Investigating the effect of translation inhibition on cellular mRNA levels

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DEDICATION

I dedicate this work to my loving family and friends. They inspire me every day and I am lucky to have their constant, unwavering support.

ABSTRACT

Eukaryotic translation is an intricate and highly regulated process by which proteins are synthesized. Dysregulation of translation can have profound consequences, allowing cancer cells to usurp regulatory mechanisms, leading to the phenotypic hallmarks of cancer. The majority of translational control is exerted at the level of initiation, making it a therapeutic target in certain cancer settings. Rocaglates are a family of compounds that target translation initiation by selectively inhibiting the DEAD-box RNA helicase, eukaryotic initiation factor 4A (eIF4A). A well-characterized rocaglate, silvestrol, has potent anti-tumour activity *in vivo* and inhibits tumour growth in several xenograft models. In some instances, inhibition of translation elongation by cycloheximide (CHX) has been reported to strongly induce cellular mRNA levels, but the exact mechanism by which this takes place is not well understood. We investigated the effect of translation inhibition by silvestrol on mRNA levels in multiple myeloma cells and observed a dramatic increase 2 h after treatment. We characterized this response and identified that silvestrol (and other inhibitors of translation) cause a change in mRNA half-life. Next, we performed RNA-seq on cells treated with silvestrol and cycloheximide (CHX) in order to uncover the *cis* and *trans* acting factors that regulate mRNA stability in response to treatment. Through a transcriptome-wide approach, we identify potential elements within the 5' UTR, 3' UTR and CDS that may play a role in stabilizing transcripts after inhibition of protein synthesis. Our data provides a promising start in developing a systematic approach to identify regulatory elements in the mRNA structure that modulate stability.

RÉSUMÉ

La synthèse protéique chez les eukaryotes est un processus complexe et hautement contrôlé. La perte de cette régulation peut entraîner des conséquences profondes, allant même jusqu'à permettre aux cellules cancéreuses d'usurper des mécanismes de contrôle qui normalement bloqueraient sa progression. La grande majorité de la régulation de la traduction s'exerce lors de l'initiation, ce qui en fait une cible thérapeutique particulièrement attrayante en oncologie. Les composés de la famille des rocaglates ciblent l'initiation de la traduction en inhibant sélectivement l'hélicase à ARN à boîte DEAD, le facteur d'initiation eucaryote 4A (eIF4A). L'étude d'un membre spécifique de cette famille, le silvestrol, a démontré qu'il possède une activité anti-tumorale in vivo et qu'il inhibe la croissance des cellules cancéreuses dans plusieurs modèles de souris dont les xénogreffes. Il a été rapporté que l'inhibition de l'élongation de la traduction par la cycloheximide (CHX) peut induire fortement les niveaux d'ARNm cellulaires, mais le mécanisme exact par lequel cela se produit n'est pas bien caractérisé. Nos travaux, ont pour objectif d'étudier l'effet de l'inhibition de la traduction par le silvestrol sur les niveaux d'ARNm dans les cellules de myélome multiple. Nous avons observé une augmentation marquée de plusieurs ARNm cellulaires deux heures suivant le traitement des cellules. Cette conséquence s'explique par le fait que le silvestrol, ainsi que d'autres inhibiteurs de la traduction, provoquent une modification de la demi-vie de l'ARNm. Ensuite, nous avons effectué des expériences de séquençage d'ARN sur des cellules traitées avec du silvestrol et du CHX afin de découvrir les facteurs cellulaires qui peuvent réguler cette stabilité en réponse au traitement. Notre approche à l'échelle du transcriptome, nous a permis d'identifier des éléments potentiels dans les régions 5 'UTR, 3' UTR et codantes des ARNm qui peuvent jouer un rôle dans leur stabilisation suite à l'inhibition de la synthèse des protéines. Nos données fournissent des informations initiales prometteuses dans le développement d'une approche systématique pour identifier les éléments de régulation dans la structure de l'ARNm qui modulent leur stabilité.

