, N. H. (2003) The transformation suppressor Pdcd4 is a novel eukaryotic translation initiation factor 4A binding protein that inhibits translation, *Molecular and cellular biology 23*, 26-37.
120. Yang, H. S., Cho, M. H., Zakowicz, H., Hegamyer, G., Sonenberg, N., and Colburn, N. H. (2004) A novel function of the MA-3 domains in transformation and translation suppressor Pdcd4 is essential for its binding to eukaryotic translation initiation factor 4A, *Molecular and cellular biology 24*, 3894-3906.
121. Meric-Bernstam, F., Chen, H., Akcakanat, A., Do, K. A., Lluch, A., Hennessy, B. T., Hortobagyi, G. N., Mills, G. B., and Gonzalez-Angulo, A. M. (2012) Aberrations in translational regulation are associated with poor prognosis in hormone receptor-positive breast cancer, *Breast cancer research : BCR 14*, R138.
122. Modelska, A., Turro, E., Russell, R., Beaton, J., Sbarrato, T., Spriggs, K., Miller, J., Graf, S., Provenzano, E., Blows, F., Pharoah, P., Caldas, C., and Le Quesne, J. (2015) The malignant phenotype in breast cancer is driven by eIF4A1-mediated changes in the translational landscape, *Cell death & disease 6*, e1603.
123. Wang, X., Wei, Z., Gao, F., Zhang, X., Zhou, C., Zhu, F., Wang, Q., Gao, Q., Ma, C., Sun, W., Kong, B., and Zhang, L. (2008) Expression and prognostic significance of PDCD4 in human epithelial ovarian carcinoma, *Anticancer research 28*, 2991-2996.
124. Yang, H. S., Jansen, A. P., Nair, R., Shibahara, K., Verma, A. K., Cmarik, J. L., and Colburn, N. H. (2001) A novel transformation suppressor, Pdcd4, inhibits AP-1 transactivation but not NF-kappaB or ODC transactivation, *Oncogene 20*, 669-676.
125. Guo, X., Li, W., Wang, Q., and Yang, H. S. (2011) AKT Activation by Pdcd4 Knockdown Up-Regulates Cyclin D1 Expression and Promotes Cell Proliferation, *Genes & cancer 2*, 818-828.
126. Yang, H. S., Matthews, C. P., Clair, T., Wang, Q., Baker, A. R., Li, C. C., Tan, T. H., and Colburn, N. H. (2006) Tumorigenesis suppressor Pdcd4 down-regulates mitogen-activated protein kinase kinase kinase kinase 1 expression to suppress colon carcinoma cell invasion, *Molecular and cellular biology 26*, 1297-1306.
127. Wang, Q., Zhang, Y., and Yang, H. S. (2012) Pdcd4 knockdown up-regulates MAP4K1 expression and activation of AP-1 dependent transcription through c-Myc, *Biochimica et biophysica acta 1823*, 1807-1814.
128. Goodarzi, H., Liu, X., Nguyen, H. C., Zhang, S., Fish, L., and Tavazoie, S. F. (2015) Endogenous tRNA-Derived Fragments Suppress Breast Cancer Progression via YBX1 Displacement, *Cell 161*, 790-802.
129. Kremer, C. L., Klein, R. R., Mendelson, J., Browne, W., Samadzedeh, L. K., Vanpatten, K., Highstrom, L., Pestano, G. A., and Nagle, R. B. (2006) Expression of mTOR signaling pathway markers in prostate cancer progression, *The Prostate 66*, 1203-1212.
130. Shang, Z. F., Yu, L., Li, B., Tu, W. Z., Wang, Y., Liu, X. D., Guan, H., Huang, B., Rang, W. Q., and Zhou, P. K. (2012) 4E-BP1 participates in maintaining spindle integrity and genomic stability via interacting with PLK1, *Cell Cycle 11*, 3463-3471.
131. Koromilas, A. E. (2015) Roles of the translation initiation factor eIF2alpha serine 51 phosphorylation in cancer formation and treatment, *Biochimica et biophysica acta 1849*, 871-880.

132. Robichaud, N., and Sonenberg, N. (2017) Translational control and the cancer cell response to stress, *Current opinion in cell biology 45*, 102-109.
133. Wang, S., Rosenwald, I. B., Hutzler, M. J., Pihan, G. A., Savas, L., Chen, J. J., and Woda, B. A. (1999) Expression of the eukaryotic translation initiation factors 4E and 2alpha in non-Hodgkin's lymphomas, *The American journal of pathology 155*, 247-255.
134. Rosenwald, I. B., Hutzler, M. J., Wang, S., Savas, L., and Fraire, A. E. (2001) Expression of eukaryotic translation initiation factors 4E and 2alpha is increased frequently in bronchioloalveolar but not in squamous cell carcinomas of the lung, *Cancer 92*, 2164-2171.
135. Rosenwald, I. B., Wang, S., Savas, L., Woda, B., and Pullman, J. (2003) Expression of translation initiation factor eIF-2alpha is increased in benign and malignant melanocytic and colonic epithelial neoplasms, *Cancer 98*, 1080-1088.
136. Kim, S. H., Gunnery, S., Choe, J. K., and Mathews, M. B. (2002) Neoplastic progression in melanoma and colon cancer is associated with increased expression and activity of the interferon-inducible protein kinase, PKR, *Oncogene 21*, 8741-8748.
137. Ye, J., Kumanova, M., Hart, L. S., Sloane, K., Zhang, H., De Panis, D. N., Bobrovnikova-Marjon, E., Diehl, J. A., Ron, D., and Koumenis, C. (2010) The GCN2-ATF4 pathway is critical for tumour cell survival and proliferation in response to nutrient deprivation, *The EMBO journal 29*, 2082-2096.
138. Denoyelle, S., Chen, T., Chen, L., Wang, Y., Klosi, E., Halperin, J. A., Aktas, B. H., and Chorev, M. (2012) In vitro inhibition of translation initiation by N,N'-diarylureas--potential anti-cancer agents, *Bioorganic & medicinal chemistry letters 22*, 402-409.
139. Schewe, D. M., and Aguirre-Ghiso, J. A. (2009) Inhibition of eIF2alpha dephosphorylation maximizes bortezomib efficiency and eliminates quiescent multiple myeloma cells surviving proteasome inhibitor therapy, *Cancer research 69*, 1545-1552.
140. Hamamura, K., Minami, K., Tanjung, N., Wan, Q., Koizumi, M., Matsuura, N., Na, S., and Yokota, H. (2014) Attenuation of malignant phenotypes of breast cancer cells through eIF2alpha-mediated downregulation of Rac1 signaling, *International journal of oncology 44*, 1980-1988.
141. Lacerda, R., Menezes, J., and Romao, L. (2017) More than just scanning: the importance of cap-independent mRNA translation initiation for cellular stress response and cancer, *Cellular and molecular life sciences : CMLS 74*, 1659-1680.
142. Zhou, J., Wan, J., Gao, X., Zhang, X., Jaffrey, S. R., and Qian, S. B. (2015) Dynamic m(6)A mRNA methylation directs translational control of heat shock response, *Nature 526*, 591-594.
143. Meyer, K. D., Patil, D. P., Zhou, J., Zinoviev, A., Skabkin, M. A., Elemento, O., Pestova, T. V., Qian, S. B., and Jaffrey, S. R. (2015) 5' UTR m(6)A Promotes Cap-Independent Translation, *Cell 163*, 999-1010.
144. Singh, C. R., Lee, B., Udagawa, T., Mohammad-Qureshi, S. S., Yamamoto, Y., Pavitt, G. D., and Asano, K. (2006) An eIF5/eIF2 complex antagonizes guanine nucleotide exchange by eIF2B during translation initiation, *The EMBO journal 25*, 4537-4546.
145. Singh, C. R., Watanabe, R., Zhou, D., Jennings, M. D., Fukao, A., Lee, B., Ikeda, Y., Chiorini, J. A., Campbell, S. G., Ashe, M. P., Fujiwara, T., Wek, R. C., Pavitt, G. D., and Asano, K. (2011) Mechanisms of translational regulation by a human eIF5-mimic protein, *Nucleic acids research 39*, 8314-8328.
146. Kozel, C., Thompson, B., Hustak, S., Moore, C., Nakashima, A., Singh, C. R., Reid, M., Cox, C., Papadopoulos, E., Luna, R. E., Anderson, A., Tagami, H., Hiraishi, H., Slone, E. A., Yoshino, K. I., Asano, M., Gillaspie, S., Nietfeld, J., Perchellet, J. P., Rothenburg, S., Masai, H., Wagner, G., Beeser, A., Kikkawa, U., Fleming, S. D., and Asano, K. (2016) Overexpression of eIF5 or its protein mimic 5MP perturbs eIF2 function and induces ATF4 translation through delayed re-initiation, *Nucleic acids research 44*, 8704-8713.

147. Li, S., Chai, Z., Li, Y., Liu, D., Bai, Z., and Situ, Z. (2009) BZW1, a novel proliferation regulator that promotes growth of salivary muocepodermoid carcinoma, *Cancer letters 284*, 86-94.
148. MacDonald, J. I., Verdi, J. M., and Meakin, S. O. (1999) Activity-dependent interaction of the intracellular domain of rat trkA with intermediate filament proteins, the beta-6 proteasomal subunit, Ras-GRF1, and the p162 subunit of eIF3, *Journal of molecular neuroscience : MN 13*, 141-158.
149. Lin, L., Holbro, T., Alonso, G., Gerosa, D., and Burger, M. M. (2001) Molecular interaction between human tumor marker protein p150, the largest subunit of eIF3, and intermediate filament protein K7, *Journal of cellular biochemistry 80*, 483-490.
150. Lee, J. Y., Kim, H. J., Rho, S. B., and Lee, S. H. (2016) eIF3f reduces tumor growth by directly interrupting clusterin with anti-apoptotic property in cancer cells, *Oncotarget 7*, 18541-18557.
151. Wang, Y. W., Lin, K. T., Chen, S. C., Gu, D. L., Chen, C. F., Tu, P. H., and Jou, Y. S. (2013) Overexpressed-eIF3I interacted and activated oncogenic Akt1 is a theranostic target in human hepatocellular carcinoma, *Hepatology 58*, 239-250.
152. Lee, A. S., Kranzusch, P. J., and Cate, J. H. (2015) eIF3 targets cell-proliferation messenger RNAs for translational activation or repression, *Nature 522*, 111-114.
153. Lee, A. S., Kranzusch, P. J., Doudna, J. A., and Cate, J. H. (2016) eIF3d is an mRNA cap-binding protein that is required for specialized translation initiation, *Nature 536*, 96-99.
154. Zoll, W. L., Horton, L. E., Komar, A. A., Hensold, J. O., and Merrick, W. C. (2002) Characterization of mammalian eIF2A and identification of the yeast homolog, *The Journal of biological chemistry 277*, 37079-37087.
155. Kwon, O. S., An, S., Kim, E., Yu, J., Hong, K. Y., Lee, J. S., and Jang, S. K. (2017) An mRNA-specific tRNAi carrier eIF2A plays a pivotal role in cell proliferation under stress conditions: stress-resistant translation of c-Src mRNA is mediated by eIF2A, *Nucleic acids research 45*, 296-310.
156. Sendoel, A., Dunn, J. G., Rodriguez, E. H., Naik, S., Gomez, N. C., Hurwitz, B., Levorse, J., Dill, B. D., Schramek, D., Molina, H., Weissman, J. S., and Fuchs, E. (2017) Translation from unconventional 5' start sites drives tumour initiation, *Nature 541*, 494-499.
157. Starck, S. R., Jiang, V., Pavon-Eternod, M., Prasad, S., McCarthy, B., Pan, T., and Shastri, N. (2012) Leucine-tRNA initiates at CUG start codons for protein synthesis and presentation by MHC class I, *Science 336*, 1719-1723.
158. Kim, J. H., Park, S. M., Park, J. H., Keum, S. J., and Jang, S. K. (2011) eIF2A mediates translation of hepatitis C viral mRNA under stress conditions, *The EMBO journal 30*, 2454-2464.
159. Starck, S. R., Tsai, J. C., Chen, K., Shodiya, M., Wang, L., Yahiro, K., Martins-Green, M., Shastri, N., and Walter, P. (2016) Translation from the 5' untranslated region shapes the integrated stress response, *Science 351*, aad3867.
160. Novoa, E. M., and Ribas de Pouplana, L. (2012) Speeding with control: codon usage, tRNAs, and ribosomes, *Trends in genetics : TIG 28*, 574-581.
161. Gingold, H., Tehler, D., Christoffersen, N. R., Nielsen, M. M., Asmar, F., Kooistra, S. M., Christophersen, N. S., Christensen, L. L., Borre, M., Sorensen, K. D., Andersen, L. D., Andersen, C. L., Hulleman, E., Wurdinger, T., Ralfkiaer, E., Helin, K., Gronbaek, K., Orntoft, T., Waszak, S. M., Dahan, O., Pedersen, J. S., Lund, A. H., and Pilpel, Y. (2014) A dual program for translation regulation in cellular proliferation and differentiation, *Cell 158*, 1281-1292.
162. Pavon-Eternod, M., Gomes, S., Geslain, R., Dai, Q., Rosner, M. R., and Pan, T. (2009) tRNA over-expression in breast cancer and functional consequences, *Nucleic acids research 37*, 7268-7280.
163. Topisirovic, I., and Sonenberg, N. (2014) Distinctive tRNA repertoires in proliferating versus differentiating cells, *Cell 158*, 1238-1239.
164. Sulima, S. O., Hofman, I. J. F., De Keersmaecker, K., and Dinman, J. D. (2017) How Ribosomes Translate Cancer, *Cancer discovery 7*, 1069-1087.

165. Bordeira-Carrico, R., Pego, A. P., Santos, M., and Oliveira, C. (2012) Cancer syndromes and therapy by stop-codon readthrough, *Trends in molecular medicine 18*, 667-678.
166. Finkel, R. S., Flanigan, K. M., Wong, B., Bonnemann, C., Sampson, J., Sweeney, H. L., Reha, A., Northcutt, V. J., Elfring, G., Barth, J., and Peltz, S. W. (2013) Phase 2a study of ataluren-mediated dystrophin production in patients with nonsense mutation Duchenne muscular dystrophy, *PloS one 8*, e81302.
167. Welch, E. M., Barton, E. R., Zhuo, J., Tomizawa, Y., Friesen, W. J., Trifillis, P., Paushkin, S., Patel, M., Trotta, C. R., Hwang, S., Wilde, R. G., Karp, G., Takasugi, J., Chen, G., Jones, S., Ren, H., Moon, Y. C., Corson, D., Turpoff, A. A., Campbell, J. A., Conn, M. M., Khan, A., Almstead, N. G., Hedrick, J., Mollin, A., Risher, N., Weetall, M., Yeh, S., Branstrom, A. A., Colacino, J. M., Babiak, J., Ju, W. D., Hirawat, S., Northcutt, V. J., Miller, L. L., Spatrick, P., He, F., Kawana, M., Feng, H., Jacobson, A., Peltz, S. W., and Sweeney, H. L. (2007) PTC124 targets genetic disorders caused by nonsense mutations, *Nature 447*, 87-91.
168. Sanvito, F., Vivoli, F., Gambini, S., Santambrogio, G., Catena, M., Viale, E., Veglia, F., Donadini, A., Biffo, S., and Marchisio, P. C. (2000) Expression of a highly conserved protein, p27BBP, during the progression of human colorectal cancer, *Cancer research 60*, 510-516.
169. Rosso, P., Cortesina, G., Sanvito, F., Donadini, A., Di Benedetto, B., Biffo, S., and Marchisio, P. C. (2004) Overexpression of p27BBP in head and neck carcinomas and their lymph node metastases, *Head & neck 26*, 408-417.
170. Gandin, V., Miluzio, A., Barbieri, A. M., Beugnet, A., Kiyokawa, H., Marchisio, P. C., and Biffo, S. (2008) Eukaryotic initiation factor 6 is rate-limiting in translation, growth and transformation, *Nature 455*, 684-688.
171. Miluzio, A., Beugnet, A., Grosso, S., Brina, D., Mancino, M., Campaner, S., Amati, B., de Marco, A., and Biffo, S. (2011) Impairment of cytoplasmic eIF6 activity restricts lymphomagenesis and tumor progression without affecting normal growth, *Cancer cell 19*, 765-775.
172. Benne, R., Brown-Luedi, M. L., and Hershey, J. W. (1978) Purification and characterization of protein synthesis initiation factors eIF-1, eIF-4C, eIF-4D, and eIF-5 from rabbit reticulocytes, *The Journal of biological chemistry 253*, 3070-3077.
173. Gutierrez, E., Shin, B. S., Woolstenhulme, C. J., Kim, J. R., Saini, P., Buskirk, A. R., and Dever, T. E. (2013) eIF5A promotes translation of polyproline motifs, *Molecular cell 51*, 35-45.
174. Pelechano, V., and Alepuz, P. (2017) eIF5A facilitates translation termination globally and promotes the elongation of many non polyproline-specific tripeptide sequences, *Nucleic acids research*.
175. Schuller, A. P., Wu, C. C., Dever, T. E., Buskirk, A. R., and Green, R. (2017) eIF5A Functions Globally in Translation Elongation and Termination, *Molecular cell 66*, 194-205 e195.
176. Nakanishi, S., and Cleveland, J. L. (2016) Targeting the polyamine-hypusine circuit for the prevention and treatment of cancer, *Amino acids 48*, 2353-2362.
177. van Riggelen, J., Yetil, A., and Felsher, D. W. (2010) MYC as a regulator of ribosome biogenesis and protein synthesis, *Nature reviews. Cancer 10*, 301-309.
178. Gomez-Roman, N., Grandori, C., Eisenman, R. N., and White, R. J. (2003) Direct activation of RNA polymerase III transcription by c-Myc, *Nature 421*, 290-294.
179. Grandori, C., Gomez-Roman, N., Felton-Edkins, Z. A., Ngouenet, C., Galloway, D. A., Eisenman, R. N., and White, R. J. (2005) c-Myc binds to human ribosomal DNA and stimulates transcription of rRNA genes by RNA polymerase I, *Nature cell biology 7*, 311-318.
180. Rosenwald, I. B., Rhoads, D. B., Callanan, L. D., Isselbacher, K. J., and Schmidt, E. V. (1993) Increased expression of eukaryotic translation initiation factors eIF-4E and eIF-2 alpha in response to growth induction by c-myc, *Proceedings of the National Academy of Sciences of the United States of America 90*, 6175-6178.

181. Jones, R. M., Branda, J., Johnston, K. A., Polymenis, M., Gadd, M., Rustgi, A., Callanan, L., and Schmidt, E. V. (1996) An essential E box in the promoter of the gene encoding the mRNA cap-binding protein (eukaryotic initiation factor 4E) is a target for activation by c-myc, *Molecular and cellular biology 16*, 4754-4764.
182. Lin, C. J., Cencic, R., Mills, J. R., Robert, F., and Pelletier, J. (2008) c-Myc and eIF4F are components of a feedforward loop that links transcription and translation, *Cancer research 68*, 5326-5334.
183. De Benedetti, A., Joshi, B., Graff, J. R., and Zimmer, S. G. (1994) CHO cells transformed by the translation factor eIF-4E display increased c-Myc expression, but require overexpression of Max for tumorigenicity, *Mol. cell. Diff 2*, 347–371.
184. Pourdehnad, M., Truitt, M. L., Siddiqi, I. N., Ducker, G. S., Shokat, K. M., and Ruggero, D. (2013) Myc and mTOR converge on a common node in protein synthesis control that confers synthetic lethality in Myc-driven cancers, *Proceedings of the National Academy of Sciences of the United States of America 110*, 11988-11993.
185. Shi, Z., Fujii, K., Kovary, K. M., Genuth, N. R., Rost, H. L., Teruel, M. N., and Barna, M. (2017) Heterogeneous Ribosomes Preferentially Translate Distinct Subpools of mRNAs Genome-wide, *Molecular cell 67*, 71-83 e77.
186. Zhou, X., Liao, W. J., Liao, J. M., Liao, P., and Lu, H. (2015) Ribosomal proteins: functions beyond the ribosome, *Journal of molecular cell biology 7*, 92-104.
187. Zhou, X., Hao, Q., Liao, J. M., Liao, P., and Lu, H. (2013) Ribosomal protein S14 negatively regulates c-Myc activity, *The Journal of biological chemistry 288*, 21793-21801.
188. Comtesse, N., Keller, A., Diesinger, I., Bauer, C., Kayser, K., Huwer, H., Lenhof, H. P., and Meese, E. (2007) Frequent overexpression of the genes FXR1, CLAPM1 and EIF4G located on amplicon 3q26-27 in squamous cell carcinoma of the lung, *International journal of cancer. Journal international du cancer 120*, 2538-2544.
189. Rosenwald, I. B., Lazaris-Karatzas, A., Sonenberg, N., and Schmidt, E. V. (1993) Elevated levels of cyclin D1 protein in response to increased expression of eukaryotic initiation factor 4E, *Molecular and cellular biology 13*, 7358-7363.
190. Cencic, R., Carrier, M., Galicia-Vazquez, G., Bordeleau, M. E., Sukarieh, R., Bourdeau, A., Brem, B., Teodoro, J. G., Greger, H., Tremblay, M. L., Porco, J. A., Jr., and Pelletier, J. (2009) Antitumor activity and mechanism of action of the cyclopenta[b]benzofuran, silvestrol, *PloS one 4*, e5223.
191. Braunstein, S., Karpisheva, K., Pola, C., Goldberg, J., Hochman, T., Yee, H., Cangiarella, J., Arju, R., Formenti, S. C., and Schneider, R. J. (2007) A hypoxia-controlled cap-dependent to cap-independent translation switch in breast cancer, *Molecular cell 28*, 501-512.
192. Badura, M., Braunstein, S., Zavadil, J., and Schneider, R. J. (2012) DNA damage and eIF4G1 in breast cancer cells reprogram translation for survival and DNA repair mRNAs, *Proceedings of the National Academy of Sciences of the United States of America 109*, 18767-18772.
193. Sonenberg, N. (1994) Regulation of translation and cell growth by eIF-4E, *Biochimie 76*, 839-846.
194. Mamane, Y., Petroulakis, E., Rong, L., Yoshida, K., Ler, L. W., and Sonenberg, N. (2004) eIF4E--from translation to transformation, *Oncogene 23*, 3172-3179.
195. Teng, T., Thomas, G., and Mercer, C. A. (2013) Growth control and ribosomopathies, *Current opinion in genetics & development 23*, 63-71.
196. Nathan, C. O., Carter, P., Liu, L., Li, B. D., Abreo, F., Tudor, A., Zimmer, S. G., and De Benedetti, A. (1997) Elevated expression of eIF4E and FGF-2 isoforms during vascularization of breast carcinomas, *Oncogene 15*, 1087-1094.
197. Scott, P. A., Smith, K., Poulsom, R., De Benedetti, A., Bicknell, R., and Harris, A. L. (1998) Differential expression of vascular endothelial growth factor mRNA vs protein isoform expression in human breast cancer and relationship to eIF-4E, *British journal of cancer 77*, 2120-2128.

