Université de Montréal

Understanding the Transcriptional Control of EIF4E and its Dysregulation in Acute Myeloid Leukemia: Role of NF-κB

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Résumé

EIF4E, le facteur d'initiation de la traduction chez les eucaryotes est un oncogène puissant et qui se trouve induit dans plusieurs types de cancers, parmi lesquels les sous-types M4 et M5 de la leucémie aiguë myéloblastique (LAM). *EIF4E* est régulé à plusieurs niveaux cependant, la régulation transcriptionnelle de ce gène est peu connue. Mes résultats montrent que *EIF4E* est une cible transcriptionnelle directe du facteur nucléaire « kappa-light- chain-enhancer of activated B cells » (NF-κB).Dans les cellules hématopoïétiques primaires et les lignées cellulaires, les niveaux de EIF4E sont induits par des inducteurs de NF-κB. En effet, l'inactivation pharmaceutique ou génétique de NF-κB réprime l'activation de EIF4E. En effet, suite à l'activation de NF-κB chez l'humain, le promoteur endogène de *EIF4E* recrute p65 (RelA) et c-Rel aux sites évolutionnaires conservés κB in vitro et in vivo en même temps que p300 ainsi que la forme phosphorylée de Pol II. De plus, p65 est sélectivement associé au promoteur de *EIF4E* dans les sous-types LAM M4/M5 mais non pas dans les autres sous-types LAM ou dans les cellules hématopoïétiques primaires normales. Ceci indique que ce processus représente un facteur essentiel qui détermine l'expression différentielle de *EIF4E* dans la LAM. Les analyses de données d'expressions par séquençage de l'ARN provenant du « Cancer Genome Atlas » (TCGA) suggèrent que les niveaux d'ARNm de *EIF4E* et *RELA* se trouvent augmentés dans les cas LAM à pronostic intermédiaire ou faible mais non pas dans les groupes cytogénétiquement favorables. De plus, des niveaux élevés d'ARNm de *EIF4E* et *RELA* sont significativement associés avec un taux de survie relativement bas chez les patients. En effet, les sites uniques κB se trouvant dans le promoteur de *EIF4E* recrutent le régulateur de transcription NF-κB p65 dans 47 nouvelles cibles prévues. Finalement, 6 nouveaux facteurs de transcription potentiellement impliqués dans la régulation du gène *EIF4E* ont été prédits par des analyses de données ChIP-Seq provenant de l'encyclopédie des éléments d'ADN (ENCODE). Collectivement, ces résultats fournissent de nouveaux aperçus sur le control transcriptionnel de *EIF4E* et offrent une nouvelle base moléculaire pour sa dérégulation dans au moins un sous-groupe de spécimens de LAM. L'étude et la compréhension de ce niveau de régulation dans le contexte de spécimens de patients s'avère important pour le développement de nouvelles stratégies thérapeutiques ciblant l'expression du gène *EIF4E* moyennant des inhibiteurs de NF-κB en combinaison avec la ribavirine.

Mots-clés : EIF4E, NF-κB, La Régulation Transcriptionnelle, La Leucémie Aiguë Myéloblastique.

Abstract

The eukaryotic translation initiation factor *EIF4E* is a powerful oncogene that is overexpressed in cancers, including the M4 and M5 subtypes of acute myeloid leukemia (AML). *EIF4E* is regulated at multiple levels; however not much is known about the transcriptional regulation of this gene. My findings show that the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) is a direct transcriptional regulator of *EIF4E*. EIF4E levels are induced in primary hematopoietic cells and in cell lines in response to NF-κB activating stimuli. Pharmacological and genetic inhibition of NF-κB suppresses EIF4E levels. NF-κB factors RelA (p65) and c-Rel are recruited to evolutionarily conserved κB sites in the *EIF4E* promoter *in vitro* and *in vivo* following NF-κB activation concurrent with the recruitment of p300 and phosphorylated Pol II. Furthermore, p65 is selectively associated with the *EIF4E* promoter in M4/M5 AML subtypes but not in other AML subtypes or normal primary hematopoietic cells and thus represents an underlying factor in determining the differential expression of *EIF4E* in AML. Analysis of gene expression RNA-Seq data from The Cancer Genome Atlas (TCGA) suggests that *EIF4E* and *RELA* mRNA levels are upregulated in intermediate and poor prognosis AML but not in the cytogenetically favorable group. Additionally, elevated *EIF4E* and *RELA* mRNA levels are significantly associated with worst patient survival outcome. Furthermore, 8 new putative NF-κB target genes that may be regulated with a pattern similar to *EIF4E* in poor prognosis AML were in silico predicted from Chip-Seq data. Finally, 6 new transcription factors that may be implicated in *EIF4E* gene regulation were predicted from the analysis of ChIP-Seq data from the encyclopedia of DNA elements (ENCODE). Collectively, these findings could offer novel insights into the transcriptional regulation of EIF4E and a novel molecular basis for its dysregulation in AML. Understanding this level of regulation within the context of patient specimens is important for the development of novel therapeutic strategies to target *EIF4E* gene expression with specific NF-κB inhibitors combined with ribavirin.

Keywords : EIF4E, NF-κB, Transcriptional Regulation, Acute Myeloid Leukemia

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Abbreviations

4E-BE	4E Basal Element
4E-BP	4E Binding Protein
4E-HP	4E Homologue Protein
4E-SE	4E Sensitivity Element
4E-T	4E Transporter
4GI	4E/4G Inhibitor
AEG	Astrocyte Elevated Gene
AhR	Aryl Hydrocarbon Receptor
AIDS	Acquired Immunedefficiency Syndrome
AKIP	PKA Interacting Protein
AKT	Aktinic Kinase
AML	Acute Myeloid Leukemia
AML/ETO2	AML/Eight Twenty One Fusion Protein
ANOVA	Analysis of Variance
AP1	Activating Protein1
APL	Acute Pro-myelocytic Leukemia
ARE	AU Rich Element
AUF1	ARE Binding Factor
BAFFR	B-Cell-Activating Factor Receptor
Bcl	B-Cell Lymphoma
BCR	B-Cell Receptor
BCR/Abl	Breakage Cluster Region/Abelson Kinase Fusion Protein
Bp	Base Pairs
C/EBP	CAAT/Enhancer Binding Protein
CARMA1	CARD-Containing MAGUK Protein
CBP	CREB Binding Protein
CCND1	CyclinD1
CCP	Consensus Cold Probe
Cdx	Caudal Box Protein

CHD9	Chromodomain Helicase DNA Binding Protein 9
CHFR	Checkpoint with Forkhead and RING domain Protein
ChIP	Chromatin Immunoprecipitation
ChIP-Seq	Chromatin Immunoprecipitation coupled to Sequencing
cIAP	Cellular Inhibitor of Apoptic Pathways
CML	Chronic Myelogenous Leukemia
COMMD1	Copper Metabolism with MURR Domain 1
COX	Cyclooxygenase
CREB	cAMP Response Element Binding Protein
CRISPR	Clustered regularly interspaced short palindromic repeats
CRM1	Chromosome Region Maintenance 1
CYLD	Cylindromatosis Deubiquitinase
CYTH4	Cytohesin4
DNA	Deoxyribonucleic Acid
DR5	Death Receptor 5
DSIF	DRB Sensitivity Inducing Factor
EGF	Epidermal Growth Factor
eIF	Eukaryotic Initiation Factor (e.g. EIF4E, EIF4G, EIF4A)
ELAM	Endothelial Leukocyte Adhesion Molecule
EMSA	Electrophoretic Mobility Shift Assay
ENCODE	Encylcopedia of DNA Elements
FAB	French American British
FBS	Fetal Bovine Serum
FDR	False Discovery Rate
GAPDH	Glyeraldehyde 3-Phosphate Dehydrogenase
GnRH	Gonadotropin Releasing Hormone
GTP	Guanosine Triphosphate
HAT	Histone Acetyl Transferase
HDAC	Histone Deacetylase
hnRNPK	Heteronuclear Ribonuclear Protein K

HOIP/HOIL	Heme-Oxidized IRP2 Ubiquitin Ligase with Interacting Protein
HoxA9	Homeodomain Box Protein A9
ICAM	Intercellular Adhesion Molecule
IgG	Immunoglobulin G
IgM	Immunoglobulin G
IGV	Integrative Genomics Viewer
IKK	IκB Kinase
IL	Interleukin
ING4	Inhibitor of Growth 4
IRE	Internal Ribosomal Entry
IRES	Internal Ribosomal Entry Site
ITAF	IRES Trans Acting Factors
IκB	Inhibitor of NF-κB
IκB-SR	IκB Super Repressor
Kbp	Kilo Base Pairs
KDa	Kilo Dalton
LRA	Luciferase Reporter Assay
LRP/PRC	Leucine-Rich Pentatricopeptide Repeat Containing
LTβ	Lymphotoxin beta
M7GDP	7-Methyl Guanosine Diphosphate
MALT1	Mucosa-Associated Lymphoid Tissue Lymphoma 1
MAPK	Mitogen Activated Protein Kinase
Mcl1	Myeloid Cell Leukemia Protein 1
MCP	Mutant Cold Probe
MEME	Multiple EM for Motif Elicitation
MLL	Mixed-Lineage Leukemia Protein
MMP	Matrix Metalloproteinase
Mnk	MAP Kinase Interacting Serine/Threonine Kinase
mTOR	Mammalian Target of Rapamycin
MYC	Myelocytomatosis Protein

MyD88	Myeloid Differentiation Primary Response 88
NCBI	National Center for Biotechnology Information
NELF	Negative Regulator of Transcriptional Elongation Factor
NEMO	NF-κB Essential Modulator
NEXT-GEN	Next Generation
NF-κB	Nuclear Factor Kappa-light-chain-enhancer of activated B-cells
NFAT	Nuclear Factor of Activated T-cells
NFκBIA	NF-κB Inhibitor alpha
NIK	NF-κB Inducing Kinase
NURR	NR4A Nuclear Receptor
OCT	Octamer Factor
ODC	Ornithine Decarboxylase
PABP	PolyA Binding Protein
PAX5	Paired Box Protein
PBMC	Peripheral Blood Mononuclear Cells
PCR	Polymerase Chain Reaction
PDLIM2	PDZ And LIM Domain Protein 2
PKC	Protein Kinase C
PLXNA1	Plexin A1
PM	Perfect Match
PMA	Phorbol-12-myristate-13-acetate
PML	Promyelocytic Leukemia
PML/RARA	PML Protein/Retinoic Acid Receptor alpha Fusion Protein
PPP1R15B	Protein Phosphatase 1, Regulatory (Inhibitor) Subunit 15B
PRH	Proline-Rich Protein HaeIII Subfamily
pTEFb	Positive Regulator Transcription Elongation Factor b
PTL	Parthenolide
PU.1	Purine Rich (PU) box Binding Protein
RANBP2	Ran-Binding Protein 2
RARG	Retinoic Acid Receptor gamma

REC	Research Ethics Committee
RHD	Rel Homology Domain
RING	Really Interesting New Gene Protein
RIP	Receptor Interacting Protein
RMA	Robust Multi-Array
RNA	Ribonucleic Acid
RNA-Seq	RNA Sequencing
RNAP	RNA Polymerase
RNGTT	RNA Guanylyl transferase triphosphatase
RNMT	RNA Methyl Transferases
RNP	Ribonuclear Protein
RPKM	Reads Per Kilobase of exon model per Kilobase mapped reads
RPS	Ribosomal Protein S
SAH	S-Adenosylhomocysteine
SAHH	S-Adenosylhomocysteine Hydrolase
SCF-βTrCP	SKP-Cullin-F-box/Beta-Transducin Repeat Containing
Shh	Sonic Hedgehog
siRNA	Small interfering RNA
SIRT	Sirtuin
SNX32	Syntaxin 32
SOD	Superoxide Distmutase
SP1	Specificity Protein 1
STAT	Signal Transducer and Activator of Transcription
SUMO	Small Ubiquitin-Like Modifier 1
TAB	TAK Binding Protein
TAD	Transactivation Domain
TAK	Transforming Growth Factor β Activated Protein Kinase
TAP/NXF1	Tip Associating Protein/Nuclear Export Factor1
TBP	TATA Binding Protein
TCGA	The Cancer Genome Atlas

TCR	T-cell Receptor
TFIIB	Transcription Factor II B
TK	Thymidine Kinase
TNF-α	Tumor Necrosis Factor alpha
TRADD	TNF Receptor Associated Death Domain
TRAF1	TNF Receptor Associated Factor1
TRED	Transcription Regulatory Element Database
TSS	Transcription Start Site
Ubc/Uev	Ubiquitin C/Ubiquitin Conjugating Enzyme Variant
UCSC	University of California, Santa Cruz
USER	Untranslated Sequence Elements for Regulation
UTR	Untranslated Region
VCAM	Vascular Cell Adhesion Molecule
VEGF	Vascular Endothelial Growth Factor
WDR33	WD Repeat Domain 33
WHO	World Health Organization

To my family,

My wife,

And all of my friends,

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Chapter 1: Introduction

It is imperative that global rates of gene expression are strictly regulated to achieve optimal spatiotemporal RNA concentrations required to elicit a biological response[1]. Eukaryotic cells regulate gene expression through transcription, mRNA stability and post-transcriptional modifications, as well as protein synthesis (translation) and post-translational modifications. The RNA regulon model offers a blueprint in which cells regulate gene expression and protein synthesis. Cis-acting elements are positioned within the untranslated regions of transcripts known as untranslated sequence elements for regulation (USER) that recruit RNA binding proteins (RNPs) to modulate mRNA stability, export and translation[1,2].

Translational control is rapid and represents an important hallmark of cell development to modulate cell growth, proliferation and differentiation. Dysregulated expression and activity of components of the translation apparatus have been linked to cell transformation and carcinogenesis[3]. One key rate-limiting component, the eukaryotic initiation factor EIF4E, is upregulated in a plethora of cancers[4,5]. The transcriptional regulation of *EIF4E* and its dysregulation in acute myeloid leukemia (AML) is the focus of this thesis.

1.1 Cap- Dependent Eukaryotic Translation Initiation

Protein synthesis or mRNA translation is comprised of three distinct stages: initiation, elongation and termination[3]. The rate-limiting step in protein synthesis is translation initiation when the target mRNA is recognized by the translation machinery which catalyzes ribosomal assembly to commence protein synthesis[3,6]. Three mechanisms have been described so far for eukaryotic mRNA translational initiation[7]. These are (1) cap-dependent scanning[8], (2) scanning-independent ribosomal shunting[7] and (3) cap-independent scanning-independent internal ribosomal entry (IRE)[9]. In this thesis, I present my research on the initiation factor EIF4E, an integral component in the cap-dependent mode of translation initiation.

1.1.1 A 7-methyl guanosine cap structure is required for cap-dependent translation initiation

Following DNA transcription, the nascent pre-mRNA transcripts undergo a series of modifications including the amendment of a 7-methyl guanosine cap structure to the 5' end of transcripts[6]. The process of mRNA cap methylation constitutes a key step that is essential for

gene expression. It involves two enzymatic reactions catalyzed by the RNA guanylyl transferase triphosphatase (RNGTT), that forms the 5' guanylylated end on transcripts, and RNA methyl transferase (RNMT), which methylates the added 5' guanosine. It is has been demonstrated that elevating c-Myc expression promotes an increase in the proportion of capped transcripts, thus leading to increased rates of protein synthesis. c-Myc enhances capping by promoting RNA pol II phosphorylation as well as the upregulation of s-Adenosyl Homocysteine Hydrolase (SAHH) which neutralizes s-Adenosyl Homocysteine (SAH), an inhibitory bi-product of methylation reactions[10].

The cap structure is essential for mRNA stability[11], splicing[12], nucleo-cytoplasmic export[13] and acts as a marker that interacts with the translation initiation machinery[14]. Evidence for cap requirement in translation initiation came from studies using protein synthesis-competent wheat germ extracts. In these studies, only capped reovirus RNAs formed an interaction with the 40S ribosomal subunit and were efficiently translated[11,14]. Addition of m^7GDP cap analogs to the *in vitro* translation reaction or removal of the m7 cap structure from the viral transcripts diminished the translation of these RNAs[15], corroborating the importance of the cap structure in mRNA translation.

In an attempt to decipher the mechanism through which the cap structure triggers mRNA translation, cap-binding complexes were identified and isolated using ribosomal washes from *Artemia salina*[16] as well as from capped viral RNA studies. These complexes were originally referred to as cap binding proteins (CBP) I and II and are now known as eukaryotic translation initiation factors 4E (EIF4E) and 4F (EIF4F) respectively[17,18]. The EIF4E-cap interaction was displaced by cap analogs suggesting specificity. Furthermore, purified preparations of the 25KDa EIF4E protein revealed an integral role in stimulating mRNA translation of only capped viral transcripts[19]. The EIF4F molecule was later shown to comprise EIF4E in complex with a 46KDa RNA helicase (EIF4A) and a 220KDa mRNA-ribosome bridging factor (EIF4G)[20].

1.1.2 Molecular mechanism of cap-dependent translation initiation

As illustrated in figure 1, the dissociation of the 80S ribosome marks the start of mRNA translation facilitated by EIF6, which binds the 60S ribosomal subunit as well as EIF3 and EIF1A that bind the 40S ribosome. The 40S subunit associated with EIF3 and EIF1A is loaded with EIF2 together with GTP and the initiator tRNA, Met-tRNA, forming the 43S pre-initiation complex[3,21]. This complex is then recruited to the 5' end of transcripts through its interaction with the EIF4F cap-binding complex.

The EIF4F cap-binding complex is formed in three stages, first, EIF4E binds the 5' capped end of transcripts; next, the scaffolding protein EIF4G binds EIF4E and recruits the 43S pre-initiation complex to mRNA through its ability to interact with EIF3. The RNA helicase EIF4A and a "scanning" protein EIF4B are then recruited through EIF4G and are required to unwind the complex 5' untranslated regions (5'UTR) and scan for the start codon positioned in the consensus "Kozak" initiator sequence[3,8,21]. The circularization of the complex is attained through the bridging properties of EIF4G that interacts with the polyA binding protein (PABP), which in turn binds the polyadenylated 3' end of the transcripts. This subsequent association of the 43S pre-initiation complex and EIF4F results in the 48S complex[3,21].

The scanning properties of EIF4F allows the 48S complex to traverse the bound transcripts in a 5' to 3' fashion until the start codon (AUG) is located. Subsequently, the initiation factors are released in a process catalyzed by EIF5, a GTPase activating protein. The dissociation of these initiation factors allows the association of the 60S subunit and the commencement of translation elongation[3]. The association of 43S complex with mRNA through EIF4E constitutes the rate-limiting step in translation initiation[3,6]. The abundance and activity of the EIF4E protein is highly regulated in a multifactorial manner and will be discussed thoroughly in this chapter.

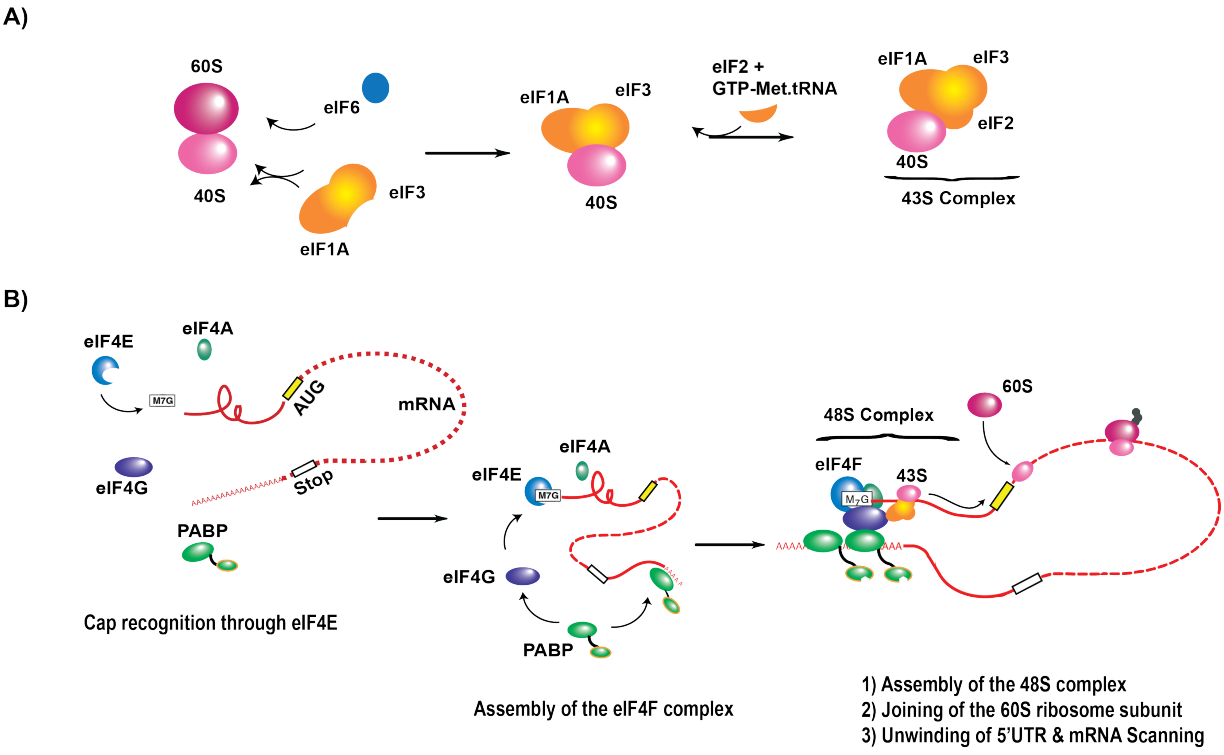

Figure 1. The mechanism of eukaryotic translation initiation. (A) Translation initiation commences with the dissociation of the 80S ribosomal subunit catalyzed by EIF1A, EIF3 and EIF6. The 40S subunit forms the 43S pre-initiation complex with EIF1A and EIF6 together with EIF2 and the Met initiator tRNA. **(B)** The 43S complex is recruited to the 5' end of transcripts through its interaction with the EIF4F complex. The EIF4F complex is formed from three main proteins: the cap binding protein EIF4E, a scaffolding protein EIF4G and an RNA helicase EIF4A (coupled to the scanning factor EIF4B). The EIF4F complex can interact with the 43S complex through EIF3 and and recruits it to the bound transcripts forming the 48S complex. Circularization is achieved through the interaction of EIF4G with the poly A binding protein (PABP). The 48S complex traverses the transcript in a 5' to 3' manner to locate the start codon. Translation initiation ends with the release of the initiation factors and the joining of the 60S ribosomal subunit.

1.2 Alternative Mechanisms for Translation Initiation

Alternative mechanisms for translation initiation have been previously described and involve cap-independent strategies[7]. Some viral and cellular mRNAs are innately uncapped and are thus translated by a process involving internal initiation. These transcripts harbor an internal ribosome entry site (IRES), a structural element in the 5'UTR. This alternative mode of initiation involves direct recruitment of the initiation factors and ribosome complex to the IRES element independent of EIF4E through IRES trans acting factors (ITAFs)[7,9,21]. Under patho-physiological and stress conditions, cap-dependent translation is impaired; however, mRNA translation of a subset of transcripts is maintained with a translation initiation re-programming in favor of IRES-mediated translation[21]. Translational profiling experiments performed under physiological and stress conditions such as, mitosis, differentiation and apoptosis as well as following heat shock and hypoxia, revealed that 10-15% of all mRNAs are translated potentially through an IRES-mediated process when cap-dependent translation is compromised[21]. This mechanism allows cells to adapt in response to various physiological and stress stimuli. Interestingly, since this process still requires most of the translational machinery, several picornaviruses have evolved strategies to hijack the host's translational machinery for viral protein synthesis through site-specific cleavage of EIF4G's amino-terminus compromising EIF4G-EIF4E interaction without altering its binding properties to other factors. This process diverts the host's EIF4G protein from cap-dependent translation in favor of viral IRES-mediated protein synthesis[22].

Finally, a role for the cap binding complex Cbc1 in mRNA translation has been demonstrated. In yeast, global translation is suppressed in response to osmotic stress; however, mRNAs encoding stress protective proteins remain selectively translated to allow survival. This process was shown to be dependent on Cbc1[23].

1.3 EIF4E Functions in mRNA Export and Translation

1.3.1 Structure of the EIF4E gene, alternative splicing and homology

The human eukaryotic initiation factor *EIF4E* gene spans more than 50 kilobase pairs (kbp) and is situated on chromosome 4. It is a functionally conserved gene encoded by 8 exons with three possible transcript variants illustrated in figure 2A. The most common transcript variant

1 (4749 bp) produces a 217 amino acid protein. Through alternative splicing, the longest transcript variant 2 (4842 bp) uses an alternative exon in the 3' coding region producing a 248 amino acid protein with an extended C-terminus. Transcript variant 3 (3406 bp) has a longer N-terminus producing a 237 amino acid protein by using an alternative exon in the 5'UTR and 5' coding region with a distinct initiation codon AUG. The alternatively spliced transcripts 2 and 3 have been predicted from cDNA libraries; however, their expression and biological functions are yet to be investigated[24].

Interestingly, two EIF4E mammalian paralogues have been described and are referred to as EIF4E-2, also known as 4E-HP, and EIF4E-3[24,25]. These proteins are distinct in their structure, function and expression pattern from the main ubiquitously expressed EIF4E-1 protein. EIF4E-2 is highly expressed in the testis whereas EIF4E-3 is mostly expressed in muscles, lung and spleen[24]. These paralogues have been shown to bind the 7-methyl cap[26,27]; however are, unlike EIF4E-1, incapable of functionally rescuing the growth-arrested phenotype in *S. cerevisiae* with a deletion in the *cdc33* gene, an *EIF4E* orthologue, suggesting that EIF4E-2 and EIF4E-3 fulfill distinct tissue-dependent functions[24]. In fact, studies have shown that EIF4E-2 and EIF4E-3 are not associated with EIF4G and are thus not part of the translation competent EIF4F complex. Furthermore, these proteins are incapable of binding known EIF4E-1 regulators such as the EIF4E binding protein 1(4E-BP1)[26,27]. These studies suggest that EIF4E paralogues may function as negative regulators of EIF4E-1 activity by competing for the same pool of capped transcripts and thus impede EIF4E-1 functions. The structure and function of the main EIF4E-1 protein will be further discussed and will be referred to as EIF4E.

1.3.2 The EIF4E structure reveals a distinct mode for cap-recognition required in mRNA export and translation

The EIF4E protein is of relatively small size at 25 KDa and is present as part of the EIF4F complex as well as in free form. The human and mouse cap-bound EIF4E structures have been elucidated with X-ray crystallography revealing a unique structure underlying EIF4E's cap binding properties[24].

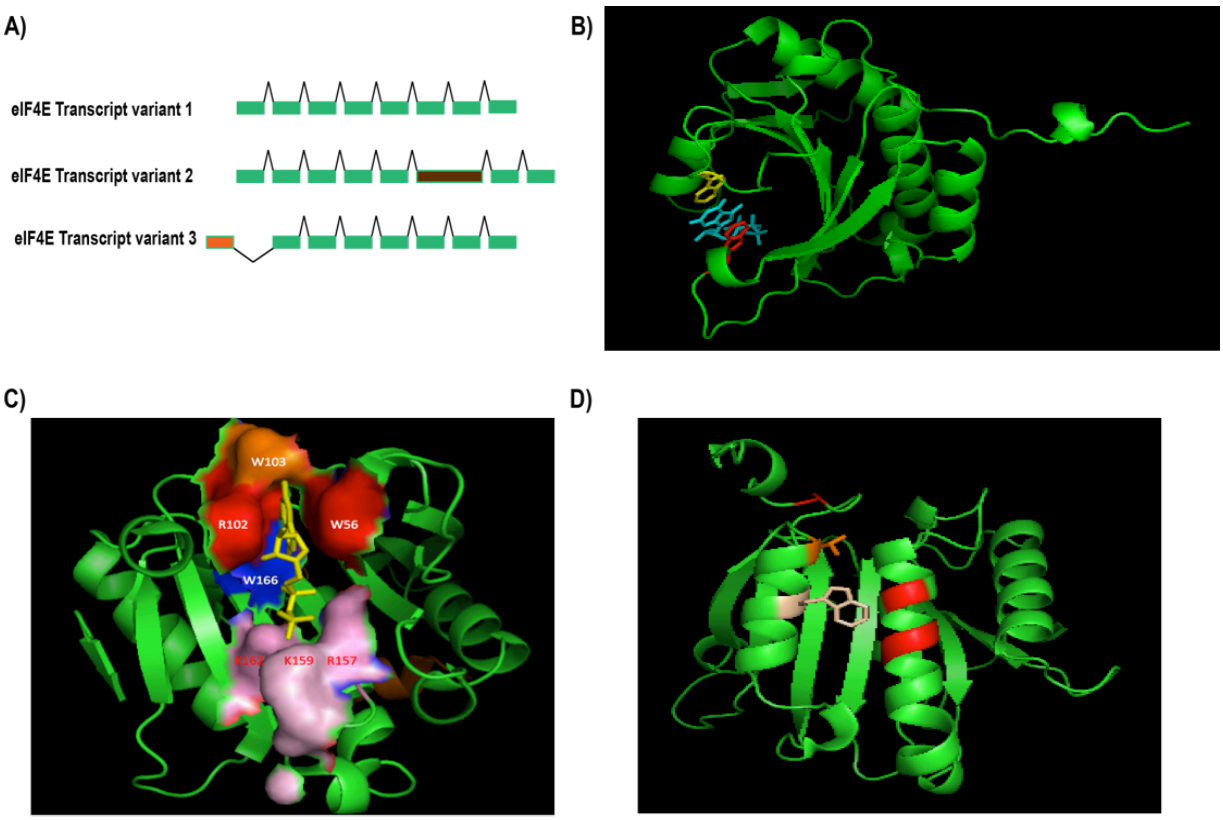

Figure 2. The initiation factor *EIF4E* gene comprises 8 exons that codes for a cap-binding protein with a unique structure. (A) Alternative splicing of the *EIF4E* gene produces three transcript variants, the most common being transcript variant 1 which produces a 217 amino acid protein. Transcript variant 2 uses an alternative exon in the 3' coding region resulting in a 248 amino acid protein with a longer C-terminus. Transcript variant 3 has a longer N-terminus producing a 237 amino acid protein by using an alternative exon in the 5' coding region. **(B)** Crystal structure of the mouse EIF4E bound the 7-methyl cap (blue) reveals a unique "cupped hand" structure composed of 8 anti-parallel beta strands supported by 3 alpha helices. Cap binding involves aromatic pi-pi stacking and requires W56 (red) and W102 (orange). **(C)** The 7-methyl cap (yellow) is nestled in a binding groove involving 7 amino acids: W56, W102, E103, W166, R157, K159 and K162 (see text for details). **(D)** The EIF4E dorsal surface binds protein partners that serve a regulatory purpose. Several key residues include H37, P38, L131, E132 and L135 (shown in red). Disruption of V69 (orange) and W73 (beige) hinders EIF4E's interaction with EIF4G and 4E-BPs. All cartoons were generated from the 1L8B structure, obtained from pdb.org, using pyMol.

The EIF4E protein consists of eight anti-parallel beta-sheets supported by three alpha-helices to form the palm and back of a cupped hand respectively[28-30] as illustrated in figure 2B. Studies have shown that EIF4E recognizes the 7-methyl cap through intercalation between two aromatic residues W56 and W102, also known as π-π stacking[31,32].

Additional residues involved in cap recognition are highlighted in figure 2C and include a polar E103 that interacts with the nitrogen moiety of the cap, a W166 residue forming a hydrophobic interaction with the methyl group of the cap, and three positively charged residues R157, K159, K162 that interact with the phosphate backbone of the cap. Nuclear magnetic resonance (NMR) solution structure of the cap-free EIF4E form, apo-EIF4E, revealed that this factor remains structured; however, key structural variations in the cap-binding pocket and the dorsal surface were noticed compared to the cap-bound EIF4E. Structural alterations in the S4-H4 loop distal to the cap binding pocket appears to be essential in regulating conformational changes in EIF4E following cap binding[33].

Structural studies with mouse EIF4E bound to EIF4G and 4E-BP1, an EIF4E regulator, revealed a requirement for the dorsal region in the EIF4E protein. Several amino acids (H37, P38, V69, W73, L131, E132 and L135) situated in the dorsal surface mediate EIF4E's interaction with its binding partners and regulators[34] (figure 2D). Disruption of two key phylogenetically conserved residues V69 and W73 hinders EIF4E's interaction with EIF4G and 4E-BPs[35,36]. Interestingly, phylogenetic alignment studies of EIF4E revealed that only around 170 amino acids representing the EIF4E core are conserved in all eukaryotes. This region includes the amino acids involved in cap recognition as well as binding to EIF4E partners and regulators[35,37]. Accordingly, EIF4E is functionally conserved whereby mammalian *EIF4E* is capable of rescuing the growth-arrested phenotype in *S. cerevisiae* with a deletion in the *cdc33* gene, an *EIF4E* orthologue, although it harbors only 30% sequence identity with its yeast counterpart[38,39].

Subcellular localization experiments revealed a dynamic nuclear and cytoplasmic localization for EIF4E. EIF4E functions at two levels: mRNA export and mRNA translation, both of which require cap recognition[24] (figure 3). Transcripts that harbor a complex GC rich highly

structured 5'UTR have been shown to be translated more efficiently, in an EIF4F dependent fashion, than transcripts with short unstructured 5'UTRs[40-42]. These transcripts have been dubbed as EIF4E translationally sensitive targets and include genes involved in cell proliferation and survival such as *PIM1, VEGF, MYC, ODC* and many more[41,43-45].