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ABBREVIATIONS

4E-BP	eIF4E-Binding Protein
ABCB1	ATP-Binding Cassette Subfamily B Member 1
ARE	AU-rich element
ARE-BP	AU-rich element binding protein
ATL	Adult T-cell Leukemia
ATP	Adenosine Triphosphate
BGSS	Bovine Growth Supplemented Serum
CDC	Cap Binding Complex
cDNA	Complementary DNA
CHX	Cycloheximide
CDS	Coding Sequence
CLL	Chronic Lymphocytic Leukemia
CRD	Coding Region Instability Determinant
CRD-BP	CRD-binding protein
CTD	C-Terminal Domain
DDX	Dead-Box Helicase Protein
DMDA-Pat A	desmethyl-desamino Pateamine A
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
eIF	Eukaryotic Initiation Factor
eIF4A$_f$	Free eIF4A
EJC	Exon Junction Complex
FBS	Fetal Bovine Serum
FDR	False Discovery Rate
GO	Gene Ontology
GPS	Genetic Perturbation Service
GMP	Guanosine Monophosphate
GTP	Guanosine Triphosphate

HEAT	Huntingtin, Elongation factor 3 (EF3), protein phosphatase 2A (PP2A), and the yeast kinase TOR1
HHT	Homoharringtonine
IRES	Internal Ribosome Entry Site
ITAF	IRES *trans*-acting factors
lncRNA	Long Non Coding RNA
m^7GDP	7-methylguanosine diphosphate
Met-$tRNA_i^{Met}$	Methionine Initiator Transfer Ribonucleic Acid
mRNA	Messenger Ribonucleic Acid
mTOR	Mammalian Target of Rapamycin
NSCLC	Non-Small Cell Lung Cancer
nts	Nucleotides
NMD	Nonsense-Mediated Decay
ORF	Open Reading Frame
PABP	Poly(A)-Binding Protein
Pat A	Pateamine A
PCR	Polymerase Chain Reaction
PDA	Pancreatic Ductal Adenocarcinoma
PDCD4	Programmed Cell Death Protein 4
PEI	Polyethylenimine
P-gp	P-glycoprotein
PIC	Pre-Initiation Complex
RNAi	RNA interference
RNA-seq	RNA sequencing
RocA	Rocaglamide A
rRNA	Ribosomal RNA
RT-qPCR	Reverse Trancsription Quantitative Polymerase Chain Reaction
SEM	Standard Error of the Mean
smFISH	Single Molecule Fluorescence In Situ Hybridization
TC	Ternary Complex

TCA	Trichloroacetic Acid
UTR	Untranslated Region
uORF	Upstream Open Reading Frame

1. INTRODUCTION

1.1 mRNA Structure and Function

Eukaryotic precursor-messenger RNA (mRNA) undergoes extensive processing before it is translated into protein. 5' capping, splicing and 3' end processing largely affect transcript fate and expression (Fig. 1). The resultant mature mRNA is exported from the nucleus to the cytoplasm where it is translated by ribosomes. Post-transcriptional mRNA processing contributes to its recognition by ribosomes as well as its stability. Apart from regulatory elements within the untranslated regions (UTRs), mRNAs also contain sequences within their coding regions that mediate their translational control and stability[1].