198. Bastide, A., Karaa, Z., Bornes, S., Hieblot, C., Lacazette, E., Prats, H., and Touriol, C. (2008) An upstream open reading frame within an IRES controls expression of a specific VEGF-A isoform, *Nucleic acids research 36*, 2434-2445.
199. Arcondeguy, T., Lacazette, E., Millevoi, S., Prats, H., and Touriol, C. (2013) VEGF-A mRNA processing, stability and translation: a paradigm for intricate regulation of gene expression at the post-transcriptional level, *Nucleic acids research 41*, 7997-8010.
200. Lang, K. J., Kappel, A., and Goodall, G. J. (2002) Hypoxia-inducible factor-1alpha mRNA contains an internal ribosome entry site that allows efficient translation during normoxia and hypoxia, *Molecular biology of the cell 13*, 1792-1801.
201. El-Naggar, A. M., Veinotte, C. J., Cheng, H., Grunewald, T. G., Negri, G. L., Somasekharan, S. P., Corkery, D. P., Tirode, F., Mathers, J., Khan, D., Kyle, A. H., Baker, J. H., LePard, N. E., McKinney, S., Hajee, S., Bosiljcic, M., Leprivier, G., Tognon, C. E., Minchinton, A. I., Bennewith, K. L., Delattre, O., Wang, Y., Dellaire, G., Berman, J. N., and Sorensen, P. H. (2015) Translational Activation of HIF1alpha by YB-1 Promotes Sarcoma Metastasis, *Cancer cell 27*, 682-697.
202. Dodd, K. M., Yang, J., Shen, M. H., Sampson, J. R., and Tee, A. R. (2015) mTORC1 drives HIF-1alpha and VEGF-A signalling via multiple mechanisms involving 4E-BP1, S6K1 and STAT3, *Oncogene 34*, 2239-2250.
203. Yi, T., Papadopoulos, E., Hagner, P. R., and Wagner, G. (2013) Hypoxia-inducible factor-1alpha (HIF-1alpha) promotes cap-dependent translation of selective mRNAs through up-regulating initiation factor eIF4E1 in breast cancer cells under hypoxia conditions, *The Journal of biological chemistry 288*, 18732-18742.
204. Sorokin, D. V., Scherbakov, A. M., Yakushina, I. A., Semina, S. E., Gudkova, M. V., and Krasil'nikov, M. A. (2016) The Mechanism of Adaptation of Breast Cancer Cells to Hypoxia: Role of AMPK/mTOR Signaling Pathway, *Bulletin of experimental biology and medicine 160*, 555-559.
205. Uniacke, J., Holterman, C. E., Lachance, G., Franovic, A., Jacob, M. D., Fabian, M. R., Payette, J., Holcik, M., Pause, A., and Lee, S. (2012) An oxygen-regulated switch in the protein synthesis machinery, *Nature 486*, 126-129.
206. Joshi, B., Cameron, A., and Jagus, R. (2004) Characterization of mammalian eIF4E-family members, *European journal of biochemistry / FEBS 271*, 2189-2203.
207. Zuberek, J., Kubacka, D., Jablonowska, A., Jemielity, J., Stepinski, J., Sonenberg, N., and Darzynkiewicz, E. (2007) Weak binding affinity of human 4EHP for mRNA cap analogs, *RNA 13*, 691-697.
208. Young, S. K., and Wek, R. C. (2016) Upstream Open Reading Frames Differentially Regulate Gene-specific Translation in the Integrated Stress Response, *The Journal of biological chemistry 291*, 16927-16935.
209. Calvo, S. E., Pagliarini, D. J., and Mootha, V. K. (2009) Upstream open reading frames cause widespread reduction of protein expression and are polymorphic among humans, *Proceedings of the National Academy of Sciences of the United States of America 106*, 7507-7512.
210. Andreev, D. E., O'Connor, P. B., Fahey, C., Kenny, E. M., Terenin, I. M., Dmitriev, S. E., Cormican, P., Morris, D. W., Shatsky, I. N., and Baranov, P. V. (2015) Translation of 5' leaders is pervasive in genes resistant to eIF2 repression, *eLife 4*, e03971.
211. Xu, Y., Li, N., Xiang, R., and Sun, P. (2014) Emerging roles of the p38 MAPK and PI3K/AKT/mTOR pathways in oncogene-induced senescence, *Trends in biochemical sciences 39*, 268-276.
212. Bartkova, J., Rezaei, N., Liontos, M., Karakaidos, P., Kletsas, D., Issaeva, N., Vassiliou, L. V., Kolettas, E., Niforou, K., Zoumpourlis, V. C., Takaoka, M., Nakagawa, H., Tort, F., Fugger, K., Johansson, F., Sehested, M., Andersen, C. L., Dyrskjot, L., Orntoft, T., Lukas, J., Kittas, C., Helleday, T., Halazonetis, T. D., Bartek, J., and Gorgoulis, V. G. (2006) Oncogene-induced senescence is part of the tumorigenesis barrier imposed by DNA damage checkpoints, *Nature 444*, 633-637.

213. Di Micco, R., Fumagalli, M., Cicalese, A., Piccinin, S., Gasparini, P., Luise, C., Schurra, C., Garre, M., Nuciforo, P. G., Bensimon, A., Maestro, R., Pelicci, P. G., and d'Adda di Fagagna, F. (2006) Oncogene-induced senescence is a DNA damage response triggered by DNA hyper-replication, *Nature 444*, 638-642.
214. Petroulakis, E., Parsyan, A., Dowling, R. J., LeBacquer, O., Martineau, Y., Bidinosti, M., Larsson, O., Alain, T., Rong, L., Mamane, Y., Paquet, M., Furic, L., Topisirovic, I., Shahbazian, D., Livingstone, M., Costa-Mattioli, M., Teodoro, J. G., and Sonenberg, N. (2009) p53-dependent translational control of senescence and transformation via 4E-BPs, *Cancer cell 16*, 439-446.
215. Avdulov, S., Herrera, J., Smith, K., Peterson, M., Gomez-Garcia, J. R., Beadnell, T. C., Schwertfeger, K. L., Benyumov, A. O., Manivel, J. C., Li, S., Bielinsky, A. K., Yee, D., Bitterman, P. B., and Polunovsky, V. A. (2015) eIF4E threshold levels differ in governing normal and neoplastic expansion of mammary stem and luminal progenitor cells, *Cancer research 75*, 687-697.
216. Larsson, O., Li, S., Issaenko, O. A., Avdulov, S., Peterson, M., Smith, K., Bitterman, P. B., and Polunovsky, V. A. (2007) Eukaryotic translation initiation factor 4E induced progression of primary human mammary epithelial cells along the cancer pathway is associated with targeted translational deregulation of oncogenic drivers and inhibitors, *Cancer research 67*, 6814-6824.
217. Musa, F., Alard, A., David-West, G., Curtin, J. P., Blank, S. V., and Schneider, R. J. (2016) Dual mTORC1/2 Inhibition as a Novel Strategy for the Resensitization and Treatment of Platinum-Resistant Ovarian Cancer, *Molecular cancer therapeutics 15*, 1557-1567.
218. Larsson, O., Perlman, D. M., Fan, D., Reilly, C. S., Peterson, M., Dahlgren, C., Liang, Z., Li, S., Polunovsky, V. A., Wahlestedt, C., and Bitterman, P. B. (2006) Apoptosis resistance downstream of eIF4E: posttranscriptional activation of an anti-apoptotic transcript carrying a consensus hairpin structure, *Nucleic acids research 34*, 4375-4386.
219. Lazaris-Karatzas, A., and Sonenberg, N. (1992) The mRNA 5' cap-binding protein, eIF-4E, cooperates with v-myc or E1A in the transformation of primary rodent fibroblasts, *Molecular and cellular biology 12*, 1234-1238.
220. Rinker-Schaeffer, C. W., Graff, J. R., De Benedetti, A., Zimmer, S. G., and Rhoads, R. E. (1993) Decreasing the level of translation initiation factor 4E with antisense RNA causes reversal of ras-mediated transformation and tumorigenesis of cloned rat embryo fibroblasts, *International journal of cancer. Journal international du cancer 55*, 841-847.
221. Rajesh, K., Papadakis, A. I., Kazimierczak, U., Peidis, P., Wang, S., Ferbeyre, G., Kaufman, R. J., and Koromilas, A. E. (2013) eIF2alpha phosphorylation bypasses premature senescence caused by oxidative stress and pro-oxidant antitumor therapies, *Aging 5*, 884-901.
222. Horiguchi, M., Koyanagi, S., Okamoto, A., Suzuki, S. O., Matsunaga, N., and Ohdo, S. (2012) Stress-regulated transcription factor ATF4 promotes neoplastic transformation by suppressing expression of the INK4a/ARF cell senescence factors, *Cancer research 72*, 395-401.
223. Bengsch, F., Tu, Z., Tang, H. Y., Zhu, H., Speicher, D. W., and Zhang, R. (2015) Comprehensive analysis of the ubiquitinome during oncogene-induced senescence in human fibroblasts, *Cell Cycle 14*, 1540-1547.
224. Vyas, S., Zaganjor, E., and Haigis, M. C. (2016) Mitochondria and Cancer, *Cell 166*, 555-566.
225. Zucal, C., D'Agostino, V. G., Casini, A., Mantelli, B., Thongon, N., Soncini, D., Caffa, I., Cea, M., Ballestrero, A., Quattrone, A., Indraccolo, S., Nencioni, A., and Provenzani, A. (2015) EIF2A-dependent translational arrest protects leukemia cells from the energetic stress induced by NAMPT inhibition, *BMC cancer 15*, 855.
226. Morita, M., Gravel, S. P., Chenard, V., Sikstrom, K., Zheng, L., Alain, T., Gandin, V., Avizonis, D., Arguello, M., Zakaria, C., McLaughlan, S., Nouet, Y., Pause, A., Pollak, M., Gottlieb, E., Larsson, O., St-Pierre, J., Topisirovic, I., and Sonenberg, N. (2013) mTORC1 controls mitochondrial activity and biogenesis through 4E-BP-dependent translational regulation, *Cell metabolism 18*, 698-711.

227. Larsson, O., Morita, M., Topisirovic, I., Alain, T., Blouin, M. J., Pollak, M., and Sonenberg, N. (2012) Distinct perturbation of the translatome by the antidiabetic drug metformin, *Proceedings of the National Academy of Sciences of the United States of America 109*, 8977-8982.
228. Tamarkin-Ben-Harush, A., Vasseur, J. J., Debart, F., Ulitsky, I., and Dikstein, R. (2017) Cap-proximal nucleotides via differential eIF4E binding and alternative promoter usage mediate translational response to energy stress, *eLife 6*.
229. Dong, K., Wang, R., Wang, X., Lin, F., Shen, J. J., Gao, P., and Zhang, H. Z. (2009) Tumor-specific RNAi targeting eIF4E suppresses tumor growth, induces apoptosis and enhances cisplatin cytotoxicity in human breast carcinoma cells, *Breast cancer research and treatment 113*, 443-456.
230. Zhou, F. F., Yan, M., Guo, G. F., Wang, F., Qiu, H. J., Zheng, F. M., Zhang, Y., Liu, Q., Zhu, X. F., and Xia, L. P. (2011) Knockdown of eIF4E suppresses cell growth and migration, enhances chemosensitivity and correlates with increase in Bax/Bcl-2 ratio in triple-negative breast cancer cells, *Med Oncol 28*, 1302-1307.
231. Boussemart, L., Malka-Mahieu, H., Girault, I., Allard, D., Hemmingsson, O., Tomasic, G., Thomas, M., Basmadjian, C., Ribeiro, N., Thuaud, F., Mateus, C., Routier, E., Kamsu-Kom, N., Agoussi, S., Eggermont, A. M., Desaubry, L., Robert, C., and Vagner, S. (2014) eIF4F is a nexus of resistance to anti-BRAF and anti-MEK cancer therapies, *Nature*.
232. Yu, Y., Tian, L., Feng, X., Cheng, J., Gong, Y., Liu, X., Zhang, Z., Yang, X., He, S., Li, C. Y., and Huang, Q. (2016) eIF4E-phosphorylation-mediated Sox2 upregulation promotes pancreatic tumor cell repopulation after irradiation, *Cancer letters 375*, 31-38.
233. Martinez, A., Sese, M., Losa, J. H., Robichaud, N., Sonenberg, N., Aasen, T., and Ramon y Cajal, S. (2015) Phosphorylation of eIF4E Confers Resistance to Cellular Stress and DNA-Damaging Agents through an Interaction with 4E-T: A Rationale for Novel Therapeutic Approaches, *PloS one 10*, e0123352.
234. Grzmil, M., Seebacher, J., Hess, D., Behe, M., Schibli, R., Moncayo, G., Frank, S., and Hemmings, B. A. (2016) Inhibition of MNK pathways enhances cancer cell response to chemotherapy with temozolomide and targeted radionuclide therapy, *Cellular signalling 28*, 1412-1421.
235. Jeon, Y. J., Kim, J. H., Shin, J. I., Jeong, M., Cho, J., and Lee, K. (2016) Salubrinal-Mediated Upregulation of eIF2alpha Phosphorylation Increases Doxorubicin Sensitivity in MCF-7/ADR Cells, *Molecules and cells 39*, 129-135.
236. De Benedetti, A., and Graff, J. R. (2004) eIF-4E expression and its role in malignancies and metastases, *Oncogene 23*, 3189-3199.
237. Nasr, Z., Robert, F., Porco, J. A., Jr., Muller, W. J., and Pelletier, J. (2013) eIF4F suppression in breast cancer affects maintenance and progression, *Oncogene 32*, 861-871.
238. Bramham, C. R., Jensen, K. B., and Proud, C. G. (2016) Tuning Specific Translation in Cancer Metastasis and Synaptic Memory: Control at the MNK-eIF4E Axis, *Trends in biochemical sciences 41*, 847-858.
239. Mujcic, H., Nagelkerke, A., Rouschop, K. M., Chung, S., Chaudary, N., Span, P. N., Clarke, B., Milosevic, M., Sykes, J., Hill, R. P., Koritzinsky, M., and Wouters, B. G. (2013) Hypoxic activation of the PERK/eIF2alpha arm of the unfolded protein response promotes metastasis through induction of LAMP3, *Clinical cancer research : an official journal of the American Association for Cancer Research 19*, 6126-6137.
240. Pinzaglia, M., Montaldo, C., Polinari, D., Simone, M., La Teana, A., Tripodi, M., Mancone, C., Londei, P., and Benelli, D. (2015) EIF6 over-expression increases the motility and invasiveness of cancer cells by modulating the expression of a critical subset of membrane-bound proteins, *BMC cancer 15*, 131.
241. Fujimura, K., Choi, S., Wyse, M., Strnadel, J., Wright, T., and Klemke, R. (2015) Eukaryotic Translation Initiation Factor 5A (EIF5A) Regulates Pancreatic Cancer Metastasis by Modulating

RhoA and Rho-associated Kinase (ROCK) Protein Expression Levels, *The Journal of biological chemistry 290*, 29907-29919.
242. Braunstein, S., Badura, M. L., Xi, Q., Formenti, S. C., and Schneider, R. J. (2009) Regulation of protein synthesis by ionizing radiation, *Molecular and cellular biology 29*, 5645-5656.
243. Chio, II, Jafarnejad, S. M., Ponz-Sarvise, M., Park, Y., Rivera, K., Palm, W., Wilson, J., Sangar, V., Hao, Y., Ohlund, D., Wright, K., Filippini, D., Lee, E. J., Da Silva, B., Schoepfer, C., Wilkinson, J. E., Buscaglia, J. M., DeNicola, G. M., Tiriac, H., Hammell, M., Crawford, H. C., Schmidt, E. E., Thompson, C. B., Pappin, D. J., Sonenberg, N., and Tuveson, D. A. (2016) NRF2 Promotes Tumor Maintenance by Modulating mRNA Translation in Pancreatic Cancer, *Cell 166*, 963-976.
244. Vezina, C., Kudelski, A., and Sehgal, S. N. (1975) Rapamycin (AY-22,989), a new antifungal antibiotic. I. Taxonomy of the producing streptomycete and isolation of the active principle, *J Antibiot (Tokyo) 28*, 721-726.
245. Heitman, J., Movva, N. R., and Hall, M. N. (1991) Targets for cell cycle arrest by the immunosuppressant rapamycin in yeast, *Science 253*, 905-909.
246. Brown, E. J., Albers, M. W., Shin, T. B., Ichikawa, K., Keith, C. T., Lane, W. S., and Schreiber, S. L. (1994) A mammalian protein targeted by G1-arresting rapamycin-receptor complex, *Nature 369*, 756-758.
247. Hara, K., Maruki, Y., Long, X., Yoshino, K., Oshiro, N., Hidayat, S., Tokunaga, C., Avruch, J., and Yonezawa, K. (2002) Raptor, a binding partner of target of rapamycin (TOR), mediates TOR action, *Cell 110*, 177-189.
248. Kim, D. H., Sarbassov, D. D., Ali, S. M., King, J. E., Latek, R. R., Erdjument-Bromage, H., Tempst, P., and Sabatini, D. M. (2002) mTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery, *Cell 110*, 163-175.
249. Yang, H., Rudge, D. G., Koos, J. D., Vaidialingam, B., Yang, H. J., and Pavletich, N. P. (2013) mTOR kinase structure, mechanism and regulation, *Nature 497*, 217-223.
250. Oshiro, N., Yoshino, K., Hidayat, S., Tokunaga, C., Hara, K., Eguchi, S., Avruch, J., and Yonezawa, K. (2004) Dissociation of raptor from mTOR is a mechanism of rapamycin-induced inhibition of mTOR function, *Genes to cells : devoted to molecular & cellular mechanisms 9*, 359-366.
251. Sarbassov, D. D., Ali, S. M., Kim, D. H., Guertin, D. A., Latek, R. R., Erdjument-Bromage, H., Tempst, P., and Sabatini, D. M. (2004) Rictor, a novel binding partner of mTOR, defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton, *Current biology : CB 14*, 1296-1302.
252. Yip, C. K., Murata, K., Walz, T., Sabatini, D. M., and Kang, S. A. (2010) Structure of the human mTOR complex I and its implications for rapamycin inhibition, *Molecular cell 38*, 768-774.
253. Kang, S. A., Pacold, M. E., Cervantes, C. L., Lim, D., Lou, H. J., Ottina, K., Gray, N. S., Turk, B. E., Yaffe, M. B., and Sabatini, D. M. (2013) mTORC1 phosphorylation sites encode their sensitivity to starvation and rapamycin, *Science 341*, 1236566.
254. Yoon, S. O., and Roux, P. P. (2013) Rapamycin resistance: mTORC1 substrates hold some of the answers, *Current biology : CB 23*, R880-883.
255. Sarbassov, D. D., Ali, S. M., Sengupta, S., Sheen, J. H., Hsu, P. P., Bagley, A. F., Markhard, A. L., and Sabatini, D. M. (2006) Prolonged rapamycin treatment inhibits mTORC2 assembly and Akt/PKB, *Molecular cell 22*, 159-168.
256. Lamming, D. W., Ye, L., Katajisto, P., Goncalves, M. D., Saitoh, M., Stevens, D. M., Davis, J. G., Salmon, A. B., Richardson, A., Ahima, R. S., Guertin, D. A., Sabatini, D. M., and Baur, J. A. (2012) Rapamycin-induced insulin resistance is mediated by mTORC2 loss and uncoupled from longevity, *Science 335*, 1638-1643.
257. Janes, M. R., and Fruman, D. A. (2010) Targeting TOR dependence in cancer, *Oncotarget 1*, 69-76.

258. Fruman, D. A., and Rommel, C. (2014) PI3K and cancer: lessons, challenges and opportunities, *Nature reviews. Drug discovery 13*, 140-156.
259. Benjamin, D., Colombi, M., Moroni, C., and Hall, M. N. (2011) Rapamycin passes the torch: a new generation of mTOR inhibitors, *Nature reviews. Drug discovery 10*, 868-880.
260. Harrington, L. S., Findlay, G. M., and Lamb, R. F. (2005) Restraining PI3K: mTOR signalling goes back to the membrane, *Trends in biochemical sciences 30*, 35-42.
261. Choo, A. Y., and Blenis, J. (2009) Not all substrates are treated equally: implications for mTOR, rapamycin-resistance and cancer therapy, *Cell Cycle 8*, 567-572.
262. Hsu, P. P., Kang, S. A., Rameseder, J., Zhang, Y., Ottina, K. A., Lim, D., Peterson, T. R., Choi, Y., Gray, N. S., Yaffe, M. B., Marto, J. A., and Sabatini, D. M. (2011) The mTOR-regulated phosphoproteome reveals a mechanism of mTORC1-mediated inhibition of growth factor signaling, *Science 332*, 1317-1322.
263. Yu, Y., Yoon, S. O., Poulogiannis, G., Yang, Q., Ma, X. M., Villen, J., Kubica, N., Hoffman, G. R., Cantley, L. C., Gygi, S. P., and Blenis, J. (2011) Phosphoproteomic analysis identifies Grb10 as an mTORC1 substrate that negatively regulates insulin signaling, *Science 332*, 1322-1326.
264. Carracedo, A., Ma, L., Teruya-Feldstein, J., Rojo, F., Salmena, L., Alimonti, A., Egia, A., Sasaki, A. T., Thomas, G., Kozma, S. C., Papa, A., Nardella, C., Cantley, L. C., Baselga, J., and Pandolfi, P. P. (2008) Inhibition of mTORC1 leads to MAPK pathway activation through a PI3K-dependent feedback loop in human cancer, *J Clin Invest 118*, 3065-3074.
265. Fan, Q. W., Knight, Z. A., Goldenberg, D. D., Yu, W., Mostov, K. E., Stokoe, D., Shokat, K. M., and Weiss, W. A. (2006) A dual PI3 kinase/mTOR inhibitor reveals emergent efficacy in glioma, *Cancer cell 9*, 341-349.
266. Thoreen, C. C., Kang, S. A., Chang, J. W., Liu, Q., Zhang, J., Gao, Y., Reichling, L. J., Sim, T., Sabatini, D. M., and Gray, N. S. (2009) An ATP-competitive mammalian target of rapamycin inhibitor reveals rapamycin-resistant functions of mTORC1, *The Journal of biological chemistry 284*, 8023-8032.
267. Feldman, M. E., Apsel, B., Uotila, A., Loewith, R., Knight, Z. A., Ruggero, D., and Shokat, K. M. (2009) Active-site inhibitors of mTOR target rapamycin-resistant outputs of mTORC1 and mTORC2, *PLoS biology 7*, e38.
268. Garcia-Martinez, J. M., Moran, J., Clarke, R. G., Gray, A., Cosulich, S. C., Chresta, C. M., and Alessi, D. R. (2009) Ku-0063794 is a specific inhibitor of the mammalian target of rapamycin (mTOR), *The Biochemical journal 421*, 29-42.
269. Hresko, R. C., and Mueckler, M. (2005) mTOR.RICTOR is the Ser473 kinase for Akt/protein kinase B in 3T3-L1 adipocytes, *The Journal of biological chemistry 280*, 40406-40416.
270. Sarbassov, D. D., Guertin, D. A., Ali, S. M., and Sabatini, D. M. (2005) Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex, *Science 307*, 1098-1101.
271. Choo, A. Y., Yoon, S. O., Kim, S. G., Roux, P. P., and Blenis, J. (2008) Rapamycin differentially inhibits S6Ks and 4E-BP1 to mediate cell-type-specific repression of mRNA translation, *Proceedings of the National Academy of Sciences of the United States of America 105*, 17414-17419.
272. Chresta, C. M., Davies, B. R., Hickson, I., Harding, T., Cosulich, S., Critchlow, S. E., Vincent, J. P., Ellston, R., Jones, D., Sini, P., James, D., Howard, Z., Dudley, P., Hughes, G., Smith, L., Maguire, S., Hummersone, M., Malagu, K., Menear, K., Jenkins, R., Jacobsen, M., Smith, G. C., Guichard, S., and Pass, M. (2010) AZD8055 is a potent, selective, and orally bioavailable ATP-competitive mammalian target of rapamycin kinase inhibitor with in vitro and in vivo antitumor activity, *Cancer research 70*, 288-298.
273. Janes, M. R., Limon, J. J., So, L., Chen, J., Lim, R. J., Chavez, M. A., Vu, C., Lilly, M. B., Mallya, S., Ong, S. T., Konopleva, M., Martin, M. B., Ren, P., Liu, Y., Rommel, C., and Fruman, D. A. (2010) Effective and selective targeting of leukemia cells using a TORC1/2 kinase inhibitor, *Nature medicine 16*, 205-213.