The nuclear localization of EIF4E (up to 68%)[46] suggests EIF4E nuclear functions. In fact, EIF4E has been shown to promote the export of transcripts containing a 50 nucleotide 4E-sensitivity element (4E-SE) in their 3'UTR[1,44,47,48]. Transcripts containing a 4E-SE element are bound by the export factor LRPPRC that interacts with EIF4E resulting in a CRM1-dependant export; unlike bulk mRNA export which is TAP/NXF1 dependent[48,49]. EIF4E export targets are also of the pro-proliferative nature and include *MYC, CCND1* (CyclinD1)*, ODC* and many more[44,47]. Interestingly, EIF4E was also shown to reprogram the nuclear pore complex to enhance the export of its target genes. Specifically, the RanBP2 cytoplasmic fibrils reduce the release and recycling of export factors to the nucleus, thereby impeding EIF4E mediated export; EIF4E circumvents this inhibitory mechanism by indirectly reducing the levels of RanBP2[50].

Eukaryotic cells organize the regulation of mRNA pools involved in the same biological process at the post-transcriptional level by altering the activities of RNPs interacting with these transcripts through USER sequences[2]. EIF4E functions to promote the nuclear export of its target genes and subsequently enhances the translation of the cytoplasmic EIF4E sensitive transcripts; these functions are independent of ongoing transcription and protein synthesis[48]. EIF4E export and translation targets are involved in cell proliferation and survival, accordingly, EIF4E is a central node of an RNA regulon that directs cell survival[48]. Importantly, not all EIF4E transcripts are sensitive at both export and translation levels[24].

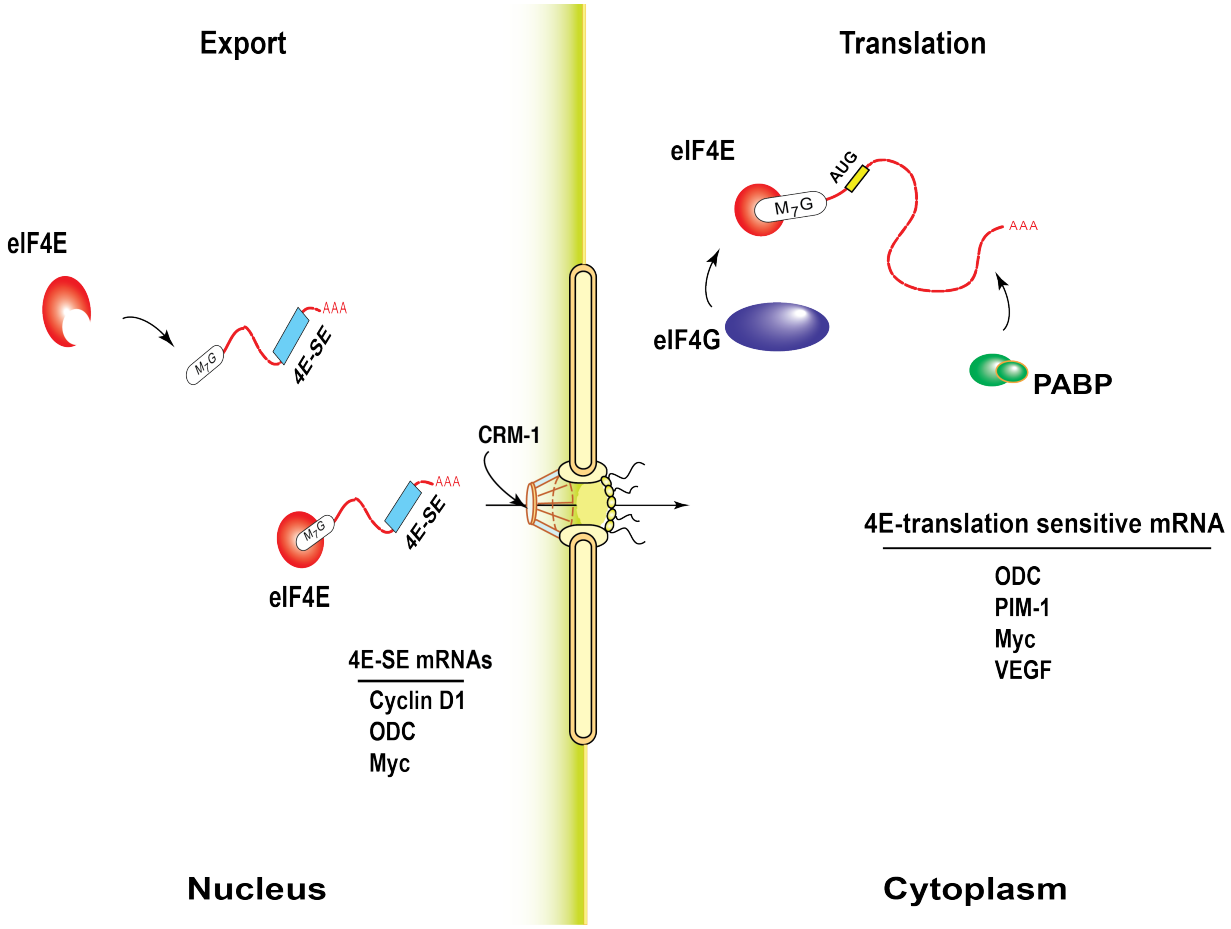

Figure 3: EIF4E functions at two levels: mRNA export and translation initiation. In the nucleus, EIF4E promotes the export of transcripts containing a unique 50 nucleotide element in their 3' UTR referred to as the 4E sensitivity element (4E-SE). EIF4E export is CRM1 dependent. In the cytoplasm, EIF4E enhances the translation of transcripts containing a complex highly structured 5'UTR. EIF4E export or translationally sensitive transcripts include genes involved in cell proliferation and survival (e.g. *ODC, MYC, CCND1, VEGF, PIM1*). EIF4E overexpression has been associated with cell transformation and tumor promotion owing to its proliferative and anti-apoptic program.

Finally, EIF4E's pro-survival program endows oncogenic properties for this initiation factor; in fact, EIF4E is overexpressed in an estimated 30% of human cancers[4,5].

1.4 EIF4E regulation is multifaceted with distinct levels of control

EIF4E expression and activity are regulated at multiple levels through transcription, mRNA stability, protein interactions as well as post-translational modifications. These distinct modes of regulation are illustrated in figures 4 and 5. Dysregulation in *EIF4E* control modules have been linked to malignant transformation[6].

1.4.1 A c-Myc centric view for EIF4E transcriptional control

The identification and cloning of the *EIF4E* promoter was achieved through screening human genomic DNA libraries using 5'c-DNA probes corresponding to *EIF4E* exons[51]. The transcription start site (TSS) of *EIF4E* was mapped with RNase protection assays[51,52]. The *EIF4E* promoter lacks a TATA box but harbors a polypyrimidine tract at position -25 distal to the TSS known as the EIF4E basal element (4EBE)[53]. This element binds the heteronuclear ribonuclear protein K (hnRNPK) that recruits the TATA binding protein (TBP) and consequently the basal transcription machinery to the *EIF4E* promoter[53,54]. Studies of the *EIF4E* promoter revealed multiple E-box elements that were shown to bind c-Myc and transactivate the *EIF4E* gene[51] as seen in figure 4. The tumor suppressor p53 was shown to repress *EIF4E* through luciferase reporter assays (LRA) possibly through sequestering c-Myc and hindering its interaction with the promoter[55]. In addition, the sonic hedgehog pathway was also shown to upregulate *EIF4E* in neural cells through Myc[56]. Accordingly, for the past 16 years *EIF4E* transcriptional regulation has been solely the purview of Myc; however, *EIF4E* transcript is still inducible in Myc null fibroblasts following serum stimulation[57] suggesting that other mechanisms are involved in *EIF4E* transcription. Consistent with this idea, a recent report suggests that *EIF4E* is also a C/EBP target[58]. In fact, the *EIF4E* promoter is enriched with binding sites for a plethora of transcription regulators including NF-κB, STAT, PU.1, PAX, NFAT, GATA, SP1 and many more[59]. This doctoral thesis focuses on the transcriptional regulation of *EIF4E* through NF-κB and its dysregulation in AML.

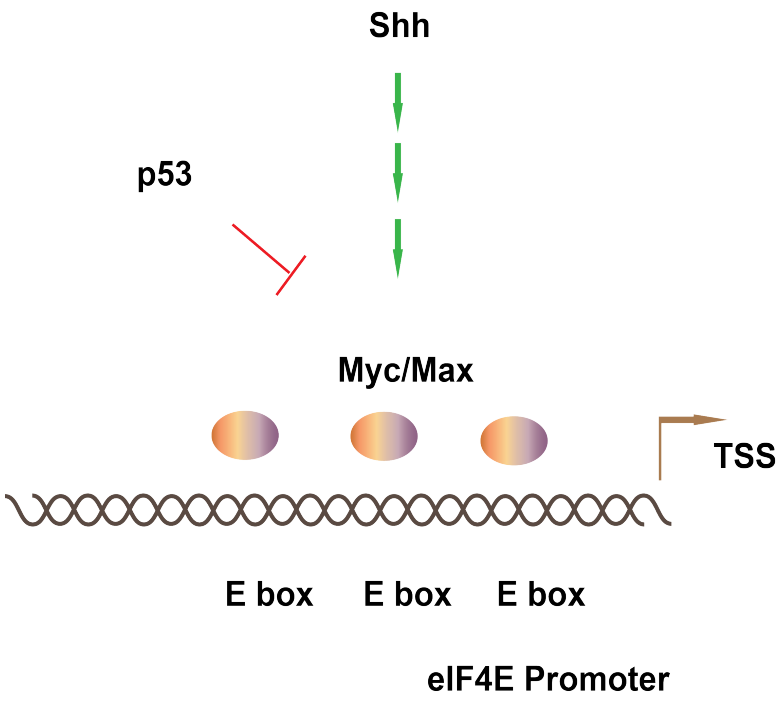

Figure 4. The Myc-centric view depicting *EIF4E* transcriptional control. The *EIF4E* promoter harbors multiple E-box elements that recruit c-Myc and upregulate *EIF4E* expression. The tumor suppressor p53 represses *EIF4E* expression by sequestering Myc and hindering its interaction with the promoter. The sonic hedgehog (SHH) pathway induces a Myc-dependent *EIF4E* expression in neuronal cells. These pathways illustrate Myc-centric mechanisms for the transcriptional regulation of *EIF4E*.

1.4.2 The stability of EIF4E mRNA is regulated by HuR and AUF1

Three conserved AU rich elements (AREs) have been identified in the 3'UTR of *EIF4E*. These elements direct HuR binding to stabilize the *EIF4E* transcript. On the other hand, a competing destabilizing protein, p42 AUF1, was shown to reduce *EIF4E* transcript stability. Furthermore, HuR is upregulated in cancer cells with elevated EIF4E expression and depletion of HuR in cancer cells results in EIF4E downregulation[60]. Thus EIF4E expression is also modulated through post-transcriptional events independent of *EIF4E* transcript levels (figure 5A).

1.4.3 EIF4E activity is modulated by protein interactions with a multitude of regulators

Another mode of EIF4E regulation involves its interaction with binding proteins that confer an activating or inhibitory effect[6] (figure 5B). Experiments conducted with Far-Western hybridization led to the isolation of two small proteins that interact with EIF4E and were referred to as EIF4E binding proteins 4E-BP1 and 4E-BP2[61]. Many proteins that bind EIF4E share the following small conserved amino acid motif YXXXXLϕ, where X is any residue and ϕ is a hydrophobic amino acid[62]. Accordingly, 4E-BPs compete with EIF4G to bind EIF4E and prohibit its access to the translational apparatus. In fact, 4E-BPs enhance the cap binding affinity in EIF4E thereby sequestering the bound EIF4E-mRNA complex to inhibit translation[61,63]. The dual cytoplasmic and nuclear localization of 4E-BPs suggests that these binding proteins can alter both EIF4E export and translation activities[64]. Importantly, 4E-BPs are regulated by phosphorylation through the mammalian target of rapamycin (mTOR); this reduces the interaction with EIF4E and increases translational activity[65].

Interestingly, mice lacking 4E-BPs were not more prone to developing cancers than controls[66-68] suggesting a redundancy in EIF4E regulators. A multitude of proteins have been identified that contain the consensus EIF4E binding motif including more than 200 homeodomain proteins; these proteins can act as positive and/or negative regulators of EIF4E activity[24]. The PRH/Hex homeodomain protein is a negative regulator of EIF4E's nuclear export functions[69,70] whereas HoxA9 was shown to promote both EIF4E export and translation functions[71].

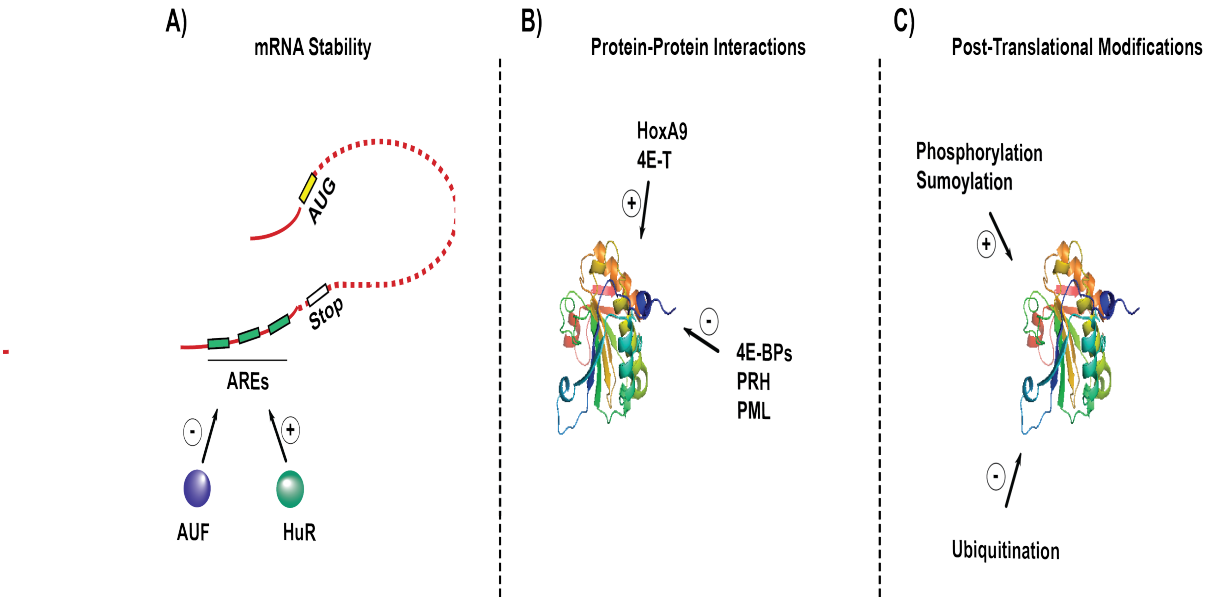

Figure 5. EIF4E is regulated at multiple levels through transcript stability, protein interactions and post-translational modifications. (A) The *EIF4E* mRNA harbors 3 AU rich elements (AREs) in the 3'UTR that bind HuR and AUF1. *EIF4E* transcript stability is enhanced by HuR and reduced with AUF1. **(B)** EIF4E activity is modulated by multiple protein-protein interactions. HoxA9 promotes the export and translation functions of EIF4E, whereas PRH and PML inhibit EIF4E-mediated export. On the other hand, 4E-BPs can inhibit both EIF4E export and translation activities. **(C)** EIF4E activity is also regulated through post-translational modifications. EIF4E sumoylation and phosphorylation enhance its translation functions. Ubiquitination of the EIF4E protein promotes its proteasomal-mediated degradation.

Additional homeodomain proteins shown to alter EIF4E activity include Otx, Engrailed 2, Emx2, Bicoid and Hox11[72].

Furthermore, two additional distinct protein families have been shown to interact with EIF4E and regulate its activity; they lack the YXXXXLϕ motif and include the RING domain containing proteins as well as the virus protein linked to the genome (VPg). Promyelocytic leukemia (PML) and arenaviral Z proteins are RING domain containing proteins that impede EIF4E activity by reducing its affinity to the 7-methyl cap (~100 fold)[24]. Nuclear PML was shown to impede EIF4E's mRNA export function[73]. The potyviral VPg was shown to form a complex with EIF4E and reduces its affinity for the mRNA cap[74].

The regulation of EIF4E through binding partners is thus multifaceted[24] involving an abundance of proteins expressed at various stages of the cell cycle and development to ensure optimal levels of EIF4E activity.

1.4.4 EIF4E activity is modulated through post-translational modifications

A fourth level of EIF4E regulation involves post-translational modifications of the EIF4E protein[6] including phosphorylation, ubiquitylation and sumoylation (figure 5C). The phosphorylation status of EIF4E reflects the translation rate and growth state of the cell[6]. The stress and cytokine activated p38 mitogen activated protein kinase (p38 MAPK) pathway converges at two EIF4E kinases, Mnk1 and Mnk2, to phosphorylate EIF4E at residue S209[75-77]. EIF4E phosphorylation enhances its export function and cell transformation capacity[78]. Furthermore, EIF4E could also be modified by ubiquitylation and SUMO1 conjugation[79-81].

This described multifactorial regulation of EIF4E ensures that ideal levels of EIF4E protein and activity are maintained to achieve an appropriate biological effect in response to physiological stimuli. Aberrant regulation of EIF4E expression and/or activity has been linked to malignancies as well as cell transformation.

1.5 EIF4E is Overexpressed in approximately 30% of Human Cancers and is a Plausible Candidate for Clinical Targeting

EIF4E target genes at the export and translation levels impart a proliferative effect[48]. In fact, microinjection of EIF4E in quiescent fibroblasts promotes DNA synthesis[82]. In addition, EIF4E overexpression decreases cell cycle transit time in HeLa cells, whereas downregulation of EIF4E using anti-sense methods increases transit time in a dose-dependent fashion[83]. These studies demonstrated a role of EIF4E in supporting cell cycle progression and cell transformation[6]. Furthermore, anti-apoptic functions have also been described for EIF4E whereby overexpression in NIH3T3 cells blocks apoptosis following serum deprivation[84]. Additionally, EIF4E overexpression impedes Myc-driven apoptosis[84]. Taken together, these studies highlight EIF4E's pro-survival properties. EIF4E is overexpressed in a multitude of cancers including hematopoietic malignancies and solid tumors[4,5].

EIF4E overexpression is underlined by several factors and is correlated with poor prognosis. EIF4E overexpression at the RNA and protein levels has been described in epithelial cancers including breast, colon, prostate, lung, cervix and squamous head and neck carcinoma. EIF4E gene amplification has also been described in head and neck as well as breast carcinomas. Hematopoietic cancers with elevated EIF4E levels include AML and Hodgkin and non-Hodgkin lymphomas[4]. In this thesis, I present a new mechanism underlying the dysregulation of *EIF4E* in AML. A brief overview on AML and its classification systems will be covered in this chapter.

Increased levels of EIF4E drive cell transformation and oncogenesis, accordingly, EIF4E represented a plausible candidate for clinical targeting[85]. Several preclinical and clinical methods have been described including synthetic peptides, anti-sense oligos, suicide gene therapy as well as a cap mimetic. Through a high throughput fluorescence polarization binding screen, the 4EGI-1 synthetic peptide was identified. This peptide inhibits the association of EIF4E with the EIF4G and blocks the formation of an active EIF4F complex. 4EGI-1 was shown to posses preferential activity in transformed cells and reduces the expression of *MYC* and *BCL2L1* (BclXL), both of which are EIF4E targets[86]. Small molecule analogs for 4EGI-1 have been described to target EIF4E in T-cell leukemia and non-small-cell lung cancer cells[85].

Anti-sense oligonucleotides targeting EIF4E have been described in breast, prostate and head and neck carcinoma xenograft models[87-89]. In addition, a suicide gene therapy strategy has effectively targeted EIF4E in a head and neck carcinoma mouse xenograft model by fusing a complex structured 5'UTR upstream of the toxic gene encoding thymidine kinase *TK*, thereby promoting its expression in cells with upregulated EIF4E[90]. Furthermore, a novel strategy in epithelial ovarian cancer with upregulated gonadotropin releasing hormone (GnRH) receptor has also been described by fusing the EIF4E negative regulator 4EBP1 to agonists of the GnRH receptor to inhibit EIF4E activity in those cells[91].

Finally, an effective EIF4E targeting strategy using a 7-methyl cap mimetic, ribavirin, has been demonstrated in AML[92,93], breast cancer[94] as well as head and neck carcinoma mouse xenograft model[93]. Ribavirin, an antiviral drug established in hepatitis C treatment, is structurally similar to the 7-methyl cap and was shown to physically interact with EIF4E[93,95,96] to impede its oncogenic functions in phase II clinical trial of poor prognosis AML, leading to clinical response with no adverse drug-related side effects[92]. This provided a novel clinical approach to target mRNA translation in cancers with elevated EIF4E. Importantly, ribavirin is the only EIF4E inhibitor to date that has provided promising clinical outcomes. Furthermore, similar exciting findings have been observed in AML patients treated with Ribavirin plus low dose cytarabine (Ara-C) with remission up to two years (Assouline et al. In press). Finally, Ribavirin resistance has been observed in AML patients and has been attributed to a GLI1 driven UGT1A-dependent glucuronidation of ribavirin. In this same study, ribavirin resistance was overcome by genetic or pharmacological inhibition of GLI1, suggesting a novel strategy to overcome ribavirin resistance in the treated patients[97].

1.6 Acute Myeloid Leukemia: A Hematopoietic Malignancy with Aberrant EIF4E Expression and Activity

Acute myeloid leukemia is a hematological malignancy of the myeloid lineage of blood cells; this malignancy affects the immature myeloid population of cells (myeloblasts) that expand at the expense of normal cells[98]. AML is a disease of older adults (> 60 years of age) with a median age at diagnosis of 67 years. The yearly incidence of new AML diagnoses in the USA

is 17.6/100,000 for individuals > 65 years of age, compared to 1.8/100,000 for individuals < 65 years (2008 statistics)[99]. The average 5-year overall survival (OS) rates, in patients receiving therapy, range from 5–15% in older adults and approach 30% in younger adults with AML[99]. The French-American-British classification (FAB) has classified AML into 9 distinct groups based on of the type of cell from which the leukemia has developed as well as the degree of maturity. The groups are: M0 (minimally differentiated), M1 (no maturation), M2 (granulocytic maturation), M3 (promyelocytic leukemia), M4 (myelomonocytic), M5a (monoblastic), M5b (monocytic), M6 (erythrocytic) and M7 (megakaryocytic)[98,100].

Several epigenetic and genetic anomalies underscore AML blasts progression. Epigenetic and/ or cytogenetic aberrations may result in the overexpression or the formation of fusion genes resulting in a blockade of myeloid differentiation and the formation of immature myeloblasts. Additionally, aberrant constitutive activation of cell receptors (e.g. Flt3, c-kit) confers a proliferative advantage to blast cells[98]. Notably, many AML cases are characterized by a normal karyotype.

1.6.1 Cytogenetic and Molecular classification of AML to predict patient prognosis

The four decades old FAB AML classification system has been subject to criticism, revisions and re-evaluations since the techniques required in classifying AML samples into FAB groups are very descriptive and rely on cell morphological features as well as simple cytochemical assays[101]. Accordingly, the World Health Organization (WHO) classifies AML into three clinical prognosis groups: favorable, intermediate and poor prognosis. The criteria required in this diagnostic classification are based on clinical data (patient history), cytogenetic analysis, immunophenotyping and biological features. This system aimed at offering a clinically relevant approach to determine prognostic parameters and plan more effective treatment regimen[102,103].

The advent of sequencing technologies has improved the WHO cytogenetic prognostic model through establishing molecular markers in all cytogenetic AML groups. This approach allowed for the classification of 5 prognostic subgroups with significant differences in OS thus

Cytogenetic Abnormality	Frequency	3-year OS%	Median OS (months)
Very Favorable PML/RARa (t(15;17)(q22;q12)) with CEPBA double mutations	< 10%	83%	NA
Favorable RUNX1/RUNX1T1 (t(8;21)(q22;q22)) or CBFB/MYH11 (inv(16)(p13q22)) with [NPM1 mutations and/or FLT3-ITD]	30%	62.6%	62.2
Intermediate Normal Karyotype with CEPBA single mutations, with [NPM1 mutations and/or FLT3-ITD]	28%	44.2%	25.6
Unfavorable Complex karyotype with MLL-PTD, RUNX1 and/or ASXL1 mutated	24%	22%	13.7
Very Unfavorable Complex karyotype with TP53 mutated	10%	0%	4.6

Table 1. Classification of AML into five clinical prognosis groups based on the underlying cytogenetic and molecular aberrations. The five distinct AML prognosis groups are shown with the underlying anomalies and frequency of occurrence. The 3-year overall survival (%) as well as the median survival rates for patients harboring the outlined anomalies are presented when applicable. NA, Not Applicable. Adapted from Grossmann et al.[104]

leading to a model based on molecular markers that is more comprehensive than standalone cytogenetics[104]. A summary of this prognostic classification system with the underlying cytogenetic and molecular aberrations and the associated OS rates is summarized in Table 1.

1.6.2 EIF4E is overexpressed in poor prognosis AML

A striking trend for *EIF4E* overexpression was observed in M4/M5 poor prognosis primary AML specimens but not in most M1/M2 specimens, with more than 100 samples tested[70]. The EIF4E export function is also augmented in M4/M5 AML; these samples show a predominant EIF4E nuclear accumulation. The nuclear function of EIF4E was shown to contribute to leukemogenesis by enhancing the export of target genes imparting a pro-proliferative and anti-differentiation program[70]. Molecular targeting of EIF4E with ribavirin (1μM) in M4/M5 primary AML specimens resulted in an EIF4E relocalization from the nucleus to the cytoplasm and inhibited EIF4E export functions leading to growth suppression. On the other hand, M1/M2 AML specimens with normal EIF4E levels were inhibited at a much higher concentration; additionally, M1 AML specimens with high EIF4E were also affected[93]. Accordingly, targeting EIF4E with ribavirin in poor prognosis AML patients led to clinical response[92]. These findings suggest that AML cells overexpressing EIF4E evolved an EIF4E dependency for proliferation and survival and thus have an oncogene addiction to EIF4E[92,93].

The molecular underpinnings for *EIF4E*'s differential regulation in AML will be discussed in chapter 2. Furthermore, the expression pattern of this oncogene has not been investigated in clinical prognosis AML groups and accordingly, this notion will be discussed in chapter 3.

1.7 Disruption of NF-κB activity in AML alters *EIF4E* expression and localization

The nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-κB) pathway is constitutively activated in primary leukemia specimens[105]. The link between NF-κB and *EIF4E* came from experiments performed in M5 primary AML and bc-CML (blast crisis Chronic Myelogenous Leukemia) specimens. In these cells, genetic NF-κB disruption with a super repressor (IκB-SR) resulted in a downregulation of EIF4E transcript and protein[70].

Furthermore, NF-κB suppression led to the re-organization of EIF4E nuclear bodies and its co-localization with PRH[70], a negative regulator of EIF4E activity[69].

These findings suggest that EIF4E expression and activity are regulated through the NF-κB pathway; however, the molecular mechanism underlying this control was not further investigated. The transcriptional regulation of *EIF4E* through NF-κB and its dysregulation in AML is the focus of this thesis.

1.8 An Overview of the NF-κB Pathway and its Dysregulation in AML

The NF-κB factors belong to a family of ubiquitous and inducible regulators first discovered by Sen and Baltimore in 1986 in the nuclei of activated B-cells[106]. They are evolutionary conserved from Cnidarians to humans but are absent in yeast and *C. elegans*, suggesting that they might have been lost during evolution[107]. They have been implicated in development as well as host defense and immune functions. Aberrations in this pathway have been linked to a variety of human diseases including arthritis, asthma, atherosclerosis, AIDS, inflammation as well as malignant transformation and oncogenesis[108].

1.8.1 NF-κB transcription factors

The mammalian NF-κB pathway is comprised of five distinct transcription regulators classified into two groups. The first group includes NF-κB1 (p105/p50) and NF-κB2 (p100/p52), whereas the second group includes RelA (p65), RelB and c-Rel. These proteins share a conserved 300 amino acid Rel homology N-terminus domain required for dimerization and DNA binding. NF-κB transcription factors exist as homo- or heterodimers and bind 10 bp cognate DNA sequences known as κB sites following the consensus motif 5'-GGGRNYYYCC-3' where R is a purine, Y is a pyrimidine and N is any nucleotide. Members of the first group are synthesized as precursor proteins (p105 and p100) containing ankyrin repeats that shield the nuclear localization signal; accordingly, these proteins must undergo limited proteolysis to yield the active subunits (p50 and p52). Members of the second NF-κB group share a C-terminus transactivation domain to regulate target gene expression[108].

In resting cells, NF-κB transcription factors reside in the cytoplasm and are kept inactive by the IκB family of inhibitors. Signals that elicit an NF-κB response trigger a cascade leading to the phosphorylation and degradation of the IκB-α inhibitor thus liberating NF-κB dimers that translocate to the nucleus and modulate gene transcription[107].

NF-κB gene deletion studies suggested a role for these transcription regulators in development and immune response[108]. *NFκB1* (p50) is critical for the survival of non-activated B-cells[109]. *NFκB2* (p52) is required for antigen presentation in dendritic cells and macrophages as well as the maintenance of lymph node and splenic architecture[110]. *RELA* (p65) knockouts are embryonic lethal due to defective fetal liver development[111]. Accordingly, p65 NF-κB has been implicated in cell survival and has been shown to promote induced lymphocyte proliferation and isotype switching[112]. The c-Rel NF-κB factor is important for B-cell proliferation in response to immunogens as well as cytokine production in T-cells and macrophages[113]. RelB has been shown to cross talk with the aryl hydrocarbon receptor (AhR) pathway and mediate an inflammatory response[114].

1.8.2 NF-κB signaling pathways

The NF-κB axis is activated by diverse stimuli and physiological conditions including cytokines and growth factors, viruses and bacteria and their products, carcinogens, tumor promoters, reactive oxygen species, stress as well as apoptosis inducers[108]. Two major NF-κB signal transduction pathways have been described: canonical and non-canonical, these are illustrated in figure 6.

The canonical pathway is the classic NF-κB activation pathway (figure 6A) that is triggered by ligand binding to cell surface receptors such as the tumor necrosis factor receptor, cytokine receptor, toll-like receptor as well as B- and T-cell receptors[107]. Receptor activation recruits scaffolding proteins (e.g. RIP, TRAF, TRADD, MyD88) and converges on an NF-κB activating module known as the IκB Kinase complex (IKK)[115]. The IKK complex is composed of three subunits: two catalytic subunits (IKKα and IKKβ) as well as a regulatory subunit IKKγ, also known as the NF-κB essential modulator (NEMO)[108]. The recruitment and

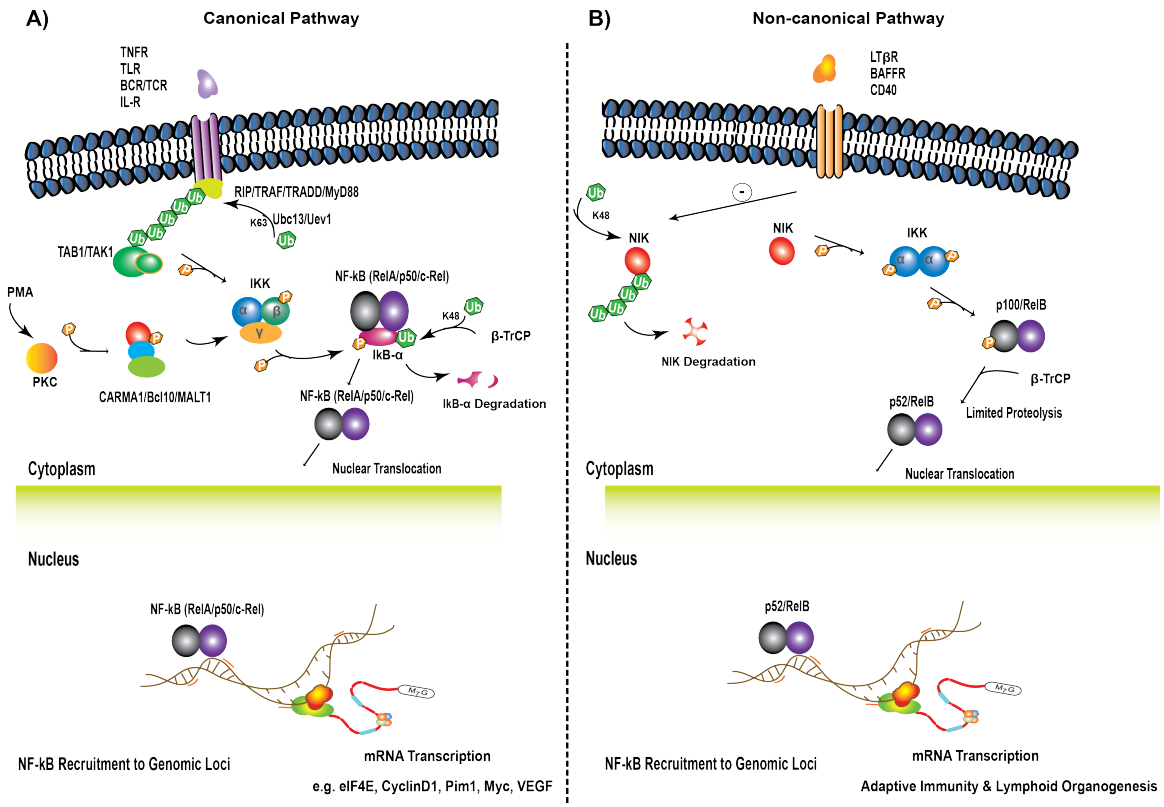

Figure 6. The NF-κB signaling pathways involve two main transduction modules: canonical and non-canonical. (A) The canonical pathway is triggered through the TNF, Toll-like, cytokine, B- and T-cell receptors to promote the activation of the NF-κB proteins p50, RelA (p65) and c-Rel. Activation leads to rapid recruitment of scaffolding proteins (e.g. RIP, TRAF, TRADD, MyD88). The kinase TAB/TAK phosphorylates and activates the IKK complex. Phosphorylation of the NF-κB inhibitor IκB-α through IKKβ promotes its degradation and subsequent translocation of NF-κB transcription factors the nucleus to induce a proliferative gene expression program. The phorbol ester PMA induces NF-κB through PKC. **(B)** The alternative non-canonical NF-κB activation pathway is triggered by ligands acting through the lymphotoxin receptor as well as the TNF receptors, CD40 and BAFFR. In the absence of a ligand, the NF-κB inducing kinase (NIK) is ubiquitinated and degraded. Following stimulation, NIK ubiquitination is arrested leading to its accumulation and the subsequent activation of IKKα. The NF-κB proteins affected in this pathway are p100 and RelB. P100 undergoes phosphorylation through IKKα, which promotes its limited proteolysis into active p52. Consequently, p52/RelB complexes translocate to the nucleus to promote the expression of genes involved in lymphoid organogenesis and adaptive immunity.

activation of the IKK complex often involves K63-linked ubiquitination of the scaffolding proteins as well as the NEMO subunit by the Ubc13/Uev1 and the HOIP/HOIL-1L ubiquitin ligases. Two deubiquitinases, A20 and CYLD, have been shown to negatively regulate NF-κB activation by reversing the activating K63-linked ubiquitination of NEMO and the recruiting scaffolding proteins[115].