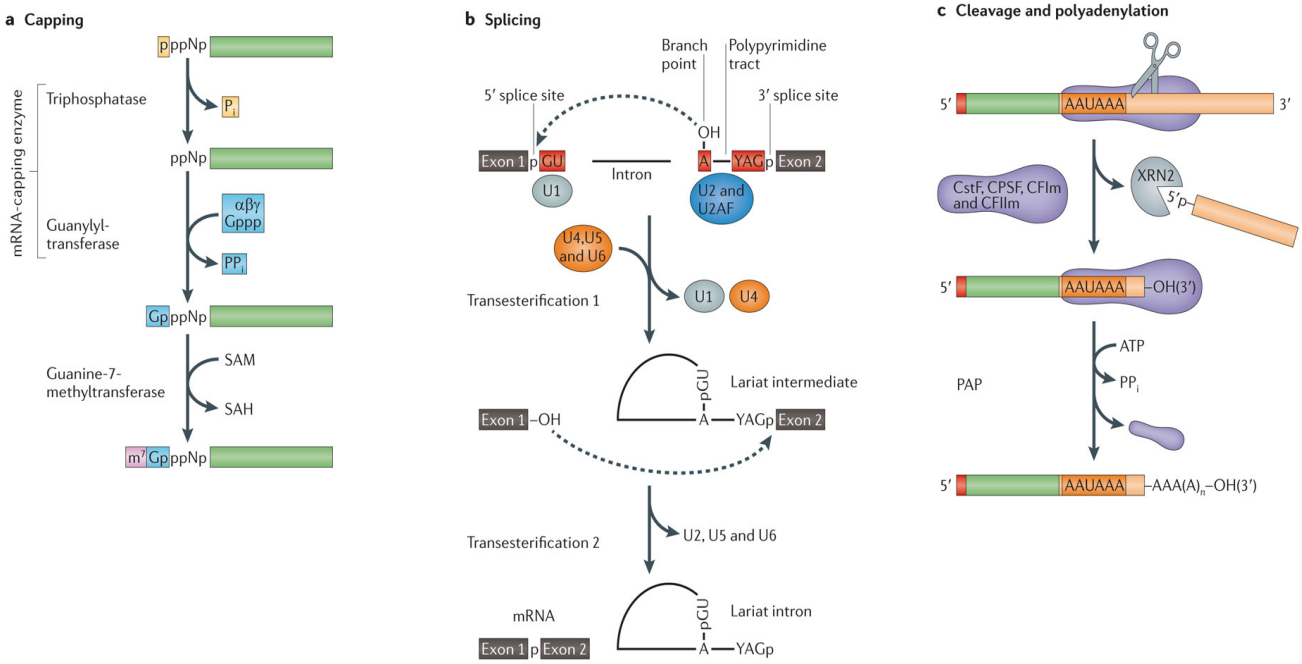


Figure 1. Major co-transcriptional processing events. (a) The RNA is shown in green; both GTP and the added guanosine cap (Gp) are shown in blue. The mRNA-capping enzyme has both triphosphatase and guanylyl-transferase activities that remove the γ-phosphate of the nascent transcript and transfer GMP from the GTP donor. The methyl donor *S*-adenosyl-l-methionine (SAM) is converted to S-adenosyl-l-homocysteine (SAH), which results in the 7-methylguanosine

cap (shown in pink). (**b)** Splicing removes an intron as a lariat and ligates the flanking exons together Spliceosomal small nuclear ribonucleoprotein particles (snRNPs) and U2 auxiliary factor (U2AF) are shown, but many spliceosomal proteins are omitted for clarity. (**c)** The 3′ ends of mRNAs are formed by coupled cleavage and polyadenylation. Cleavage of mammalian pre-mRNAs occurs ~25 bases downstream of a consensus sequence (AAUAAA) and is carried out by a multi-subunit complex (shown in purple). Poly(A) polymerase (PAP) adds the poly(A) tail. The 5′-to-3′ RNA exonuclease 2 (XRN2) degrades RNA downstream of the cleavage site and facilitates transcription termination. Adapted by permission from Nature Springer: **[**Nature Reviews Molecular Biology], *Coupling mRNA processing with transcription in time and space,* Bentley *et al.* Copyright © 2017.

1.1.1 The mRNA Cap Structure

The mRNA cap is a methylated modification of the 5' end of RNA Pol-II transcribed RNAs. The cap structure protects mRNAs from 5'-3' exonuclease mediated degradation, recruits protein complexes involved in RNA processing, export, translation and stability. It is also a cellular mark to identify the RNA as 'self' to avoid invoking an innate immune response[3]. mRNA cap formation occurs during transcription and protects pre-mRNA from decay. The nuclear cap binding complex (CBC) binds to the mRNA cap and allows recruitment of proteins which play a role in post-transcriptional modifications such as splicing and polyadenylation, subsequently allowing export into the cytoplasm. Eukaryotic initiation factor 4F (eIF4F) complex associates with the cap structure by direct bind of the eIF4E subunit to the cap, allowing recruitment of the 43S pre-initiation complex (PIC) and initiation of translation. The mature mRNA cap continues to protect the RNA from 5′-3′ exonucleases until it is removed by decapping enzymes[3].