274. Janes, M. R., Vu, C., Mallya, S., Shieh, M. P., Limon, J. J., Li, L. S., Jessen, K. A., Martin, M. B., Ren, P., Lilly, M. B., Sender, L. S., Liu, Y., Rommel, C., and Fruman, D. A. (2013) Efficacy of the investigational mTOR kinase inhibitor MLN0128/INK128 in models of B-cell acute lymphoblastic leukemia, *Leukemia 27*, 586-594.
275. Alain, T., Morita, M., Fonseca, B. D., Yanagiya, A., Siddiqui, N., Bhat, M., Zammit, D., Marcus, V., Metrakos, P., Voyer, L. A., Gandin, V., Liu, Y., Topisirovic, I., and Sonenberg, N. (2012) eIF4E/4E-BP ratio predicts the efficacy of mTOR targeted therapies, *Cancer research 72*, 6468-6476.
276. Satheesha, S., Cookson, V. J., Coleman, L. J., Ingram, N., Madhok, B., Hanby, A. M., Suleman, C. A., Sabine, V. S., Macaskill, E. J., Bartlett, J. M., Dixon, J. M., McElwaine, J. N., and Hughes, T. A. (2011) Response to mTOR inhibition: activity of eIF4E predicts sensitivity in cell lines and acquired changes in eIF4E regulation in breast cancer, *Molecular cancer 10*, 19.
277. Ilic, N., Utermark, T., Widlund, H. R., and Roberts, T. M. (2011) PI3K-targeted therapy can be evaded by gene amplification along the MYC-eukaryotic translation initiation factor 4E (eIF4E) axis, *Proceedings of the National Academy of Sciences of the United States of America 108*, E699-708.
278. Cope, C. L., Gilley, R., Balmanno, K., Sale, M. J., Howarth, K. D., Hampson, M., Smith, P. D., Guichard, S. M., and Cook, S. J. (2014) Adaptation to mTOR kinase inhibitors by amplification of eIF4E to maintain cap-dependent translation, *Journal of cell science 127*, 788-800.
279. Mallya, S., Fitch, B. A., Lee, J. S., So, L., Janes, M. R., and Fruman, D. A. (2014) Resistance to mTOR kinase inhibitors in lymphoma cells lacking 4EBP1, *PloS one 9*, e88865.
280. Martineau, Y., Azar, R., Muller, D., Lasfargues, C., El Khawand, S., Anesia, R., Pelletier, J., Bousquet, C., and Pyronnet, S. (2014) Pancreatic tumours escape from translational control through 4E-BP1 loss, *Oncogene 33*, 1367-1374.
281. Alain, T., Sonenberg, N., and Topisirovic, I. (2012) mTOR inhibitor efficacy is determined by the eIF4E/4E-BP ratio, *Oncotarget 3*, 1491-1492.
282. Coleman, L. J., Peter, M. B., Teall, T. J., Brannan, R. A., Hanby, A. M., Honarpisheh, H., Shaaban, A. M., Smith, L., Speirs, V., Verghese, E. T., McElwaine, J. N., and Hughes, T. A. (2009) Combined analysis of eIF4E and 4E-binding protein expression predicts breast cancer survival and estimates eIF4E activity, *British journal of cancer 100*, 1393-1399.
283. Muranen, T., Selfors, L. M., Worster, D. T., Iwanicki, M. P., Song, L., Morales, F. C., Gao, S., Mills, G. B., and Brugge, J. S. (2012) Inhibition of PI3K/mTOR leads to adaptive resistance in matrix-attached cancer cells, *Cancer cell 21*, 227-239.
284. Larsson, S. C., Orsini, N., and Wolk, A. (2005) Diabetes mellitus and risk of colorectal cancer: a meta-analysis, *Journal of the National Cancer Institute 97*, 1679-1687.
285. Larsson, S. C., Mantzoros, C. S., and Wolk, A. (2007) Diabetes mellitus and risk of breast cancer: a meta-analysis, *International journal of cancer. Journal international du cancer 121*, 856-862.
286. Pollak, M. (2013) Potential applications for biguanides in oncology, *The Journal of clinical investigation 123*, 3693-3700.
287. Pollak, M. N. (2012) Investigating metformin for cancer prevention and treatment: the end of the beginning, *Cancer discovery 2*, 778-790.
288. Dowling, R. J., Zakikhani, M., Fantus, I. G., Pollak, M., and Sonenberg, N. (2007) Metformin inhibits mammalian target of rapamycin-dependent translation initiation in breast cancer cells, *Cancer research 67*, 10804-10812.
289. Kordes, S., Pollak, M. N., Zwinderman, A. H., Mathot, R. A., Weterman, M. J., Beeker, A., Punt, C. J., Richel, D. J., and Wilmink, J. W. (2015) Metformin in patients with advanced pancreatic cancer: a double-blind, randomised, placebo-controlled phase 2 trial, *The Lancet. Oncology 16*, 839-847.
290. Konicek, B. W., Stephens, J. R., McNulty, A. M., Robichaud, N., Peery, R. B., Dumstorf, C. A., Dowless, M. S., Iversen, P. W., Parsons, S., Ellis, K. E., McCann, D. J., Pelletier, J., Furic, L., Yingling,

J. M., Stancato, L. F., Sonenberg, N., and Graff, J. R. (2011) Therapeutic inhibition of MAP kinase interacting kinase blocks eukaryotic initiation factor 4E phosphorylation and suppresses outgrowth of experimental lung metastases, *Cancer research 71*, 1849-1857.

291. Tschopp, C., Knauf, U., Brauchle, M., Zurini, M., Ramage, P., Glueck, D., New, L., Han, J., and Gram, H. (2000) Phosphorylation of eIF-4E on Ser 209 in response to mitogenic and inflammatory stimuli is faithfully detected by specific antibodies, *Molecular cell biology research communications : MCBRC 3*, 205-211.
292. Knauf, U., Tschopp, C., and Gram, H. (2001) Negative regulation of protein translation by mitogen-activated protein kinase-interacting kinases 1 and 2, *Molecular and cellular biology 21*, 5500-5511.
293. Bain, J., Plater, L., Elliott, M., Shpiro, N., Hastie, C. J., McLauchlan, H., Klevernic, I., Arthur, J. S., Alessi, D. R., and Cohen, P. (2007) The selectivity of protein kinase inhibitors: a further update, *The Biochemical journal 408*, 297-315.
294. Diab, S., Kumarasiri, M., Yu, M., Teo, T., Proud, C., Milne, R., and Wang, S. (2014) MAP Kinase-Interacting Kinases-Emerging Targets against Cancer, *Chemistry & biology*.
295. Diab, S., Teo, T., Kumarasiri, M., Li, P., Yu, M., Lam, F., Basnet, S. K., Sykes, M. J., Albrecht, H., Milne, R., and Wang, S. (2014) Discovery of 5-(2-(Phenylamino)pyrimidin-4-yl)thiazol-2(3H)-one Derivatives as Potent Mnk2 Inhibitors: Synthesis, SAR Analysis and Biological Evaluation, *ChemMedChem 9*, 962-972.
296. Xu, J., Chen, A., Joy, J., Xavier, V. J., Ong, E. H., Hill, J., and Chai, C. L. (2013) Rational design of resorcylic acid lactone analogues as covalent MNK1/2 kinase inhibitors by tuning the reactivity of an enamide Michael acceptor, *ChemMedChem 8*, 1483-1494.
297. Ramalingam, S., Gediya, L., Kwegyir-Afful, A. K., Ramamurthy, V. P., Purushottamachar, P., Mbatia, H., and Njar, V. C. (2014) First MNKs degrading agents block phosphorylation of eIF4E, induce apoptosis, inhibit cell growth, migration and invasion in triple negative and Her2-overexpressing breast cancer cell lines, *Oncotarget 5*, 530-543.
298. Adesso, L., Calabretta, S., Barbagallo, F., Capurso, G., Pilozzi, E., Geremia, R., Delle Fave, G., and Sette, C. (2013) Gemcitabine triggers a pro-survival response in pancreatic cancer cells through activation of the MNK2/eIF4E pathway, *Oncogene 32*, 2848-2857.
299. Sun, S. Y., Rosenberg, L. M., Wang, X., Zhou, Z., Yue, P., Fu, H., and Khuri, F. R. (2005) Activation of Akt and eIF4E survival pathways by rapamycin-mediated mammalian target of rapamycin inhibition, *Cancer research 65*, 7052-7058.
300. Sale, M. J., and Cook, S. J. (2014) Intrinsic and acquired resistance to MEK1/2 inhibitors in cancer, *Biochemical Society transactions 42*, 776-783.
301. Wagner, C. R., Iyer, V. V., and McIntee, E. J. (2000) Pronucleotides: toward the in vivo delivery of antiviral and anticancer nucleotides, *Medicinal research reviews 20*, 417-451.
302. Ghosh, B., Benyumov, A. O., Ghosh, P., Jia, Y., Avdulov, S., Dahlberg, P. S., Peterson, M., Smith, K., Polunovsky, V. A., Bitterman, P. B., and Wagner, C. R. (2009) Nontoxic chemical interdiction of the epithelial-to-mesenchymal transition by targeting cap-dependent translation, *ACS chemical biology 4*, 367-377.
303. Li, S., Jia, Y., Jacobson, B., McCauley, J., Kratzke, R., Bitterman, P. B., and Wagner, C. R. (2013) Treatment of breast and lung cancer cells with a N-7 benzyl guanosine monophosphate tryptamine phosphoramidate pronucleotide (4Ei-1) results in chemosensitization to gemcitabine and induced eIF4E proteasomal degradation, *Molecular pharmaceutics 10*, 523-531.
304. Jia, Y., Polunovsky, V., Bitterman, P. B., and Wagner, C. R. (2012) Cap-dependent translation initiation factor eIF4E: an emerging anticancer drug target, *Medicinal research reviews 32*, 786-814.
305. Chen, X., Kopecky, D. J., Mihalic, J., Jeffries, S., Min, X., Heath, J., Deignan, J., Lai, S., Fu, Z., Guimaraes, C., Shen, S., Li, S., Johnstone, S., Thibault, S., Xu, H., Cardozo, M., Shen, W., Walker,

N., Kayser, F., and Wang, Z. (2012) Structure-guided design, synthesis, and evaluation of guanine-derived inhibitors of the eIF4E mRNA-cap interaction, *Journal of medicinal chemistry 55*, 3837-3851.

306. Zochowska, M., Piguet, A. C., Jemielity, J., Kowalska, J., Szolajska, E., Dufour, J. F., and Chroboczek, J. (2014) Virus-like particle-mediated intracellular delivery of mRNA cap analog with in vivo activity against hepatocellular carcinoma, *Nanomedicine : nanotechnology, biology, and medicine*.
307. Kentsis, A., Topisirovic, I., Culjkovic, B., Shao, L., and Borden, K. L. (2004) Ribavirin suppresses eIF4E-mediated oncogenic transformation by physical mimicry of the 7-methyl guanosine mRNA cap, *Proceedings of the National Academy of Sciences of the United States of America 101*, 18105-18110.
308. Yan, Y., Svitkin, Y., Lee, J. M., Bisaillon, M., and Pelletier, J. (2005) Ribavirin is not a functional mimic of the 7-methyl guanosine mRNA cap, *Rna 11*, 1238-1244.
309. Westman, B., Beeren, L., Grudzien, E., Stepinski, J., Worch, R., Zuberek, J., Jemielity, J., Stolarski, R., Darzynkiewicz, E., Rhoads, R. E., and Preiss, T. (2005) The antiviral drug ribavirin does not mimic the 7-methylguanosine moiety of the mRNA cap structure in vitro, *Rna 11*, 1505-1513.
310. Assouline, S., Culjkovic, B., Cocolakis, E., Rousseau, C., Beslu, N., Amri, A., Caplan, S., Leber, B., Roy, D. C., Miller, W. H., Jr., and Borden, K. L. (2009) Molecular targeting of the oncogene eIF4E in acute myeloid leukemia (AML): a proof-of-principle clinical trial with ribavirin, *Blood 114*, 257-260.
311. Malina, A., Cencic, R., and Pelletier, J. (2011) Targeting translation dependence in cancer, *Oncotarget 2*, 76-88.
312. Moerke, N. J., Aktas, H., Chen, H., Cantel, S., Reibarkh, M. Y., Fahmy, A., Gross, J. D., Degterev, A., Yuan, J., Chorev, M., Halperin, J. A., and Wagner, G. (2007) Small-molecule inhibition of the interaction between the translation initiation factors eIF4E and eIF4G, *Cell 128*, 257-267.
313. Cencic, R., Desforges, M., Hall, D. R., Kozakov, D., Du, Y., Min, J., Dingledine, R., Fu, H., Vajda, S., Talbot, P. J., and Pelletier, J. (2011) Blocking eIF4E-eIF4G interaction as a strategy to impair coronavirus replication, *Journal of virology 85*, 6381-6389.
314. Chen, L., Aktas, B. H., Wang, Y., He, X., Sahoo, R., Zhang, N., Denoyelle, S., Kabha, E., Yang, H., Freedman, R. Y., Supko, J. G., Chorev, M., Wagner, G., and Halperin, J. A. (2012) Tumor suppression by small molecule inhibitors of translation initiation, *Oncotarget 3*, 869-881.
315. Papadopoulos, E., Jenni, S., Kabha, E., Takrouri, K. J., Yi, T., Salvi, N., Luna, R. E., Gavathiotis, E., Mahalingam, P., Arthanari, H., Rodriguez-Mias, R., Yefidoff-Freedman, R., Aktas, B. H., Chorev, M., Halperin, J. A., and Wagner, G. (2014) Structure of the eukaryotic translation initiation factor eIF4E in complex with 4EGI-1 reveals an allosteric mechanism for dissociating eIF4G, *Proceedings of the National Academy of Sciences of the United States of America 111*, E3187-3195.
316. Redondo, N., Garcia-Moreno, M., Sanz, M. A., and Carrasco, L. (2013) Translation of viral mRNAs that do not require eIF4E is blocked by the inhibitor 4EGI-1, *Virology 444*, 171-180.
317. Willimott, S., Beck, D., Ahearne, M. J., Adams, V. C., and Wagner, S. D. (2013) Cap-translation inhibitor, 4EGI-1, restores sensitivity to ABT-737 apoptosis through cap-dependent and -independent mechanisms in chronic lymphocytic leukemia, *Clinical cancer research : an official journal of the American Association for Cancer Research 19*, 3212-3223.
318. Ko, S. Y., Guo, H., Barengo, N., and Naora, H. (2009) Inhibition of ovarian cancer growth by a tumor-targeting peptide that binds eukaryotic translation initiation factor 4E, *Clinical cancer research : an official journal of the American Association for Cancer Research 15*, 4336-4347.
319. Malina, A., Mills, J. R., and Pelletier, J. (2012) Emerging therapeutics targeting mRNA translation, *Cold Spring Harbor perspectives in biology 4*, a012377.
320. Bordeleau, M. E., Mori, A., Oberer, M., Lindqvist, L., Chard, L. S., Higa, T., Belsham, G. J., Wagner, G., Tanaka, J., and Pelletier, J. (2006) Functional characterization of IRESes by an inhibitor of the RNA helicase eIF4A, *Nature chemical biology 2*, 213-220.

321. Bordeleau, M. E., Matthews, J., Wojnar, J. M., Lindqvist, L., Novac, O., Jankowsky, E., Sonenberg, N., Northcote, P., Teesdale-Spittle, P., and Pelletier, J. (2005) Stimulation of mammalian translation initiation factor eIF4A activity by a small molecule inhibitor of eukaryotic translation, *Proceedings of the National Academy of Sciences of the United States of America 102*, 10460-10465.
322. Bordeleau, M. E., Robert, F., Gerard, B., Lindqvist, L., Chen, S. M., Wendel, H. G., Brem, B., Greger, H., Lowe, S. W., Porco, J. A., Jr., and Pelletier, J. (2008) Therapeutic suppression of translation initiation modulates chemosensitivity in a mouse lymphoma model, *J Clin Invest 118*, 2651-2660.
323. Low, W. K., Dang, Y., Schneider-Poetsch, T., Shi, Z., Choi, N. S., Merrick, W. C., Romo, D., and Liu, J. O. (2005) Inhibition of eukaryotic translation initiation by the marine natural product pateamine A, *Molecular cell 20*, 709-722.
324. Low, W. K., Li, J., Zhu, M., Kommaraju, S. S., Shah-Mittal, J., Hull, K., Liu, J. O., and Romo, D. (2014) Second-generation derivatives of the eukaryotic translation initiation inhibitor pateamine A targeting eIF4A as potential anticancer agents, *Bioorganic & medicinal chemistry 22*, 116-125.
325. Kuznetsov, G., Xu, Q., Rudolph-Owen, L., Tendyke, K., Liu, J., Towle, M., Zhao, N., Marsh, J., Agoulnik, S., Twine, N., Parent, L., Chen, Z., Shie, J. L., Jiang, Y., Zhang, H., Du, H., Boivin, R., Wang, Y., Romo, D., and Littlefield, B. A. (2009) Potent in vitro and in vivo anticancer activities of des-methyl, des-amino pateamine A, a synthetic analogue of marine natural product pateamine A, *Molecular cancer therapeutics 8*, 1250-1260.
326. Tsumuraya, T., Ishikawa, C., Machijima, Y., Nakachi, S., Senba, M., Tanaka, J., and Mori, N. (2011) Effects of hippuristanol, an inhibitor of eIF4A, on adult T-cell leukemia, *Biochemical pharmacology 81*, 713-722.
327. Cencic, R., Robert, F., Galicia-Vazquez, G., Malina, A., Ravindar, K., Somaiah, R., Pierre, P., Tanaka, J., Deslongchamps, P., and Pelletier, J. (2013) Modifying chemotherapy response by targeted inhibition of eukaryotic initiation factor 4A, *Blood cancer journal 3*, e128.
328. Sadlish, H., Galicia-Vazquez, G., Paris, C. G., Aust, T., Bhullar, B., Chang, L., Helliwell, S. B., Hoepfner, D., Knapp, B., Riedl, R., Roggo, S., Schuierer, S., Studer, C., Porco, J. A., Jr., Pelletier, J., and Movva, N. R. (2013) Evidence for a Functionally Relevant Rocaglamide Binding Site on the eIF4A-RNA Complex, *ACS chemical biology*.
329. Liu, T., Nair, S. J., Lescarbeau, A., Belani, J., Peluso, S., Conley, J., Tillotson, B., O'Hearn, P., Smith, S., Slocum, K., West, K., Helble, J., Douglas, M., Bahadoor, A., Ali, J., McGovern, K., Fritz, C., Palombella, V., Wylie, A., Castro, A., and Tremblay, M. (2012) Synthetic silvestrol analogues as potent and selective protein synthesis inhibitors, *Journal of medicinal chemistry*.
330. Wolfe, A. L., Singh, K., Zhong, Y., Drewe, P., Rajasekhar, V. K., Sanghvi, V. R., Mavrakis, K. J., Jiang, M., Roderick, J. E., Van der Meulen, J., Schatz, J. H., Rodrigo, C. M., Zhao, C., Rondou, P., de Stanchina, E., Teruya-Feldstein, J., Kelliher, M. A., Speleman, F., Porco, J. A., Pelletier, J., Ratsch, G., and Wendel, H. G. (2014) RNA G-quadruplexes cause eIF4A-dependent oncogene translation in cancer, *Nature*.
331. Rubio, C. A., Weisburd, B., Holderfield, M., Arias, C., Fang, E., DeRisi, J. L., and Fanidi, A. (2014) Transcriptome-wide characterization of the eIF4A signature highlights plasticity in translation regulation, *Genome biology 15*, 476.
332. Gupta, S. V., Sass, E. J., Davis, M. E., Edwards, R. B., Lozanski, G., Heerema, N. A., Lehman, A., Zhang, X., Jarjoura, D., Byrd, J. C., Pan, L., Chan, K. K., Kinghorn, A. D., Phelps, M. A., Grever, M. R., and Lucas, D. M. (2011) Resistance to the Translation Initiation Inhibitor Silvestrol is Mediated by ABCB1/P-Glycoprotein Overexpression in Acute Lymphoblastic Leukemia Cells, *AAPS J 13*, 357-364.