The ubiquitinated scaffolding proteins would then serve as a platform to recruit the activating kinase complex composed of the transforming growth factor β activated protein kinase 1 (TAK1) and the TAK1 binding protein (TAB). The TAB/TAK1 complex phosphorylates and activates the IKK complex through IKKβ[115]. Activated IKKβ then phosphorylates the NF-κB inhibitor IκB-α at S32 and S36. Subsequently, the phosphorylated IκB-α undergoes K48-linked ubiquitination at K21 and K22 by the SCF-βTrCP ubiquitin ligase leading to degradation in the 26S proteasome[108]. IκB-α degradation allows NF-κB dimers containing NF-κB1 (p50), RelA (p65) and c-Rel to translocate to the nucleus and modulate target gene expression[115]. Furthermore, NF-κB activation independent of IκB-α degradation has been described in response to stress signals including hypoxia and exposure to ionizing irradiation as well as in response to erythropoietin and the epidermal growth factor (EGF). These signals promote NF-κB activation through Y42 phosphorylation of IκB-α without its degradation[108].

Small cell permeable molecules including the tumor promoter phorbol ester, PMA, can also trigger the canonical NF-κB pathway[106]. PMA binds and activates protein kinase C, PKC[116], which in turn activates IKK through the mucosa associated lymphoid tissue lymphoma complex, CARMA1/Bcl10/MALT1[117,118]. Importantly, PMA is a pleiotropic agent that triggers additional signaling pathways and thus proper experimental design must be implemented when studying PMA-induced NF-κB activation; for instance, coupling PMA treatment with NF-κB inhibitors.

The alternative non-canonical NF-κB activation pathway (figure 6B) is required during B- and T-cell development. This pathway converges on NF-κB2/RelB and is triggered by specific receptor signals including lymphotoxin (LTβ), B-cell activating factor (BAFF) and CD40[107]. In unstimulated cells, the NF-κB inducing kinase (NIK) is kept in an inactive state through

K48-linked ubiquitination and degradation. However, in response to signals triggering the alternative NF-κB pathway, NIK accumulates and is activated through auto-phosphorylation. Activated NIK phosphorylates IKKα homodimers, which in turn phosphorylates NF-κB2 (p100) leading to proteasome-mediated limited proteolysis of p100 into active p52. RelB/p52 heterodimers are formed and undergo nuclear import to promote a gene expression program underlying adaptive immunity and lymphoid organogenesis[115].

1.8.3 NF-κB factors exert a bimodal transcriptional activity

NF-κB factors exert their transcriptional activity through a transactivation domain (TAD) to modulate diverse gene expression programs. The NF-κB proteins p50 and p52 lack a TAD and thus must exist in complex with p65, c-Rel and RelB. In fact, homodimers formed from p50 or p52 subunits act as repressors of gene transcription[119]. The TAD of NF-κB proteins interact with different components of the basal transcriptional machinery including the TATA binding protein (TBP) and transcription factor IIB (TFIIB)[119]. NF-κB factors regulate gene expression through histone modification and chromatin remodeling as well as through regulation of transcription elongation. The p300 histone acetyltransferase (HAT) as well as histone deacetylases (HDACs) are recruited by NF-κB factors to exert positive and negative effects on gene expression respectively[119]. Furthermore, NF-κB proteins modulate transcription by recruiting regulators of transcriptional elongation: the DRB sensitivity-inducing factor (DSIF), the negative regulator of transcription elongation (NELF) and the positive regulator of transcriptional elongation (pTEFb). Recruitment of DSIF and NELF negatively modulates RNA polymerase (RNAP); on the other hand, pTEFb recruitment promotes the phosphorylation of the RNAP C-terminal domain and subsequently transcriptional activation[120,121]. This differential mode of regulation through elongation factors is largely dependent on the core promoter type in target genes[121].

NF-κB transcription factors modulate gene expression by acting as activators or repressors; this bimodal transcriptional activity is dependent on the inducing stimulus and physiologic context, post-translational modifications of NF-κB factors as well as the interaction with nuclear non-Rel proteins that modulate the selectivity of the NF-κB response[119,122].

NF-κB signaling is triggered by a variety of stimuli under distinct physiologic contexts ranging from growth promoting stimuli to stress, DNA damaging agents and death receptors[119]. Accordingly, two facets of NF-κB mediated transcriptional regulation are invoked in response to divergent stimuli. Growth promoting agents such as EGF harness the proliferative "face" of NF-κB activity which upregulates the anti-apoptic genes *MCL1, BCL2* and *BCL2L1* (BclXL) as well as repress cell death by downregulating the pro-apoptic genes *TNFRSF10B* (DR5), *FAS*, and *FASL*. On the other hand, exposure to stress causing stimuli utilizes a different facet of NF-κB activity, which increases the expression of pro-apoptic genes and downregulates the anti-apoptic program. These divergent facets of NF-κB activity involve NF-κB mediated chromatin remodeling through the histone modifying enzymes p300 and HDACs[119]. Furthermore, some NF-κB target gene promoters are pre-bound by NF-κB proteins, which are coordinately replaced by other NF-κB containing complexes in response to an activating stimulus[123]. Thus, NF-κB target genes can be positively and negatively regulated dependent on the physiologic context.

NF-κB transcriptional activity is essentially dependent on post-translational modifications including phosphorylation, ubiquitination, acetylation, nitrosylation, prolyl isomerization and mono-methylation. These modification patterns act as "barcodes" for dictating NF-κB transcriptional activity. NF-κB p65 phosphorylation occurs at multiple serine and threonine residues including S468, S536, S276, S311, T505 and T254. These phosphorylation patterns modulate p65 activity and can promote either transactivation or repression depending on the cell cycle and stimulus[122,124,125]. For instance, phosphorylated p65 upregulates the expression of the matrix metalloproteinase genes *MMP3* and *MMP13*; on the other hand, the same phosphorylation pattern represses the expression of the adhesion genes *ICAM* and *VCAM*[125]. Phosphorylation patterns that impart a transactivating potential promote the recruitment of p300; conversely, patterns that elicit a repressive effect promote the recruitment of HDACs. NF-κB p65 is also acetylated at five key lysine residues. Acetylation at residues K218, K221 and K310 enhances DNA binding activity and transactivation; on the other hand, acetylation at K122 and K123 impairs p65-mediated transactivation[119]. A summary of the post-translational modifications of NF-κB subunits is shown in table 2.

Recent studies suggest that specific nuclear signaling is integral in regulating NF-κB activity. This involves the interaction with non-Rel (i.e. non- NF-κB) nuclear proteins that selectively modulate DNA recognition and transactivation of NF-κB factors. Non-Rel proteins have been shown to either positively or negatively regulate NF-κB activity. Positive regulators include RPS3, AKT1, CD40, CD30, BAFFR, AKIRINs, AEG1 and AKIP1. These non-Rel proteins enhance NF-κB activity by promoting positive NF-κB post-translational modifications as well as the recruitment of p300 HAT. On the other hand, non-Rel negative regulators of NF-κB activity include CHFR, ING4, SIRT1/6, NURR1, PDLIM2 and COMMD1, which favor the recruitment of HDACs and promote gene repression of NF-κB targets. Furthermore, non-Rel nuclear proteins modulate the selectivity and affinity of NF-κB factors to κB sites in the genome[122].

1.8.4 NF-κB is constitutively activated in cancer

Genetic and cytogenetic anomalies as well as aberrations in the tissue microenvironment underlie carcinogenesis. NF-κB is constitutively activated in tumors with persistent nuclear localization. This constitutive activity is triggered by the tumor microenvironment (e.g. stress, pH), augmented signaling cascades that induce NF-κB as well as alterations in NF-κB protein expression or dysregulation of NF-κB inhibitors. Alterations in NF-κB proteins occur in a variety of cancers as a result of gene amplification as well as chromosomal rearrangement. Increased expression of NF-κB factors contributes to their constitutive activity by crowding the inhibitor IκB-α and thus spontaneously undergoes nuclear translocation in the absence of a stimulus. Inactivating mutations in the inhibitor IκB-α has also been linked to constitutive NF-κB activation. Cancers with aberrant NF-κB regulation include AML, Hodgkin's lymphoma, B- and T-cell leukemias and lymphomas, breast, liver, prostate, thyroid, bladder, ovarian, colon and lung cancer[108]. In AML, the NF-κB factor p65 is upregulated owing to a gene amplification as a result of a trisomy in chromosome 11[102]. Furthermore, NF-κB is constitutively activated in all AML subtypes. Interestingly, aberrant *EIF4E* and *NFκB* expression levels overlap in a multitude of cancers including AML.

Constitutive NF-κB activity promotes cell transformation, tumor initiation and promotion as well as angiogenesis, cell invasion and metastasis. This is achieved through NF-κB mediated upregulation of pro-proliferative and anti-apoptic gene expression programs. Increased cell survival is achieved by upregulating the anti-apoptic genes *BCL2, BIRC* (cIAP), *SOD* and *TRAF*. In addition, the tumor microenvironment is maintained by NF-κB mediated upregulation of *COX2, TNF, IL1A, MMP9* and chemokines. Also, NF-κB contributes to angiogenesis and metastasis by enhancing the expression of *ICAM, VCAM, ELAM* and *VEGF*. Furthermore, NF-κB activity has been linked to resistance to radiation treatment and chemotherapeutic agents through its anti-apoptic activity as well the upregulation of the multidrug resistance gene. Thus constitutive NF-κB activity causes perpetuating tumorigenesis[108,126].

1.8.5 Strategies to target NF-κB activity

Altered NF-κB activity contributes to carcinogenesis; accordingly, numerous strategies have been investigated to target this pathway. Several natural and synthetic compounds have been identified to inhibit NF-κB activation pathways as well as DNA binding activity[108]. Agents that block NF-κB DNA binding activity act as transcription factor decoys that bind the consensus κB motif and include synthetic peptides, heavy metals as well as natural compounds[126-128]. Other agents that block NF-κB activation do so by promoting the upregulation of the inhibitor IκB-α[129] as well as inhibit its phosphorylation[130] and proteasomal degradation[131] through steroidal compounds, IKK inhibitors and proteasome inhibitors respectively.

Several studies have focused on targeting NF-κB activity in AML using the proteasomal inhibitor MG-132 as well as a non-specific IKK inhibitor parthenolide (PTL). Primary AML specimens with high NF-κB activity that were treated with these inhibitors displayed a rapid induction of cell death while causing no significant toxicity to normal hematopoietic cells[131,132]. The efficiency of targeting NF-κB activity through current available inhibitors in AML will be further discussed in chapter 4 with emphasis on new plausible and more effective strategies.

Protein	Modification	Residue(s)	Enzyme (s)	Effect
NF-κB1	S-nitrosylation	C62	Unknown	Inhibition of DNA-binding
NF-κB1	Phosphorylation	S337	PKAc	Enhanced DNA-binding
NF-κB1	Acetylation	K431, K440 and K441	P300	Enhanced DNA-binding
NF-κB1	Phosphorylation	S927 and S932	IKKβ	Ubiquitination
NF-κB1	Phosphorylation	S903 and 907	GSK3β	Stabilizes
NF-κB1	Ubiquitination	Multiple lysines	βTrCP	Degradation
NF-κB2	Phosphorylation	S99, S108, S115, S123 S866, S870 and S872	IKKα	Ubiquitination
NF-κB2	Ubiquitination	K856	βTrCP	Degradation
NF-κB2	Acetylation	Unknown	P300	Increased DNA binding and processing
RelA	Oxidation	C38	Non-enzymatic	Inhibition
RelA	Nitration	Y66 and Y152	Nitric oxide	Inhibition
RelA	Acetylation	K122 and K123	P300/CBP, PCAF	Inhibition
RelA	Phosphorylation	S205	Unknown	Transcriptional activity
RelA	Acetylation	K218 and K221	P300/CBP	Inhibits IkBα binding, enhances DNA-binding (K221)
RelA	Phosphorylation	T254	Unknown	Prolyl isomerization
RelA	Proline isomerization	Pro255	Pin1	Stabilization and nuclear localization
RelA	Ubiquitination	aa220–335	SOCS-1	Degradation
RelA	Phosphorylation	S276	PKAc, MSK1	Activation/transcriptional activity
RelA	Phosphorylation	S281	Unknown	Transcriptional activity
RelA	Acetylation	K310	P300/CBP	Transcriptional activity
RelA	Phosphorylation	S311	PKCζ	Transcriptional activity
RelA	Phosphorylation	T435	Unknown	Transcriptional activity
RelA	Phosphorylation	S468	IKKε, IKKβ, GSK3β	Transcriptional activity
RelA	Phosphorylation	T505	Chk1	Transcriptional activity
RelA	Phosphorylation	S529	CK2	Transcriptional activity
RelA	Phosphorylation	S536	IKKβ, IKKα , RSK1	Transcriptional activity, nuclear localization, stability
RelB	Phosphorylation	T84 and S552	Unknown	Degradation
RelB	Phosphorylation	S368	Unknown	Dimerization
cRel	Oxidation/alkylation	C27	Non-enzymatic	Inhibition
cRel	Phosphorylation	S267	PKA	Nuclear localization, transcriptional activity
cRel	Ubiquitination	aa427–480	Unknown	Degradation
cRel	Phosphorylation	S454 and S460	Unknown	Transcriptional activity
cRel	Phosphorylation	S471	PKCζ, NIK	Transcriptional activity

Table 2. Post-translational modifications of NF-κB transcription factors have diverse impacts on functionality. NF-κB proteins are subject to a multitude of modifications that serve as "barcodes" dictating different transcriptional outcomes[133].

1.9 Hypothesis and Main Objectives

The inhibition of NF-κB activity with IκB-SR in primary AML and bc-CML specimens resulted in a substantial reduction in *EIF4E* transcript and protein levels[70] suggesting that *EIF4E* is downstream to NF-κB. However, the molecular underpinnings of this plausible regulation were not investigated. Accordingly, I hypothesized that *EIF4E* is a direct NF-κB transcriptional target and that aberrant NF-κB activity contributes to EIF4E dysregulation in AML. To that end, two aims were set.

Aim1: Investigate the mechanism underlying NF-κB mediated regulation of *EIF4E* using hematopoietic cell lines and primary specimens. I also aimed at examining a correlation between constitutive NF-κB activity and selective *EIF4E* dysregulation in AML. This aim will be discussed in chapter 2, which outlines my published manuscript on NF-κB mediated *EIF4E* regulation.

Aim2: Perform a bioinformatics analysis using data from public repositories to identify a correlation between elevated *EIF4E* and NF-κB *RELA* (p65) mRNA expression in poor prognosis AML as well as provide an in depth understanding of *EIF4E*'s intricate transcriptional control mechanisms. This aim will be discussed in chapter 3, which outlines a manuscript in preparation on new insights into *EIF4E* regulation and the association of elevated *EIF4E* and *RELA* expression with poor patient prognosis in AML.

This thesis delineates novel insights into understanding the transcriptional regulation of *EIF4E* through NF-κB, as well as describes a prognostic association of *EIF4E* and NF-κB *RELA* expression with clinical outcome in AML. These findings will provide an additional level of EIF4E targeting through NF-κB where targeting EIF4E activity with ribavirin is coupled to modulating *EIF4E* expression through NF-κB suppression in AML.

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Chapter 2: The eukaryotic translation initiation factor *EIF4E* is a direct transcriptional target of NF-κB and is aberrantly regulated in acute myeloid leukemia

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Synopsis: The transcriptional control of *EIF4E* has been viewed, for the past 16 years, as Myc-centric; however, *EIF4E* is still inducible in Myc null fibroblasts. Loss of NF-κB activity in M5 acute myeloid leukemia specimens resulted in a downregulation of EIF4E suggesting a link between NF-κB activity and EIF4E regulation. In this chapter, I present data supporting an NF-κB mediated transcriptional modulation of EIF4E and the molecular mechanisms of this control. Furthermore, I present evidence suggesting an NF-κB dependent mechanism underlying the differential upregulation of EIF4E in AML.

Contribution:

All the data in **Figures 1, 2, 3, 4, 5A, 5B, 6, 7** were generated by **Fadi Hariri (100%)**

All the data in **Supplementary Figures 1A, 1B, 2, 3, 4C** were generated by **Fadi Hariri (100%)**

Data in **Figure 5C** and **Supplementary Figure 1C and 1D** were generated by **Fadi Hariri (50%)** and **Meztli Arguello (50%)**

Data in **Supplementary Figure 4A and 4B** were generated by **Biljana Culjkovic (100%)**

The eukaryotic translation initiation factor *EIF4E* is a direct transcriptional target of NF-κB and is aberrantly regulated in Acute Myeloid Leukemia

Authors: Fadi Hariri, Meztli Arguello, Laurent Volpon, Biljana Culjkovic-Kraljacic, Torsten Holm Nielsen, John Hiscott, Koren K. Mann and Katherine L.B. Borden

Abbreviations Footnote: NF-κB: Nuclear factor κ in activated B Lymphocytes, EIF4E: eukaryotic translation initiation factor 4E, PMA: phorbol 12-myristate-13-acetate, PBMCs: peripheral blood mononuclear cells; Acute Myeloid Leukemia, AML

Manuscript: Text, Figures; Supplementary text and figures **(No Data Deposition)**

Key Points:

1) *EIF4E* is a direct inducible transcriptional target of NF-κB. Its upregulation is abrogated with genetic or pharmacological NF-κB inhibition.
2) NF-κB in M4/M5 AML is strongly and constitutively associated with the *EIF4E* promoter, contributing to its transcriptional upregulation.

Abstract

The eukaryotic translation initiation factor *EIF4E* is a potent oncogene elevated in many cancers including the M4 and M5 subtypes of acute myeloid leukemia (AML). Although *EIF4E* RNA levels are elevated 3-10 fold in M4/M5 AML, the molecular underpinnings of this dysregulation were unknown. Here, we demonstrate that *EIF4E* is a direct transcriptional target of nuclear factor kappa-light- chain-enhancer of activated B cells (NF-κB) that is dysregulated preferentially in M4 and M5 AML. In primary hematopoietic cells and in cell lines, *EIF4E* levels are induced by NF-κB activating stimuli. Pharmacological or genetic inhibition of NF-κB represses activation. The endogenous human *EIF4E* promoter recruits p65 and cRel to evolutionarily conserved κB sites *in vitro* and *in vivo* following NF-κB activation. Transcriptional activation is demonstrated by recruitment of p300 to the κB sites and phosphorylated Pol II to the transcriptional start site. In primary AML specimens, generally we observe that substantially more NF-κB complexes associate with *EIF4E* promoter elements in M4 and M5 AML specimens examined than in other subtypes or unstimulated normal primary hematopoietic cells. Consistently, genetic inhibition of NF-κB abrogates *EIF4E* RNA levels in this same population. These findings provide novel insights into the transcriptional control of *EIF4E* and a novel molecular basis for its dysregulation in at least a subset of M4/M5 AML specimens.

Key Words: AML, EIF4E, NF-κB

2.1 Introduction

The eukaryotic translation initiation factor 4E (*EIF4E*) is a potent oncogene which is inappropriately elevated in about 30% of human cancers including the M4 and M5 subtypes of Acute Myelogenous Leukemia (AML) and in blast crisis, but not chronic phase, CML[1]. EIF4E overexpression leads to increased proliferation, evasion of apoptosis, oncogenic transformation, tumor invasion and metastases [1-5]. EIF4E interacts with the methyl-7-guanosine cap moiety on the 5' end of mRNAs [6] and via this activity plays a central role in cap dependent translation and in nucleo-cytoplasmic export of a subset of transcripts encoding proteins involved in cellular growth, survival and transformation such as Cyclin D1, VEGF, c-myc, Mcl1 and Pim1 [7,8]. Both the translation and export activities of EIF4E contribute to its transformation potential [9]. Depletion of EIF4E in cancer cells using siRNA, anti-sense oligonucleotides or pharmacological inhibitors leads to cell cycle arrest and decreased tumorigenicity [10-13]. Targeting of EIF4E with a competitive inhibitor of the m^7G cap moiety, ribavirin, led to clinical responses in poor prognosis M4 and M5 AML patients, including remissions in a phase II trial [10].

Few studies have focused on how EIF4E RNA levels become elevated in malignant cells. In M4 and M5 AML specimens, EIF4E RNA and protein levels are elevated by ~ 3-10 fold relative to primary hematopoietic cells from healthy volunteers [10]. Traditionally, transcription of *EIF4E* was thought to be controlled only by c-Myc [14]. Other studies have implicated Sonic hedgehog signaling [15] and p53 [16] in the control of *EIF4E* transcription, but these too are ultimately considered to be mediated by c-Myc interaction with the *EIF4E* promoter. Interestingly, *EIF4E* expression is still stimulated in response to serum in Myc null fibroblasts indicating that there are other mechanisms to control *EIF4E* transcription [17]. Consistent with this idea, a recent report suggests that *EIF4E* is also a C/EBP target [18]. Our previous studies in primary M4 and M5 AML specimens suggested a tantalizing link between NF-κB and the transcription of *EIF4E*. Introduction of the NF-κB inhibitor IκB-super repressor (IκB-SR) into primary M4 or M5 AML specimens, which are characterized by constitutive NF-κB activity, resulted in a substantial reduction in EIF4E transcript and protein levels [19]. However to date, whether the link between NF-κB and EIF4E was a direct one, had not been investigated.

The NF-κB family of transcription factors plays a central role in the regulation of growth, proliferation, inflammation and apoptosis [20]. Importantly, NF-κB is constitutively active in primary AML specimens and this elevation contributes to the leukemogenic phenotype[21]. NF-κB members include NF-κB1 (p50), NF-κB2 (p52), RelA (p65), RelB and cRel and they may form homo- or hetero-dimers. In resting cells, NF-κB subunits reside in the cytoplasm, kept inactive by the IκB family of inhibitors. Receptor stimulation leads to a signaling cascade that culminates in the activation of the IKK kinase complex, which phosphorylates the IκB molecules leading to their proteasomal degradation. Free NF-κB dimers translocate into the nucleus, where they bind cognate DNA sequences known as κB sites (consensus 5'-GGGRNYYYCC-3') *via* their Rel homology domain (RHD) to regulate gene transcription [22,23]. Importantly, introduction of a dominant negative repressor of NF-κB, IκB-SR, which blocks its nuclear translocation leads to reduced growth of primary AML cells [19].

In this study, we establish that *EIF4E* is a direct transcriptional target of NF-κB in hematopoietic cell lines and primary normal hematopoietic cells. Further, studies in primary M4/M5 AML specimens indicate that at least for the specimens examined the *EIF4E* promoter elements are preferentially occupied relative to M1 and M2 AML subtypes with normal EIF4E levels or to healthy, unstimulated, hematopoietic cells. These studies suggest that NF-κB activation can differentially target subsets of genes in specific AML contexts. These findings provide a novel control mechanism for *EIF4E* expression and a novel basis for its dysregulation in AML, and likely in other malignancies characterized by activated NF-κB.

2.2 Materials and Methods

2.2.1 Primary cell isolation and treatments

Primary B cells were purchased from Stem Cell Research. Primary AML samples from anonymous patients (M1, M2, M4 and M5) were obtained from the BCLQ (Banque de Cellules Leucémiques du Québec) with ethics committee approval from the University of Montreal (Comité d'éthique de la recherche en santé CEFRM#195). Leukaphereses from healthy donors were obtained at the Royal Victoria Hospital, Montreal, Quebec, Canada with

ethics committee approval from the Jewish General Hospital and McGill University Research Ethics Committee (REC) board of the SMBD-Jewish General Hospital (protocol number# 06-103). Written informed consent was obtained from both healthy donors and AML patients in accordance with the Declaration of Helsinki. Characteristics of the AML primary specimens used in this study are presented in Supplementary Table 1. AML cells were thawed in RPMI 1640 (Invitrogen, Life Technologies, Burlington, ON, Canada) supplemented with 10% heat inactivated FBS and 100 U of penicillin/streptomycin (Invitrogen). Nuclear lysates were prepared from 4 million cells respectively.

Peripheral blood mononuclear cells (PBMCs) were isolated with Ficoll-Paque PLUS (Invitrogen) according to the manufacturer's instructions. Cells were re-suspended in RPMI 1640 (Invitrogen) supplemented with 15% heat inactivated FBS and 100 U of penicillin/streptomycin (Invitrogen). Cells were plated at a density of 30 x 10^6 cells in T75 flasks and treated with PMA (Phorbol 12-myristate 13-acetate, Sigma, Oakville, ON, Canada) at 20 ng/ml for the described time points. For NF-κB inhibition, cells were pretreated for 1 hour with 10 μM Bay 11-7082 (Sigma) prior to PMA stimulation and the inhibitor was kept in the media throughout the experiment.

2.2.2 Cell culture

BJAB (Burkitt's cell lymphoma), KG1a (M0 AML) and THP1 (M5 AML) cells were obtained from and cultured according to the American Type Culture Collection (ATCC). KM-H2 (Hodgkin's) lymphoma cell line was a kind gift from Dr Sigrun Smola. The BJAB cell line used was always cultured at low density, maintained at ~70% confluency and kept from reaching full confluency. BJAB cells (10^6 cells in six-well plates) were treated with 20 ng/ml PMA (Sigma). NF-kB inhibition was carried out using the Bay 11-7082 (Sigma) by pretreating the cells with 10 μM for 1 h before stimulation with PMA.

2.2.3 Antibodies and Primers

The following antibodies were used: p50 (Rockland Immunochemicals, 100-4164; Cedarlane Laboratories, Burlington, ON, Canada), RelA (p65) (Rockland Immunochemicals, 100-4165), cRel (Cell Signaling no. 4727S, New England Biolabs, Whitby, ON, Canada), p300 (Rockland Immuno- chemicals, 100-301-76), EIF4E (BD Transduction laboratory, 610270, Mississauga, ON, Canada), S2/5 phospho-RNA polymerase II (Cell Signaling no. 4735S) and β-actin (Sigma, A5441). Specific primers designed for gel shift assays, chromatin immunoprecipitation (ChIP) and expression analysis are summarized in Supplementary Table 2.

2.2.4 Promoter Analysis and validation of NF-κB sites

The human *EIF4E* promoter sequence was obtained from the Transcriptional Regulatory Element Database (TRED, Cold Spring Harbor, http:// rulai.cshl.edu/cgi-bin/TRED/tred.cgi?process=home). Promoter analysis for transcription factor binding sites was performed using MatInspector[24] (http://www.genomatix.de/cgi-bin/matinspector_prof/). Validation of the putative NF-κB elements was carried out with ChIP and electrophoretic mobility shift assays (EMSA). These protocols are described in detail in the supporting text.

2.2.5 Expression Analysis

Total RNA was isolated from control and treated cells using Trizol (Invitrogen) according to the manufacturer's instructions. RNA was additionally treated with RNase-free DNase I (Ambion, Life Technologies). Complementary DNA was synthesized using M-MLV Reverse Transcriptase (Invitrogen) and expression analysis was then performed with quantitative real-time PCR using the StepONE real-time PCR (Applied Biosystems, Life Technologies). Ct values were analyzed using the DDCT method[25] and normalized to β-Actin, Histone 2B and Ubiquitin. Total protein was extracted with RIPA buffer (Tris 50 mM, NaCl 150 mM, SDS 0.1%, sodium deoxycholate 0.5% and NP-40 1%) and analyzed by western blot using chemiluminescence (Thermoscientific, Fisher, Ottawa, ON, Canada). Western blot band densities were analyzed with ImageJ software (ImageJ, US National Institutes of Health, Bethesda, MD, USA).

2.3 Results

2.3.1 NF-κB activation stimulates EIF4E expression in hematopoietic cell lines

Our previous studies indicated that EIF4E is highly elevated (~3- to 10-fold) in M4/M5 AML specimens but generally not in other AML subtypes or in a variety of primary hematopoietic cells isolated from healthy volunteers, including $CD34^+$ cells, granulocytes and monocytes[10,19,26]. Given our previous finding that IκB-SR expression in a primary M5 AML specimen repressed *EIF4E* RNA levels, we investigated the potential role of NF-κB activity in the direct transcriptional control of *EIF4E*. In M4/M5 AML cells, NF-κB activity is constitutive thus, we used a hematopoietic cell line BJAB as a model system because of its low-basal NF-kB activity, which enabled us to examine the effects of NF-kB activation [22,23,27]. Cells treated with PMA, an NF-κB stimulator, exhibited a rapid increase in *EIF4E* mRNA, with levels doubling at 3 h (Figure 1A), as determined by quantitative real-time PCR and a parallel increase in EIF4E protein levels up to sevenfold was also observed (Figure 1D). Known NF-κB targets, *MYC* and *CCND1*, also displayed a similar increase (two- to threefold) upon PMA stimulation of these cells (Figures 1B and 1C). Given that PMA is a pleiotropic agent, we assessed the effects of the pharmacological IKK complex inhibitor Bay 11-7082,[28] which blocks IκBα phosphorylation. The observed induction of *EIF4E* mRNA expression was completely abrogated when samples were treated with Bay 11-7082, consistent with the hypothesis that the increase in *EIF4E* mRNA observed is due to NF-κB activation (Figure 1A).

2.3.2 The NF-κB subunits cRel and p65 directly alter EIF4E promoter activity.

We analyzed the human *EIF4E* promoter (up to 863 bp upstream of the transcriptional start site) using bioinformatics (MatInspector[24]) for the presence of κB sites. Previous analysis of the rat *EIF4E* promoter had suggested the presence of two putative Rel elements but these were never examined for activity[29]. We identified four κB sites (denoted κB1 through 4 in the text) centered on positions -836, -808, -630 and -348 relative to the transcriptional start site (Figure 2A and Supplementary Figure 2A).

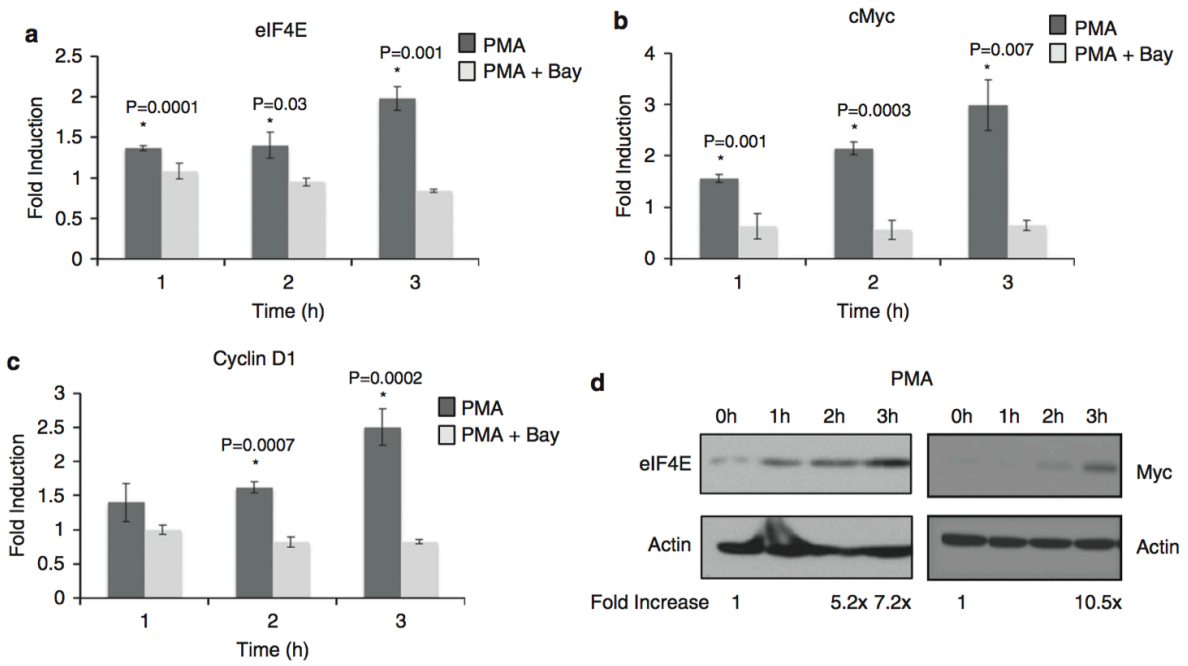

Figure 1. Stimulation of BJAB cells with PMA leads to NF-κB dependent *EIF4E* transcriptional upregulation. BJAB cells at approximately 50% confluency were stimulated with PMA (20 ng/mL) for the indicated time points, in the presence (black) or absence (grey) of BAY11-7082 (10 μM). **A, B, C**) Expression of EIF4E (*EIF4E*), cMyc (*MYC*) and cyclinD1 (*CCND1*) mRNA was analyzed by Q-PCR. Results shown are the average of 3 independent experiments each performed in triplicate (error bars are s.e.m, p values were calculated using the student t-test). **D**) Immunoblot of EIF4E and cMyc expression following PMA stimulation of BJAB cells for the indicated times; β-actin is provided as loading control. Band intensity was quantified with ImageJ.