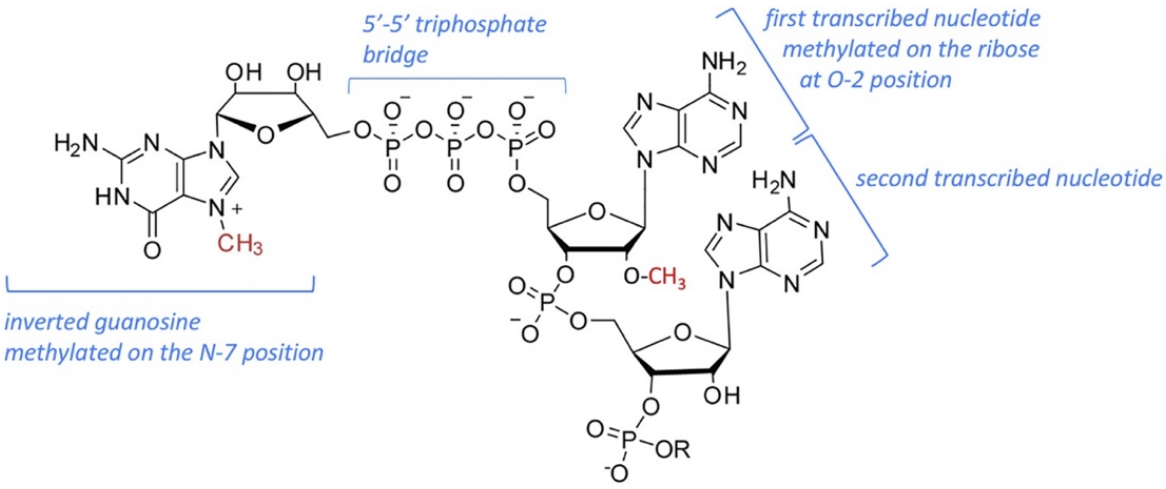


Figure 2. Structure of the mRNA cap. Figure obtained from Galloway *et al.* (2019) (CC BY).

1.1.2 The 5' Untranslated Region (UTR)

RNA molecules are known for their intricate folding which can contribute to a multitude of regulatory elements controlling gene expression. The 5' UTR is a region of the mRNA located between the transcription start site and the first translation initiation codon. The 5' UTR may regulate cap-dependent translation initiation through helicase mediated unwinding of RNA structure and higher-order RNA interactions[4]. Additionally, 5' UTR structures are known to regulate cap-independent translation initiation through internal ribosome entry sites (IRESs) and mRNA modifications. During eukaryotic evolution, the 5′ UTR has maintained a median length of approximately 53–218 nucleotides[12]. Prediction of 5' UTR structure is usually based on parameters such as high GC content and a negative folding free energy (ΔG). Canonical translation initiation requires scanning of the 5' leader region by the 43S PIC until it reaches the start codon. High GC content and more secondary structure has been thought to cause inefficient ribosome scanning and lower rates of initiation[30]. Interestingly, some human mRNAs have extremely short 5' UTRs (about 12 nucleotides) with a specific motif ($^{5'}$SAASATGGCGGC$^{3'}$, in which S is C or G) known as translation initiator of short 5' UTR (TISU) which undergo scanning-free initiation[165]. Genes that contain TISU elements are typically enriched in mRNAs that encode proteins involved in basic cellular functions such as RNA synthesis, protein metabolism and respiration[165]. There are also *cis*-acting regulatory elements and structures in the 5' UTR that

influence translation (Fig. 3). These include RNA G-quadruplexes, which are four-stranded G-quadruplex (G4) structures that form through self-recognition of guanines into stacked tetrads. Most examples of RG4s in 5′ UTRs are linked to translation repression in *cis* presumably by preventing the 43S pre-initiation complex from binding to mRNA or by slowing down scanning[166]. Additionally, short AUG-initiated upstream ORFS (uORF) located in the 5' UTR of eukaryotic mRNAs are typically translated by a cap-dependent mechanism and in many instances repress translation of any major downstream ORF present[12]. RNAs are able to form higher-order interactions, complexes with trans-acting and long non-coding RNAs (lncRNAs) for post-transcriptional control. For example, in the mouse ubiquitin carboxyl terminal hydrolase L1 (*Uchl1*) mRNA, the antisense lncRNA *Uchl1AS* undergoes partial base pairing with the *Uchl1* mRNA and a repeat region of the lncRNA increases ribosome binding and translation by an unknown mechanism[167].

There is evidence that suggests secondary and tertiary structures at the 5' end may physically occlude 5' caps and lead to weaker eIF4E-cap interactions[5]. Furthermore, initiation factor eIF3 can directly bind to modified 5' UTRs in target mRNAs to allow selective internal initiation. In summary, mRNAs may be differentially translated as a result of 5' UTR structure variation[5, 6, 7].