333. Hawkins, B. C., Lindqvist, L. M., Nhu, D., Sharp, P. P., Segal, D., Powell, A. K., Campbell, M., Ryan, E., Chambers, J. M., White, J. M., Rizzacasa, M. A., Lessene, G., Huang, D. C., and Burns, C. J. (2014) Simplified Silvestrol Analogues with Potent Cytotoxic Activity, *ChemMedChem*.
334. Koromilas, A. E., and Mounir, Z. (2013) Control of oncogenesis by eIF2alpha phosphorylation: implications in PTEN and PI3K-Akt signaling and tumor treatment, *Future Oncol 9*, 1005-1015.
335. Donze, O., Jagus, R., Koromilas, A. E., Hershey, J. W., and Sonenberg, N. (1995) Abrogation of translation initiation factor eIF-2 phosphorylation causes malignant transformation of NIH 3T3 cells, *The EMBO journal 14*, 3828-3834.
336. Donze, O., Deng, J., Curran, J., Sladek, R., Picard, D., and Sonenberg, N. (2004) The protein kinase PKR: a molecular clock that sequentially activates survival and death programs, *The EMBO journal 23*, 564-571.
337. Ron D, H. H. C. S. H. L. P., Cold Spring Harbor, NY (2007) *eIF2α phosphorylation in cellular stress-responses and disease. In Translational control in biology and medicine* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
338. Chen, T., Ozel, D., Qiao, Y., Harbinski, F., Chen, L., Denoyelle, S., He, X., Zvereva, N., Supko, J. G., Chorev, M., Halperin, J. A., and Aktas, B. H. (2011) Chemical genetics identify eIF2alpha kinase heme-regulated inhibitor as an anticancer target, *Nat Chem Biol 7*, 610-616.
339. Boyce, M., Bryant, K. F., Jousse, C., Long, K., Harding, H. P., Scheuner, D., Kaufman, R. J., Ma, D., Coen, D. M., Ron, D., and Yuan, J. (2005) A selective inhibitor of eIF2alpha dephosphorylation protects cells from ER stress, *Science 307*, 935-939.
340. Moenner, M., Pluquet, O., Bouchecareilh, M., and Chevet, E. (2007) Integrated endoplasmic reticulum stress responses in cancer, *Cancer research 67*, 10631-10634.
341. Hetz, C., Chevet, E., and Harding, H. P. (2013) Targeting the unfolded protein response in disease, *Nature reviews. Drug discovery 12*, 703-719.
342. Tsaytler, P., Harding, H. P., Ron, D., and Bertolotti, A. (2011) Selective inhibition of a regulatory subunit of protein phosphatase 1 restores proteostasis, *Science 332*, 91-94.
343. Robert, F., Kapp, L. D., Khan, S. N., Acker, M. G., Kolitz, S., Kazemi, S., Kaufman, R. J., Merrick, W. C., Koromilas, A. E., Lorsch, J. R., and Pelletier, J. (2006) Initiation of protein synthesis by hepatitis C virus is refractory to reduced eIF2.GTP.Met-tRNA(i)(Met) ternary complex availability, *Molecular biology of the cell 17*, 4632-4644.
344. Ollenschlager, G., Roth, E., Linkesch, W., Jansen, S., Simmel, A., and Modder, B. (1988) Asparaginase-induced derangements of glutamine metabolism: the pathogenetic basis for some drug-related side-effects, *European journal of clinical investigation 18*, 512-516.
345. Hill, J. M., Roberts, J., Loeb, E., Khan, A., MacLellan, A., and Hill, R. W. (1967) L-asparaginase therapy for leukemia and other malignant neoplasms. Remission in human leukemia, *JAMA : the journal of the American Medical Association 202*, 882-888.
346. Buaboonnam, J., Cao, X., Pauley, J. L., Pui, C. H., Ribeiro, R. C., Rubnitz, J. E., and Inaba, H. (2013) Sequential administration of methotrexate and asparaginase in relapsed or refractory pediatric acute myeloid leukemia, *Pediatric blood & cancer 60*, 1161-1164.
347. Bunpo, P., Dudley, A., Cundiff, J. K., Cavener, D. R., Wek, R. C., and Anthony, T. G. (2009) GCN2 protein kinase is required to activate amino acid deprivation responses in mice treated with the anti-cancer agent L-asparaginase, *The Journal of biological chemistry 284*, 32742-32749.
348. Tscherne, J. S., and Pestka, S. (1975) Inhibition of protein synthesis in intact HeLa cells, *Antimicrobial agents and chemotherapy 8*, 479-487.
349. Huang, M. T. (1975) Harringtonine, an inhibitor of initiation of protein biosynthesis, *Molecular pharmacology 11*, 511-519.

350. O'Brien, S., Kantarjian, H., Keating, M., Beran, M., Koller, C., Robertson, L. E., Hester, J., Rios, M. B., Andreeff, M., and Talpaz, M. (1995) Homoharringtonine therapy induces responses in patients with chronic myelogenous leukemia in late chronic phase, *Blood 86*, 3322-3326.
351. White, R. J. (2005) RNA polymerases I and III, growth control and cancer, *Nature reviews. Molecular cell biology 6*, 69-78.
352. Drygin, D., Lin, A., Bliesath, J., Ho, C. B., O'Brien, S. E., Proffitt, C., Omori, M., Haddach, M., Schwaebe, M. K., Siddiqui-Jain, A., Streiner, N., Quin, J. E., Sanij, E., Bywater, M. J., Hannan, R. D., Ryckman, D., Anderes, K., and Rice, W. G. (2011) Targeting RNA polymerase I with an oral small molecule CX-5461 inhibits ribosomal RNA synthesis and solid tumor growth, *Cancer research 71*, 1418-1430.
353. Bywater, M. J., Poortinga, G., Sanij, E., Hein, N., Peck, A., Cullinane, C., Wall, M., Cluse, L., Drygin, D., Anderes, K., Huser, N., Proffitt, C., Bliesath, J., Haddach, M., Schwaebe, M. K., Ryckman, D. M., Rice, W. G., Schmitt, C., Lowe, S. W., Johnstone, R. W., Pearson, R. B., McArthur, G. A., and Hannan, R. D. (2012) Inhibition of RNA polymerase I as a therapeutic strategy to promote cancer-specific activation of p53, *Cancer cell 22*, 51-65.
354. Jasiulionis, M. G., Luchessi, A. D., Moreira, A. G., Souza, P. P., Suenaga, A. P., Correa, M., Costa, C. A., Curi, R., and Costa-Neto, C. M. (2007) Inhibition of eukaryotic translation initiation factor 5A (eIF5A) hypusination impairs melanoma growth, *Cell biochemistry and function 25*, 109-114.
355. Park, M. H., Nishimura, K., Zanelli, C. F., and Valentini, S. R. (2010) Functional significance of eIF5A and its hypusine modification in eukaryotes, *Amino acids 38*, 491-500.
356. Martinez-Saez, E., Peg, V., Ortega-Aznar, A., Martinez-Ricarte, F., Camacho, J., Hernandez-Losa, J., Ferreres Pinas, J. C., and Ramon y Cajal, S. (2016) peIF4E as an independent prognostic factor and a potential therapeutic target in diffuse infiltrating astrocytomas, *Cancer medicine 5*, 2501-2512.
357. Signer, R. A., Qi, L., Zhao, Z., Thompson, D., Sigova, A. A., Fan, Z. P., DeMartino, G. N., Young, R. A., Sonenberg, N., and Morrison, S. J. (2016) The rate of protein synthesis in hematopoietic stem cells is limited partly by 4E-BPs, *Genes & development 30*, 1698-1703.
358. Tahmasebi, S., Jafarnejad, S. M., Tam, I. S., Gonatopoulos-Pournatzis, T., Matta-Camacho, E., Tsukumo, Y., Yanagiya, A., Li, W., Atlasi, Y., Caron, M., Braunschweig, U., Pearl, D., Khoutorsky, A., Gkogkas, C. G., Nadon, R., Bourque, G., Yang, X. J., Tian, B., Stunnenberg, H. G., Yamanaka, Y., Blencowe, B. J., Giguere, V., and Sonenberg, N. (2016) Control of embryonic stem cell self-renewal and differentiation via coordinated alternative splicing and translation of YY2, *Proceedings of the National Academy of Sciences of the United States of America*.
359. Tahmasebi, S., Alain, T., Rajasekhar, V. K., Zhang, J. P., Prager-Khoutorsky, M., Khoutorsky, A., Dogan, Y., Gkogkas, C. G., Petroulakis, E., Sylvestre, A., Ghorbani, M., Assadian, S., Yamanaka, Y., Vinagolu-Baur, J. R., Teodoro, J. G., Kim, K., Yang, X. J., and Sonenberg, N. (2014) Multifaceted regulation of somatic cell reprogramming by mRNA translational control, *Cell stem cell 14*, 606-616.
360. Zismanov, V., Chichkov, V., Colangelo, V., Jamet, S., Wang, S., Syme, A., Koromilas, A. E., and Crist, C. (2016) Phosphorylation of eIF2alpha Is a Translational Control Mechanism Regulating Muscle Stem Cell Quiescence and Self-Renewal, *Cell stem cell 18*, 79-90.
361. Llorens-Bobadilla, E., Zhao, S., Baser, A., Saiz-Castro, G., Zwadlo, K., and Martin-Villalba, A. (2015) Single-Cell Transcriptomics Reveals a Population of Dormant Neural Stem Cells that Become Activated upon Brain Injury, *Cell stem cell 17*, 329-340.
362. Sugiyama, H., Takahashi, K., Yamamoto, T., Iwasaki, M., Narita, M., Nakamura, M., Rand, T. A., Nakagawa, M., Watanabe, A., and Yamanaka, S. (2017) Nat1 promotes translation of specific proteins that induce differentiation of mouse embryonic stem cells, *Proceedings of the National Academy of Sciences of the United States of America 114*, 340-345.

363. Liberman, N., Gandin, V., Svitkin, Y. V., David, M., Virgili, G., Jaramillo, M., Holcik, M., Nagar, B., Kimchi, A., and Sonenberg, N. (2015) DAP5 associates with eIF2beta and eIF4AI to promote Internal Ribosome Entry Site driven translation, *Nucleic acids research 43*, 3764-3775.
364. Blanco, S., Bandiera, R., Popis, M., Hussain, S., Lombard, P., Aleksic, J., Sajini, A., Tanna, H., Cortes-Garrido, R., Gkatza, N., Dietmann, S., and Frye, M. (2016) Stem cell function and stress response are controlled by protein synthesis, *Nature 534*, 335-340.
365. Bell, J. B., Eckerdt, F. D., Alley, K., Magnusson, L. P., Hussain, H., Bi, Y., Arslan, A. D., Clymer, J., Alvarez, A. A., Goldman, S., Cheng, S. Y., Nakano, I., Horbinski, C., Davuluri, R. V., James, C. D., and Platanias, L. C. (2016) MNK Inhibition Disrupts Mesenchymal Glioma Stem Cells and Prolongs Survival in a Mouse Model of Glioblastoma, *Molecular cancer research : MCR 14*, 984-993.
366. Lim, S., Saw, T. Y., Zhang, M., Janes, M. R., Nacro, K., Hill, J., Lim, A. Q., Chang, C. T., Fruman, D. A., Rizzieri, D. A., Tan, S. Y., Fan, H., Chuah, C. T., and Ong, S. T. (2013) Targeting of the MNK-eIF4E axis in blast crisis chronic myeloid leukemia inhibits leukemia stem cell function, *Proceedings of the National Academy of Sciences of the United States of America 110*, E2298-2307.
367. Altman, J. K., Szilard, A., Konicek, B. W., Iversen, P. W., Kroczynska, B., Glaser, H., Sassano, A., Vakana, E., Graff, J. R., and Platanias, L. C. (2013) Inhibition of Mnk kinase activity by cercosporamide and suppressive effects on acute myeloid leukemia precursors, *Blood 121*, 3675-3681.
368. Kalluri, R. (2016) The biology and function of fibroblasts in cancer, *Nature reviews. Cancer 16*, 582-598.
369. Nishimura, K., Kumazawa, T., Kuroda, T., Katagiri, N., Tsuchiya, M., Goto, N., Furumai, R., Murayama, A., Yanagisawa, J., and Kimura, K. (2015) Perturbation of ribosome biogenesis drives cells into senescence through 5S RNP-mediated p53 activation, *Cell reports 10*, 1310-1323.
370. Laberge, R. M., Sun, Y., Orjalo, A. V., Patil, C. K., Freund, A., Zhou, L., Curran, S. C., Davalos, A. R., Wilson-Edell, K. A., Liu, S., Limbad, C., Demaria, M., Li, P., Hubbard, G. B., Ikeno, Y., Javors, M., Desprez, P. Y., Benz, C. C., Kapahi, P., Nelson, P. S., and Campisi, J. (2015) MTOR regulates the pro-tumorigenic senescence-associated secretory phenotype by promoting IL1A translation, *Nature cell biology 17*, 1049-1061.
371. Herranz, N., Gallage, S., Mellone, M., Wuestefeld, T., Klotz, S., Hanley, C. J., Raguz, S., Acosta, J. C., Innes, A. J., Banito, A., Georgilis, A., Montoya, A., Wolter, K., Dharmalingam, G., Faull, P., Carroll, T., Martinez-Barbera, J. P., Cutillas, P., Reisinger, F., Heikenwalder, M., Miller, R. A., Withers, D., Zender, L., Thomas, G. J., and Gil, J. (2015) mTOR regulates MAPKAPK2 translation to control the senescence-associated secretory phenotype, *Nature cell biology 17*, 1205-1217.
372. Martel, R. R., Klicius, J., and Galet, S. (1977) Inhibition of the immune response by rapamycin, a new antifungal antibiotic, *Canadian journal of physiology and pharmacology 55*, 48-51.
373. So, L., Lee, J., Palafox, M., Mallya, S., Woxland, C. G., Arguello, M., Truitt, M. L., Sonenberg, N., Ruggero, D., and Fruman, D. A. (2016) The 4E-BP-eIF4E axis promotes rapamycin-sensitive growth and proliferation in lymphocytes, *Science signaling 9*, ra57.
374. Araki, K., Morita, M., Bederman, A. G., Konieczny, B. T., Kissick, H. T., Sonenberg, N., and Ahmed, R. (2017) Translation is actively regulated during the differentiation of CD8+ effector T cells, *Nature immunology*.
375. Yoo, H. S., Lee, K., Na, K., Zhang, Y. X., Lim, H. J., Yi, T., Song, S. U., and Jeon, M. S. (2017) Mesenchymal stromal cells inhibit CD25 expression via the mTOR pathway to potentiate T-cell suppression, *Cell death & disease 8*, e2632.
376. Linke, M., Fritsch, S. D., Sukhbaatar, N., Hengstschlager, M., and Weichhart, T. (2017) mTORC1 and mTORC2 as regulators of cell metabolism in immunity, *FEBS letters*.
377. Welte, T., Kim, I. S., Tian, L., Gao, X., Wang, H., Li, J., Holdman, X. B., Herschkowitz, J. I., Pond, A., Xie, G., Kurley, S., Nguyen, T., Liao, L., Dobrolecki, L. E., Pang, L., Mo, Q., Edwards, D. P., Huang, S.,

Xin, L., Xu, J., Li, Y., Lewis, M. T., Wang, T., Westbrook, T. F., Rosen, J. M., and Zhang, X. H. (2016) Oncogenic mTOR signalling recruits myeloid-derived suppressor cells to promote tumour initiation, *Nature cell biology 18*, 632-644.

378. Chen, X., Zhang, Z., Bi, Y., Fu, Z., Gong, P., Li, Y., Yu, Q., Jia, A., Wang, J., Xue, L., Yang, H., and Liu, G. (2016) mTOR signaling disruption from myeloid-derived suppressive cells protects against immune-mediated hepatic injury through the HIF1alpha-dependent glycolytic pathway, *Journal of leukocyte biology 100*, 1349-1362.
379. Zhang, C., Wang, S., Li, J., Zhang, W., Zheng, L., Yang, C., Zhu, T., and Rong, R. (2017) The mTOR signal regulates myeloid-derived suppressor cells differentiation and immunosuppressive function in acute kidney injury, *Cell death & disease 8*, e2695.
380. Bjur, E., Larsson, O., Yurchenko, E., Zheng, L., Gandin, V., Topisirovic, I., Li, S., Wagner, C. R., Sonenberg, N., and Piccirillo, C. A. (2013) Distinct translational control in CD4+ T cell subsets, *PLoS genetics 9*, e1003494.
381. Andersson, K., and Sundler, R. (2006) Posttranscriptional regulation of TNFalpha expression via eukaryotic initiation factor 4E (eIF4E) phosphorylation in mouse macrophages, *Cytokine 33*, 52-57.
382. Rowlett, R. M., Chrestensen, C. A., Nyce, M., Harp, M. G., Pelo, J. W., Cominelli, F., Ernst, P. B., Pizarro, T. T., Sturgill, T. W., and Worthington, M. T. (2008) MNK kinases regulate multiple TLR pathways and innate proinflammatory cytokines in macrophages, *American journal of physiology. Gastrointestinal and liver physiology 294*, G452-459.
383. Salvador-Bernaldez, M., Mateus, S. B., Del Barco Barrantes, I., Arthur, S. C., Martinez, A. C., Nebreda, A. R., and Salvador, J. M. (2017) p38alpha regulates cytokine-induced IFNgamma secretion via the Mnk1/eIF4E pathway in Th1 cells, *Immunology and cell biology*.
384. Herdy, B., Jaramillo, M., Svitkin, Y. V., Rosenfeld, A. B., Kobayashi, M., Walsh, D., Alain, T., Sean, P., Robichaud, N., Topisirovic, I., Furic, L., Dowling, R. J., Sylvestre, A., Rong, L., Colina, R., Costa-Mattioli, M., Fritz, J. H., Olivier, M., Brown, E., Mohr, I., and Sonenberg, N. (2012) Translational control of the activation of transcription factor NF-kappaB and production of type I interferon by phosphorylation of the translation factor eIF4E, *Nature immunology 13*, 543-550.
385. Gorentla, B. K., Krishna, S., Shin, J., Inoue, M., Shinohara, M. L., Grayson, J. M., Fukunaga, R., and Zhong, X. P. (2013) Mnk1 and 2 Are Dispensable for T Cell Development and Activation but Important for the Pathogenesis of Experimental Autoimmune Encephalomyelitis, *Journal of Immunology 190*, 1026-1037.
386. Yoshizawa, A., Fukuoka, J., Shimizu, S., Shilo, K., Franks, T. J., Hewitt, S. M., Fujii, T., Cordon-Cardo, C., Jen, J., and Travis, W. D. (2010) Overexpression of phospho-eIF4E is associated with survival through AKT pathway in non-small cell lung cancer, *Clinical cancer research : an official journal of the American Association for Cancer Research 16*, 240-248.
387. Ferrandiz-Pulido, C., Masferrer, E., Toll, A., Hernandez-Losa, J., Mojal, S., Pujol, R. M., Ramon y Cajal, S., de Torres, I., and Garcia-Patos, V. (2013) mTOR signaling pathway in penile squamous cell carcinoma: pmTOR and peIF4E over expression correlate with aggressive tumor behavior, *The Journal of urology 190*, 2288-2295.
388. Zheng, J., Li, J., Xu, L., Xie, G., Wen, Q., Luo, J., Li, D., Huang, D., and Fan, S. (2014) Phosphorylated Mnk1 and eIF4E Are Associated with Lymph Node Metastasis and Poor Prognosis of Nasopharyngeal Carcinoma, *PloS one 9*, e89220.
389. Pettersson, F., Yau, C., Dobocan, M. C., Culjkovic-Kraljacic, B., Retrouvey, H., Puckett, R., Flores, L. M., Krop, I. E., Rousseau, C., Cocolakis, E., Borden, K. L., Benz, C. C., and Miller, W. H., Jr. (2011) Ribavirin treatment effects on breast cancers overexpressing eIF4E, a biomarker with prognostic specificity for luminal B-type breast cancer, *Clinical cancer research : an official journal of the American Association for Cancer Research 17*, 2874-2884.

390. Friedl, P., and Alexander, S. (2011) Cancer invasion and the microenvironment: plasticity and reciprocity, *Cell 147*, 992-1009.
391. Lochter, A., Galosy, S., Muschler, J., Freedman, N., Werb, Z., and Bissell, M. J. (1997) Matrix metalloproteinase stromelysin-1 triggers a cascade of molecular alterations that leads to stable epithelial-to-mesenchymal conversion and a premalignant phenotype in mammary epithelial cells, *The Journal of cell biology 139*, 1861-1872.
392. Grzmil, M., Morin, P., Jr., Lino, M. M., Merlo, A., Frank, S., Wang, Y., Moncayo, G., and Hemmings, B. A. (2011) MAP kinase-interacting kinase 1 regulates SMAD2-dependent TGF-beta signaling pathway in human glioblastoma, *Cancer research 71*, 2392-2402.
393. DaSilva, J., Xu, L., Kim, H. J., Miller, W. T., and Bar-Sagi, D. (2006) Regulation of sprouty stability by Mnk1-dependent phosphorylation, *Molecular and cellular biology 26*, 1898-1907.
394. Hefner, Y., Borsch-Haubold, A. G., Murakami, M., Wilde, J. I., Pasquet, S., Schieltz, D., Ghomashchi, F., Yates, J. R., 3rd, Armstrong, C. G., Paterson, A., Cohen, P., Fukunaga, R., Hunter, T., Kudo, I., Watson, S. P., and Gelb, M. H. (2000) Serine 727 phosphorylation and activation of cytosolic phospholipase A2 by MNK1-related protein kinases, *The Journal of biological chemistry 275*, 37542-37551.
395. Guil, S., Long, J. C., and Caceres, J. F. (2006) hnRNP A1 relocalization to the stress granules reflects a role in the stress response, *Molecular and cellular biology 26*, 5744-5758.
396. Buxade, M., Parra, J. L., Rousseau, S., Shpiro, N., Marquez, R., Morrice, N., Bain, J., Espel, E., and Proud, C. G. (2005) The Mnks are novel components in the control of TNF alpha biosynthesis and phosphorylate and regulate hnRNP A1, *Immunity 23*, 177-189.
397. Li, L., Price, J. E., Fan, D., Zhang, R. D., Bucana, C. D., and Fidler, I. J. (1989) Correlation of growth capacity of human tumor cells in hard agarose with their in vivo proliferative capacity at specific metastatic sites, *Journal of the National Cancer Institute 81*, 1406-1412.
398. Byers, H. R., Etoh, T., Doherty, J. R., Sober, A. J., and Mihm, M. C., Jr. (1991) Cell migration and actin organization in cultured human primary, recurrent cutaneous and metastatic melanoma. Time-lapse and image analysis, *The American journal of pathology 139*, 423-435.
399. Albini, A., Iwamoto, Y., Kleinman, H. K., Martin, G. R., Aaronson, S. A., Kozlowski, J. M., and McEwan, R. N. (1987) A rapid in vitro assay for quantitating the invasive potential of tumor cells, *Cancer research 47*, 3239-3245.
400. Sommers, C. L., Byers, S. W., Thompson, E. W., Torri, J. A., and Gelmann, E. P. (1994) Differentiation state and invasiveness of human breast cancer cell lines, *Breast cancer research and treatment 31*, 325-335.
401. Huang da, W., Sherman, B. T., and Lempicki, R. A. (2009) Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources, *Nature protocols 4*, 44-57.
402. Huang da, W., Sherman, B. T., and Lempicki, R. A. (2009) Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists, *Nucleic acids research 37*, 1-13.
403. Kalluri, R., and Weinberg, R. A. (2009) The basics of epithelial-mesenchymal transition, *The Journal of clinical investigation 119*, 1420-1428.
404. Xu, J., Lamouille, S., and Derynck, R. (2009) TGF-beta-induced epithelial to mesenchymal transition, *Cell research 19*, 156-172.
405. Zeisberg, M., and Neilson, E. G. (2009) Biomarkers for epithelial-mesenchymal transitions, *The Journal of clinical investigation 119*, 1429-1437.
406. Zhang, Y. E. (2009) Non-Smad pathways in TGF-beta signaling, *Cell research 19*, 128-139.
407. Bakin, A. V., Rinehart, C., Tomlinson, A. K., and Arteaga, C. L. (2002) p38 mitogen-activated protein kinase is required for TGFbeta-mediated fibroblastic transdifferentiation and cell migration, *Journal of cell science 115*, 3193-3206.