These sites are evolutionary conserved in human, cow, monkey, mouse and rat (Supplementary Figure 2B). Note that the NF-κB pathway is absent in yeast and worms [20] although both express *EIF4E* suggesting an evolutionary emergence of an NF-κB mediated mechanism to induce *EIF4E* expression in higher organisms. Our analysis also revealed the presence of binding sites for several other transcription factors (PU.1, PAX5, Octamer, NF-AT) (not shown). Although the significance of these novel binding sites needs further investigation, they are suggestive of a more intricate transcriptional control of *EIF4E* than the prevailing model of transcriptional regulation solely through Myc.

In order to determine whether activating signals induce binding of NF-κB complexes to the putative κB elements in the *EIF4E* promoter, we carried out electrophoretic mobility shift assays (EMSA) using nuclear extracts from BJAB cells in the presence and absence of PMA. These cells exhibited low basal levels of NF-κB activity, which could be dramatically induced by PMA treatment as determined by EMSA using the kappa light chain consensus motif (Figure 2B). At 90 minutes post-stimulation, all four of the predicted κB elements yielded inducible complexes when incubated with nuclear extracts (Figure 2C and Supplementary Figure 3). The complexes were specific, as they could be competed by excess cold probe corresponding to the consensus kappa light chain *κB* motif [30] (Figure 2C, lane 6), but not with a cold probe corresponding to mutant sites for κB3 and κB4 (Figure 2D). Supershift analysis with antibodies against p50, p65 and c-Rel revealed cRel/p65 heterodimers bound the promoter elements (Figure 2C, lanes 2 and 4). Although some p50 protein was supershifted (Figure 2C, lane 3), specific bands corresponding to p50-containing complexes could not be detected, suggesting that p50 is not a central component of the bound complexes. These experiments demonstrated that all four κB sites in the *EIF4E* promoter recruit cRel/p65 complexes.

2.3.3 NF-κB recruits p300 and Pol II to the EIF4E promoter in vivo

To establish that the NF-κB complexes detected by EMSA formed not only *in vitro* on *EIF4E* promoter fragments but also in cells, we carried out chromatin immunoprecipitation (ChIP) experiments in BJAB cells treated with PMA for one or two hours (Figure 3).

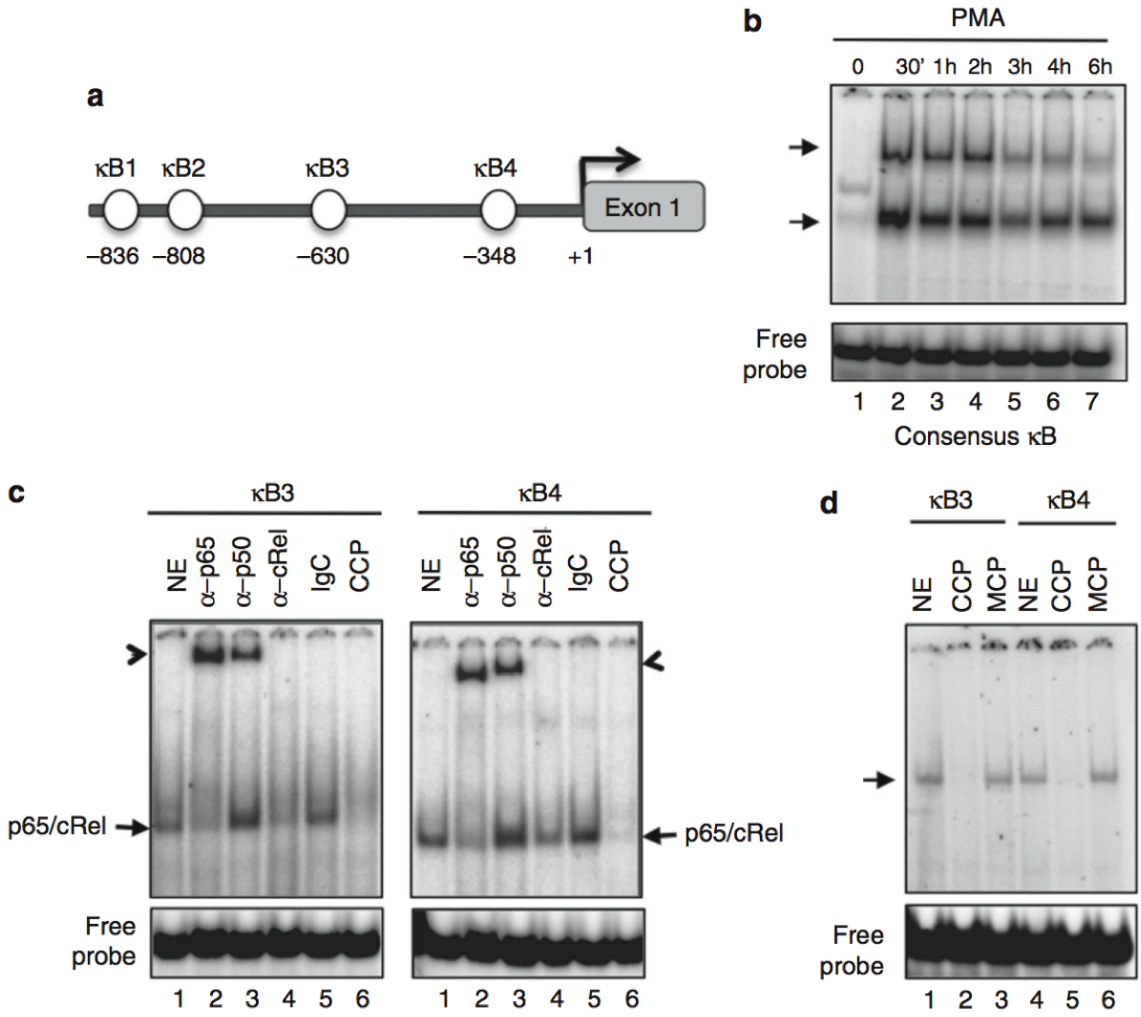

Figure 2. The *EIF4E* promoter contains four κB sites preferentially bound by cRel-p65 NF-κB complexes. A) Schematic representation of the *EIF4E* promoter. Predicted κB sites (open circles) are indicated. **B**) Kinetics of NF-κB binding to the consensus κB following PMA stimulation of BJAB cells. **C**) EMSA of nuclear extracts (NE) from PMA-stimulated BJAB (90 min) using probes corresponding to κB3 and κB4 sites in the *EIF4E* promoter. Supershift analysis using antibodies against p65, p50, cRel and IgG control as well as competition with consensus cold probe (CCP) are indicated. Protein/DNA complexes are indicated by arrows, supershifted complexes by arrowheads. Free probe is also shown. **D)** EMSA of nuclear extracts from PMA-stimulated BJAB (90 min) using probes corresponding to κB3 and κB4 sites in the *EIF4E* promoter with cold probe competition using consensus cold probe (CCP) or mutant cold probe (MCP).

Our results revealed maximum cRel binding at 1 hour post-treatment, with 3-fold enrichment at the κB3 site and a 2-fold enrichment at the κB4 site (Figure 3B). The p65 subunit was recruited to both sites following 1 hour of treatment with maximal 9-fold and 10-fold enrichment at the κB3 and κB4 sites, respectively (Figure 3A). Activated NF-κB (cRel and p65) dimers bound to the *EIF4E* promoter effectively recruited p300 histone acetyl transferase, a marker of transcriptionally active NF-κB complexes [31]. Maximum p300 enrichment was again observed one hour post-treatment: 9-fold for κB3 and 7-fold for κB4 (Figure 3C). In contrast, no binding of NF-κB subunits was detected at the κB1 and κB2 sites by realtime PCR at 1 or 2 hours post-PMA treatment (data not shown). This suggests that the κB1 and κB2 sites are not active *in vivo* in this context, possibly due to the chromatin status at the EIF4E locus. Phosphorylated Pol II was significantly enriched at the *EIF4E* coding region with 2-fold at 1h and 5-fold at 2h (Figure 3D) confirming enhanced *EIF4E* gene transcription. Thus, shortly after NF-κB stimulation, binding of cRel/p65 dimers at the κB3 and κB4 but not κB1 and κB2 sites of the *EIF4E* promoter results in transcriptional upregulation of *EIF4E* expression *via* recruitment of p300.

2.3.4 NF-κB activation induces EIF4E transcription in primary human cells

To examine the biological relevance of our findings to the normal control of *EIF4E* transcription, we assessed EIF4E levels as a function of PMA stimulation in primary PBMCs isolated from two healthy individuals (Figure 4, Supplementary Figure 1). PMA induced *EIF4E* mRNA expression as early as two hours with maximal induction at 4 hours (4-fold upregulation relative to the untreated controls, Figure 4A). Increased *EIF4E* mRNA levels were observed up to 12 h post-treatment (3-fold). Established NF-κB targets *MYC* and *CCND1* mRNAs, had their RNA levels induced following PMA stimulation (Supplementary Figure 1A and 1B). Results were similar for *EIF4E* transcript from another healthy volunteer (Figure 4B). Flow cytometry analysis of the same samples revealed a significant increase in the population of cells with elevated EIF4E protein levels (Supplementary Figure 1C and 1D).

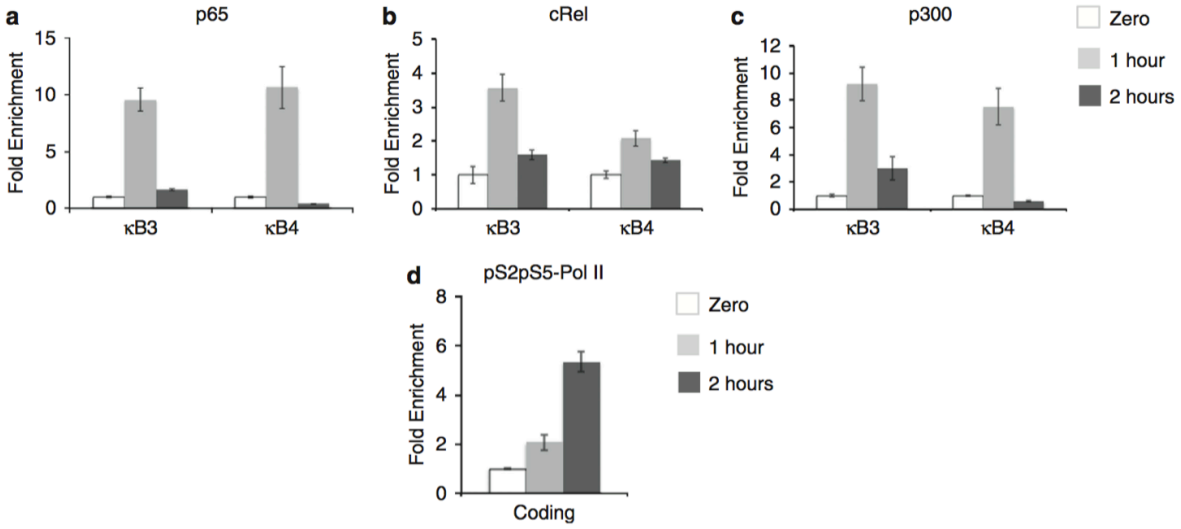

Figure 3. NF-κB complexes are recruited to the *EIF4E* promoter and promote transactivation. BJAB cells were stimulated with PMA for 0, 1 or 2 hours and subjected to chromatin immunoprecipitation using antibodies specific for p65 **(A)**, cRel **(B)**, p300 **(C)** and phosphorylated Pol II (Ser2/Ser5) **(D)**. Data were normalized to IgG control and represented as fold enrichment with respect to untreated cells. Error bars represent standard deviations from triplicate measurements of a representative experiment.

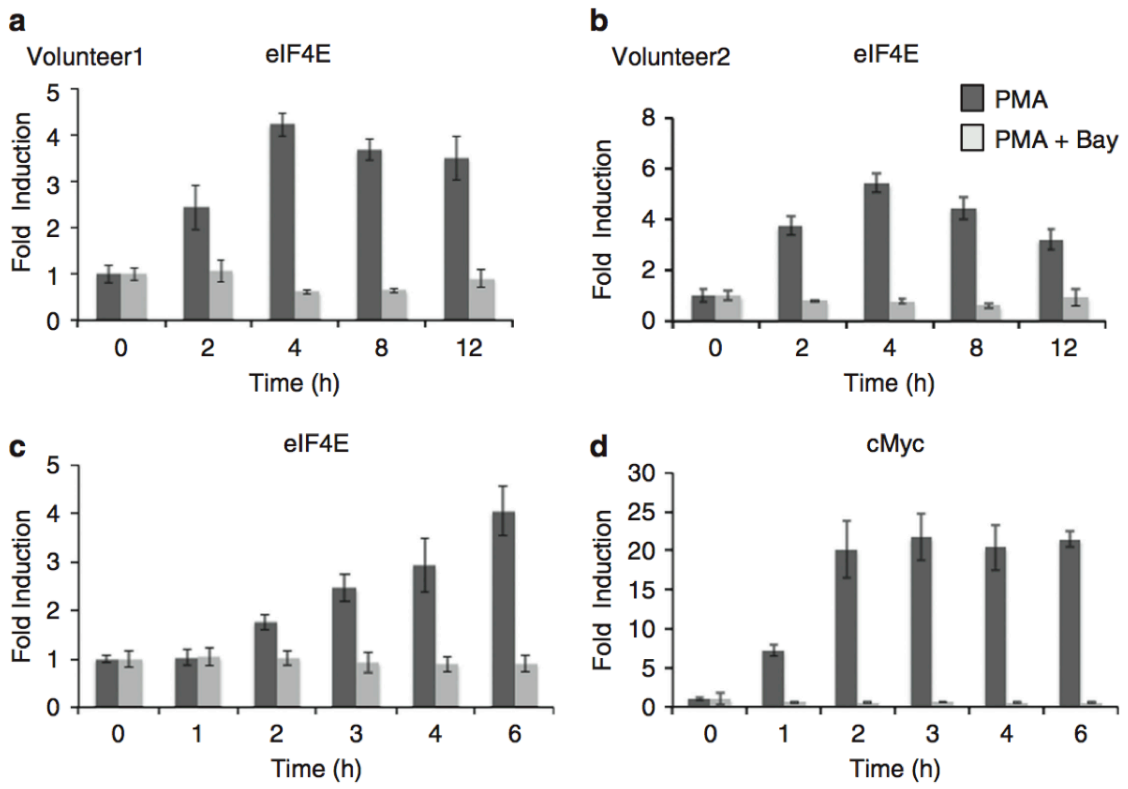

Figure 4. PMA Stimulation of primary human PBMCs increases EIF4E expression in an NF-κB dependent manner. (**A**) PBMCs (volunteer1) were stimulated with PMA (20 ng/mL) for the indicated time points in the presence or absence of the NF-κB inhibitor Bay 11-7082 (10 μM). Expression of EIF4E (*EIF4E*) was assessed at the mRNA level by realtime Q-PCR. (**B**) Same as **(A)** using PBMCs of a second healthy volunteer. Error bars indicate standard deviation. **(C, D)** Primary B lymphocytes were stimulated with PMA (20 ng/mL) for the indicated time points in the presence or absence of the NF-κB inhibitor Bay 11-7082 (10 μM). Expression of EIF4E and cMyc (*MYC*) were assessed at the mRNA level by realtime Q-PCR. Results shown are from cells obtained from one healthy donor in triplicate.

Upon treatment with the NF-κB inhibitor, Bay 11-7082, we observe that both EIF4E mRNA and protein expression were no longer induced by PMA consistent with these effects being mediated through NF-κB. Furthermore, *EIF4E* mRNA was monitored as a function of PMA stimulation in primary B lymphocytes as a direct primary companion to BJABs. *EIF4E* transcript doubled at two hours and reached a maximum of 4-fold at 6 hours in an NF-κB dependent manner (Figure 4C and 4D). Thus *EIF4E* is an NF-κB inducible gene in primary hematological cells.

2.3.5 EIF4E transcription is elevated in cells with constitutively active NF-κB

Many cancers including AML are characterized by constitutively active NF-κB[21]. Thus, we monitored EIF4E in KM-H2 cells which are hematopoietic cells characterized by constitutively active NF-κB, owing to a somatic mutation in the IκBα gene [32]. In these cells, EMSA analysis revealed constitutive binding of NF-κB to the κB3 and κB4 sites of the *EIF4E* promoter (Figure 5A). Supershift analysis demonstrated the presence of cRel/cRel homodimers as well as cRel/p65 heterodimers; some p65/p50 complexes were also detected with κB4, although their unusually slow migration pattern was suggestive of the presence of an additional unidentified factor(s). To further demonstrate the direct role of NF-κB in this system, we transduced KM-H2 cells with a retroviral vector expressing IκB–SR to block NF-κB activity. Consistently, IκB-SR reduced EIF4E transcript and protein levels as well as those for the c-Myc control (Figure 5B and 5C). Thus, *EIF4E* transcription is elevated in the context of constitutively active NF-κB.

2.3.6 Elevated NF-κB activity in M4 and M5 AML specimens underlies, at least in part, EIF4E dysregulation

To determine whether NF-κB transcriptional activity could underlie elevation of EIF4E in primary M4 and M5 AML, we examined NF-κB activity in the AML-M5 cell line, THP1 [33], characterized by elevated EIF4E [26]. Consistent with previous findings [34], THP1 cells harbor constitutively active NF-κB.

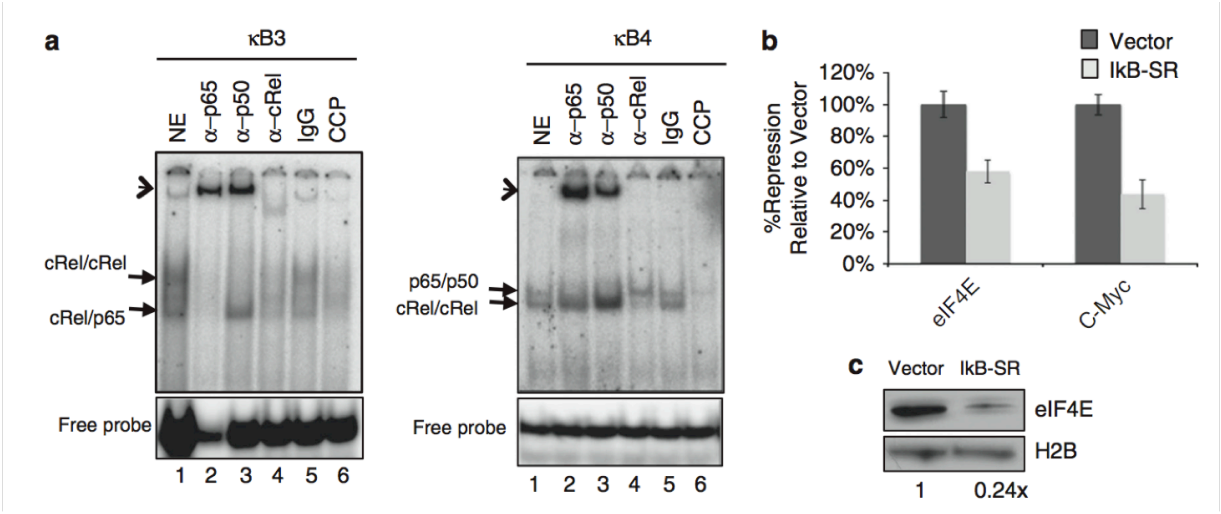

Figure 5. Constitutively active NF-κB regulates EIF4E expression in KM-H2 cells. (A) EMSA analysis of KM-H2 nuclear extracts using probes corresponding to the κB3 and κB4 sites. Supershift analysis using antibodies against p65, p50, cRel and IgG control as well as competition with consensus cold probe (CCP) were done. Protein/DNA complexes are indicated by arrows and supershifted complexes by arrowheads. Free probe is also shown. **(B)** KM-H2 cells were transduced with IκB-SR or vector control and EIF4E and cMyc (positive control) RNA levels were assessed by Q-PCR. Error bars represent standard deviations. **(C)** Same samples as in **(B)** were analyzed by immunoblot.

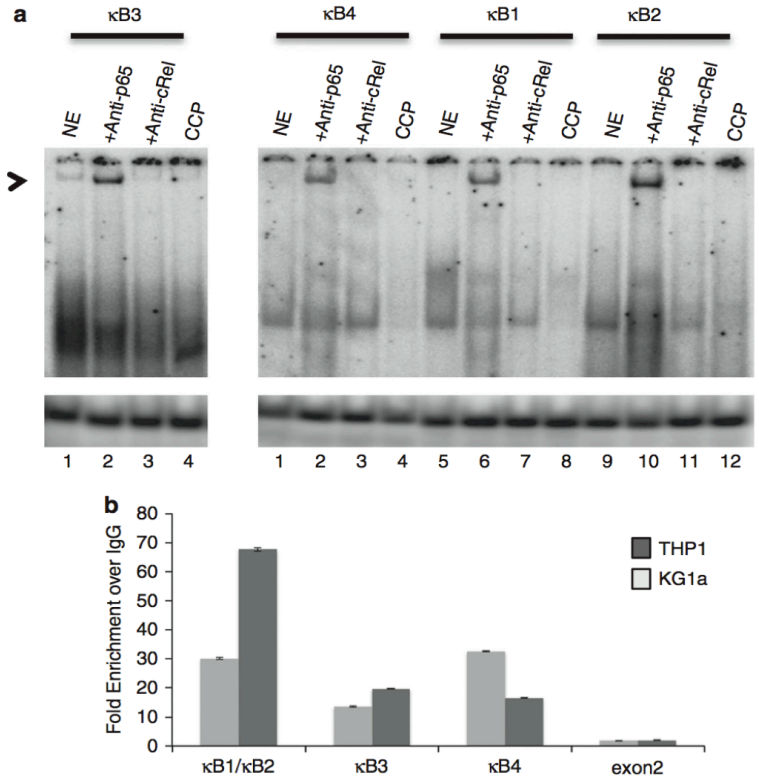

Figure 6. NF-κB recognition of the *EIF4E* promoter elements in AML cell lines. (A) EMSA of nuclear extracts prepared from unstimulated THP1 cells (M5 AML) that were incubated with the κB elements in the EIF4E promoter. Supershift analysis (arrowheads) using antibodies against p65 and cRel as well as competition with consensus cold probe (CCP) are indicated. Free probe is also shown. **(B)** NF-κB complexes are recruited to the *EIF4E* promoter in THP1 and KG1a (M0 AML with high EIF4E) cell lines. Chromatin immunoprecipitation was carried out with RelA antibody using chromatin from THP1 and KG1a cells. Recruitment to the κB elements as well as a non-specific control region in exon 2 of EIF4E was monitored by Q-PCR. Data is represented as fold enrichment over IgG. Error bars represent standard deviations from triplicate measurements.

Nuclear lysates prepared from THP1 cells exhibited NF-κB specific binding for the four identified promoter elements (Figure 6A). ChIP analysis performed on these cells revealed RelA (p65) enrichment on all NF-κB elements in the *EIF4E* promoter as seen in Figure 6B but not to a random region in exon 2 of *EIF4E*. Note that given the proximity of the κB1 and κB2 elements, the ChIP experiment cannot differentiate between these 2 sites and thus is referred to as κB1/κB2. Thus, NF-κB is constitutively found on the *EIF4E* promoter.

Further, we examined NF-κB activity in KG1a cells, a variant AML M0 cell line derived from the AML M1 cell line KG1[35]. KG1a cells are characterized by highly elevated EIF4E that is mainly nuclear (Supplementary Figures 4A and B). Similar to THP1, nuclear lysates from KG1a cells demonstrated NF-κB binding for all four identified promoter elements (Supplementary Figure 4C) and ChIP analysis indicated RelA enrichment for all the elements (Figure 6B). Thus, elevation of EIF4E by NF-κB activity is not necessarily lineage restricted and could also explain how EIF4E levels become elevated in a fraction of other AML subtypes characterized by elevated EIF4E.

To assess whether our findings could be translated to patient specimens, we extended our studies to primary AML specimens. We observed no substantial differences in constitutive NF-κB activity across AML subtypes as determined by EMSA experiments using the consensus kappa light chain element consistent with previous reports [21] (Figure 7A). To assess if the *EIF4E* promoter was specifically enriched for NF-κB proteins we carried out EMSA experiments with all four promoter elements in these AML specimens. We observed complexes highly enriched on the κB3 promoter elements in M4 and M5 specimens (in 2/2 M4 and 2/2 M5) relative to the M1 and M2 specimens (0/1 M1 and 0/2 M2 specimens), which were previously characterized by normal EIF4E levels [26] (Figure 7A and Supplementary Table 1). Consistently, the κB4 element was preferentially occupied in M4 and M5 specimens relative to the M1 and M2 specimens (Figure 7A). Further, we observed supershifts using antibodies to RelA and c-Rel consistent with these being NF-κB complexes with predominant RelA species (Figure 7B). Additionally, M4/M5 nuclear lysates strongly bound the κB1 and κB2 elements (Figure 7C).

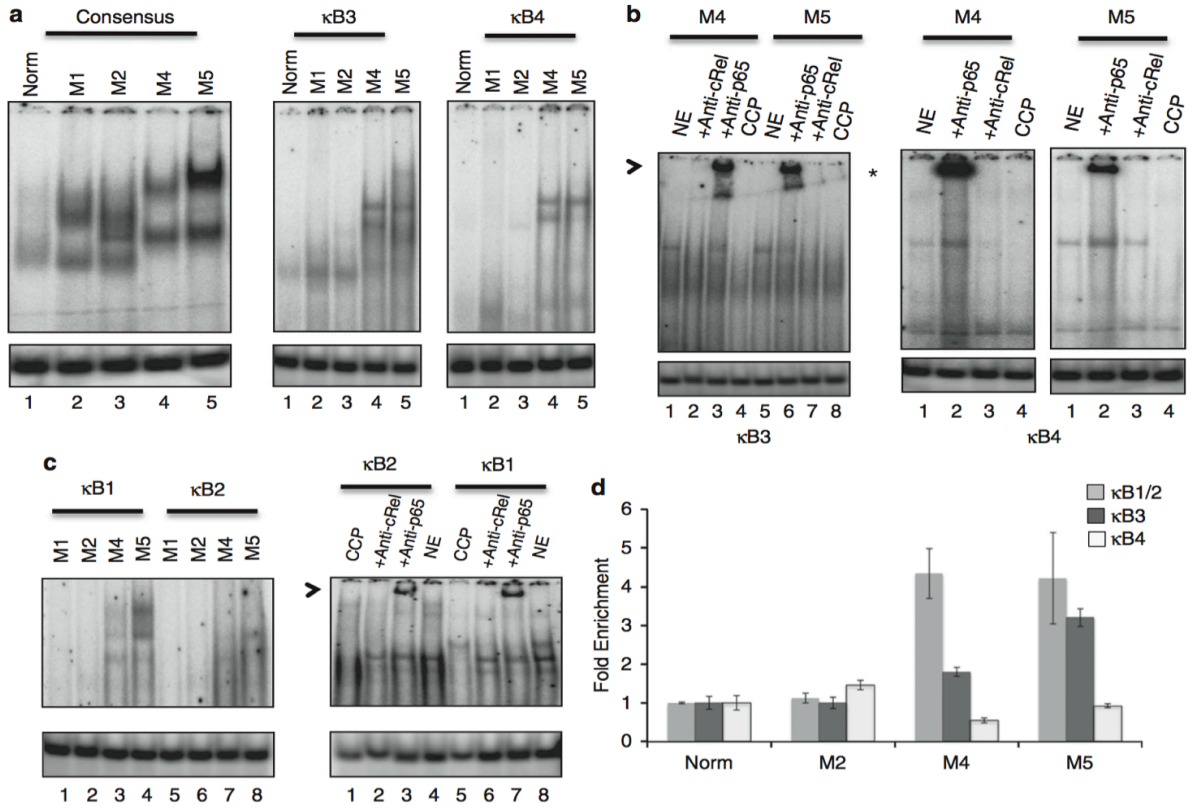

Figure 7. Selective NF-κB recognition of the *EIF4E* promoter elements in M4/M5 AML. EMSA of nuclear extracts prepared from unstimulated PBMCs from a healthy volunteer (Norm) and different M1, M2, M4 and M5 AML primary specimen (corresponding to UPN samples 1-4 as presented in supplemental table1) incubated with consensus κB motif or with the κB3 and κB4 elements **(A)**. **(B)** Supershift analysis (κB3 /κB4) (arrowheads) using antibodies against p65 and cRel as well as competition with consensus cold probe (CCP) are indicated. The asterisk (*) indicates a break in the gel during drying. **(C)** EMSA of nuclear extracts prepared from M2, M4 and M5 AML primary specimen incubated with κB1/κB2 elements. Supershift analysis (arrowheads) using antibodies against p65 and cRel as well as competition with consensus cold probe (CCP) are indicated. Free probe is also shown. **(D)** Chromatin immunoprecipitation was carried out with Rel A antibody using chromatin from the different specimen (Norm, M2, M4 and M5) used in (A). Recruitment to the κB elements was monitored by Q-PCR. Data were normalized to the IgG control and depicted as fold enrichment with respect to the normal healthy specimen (Norm). Error bars represent standard deviations from triplicate measurements.

This is consistent with our previous findings that EIF4E RNA and protein levels are preferentially elevated in M4 and M5 relative to M1 and M2 AML subtypes with approximately 44/44 M4/M5 AML specimens and 2/22 M1/M2 AML specimens relative to 5 healthy controls [1,10,19,26].

To assess the functional relevance of these associations we used ChIP analysis performed to monitor RelA recruitment in the examined AML samples. RelA was recruited to κB1/κB2 and κB3 but not κB4 in the M4/M5 samples but not the M2 (Figure 7D). Although all four elements were bound by nuclear lysates from PMA-induced BJABs as well as the NF-κB constitutive AML cell line (THP1) and primary specimens through EMSA assays, ChIP assays revealed RelA recruitment to the κB1/κB2 elements in only the AML primary specimens and cell line but not in BJAB cells implying a lineage specific recognition for NF-κB proteins on the *EIF4E* promoter. Importantly, κB3 elements were also bound in the ChIP assays indicating these likely play important roles as well.

Thus, *EIF4E* promoter elements specifically recruit NF-κB complexes enabling increased *EIF4E* transcription relative to other AML subtypes. Consistently, previous studies showed that introduction of IκB-SR into primary M4 and M5 AML specimens resulted in reduced EIF4E mRNA and protein levels [19]. It seems likely that NF-κB dysregulation in at least a subset of M4/M5 AML underlies, at least in part, aberrant elevation of *EIF4E* RNA levels in the evaluated samples. Clearly, more specimens will need to be examined to determine the generality of these findings.

2.4 Discussion

This study reveals novel insights into the control of *EIF4E* transcription in primary hematopoietic cells as well as its dysregulation in AML specimens. These are the first studies to show that *EIF4E* is a transcriptional target of NF-κB. Recently, C/EBP has also been shown to regulate *EIF4E* transcription [18] and thus NF-κB and C/EBP serve as examples that the transcriptional control of *EIF4E*, thought for nearly 16 years to be solely the purview of c-

Myc, is more complicated. These findings suggest that there could be eventual clinical utility in controlling the transcription of *EIF4E* with the use of NF-κB inhibitors in addition to directly inhibiting EIF4E activity with ribavirin [10,12,26,36,37]. Interestingly, many NF-κB target genes are in fact EIF4E mRNA export and/or translation targets (e.g. *MYC* and *CCND1*) suggesting these pathways cooperate to drive proliferative gene expression. Our results have shown that genetic and pharmacological inhibition of NF-κB result in downregulation of EIF4E targets suggesting that there is a nexus between transcriptional and post-transcriptional gene expression networks to modulate cell proliferation.

Beyond the control of *EIF4E*, our findings strongly suggest that NF-κB activity is likely heterogeneous amongst AML specimens with regard to other targeted promoters. In other words, the NF-κB dependent transcription of factors besides EIF4E may be differentially regulated between AML subtypes, potentially contributing to differences in leukemogenic potential. In this way, the kappa light chain occupancy may not be altered amongst subtypes as it is dependent on Rel components, but differences in the sequences of other promoters may lead to increased dependency of these promoters on non-Rel components for transcriptional activation. Furthermore, the selective in vivo recruitment of RelA to the κB1/2 region in the M4/M5 AML specimens as well as the M5 AML THP1 cell line but not in the lymphocytic BJAB cells could be due to lineage differences. However, this is not strictly lineage restricted as ChIP studies indicate that in the high EIF4E AML cell line KG1a, enrichment on the κB1/2 binding elements is also observed. These differences could underlie the constitutive EIF4E upregulation in M4/M5 AML and suggest that non-Rel components play a role in this process. Finally, we did not have access to sufficient primary M3 AML (APL) specimens to examine NF-κB activity and thus cannot exclude that this is relevant to this leukemia subtype. However, primary APL specimens and NB4 cells are characterized by normal EIF4E levels and localization suggesting this may not be critical [19,38].