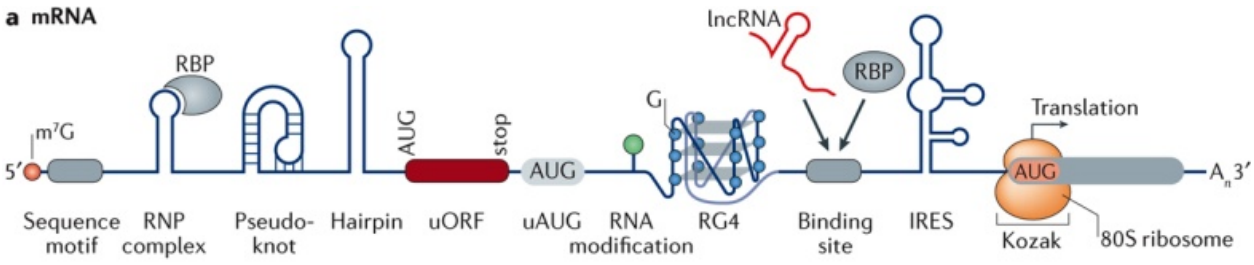


Figure 3. *Cis*-acting elements in the 5' UTR mediate translation. Structures in the 5' UTR such as pseudoknots, hairpins, RNA G-quadruplexes (RG4s), upstream open reading frames (uORFs) and upstream start codons (uAUGs), play an inhibitory role in translation. IRESs mediate cap-independent translation. RNA modifications, RNA-binding proteins (RBPs) and long non-coding RNAs (lncRNAs) that interact with sequences in the RNA may form ribonucleoprotein (RNP) complexes that can also regulate translation initiation and mRNA stability[4]. Adapted by permission

1.1.3 The Coding Region

The resultant mature mRNA also consists of a coding sequence (CDS), which is the sequence translated into protein. It is characterized by an initiation codon, usually an AUG, where translation commences and ends with a stop codon (TAA/TAG/TGA within the same frame). The sequence directly preceding the AUG codon is highly important for translation initiation site recognition and is termed the Kozak consensus sequence[8]. The Kozak consensus sequence for initiation of translation in vertebrates is $^{5'}$(GCC)GCCRCCATGG$^{3'}$, where R is a purine (A or G)[8]. There is evidence that suggests some nucleotides of the Kozak sequence are more important than others, particularly the − 3 and the + 4 nucleotides (numbering is relative to the A of the AUG codon)[8]. In addition to the 5' UTR, the CDS of certain transcripts may also contribute to translational efficiency and mRNA stability. It has been shown that a 249-nucleotide coding region instability determinant (CRD) destabilizes *c-myc* by causing ribosome pausing, which leaves a ribosome-deficient region downstream of the pause-site; exposing it to endonucleolytic attack[9]. RNA-binding sites for proteins within the coding region can also contribute to translational repression[116].

1.1.4 The 3' UTR and Poly(A) tail

The 3' UTR of a transcript is downstream of the translation stop codon and contains *cis*-acting regulatory elements similar to the 5' leader region. A well-characterized element is the AU-rich element (ARE) which is a platform for RNA-binding proteins such as the HuR and AUF1 proteins which contribute to mRNA stability and mRNP-complex formation[10]. Additionally, there exists a consensus sequence in the 3' UTR which directs polyadenylation of mRNA by the enzyme polyadenylate polymerase (PAP). With a few exceptions, all RNA-pol II transcripts are polyadenylated at the 3' end with anywhere from 50 - 200 adenosine nucleotides added during nuclear pre-mRNA processing. Poly(A) tail length is proposed to mediate mRNA stability and

poly(A) tail shortening serves as a timer for mRNA turnover[151]. This modification also serves to protect the 3' end from 3'-5' exonuclease directed degradation. Poly(A)-binding proteins (PABPs) may bind to the poly(A) tail while simultaneously interacting with eIF4G at the 5' end of the transcript, allowing circularization of the mRNA[11, 12]. This mechanism is postulated to promote translation initiation by bringing terminating ribosomes in close proximity to the 5' end to re-initiate[11, 12].

1.2 Eukaryotic mRNA Translation

Protein synthesis is a fundamental process whereby amino acid sequence is determined by decoding triplet-codons. In both prokaryotes and eukaryotes, this is done by complex, macromolecular machines known as ribosomes. Ribosomes consist of two subunits- either the 30S and 50S in prokaryotes or the 40S and 60S in eukaryotes. The focus of this work will strictly consider eukaryotic translation, unless stated otherwise. Translation has four main stages: initiation, elongation, termination and ribosome recycling. Each of the four stages is complex and regulated, and dysregulation can lead to disease. For example, many cancers have an increased dependence on translation upregulation in order to maintain their rapid growth rates. It is therefore crucial to tightly regulate protein synthesis to maintain physiological equilibrium. Translation initiation is the rate-limiting step and the central target for translation regulation. In eukaryotes, translation can occur in a cap-dependent or cap-independent fashion, with the former being most typical in cells. Numerous initiation factor proteins participate in this process, and the work herein will focus on this initiation step[13].