408. Inman, G. J., Nicolas, F. J., Callahan, J. F., Harling, J. D., Gaster, L. M., Reith, A. D., Laping, N. J., and Hill, C. S. (2002) SB-431542 is a potent and specific inhibitor of transforming growth factor-beta superfamily type I activin receptor-like kinase (ALK) receptors ALK4, ALK5, and ALK7, *Molecular pharmacology 62*, 65-74.
409. Imamura, T., Hikita, A., and Inoue, Y. (2012) The roles of TGF-beta signaling in carcinogenesis and breast cancer metastasis, *Breast Cancer 19*, 118-124.
410. Scheper, G. C., Morrice, N. A., Kleijn, M., and Proud, C. G. (2001) The mitogen-activated protein kinase signal-integrating kinase Mnk2 is a eukaryotic initiation factor 4E kinase with high levels of basal activity in mammalian cells, *Molecular and cellular biology 21*, 743-754.
411. Aslakson, C. J., and Miller, F. R. (1992) Selective events in the metastatic process defined by analysis of the sequential dissemination of subpopulations of a mouse mammary tumor, *Cancer research 52*, 1399-1405.
412. Guy, C. T., Cardiff, R. D., and Muller, W. J. (1992) Induction of mammary tumors by expression of polyomavirus middle T oncogene: a transgenic mouse model for metastatic disease, *Molecular and cellular biology 12*, 954-961.
413. Dilworth, S. M. (2002) Polyoma virus middle T antigen and its role in identifying cancer-related molecules, *Nature reviews. Cancer 2*, 951-956.
414. Franci, C., Takkunen, M., Dave, N., Alameda, F., Gomez, S., Rodriguez, R., Escriva, M., Montserrat-Sentis, B., Baro, T., Garrido, M., Bonilla, F., Virtanen, I., and Garcia de Herreros, A. (2006) Expression of Snail protein in tumor-stroma interface, *Oncogene 25*, 5134-5144.
415. Heppner, K. J., Matrisian, L. M., Jensen, R. A., and Rodgers, W. H. (1996) Expression of most matrix metalloproteinase family members in breast cancer represents a tumor-induced host response, *The American journal of pathology 149*, 273-282.
416. Bronson, M. W., Hillenmeyer, S., Park, R. W., and Brodsky, A. S. (2010) Estrogen coordinates translation and transcription, revealing a role for NRSF in human breast cancer cells, *Mol Endocrinol 24*, 1120-1135.
417. Muzzey, D., Sherlock, G., and Weissman, J. S. (2014) Extensive and coordinated control of allele-specific expression by both transcription and translation in Candida albicans, *Genome research 24*, 963-973.
418. Tamarkin-Ben-Harush, A., Schechtman, E., and Dikstein, R. (2014) Co-occurrence of transcription and translation gene regulatory features underlies coordinated mRNA and protein synthesis, *BMC genomics 15*, 688.
419. He, T. C., Sparks, A. B., Rago, C., Hermeking, H., Zawel, L., da Costa, L. T., Morin, P. J., Vogelstein, B., and Kinzler, K. W. (1998) Identification of c-MYC as a target of the APC pathway, *Science 281*, 1509-1512.
420. West, M. J., Sullivan, N. F., and Willis, A. E. (1995) Translational upregulation of the c-myc oncogene in Bloom's syndrome cell lines, *Oncogene 11*, 2515-2524.
421. Robert, F., and Pelletier, J. (2009) Translation initiation: a critical signalling node in cancer, *Expert opinion on therapeutic targets 13*, 1279-1293.
422. Beggs, J. E., Tian, S., Jones, G. G., Xie, J., Iadevaia, V., Jenei, V., Thomas, G., and Proud, C. G. (2015) The MAP kinase-interacting kinases regulate cell migration, vimentin expression and eIF4E/CYFIP1 binding, *The Biochemical journal 467*, 63-76.
423. Kumar, K., Chow, C. R., Ebine, K., Arslan, A. D., Kwok, B., Bentrem, D. J., Eckerdt, F. D., Platanias, L. C., and Munshi, H. G. (2016) Differential Regulation of ZEB1 and EMT by MAPK-Interacting Protein Kinases (MNK) and eIF4E in Pancreatic Cancer, *Molecular cancer research : MCR 14*, 216-227.
424. Carter, J. H., Deddens, J. A., Spaulding Iv, N. R., Lucas, D., Colligan, B. M., Lewis, T. G., Hawkins, E., Jones, J., Pemberton, J. O., Douglass, L. E., and Graff, J. R. (2016) Phosphorylation of eIF4E serine

209 is associated with tumour progression and reduced survival in malignant melanoma, *British journal of cancer 114*, 444-453.
425. Yin, B., Morgan, K., Hasz, D. E., Mao, Z., and Largaespada, D. A. (2006) Nfl gene inactivation in acute myeloid leukemia cells confers cytarabine resistance through MAPK and mTOR pathways, *Leukemia 20*, 151-154.
426. Wen, Q., Wang, W., Luo, J., Chu, S., Chen, L., Xu, L., Zang, H., Alnemah, M. M., Ma, J., and Fan, S. (2016) CGP57380 enhances efficacy of RAD001 in non-small cell lung cancer through abrogating mTOR inhibition-induced phosphorylation of eIF4E and activating mitochondrial apoptotic pathway, *Oncotarget 7*, 27787-27801.
427. (2006) RIPA buffer (05-01), *Cold Spring Harbor Protocols 2006*, pdb.rec10617.
428. Ling, C., Su, V. M., Zuo, D., and Muller, W. J. (2012) Loss of the 14-3-3sigma tumor suppressor is a critical event in ErbB2-mediated tumor progression, *Cancer discovery 2*, 68-81.
429. Larsson, O., Sonenberg, N., and Nadon, R. (2011) anota: Analysis of differential translation in genome-wide studies, *Bioinformatics 27*, 1440-1441.
430. Larsson, O., Sonenberg, N., and Nadon, R. (2010) Identification of differential translation in genome wide studies, *Proceedings of the National Academy of Sciences of the United States of America 107*, 21487-21492.
431. Huang da, W., Sherman, B. T., Zheng, X., Yang, J., Imamichi, T., Stephens, R., and Lempicki, R. A. (2009) Extracting biological meaning from large gene lists with DAVID, *Current protocols in bioinformatics / editoral board, Andreas D. Baxevanis ... [et al.] Chapter 13*, Unit 13 11.
432. Hanahan, D., and Coussens, L. M. (2012) Accessories to the crime: functions of cells recruited to the tumor microenvironment, *Cancer cell 21*, 309-322.
433. Kouidhi, S., Elgaaied, A. B., and Chouaib, S. (2017) Impact of Metabolism on T-Cell Differentiation and Function and Cross Talk with Tumor Microenvironment, *Frontiers in immunology 8*, 270.
434. Sidrauski, C., Acosta-Alvear, D., Khoutorsky, A., Vedantham, P., Hearn, B. R., Li, H., Gamache, K., Gallagher, C. M., Ang, K. K., Wilson, C., Okreglak, V., Ashkenazi, A., Hann, B., Nader, K., Arkin, M. R., Renslo, A. R., Sonenberg, N., and Walter, P. (2013) Pharmacological brake-release of mRNA translation enhances cognitive memory, *eLife 2*, e00498.
435. Gkogkas, C. G., Khoutorsky, A., Ran, I., Rampakakis, E., Nevarko, T., Weatherill, D. B., Vasuta, C., Yee, S., Truitt, M., Dallaire, P., Major, F., Lasko, P., Ruggero, D., Nader, K., Lacaille, J. C., and Sonenberg, N. (2013) Autism-related deficits via dysregulated eIF4E-dependent translational control, *Nature 493*, 371-377.
436. Gkogkas, C. G., Khoutorsky, A., Cao, R., Jafarnejad, S. M., Prager-Khoutorsky, M., Giannakas, N., Kaminari, A., Fragkouli, A., Nader, K., Price, T. J., Konicek, B. W., Graff, J. R., Tzinia, A. K., Lacaille, J. C., and Sonenberg, N. (2014) Pharmacogenetic inhibition of eIF4E-dependent Mmp9 mRNA translation reverses fragile X syndrome-like phenotypes, *Cell reports 9*, 1742-1755.
437. Khoutorsky, A., Bonin, R. P., Sorge, R. E., Gkogkas, C. G., Pawlowski, S. A., Jafarnejad, S. M., Pitcher, M. H., Alain, T., Perez-Sanchez, J., Salter, E. W., Martin, L., Ribeiro-da-Silva, A., De Koninck, Y., Cervero, F., Mogil, J. S., and Sonenberg, N. (2015) Translational control of nociception via 4E-binding protein 1, *eLife 4*.
438. Aguilar-Valles, A., Matta-Camacho, E., Khoutorsky, A., Gkogkas, C., Nader, K., Lacaille, J. C., and Sonenberg, N. (2015) Inhibition of Group I Metabotropic Glutamate Receptors Reverses Autistic-Like Phenotypes Caused by Deficiency of the Translation Repressor eIF4E Binding Protein 2, *The Journal of neuroscience : the official journal of the Society for Neuroscience 35*, 11125-11132.
439. Nagaleekar, V. K., Sabio, G., Aktan, I., Chant, A., Howe, I. W., Thornton, T. M., Benoit, P. J., Davis, R. J., Rincon, M., and Boyson, J. E. (2011) Translational control of NKT cell cytokine production by p38 MAPK, *J Immunol 186*, 4140-4146.

440. Joshi, S., and Platanias, L. C. (2012) Mnk Kinases in Cytokine Signaling and Regulation of Cytokine Responses, *Biomolecular concepts 3*, 127-139.
441. Colina, R., Costa-Mattioli, M., Dowling, R. J., Jaramillo, M., Tai, L. H., Breitbach, C. J., Martineau, Y., Larsson, O., Rong, L., Svitkin, Y. V., Makrigiannis, A. P., Bell, J. C., and Sonenberg, N. (2008) Translational control of the innate immune response through IRF-7, *Nature 452*, 323-328.
442. Di Mitri, D., Sambucci, M., Loiarro, M., De Bardi, M., Volpe, E., Cencioni, M. T., Gasperini, C., Centonze, D., Sette, C., Akbar, A. N., Borsellino, G., and Battistini, L. (2015) The p38 mitogen-activated protein kinase cascade modulates T helper type 17 differentiation and functionality in multiple sclerosis, *Immunology 146*, 251-263.
443. Noske, A., Lindenberg, J. L., Darb-Esfahani, S., Weichert, W., Buckendahl, A. C., Roske, A., Sehouli, J., Dietel, M., and Denkert, C. (2008) Activation of mTOR in a subgroup of ovarian carcinomas: correlation with p-eIF-4E and prognosis, *Oncology reports 20*, 1409-1417.
444. Rautela, J., Baschuk, N., Slaney, C. Y., Jayatilleke, K. M., Xiao, K., Bidwell, B. N., Lucas, E. C., Hawkins, E. D., Lock, P., Wong, C. S., Chen, W., Anderson, R. L., Hertzog, P. J., Andrews, D. M., Moller, A., and Parker, B. S. (2015) Loss of Host Type-I IFN Signaling Accelerates Metastasis and Impairs NK-cell Antitumor Function in Multiple Models of Breast Cancer, *Cancer immunology research 3*, 1207-1217.
445. Kowanetz, M., Wu, X., Lee, J., Tan, M., Hagenbeek, T., Qu, X., Yu, L., Ross, J., Korsisaari, N., Cao, T., Bou-Reslan, H., Kallop, D., Weimer, R., Ludlam, M. J., Kaminker, J. S., Modrusan, Z., van Bruggen, N., Peale, F. V., Carano, R., Meng, Y. G., and Ferrara, N. (2010) Granulocyte-colony stimulating factor promotes lung metastasis through mobilization of Ly6G+Ly6C+ granulocytes, *Proceedings of the National Academy of Sciences of the United States of America 107*, 21248-21255.
446. Hanks, B. A., Holtzhausen, A., Evans, K. S., Jamieson, R., Gimpel, P., Campbell, O. M., Hector-Greene, M., Sun, L., Tewari, A., George, A., Starr, M., Nixon, A., Augustine, C., Beasley, G., Tyler, D. S., Osada, T., Morse, M. A., Ling, L., Lyerly, H. K., and Blobe, G. C. (2013) Type III TGF-beta receptor downregulation generates an immunotolerant tumor microenvironment, *The Journal of clinical investigation 123*, 3925-3940.
447. Granot, Z., and Jablonska, J. (2015) Distinct Functions of Neutrophil in Cancer and Its Regulation, *Mediators of inflammation 2015*, 701067.
448. Graff, J. R., Konicek, B. W., Vincent, T. M., Lynch, R. L., Monteith, D., Weir, S. N., Schwier, P., Capen, A., Goode, R. L., Dowless, M. S., Chen, Y., Zhang, H., Sissons, S., Cox, K., McNulty, A. M., Parsons, S. H., Wang, T., Sams, L., Geeganage, S., Douglass, L. E., Neubauer, B. L., Dean, N. M., Blanchard, K., Shou, J., Stancato, L. F., Carter, J. H., and Marcusson, E. G. (2007) Therapeutic suppression of translation initiation factor eIF4E expression reduces tumor growth without toxicity, *The Journal of clinical investigation 117*, 2638-2648.
449. Fortin, C. F., Mayer, T. Z., Cloutier, A., and McDonald, P. P. (2013) Translational control of human neutrophil responses by MNK1, *Journal of leukocyte biology 94*, 693-703.
450. Kosciuczuk, E. M., Saleiro, D., Kroczynska, B., Beauchamp, E. M., Eckerdt, F., Blyth, G. T., Abedin, S. M., Giles, F. J., Altman, J. K., and Platanias, L. C. (2016) Merestinib blocks Mnk kinase activity in acute myeloid leukemia progenitors and exhibits antileukemic effects in vitro and in vivo, *Blood 128*, 410-414.
451. Wu, W., Bi, C., Credille, K. M., Manro, J. R., Peek, V. L., Donoho, G. P., Yan, L., Wijsman, J. A., Yan, S. B., and Walgren, R. A. (2013) Inhibition of tumor growth and metastasis in non-small cell lung cancer by LY2801653, an inhibitor of several oncokinases, including MET, *Clinical cancer research : an official journal of the American Association for Cancer Research 19*, 5699-5710.
452. Yan, S. B., Peek, V. L., Ajamie, R., Buchanan, S. G., Graff, J. R., Heidler, S. A., Hui, Y. H., Huss, K. L., Konicek, B. W., Manro, J. R., Shih, C., Stewart, J. A., Stewart, T. R., Stout, S. L., Uhlik, M. T., Um, S. L., Wang, Y., Wu, W., Yan, L., Yang, W. J., Zhong, B., and Walgren, R. A. (2013) LY2801653 is an

orally bioavailable multi-kinase inhibitor with potent activity against MET, MST1R, and other oncoproteins, and displays anti-tumor activities in mouse xenograft models, *Investigational new drugs 31*, 833-844.

453. Tabaries, S., Ouellet, V., Hsu, B. E., Annis, M. G., Rose, A. A., Meunier, L., Carmona, E., Tam, C. E., Mes-Masson, A. M., and Siegel, P. M. (2015) Granulocytic immune infiltrates are essential for the efficient formation of breast cancer liver metastases, *Breast cancer research : BCR 17*, 45.
454. Granot, Z., Henke, E., Comen, E. A., King, T. A., Norton, L., and Benezra, R. (2011) Tumor entrained neutrophils inhibit seeding in the premetastatic lung, *Cancer cell 20*, 300-314.
455. Coffelt, S. B., Kersten, K., Doornebal, C. W., Weiden, J., Vrijland, K., Hau, C. S., Verstegen, N. J., Ciampricotti, M., Hawinkels, L. J., Jonkers, J., and de Visser, K. E. (2015) IL-17-producing gammadelta T cells and neutrophils conspire to promote breast cancer metastasis, *Nature 522*, 345-348.
456. Mouchemore, K. A., Anderson, R. L., and Hamilton, J. A. (2017) Neutrophils, G-CSF and their contribution to breast cancer metastasis, *The FEBS journal*.
457. McCracken, J. M., and Allen, L. A. (2014) Regulation of human neutrophil apoptosis and lifespan in health and disease, *Journal of cell death 7*, 15-23.
458. Joshi, S., and Platanias, L. C. (2014) Mnk kinase pathway: Cellular functions and biological outcomes, *World journal of biological chemistry 5*, 321-333.
459. Webster, K. R., Goel, V. K., Hung, I. N. J., Parker, G. S., Staunton, J., Neal, M., Molter, J., Chiang, G. G., Jessen, K. A., Wegerski, C. J., Sperry, S., Huang, V., Chen, J., Thompson, P. A., Appleman, J. R., Webber, S. E., Sprengeler, P. A., and Reich, S. H. (2015) eFT508, a Potent and Selective Mitogen-Activated Protein Kinase Interacting Kinase (MNK) 1 and 2 Inhibitor, Is Efficacious in Preclinical Models of Diffuse Large B-Cell Lymphoma (DLBCL), *Blood 126*.
460. Kawada, I., Hasina, R., Arif, Q., Mueller, J., Smithberger, E., Husain, A. N., Vokes, E. E., and Salgia, R. (2014) Dramatic antitumor effects of the dual MET/RON small-molecule inhibitor LY2801653 in non-small cell lung cancer, *Cancer research 74*, 884-895.
461. Granger, J. M., and Kontoyiannis, D. P. (2009) Etiology and outcome of extreme leukocytosis in 758 nonhematologic cancer patients: a retrospective, single-institution study, *Cancer 115*, 3919-3923.
462. Sharma, P., Hu-Lieskovan, S., Wargo, J. A., and Ribas, A. (2017) Primary, Adaptive, and Acquired Resistance to Cancer Immunotherapy, *Cell 168*, 707-723.
463. TheCancerGenomeAtlasNetwork. (2015) Genomic Classification of Cutaneous Melanoma, *Cell 161*, 1681-1696.
464. Lock, R., Ingraham, R., Maertens, O., Miller, A. L., Weledji, N., Legius, E., Konicek, B. M., Yan, S. C., Graff, J. R., and Cichowski, K. (2016) Cotargeting MNK and MEK kinases induces the regression of NF1-mutant cancers, *The Journal of clinical investigation 126*, 2181-2190.
465. Shi, Y., Frost, P., Hoang, B., Yang, Y., Fukunaga, R., Gera, J., and Lichtenstein, A. (2013) MNK kinases facilitate c-myc IRES activity in rapamycin-treated multiple myeloma cells, *Oncogene 32*, 190-197.
466. Conrad, P. W., Rust, R. T., Han, J., Millhorn, D. E., and Beitner-Johnson, D. (1999) Selective activation of p38alpha and p38gamma by hypoxia. Role in regulation of cyclin D1 by hypoxia in PC12 cells, *The Journal of biological chemistry 274*, 23570-23576.
467. Goetz, C., Everson, R. G., Zhang, L. C., and Gromeier, M. (2010) MAPK signal-integrating kinase controls cap-independent translation and cell type-specific cytotoxicity of an oncolytic poliovirus, *Molecular therapy : the journal of the American Society of Gene Therapy 18*, 1937-1946.
468. Brown, M. C., Bryant, J. D., Dobrikova, E. Y., Shveygert, M., Bradrick, S. S., Chandramohan, V., Bigner, D. D., and Gromeier, M. (2014) Induction of viral, 7-methyl-guanosine cap-independent translation and oncolysis by mitogen-activated protein kinase-interacting kinase-mediated effects on the serine/arginine-rich protein kinase, *Journal of virology 88*, 13135-13148.

469. Waskiewicz, A. J., Flynn, A., Proud, C. G., and Cooper, J. A. (1997) Mitogen-activated protein kinases activate the serine/threonine kinases Mnk1 and Mnk2, *The EMBO journal 16*, 1909-1920.
470. Landon, A. L., Muniandy, P. A., Shetty, A. C., Lehrmann, E., Volpon, L., Houng, S., Zhang, Y., Dai, B., Peroutka, R., Mazan-Mamczarz, K., Steinhardt, J., Mahurkar, A., Becker, K. G., Borden, K. L., and Gartenhaus, R. B. (2014) MNKs act as a regulatory switch for eIF4E1 and eIF4E3 driven mRNA translation in DLBCL, *Nature communications 5*, 5413.
471. Osborne, M. J., Volpon, L., Kornblatt, J. A., Culjkovic-Kraljacic, B., Baguet, A., and Borden, K. L. (2013) eIF4E3 acts as a tumor suppressor by utilizing an atypical mode of methyl-7-guanosine cap recognition, *Proceedings of the National Academy of Sciences of the United States of America 110*, 3877-3882.
472. Muller, D., Lasfargues, C., El Khawand, S., Alard, A., Schneider, R. J., Bousquet, C., Pyronnet, S., and Martineau, Y. (2013) 4E-BP restrains eIF4E phosphorylation, *Translation (Austin) 1*, e25819.
473. Shveygert, M., Kaiser, C., Bradrick, S. S., and Gromeier, M. (2010) Regulation of eukaryotic initiation factor 4E (eIF4E) phosphorylation by mitogen-activated protein kinase occurs through modulation of Mnk1-eIF4G interaction, *Molecular and cellular biology 30*, 5160-5167.
474. Ueda, T., Watanabe-Fukunaga, R., Fukuyama, H., Nagata, S., and Fukunaga, R. (2004) Mnk2 and Mnk1 are essential for constitutive and inducible phosphorylation of eukaryotic initiation factor 4E but not for cell growth or development, *Molecular and cellular biology 24*, 6539-6549.
475. Chen, Y. J., Tan, B. C., Cheng, Y. Y., Chen, J. S., and Lee, S. C. (2010) Differential regulation of CHOP translation by phosphorylated eIF4E under stress conditions, *Nucleic acids research 38*, 764-777.
476. Chapat, C., Jafarnejad, S. M., Matta-Camacho, E., Hesketh, G. G., Gelbart, I. A., Attig, J., Gkogkas, C. G., Alain, T., Stern-Ginossar, N., Fabian, M. R., Gingras, A. C., Duchaine, T. F., and Sonenberg, N. (2017) Cap-binding protein 4EHP effects translation silencing by microRNAs, *Proceedings of the National Academy of Sciences of the United States of America 114*, 5425-5430.
477. Rau, M., Ohlmann, T., Morley, S. J., and Pain, V. M. (1996) A reevaluation of the cap-binding protein, eIF4E, as a rate-limiting factor for initiation of translation in reticulocyte lysate, *The Journal of biological chemistry 271*, 8983-8990.
478. Hiremath, L. S., Hiremath, S. T., Rychlik, W., Joshi, S., Domier, L. L., and Rhoads, R. E. (1989) In vitro synthesis, phosphorylation, and localization on 48 S initiation complexes of human protein synthesis initiation factor 4E, *The Journal of biological chemistry 264*, 1132-1138.
479. Archer, S. K., Shirokikh, N. E., Beilharz, T. H., and Preiss, T. (2016) Dynamics of ribosome scanning and recycling revealed by translation complex profiling, *Nature 535*, 570-574.
480. Zhang, Y. Y., Mei, Z. Q., Wu, J. W., and Wang, Z. X. (2008) Enzymatic activity and substrate specificity of mitogen-activated protein kinase p38alpha in different phosphorylation states, *The Journal of biological chemistry 283*, 26591-26601.
481. Li, Y., Yue, P., Deng, X., Ueda, T., Fukunaga, R., Khuri, F. R., and Sun, S. Y. (2010) Protein phosphatase 2A negatively regulates eukaryotic initiation factor 4E phosphorylation and eIF4F assembly through direct dephosphorylation of Mnk and eIF4E, *Neoplasia 12*, 848-855.
482. Nho, R. S., and Peterson, M. (2011) Eukaryotic translation initiation factor 4E binding protein 1 (4EBP-1) function is suppressed by Src and protein phosphatase 2A (PP2A) on extracellular matrix, *The Journal of biological chemistry 286*, 31953-31965.
483. Hertz, E. P. T., Kruse, T., Davey, N. E., Lopez-Mendez, B., Sigurethsson, J. O., Montoya, G., Olsen, J. V., and Nilsson, J. (2016) A Conserved Motif Provides Binding Specificity to the PP2A-B56 Phosphatase, *Molecular cell 63*, 686-695.
484. Frederickson, R. M., Mushynski, W. E., and Sonenberg, N. (1992) Phosphorylation of translation initiation factor eIF-4E is induced in a ras-dependent manner during nerve growth factor-mediated PC12 cell differentiation, *Molecular and cellular biology 12*, 1239-1247.