Thus, specific non-Rel transcriptional co-factors of NF-κB may be specifically dysregulated in M4 and M5 AML, allowing preferential dysregulation of *EIF4E*, and potentially other promoters. These factors may be involved in the selective NF-κB recruitment to the *EIF4E*

promoter. Examples of non-Rel proteins shown to selectively modulate DNA recognition and transactivation of NF-κB proteins in other contexts include: RPS3, CD40, BAFFR, Akirins, CHFR, PKAIP, AEG-1, ING-4 and others (Reviewed in [39]). Future studies should reveal the identity of non-Rel co-factors, which likely preferentially drive transcription of *EIF4E* in M4 and M5 AML. In addition to its role in the control of *EIF4E* expression as outlined here, NF-κB can also regulate EIF4E activity indirectly by modulating its subcellular localization and thus affecting EIF4E levels and activity [19].

2. 5 Conclusion

In summary, we observe in normal primary hematopoietic cells and in cell lines with normal NF-κB activity, that *EIF4E* transcription is stimulated by NF-κB activation. We demonstrate that *EIF4E* is a direct transcriptional target of NF-κB. We observe that in the M4 and M5 AML specimens examined, the *EIF4E* promoter is highly occupied by NF-κB complexes. These studies elucidate a novel mechanism of transcriptional control for *EIF4E* and thus potentially a new point at which to target it.

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Supplementary Material

Preparation of nuclear extracts

Cell fractionation was performed with a modification from Dignam et al (Dignam JD et al. 1983. Nucleic Acid Research). Cells were pelleted and washed twice with PBS. Lysis was performed for 10 minutes on ice in hypertonic buffer A (10mM HEPES, pH 7.9, 1.5 mM MgCl2, 10mM KCl, 0.5 mM DTT, 0.1 % NP-40 and complete protease inhibitors). The lysate was then cleared by centrifugation at 14,000 rpm for 10 minutes at 4° C. The nuclear pellet was washed once with buffer A without NP-40 to remove any cytoplasmic contaminants and lysed for 10 minutes on ice in high salt buffer B (20mM HEPES, PH 7.9, 420mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM DTT, 10 % glycerol and complete protease inhibitor). Nuclear proteins were collected in the supernatant after centrifugation at 14,000 rpm for 10 minutes at 4° C. The samples were then diluted in ice cold buffer C (20mM HEPES, pH7.9, 20% glycerol, 0.2mM EDTA, 50 mM KCl, 0.5 mM DTT and complete protease inhibitor), aliquoted and stored at -80° C.

EMSA

*Oligonucleotide labeling***:** Double stranded oligonucleotides corresponding to the consensus NF-κB motif and the putative NF-κB elements identified in the *EIF4E* promoter were prepared by annealing of the sense and antisense sequences at 10 pmol/ul final concentration in TEN buffer (10mM Tris, pH 7.8, 1mM EDTA and 50mM NaCl) at 95° C for 5 minutes, followed by cooling down to room temperature. The labeling reaction was then carried out with T4 kinase (Invitrogen) for 90 minutes using 15pmol of double stranded oligonucleotides and 25uCi of γ32 ATP.

Preparation and resolving the binding reaction: 5 ug of nuclear extracts were incubated with the labeled double stranded probes (at 200,000 cpm) in NF-κB binding buffer (10mM Tris, pH 7.8, 50mM NaCl, 0.5 mM EDTA, 1mM DTT, 10% glycerol, 2mM ATP and 2mM GTP) for 20 minutes at room temperature. For supershift experiments, 2ug of antibody (p65, p50 and c-Rel) was added to the reaction mixture. Cold probe competition was performed with 200x excess of unlabeled oligonucleotides. The reactions were resolved on a 5% non-denaturing polyacrylamide gel (19:1 crosslink, 0.5x TAE and 5%glycerol) in Tris-actetic-EDTA (TAE)

buffer. The gel was run for 3 hours at 150 volts after which it was dried for 2 hours using a BioRad gel dryer and then exposed overnight on a Fugifilm imaging plate for detection.

ChIP

Crosslinking: Cells were crosslinked for 20 minutes at room temperature on a rocking platform with 1/10 volume Formaldehyde solution (11% Formaldehyde, 100mM NaCl, 1mM EDTA, 0.5mM EGTA and 50mM HEPES pH 7.4). Quenching was performed for 5 minutes with 1/20 volume 2.5 M Glycine. The crosslinked samples were then pelleted by centrifugation at 1,200 rpm for 5 minutes at 4° C and washed twice with PBS.

Cell lysis and Sonication: The pelleted cells were first lysed in cytoplasmic lysis buffer for 10 minutes on ice (0.005 mM PIPES, 85 mM KCl, 0.5% NP-40 and complete protease inhibitor). Following clarification at 4,000 rpm for 5 minutes at 4° C, the nuclear pellet was washed once with cytoplasmic lysis buffer with out NP-40 to remove cytoplasmic contaminants. Nuclear chromatin was then harvested by incubating with nuclear lysis buffer for 10 minutes on ice (50mM Tris, pH 8, 10mM EDTA, 1% SDS and complete protease inhibitor). Sonication was then carried out using a waterbath Bioruptor at 4° C. Four rounds were performed: eight minutes each at 30 seconds intervals on medium power. The samples were then cleared by centrifugation at 10,000 rpm for 10 minutes at 4° C. This sonication protocol produces sheared chromatin between 200 and 500 bp.

Pre-clearing and Chromatin Immunoprecipitation: Protein A/G beads were first pre- blocked with 200 ug salmon sperm DNA (Sigma) and 500 ug BSA (Sigma) for 3 hours at 4° C with rocking. 50 ug of chromatin was diluted in Immunoprecipitation buffer to a final volume of 1ml (IP buffer: 10% Triton-X, 1.2 mM EDTA, 16.7mM Tris pH 8, 167 mM NaCl and complete protease inhibitor). 10ul of cell lysate was conserved as 1% Input. Chromatin pre-clearing was performed with pre-blocked Protein A/G agarose beads (Santa Cruz biotechnologies) for 1hour at 4° C with rocking. Pre-cleared chromatin was then incubated with 10ug of antibody (p65, c-Rel and p300) overnight at 4° C with rocking. The samples were then centrifuged at 13,000 rpm for 10 minutes (this step ensures the removal of insoluble

chromatin material) and the supernatant was incubated with pre-blocked protein A/G agarose beads for 2 hours at 4° C with rocking.

Sample elution and realtime PCR: Following immunoprecipitation, the beads were washed twice with wash buffer 1 (2 mM EDTA, 50 mM Tris pH 8, 0.2% Sarkosyl) followed by two washes with wash buffer 2 (500 mM LiCl, 1% NP-40, 1% Sodium deoxycholate and 33 mM Tris, pH 8). Elution was carried out with 100 ul of 10% chellex slurry (BioRad) at 95° C for 15 minutes. The samples were then cleared at 13,000 rpm for 1 minute and treated with 20 ug of Proteinase K (Sigma) for 1 hour at 55° C with shaking. Final purification with Qiagen PCR purification kit was then performed. Input DNA was purified with 10% chellex beads at 95° C followed by proteinase K treatment and sample purification. Realtime quantitative PCR was then perfomed with Syber Green (Invitrogen) and primers flanking the putative NF-κB elements in the *EIF4E* promoter using the Step one thermal cycler from Applied Biosystems. CT values were analyzed with the DDCT method and normalized relative to IgG (Haring M et al. 2007. Plant Methods).

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Supplemental Figure 1. (A) Expression of positive controls cMyc (*MYC*) and cyclinD1 (*CCND1*) were assessed at the mRNA level by realtime Q-PCR. **(B)** Same as **(A)** but from PBMCs from a second volunteer. (C, D) EIF4E protein levels from the PBMC samples (Figure 4) were analyzed by flow cytometry to assess cell populations with high EIF4E protein levels.

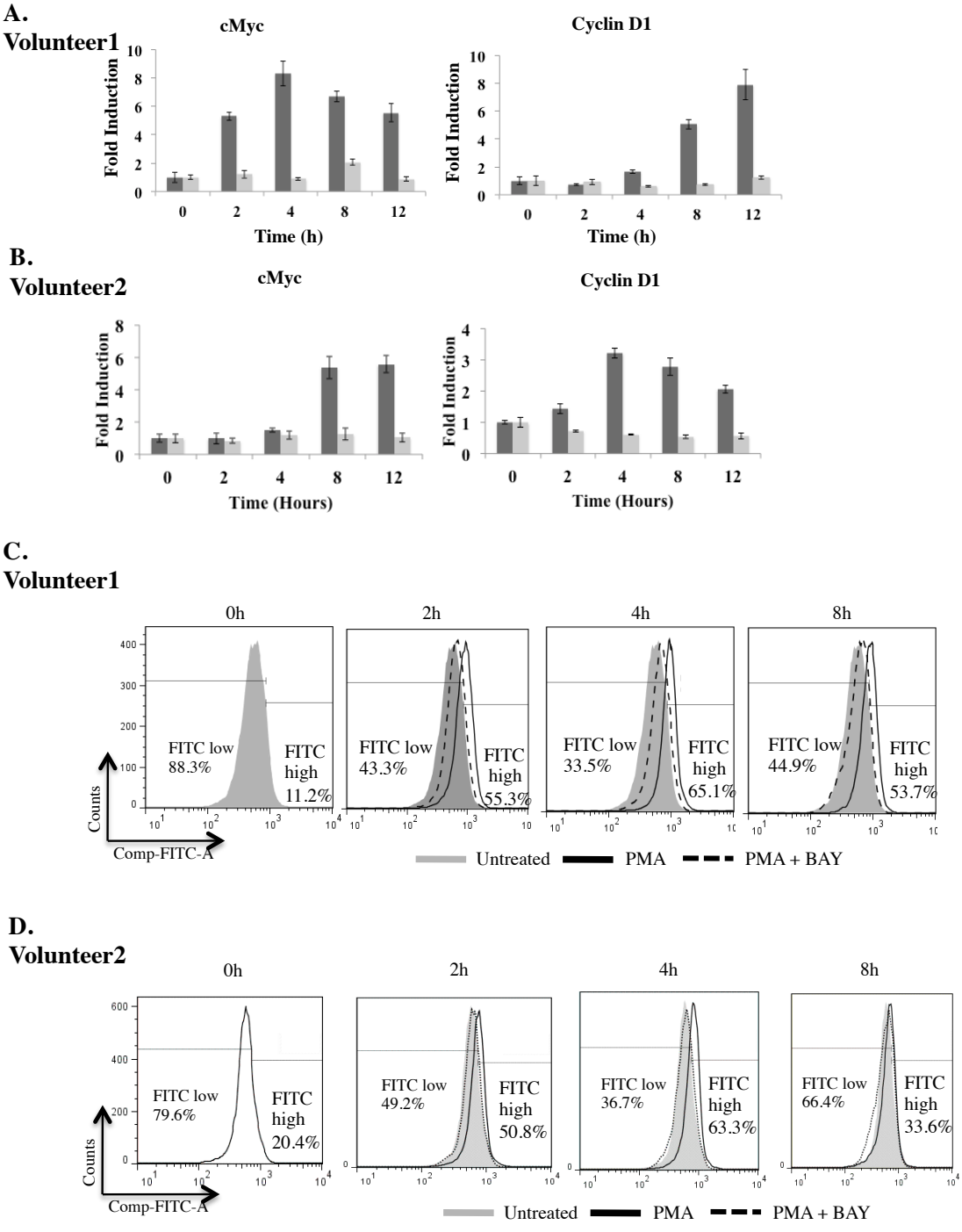
Supplemental Figure 2. The *EIF4E* promoter harbors 4 NF-κB elements. A) Nucleotide sequence and position relative to the transcriptional start site (+1) of the four κB sites predicted in the *EIF4E* promoter. **B**) Sequence alignment showing species conservation of κB elements.

Supplemental Figure 3. Electromobility shift assay of BJAB nuclear extracts stimulated with PMA (20 ng/mL). EMSA of BJAB cells stimulated with PMA for 90 minutes using κB1 and κB2 probes. Supershift analysis was done with the indicated antibodies.

Supplemental Figure 4. EIF4E is elevated with nuclear localization in the M0 AML cell line KG1a. A) Western blot from whole cell lysates obtained from: a normal volunteer as well as THP1 (M5), KG1a (M0) and an M1 AML specimen. The panels are obtained from the same western blot but with intervening unrelated samples cropped out. Thus there is a space between the two parts of the gel. **B)** Confocal imaging performed on KG1a and THP1 cells as well as a normal and an M5 AML specimen. **C)** EMSA of nuclear extracts prepared from unstimulated THP1 and KG1a that were incubated with the κB elements in the *EIF4E* promoter. Free probe is shown. PC is phase contrast.

Supplementary Table 1. List of the AML patient specimens and cell lines used in this study. ITD, internal tandem duplications. UPN, unidentified patient number. Not available NA. THP1 (ATCC-TIB202). KG1a (ATCC-CCL246.1) referenced in [Koeffler HP, Billing R, Lusis AJ, Sparkes R, Golde DW. An undifferentiated variant derived from the human acute myelogenous leukemia cell line (KG-1). Blood. 1980 Aug;56(2):265-73.]
(*) Referenced in [Odero MD, Zeleznik-Le NJ, Chinwalla V, Rowley JD. Cytogenetic and molecular analysis of the acute monocytic leukemia cell line THP-1 with an MLL-AF9 translocation. Genes Chromosomes Cancer. 2000 Dec;29(4):333-8.]

Supplementary Table 2. List of oligonucleotide sequences used in this study.

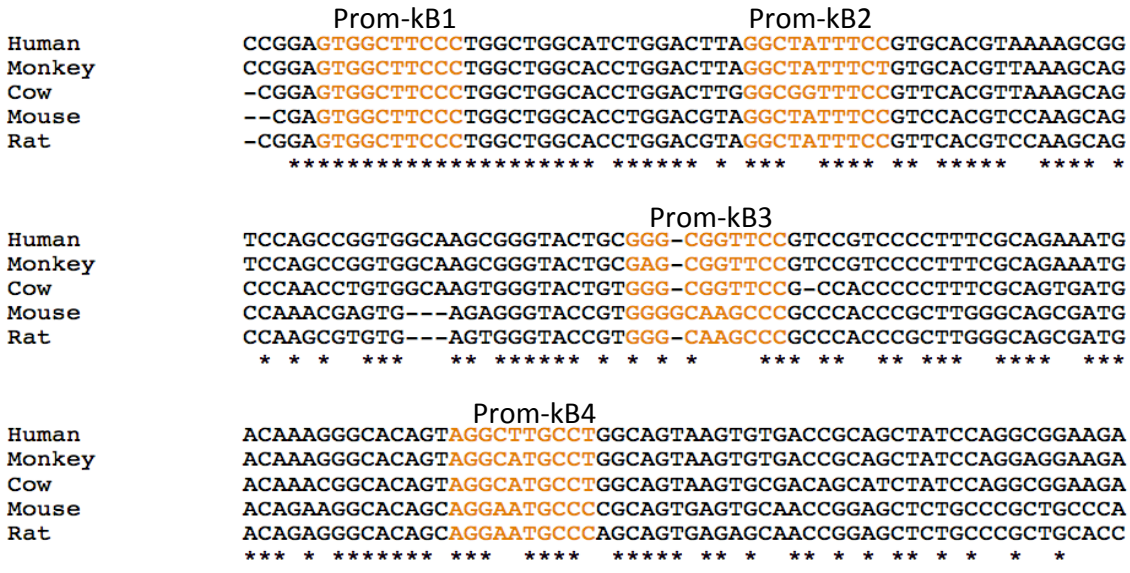


Supplemental Figure 1

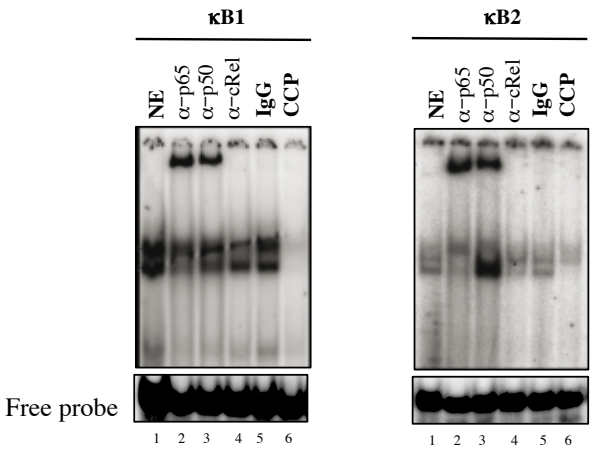
A.

κB Element	Sequence	Position
Prom-κB1	GTGGCTTCCC	-836
Prom-κB2	GGCTATTTCC	-808
Prom-κB3	GGGCGGTTCC	-630
Prom-κB4	AGGCTTGCCT	-348

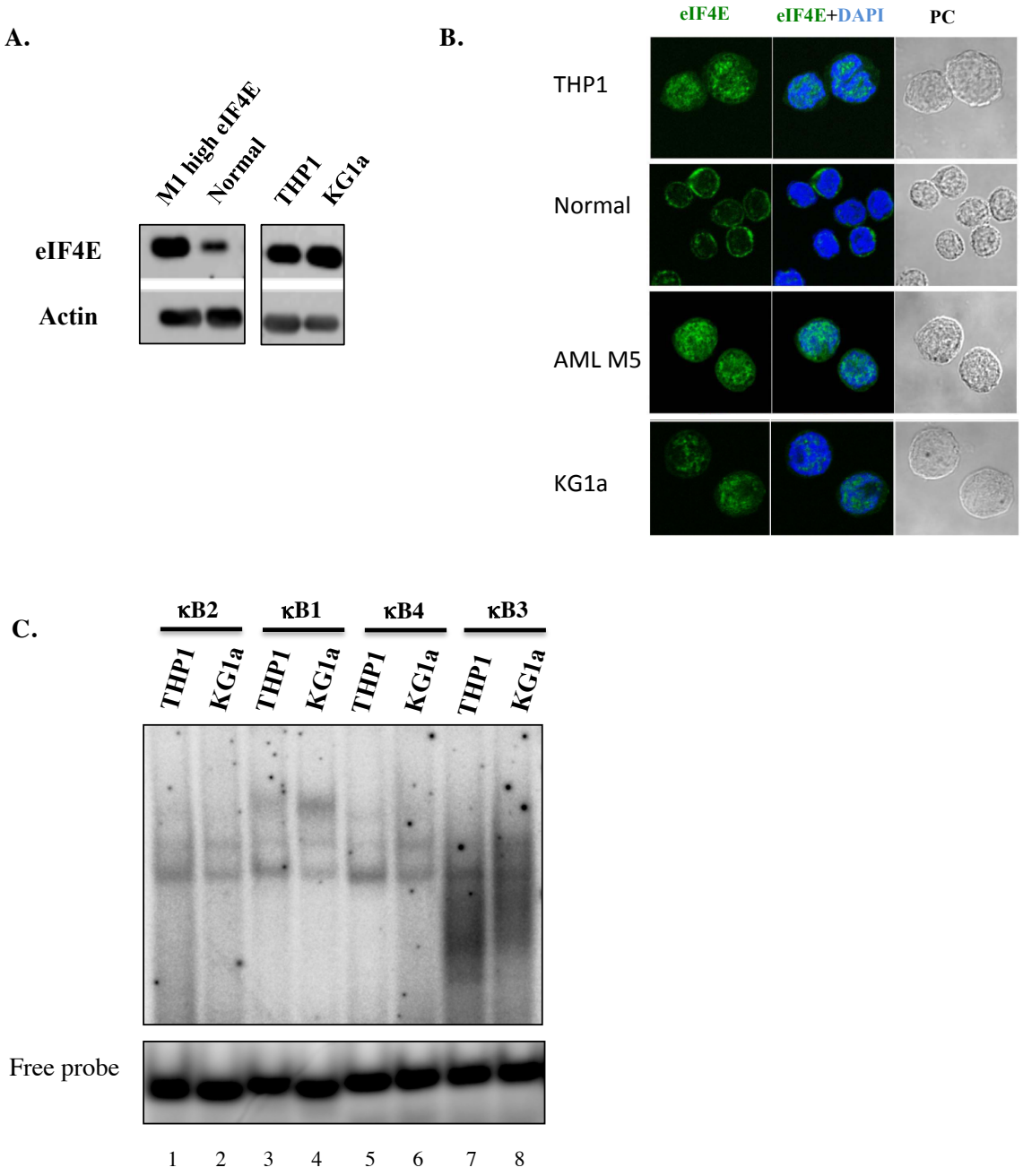
B.



Supplemental Figure 2.



Supplemental Figure 3.



Supplemental Figure 4.

Patient	Subtype	Age	eIF4E (RNA/Protein)	Cytogenetics	Flt3	NPM	Other
UPN1	M4	61	High	46,XX	ITD	Mutated	-
UPN2	M5	50	High	46,XY	None	Mutated	-
UPN3	M1	45	Normal	46,XX	None	Mutated	-
UPN4	M2	47	Normal	46, XX	ITD	Mutated	-
UPN5	M4	38	High	46, XX	NA	NA	-
UPN6	M5	48	High	46, XY	ITD	Mutated	-
UPN7	M2	73	Normal	46, XY	ITD	Mutated	-
THP1	M5	1	High	46, XY	NA	NA	MLL-AF9*
KG1a	M0	59	High	46, XY	NA	NA	-

Supplemental Table1.

Primer	Sequence	T annealing
EMSA		
Consensus Fw	AGTTGAGGGGACTTTCCCAGG	
Consensus Rev	CCTGGGAAAGTCCCCTCAACT	NA
Prom-kB1 Fw	CCGGAGTGGCTTCCCTGGCT	
Prom-kB1 Rev	AGCCAGGGAAGCCACTCCGG	NA
Prom-kB2 Fw	ACTTAGGCTATTTCCGTGCA	
Prom-kB2 Rev	TGCACGGAAATAGCCTAAGT	NA
Prom-kB3 Fw	GTACTGCGGGCGGTTCCGTCC	
Prom-kB3 Rev	GGACGGAACCGCCCGCAGTAC	NA
Prom-kB4 Fw	ACAGTAGGCTTGCCTGGCAGT	
Prom-kB4 Rev	ACTGCCAGGCAAGCCTACTGT	NA
ChIP		
Coding Fw	TTCAACTCCCGACCTCAGGT	
Coding Rev	ATAACTAGCCTGTGCACAAG	56 °C
Prom-kB1/2 Fw	AGTGGCTTCCCTGGCTGGCAT	
Prom-kB1/2 Rev	GAGATTAAGACCTCAGGGCTA	56°C
Prom-kB3 Fw	TCAGGATTTGGGACAGTAAAAGCTG	
Prom-kB3 Rev	CATTTCTGCGAAAGGGGACG	56°C
Prom-kB4- Fw	TGCTGAGCCTGCAGTTCCCA	
Prom-kB4 Rev	CTCTGCTCTTCCGCCTGGAT	56°C
eIF4E-exon2-Fw	GAAACCACCCCTACTCCTAA	56°C
eIF4E-exon2-Rev	TACCTGTTCTGTAGGGGATG	56°C
mRNA expression		
Actin Fw	GCATGGAGTCCTGTGGCAACCACG	
Actin Rev	GGTGTAACGCAACTAAGTCATAG	60 °C
eIF4E Fw	CTGTGCCTTATTGGAGAAT	
eIF4E Rev	GGAGGAAGTCCTAACCTTT	60 °C
Myc Fw	TCAAGAGGCGAACACACAACG	
Myc Rev	TGGACGGACAGGATGTATGCTG	60 °C
Pim1 Fw	ACAGGTTGGGATGGGATAGGAC	
Pim Rev	GAGAAGCAGCAGGTAAAAGAGGC	60 °C
H2B Fw	GATGCCTGAACCTACCAAGTC	60 °C
H2B Rev	ACTGAATAGCTCTCCTTGCG 60 °C	
CyclinD1 Fw	CAGCGAGCAGCAGAGTCCGC	60°C
CyclinD1 Rev	ACAGGAGCTGGTGTTCCATGGC	

Supplemental Table 2. Primer sequences.

Chapter 3: Analysis of public gene expression and transcription factor binding data reveals a correlation between NF-κB and EIF4E mRNA expression levels in AML and unravels an intricate control mechanism for EIF4E

Synopsis: This chapter outlines a bioinformatics analysis of data available from public repositories. It constitutes a follow-up on my previous findings that demonstrate an NF-κB mediated regulation of *EIF4E*. Here, I describe an association between NF-κB *RELA* and *EIF4E* mRNA expression in poor prognosis AML patients as well as its impact on survival outcomes. Furthermore, I present an analysis of ChIP-Seq datasets to predict putative *EIF4E* regulators as well as predict new putative NF-κB target genes that may be regulated in an *EIF4E*-like manner. This chapter is presented as a manuscript in preparation.

Contributions:

All of the original data used in this manuscript have been cited appropriately.

The analysis presented in all of the Figures was performed by **Fadi Hariri (100%).**

Abstract

EIF4E is a powerful oncogene that is overexpressed in 30% of human cancers including hematopoietic malignancies. Our previous findings suggest that *EIF4E* is a direct NF-κB transcriptional target that is dysregulated in acute myeloid leukemia (AML). *EIF4E* and NF-κB *RELA* mRNA levels are upregulated in AML; however, the correlation between the expression patterns of these oncogenes has not been studied in AML risk groups. Analysis of gene expression RNA-Seq data from The Cancer Genome Atlas (TCGA) suggests that *EIF4E* and *RELA* mRNA levels are upregulated in intermediate and poor prognosis AML but not in the cytogenetically favorable group. Additionally, elevated *EIF4E* and *RELA* mRNA levels are significantly associated with worst patient survival outcome. Using the κB sites in the *EIF4E* promoter, 8 new putative NF-κB target genes were *in silico* predicted from RelA ChIP-Seq datasets available from the Encyclopedia of DNA Elements (ENCODE). These genes are upregulated in poor prognosis AML similar to *EIF4E*. Finally, 6 new transcription factors that may be implicated in *EIF4E* gene regulation were predicted from the analysis of ChIP-Seq ENCODE data. Collectively, these findings provide insights into the transcriptional control of *EIF4E* and the molecular basis for its dysregulation in poor prognosis AML specimens.

Keywords: Acute myeloid leukemia (AML), microarrays, RNA-Seq, ChIP-Seq, EIF4E, NF-κB RelA.

3.1 Introduction

The oncogenic translation initiation factor EIF4E can be thought of as a pivotal molecular bridge that facilitates the functional expression of growth-promoting genes (e.g. *MYC, CCND1, PIM1* and many more). The molecular basis of EIF4E functions at the mRNA export and protein synthesis levels has been extensively studied accelerating the route to EIF4E clinical targeting in cancer[1,2]. However, the body of data describing the regulation of *EIF4E* expression in normal cells and how it becomes dysregulated in cancer has not been extensive. Furthermore, it offered a c-Myc centric-transcriptional control model[3]. We have identified an alternative mechanism for *EIF4E* transcriptional regulation that was supported by data suggesting an NF-κB mediated regulation[4].

EIF4E is overexpressed in approximately 30% of human cancers including hematological malignancies and solid tumors. This aberrant upregulation of *EIF4E* expression has been shown to involve EIF4E transcript and protein[5,6]. In addition, NF-κB is constitutively activated in a plethora of cancers, which has been linked to increased expression of NF-κB transcription factors[7]. In acute myeloid leukemia (AML), the NF-κB factor RelA (p65) is upregulated owing to a gene amplification as a result of a trisomy in chromosome 11. This has been linked to poor prognosis in AML as the overall 10-year survival outcome for AML patients with this anomaly was 10%; the overall survival outcome for AML patients in the same study group with a non-complex karyotype was 37%[8]. Interestingly, aberrant *EIF4E* and *RELA* expression levels overlap in cancer including acute myeloid leukemia.

Acute myeloid leukemia is a hematological malignancy affecting the myeloid lineage of blood cells; this malignancy affects the immature myeloid population of cells (myeloblasts) that expand at the expense of normal cells[9]. For the past four decades, AML has been classified on the basis of the type of cell from which the leukemia has developed into 9 distinct groups (M0 through M7); this classification is referred to as the French-American-British classification (FAB)[10]. However, this system has been subject to criticism, revisions and re-assessment since the techniques required in classifying AML samples into FAB groups are very descriptive and rely on cell morphological features as well as simple cytochemical assays[11]. On the other hand, AML can further classified into three clinical prognosis groups (favorable, intermediate

and poor prognosis) based on cytogenetic analysis[8]. This cytogenetic classification was further diversified by molecular mutations into five prognosis groups (very favorable, favorable, intermediate, unfavorable and very unfavorable)[12] [reviewed in Chapter 1.6.1]. Accordingly, the combined cytogenetic and molecular classification offer a more reliable and clinically relevant approach to determine prognostic parameters and plan more effective treatment regimen[8,13]. The predicted overall survival outcome in AML patients from a TCGA-AML study[14] is shown in Supplementary Figure 1A.

Our findings in AML revealed that EIF4E is overexpressed in most M4/M5 AML primary specimens but not in most M1/M2 specimens[15-17]. This trend may be underlined by a selective NF-κB activity on the *EIF4E* promoter in those subtypes[4]. Both *EIF4E* and *RELA* expression are upregulated in AML[8,18,19]; however, the correlation between the expression patterns of these oncogenes has not been investigated in clinical prognosis AML groups.

The NF-κB mediated regulation of *EIF4E* is the product of direct transcriptional control inherent in the recruitment of NF-κB transcription factors: RelA (p65), cRel and NFκB1 (p50) to four conserved κB elements in the *EIF4E* promoter. Interestingly, a lineage specific requirement for κB elements in the *EIF4E* promoter exists with a myeloid requirement for all binding elements and a preference for κB3 and κB4 sites in mature B-lymphocytes[4]. Additionally, two intronic κB elements were identified and validated by gel shift (EMSA) experiments to engage NF-κB proteins. This unpublished observation may imply a more elaborate control of *EIF4E* in response to different NF-κB inducing stimuli as NF-κB dependent regulation of target genes has been shown to occur through promoter binding sites as well as intronic regions[20-22]. Finally, the *EIF4E* promoter is enriched for a plethora of transcription factor binding sites[23] (e.g. PU.1, NFAT, GATA, SP1) further re-enforcing that *EIF4E* transcriptional control is not "factor" centric and likely involves an interplay of transcriptional regulators.

With the advancement of high throughput technology, many databases provide large datasets for gene expression and transcription factor binding studies. Accordingly, **We hypothesized that the analysis of these public repositories will identify a correlation between elevated**

***EIF4E* and *RELA* mRNA expression in poor prognosis AML as well as provide an in depth understanding of *EIF4E*'s intricate transcriptional control mechanisms.** To that end, two aims were set:

Aim1: Analyze microarray and RNA-Seq gene expression profiles from AML to determine a correlation between *EIF4E* and *RELA* gene expression in different clinical prognosis groups. Furthermore, the survival outcomes for AML patients with high versus low *EIF4E/ RELA* expression levels will be determined.

Aim2: Analyze NF-κB ChIP-Seq data to establish an NF-κB binding profile across the *EIF4E* locus in the promoter and introns. In addition, this study aims at performing a genome wide motif scanning using the identified NF-κB binding sites in the *EIF4E* promoter and intron to predict new candidate genes that may be regulated through NF-κB in a manner similar to *EIF4E*. Given the transcription factor wide nature of the Encyclopedia of DNA Elements (ENCODE) ChIP-Seq experiments, we will predict, if possible, the recruitment of other transcription factors to the *EIF4E* regulatory locus.

3.1.1 Public Databases and Limitations

Microarrays and RNA-Seq have been implemented for the diagnosis and prognosis of AML. This area of research has been critical in reshaping our understanding of the development and pathogenesis of AML as well as predicting therapeutic response and identifying the underlying mechanism of action[24,25]. Furthermore, the advent of DNA sequencing technologies (NextGEN sequencing) and transcription factor association experiments gave rise to Chromatin Immunoprecipitation (ChIP) followed by high-throughput sequencing (ChIP-Seq), enabling the large-scale identification of transcription factor localization and histone modification across the genome[26].

The available gene expression databases include MedSapiens[27], the Gene Expression Omnibus (GEO) and The Cancer Genome Atlas (TCGA)[14] and ENCODE[28]. Despite the wealth of information available from these databases, several limitations must be considered as they can

largely interfere with the analysis of these data as well as impact the drawn conclusions. For instance, the available gene expression data reflects the mRNA pool and not protein levels. Similarly, data regarding protein activity (NF-κB for instance) is also lacking. The technical limitations must also be considered. For instance, the microarray technology is limited by high background owing to cross hybridization, probe specificity, and a limited dynamic range of detection (compressed expression levels) due to signal saturation; however, these limitations are overcome in RNA-Seq experiments. On the other hand, RNA-Seq data is also affected by the sequencing depth and bioinformatics analysis and sequence mapping[29]. The available ChIP-Seq data is limited to particular cell lines and transcription factors as well as antibody specificity; additionally, technical analytical limitations exist in the process of transcription factor peak calling[30,31]. Finally, not all public datasets provide detailed sample information; in addition, the raw data for the deposited experiments is not always available. These limitations must be taken into consideration and controlled for when applicable to derive critical conclusions.