1.2.1 Cap dependent translation initiation

The very first step of cap dependent translation initiation is the binding of eIF4E, a small cap-binding protein and member of the eIF4F complex (eIF4E, eIF4A and eIF4G), to the 5' cap structure of the mRNA. Interactions between eIF4G, a large scaffolding protein, and the mRNA stabilize the eIF4F complex onto the 5' end. Next, there is recruitment of a ternary complex (TC) which consists of the eukaryotic initiation factor (eIF) 2, GTP and a Met-initiator transfer RNA

(Met-tRNA$_i^{Met}$)[14]. This complex joins the small 40S ribosomal subunit along with other essential initiator factors 1, 1A, 3 and 5, leading to the formation of a 43S pre-initiation complex (PIC). In the presence of ATP, secondary structures in the 5' UTR are resolved by the helicase eIF4A with the help of RNA chaperones eIF4B and 4H, creating an accessible landing platform for 43S PIC binding. Once the 43S PIC binds the leader region, GTP is hydrolyzed, but not yet released. The 43S PIC scans the leader region in the 5'→3' direction until it encounters an AUG start codon, and eIF1, eIF2-GDP-eIF5 complex and P_i are released. Next, eIF5B-GTP joins and recruits the large 60S ribosomal subunit, GTP is hydrolyzed and the eIF5B-GDP complex along with eIF4A and P_i are released. This results in an elongation-competent 80S ribosome (Figure 4)[13, 14].