485. Rychlik, W., Russ, M. A., and Rhoads, R. E. (1987) Phosphorylation site of eukaryotic initiation factor 4E, *The Journal of biological chemistry 262*, 10434-10437.
486. Joshi, B., Cai, A. L., Keiper, B. D., Minich, W. B., Mendez, R., Beach, C. M., Stepinski, J., Stolarski, R., Darzynkiewicz, E., and Rhoads, R. E. (1995) Phosphorylation of eukaryotic protein synthesis initiation factor 4E at Ser-209, *The Journal of biological chemistry 270*, 14597-14603.
487. Flynn, A., and Proud, C. G. (1995) Serine 209, not serine 53, is the major site of phosphorylation in initiation factor eIF-4E in serum-treated Chinese hamster ovary cells, *The Journal of biological chemistry 270*, 21684-21688.
488. Joshi-Barve, S., Rychlik, W., and Rhoads, R. E. (1990) Alteration of the major phosphorylation site of eukaryotic protein synthesis initiation factor 4E prevents its association with the 48 S initiation complex, *The Journal of biological chemistry 265*, 2979-2983.
489. De Benedetti, A., and Rhoads, R. E. (1990) Overexpression of eukaryotic protein synthesis initiation factor 4E in HeLa cells results in aberrant growth and morphology, *Proceedings of the National Academy of Sciences of the United States of America 87*, 8212-8216.
490. Koromilas, A. E., Lazaris-Karatzas, A., and Sonenberg, N. (1992) mRNAs containing extensive secondary structure in their 5' non-coding region translate efficiently in cells overexpressing initiation factor eIF-4E, *The EMBO journal 11*, 4153-4158.
491. Jagus, R., Huang, W., Hiremath, L. S., Stern, B. D., and Rhoads, R. E. (1993) Mechanism of action of developmentally regulated sea urchin inhibitor of eIF-4, *Developmental genetics 14*, 412-423.
492. Morley, S. J., and Naegele, S. (2002) Phosphorylation of eukaryotic initiation factor (eIF) 4E is not required for de novo protein synthesis following recovery from hypertonic stress in human kidney cells, *The Journal of biological chemistry 277*, 32855-32859.
493. McKendrick, L., Morley, S. J., Pain, V. M., Jagus, R., and Joshi, B. (2001) Phosphorylation of eukaryotic initiation factor 4E (eIF4E) at Ser209 is not required for protein synthesis in vitro and in vivo, *European journal of biochemistry / FEBS 268*, 5375-5385.
494. Reiling, J. H., Doepfner, K. T., Hafen, E., and Stocker, H. (2005) Diet-dependent effects of the Drosophila Mnk1/Mnk2 homolog Lk6 on growth via eIF4E, *Current biology : CB 15*, 24-30.
495. Wculek, S. K., and Malanchi, I. (2015) Neutrophils support lung colonization of metastasis-initiating breast cancer cells, *Nature 528*, 413-417.
496. Cencic, R., Carrier, M., Trnkus, A., Porco, J. A., Jr., Minden, M., and Pelletier, J. (2010) Synergistic effect of inhibiting translation initiation in combination with cytotoxic agents in acute myelogenous leukemia cells, *Leukemia research 34*, 535-541.
497. Bordeleau, M. E., Cencic, R., Lindqvist, L., Oberer, M., Northcote, P., Wagner, G., and Pelletier, J. (2006) RNA-mediated sequestration of the RNA helicase eIF4A by Pateamine A inhibits translation initiation, *Chemistry & biology 13*, 1287-1295.
498. Chapman, N. M., and Chi, H. (2014) mTOR signaling, Tregs and immune modulation, *Immunotherapy 6*, 1295-1311.
499. Stallone, G., Schena, A., Infante, B., Di Paolo, S., Loverre, A., Maggio, G., Ranieri, E., Gesualdo, L., Schena, F. P., and Grandaliano, G. (2005) Sirolimus for Kaposi's sarcoma in renal-transplant recipients, *The New England journal of medicine 352*, 1317-1323.
500. Martin, D., Nguyen, Q., Molinolo, A., and Gutkind, J. S. (2014) Accumulation of dephosphorylated 4EBP after mTOR inhibition with rapamycin is sufficient to disrupt paracrine transformation by the KSHV vGPCR oncogene, *Oncogene 33*, 2405-2412.
501. Guthrie, L. N., Abiraman, K., Plyler, E. S., Sprenkle, N. T., Gibson, S. A., McFarland, B. C., Rajbhandari, R., Rowse, A. L., Benveniste, E. N., and Meares, G. P. (2016) Attenuation of PKR-like ER Kinase (PERK) Signaling Selectively Controls Endoplasmic Reticulum Stress-induced Inflammation Without Compromising Immunological Responses, *The Journal of biological chemistry 291*, 15830-15840.

502. Takigawa, S., Chen, A., Nishimura, A., Liu, S., Li, B. Y., Sudo, A., Yokota, H., and Hamamura, K. (2016) Guanabenz Downregulates Inflammatory Responses via eIF2alpha Dependent and Independent Signaling, *International journal of molecular sciences 17*.
503. Scheu, S., Stetson, D. B., Reinhardt, R. L., Leber, J. H., Mohrs, M., and Locksley, R. M. (2006) Activation of the integrated stress response during T helper cell differentiation, *Nature immunology 7*, 644-651.
504. Mao, X., Green, J. M., Safer, B., Lindsten, T., Frederickson, R. M., Miyamoto, S., Sonenberg, N., and Thompson, C. B. (1992) Regulation of translation initiation factor gene expression during human T cell activation, *The Journal of biological chemistry 267*, 20444-20450.
505. Boal, T. R., Chiorini, J. A., Cohen, R. B., Miyamoto, S., Frederickson, R. M., Sonenberg, N., and Safer, B. (1993) Regulation of eukaryotic translation initiation factor expression during T-cell activation, *Biochimica et biophysica acta 1176*, 257-264.
506. Li, B. D., McDonald, J. C., Nassar, R., and De Benedetti, A. (1998) Clinical outcome in stage I to III breast carcinoma and eIF4E overexpression, *Annals of surgery 227*, 756-756l; discussion 761-753.
507. Nathan, C. O., Franklin, S., Abreo, F. W., Nassar, R., De Benedetti, A., and Glass, J. (1999) Analysis of surgical margins with the molecular marker eIF4E: a prognostic factor in patients with head and neck cancer, *Journal of clinical oncology : official journal of the American Society of Clinical Oncology 17*, 2909-2914.
508. Wang, X. L., Cai, H. P., Ge, J. H., and Su, X. F. (2012) Detection of eukaryotic translation initiation factor 4E and its clinical significance in hepatocellular carcinoma, *World journal of gastroenterology : WJG 18*, 2540-2544.
509. Crew, J. P., Fuggle, S., Bicknell, R., Cranston, D. W., de Benedetti, A., and Harris, A. L. (2000) Eukaryotic initiation factor-4E in superficial and muscle invasive bladder cancer and its correlation with vascular endothelial growth factor expression and tumour progression, *British journal of cancer 82*, 161-166.
510. Chen, C. N., Hsieh, F. J., Cheng, Y. M., Lee, P. H., and Chang, K. J. (2004) Expression of eukaryotic initiation factor 4E in gastric adenocarcinoma and its association with clinical outcome, *Journal of surgical oncology 86*, 22-27.
511. Heikkinen, T., Korpela, T., Fagerholm, R., Khan, S., Aittomaki, K., Heikkila, P., Blomqvist, C., Carpen, O., and Nevanlinna, H. (2013) Eukaryotic translation initiation factor 4E (eIF4E) expression is associated with breast cancer tumor phenotype and predicts survival after anthracycline chemotherapy treatment, *Breast cancer research and treatment 141*, 79-88.
512. Matthews-Greer, J., Caldito, G., de Benedetti, A., Herrera, G. A., Dominguez-Malagon, H., Chanona-Vilchis, J., and Turbat-Herrera, E. A. (2005) eIF4E as a marker for cervical neoplasia, *Applied immunohistochemistry & molecular morphology : AIMM / official publication of the Society for Applied Immunohistochemistry 13*, 367-370.
513. Salehi, Z., and Mashayekhi, F. (2006) Expression of the eukaryotic translation initiation factor 4E (eIF4E) and 4E-BP1 in esophageal cancer, *Clinical biochemistry 39*, 404-409.
514. Salehi, Z., Mashayekhi, F., and Shahosseini, F. (2007) Significance of eIF4E expression in skin squamous cell carcinoma, *Cell biology international 31*, 1400-1404.
515. Seki, N., Takasu, T., Sawada, S., Nakata, M., Nishimura, R., Segawa, Y., Shibakuki, R., Hanafusa, T., and Eguchi, K. (2010) Prognostic significance of expression of eukaryotic initiation factor 4E and 4E binding protein 1 in patients with pathological stage I invasive lung adenocarcinoma, *Lung Cancer 70*, 329-334.
516. Martin, M. E., Perez, M. I., Redondo, C., Alvarez, M. I., Salinas, M., and Fando, J. L. (2000) 4E binding protein 1 expression is inversely correlated to the progression of gastrointestinal cancers, *The international journal of biochemistry & cell biology 32*, 633-642.

517. Rojo, F., Najera, L., Lirola, J., Jimenez, J., Guzman, M., Sabadell, M. D., Baselga, J., and Ramon y Cajal, S. (2007) 4E-binding protein 1, a cell signaling hallmark in breast cancer that correlates with pathologic grade and prognosis, *Clinical cancer research : an official journal of the American Association for Cancer Research 13*, 81-89.
518. Zhou, X., Tan, M., Stone Hawthorne, V., Klos, K. S., Lan, K. H., Yang, Y., Yang, W., Smith, T. L., Shi, D., and Yu, D. (2004) Activation of the Akt/mammalian target of rapamycin/4E-BP1 pathway by ErbB2 overexpression predicts tumor progression in breast cancers, *Clinical cancer research : an official journal of the American Association for Cancer Research 10*, 6779-6788.
519. Castellvi, J., Garcia, A., Rojo, F., Ruiz-Marcellan, C., Gil, A., Baselga, J., and Ramon y Cajal, S. (2006) Phosphorylated 4E binding protein 1: a hallmark of cell signaling that correlates with survival in ovarian cancer, *Cancer 107*, 1801-1811.
520. Liang, S., Zhou, Y., Chen, Y., Ke, G., Wen, H., and Wu, X. (2014) Decreased Expression of EIF4A1 After Preoperative Brachytherapy Predicts Better Tumor-Specific Survival in Cervical Cancer, *International journal of gynecological cancer : official journal of the International Gynecological Cancer Society 24*, 908-915.
521. Tu, L., Liu, Z., He, X., He, Y., Yang, H., Jiang, Q., Xie, S., Xiao, G., Li, X., Yao, K., and Fang, W. (2010) Over-expression of eukaryotic translation initiation factor 4 gamma 1 correlates with tumor progression and poor prognosis in nasopharyngeal carcinoma, *Molecular cancer 9*, 78.
522. Chen, Y., Knosel, T., Kristiansen, G., Pietas, A., Garber, M. E., Matsuhashi, S., Ozaki, I., and Petersen, I. (2003) Loss of PDCD4 expression in human lung cancer correlates with tumour progression and prognosis, *The Journal of pathology 200*, 640-646.
523. Mudduluru, G., Medved, F., Grobholz, R., Jost, C., Gruber, A., Leupold, J. H., Post, S., Jansen, A., Colburn, N. H., and Allgayer, H. (2007) Loss of programmed cell death 4 expression marks adenoma-carcinoma transition, correlates inversely with phosphorylated protein kinase B, and is an independent prognostic factor in resected colorectal cancer, *Cancer 110*, 1697-1707.
524. Gao, F., Wang, X., Zhu, F., Wang, Q., Zhang, X., Guo, C., Zhou, C., Ma, C., Sun, W., Zhang, Y., Chen, Y. H., and Zhang, L. (2009) PDCD4 gene silencing in gliomas is associated with 5'CpG island methylation and unfavourable prognosis, *Journal of cellular and molecular medicine 13*, 4257-4267.
525. Li, X., Xin, S., Yang, D., He, Z., Che, X., Wang, J., Chen, F., Wang, X., and Song, X. (2012) Down-regulation of PDCD4 expression is an independent predictor of poor prognosis in human renal cell carcinoma patients, *Journal of cancer research and clinical oncology 138*, 529-535.
526. Tunca, B., Tezcan, G., Cecener, G., Egeli, U., Zorluoglu, A., Yilmazlar, T., Ak, S., Yerci, O., Ozturk, E., Umut, G., and Evrensel, T. (2013) Overexpression of CK20, MAP3K8 and EIF5A correlates with poor prognosis in early-onset colorectal cancer patients, *Journal of cancer research and clinical oncology 139*, 691-702.
527. He, L. R., Zhao, H. Y., Li, B. K., Liu, Y. H., Liu, M. Z., Guan, X. Y., Bian, X. W., Zeng, Y. X., and Xie, D. (2011) Overexpression of eIF5A-2 is an adverse prognostic marker of survival in stage I non-small cell lung cancer patients, *International journal of cancer. Journal international du cancer 129*, 143-150.
528. Lee, N. P., Tsang, F. H., Shek, F. H., Mao, M., Dai, H., Zhang, C., Dong, S., Guan, X. Y., Poon, R. T., and Luk, J. M. (2010) Prognostic significance and therapeutic potential of eukaryotic translation initiation factor 5A (eIF5A) in hepatocellular carcinoma, *International journal of cancer. Journal international du cancer 127*, 968-976.
529. Flavin, R. J., Smyth, P. C., Finn, S. P., Laios, A., O'Toole, S. A., Barrett, C., Ring, M., Denning, K. M., Li, J., Aherne, S. T., Aziz, N. A., Alhadi, A., Sheppard, B. L., Loda, M., Martin, C., Sheils, O. M., and O'Leary, J. J. (2008) Altered eIF6 and Dicer expression is associated with clinicopathological

features in ovarian serous carcinoma patients, *Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc 21*, 676-684.
530. Finch, A. J., Hilcenko, C., Basse, N., Drynan, L. F., Goyenechea, B., Menne, T. F., Gonzalez Fernandez, A., Simpson, P., D'Santos, C. S., Arends, M. J., Donadieu, J., Bellanne-Chantelot, C., Costanzo, M., Boone, C., McKenzie, A. N., Freund, S. M., and Warren, A. J. (2011) Uncoupling of GTP hydrolysis from eIF6 release on the ribosome causes Shwachman-Diamond syndrome, *Genes & development 25*, 917-929.
531. Burwick, N., Coats, S. A., Nakamura, T., and Shimamura, A. (2012) Impaired ribosomal subunit association in Shwachman-Diamond syndrome, *Blood 120*, 5143-5152.
532. Bachmann, F., Banziger, R., and Burger, M. M. (1997) Cloning of a novel protein overexpressed in human mammary carcinoma., *Cancer Res. 57*, 988-994.
533. Dellas, A., and al., e. (1998) Expression of p150 in cervical neoplasia and its potential value in predicting survival. , *Cancer 83*, 1376-1383.
534. Chen, G., and Burger, M. M. (1999) p150 expression and its prognostic value in squamous-cell carcinoma of the esophagus. , *Int. J. Cancer 84*, 95-100.
535. Pincheira, R., Chen, Q., and Zhang, J. T. (2001) Identification of a 170-kDa protein over-expressed in lung cancers. , *Br. J. Cancer 84*, 1520-1527.
536. Chen, G., and Burger, M. M. (2004) p150 overexpression in gastric carcinoma: the association with p53, apoptosis and cell proliferation., *Int. J. Cancer 112*, 393-398.
537. Wang, H., Ru, Y., Sanchez-Carbayo, M., Wang, X., Kieft, J. S., and Theodorescu, D. (2013) Translation Initiation Factor eIF3b Expression in Human Cancer and its Role in Tumor Growth and Lung Colonization., *Clin. Cancer Res. 19*, 2850-2860.
538. Lin, L., Holbro, T., Alonso, G., Gerosa, D., and Burger, M. M. (2001) Molecular interaction between human tumor marker protein p150, the largest subunit of eIF3, and intermediate filament protein K7., *J. Cell. Biochem. 80*, 483-490.
539. Scoles, D. R., Yong, W. H., Qin, Y., Wawrowsky, K., and Pulst, S. M. (2006) Schwammonin inhibits tumorigenesis through direct interaction with the eukaryotic initiation factor subunit c (eIF3c). *Hum. Mol. Genet. 15*, 1059-1070.
540. Rothe, M., Ko, Y., Albers, P., and Wernert, N. (2000) Eukaryotic initiation factor 3 p110 mRNA is overexpressed in testicular seminomas. , *Am. J. Pathol. 157*, 1597-1604.
541. Nupponen, N. N., Porkka, K., Kakkola, L., Tanner, M., Peersson, K., Borg, A., Isola, J., and Visakorpi, T. (1999) Amplification and overexpression of p40 subunit of eukaryotic translation initiation factor 3 in breast and prostate cancer., *Am. J. Pathol. 154*, 1777-1783.
542. Tomlinson, I. P., Webb, E., Carvajal-Carmona, L., Broderick, P., Howarth, K., and al., e. (2008) A genome-wide association study identifies colorectal cancer susceptibility loci on chromosomes 10p14 and 8q23.3., *Nat. Genet. 40*, 623-630.
543. Okamoto, H., Yasui, K., Zhao, C., Arii, S., and Inazawa, J. (2003) PTK2 and EIF3S3 genes may be amplification targets at 8q23-q24 and are associated with large hepatocellular carcinomas, *Hepatology 38*, 1242-1249.
544. Saramaki, O., and al., e. (2001) Amplication of EIF3S3 gene is associated with advanced stage in prostate cancer., *Am. J. Pathol. 159*, 2089-2094.
545. Matsuda, S., Kasumata, R., Okuda, T., Yamamoto, T., Miyazaki, K., Senga, T., Machida, K., Thant, A. A., Nakatsugawa, S., and Hamaguchi, M. (2000) Molecular cloning and characterization of human MAWD, a novel protein containing WD-40 repeats frequently overexpressed in breast cancer., *Cancer Res. 60*, 13-17.
546. Rauch, J., Ahlemann, M., Schaffrik, M., Mack, B., Ertongur, S., Andratschke, M., and al., e. (2004) Allogenic antibody-mediated identification of head and neck cancer antigens., *Biochem. Biophys. Res. Commun. 323*, 156-162.

547. Gray, S. G., Kytola, S., Liu, W. O., Larsson, C., and Elkstrom, T. J. (2000) Modulating IGFBP-3 expression by trichostatin A: potential therapeutc role on the treatment of hepatocellular carcinoma., *Int. J. Mol. Med. 5*, 33-41.
548. Bernardini, S., Melino, G., Saura, F., Anniechiarico-Peruzzelli, M., Motti, C., Cortese, C., and al., e. (1997) Expression of co-factors (SMRT and Trip-1) for retinoic acid receptors in human neuroectodermal cell lines., *Biochem. Biophys. Res. Commun. 234*, 278-282.
549. Goh, S. H., Hong, S. H., Lee, B. C., Ju, M. H., Jeong, J. S., Cho, Y. R., Kim, I. H., and Lee, Y. S. (2011) eIF3m expression influences the regulation of tumorigenesis-related genes in human colon cancer., *Oncogene 30*, 398-409.
550. Marchetti, A., Buttitta, F., Pellegrini, S., Bertacca, G., and Callahan, R. (2001) Reduced expression of INT-6/eIF3-p48 in human tumors. , *Int J. Oncol. 18*, 175-179.
551. Buttitta, F., C., M., Barassi, F., Felicioni, L., Salvatore, S., and Rosini, S. (2005) Int6 expression an predict survival in early-stage non-small cell lung cancer patients., *Clin. Cancer Res. 11*, 3198-3204.
552. Shi, J., Kahle, A., Hershey, J. W. B., Honchak, B. M., Warneke, J. A., Leong, S. P., and Nelson, M. A. (2006) Decreased expression of eukaryotic initiation factor 3f deregulates translation and apoptosis in tumor cells., *Oncogene 25*, 4923-4936.
553. Doldan, A., Chandramouli, A., Shanas, R., Bhattacharyya, A., Leong, S. P., Nelson, M. A., and Shi, J. (2008) Loss of the eukaryotic initiation factor 3f in melanoma., *Mol. Carcinog. 47*.
554. Doldan, A., Chandramouli, A., Shanas, R., Bhattacharyya, A., Cunningham, J. T., Nelson, M. A., and Shi, J. (2008) Loss of the eukaryotic initiation factor 3f in pancreatic cancer., *Mol. Carcinog. 47*, 235-244.