In this study, a comprehensive analysis of microarray datasets in AML determined a positive correlation between *EIF4E* and NF-κB *RELA* mRNA levels. Furthermore, the median *EIF4E* and *RELA* expression is upregulated in intermediate and poor prognosis AML compared to favorable prognosis samples and predicts worst overall survival outcomes in those samples. NF-κB ChIP-Seq analysis revealed a complex pattern of NF-κB RelA recruitment to the *EIF4E* promoter. Using the κB sites in the *EIF4E* regulatory regions, 46 new putative NF-kB target genes were *in silico* predicted from RelA ChIP-Seq datasets available from ENCODE. Finally, 6 new transcription factors that may be implicated in *EIF4E* gene regulation were predicted from the analysis of ChIP-Seq ENCODE data. Collectively, these findings provide novel highlight the complexity of *EIF4E* transcriptional control as well as implicate elevated *EIF4E* and *RELA* mRNA levels in poor patient prognosis in AML.

3.2 Methods for acquisition and processing of public gene expression and transcription factor binding data

3.2.1 Choice and mining of datasets

The gene expression data discussed in this chapter was obtained from two online public repositories. The public microarray raw data used in my analysis was downloaded from the National Center for Biotechnology Information (NCBI) GEO website (http://www.ncbi.nlm.nih.gov/geo) as well as the TCGA. Two datasets were considered: GSE10358[32] (microarray) and TCGA_LAML_exp_GA[14] (RNA-Seq). ChIP-Seq datasets were obtained from the ENCODE repository (http://genome.ucsc.edu/ENCODE/).

3.2.2 Microarray data analysis in R Bioconductor

The raw microarray dataset was analyzed using Bioconductor packages[33] in the R programming language. All datasets used were generated with Affymetrix gene chips (U133A). Initially, an assessment for the quality was performed using the SimpleAffy package (http://bioinformatics.picr.man.ac.uk/simpleaffy) to ensure a comprehensive and unbiased interpretation of the microarray data. Several factors can affect the quality of the data generated by each array and these include: mRNA degradation, errors in sample loading across different arrays in an experiment and bias arising during array scanning. Accordingly, any sample or array that did not match proper quality standards was discarded as no valid interpretation can be obtained from a degraded or compromised sample[34].

Next, data preprocessing and normalization was performed using the Affy package[35] with the popular Robust Multi-Array Analysis (RMA) method to account for systematic differences across arrays. This method uses a linear model to correct the background, normalizes and logs (base 2) the Perfect Match (PM) probe intensities[34]. Finally, each array is then annotated with the human gene names corresponding to each probe using the Annotate package. Statistical analysis was then performed to determine gene correlations with Pearson's correlation coefficient. The statistical significance of these correlations was determined with the Analysis of Variance test (ANOVA). Plots were generated in R using the lattice and ggplot2 packages.

3.2.3 RNA-Seq data analysis in R Bioconductor

Gene-level transcription estimates in RPKM values (Reads Per Kilobase of exon model per Million mapped reads) were obtained for each RNA-Seq dataset. Genes were mapped onto the human genome coordinates using UCSC human genome hg19. The data was parsed in R to extract the RPKM values for specific genes (e.g. *EIF4E, RELA*) and generate correlations. Phenotypical features of the samples in the TCGA dataset included information regarding the clinical prognosis for each AML sample as well as overall survival. Kaplan-Meier overall survival analysis was performed using the Survival R package and log-rank p values were calculated using the Mantel-Cox test. Heat maps were plotted with the gplots R package.

3.2.4 ChIP-Seq quality control and data analysis with IGV, MEME, TFSEARCH, R Bioconductor and Panther

ENCODE has implemented several statistical and quality control algorithms to ensure reporting of statistically significant enrichments with high consistency and reproducibility. An overview of the NF-κB RelA ChIP-Seq experiments is shown in Supplementary Figure 2. A numeric integrated quality flag was curated to reflect the quality and reliability of the experiments; a value of -1 is indicative of poor quality scores, whereas values of 0 and 1 are indicative of moderate and high/satisfactory qualities respectively. Furthermore, functional genomics experiments (ChIP-Seq) often produce artifact signals in particular regions of the genome (e.g. repeats). These regions are referred to as blacklisted regions; a percentage score of the blacklisted reads is usually reported. A summary of the quality control flag and blacklist percentages for all the experimental datasets described.

NarrowPeak files were downloaded from ENCODE and represent statistically significant enriched peaks calculated by the SPP peak caller, these were analyzed using several dedicated tools. The files were loaded in the integrative genomics viewer (IGV)[36] to visualize the transcription factor enrichment across the genome. The DNA sequence files corresponding to enriched genomic regions were obtained using the custom tracks tool from the UCSC Genome Browser (http://genome.ucsc.edu). Global NF-κB motif enrichment was performed with MEME-ChIP (http://meme.nbcr.net/meme/cgi-bin/meme-chip.cgi)[37]. Enriched NF-κB peaks

lacking a consensus NF-κB binding motif were searched with TFSEARCH[38] for putative transcription factors that could tether NF-κB to those regions. In an attempt to discover new NF-κB target genes with a regulation mode similar to that of *EIF4E*, the NF-κB enriched DNA sequences were parsed in the R programming language to determine genomic regions containing the NF-κB binding sites that have been identified and validated in the *EIF4E* promoter[4]. The identified regions were annotated using the ChIPpeakAnno[39] and org.Hs.eg.db (human genome hg19) packages. Functional gene annotation was then performed with Panther[40]. The median expression for these genes in different AML prognosis groups was determined from the TCGA RNA-Seq dataset. Statistical significance for the differences in median gene expression between favorable and poor AML prognosis groups was determined by the non-parametric Wilcoxon-Whitney-Mann test. False discovery rate (FDR) was determined according to Benjamini and Hochberg.

3.3 Results

3.3.1 EIF4E and NF-κB RELA mRNA expression levels follow a positive Pearson correlation in AML

Our initial findings suggested that *EIF4E* is an NF-κB inducible gene[4]. Here, we aimed at extending our original observations and explore a correlation between *EIF4E* and *RELA* mRNA levels in AML primary specimen from a large sample population. Accordingly, a large microarray gene expression dataset was analyzed and the Pearson correlation was determined. The data shown in Figure 1 suggests a statistically significant ($p < 0.01$) positive correlation ($r = 0.172$) between *EIF4E* and *RELA* mRNA levels from 283 AML samples[32]. This finding suggests that increased *RELA* mRNA expression in AML is likely linked to upregulated *EIF4E* expression. The converse also holds true, whereby increased *EIF4E* expression may be linked to elevated *RELA* expression.

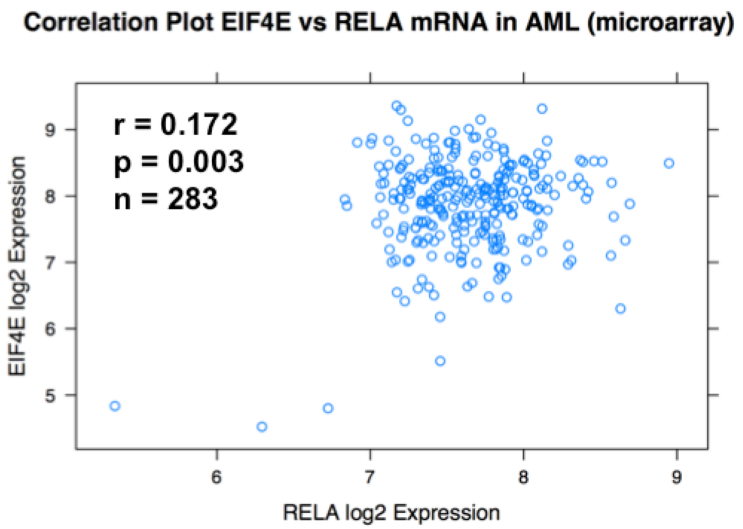


Figure 1. *EIF4E* and NF-κB *RELA* mRNA expression levels are positively correlated in an AML microarray gene expression study. Correlation plots between *EIF4E* and *RELA* mRNA levels are shown from a microarray AML dataset [Walter MJ et al. 2009]. Each open circle represents a patient. Gene expression is denoted as log2 expression for microarray data The Pearson correlation coefficient is represented as (r). Statistical significance was determined with ANOVA (analysis of variance).

3.3.2 EIF4E and RELA mRNA expression levels predict poor overall survival outcomes

The prognostic classification of AML links cytogenetic anomalies and molecular aberrations to predict patient outcome and response to therapy[8]. Since *EIF4E* and *RELA* mRNA expressions are upregulated in AML, we sought to determine whether high levels of *EIF4E* and *RELA* are indicative of poor patient prognosis. Accordingly, we analyzed a TCGA RNA-Seq dataset from 167 AML patients with relevant overall survival data. These patients were classified into the three clinical cytogenetic prognosis groups (Favorable, Intermediate, poor).

In order to determine whether this elevated expression correlates with worse patient survival outcomes, Kaplan-Meier overall survival was determined for these AML patients stratified into two groups: *EIF4E* and *RELA* high (sample size = 10) versus *EIF4E* and *RELA* low (sample size = 53). *EIF4E* and *RELA* high samples reflect RPKM values that are higher than the lowest median expression for those genes observed in the clinically favorable AML samples (*EIF4E* RPKM > 3 and *RELA* RPKM > 5). The survival plot shown in Figure 2 represents the overall survival probability as a function of time (days) and indicates that elevated *EIF4E* and *RELA* mRNA levels predict a significantly (log-rank $p < 0.05$) worse trend of patient survival outcomes than low levels of these respective oncogenes. Furthermore, survival data from patients with near threshold RPKM values as well as RPKM high values for either *EIF4E* or *RELA* (sample size = 104 labeled as "Other") revealed a survival outcome similar to that of the *EIF4E* and *RELA* low group (Supplementary Figure 1B). Taken together, these findings suggest that elevation of both *EIF4E* and *RELA* mRNA expressions predict inferior survival outcomes. Accordingly, elevated *EIF4E* and *RELA* mRNA levels may serve as prognostic markers in AML.

3.3.3 The RelA and p50 consensus binding sites are significantly enriched in the ENCODE NFkB ChIP-Seq datasets from B-lymphoblast cells

In an attempt to provide an in depth understanding of *EIF4E*'s complex transcriptional control, we analyzed ChIP-Seq datasets from ENCODE. RelA (p65) ChIP-Seq datasets were available from 10 B-lymphoblast cell lines (immature B-cells) stimulated with TNF-α, a known NF-κB activator[41] (Supplementary Table 1). Initial characterization of these datasets was conducted to

ensure quality and reliability of the drawn conclusions. A summary of the quality control metrics is shown in Supplementary Table 2; the data is indicative of high and reliable quality with less than 1% of the enriched sequence reads in blacklist regions. Analysis for NF-κB motif enrichment in these datasets with MEME-ChIP indicates a statistical significant enrichment of the RelA and NF-κB1 (p50) consensus motifs (Figure 3A, 3B and Supplementary Table 3). RelA has been shown to homodimerize or heterodimerize with NF-κB1[42]; this is reflected in the co-enrichment of the binding motifs for both RelA and p50 in the ENCODE NF-κB ChIP-Seq datasets. The observed sequence similarity in the reported consensus sites can be attributed to a conserved DNA binding domain (Rel homology) in NF-κB proteins[7].

Furthermore, since RelA protein has been demonstrated to form homodimers as well as heterodimers with all other NF-κB species, it is quite striking that the predominant species that are possibly formed are RelA-RelA homodimers and RelA-p50 heterodimers. This may suggest a lineage dependent observation as the samples are of the immature B-cell lineage.

Additionally, RelA is enriched across the entire genome (Figure 3C) suggesting a genome wide regulatory mode for NF-κB. Furthermore, the enrichment pattern is consistent across the 10 datasets suggesting robust and reproducible immunoprecipitations as well as peak calling. Taken together, these datasets can be used in the comprehensive analysis for *EIF4E* transcriptional regulation.

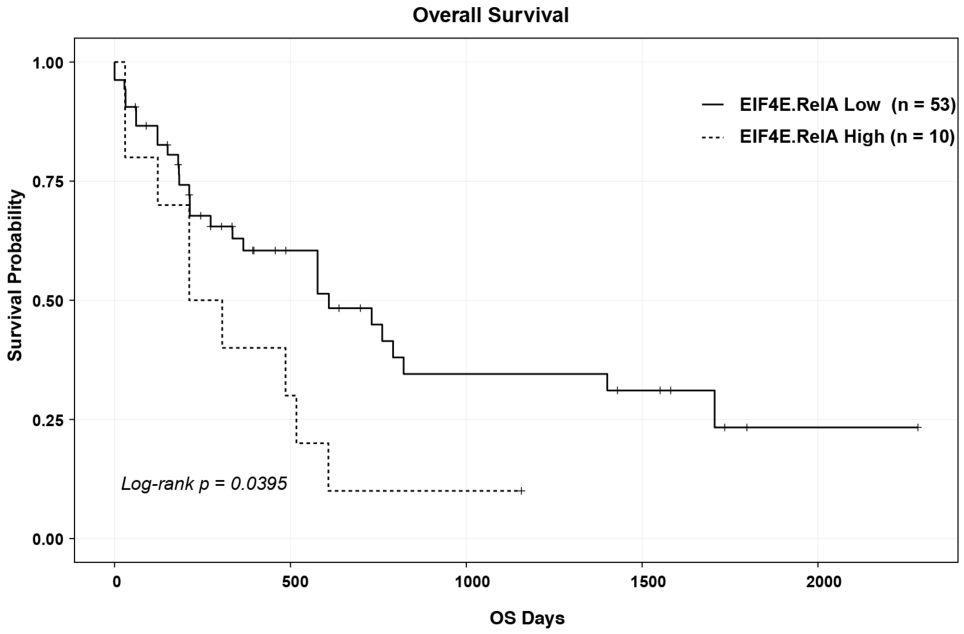


Figure 2. Elevated *EIF4E* and NF-κB *RELA* mRNAs predict worse survival outcomes in AML. Kaplan–Meier plots of overall survival from AML patients stratified by *EIF4E* and *RELA* levels. *EIF4E* and *RELA* high samples reflect RPKM values that are higher than the lowest median expression for those genes observed in the clinically favorable AML samples (*EIF4E high* RPKM > 3 and *RELA high* RPKM > 5). Each mark on the survival curve represents a censored event where the patient is still alive at the end of the study. Mantel–Cox test was performed to calculate log-rank P values. Data source: AML TCGA study.

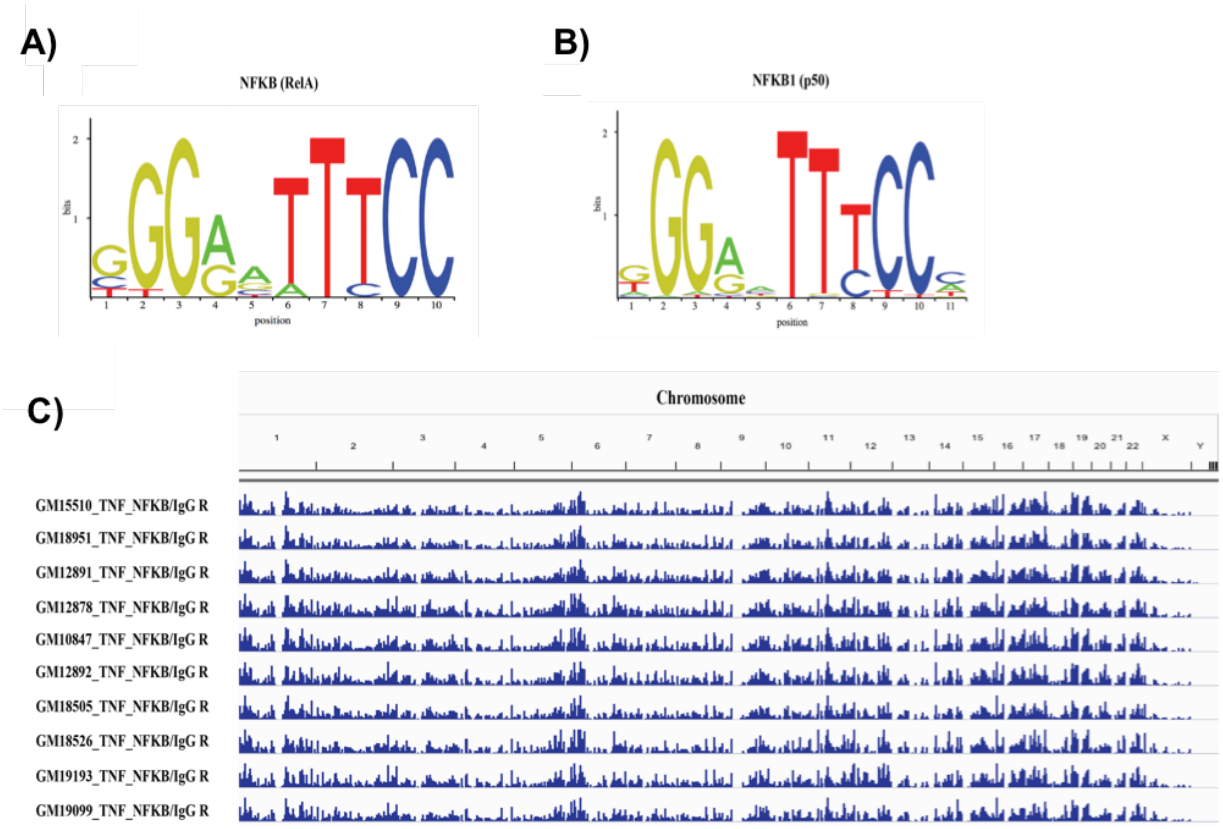
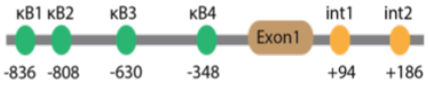


Figure 3. The RelA and p50 consensus binding sites are significantly enriched in the ENCODE NF-κB ChIP-Seq datasets suggesting the formation of RelA and p50 complexes in the regulatory regions of target genes. Sequence logo representation of RelA **(A)** and NF-κB1 (p50) **(B)** consensus motifs enriched in the reported ENCODE NF-κB ChIP-Seq datasets were generated with the MEME-ChIP tool. These are position-specific probability matrices that specify the probability of each possible nucleotide, in bits, appearing at each possible position in an occurrence of the motif. **(C)** Graphical representation with IGV of statistically significant RelA peaks across the genome from 10 B-lymphoblast cell lines reflects a consistent genome wide enrichment of RelA across all samples in this dataset.

3.3.4 RelA (p65) is enriched in the EIF4E promoter and intron and could be recruited indirectly to the EIF4E locus

We have previously identified and validated four κB binding sites in the *EIF4E* promoter (Figure 4A and 4B) that recruit NF-κB factors in a lineage dependent fashion. In mature B-cells, κB3/4 are accessible to NF-κB proteins but not κB1/2; on the other hand, all sites are accessible to NF-κB factors in myeloid cell lines and primary specimens[4]. Interestingly, we have also identified two conserved κB intronic elements that can bind NF-κB complexes *in vitro* (unpublished findings, Chapter 4); however, the accessibility of these elements and their capacity to bind NF-κB proteins *in vivo* was not investigated. Given the wealth of data that could be mined from the ChIP-Seq datasets, we sought to monitor the enrichment pattern of RelA in the *EIF4E* promoter and intron. In the investigated datasets, RelA peaks were significantly enriched in the *EIF4E* promoter and intron in 4 out of the 10 datasets (Figure 4C). As a control, we examined RelA enrichment in other known NF-κB target genes *BCL2*[43] and *NFκBIA*[44] (IκB- α) and the results show enrichment with RelA peaks in all of the 10 datasets (Supplementary Figure 3A). These controls further attest to the quality of the examined ChIP-Seq datasets as these genes represent validated NF-κB targets. It is important to note that no RelA peaks were present in the *CCND1* (CyclinD1) promoter[45], whereas RelA peaks were present in 3 and 4 datasets for the NF-κB targets *PIM1*[46] and *MYC*[47] respectively.

A)



B)

NFKB-Site	Sequence	Position	Validated
NFKB-prom1	GTGGCTTCCC	-836	EMSA/ChIP
NFKB-prom2	GGCTATTTCC	-808	EMSA/ChIP
NFKB-prom3	GGGCGGTTCC	-630	EMSA/ChIP
NFKB-prom4	AGGCTTGCCT	-348	EMSA/ChIP
NFKB-Intronic1	GGGGGACTCC	+94	EMSA
NFKB-Intronic2	GGGACGTCCC	+186	EMSA

C)

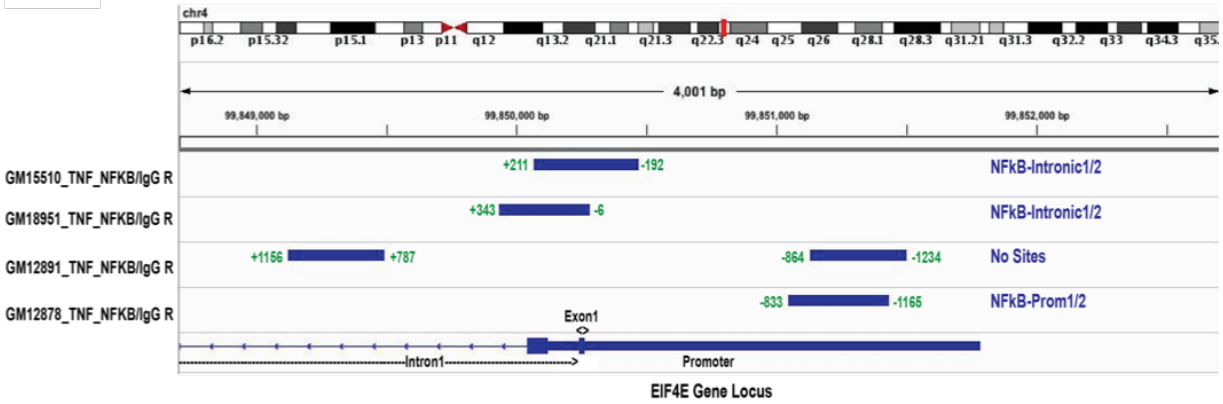


Figure 4. NF-κB (RelA) is enriched in the *EIF4E* promoter and first intron. (A) Schematic representation of the *EIF4E* regulatory locus. Promoter and intronic κB sites are indicated in green and orange respectively. **(B)** The distribution**,** sequence and validation methods for the different κB elements in the *EIF4E* promoter and intron are presented. **(C)** NF-κB RelA enrichment peaks across the *EIF4E* promoter and intron were generated with IGV from four B-lymphoblast datasets and reveal RelA enrichment in the *EIF4E* promoter and intron. The position of these peaks with respect to the *EIF4E* transcriptional start site is shown in green. Previously validated κB sites are referenced for each peak in blue lettering.

RelA peaks in the *EIF4E* regulatory region were localized to the promoter and the first intron (Figure 4C and Supplementary Sequences). In the GM15510 and GM18951 datasets, the RelA peaks contained the two intronic elements, which we previously validated with gel shift experiments. In the GM12878 dataset, the RelA peak contained two NF-κB promoter sites 1 and 2, which we have previously validated with gel shift and ChIP experiments. Importantly, no additional NF-κB binding sites were identified in these datasets. These findings suggest that the intronic κB elements are accessible in cell lines; however, further investigation is required to elucidate whether these elements are functional in promoting NF-κB-dependent transcriptional control of *EIF4E.* Furthermore, κB1/2 elements enrichment in this immature B-cell backbone suggests that these elements might be accessible during B-cell development but not in mature B-lymphocytes as previously determined from ChIP experiments in mature BJAB cells[4].

Interestingly, in the GM12891 cell line, RelA peaks were present in both the *EIF4E* promoter and first intron; however, sequence analysis revealed no NF-κB binding sites. Given that the promoter peak was shifted by 31 base pairs from the κB-containing peak in GM12878, it is most likely the product of peak calling stringency; however, the intronic peak in GM12891 is an independent peak that suggests an indirect RelA recruitment to the *EIF4E* intron through other transcription factors and/or complexes. Accordingly, we analyzed the sequence underlying this RelA peak in the GM12891 cell line using TFSEARCH and identified 3 binding sites for proteins known to interact with RelA: Cdx, SP1 and STAT. These factors have been shown to interact with RelA and potentiate or alleviate its transcriptional power. A brief overview of these factors is presented below.

a) Cdx is a caudal type homeobox factor that has been identified as a colonic tumor suppressor. It was shown to interact with RelA/p50 and impede the complex's transcriptional activity[48].
b) SP1 is a zinc finger transcription factor involved in various cellular processes. It has been shown to interact with various NF-κB factors including RelA to promote leukemogenesis[49].

c) STAT is a signal transducer and activator of transcription with diverse functions. STAT proteins mediate hematopoiesis and hematopoietic functions[50], it interacts with RelA to enhance[51] (STAT3) or suppress[52] (STAT1) its activities.

Currently, there is no evidence supporting a role for these factors in the control of protein synthesis. Given the ability of these factors to interact with and modulate RelA activity, it would be plausible to consider an indirect RelA recruitment to the *EIF4E* regulatory regions through tethering with one or more of these factors. Further experiments are needed to investigate this hypothesis.

3.3.5 In silico prediction of 8 new putative NF-κB target genes that may be regulated with a pattern similar to EIF4E in poor prognosis AML

My research on the transcriptional regulation of *EIF4E* in AML suggests that the selective overexpression of *EIF4E* across different AML groups may be the product of selective NF-κB recognition of its binding sites in the *EIF4E* promoter[4]. This intriguing notion sparkled an interest for new NF-κB target gene prediction that may be regulated in a manner similar to that of *EIF4E* through the same κB elements. Accordingly, we screened the NF-κB ChIP-Seq datasets for genes with NF-κB enrichment in promoter and/or intronic regions containing any of the previously validated κB binding sites present in the *EIF4E* regulatory regions (Figure 4A and 4B). 47 genes were predicted as putative targets with NF-κB RelA enrichment in their regulatory regions containing one or more of the previously identified κB sites in the *EIF4E* promoter and intron (Supplementary Table 4); these regions are evolutionary conserved in monkey and chimpanzee. Interestingly, one of these genes, *IL2RA*, has been previously validated as an NF-κB target[53]. The biological process annotation was performed using the Panther database[40] (Figure 5A and Supplementary Table 5) which suggests that these new candidate genes are part of diverse cellular processes most of which are dysregulated in cancer.

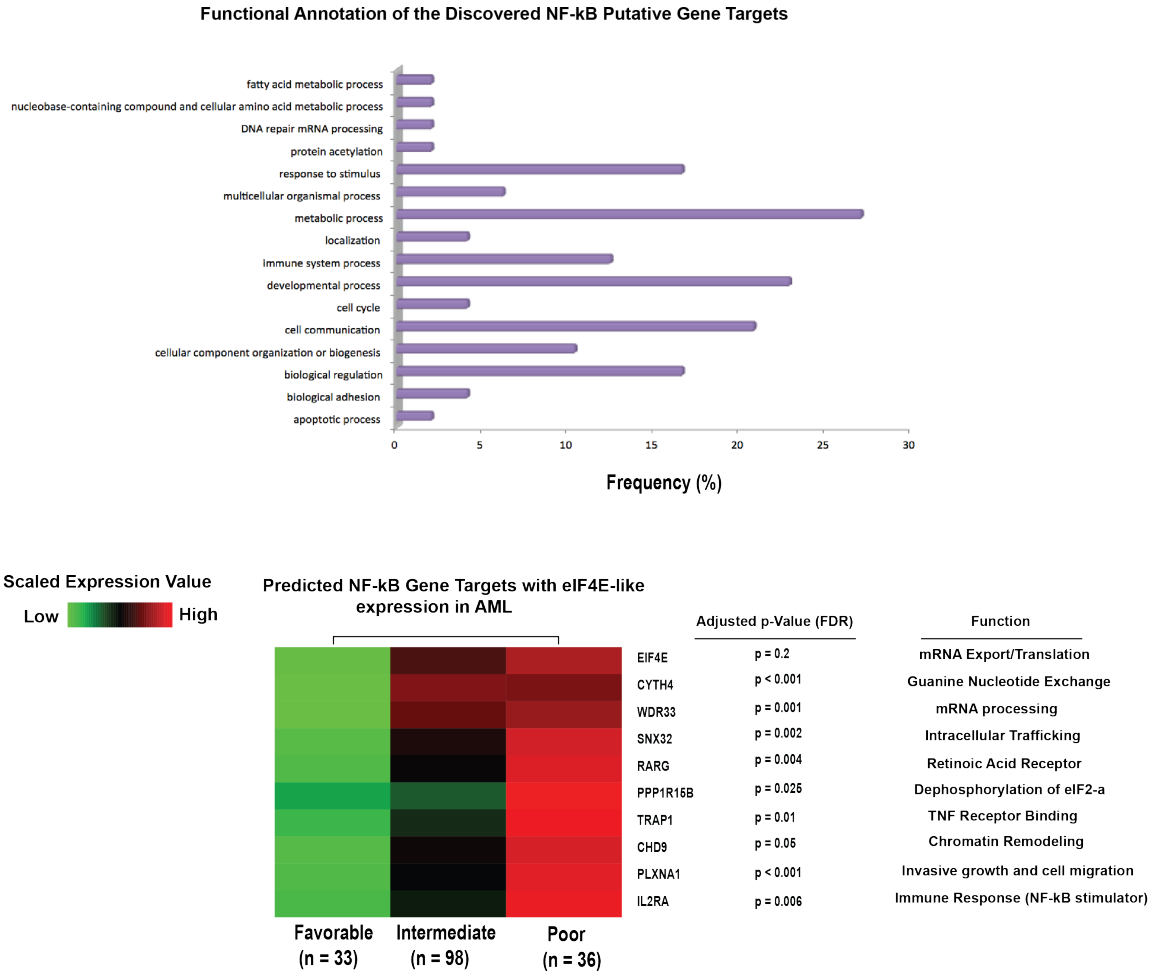


Figure 5. In silico prediction of new putative NF-κB target genes that are upregulated in poor prognosis AML specimens. (A) New genes with NF- κB RelA enrichment at regulatory regions containing one or more *EIF4E* κB elements. These sequences and their respective position in the identified target genes are evolutionary conserved in monkey and chimpanzee. Functional annotation of 46 new putative NF-κB targets was performed with Panther and is graphically presented as frequency of genes associated with a biological process. **(B)** Heat map depicting the median expression signatures of 9 predicted targets across AML risk groups. The color intensity is proportional to the scaled gene expression values with red as high and green as low. Non-parametric Wilcoxon-Whitney-Mann test was used to analyze the differences between groups and the false discovery rate (FDR) was determined according to Benjamini and Hochberg. The biological function for these targets is also shown.

Next, we sought to determine whether any of these newly identified genes is dysregulated in AML in a manner similar to that of *EIF4E*. Accordingly, we analyzed the expression pattern of these candidate genes in the TCGA RNA-Seq[14]. Only genes with significant median expression pattern similar to *EIF4E* that is higher in the poor prognosis group were selected and are shown in the heat map in Figure 5B with the adjusted p value and biological function.

Nine genes presented an expression pattern that is similar to EIF4E as determined from 167 AML specimens. This pattern depicts a poor prognosis high and favorable prognosis low median expression; these genes were: *CYTH4, WDR33, SNX32, RARG, PPP1R15B, TRAP1, CHD9, PLXNA1* and *IL2RA*. However, these findings are of preliminary nature and further studies are required to investigate the functional relevance of these new targets in AML and whether their transcriptional regulation is NF-κB dependent.

3.3.6 In silico prediction of 6 new putative EIF4E transcriptional regulators

The *EIF4E* promoter contains an abundance of transcription factor binding sites including Myc, NF-κB, AP1, PU.1, SP1, GATA, OCT1, CREB, PAX5, E2F, STAT5, NFATC1 and many others. These have been predicted through MatInspector analysis[23] and would suggest a complex mode of lineage-dependent and/or stimulus-dependent transcriptional regulation. However, with a predominant Myc-centric view, not much has been done to investigate the requirement of these putative regulatory sites. Accordingly, we analyzed ChIP-Seq datasets for a number of these factors in the available hematopoietic cell lines from ENCODE. These included the B-lymphoblast cell lines: GM12878, GM12891 and GM12892, as well as the chronic myelogenous leukemia (CML) cell line K562. The relevant transcription factor peaks across the *EIF4E* promoter and intron in the available cell lines are delineated in Figure 6. A brief overview of these factors and their enrichment patterns is presented below.