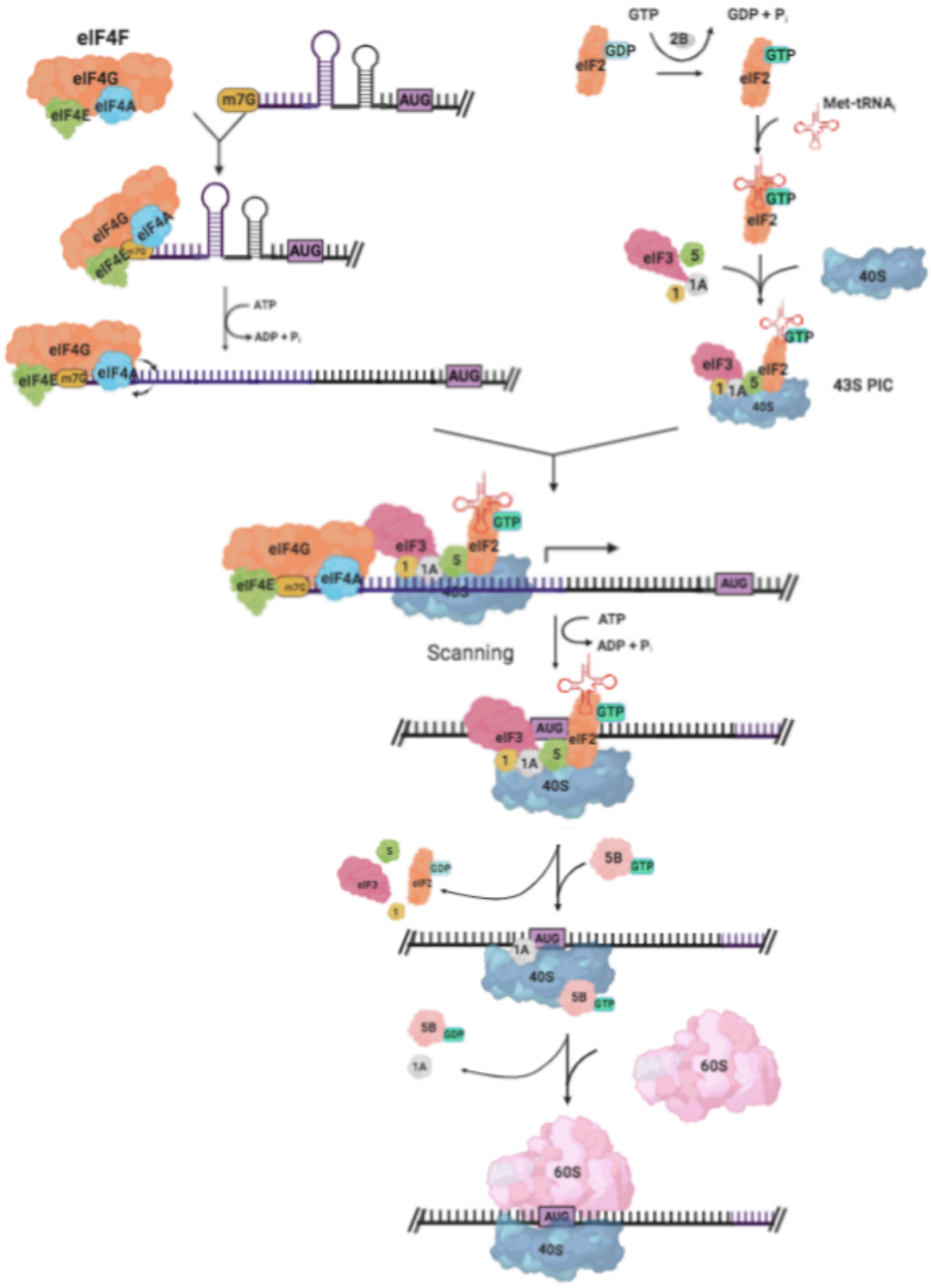


Figure 4. Overview of cap-dependent translation initiation. See text for details. Please note that PABP:4G interaction is not shown.

1.2.2 Cap independent translation initiation

An alternative mechanism for the recruitment of 40S ribosomes exists whereby binding of eIF4E to the 5' cap is entirely bypassed. This mechanism can occur via highly structured regulatory elements in the 5' UTR and is characterized as an internal ribosome entry site (IRES). This alternative mode of initiation was first discovered in polioviruses[15], encephalomyocarditis virus (EMCV)[168], and in Hepatitis C virus[18]. In these viruses, translation initiation occurs through an IRES which may vary in length and sequence but share the ability to internally recruit ribosomes. Additionally, it has been reported than 10% of cellular mRNAs also use IRESes for initiation[169]. These cellular transcripts are involved in stress-related pathways such as apoptosis, environmental stress response and endoplasmic reticulum stress. IRESes in these mRNAs potentially serve as a way for cells to maintain translation of a subset of mRNAs when global translation is off[16, 17, 18]. To date, four types of IRESes have been identified and they differ in their phylogeny, nucleotide sequence and level of dependence on initiation factors. Type I IRESes are found in enteroviruses, coxsackievirus B3, and rhinoviruses[16]. All initiation factors are required for Type I IRES activity except for eIF4E. Type II IRESes are found in cardioviruses and aphthoviruses, they form complex secondary structures, with structural domains contributing to IRES functionality and eIF4G binding[16]. Type III IRESes, such as those found in Hepatitis C virus are able to bind directly to the 43S PIC through interactions involving ribosomal protein S5 and eIF3, and independent of eIF4F, eIF1, eIF1A and eIF4B. Type IV IRESes require no initiation factors[19, 20, 21].

1.2.3 The eIF4F complex: eIF4E, eIF4G, eIF4A

eIF4F is a multi-protein complex comprised of eIF4E, a cap-binding protein, eIF4G, a large scaffolding protein and eIF4A, an ATP-dependent DEAD box RNA helicase. eIF4F is primarily responsible for recruiting ribosomes to the 5'UTR.

eIF4E

eIF4E, the small cap-binding subunit of the eIF4F complex, is a 24 kDa protein that is conserved and essential across eukaryotes[22]. A 2.2 Å crystal structure of eIF4E bound to 7-methylguanosine diphosphate (m^7GDP) revealed that the protein resembles a 'cupped-hand' and consists of 8-stranded antiparallel beta sheets, with 3 long alpha helices[23]. Structural analysis has additionally shown that the m^7G base is sandwiched between two conserved tryptophans within eIF4E and that the positive charge of the N7-methyl residue contributes to binding energy by creating cation-π interactions with tryptophan residues[24, 25]. The binding of eIF4G to eIF4E in the eIF4F complex plays a role in the recruitment of eIF4A to the 5' UTR. eIF4G is able to bind eIF4E on the dorsal surface. Importantly, it has been shown that eIF4E's interaction with eIF4G increases the affinity of eIF4E for the cap, promoting translation[26]. eIF4E is the rate-limiting component of translation initiation, and therefore, in many instances this step determines the translation efficiency of mRNAs. For this reason, eIF4E levels are highly regulated[36]. Since levels of eIF4E under normal conditions are low, mRNAs must compete for it in order to be translated. mRNAs with longer, more structured 5' UTRs are 'weak mRNAs' because they are kinetically less favoured in terms of binding and recruiting eIF4F [32]. For example, *c-myc* has a long and highly structured 5' UTR and is highly sensitive to changes in eIF4E and as a whole, eIF4F levels. Interestingly, c-Myc is a transcription factor that regulates eIF4E mRNA synthesis and this relationship establishes a strong positive feed-forward loop between eIF4E and c-Myc[37].