APPENDIX A

SUPPLEMENTAL TABLES AND FIGURES

Supplemental Table 1.1: Abbreviations

Abbreviation	Explanation
eIF…	Eukaryotic translation initiation factor… followed by numbers and letters for individual identification (as in eIF3, eIF4E, etc.)
PIC	Pre-initiation complex containing 40S ribosome and eIFs
4E-BP	eIF4E-binding protein, followed by a number to distinguish homologs or by "s" to refer to several (e.g. 4E-BP1, 4E-BPs)
PDCD4	Programmed cell death 4
MNK	MAPK integrating kinase
MAPK	Mitogen-activated protein kinase
RNA	Ribonucleic acid
mRNA	Messenger RNA
rRNA	Ribosomal RNA
tRNA	Transfer RNA
Met-tRNAi	Methionine initiator tRNA
TC	Ternary complex composed of eIF2, GTP, Met-tRNAi
miRNA	Micro RNA
UTR	Untranslated region preceding (5') or following (3') the ORF
ORF	Open reading frame, the section of mRNA encoding a protein
uORF	Upstream ORF, an ORF situated 5' of the main ORF
mTOR	Mammalian target of rapamycin
mTORC1	mTOR complex 1, sensitive to rapamycin
asTORi	Active-site mTOR inhibitor
S6K	Ribosomal protein S6 kinase
Phospho-	The phosphorylated form of a protein
PABP	Poly-A binding protein
IRES	Internal ribosome entry site
VEGFA	Vascular endothelial growth factor A
BCL2	B-cell lymphoma 2, an anti-apoptotic protein
MCL1	Myeloid cell leukemia 1, an anti-apoptotic protein
ATF4	Activating transcription factor 4, a stress-response factor
MYC	V-Myc Avian Myelocytomatosis Viral Oncogene Homolog
HIF1α	Hypoxia-inducible factor 1-alpha
IFNγ	Interferon gamma, a cytokine
TNFα	Tumor necrosis factor alpha, a cytokine
TGFβ	Transforming growth factor beta, a cytokine
MDSC	Myeloid derived suppressor cell
TME	Tumor microenvironment

Supplemental Table 1.2: Dysregulation of translation initiation factors in human cancers

Factor	Dysregulation	Clinical correlates in cancers
eIF4E	Overexpression	Decreased survival in breast (*506*), head and neck (*507*), liver (*508*), prostate (*116*), bladder (*509*), stomach (*510*). Correlates with disease progression and aggressive subtypes in many cancers (*133, 134, 511-514*) and resistance to chemotherapy (*511*)
eIF4E	Phosphorylation	Elevated in early stages of development of breast, colon, gastric, and lung cancers (*115*). Increased in prostate cancer and correlates with androgen independence (*55, 116*). Poor prognosis marker in non-small cell lung cancer (*386*)
4E-BP1	Overexpression	Inversely correlates with tumor grade (*282, 513*) Correlates with better survival in lung and prostate cancers (*116, 515*) Correlates with absence of lymph node and distant metastases in gastric cancer (*516*)
4E-BP1	Loss	Possibly responsible for loss of translational control in 50% of pancreatic tumors (*280*)
4E-BP1	Phosphorylation	Correlates with tumor grade and poor prognosis in breast, lung, ovarian and prostate cancers (*116, 282, 517-519*)
eIF4G	Increased expression	Amplification correlates with aggressive stages in lung cancer (*188*). Overexpressed in inflammatory breast cancer (*108*) and cervical cancer (*520*). Correlates with poor prognosis in nasopharyngeal carcinoma (*521*).
eIF4A	Increased expression	Overexpressed in lung (*188*) and cervical cancer (*520*). Lowered expression after radiation predicts better survival in cervical cancer (*520*).
PDCD4	Decreased expression	Associated with poor prognosis in breast (*121*), lung (*522*), colon (*523*) and ovarian cancers (*123*) and gliomas (*524*). Inversely correlated with advanced tumor stage in RCC (*525*)
eIF2α	Increased expression	Correlates with aggressive lymphoma subtypes (*133*).
eIF5A	Increased expression and hypusination	Correlates with poor prognosis in early-onset colorectal cancer (*526*). Overexpression of eIF5A2 correlates with local invasion in NSCLC (*527*) and HCC (*528*).

eIF6	Altered expression and function	Elevated in colorectal cancer (*168*), head and neck carcinomas (*169*) and ovarian serous carcinoma (*529*); low expression correlates with reduced disease-free survival in ovarian serous carcinoma (*529*); mediates lymphomagenesis in Shwachman-Diamond syndrome (*171, 530, 531*)
eIF3a	Increased expression	breast (*532*), cervix (*533*), esophagus (*534*), lung (*535*), stomach (*536*)
eIF3b	Increased expression	bladder (*537*), breast (*538*), prostate (*537*)
eIF3c	Increased expression	meningioma (*539*), testicular seminoma (*540*)
eIF3h	Increased expression	breast (*541*), colon (*542*), liver (*543*), prostate (*541, 544*)
eIF3i	Increased expression	breast (*545*), head and neck (*546*), liver (*547*), melanoma (*548*), neuroblastoma (*548*)
eIF3m	Increased expression	colon (*549*)
eIF3e	Decreased expression	breast (*550*), lung (*551*), prostate (*550*)
eIF3f	Decreased expression	breast (*552*), colon (*552*), small intestine (*552*), melanoma (*553*), ovary (*552*), pancreas (*554*), vulva (*552*)

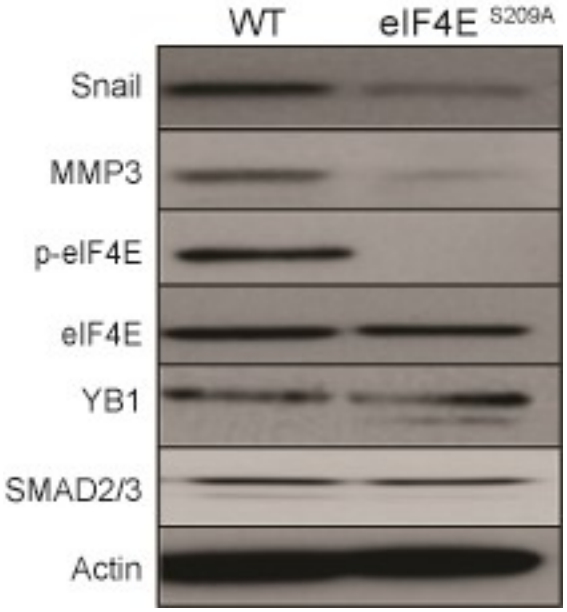


Figure S2.1. YB1 cannot explain invasion differences. Western blot analysis of indicated proteins in WT and Myc/Ras-transformed WT and eIF4E^{S209A} MEFs.

DAVID Bioinformatics Resources 6.7
National Institute of Allergy and Infectious Diseases (NIAID), NIH

Functional Annotation Clustering

Help and Manual

Current Gene List: List_1
Current Background: Mus musculus
99 DAVID IDs

Options **Classification Stringency** Medium

Rerun using options | Create Sublist

36 Cluster(s)

Download File

Annotation Cluster 1	Enrichment Score: 1.83			Count	P_Value	Benjamini
PIR_SUPERFAMILY	PIRSF000503:glutathione transferase	RT		3	6.2E-3	3.3E-1
GOTERM_MF_FAT	glutathione transferase activity	RT		3	8.2E-3	8.1E-1
INTERPRO	Glutathione S-transferase, N-terminal	RT		3	8.9E-3	8.9E-1
INTERPRO	Glutathione S-transferase, C-terminal	RT		3	9.6E-3	7.0E-1
INTERPRO	Glutathione S-transferase, C-terminal-like	RT		3	1.1E-2	6.0E-1
INTERPRO	Glutathione S-transferase/chloride channel, C-terminal	RT		3	1.4E-2	5.8E-1
GOTERM_MF_FAT	transferase activity, transferring alkyl or aryl (other than methyl) groups	RT		3	3.2E-2	7.2E-1
INTERPRO	Thioredoxin fold	RT		3	1.1E-1	9.8E-1
Annotation Cluster 2	Enrichment Score: 1.24			Count	P_Value	Benjamini
SP_PIR_KEYWORDS	chemotaxis	RT		4	4.7E-3	3.8E-1
GOTERM_MF_FAT	chemokine activity	RT		3	1.7E-2	8.2E-1
KEGG_PATHWAY	Chemokine signaling pathway	RT		6	1.7E-2	5.1E-1
GOTERM_MF_FAT	chemokine receptor binding	RT		3	1.8E-2	7.0E-1
GOTERM_BP_FAT	taxis	RT		4	2.2E-2	1.0E0
GOTERM_BP_FAT	chemotaxis	RT		4	2.2E-2	1.0E0
GOTERM_CC_FAT	extracellular space	RT		7	4.6E-2	7.9E-1

SP_PIR_KEYWORDS	metalloprotease	RT		3	1.9E-1	8.7E-1
SP_PIR_KEYWORDS	hydrolase	RT		12	1.9E-1	8.5E-1
GOTERM_MF_FAT	endopeptidase activity	RT		4	3.8E-1	9.9E-1
Annotation Cluster 7	Enrichment Score: 1.11			Count	P_Value	Benjamini
UP_SEQ_FEATURE	repeat:LRR 12	RT		4	1.2E-2	9.8E-1
UP_SEQ_FEATURE	repeat:LRR 11	RT		4	1.5E-2	9.3E-1
UP_SEQ_FEATURE	repeat:LRR 10	RT		4	2.3E-2	9.3E-1
UP_SEQ_FEATURE	repeat:LRR 9	RT		4	3.6E-2	9.6E-1
UP_SEQ_FEATURE	repeat:LRR 8	RT		4	4.5E-2	9.6E-1
UP_SEQ_FEATURE	repeat:LRR 7	RT		4	6.7E-2	9.8E-1
INTERPRO	Leucine-rich repeat	RT		4	9.2E-2	9.8E-1
UP_SEQ_FEATURE	repeat:LRR 6	RT		4	9.7E-2	9.9E-1
SMART	LRR_TYP	RT		3	1.2E-1	1.0E0
UP_SEQ_FEATURE	repeat:LRR 5	RT		4	1.2E-1	9.9E-1
UP_SEQ_FEATURE	repeat:LRR 4	RT		4	1.4E-1	9.9E-1
INTERPRO	Leucine-rich repeat, typical subtype	RT		3	1.5E-1	9.9E-1
UP_SEQ_FEATURE	repeat:LRR 3	RT		4	1.8E-1	9.9E-1
SP_PIR_KEYWORDS	leucine-rich repeat	RT		4	1.9E-1	8.6E-1
UP_SEQ_FEATURE	repeat:LRR 1	RT		4	2.1E-1	1.0E0
UP_SEQ_FEATURE	repeat:LRR 2	RT		4	2.1E-1	1.0E0
Annotation Cluster 8	Enrichment Score: 1.05			Count	P_Value	Benjamini
GOTERM_BP_FAT	immune response-activating signal transduction	RT		3	2.9E-2	1.0E0
GOTERM_BP_FAT	immune response-regulating signal transduction	RT		3	3.3E-2	1.0E0
GOTERM_BP_FAT	positive regulation of defense response	RT		3	3.9E-2	1.0E0
GOTERM_BP_FAT	regulation of transcription factor activity	RT		3	4.2E-2	1.0E0
GOTERM_BP_FAT	regulation of DNA binding	RT		3	6.3E-2	1.0E0
GOTERM_BP_FAT	activation of immune response	RT		3	8.2E-2	1.0E0
GOTERM_BP_FAT	positive regulation of response to stimulus	RT		4	8.3E-2	9.9E-1
GOTERM_BP_FAT	regulation of binding	RT		3	8.8E-2	9.9E-1

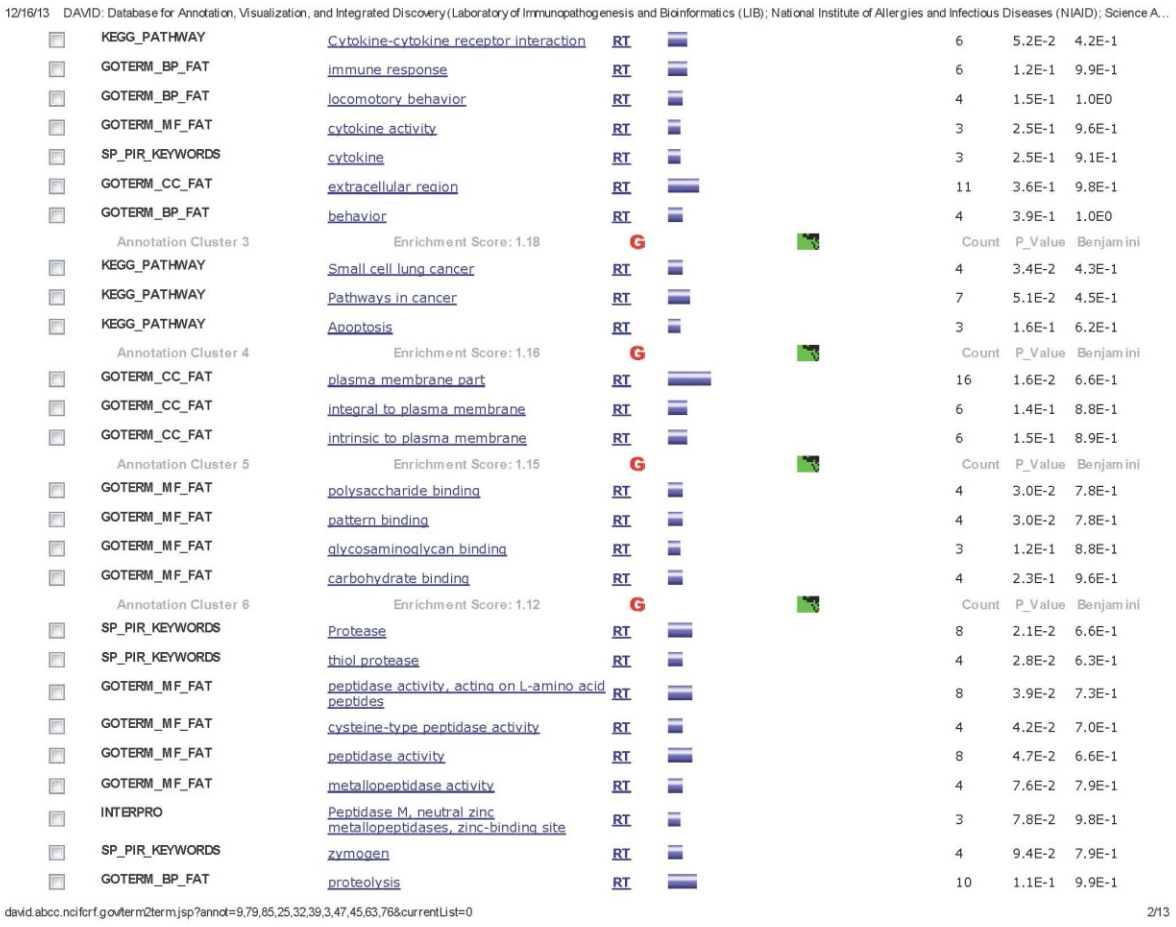


Figure S2.2. Clustered pathways of eIF4E phosphorylation regulated mRNAs. Screenshots of the complete output of clustering from the DAVID software of significantly enriched pathways from a previously generated list of phospho-eIF4E translationally-regulated mRNAs.

Glutathione metabolism

glutathione S-transferase, alpha 4
glutathione S-transferase, theta 3
predicted gene 6665; glutathione S-transferase, mu 2

Chemotaxis

a disintegrin and metallopeptidase domain 17
arginyl aminopeptidase (aminopeptidase B)
chemokine (C-C motif) ligand 7
chemokine (C-C motif) ligand 9
chemokine (C-X-C motif) ligand 16
decorin
ectonucleotide pyrophosphatase/phosphodiesterase 2
ectonucleotide pyrophosphatase/phosphodiesterase 5
guanine nucleotide binding protein (G protein), beta 4
interferon gamma receptor 1
matrix metallopeptidase 9
nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
p21 protein (Cdc42/Rac)-activated kinase 1
platelet derived growth factor receptor, alpha polypeptide
prolactin family 2, subfamily c, member 3; prolactin family 2, subfamily c, member 4
toll-like receptor 2
transformation related protein 53
vascular endothelial growth factor C

Cancer

TNF receptor-associated factor 1
baculoviral IAP repeat-containing 3
matrix metallopeptidase 9
nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
platelet derived growth factor receptor, alpha polypeptide
transformation related protein 53
vascular endothelial growth factor C

Plasma membrane

Bardet-Biedl syndrome 7 (human)
claudin 1
ectonucleotide pyrophosphatase/phosphodiesterase 2
ectonucleotide pyrophosphatase/phosphodiesterase 5
glycoprotein (transmembrane) nmb
guanine nucleotide binding protein (G protein), beta 4
immunoglobulin superfamily, member 9
islet cell autoantigen 1
p21 protein (Cdc42/Rac)-activated kinase 1
platelet derived growth factor receptor, alpha polypeptide
receptor-associated protein of the synapse
similar to UDP glycosyltransferase 1 family polypeptide A13
solute carrier family 16 (monocarboxylic acid transporters), member 10
solute carrier organic anion transporter family, member 4a1
toll-like receptor 2
translocase of outer mitochondrial membrane 34

Carbohydrate binding

chemokine (C-C motif) ligand 7
decorin
ectonucleotide pyrophosphatase/phosphodiesterase 2
glycoprotein (transmembrane) nmb

Protease

a disintegrin and metallopeptidase domain 17
arginyl aminopeptidase (aminopeptidase B)
caspase 4, apoptosis-related cysteine peptidase; hypothetical protein LOC100044206
cathepsin K
ectonucleotide pyrophosphatase/phosphodiesterase 2
ectonucleotide pyrophosphatase/phosphodiesterase 5
matrix metallopeptidase 9
methionine aminopeptidase 2
seven in absentia 1B
similar to Adenosylhomocysteinase (S-adenosyl-L-homocysteine hydrolase) (AdoHcyase) (Liver copper-binding protein) (CUBP); S-adenosylhomocysteine hydrolase
thymine DNA glycosylase; predicted gene 5597; predicted gene 9855; predicted gene 5806
tumor necrosis factor, alpha-induced protein 3
ubiquitin specific peptidase 3
ubiquitin-conjugating enzyme E2N; similar to ubiquitin-conjugating enzyme E2 UbcH-ben; similar to ubiquitin-conjugating enzyme E2N; predicted gene 5943

Leucine-rich repeat

decorin
leucine rich repeat containing 8D
leucine-rich repeat kinase 2
toll-like receptor 2

Immune response

a disintegrin and metallopeptidase domain 17
adenosine A2b receptor
guanine nucleotide binding protein (G protein), beta 4
nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
p21 protein (Cdc42/Rac)-activated kinase 1
platelet derived growth factor receptor, alpha polypeptide
toll-like receptor 2
transformation related protein 53
ubiquitin-conjugating enzyme E2N; similar to ubiquitin-conjugating enzyme E2 UbcH-ben; similar to ubiquitin-conjugating enzyme E2N; predicted gene 5943
vascular endothelial growth factor C
zinc finger and BTB domain containing 33

Zinc-binding

TNF receptor-associated factor 1
a disintegrin and metallopeptidase domain 17
alcohol dehydrogenase 7 (class IV), mu or sigma polypeptide
arginyl aminopeptidase (aminopeptidase B)
baculoviral IAP repeat-containing 3
caseinolytic peptidase X (E.coli)
cytochrome P450, family 7, subfamily b, polypeptide 1

cytochrome b-245, alpha polypeptide
ectonucleotide pyrophosphatase/phosphodiesterase 2
elongation factor 1 homolog (ELF1, S. cerevisiae)
enolase 2, gamma neuronal
matrix metallopeptidase 9
methionine aminopeptidase 2
p21 protein (Cdc42/Rac)-activated kinase 3
protocadherin 20
receptor-associated protein of the synapse
seven in absentia 1B
similar to Adenosylhomocysteinase (S-adenosyl-L-homocysteine hydrolase) (AdoHcyase) (Liver copper-binding protein) (CUBP); S-adenosylhomocysteine hydrolase
solute carrier family 24 (sodium/potassium/calcium exchanger), member 3
transformation related protein 53
tumor necrosis factor, alpha-induced protein 3
ubiquitin specific peptidase 3
zinc finger and BTB domain containing 33
zinc finger protein 280C
zinc finger protein 36, C3H type-like 1
zinc finger protein, multitype 2

Figure S2.3. Complete list of mRNAs clustered by the DAVID software.

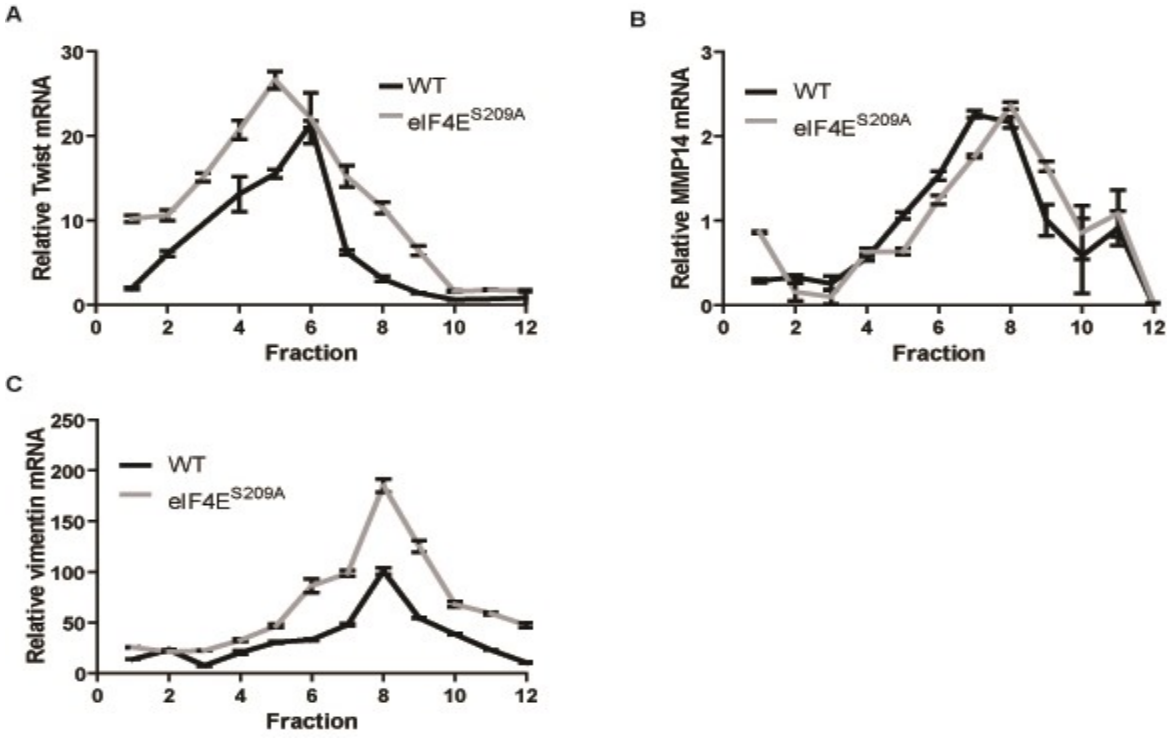


Figure S2.4. Non phospho-eIF4E regulated mRNAs involved in EMT/invasion and metastasis. qRT-PCR analysis of polysomal fractions of **(A)** *Twist* **(B)** *MPP14,* **(C)** *vimentin*, in Myc/Ras-transformed WT and eIF4E^{S209A} MEFs. Concentrations of mRNA in each fraction are normalized to GAPDH. Error bars represent standard deviations of each fraction. Results are representative of at least 2 experiments.

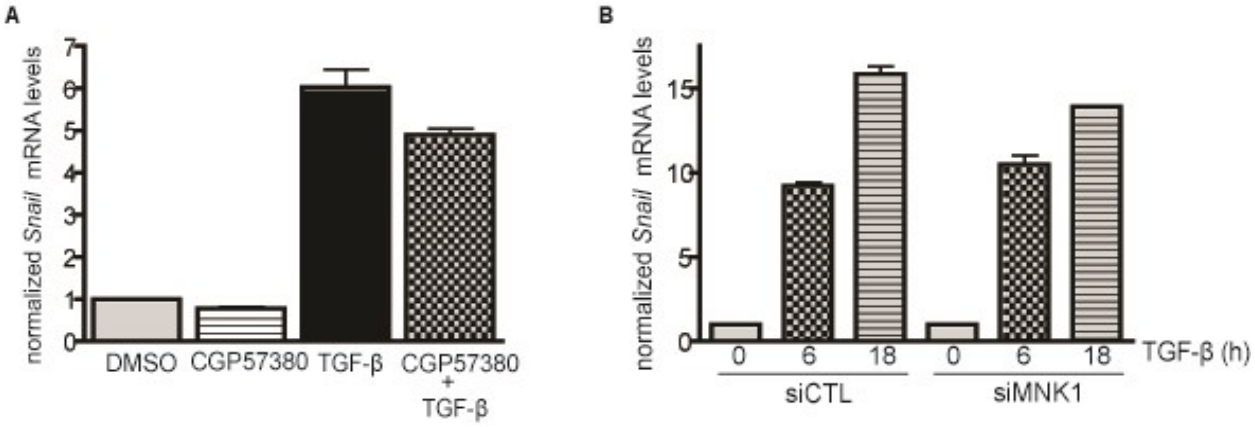


Figure S2.5. eIF4E phosphorylation does not affect *Snail* mRNA levels. qRT-PCR analysis of *Snail* mRNA in NMuMG cells treated with 5ng/mL TGFβ, **(A)** with or without 20μM CGP57380 or **(B)** in the presence of scramble siRNA or siMNK1.