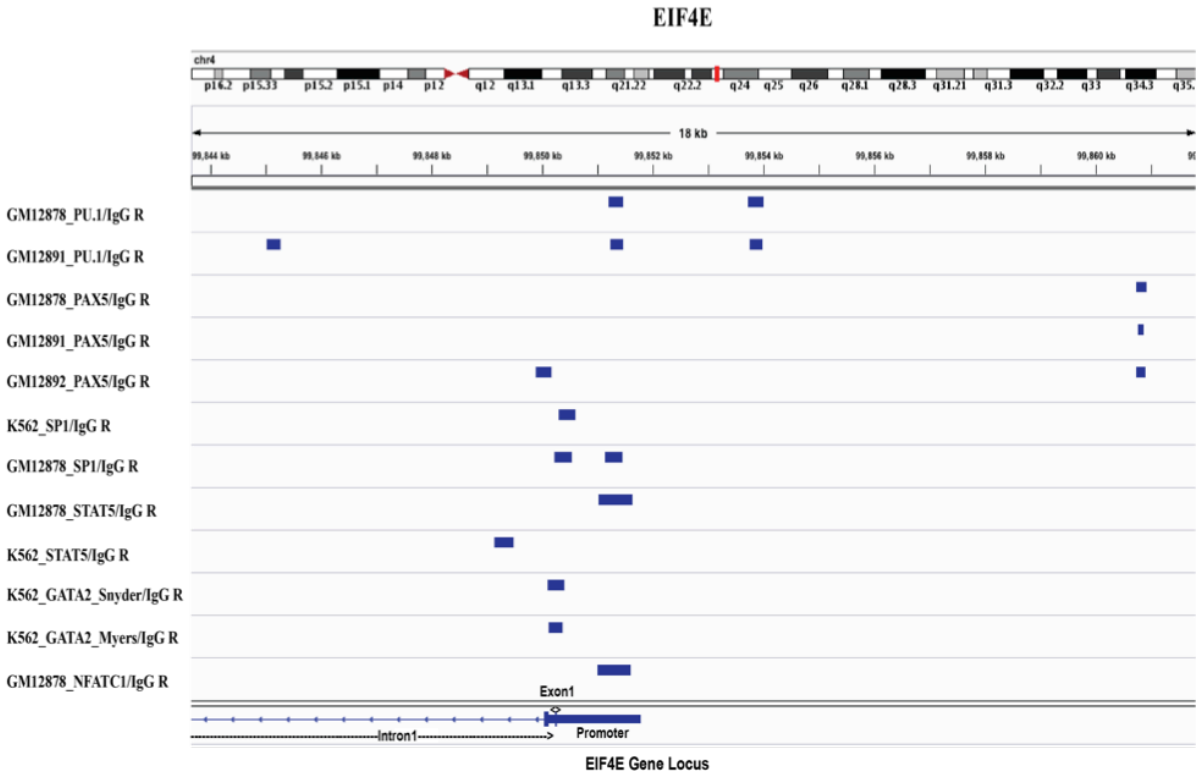


Figure 6. In silico prediction of 6 new putative *EIF4E* transcriptional regulators. Graphical representation with IGV of the *EIF4E* regulatory regions with SP1, STAT5, PU.1, PAX5, GATA2 and NFATC1 peaks in lymphoblast (GM12878, GM12891, GM12892) and chronic myelogenous leukemia (K562) cell lines.

a) PU.1 is a transcription factor important in myeloid and B-lymphoid cell development. ChIP-Seq experiments were performed in the GM12878, GM12891 and K562 cell lines. In GM12878, PU.1 enrichment peaks were present in the *EIF4E* promoter proximal and distal to the transcription start site. In GM12891, PU.1 enrichment peaks were present in the *EIF4E* promoter and intron. No peaks were detected in the K562 cell line.
b) PAX5 is a transcription factor that functions as a B-cell lineage specific activator protein expressed during B-cell differentiation. The available experiments were conducted in the GM12878, GM12891 and GM12892 lymphoid cell lines. PAX5 peaks were observed at a distance upstream of the *EIF4E* promoter in GM12878 and GM12891 whereas in the GM12892 cells, the peaks were detected in the promoter and intron regions of *EIF4E*.
c) The SP1 and STAT factors were discussed in the previous section. The available ENCODE data suggests SP1 recruitment to the *EIF4E* promoter in the GM12878 and K562 cell lines. STAT5 peaks were detected in the *EIF4E* promoter and intron in the GM12878 and K562 cell lines respectively.
d) The GATA zinc finger transcription factors belong to a diverse and conserved family of transcriptional regulators involved in cardiac and hematopoietic development. GATA1 and GATA2 ChIP-Seq experiments were analyzed in the K562 CML cell line. No GATA1 peaks were detected in the *EIF4E* regulatory regions, whereas GATA2 peaks were detected in the *EIF4E* promoter in two different experiments conducted by the Snyder and Myers groups.
e) The NFATC1 transcription factor plays an integral role in modulating inducible gene expression during an immune response. An NFATC1 ChIP-Seq experiment was analyzed in the GM12878 cell line revealing peaks in the *EIF4E* promoter.

The aforementioned data predicts 6 new putative *EIF4E* regulators and supports an intricate, dynamic and most importantly a non-nodal regulation of *EIF4E*. The identified factors are important for myeloid or lymphoid (or both) development and functions. In fact, aberrations in these factors have been linked to hematopoietic malignancies. GATA2 and SP1 have been linked to myeloid transformation[49,54]. PAX5 and PU.1 have been linked to B-cell[55,56] as well

as myeloid leukemias[57,58]. Accordingly, this data suggests that *EIF4E* could be regulated by these additional transcription factors and would be dysregulated in hematopoietic anomalies associated with aberrations in these proteins; however, further work is needed to evaluate this hypothesis since there is no current evidence linking any of the aforementioned factors to mRNA translation and protein synthesis.

3.4 Discussion

In this study, we described a positive correlation between *EIF4E* and *RELA* mRNA expression in AML. In addition, elevated mRNA levels for these genes predict a worse trend for patient survival. However, several caveats must be considered, first, these conclusions were drawn from experiments that reflect mRNA expression for these oncogenes (microarray) with no data regarding protein levels. Second, although elevated *RELA* expression levels have been linked to constitutive NF-κB activity in cancer[7], additional experimentation is required to establish this activity in the samples tested. Taken together, these findings offer a new venue for research into *EIF4E* and *RELA* as poor prognosis markers in AML; however further investigation is required. The significance of this association is inherent in the proper planning of treatment protocols for patients with high *EIF4E* and NF-κB by targeting EIF4E with Ribavirin[16] and NF-κB with current pathway inhibitors[19,59].

My previous findings show that NF-κB proteins are directly recruited to conserved κB elements in the *EIF4E* promoter with lineage preference to regulate *EIF4E* gene expression[4]. In an attempt to further dissect the transcriptional regulation of *EIF4E*, we analyzed ENCODE NF-κB ChIP-Seq data obtained from 10 B-lymphoblast cell lines. NF-κB (Rel A) peaks were detected in 4/10 samples engaging 2 promoter (κB1/2) and 2 intronic elements. However, it is important to keep in mind that peak detection is affected by several factors including the stringency of the peak detection algorithm[31]. Furthermore, the cell lines under study were EBV+, which imparts constitutive NF-κB activity in those samples. These facts together with the chosen treatment conditions (TNF-α) present caveats that may underlie the observed NF-κB enrichment in only 4/10 cell lines.

Interestingly, the NF-κB enrichment in the *EIF4E* intron suggests that these elements might be active in vivo and are potentially functional in driving EIF4E transcriptional control. However, it remains intriguing as to what cellular conditions are decisive of NF-κB recruitment to the intronic and/or promoter regions; this could be the result of differences in the chromatin status at the *EIF4E* locus which may either hinder or favor NF-κB recruitment to different regions in the *EIF4E* promoter and/or intron. Additionally, NF-κB recruitment to the κB1/2 sites in the tested immature B-lymphoblast cell lines may suggest that NF-κB accessibility to these elements is selective to B-cell developmental stages since ChIP experiments suggested that these sites were not bound by NF-κB proteins in a mature B-cell line[4]. Furthermore, the results obtained with the GM12891 cell line suggest that NF-κB could be recruited to the *EIF4E* locus through other transcriptional regulators (Sp1, STAT, Cdx); additional work is needed to validate this observation.

In this study, we provided evidence that *EIF4E* could be regulated through additional transcription factors (e.g. GATA2, SP1, PAX5, PU.1). The data suggests that these factors could be recruited to the *EIF4E* promoter; however further experiments are required to examine whether the recruitment of these factors is coupled to *EIF4E* transcription. As some of these modulators are dysregulated in AML and lymphoma, it would be interesting to study the effect of those factors on *EIF4E* regulation in those cancers. Finally, 8 new putative NF-κB target genes were predicted that are possibly upregulated in poor prognosis AML similar to *EIF4E* and accordingly, maybe considered as risk markers in AML. However, further investigation is required to validate these genes. Finally, it would be interesting to investigate whether any of these targets are also regulated by EIF4E, given that EIF4E has been shown to amplify the "NF-κB effect" by enhancing the mRNA export and/or translation of NF-κB targets (e.g. *CCND1, PIM1, MYC*)[4,60].

3.5 Conclusion

In conclusion, this study presents putative insights into the association of *EIF4E* and *RELA* expression in AML poor risk group as well as the complexity of *EIF4E*'s transcriptional control. The analysis presented in this chapter may inspire new research focused on targeting

AML poor prognosis patients with elevated *EIF4E* and NF-κB with Ribavirin and NF-κB inhibitors. Additional research perspectives include understanding *EIF4E*'s transcriptional control through new transcription factors as well as investigate an NF-κB dependent mechanism of regulation for the newly discovered putative targets in poor prognosis AML that were reported in this study.

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Supplementary Material

Supplementary Figure1. Intermediate and poor prognosis AML groups predict inferior survival outcomes. Kaplan–Meier plots of overall survival from 167 AML patients stratified by clinical risk groups: favorable (n = 33), intermediate (n = 98) and poor (n = 36). Each mark on the survival curve represents a censored event where the patient is still alive at the end of the study. Mantel–Cox test was performed to calculate log-rank P values. Data source: AML TCGA study.

Supplementary Figure2. Overview of the various stages in an NF-κB ChIP-Seq experiment. The cells are fixed with formaldehyde to generate a snapshot of the cellular chromatin status. The isolated chromatin is than fragmented and immunoprecipitated with an NF-κB antibody (RelA). Each ChIP experiment is performed with at least two biological replicates derived from independent cell cultures. Following immunoprecipitation, the purified DNA is sequenced with Illumina NextGEN sequencing. The sequenced reads are then mapped to a reference genome and only statistically significant signal/background enrichments from biological replicates will represent peaks. Peak calling is performed by overlapping statistically significant mapped reads over genomic regions. Additional post-filtering algorithms are applied for motif discovery and to eliminate artifacts.

Supplementary Figure3. NF-κB (RelA) is enriched in the promoter and intron of two validated NF-κB target genes: *NFκBIA* and *BCL2*. NF-κB RelA enrichment peaks across the *NFκBIA* **(A)** and *BCL2* **(B)** promoter and intron were generated with IGV from 10 B-lymphoblast datasets and reveal RelA enrichment.

Supplementary Table1. An overview of the 10 B-lymphoblast cell lines presented in this study.

Supplementary Table2. List of the cell lines and the ChIP-Seq datasets used in this study. An integrated quality control flag denotes the reliability of the chosen dataset with poor (-1), moderate (0) and high quality (1) metrics.

Supplementary Table3. NF-κB RelA and NF-κB1 (p50) motif enrichment in the investigated samples are statistically significant. E-Values were calculated with MEME-ChIP tool and presented for the 10 B-lymphoblast cell lines used in this study.

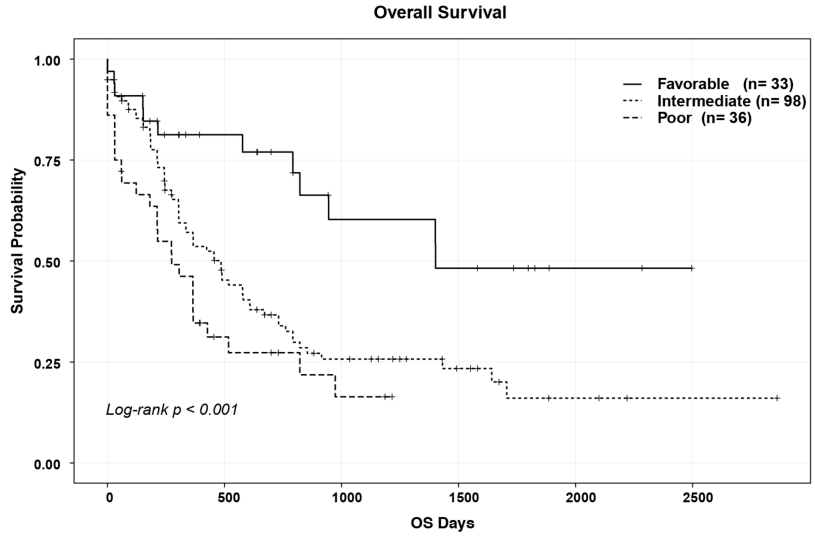
Supplementary Table4. List of 47 predicted NF-κB target genes containing *EIF4E* κB sites in their promoter and/ or intron.

Supplementary Table5. List of the functional annotation groups and their underlying genes as determined by Panther.

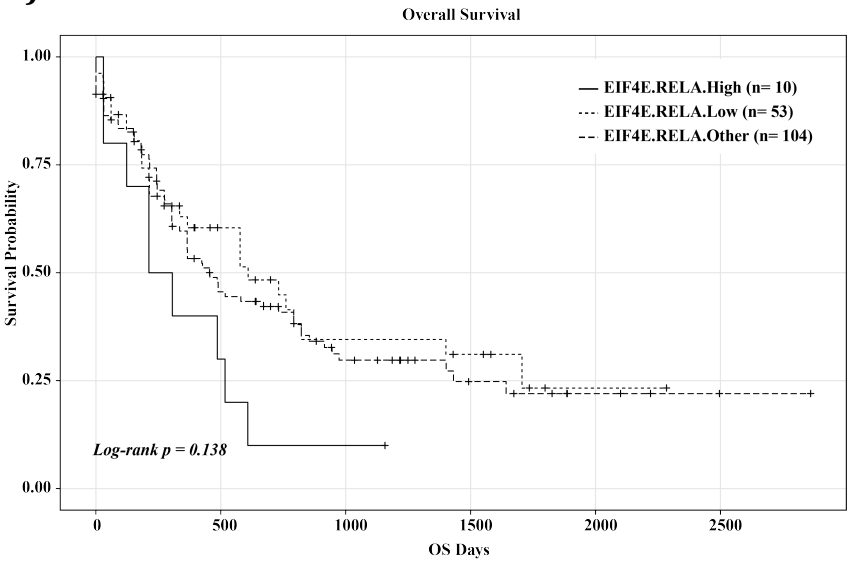
Supplementary Sequences. Base pair DNA sequences of the NF-κB RelA peaks identified in the *EIF4E* promoter and intron. Highlighted sequences represent previously validated κB sites.

Supplementary Figure 1

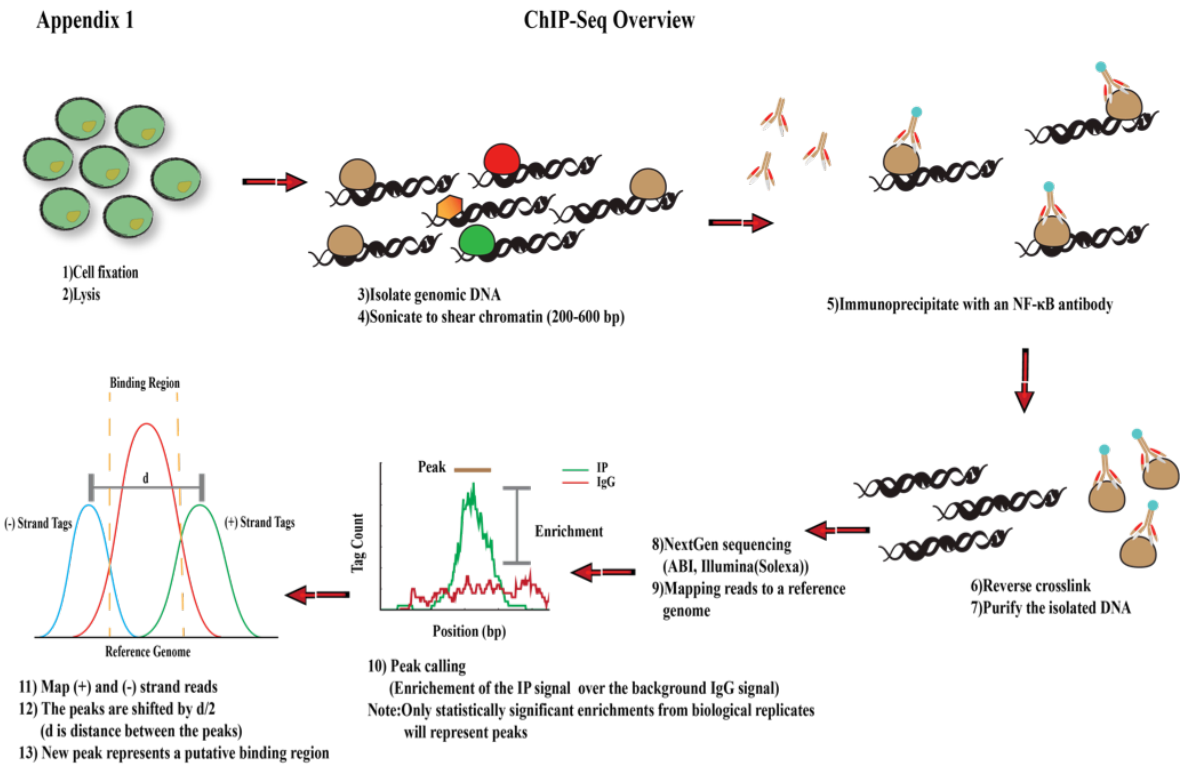
A)



B)

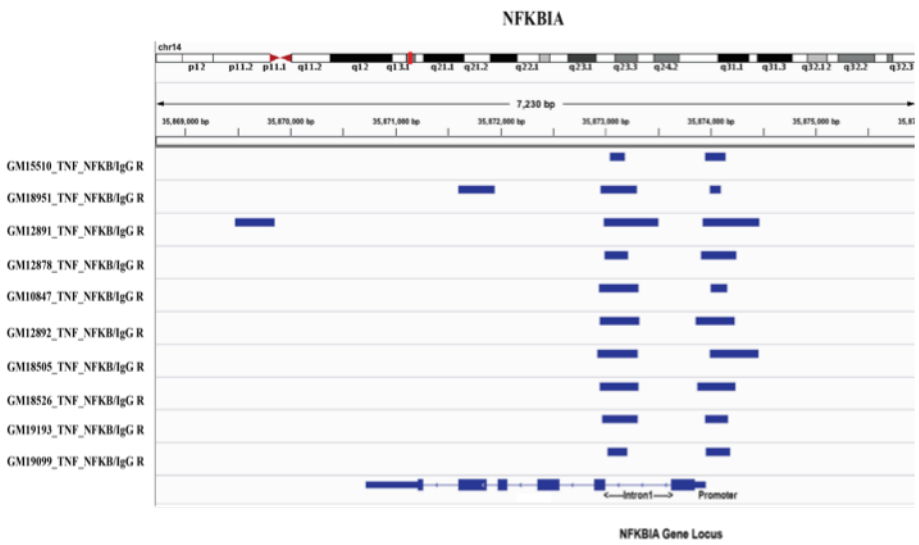


Supplementary Figure 2

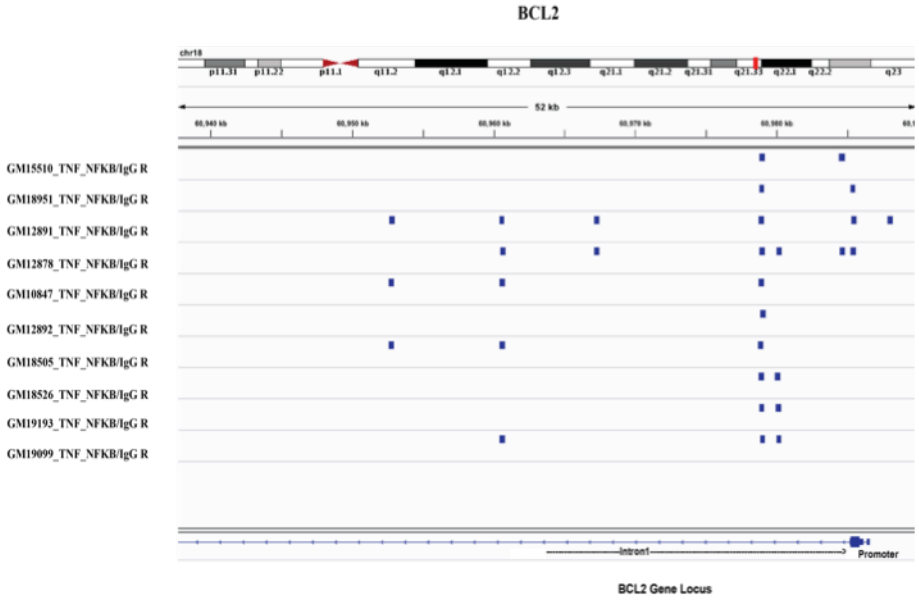


Supplementary Figure 3

A)



B)



Supplementary Table 1

Cell_Line	Origin	Source	karyotype	Treatment	RelA peak on eIF4E locus
GM12878	B_lymphocyte, lymphoblastoid	Blood	Normal	TNF-a	YES
GM10847	B_lymphocyte, lymphoblastoid	Blood	Normal	TNF-a	NO
GM12891	B_lymphocyte, lymphoblastoid	Blood	Normal	TNF-a	YES
GM12892	B_lymphocyte, lymphoblastoid	Blood	Normal	TNF-a	NO
GM15510	B_lymphocyte, lymphoblastoid	Blood	Normal	TNF-a	YES
GM18505	B_lymphocyte, lymphoblastoid	Blood	Normal	TNF-a	NO
GM18526	B_lymphocyte, lymphoblastoid	Blood	Normal	TNF-a	NO
GM18951	B_lymphocyte, lymphoblastoid	Blood	Normal	TNF-a	YES
GM19099	B_lymphocyte, lymphoblastoid	Blood	Normal	TNF-a	NO
GM19193	B_lymphocyte, lymphoblastoid	Blood	Normal	TNF-a	NO

Supplementary Table 2

CELLTYPE	TARGET NAME	Antibody	Source	INTEGRATED QUALITY FLAG (-1/0/1)	BLACKLIST PERCENTAGE
GM10847	NFKB (RelA)	Santa Cruz (sc-372)	Snyder (Stanford)	1	0.204719319
GM12878	NFKB (RelA)	Santa Cruz (sc-372)	Snyder (Stanford)	1	0.39756782
GM12891	NFKB (RelA)	Santa Cruz (sc-372)	Snyder (Stanford)	1	0.013943598
GM12892	NFKB (RelA)	Santa Cruz (sc-372)	Snyder (Stanford)	1	0.073547438
GM15510	NFKB (RelA)	Santa Cruz (sc-372)	Snyder (Stanford)	1	0.210444271
GM18505	NFKB (RelA)	Santa Cruz (sc-372)	Snyder (Stanford)	1	0.113442995
GM18526	NFKB (RelA)	Santa Cruz (sc-372)	Snyder (Stanford)	1	0.486223663
GM18951	NFKB (RelA)	Santa Cruz (sc-372)	Snyder (Stanford)	1	0.16227779
GM19099	NFKB (RelA)	Santa Cruz (sc-372)	Snyder (Stanford)	1	0.192505133
GM19193	NFKB (RelA)	Santa Cruz (sc-372)	Snyder (Stanford)	1	0.15128593
GM12878	PU.1	Santa Cruz (sc-22805)	Myers (HudsonAlpha)	1	0.132573555
GM12891	PU.1	Santa Cruz (sc-22805)	Myers (HudsonAlpha)	1	0.14110718
K562	PU.1	Santa Cruz (sc-22805)	Myers (HudsonAlpha)	1	0.347499739
GM12878	Sp1	Santa Cruz (sc-17824)	Myers (HudsonAlpha)	1	0.469073852
K562	Sp1	Santa Cruz (sc-17824)	Myers (HudsonAlpha)	1	0.45586407
GM12878	PAX5	Santa Cruz (sc-1974)	Myers (HudsonAlpha)	1	0.165458557
GM12891	PAX5	Santa Cruz (sc-1974)	Myers (HudsonAlpha)	1	1.498127341
GM12892	PAX5	Santa Cruz (sc-1974)	Myers (HudsonAlpha)	1	0.43499275
GM12878	STAT5	Santa Cruz (sc-74442)	Myers (HudsonAlpha)	1	0.322277427
K562	STAT5	Santa Cruz (sc-74442)	Myers (HudsonAlpha)	1	0.668219095
GM12878	NFATC1	Santa Cruz (sc-17834)	Myers (HudsonAlpha)	1	0.166204986
K562	GATA1	Santa Cruz (sc-266)	Snyder (Stanford)	1	1.020408163
K562	GATA2	In house	Snyder (Stanford)	1	0.327623327
K562	GATA2	Santa Cruz (sc-267)	Myers (HudsonAlpha)	1	0.309565576

Supplementary Table 3

Cell_Line	RELA E-Value	NFkB1 E-Value
GM10847	9.6e-765	1.3e-677
GM12892	8.9e-689	2.3e-635
GM18505	3.7e-774	5.6e-694
GM18526	2.6e-380	7.9e-351
GM19099	4.7e-957	1.8e-908
GM19193	1.7e-648	2.4e-598
GM12878	4.1e-1023	3.2e-939
GM12891	3.3e-2241	3.1e-2102
GM15510	3.4e-490	6.1e-416
GM18951	2.6e-774	2.0e-687

Supplementary Table 4

Gene_Symbol	Gene_Name
PLXNA1	plexin A1
FFAR2	free fatty acid receptor 2
CYTH4	cytohesin 4
RPL39	ribosomal protein L39
TNFRSF1A	tumor necrosis factor receptor superfamily, member 1A
NRG2	neuregulin 2
WDR33	WD repeat domain 33
PDK2	pyruvate dehydrogenase kinase, isozyme 2
IL2RA	interleukin 2 receptor, alpha
DTX1	deltex homolog 1 (Drosophila) E3 Ubiquitin ligase
IRF9	interferon regulatory factor 9
ANXA11	annexin A11
ALDH2	aldehyde dehydrogenase 2 family (mitochondrial)
TRMT112	tRNA methyltransferase 11-2 homolog (S. cerevisiae); similar to CG12975
SMIM14	small integral membrane protein 14
SEMA4A	semaphorin 4A
PPP1R18	protein phosphatase 1, regulatory subunit 18
MCMBP	minichromosome maintenance complex binding protein
MSL2	male-specific lethal 2 homolog (Drosophila)
DUSP4	dual specificity phosphatase 4
ZC3H4	zinc finger CCCH-type containing 4
LMNB1	lamin B1
ARPP19	cAMP-regulated phosphoprotein 19kDa
CSRP2	cysteine and glycine-rich protein 2
NAA35	MAK10 homolog, amino-acid N-acetyltransferase subunit (S. cerevisiae)
SNX32	sorting nexin 32
RBM39	RNA binding motif protein 39
ZNF408	zinc finger protein 408
DHTKD1	dehydrogenase E1 and transketolase domain containing 1
NDC1	NDC1 transmembrane nucleoporin
AKNA	AT-hook transcription factor
HSPA2	heat shock 70kDa protein 2
ARPC2	actin related protein 2/3 complex, subunit 2, 34kDa
MS4A6E	membrane-spanning 4-domains, subfamily A, member 6E
PADI2	peptidyl arginine deiminase, type II
TESC	tescalcin
DENND5A	DENN/MADD domain containing 5A
RARG	retinoic acid receptor, gamma
HIVEP2	human immunodeficiency virus type I enhancer binding protein 2
PPP1R15B	protein phosphatase 1, regulatory (inhibitor) subunit 15B
BAZ2B	bromodomain adjacent to zinc finger domain, 2B
ALCAM	activated leukocyte cell adhesion molecule
TRAP1	TNF receptor-associated protein 1
PHLDB1	pleckstrin homology-like domain, family B, member 1
CHD9	chromodomain helicase DNA binding protein 9
GNG4	guanine nucleotide binding protein (G protein), gamma 4
FBXL17	F-box and leucine-rich repeat protein 17

Supplementary Table 5

GO Biological Process	Gene_Symbol
apoptotic process	TNFRSF1A
biological adhesion	CYTH4, ALCAM
biological regulation	TNFRSF1A, PDK2, RARG, CHD9, IRF9, ZNF408, CYTH4, HIVEP2
cellular component organization or biogenesis	HSPA2, CHD9, LMNB1, BAZ2B
cell communication	IL2RA, PDK2, TESC, FFAR2, PHLDB1, SEMA4A, NRG2, TNFRSF1A, DTX1, GNG4
cell cycle	RBM39, MS4A6E
developmental process	DUSP4, RBM39, PHLDB1, SEMA4A, NRG2, TNFRSF1A, DTX1, CSRP2, ALCAM, LMNB1, PLXNA1
immune system process	IL2RA, PHLDB1, SEMA4A, TNFRSF1A, IRF9, CSRP2
localization	SNX32, DENND5A
metabolic process	GNG4, CHD9, RARG, DTX1, TRAP1, NAA35, RPL39,PADI2, RBM39, TESC, HSPA2, PDK2, DUSP4
multicellular organismal process	DUSP4, RBM39, SEMA4A
response to stimulus	IL2RA, HSPA2, PHLDB1, MS4A6E, TNFRSF1A, TRAP1, IRF9, DENND5A
protein acetylation	NAA35
DNA repair mRNA processing	WDR33
nucleobase-containing compound and cellular amino acid metabolic process	ALDH2
fatty acid metabolic process	ANXA11
No annottation	TRMT112, SMIM14, PPP1R18, MCMBP, MSL2, ZC3H4, ARPP19, PPP1R15B, DHTKD1, NDC1, AKNA, FBXL17, ARPC2

Supplementary Sequences

(NFκB ChIPed regions and binding sites)

>GM12878 (384bp) {Promoter regions:κB1 & κB2}

GTACATCATAATCAAACAAAGTAAACAGAAAAAAATTTAAACTTTGCTAAAAAAA
AAAAGCAGAAGCACTTGATCTTTAGGAAGGCACGCAGTTGCTTATTATGAATCAT
TTCTAGAGTCCGATGCATTTTCAAAGCCGGTTACAGTCATTACGAAGCACACCCT
TGTGAGGTAAGTGTATCATCACCTTTGGTTCATAAATAAAAAAGCTGAGACGCCG
AGCGATTAAGTCACTCGCCTAAGGAGAATGAGTCAACGTCAAGAGTCATAGTTGA
CCCGGCCTAAAGACTCCAGACCATCAGTCCAGGGCTTAGTCAGCGGGGCCCGGAG
TGGCTTCCCTGGCTGGCATCTGGACTTAGGCTATTTCCGTGCACGTAAAAGCGG

>GM12891_REGION1 (370bp) {No consensus or putative site}

GCCTCTATTGAGGGCAGCTGCAGTTGTAAAGGAAAAAAAATGGTCCTGAACATTT
AAAACTACACTGGTGTACATCATAATCAAACAAAGTAAACAGAAAAAAATTTAAA
CTTTGCTAAAAAAAAAAAGCAGAAGCACTTGATCTTTAGGAAGGCACGCAGTTGC
TTATTATGAATCATTTCTAGAGTCCGATGCATTTTCAAAGCCGGTTACAGTCATT
ACGAAGCACACCCTTGTGAGGTAAGTGTATCATCACCTTTGGTTCATAAATAAAA
AAGCTGAGACGCCGAGCGATTAAGTCACTCGCCTAAGGAGAATGAGTCAACGTCA
AGAGTCATAGTTGACCCGGCCTAAAGACTCCAGACCATCA

>GM12891_REGION2 (370bp) {No consensus or putative site}

GGGTATCACATTGTCCTCGCCCCTCCATGCTTGGGACTGGGCTGTCATGGAGGAG
GGAAGGGGGAGGAGCGAGGCCTACAGATCGCTGTAGCCGGTCGGCTTGCCGGCCG
GACCTGCTGCATTTCACACGCGCCCGTCTTCCATTCATTCATCCTTTCCGCGTAG
TGCGCACGCCTCAGAGAATGCGAATGGGTCTCAATCCCTCTGTTCTTCCGAGTCT
TTATCTCCTGGAAGATTTCTGAGACGCAGTTTATTGGAAATAGAAACTTTTAAAT
TTACTTTTCCATTTGACTTTTTTTTTCTCTTTATCTATGCTACAGACTTTCATTT
CAATTTAGGCACCATTTTAAACTTAACTGAAGCAAGCTGG

>GM15510 (404bp) {Intronic regions: κB-Int1 & κB-Int2}

TAAAAAAAAAAAAAAAAAAAAGGTGGGGGAGAGACTCCACTTCCCAGAAGCCTCT
CGTTACTCACGCAGCCGCAGTCTTGCGCAGGTGCCGCCAGGGCCAAACGGACATA
TCCGTCACGTGGCCAGAAGCTGGCCAATCCGGTTTGAATCTCATTTTTTTCCTCT
TACCCCCCCTTCTGGAGCGGTTGTGCGATCAGATCGATCTAAGATGGCGACTGTC
GAACCGGTGAGTATTGCCTTTGGCCCCCACCCCCACGGGTCCCCGCGCTCCGTCT
TCCTTCTGACTGGGGGACTCCGCGGGACGGCGTTCCCGGCGCGCACTGTACCCCT
TGCCGCCCCTTCCCCTTCATGTTGGACCTGACCTCCCGCGGACAAGTGGGGACGT
CCCGGAGGATGGCCGAGGC

Supplementary Sequences (Continued)

>GM18951 (350bp) {Intronic regions: κB-Int1 & κB-Int2}

TGTGCGATCAGATCGATCTAAGATGGCGACTGTCGAACCGGTGAGTATTGCCTTT
GGCCCCCACCCCCACGGGTCCCCGCGCTCCGTCTTCCTTCTGACTGGGGGACTCC
GCGGGACGGCGTTCCCGGCGCGCACTGTACCCCTTGCCGCCCCTTCCCCTTCATG
TTGGACCTGACCTCCCGCGGACAAGTGGGGACGTCCCGGAGGATGGCCGAGGCGG
CGTGTAGCGCACACTTTCTGGTGGGGGCCCGAGAACTGGGGGAGCGGTGTGGGGG
AGGGGCGGCGTGGCGCCTCCTGCGGAACCGCGAGGTGTCTACAGGGTGGAGGGTA
GCTGGGGTTCTGGGCTGGGT

Chapter 4: Discussion and Future Perspectives

Contribution: All the data presented in this chapter was generated by Fadi Hariri (100%)

4.1 EIF4E is a Direct NF-κB Transcriptional Target and Functions as an Amplifier of NF-κB Activity

My work has shown the EIF4E transcript and protein were inducible in an NF-κB-dependent manner in a B-cell lymphoma cell line, BJAB, following stimulation with the phorbol ester PMA reaching a maximum of 2-fold transcript induction (Chapter 2, Figure 1). Furthermore, EIF4E transcript and protein were also inducible in PMA stimulated primary peripheral blood mononuclear cells (PBMCs) reaching a max of 4-fold transcript induction (Chapter 2, Figure 4)[1]. This observed discrepancy in the intensity of *EIF4E* induction is inherent in the physiologic nature of the used systems. Primary human cells are quiescent with low basal rates of gene expression; accordingly, following stimulation with a growth-promoting agent (e.g. PMA), these cells proliferate and display a significant amplification in gene expression rates[2,3]. Furthermore, although PMA stimulation in BJAB cells promoted a 2-fold transcript induction, it resulted in a 7.2 fold increase in EIF4E protein levels suggesting the possible implication of other regulatory mechanisms under these conditions. The higher levels of the EIF4E protein are likely a result of an increase in translation efficiency of the EIF4E transcript in response to PMA stimulation. Accordingly, quantitative polysomal loading experiments could be performed to evaluate this hypothesis following PMA stimulation in the presence and absence of an NF-κB inhibitor to determine whether the observed effects are NF-κB specific.

NF-κB mediated regulation of *EIF4E* involves components of the canonical pathway as determined by transcription factor binding studies (EMSA and ChIP). NF-κB proteins are directly recruited to four evolutionary conserved κB sites in the *EIF4E* promoter (Chapter 2, Figure 2). Although EMSA studies demonstrated the formation of p65/c-Rel containing dimers on κB sites in the *EIF4E* promoter, supershift experiments with a p50 antibody revealed a shifted complex as well; however, no specific p50-containing band could be discerned (except in KM-H2 lysates in Chapter 2, Figure 5) leading us to believe that p50 may not be a core component. Given that EMSA experiments are performed in an *in vitro* context and thus do not take into consideration the chromatin status at the studied loci, it would not be unlikely that p50 protein is recruited to the *EIF4E* promoter. In fact, both RelA (p65) and c-Rel are known to heterodimerize with p50. ChIP experiments performed with an NF-κB1 (p50) antibody would be conclusive in determining whether this subunit is recruited to the

EIF4E promoter in response to a growth-promoting stimulus (e.g. PMA). Furthermore, our ChIP experiments demonstrated that RelA (p65) fold enrichment is 2.5 and 5 times more than c-Rel at the κB3 and κB4 sites respectively in the *EIF4E* promoter. This observation may likely be due to the immunoprecipitation efficiency of the antibodies used. It is important to note that the selectivity of our RelA antibody was confirmed through western blot experiments using total protein lysates from RelA null fibroblasts (Figure 3D). Thus our findings from ChIP experiments are reliable and specific. Finally, gene silencing studies could be performed to determine whether RelA (p65) recruitment to the κB sites was dependent or not on its ability to form a heterodimer with cRel or p50. Such experiments would involve knockdown of the p50 and c-Rel NF-κB subunits followed by ChIP experiments performed with a RelA (p65) antibody in response to PMA stimulation.

Interestingly, the recruitment of NF-κB proteins to these elements is lineage dependent. In BJAB cells reflecting a mature B-cell lineage, NF-κB factors are recruited to only κB3 and κB4 elements (Chapter 2, Figure 3) and not κB1 and κB2. On the other hand, NF-κB factors are recruited to all κB sites in the *EIF4E* promoter in the myeloid cell lines THP1 and KG1a (Chapter 2, Figure 6)[1]. Furthermore, the level of B-cell maturity may alter the recruitment patterns of NF-κB factors to κB sites. In fact, RelA ChIP-Seq data from ENCODE revealed NF-κB enrichment in 4/10 B-lymphoblast samples at regions containing κB1 and κB2 sites (Chapter 3, Figure 4C). Taken together, these data suggest that NF-κB accessibility to these elements in a B-cell lineage is also dependent on B-cell developmental stages.

Analysis of the *EIF4E* locus with MatInspector[4] and TFSearch[5] identified two additional evolutionary conserved NF-κB binding sites in the *EIF4E* intron (Chapter 3, Figure 4A). These elements were validated to bind NF-κB canonical proteins using *in vitro* gel shift assays (Figure 1). Furthermore, RelA ChIP-Seq data from ENCODE revealed NF-κB enrichment in intronic regions containing the identified sites (Chapter 3, Figure 4C). Interestingly, the NF-κB enrichment in the *EIF4E* intron suggests that these elements could be functional in driving *EIF4E* transcriptional control. The physiologic significance of these intronic regions remains a mystery. It would of interest to study the regulatory effects that these elements impart in response to various stimuli and in malignant tissues to further our understanding of the

complex mechanisms governing EIF4E transcriptional control. Importantly, NF-κB dependent regulation of target genes through intronic binding sites is not uncommon. In fact, NF-κB transcription factors have been shown to regulate the immunoglobulin κ-light chain[6] as well as the *FOS* gene[7] through direct recruitment to intronic elements. Finally, ChIP-Seq data analysis from ENCODE suggests that RelA (p65) could be indirectly recruited to the *EIF4E* promoter through Sp1, STAT or Cdx; however, further investigation is needed to validate this observation.

Furthermore, it would be interesting to determine the importance of each κB site (promoter and intron) in the transcriptional regulation of *EIF4E* and whether these elements function cooperatively under the investigated context. In fact, we have performed luciferase reporter assays (LRA) in HEK293 cells co-transfected with a plasmid harboring the human *EIF4E* promoter containing the identified κB elements together with plasmids carrying the different NF-κB transcription factors (e.g. RelA, c-Rel). Our findings revealed that the EIF4E reporter activity was upregulated following the introduction of RelA (p65) and c-Rel; however, this activity was diminished but not completely abrogated when all elements were mutated. Since only a fragment of the *EIF4E* promoter (~1KB) was analyzed, it is not truly a reflective template of the cellular context and thus LRAs do not constitute the ideal experiments to address this question. A better-suited alternative would involve the use of the RNA-guided Cas9 nuclease from the microbial clustered regularly interspaced short palindromic repeats system (CRIPSR) for genome editing[8]. Briefly, two guide RNAs that are complementary to regions flanking each κB binding site in the *EIF4E* regulatory region would be introduced together with a Cas9n nickase enzyme into the cell line of choice (e.g BJAB); this strategy would allow the deletion of the each κB element at a time (can also be multiplexed). Edited cells would then be stimulated (e.g. PMA) followed by the analysis of *EIF4E* gene expression as well as ChIP to evaluate the recruitment of NF-κB factors to the *EIF4E* regulatory regions. These experiments would highlight the requirement of each individual element in the regulation of *EIF4E* expression.

Finally, in addition to its role in *EIF4E* transcriptional regulation, NF-κB proteins can also modulate EIF4E activity indirectly by altering its subcellular localization[9]. Moreover, several

NF-κB target genes are in fact EIF4E mRNA export and/or translation targets (e.g. *MYC* and *CCND1*)[10-12] suggesting that EIF4E may act as an amplifier of NF-κB activity to drive proliferative gene expression (Figure 2). EIF4E was also shown to enhance translation of the *Nfkbi* gene encoding for the NF-κB inhibitor IκB-α[13], which is also an NF-κB target gene[14]. This NF-κB/EIF4E feedback permits a rapid proliferative response concomitant with a rapid shutdown upon signal termination by upregulating the NF-κB inhibitor IκB-α. Furthermore, the cyclin dependent kinase inhibitor p19ARF has been shown to bind the *EIF4E* promoter and repress transactivation[15]; p19ARF is in turn inactivated through NF-κB[16]. Given the pro-proliferative advantages conferred by *EIF4E*, these observations suggest a role for NF-κB in combating senescence via the alleviation of p19ARF dependent inhibition of *EIF4E* transcription. Collectively, these findings highlight a nexus between transcriptional and post-transcriptional gene expression networks to modulate cell proliferation that could be targeted in malignant tissue.

4.2 NF-κB as a Complex Regulator of *EIF4E* expression: Beyond Transcriptional Control

The coordinated regulation of *EIF4E* expression goes beyond the conventional transcriptional control model. In particular, the RNA regulon model emphasizes the role of ribonucleoprotein-driven regulation of transcripts through modulating transcript stability, export and/or translational efficiency[17]. For instance, elevated levels of the RNA binding protein HuR has been shown to enhance *EIF4E* transcript stability in the head and neck carcinoma FaDu cells but not in Detroit-551 cells with low HuR[18]. This suggests that different cell lineages may differentially express *EIF4E* through a mechanism that is not transcriptionally dependent and would involve post-transcriptional events.

Throughout the course of my PhD studies, I investigated the effect of several NF-κB activating agents on *EIF4E* expression including the tumor necrosis factor TNF-α and immunoglobulin M (IgM). Two systems were used, mouse embryonic fibroblasts (MEFs) with a genetic knockout for RelA (p65) as well as BJAB cells. Only the EIF4E protein was upregulated in wildype MEFs but not in the p65 -/- cells in response to TNF-α treatment.

Similarly, in BJAB cells, EIF4E protein was upregulated following TNF-α and IgM stimulation with minor changes in mRNA expression levels (Figure 3).

These findings suggest an NF-κB dependent post-transcriptional and/or post-translational control of EIF4E expression; however, further experiments are needed to investigate this hypothesis. Accordingly, cells would be treated with ActinomycinD or cycloheximide to block gene transcription[19] and mRNA translation[20] respectively. Cells will then be treated with NF-κB activating agents and total mRNA and protein would be extracted. EIF4E transcript and protein would then be assayed with quantitative realtime PCR and western blot respectively. The effect of NF-κB activating stimuli on EIF4E mRNA and protein stability would then be determined.

These preliminary findings constitute lead points for future investigation. Establishing an NF-κB role in the post-transcriptional and/ or post-translational control of eI4FE would suggest that NF-κB is a complex modulator of *EIF4E* expression that functions at all levels of genetic control to maintain elevated EIF4E levels under proliferative conditions.

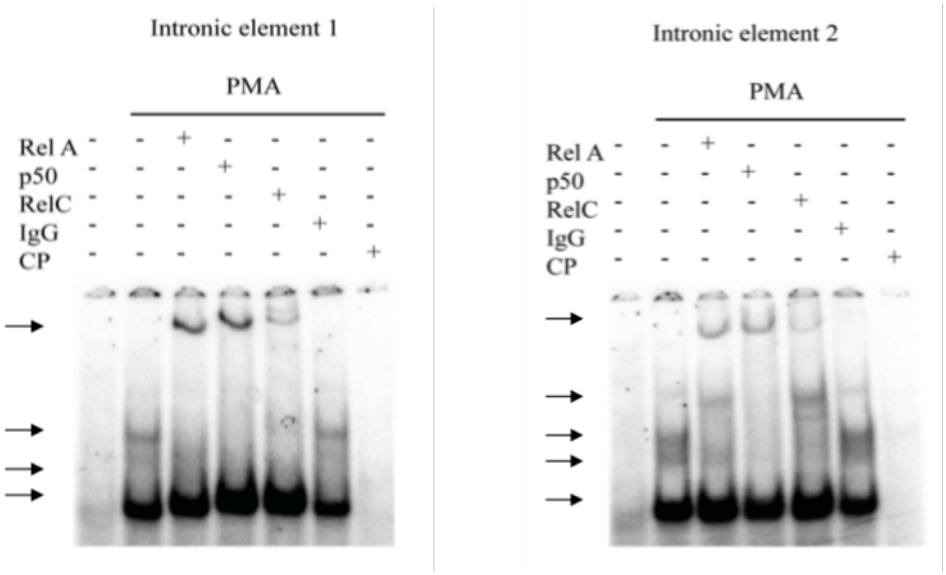


Figure 1. Two intronic κB elements are bound by NF-κB complexes. Electrophoretic mobility shift assay (EMSA) of nuclear extracts prepared from PMA-stimulated BJAB cells (90 min) using probes corresponding to the intronic κB sites. Supershift analysis using antibodies against p65, p50, cRel and immunoglobulin G (IgG) control as well as competition with consensus cold probe (CP) are indicated. Protein/DNA complexes are indicated by arrows. Supershifted complexes are indicated by an asterisk. These binding studies were performed at least three times using nuclear extracts prepared from cell lines and primary specimens.

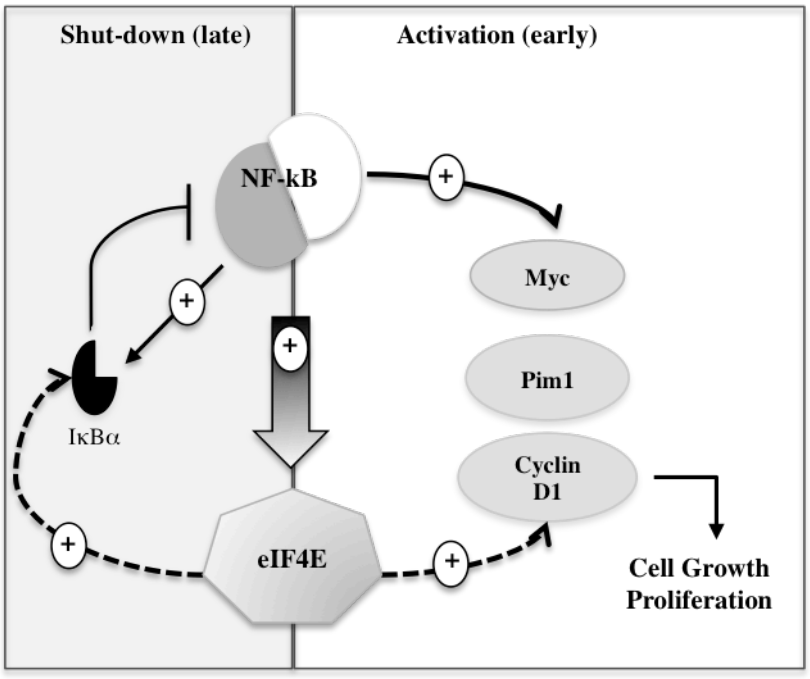


Figure 2. EIF4E is a downstream NF-κB target that acts as an amplifier of NF-κB activity to drive proliferative gene expression. EIF4E is upregulated in response to proliferative stimuli. EIF4E export and/or translation targets are also NF-κB transcription targets, thus EIF4E functions to enhance the NF-κB response. EIF4E was also shown to enhance translation of the IκB-α; this feedback allows a rapid proliferative response followed by a rapid shutdown upon signal termination by upregulating the NF-κB inhibitor IκB-α.

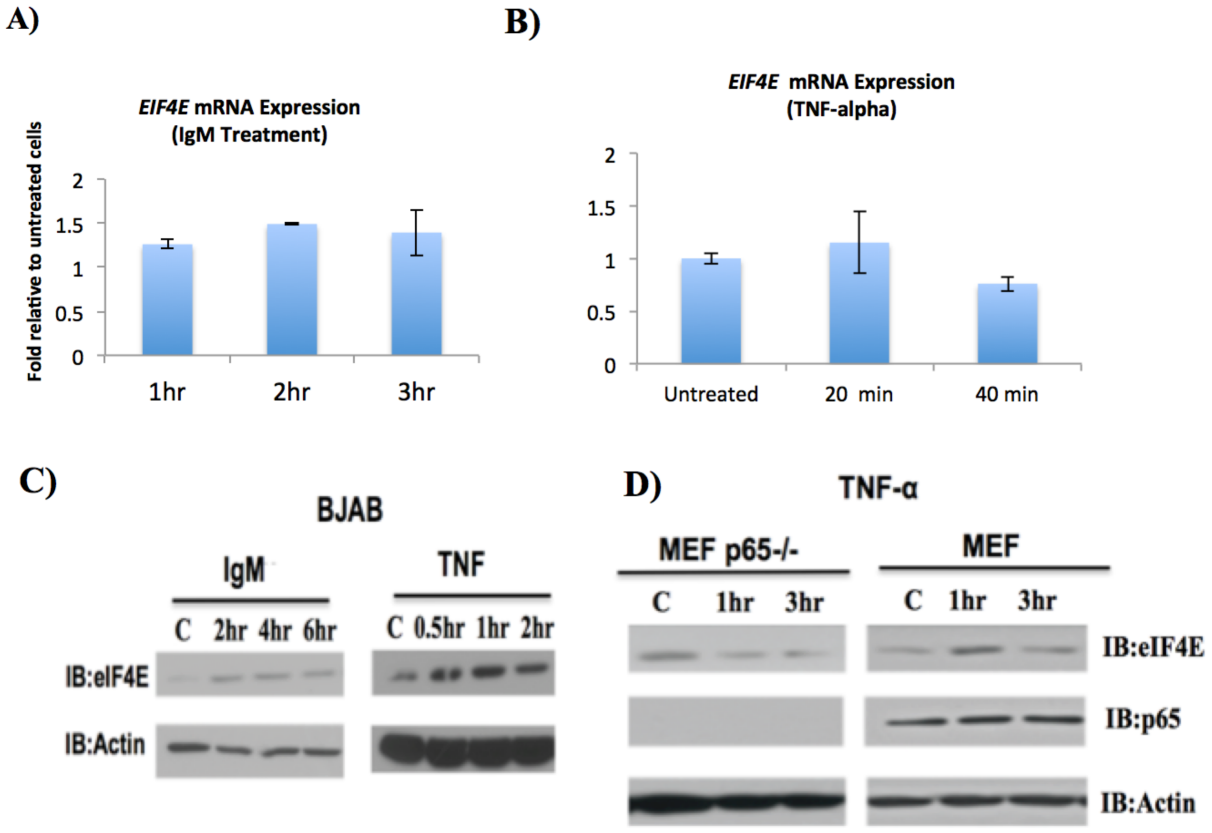


Figure 3. Regulation of EIF4E gene expression through NF-κB may involve post-transcriptional or post-translational events. (A, B) Minimal changes in EIF4E mRNA following IgM and TNF-α stimulation in BJAB cells. **(C)** EIF4E protein is upregulated in response to IgM and TNF-α in BJAB cells. **(D)** EIF4E protein is upregulated in wild type mouse embryonic fibroblasts (MEFs) but not in the NF-κB p65 -/- cells following TNF-α treatment. C refers to control untreated cells. IgM and TNF-α were performed twice.

4.3 *EIF4E* gene regulation is multifaceted and involves several transcription factors

The *EIF4E* promoter harbors a plethora of transcription factor binding sites including AP1, PU.1, SP1, GATA, OCT1, CREB, PAX5, E2F, STAT5, NFATC1 and many others[21]. Recently, C/EBP has also been shown to regulate *EIF4E* transcription[22] consistent with the fact that *EIF4E* transcriptional control is not solely dependent on Myc. Analysis of ENCODE ChIP-Seq datasets to discern the genomic binding patterns of the aforementioned transcription factors was performed. Accordingly, six new regulators were predicted to be recruited to the *EIF4E* regulatory regions. These factors are PU.1, PAX5, SP1, STAT, GATA2 and NFATC1 (Chapter 3, Figure 6). These factors are integral to myeloid and lymphoid development and functions whereby aberrations in these proteins have been linked to hematopoietic anomalies. GATA2 and SP1 were implicated in myeloid transformation[23,24], whereas PAX5 and PU.1 have been implicated in B-cell[25,26] as well as myeloid leukemias[27,28].

Interestingly, the phorbol ester PMA regulates the activity of STAT[29], GATA[30], NFAT[31], PAX[32] and PU.1[33]. BJAB cells co-treated with an NF-κB inhibitor failed to upregulate *EIF4E* in response to PMA (Chapter 2, Figure 1) suggesting that the observed *EIF4E* upregulation through PMA is strictly dependent on NF-κB and does not involve these factors; however, these proteins may still function as putative regulators of EIF4E under different conditions an in distinct cell contexts.

Investigating *EIF4E* transcriptional control through these factors constitutes future perspectives for follow-up studies further reasserting the complex and dynamic regulation of *EIF4E*. Accordingly, the expression levels as well as activity of these transcription factors will be evaluated in normal and malignant hematopoietic cells with western blot and gel shift experiments respectively. *EIF4E* expression would be evaluated following treatments with activating agents, as well as gain or loss of function in each of the aforementioned factors. The *in vivo* recruitment of these proteins to the *EIF4E* promoter would also be monitored with ChIP in normal and malignant cells. These studies would implicate the identified putative regulators in EIF4E transcriptional control.

4.4 Selective Recruitment of NF-κB Proteins to the *EIF4E* Promoter Underlies its Differential Regulation in AML

My gel shift and chromatin immunoprecipitation (ChIP) experiments performed in AML primary specimens have shown that the NF-κB factor RelA (p65) selectively binds to three elements in the *EIF4E* promoter in M4/M4 primary AML specimens but not in M2 AML or normal control (Chapter 2, Figure 7)[1]. Given that *EIF4E* is an NF-κB target, this novel finding was the first to explain the underlying preferential regulation of *EIF4E* in AML.

Recent research has identified nuclear NF-κB proteins known as non-Rel factors that selectively regulate NF-κB affinity to binding sites as well as its activity across gene promoters [Reviewed in Chapter 1.8.3]. The mechanism through which these non-Rel factors regulate NF-κB activity involves the crosstalk with other transcription factors, chromatin remodeling as well as NF-κB turnover. This body of research suggests that the selective recruitment of NF-κB complexes to the *EIF4E* promoter across different AML subtypes[1] is possibly due to nuclear non-Rel protein components.

Investigating the selective recruitment of NF-κB complexes to the *EIF4E* promoter through non-Rel nuclear proteins constitutes a future perspective for follow-up studies to delineate the full mechanism underlying the differential *EIF4E* regulation in AML subtypes. In order to identify these protein components underlying the differential NF-κB activity on the *EIF4E* promoter in AML, biotinylated *EIF4E* promoter fragments corresponding to the validated NF-κB elements would be prepared. These will be immobilized on streptavidin coated magnetic beads [34]. Nuclear extracts from the different AML subtypes (M1, M2, M4 and M5) as well as normal controls will then be prepared; these extracts will be incubated with the immobilized *EIF4E* promoter elements. The proteins bound to the immobilized fragments will be recovered and subsequently analyzed by mass spectrometry to determine their composite members. ChIP and gel shift EMSA experiments will also be carried out to validate the identified proteins on the *EIF4E* promoter in vivo and in vitro respectively. These studies would thus elucidate non-Rel protein complexes that are recruited the κB sites in the *EIF4E* promoter. Furthermore, genetic gain and loss of function experiments performed with the identified proteins would

highlight the role of these factors in regulating *EIF4E* expression concomitantly with NF-κB in myeloid cells.

The observed selective recognition of NF-κB complexes on the κB sites in the *EIF4E* promoter sparkled an interest in new NF-κB target gene prediction that may be regulated in a manner similar to that of *EIF4E* in AML through the same unique κB elements. Accordingly, NF-κB ChIP-Seq datasets were analyzed to retrieve genes with NF-κB enrichment on one or more κB sites that have been previously validated in the *EIF4E* promoter. 47 genes implicated in diverse biological processes were predicted as putative NF-κB targets (Chapter 3, Figure 5A). This finding offers a new directive for future investigations involving NF-κB research to validate new genes that may be selectively regulated through their unique κB sites. In fact, 8 of the identified genes show an expression signature similar to that of EIF4E in adverse prognosis AML samples suggesting that they may also be differentially regulated in AML (Chapter 3, Figure 5B). The validation of these genes as NF-κB targets that are differentially regulated in AML would serve, together with *EIF4E*, as prognostic risk gene signature for poor prognosis AML.

4.5 Elevated NF-κB (*RELA)* and *EIF4E* levels in AML Serve as Poor Risk Markers and Suggest a New approach of Targeting to Inhibit EIF4E Expression

NF-κB is constitutively activated in a plethora of cancers and this activity is largely due to gene amplification, mutations as well as rearrangements in elements of the pathway[35]. In AML, constitutive NF-κB activity is underlined by an aberration in the signaling cascade as well as an upregulation of *RELA* (p65) due to amplification in chromosome 11[36,37]. Analysis of a TCGA RNA-Seq dataset from AML patients suggested a correlation between elevated *RELA* and *EIF4E* expression in poor risk patient specimens (Chapter 3, Figure 2). Furthermore, elevated *RELA* and *EIF4E* mRNA levels predict worse survival outcomes in AML. These findings suggest that both *EIF4E* and *RELA* may serve as prognostic markers in AML. EIF4E targeting in AML has been successfully achieved with ribavirin[38,39].

My findings regarding NF-κB dependent *EIF4E* regulation in AML suggest a clinical utility in controlling the transcription of *EIF4E* with the use of NF-κB inhibitors, in addition to directly

inhibiting EIF4E activity with ribavirin. The most extensively used NF-κB targeting approach relies on blocking its activation at the level of nuclear entry. This strategy employed the use of IκB kinase (IKK) as well as proteasomal inhibitors and has been shown successful in some contexts. For instance, targeting NF-κB in acute myeloid leukemia (AML) using proteasomal inhibitors have shown to inhibit proliferation in CD34+ populations[37]. However, constitutive NF-κB activity is contributed for by multiple factors; the efficacy of these pathway inhibitors is largely determined by the genomic aberrations that resulted in an amplified NF-κB activity.

We have experienced this drawback with NF-κB targeting in several M4/M5 AML primary specimen as well as an M5 AML cell line, Thp1 in an attempt to block *EIF4E* transcription in the tested samples. However, only two samples out of 6 M4/M5 AML primary specimens and the M5 AML cell line responded to an NF-κB inhibitor, Bay 11-7082 that targets IκB kinase, despite the fact that the samples tested harbored constitutive NF-κB activity (Figure 4).

These findings highlight the importance of specific targeting of NF-κB transcription factors to circumvent this limitation. The identification of NF-κB small molecule inhibitors represents a future directive for NF-κB research that would allow for specific targeting of NF-κB transcription factors in a multitude of cancers where constitutive activity is endowed by more than one aberration in the pathway. Accordingly, an *in silico* screening approach combined with NMR to identify inhibitors of the DNA binding domains of NF-κB proteins p65, p50 and c-Rel would be followed. *In silico* screening approaches have been effective in identifying small protein inhibitors[40,41]. Thus the proposed strategy presents an effective tool for the identification of molecules to use as leads in drug discovery. The identified pharmacological candidates would then be investigated in AML primary specimen and a panel of cancer line lines for their capacity to inhibit NF-κB DNA binding properties and thus its activity.

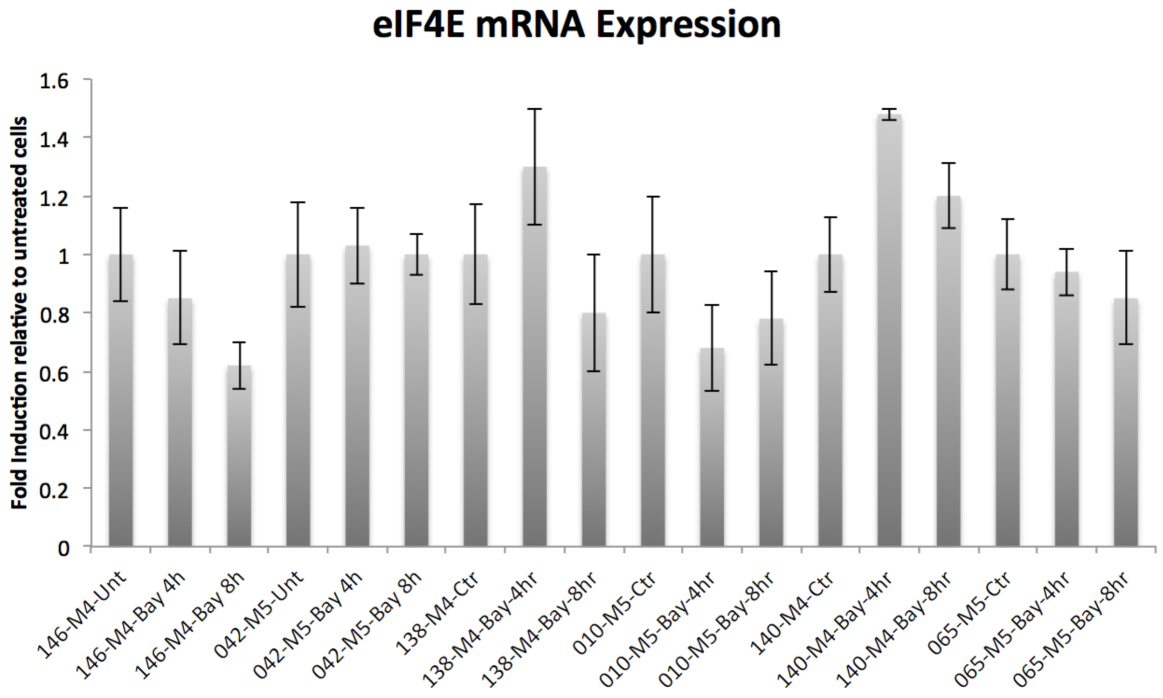


Figure 4. The NF-κB inhibitor Bay11-7082 results in a downregulation of *EIF4E* expression in 2 out of 6 AML primary specimens. *EIF4E* mRNA levels were measured by quantitative realtime PCR in primary M4/M5 AML specimens treated with Bay11-7082. Transcript levels were normalized to the histone 2B housekeeping gene. Result are shown as fold induction relative to untreated control samples. Bay refers to Bay 11-7082. Unt refers to untreated. Error bars represent standard deviations from three technical replicates.

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Conclusion

EIF4E is overexpressed in approximately 30% of cancers including M4 and M5 subtypes of acute myeloid leukemia (AML). *EIF4E* overexpression is correlated with oncogenic transformation, metastasis, tumor invasion and poor prognosis. Our group has demonstrated clinical improvement in poor prognosis AML patients through the inhibition of EIF4E with ribavirin in two clinical studies. This provided the first clinical evidence that EIF4E is a therapeutic target. *EIF4E* is regulated at multiple levels but not much is known about the transcriptional regulation of *EIF4E*. In fact, for the past 16 years, *EIF4E* transcriptional regulation has been thought of as Myc-centric. Expression of the NF-κB super repressor (IκB-SR) suppresses *EIF4E* transcript levels in CD34+ M4/M5 AML patient specimens. My findings show that *EIF4E* is a direct transcriptional target of NF-κB. Given that many EIF4E export and/or translation targets are also NF-κB targets, EIF4E thus acts as an amplifier of NF-κB activity.

NF-κB canonical complexes are directly recruited to conserved sites in the *EIF4E* promoter to direct transcription. Furthermore, our findings also suggest that NF-κB proteins can also be recruited to intronic elements at the *EIF4E* locus, although the physiological significance of this event remains to be investigated. The requirement of κB sites in the *EIF4E* promoter is dependent on cell lineage and developmental stage. Moreover, our unpublished observations suggest an NF-κB mediated post-transcriptional and/or post-translational regulation of *EIF4E*; however, further experimentation is required to validate this hypothesis.

Constitutive NF-κB activity and elevated *EIF4E* expression levels are observed in a plethora of cancers including acute myeloid leukemia. We have shown that selective NF-κB activity on the *EIF4E* promoter represents an underlying factor in determining *EIF4E*'s differential expression in M4/M5 AML subtypes but not in the M1/M2 tested samples. Non-Rel nuclear proteins determine the selectivity of NF-κB factors; accordingly, specific non-Rel proteins may be dysregulated in M4/M5 AML owing for the observed preferential *EIF4E* expression. Identifying the non-Rel components that dictate NF-κB recruitment and the preferential upregulation of *EIF4E* in AML constitutes a novel future research direction to further our understanding of *EIF4E* dysregulation in AML and the potential means to target it. Furthermore, the unique κB sites in the *EIF4E* promoter are also present and functionally

recruit the NF-κB p65 transcription regulator in 47 target genes. These genes present novel putative NF-κB targets for prospective validation and investigation.

Finally, elevated *EIF4E* and NF-κB *RELA* expression is correlated with adverse AML prognosis and predicts a poor survival trend thus may serve as clinical prognostic markers in this hematological anomaly. These findings suggest a potential clinical utility in targeting *EIF4E* expression in combination with ribavirin that targets EIF4E activity. Current NF-κB pathway inhibitors are not fully capable of inhibiting NF-κB transcription factors and their activity as their efficacy is determined by the genomic anomaly associated with the pathway's aberration. My preliminary data in AML primary specimens treated with the NF-κB inhibitor Bay11-7082 reinforces this notion. Thus investigating specific inhibitors of NF-κB transcription factors can be considered for future studies providing a more precise approach to target this pathway in cancer.

This study was the first to explore the mechanistic link between NF-κB and EIF4E activity. Understanding this level of regulation within the context of patient specimens is important for the development of novel therapeutic strategies, such as combining ribavirin treatment with specific NF-κB inhibitors in leukemia patients, in an attempt to more effectively target EIF4E and NF-κB networks as well as alleviate symptoms of poor prognosis AML patients to further improve the efficacy of patient care.

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