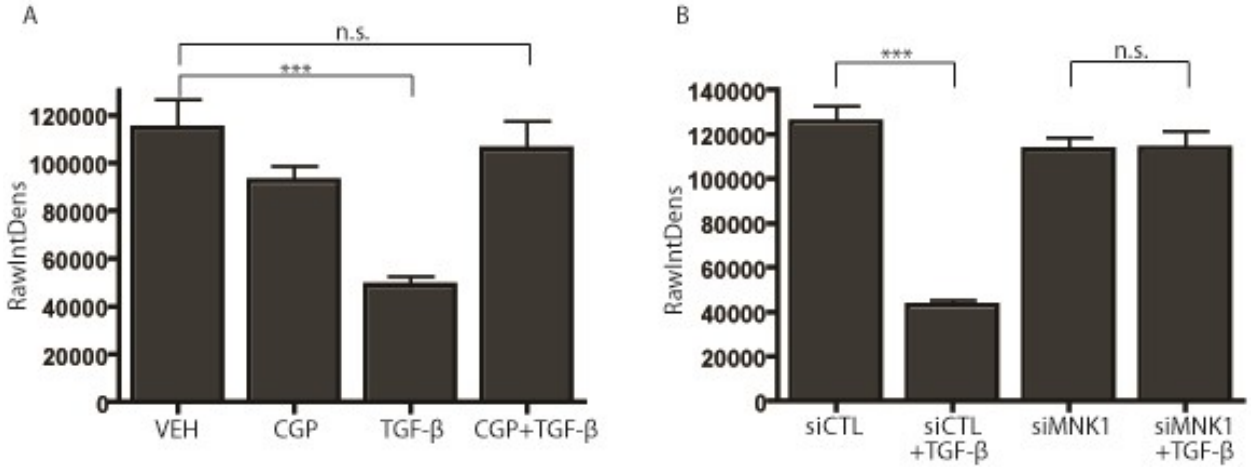


Figure S2.6. Inhibition of eIF4E phosphorylation maintains tight junctions during TGFβ induced EMT. Quantification of ZO1 immunofluorescence in NMuMG cells treated with 5ng/mL TGFβ, **(A)** with or without 20μM CGP57380 or **(B)** in the presence of scramble siRNA or siMNK1.

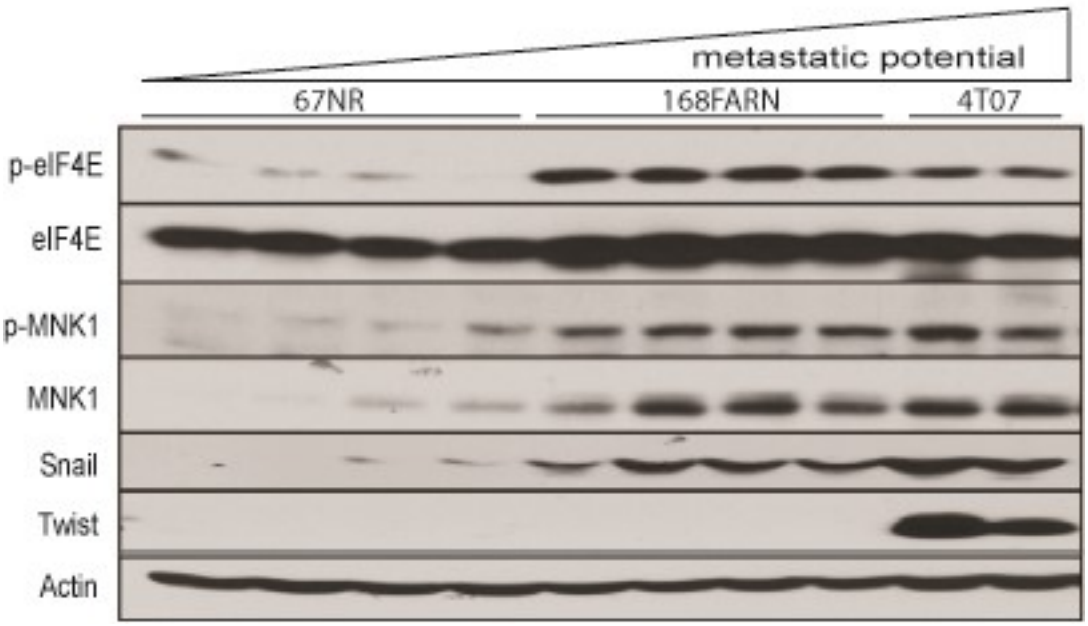
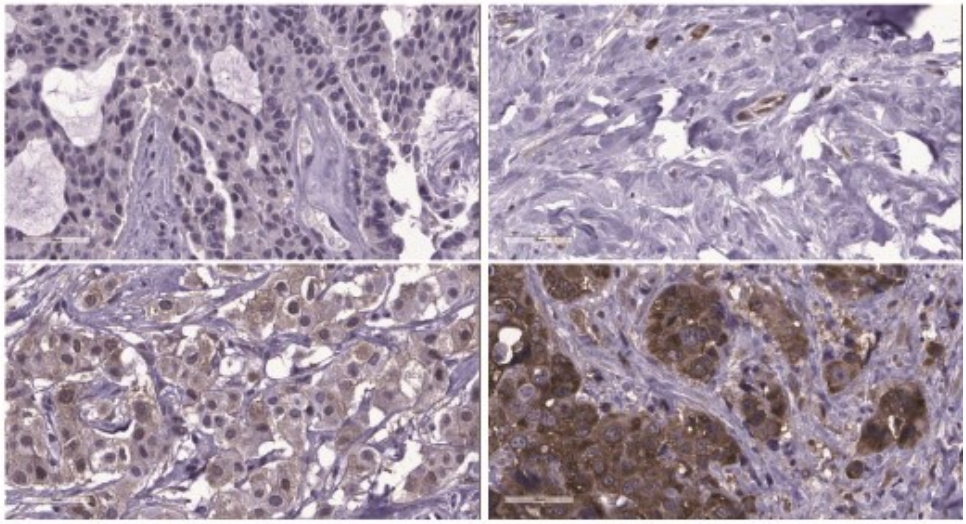
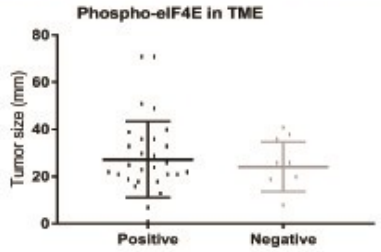


Figure S2.7. eIF4E phosphorylation correlates with EMT inducers and metastatic potential *in vivo*. Western blot analysis of eIF4E phosphorylation and transcription factors that induce EMT in tumor lysates formed by indicated isogenic cell lines.

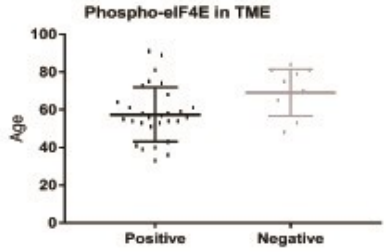
A.



B.



C.



D.

p-eIF4E	Hormone receptor status			
	ER+	PR+	HER2+	TN
positive	9	3	4	14
negative	9	4	3	1

E.

p-eIF4E	Tumor Grade			
	I	II	III	N/D
positive	1	5	22	2
negative	0	5	3	2

F.

p-eIF4E	Lymph node status		
	Positive	Negative	N/D
positive	13	12	5
negative	2	5	3

Figure S3.1. Scoring and histopathology of breast cancer samples. (A) Examples of phospho-eIF4E scoring in the epithelial cells and TME. Phospho-eIF4E expression in cancer cells was categorized as “low” if undetectable or if expression was low and sparse; it was categorized as “high” if expression was uniformly detectable or if expression was sparse but very high. Phospho-eIF4E expression in the TME was categorized as “positive” if at least 5 cells outside the tumor bed expressed phospho-eIF4E, otherwise it was categorized as “negative”. Shown are examples of a tumor where expression in cancer cells was low and negative in the TME (top left), low in the cancer cells and positive in the TME (top right), high in the cancer cells but negative in the TME (bottom left), high in the cancer cells and positive in the TME (bottom right). (B-F) Clinical correlates of samples stratified by phosphorylation of eIF4E in the TME; no statistical analysis is provided as the sample numbers do not provide the power to perform the multiple tests required. Show are tumor size (B), patient age at biopsy (C), hormone receptor status (D), tumor grade (E) and lymph node status (F). Not all information was available for all samples. For hormone receptor status, some samples are counted more than once if positive for multiple receptors. N/D not determined

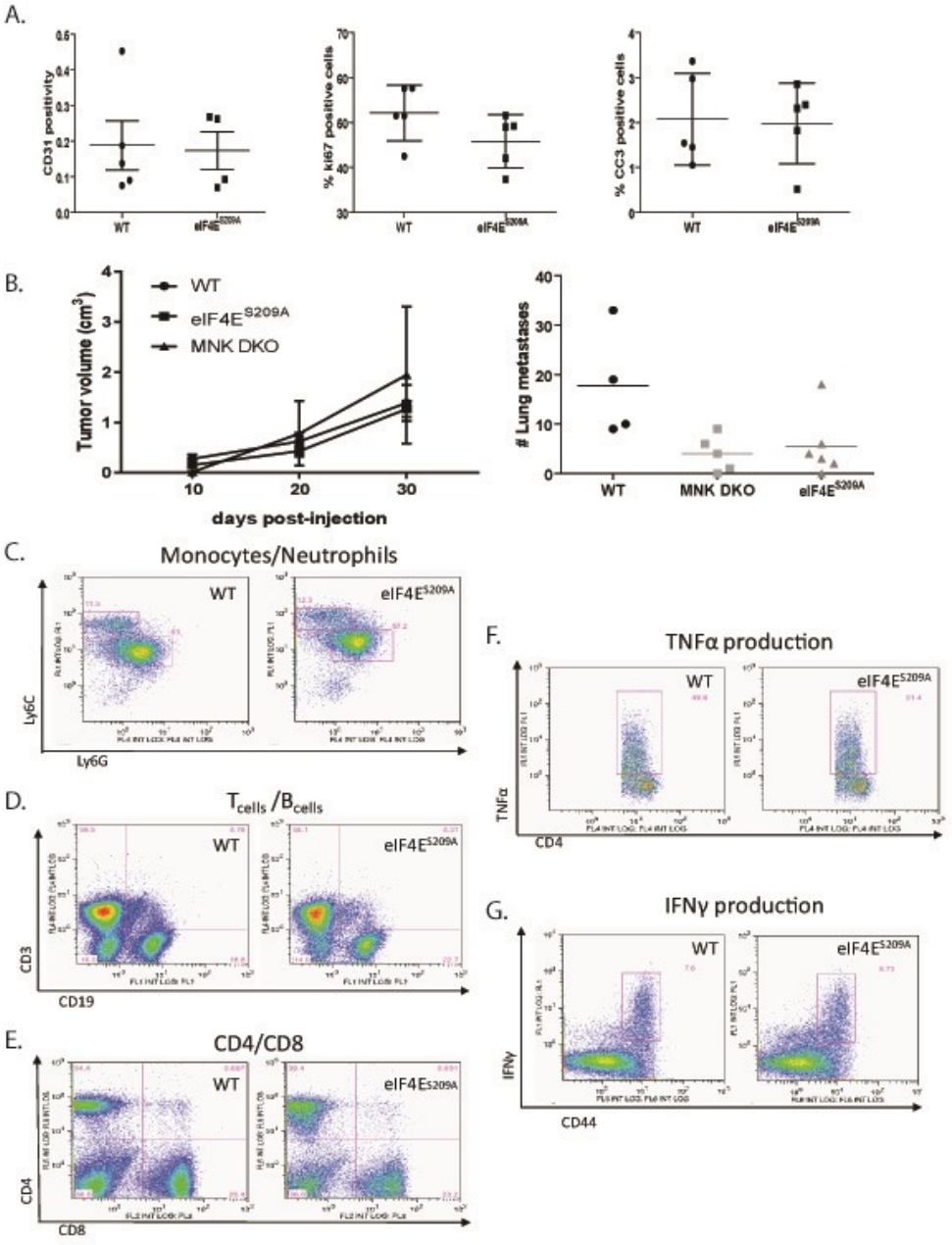


Figure S3.2. Similarities between primary tumors and healthy immune systems of WT and eIF4E^{S209A} mice. (A) Quantification of CD31, ki67 and cleaved caspase 3 in tumors from WT and eIF4E^{S209A} mice from immunohistochemical staining as in Figure 3.1C. (B) Tumor growth and lung metastatic burden of C57/BL6 mice of the indicated genotype bearing tumors derived from EO771 cells. (C-G) Spleens of WT and eIF4E^{S209A} mice were dissociated and by flow cytometry using the indicated markers. (B) Quantification of monocytes and neutrophils based on Ly6C and Ly6G expression. (C) Quantification of T cells and B cells based on CD3 and CD19 expression. (D) Quantification of T cell subtypes based on CD4 and CD8 expression. (E-F) T cells isolated from WT and eIF4E^{S209A} spleens were incubated with anti-CD3 to stimulate cytokine production, followed by flow cytometric analysis of TNFα (E) and IFNγ (F).

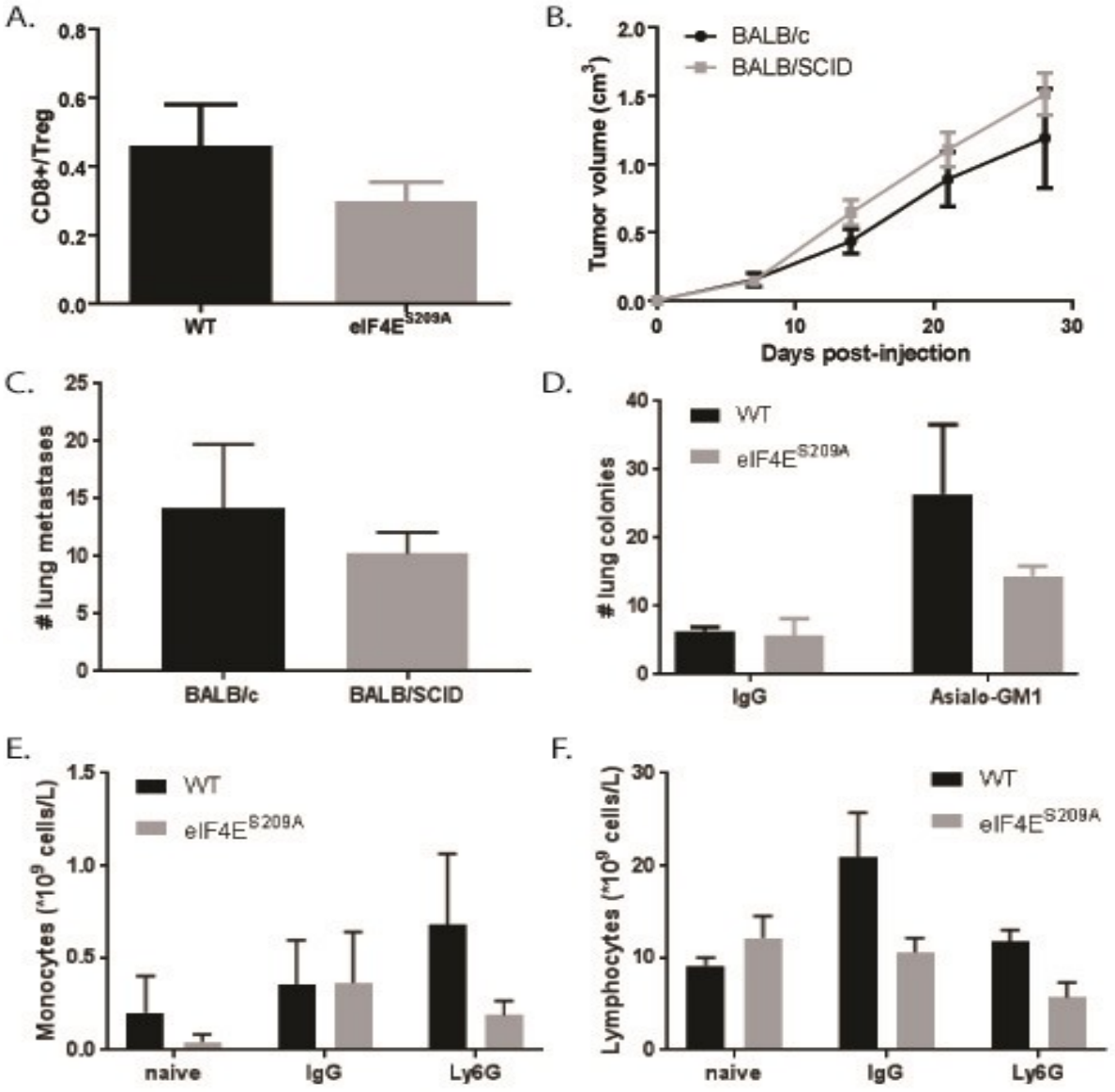


Figure S3.3. T cells and NK cells are unlikely candidates to mediate the effects of eIF4E phosphorylation on the TME. (A) Primary tumors were dissociated and single cell suspensions were analyzed by flow cytometry using markers for cytotoxic T cells and regulatory T cells, based on the expression of CD3, CD4, CD8 and FOXP3. The ratio of $CD8^+$ T cells to $FOXP3^+$ T_{regs} is presented in (A). (B-C) 66cl4 cells were injected into the fat pad of WT BALB/c mice and BALB/SCID mice. No differences were observed in tumor growth (B) or metastasis to the lung (C). (D) NK cell-mediated protection from lung colonization was assayed in WT and $eIF4E^{S209A}$ mice: NK cells were depleted by intraperitoneal injections of anti-ASIALO-GM1 2 days prior to tail vein injections of 66cl4 cells, on the day of injection and every other day for the following 12 days. The relative increase in lung colonization from control-treated to NK cell-depleted mice was compared between WT and $eIF4E^{S209A}$ mice (D). (E-F) Neutrophil depletion with Ly6G antibodies had no effect on levels of circulating monocytes (E) and lymphocytes (F), as determined in complete blood counts using an automated analyzer.

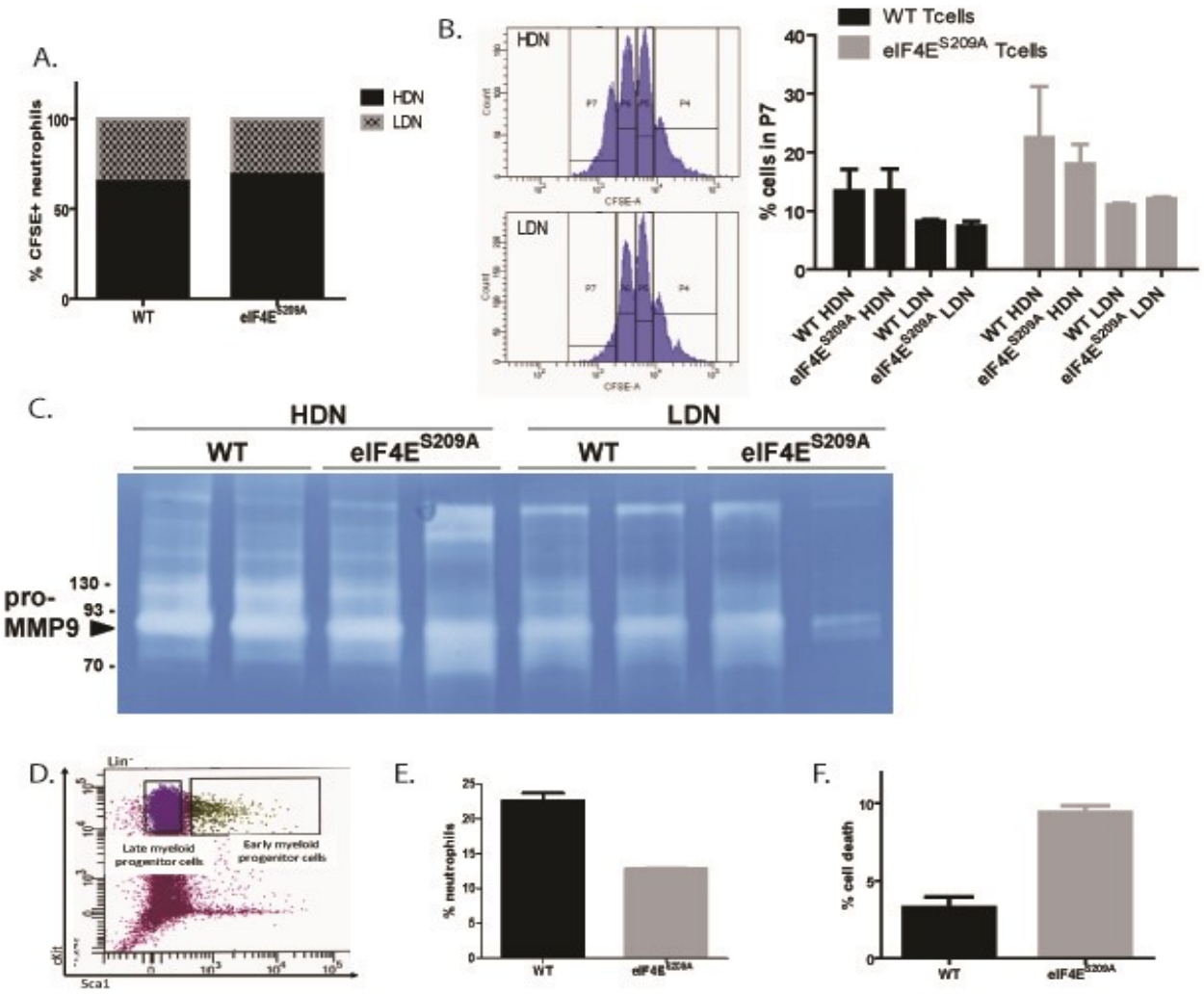


Figure S3.4. Neutrophil function and differentiation. Polarization of high density neutrophils (HDN) to pro-metastatic low density neutrophils (LDN) by the serum of tumor-bearing mice was assessed (A). Briefly, HDNs and LDNs from WT and eIF4E^{S209A} mice were isolated by density gradient centrifugation, stained with CD11b and Ly6G and sorted using a BD Aria Fusion flow cytometer. HDNs were stained with CFSE and incubated for 4 hours in whole blood from WT tumor-bearing mice. The resulting cell population was separated again by density gradient centrifugation and the proportion of CFSE positive cells in the HDN and LDN populations was assessed by flow cytometry. In addition, FACS sorted HDNs and LDNs, as in (A), from n=4 mice, were incubated in the presence of CFSE-stained WT and eIF4E^{S209A} T cells, and T cell proliferation was induced by incubation with recombinant CD3/CD28 for 3 days. T cell proliferation was then quantified by flow cytometric analysis of CFSE content: the proportion of T cells in the fastest dividing population (P7) is quantified in (B). Phosphorylation of eIF4E in both neutrophils and T cells had no significant effect of T cell proliferation. (C) Gelatin zymogram depicting MMP9 expression in HDNs and LDNs from WT and eIF4E^{S209A} mice. (D) Lin$^-$/Sca$^+$/cKit$^+$ cells were isolated from the bone marrow of naïve mice and differentiated *in vitro* in the presence of IL3, SCF and G-CSF; neutrophil differentiation and viability was monitored by flow cytometric analysis of Ly6G/Ly6C and 7-AAD (E-F).