eIF4E is regulated by three eIF4E binding proteins (4E-BP) 1, 2 and 3. When hypophosphorylated, 4E-BPs bind to eIF4E in a competitive manner with eIF4G thus blocking its binding to the latter and repressing translation. mTORC1 activation which is mediated by the PI3K/Akt pathway, leads to phosphorylation of 4E-BPs[39, 40], and this releases 4E-BPs from eIF4E enabling eIF4E to bind to eIF4G – generating more eIF4F complex and stimulating initiation of translation[28, 30, 31] (Figure 5).

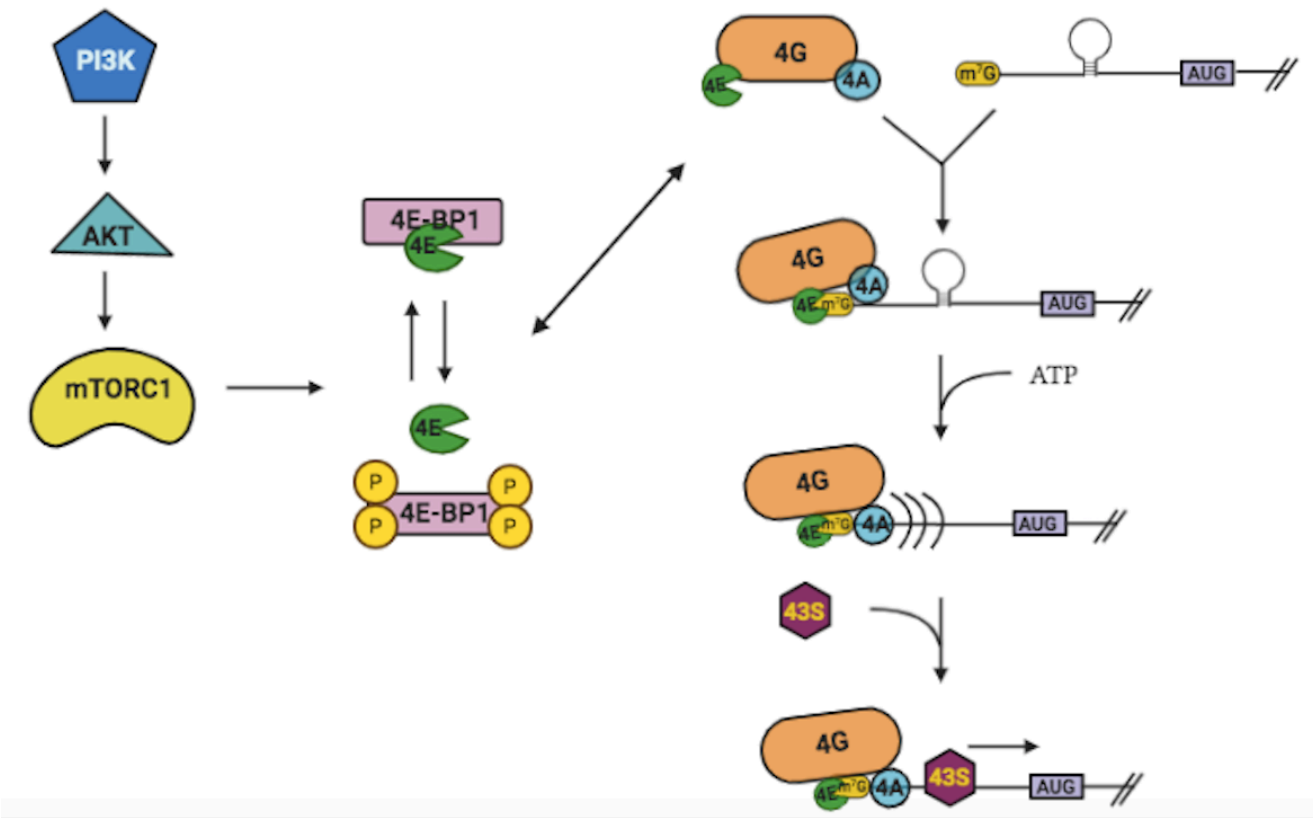


Figure 5. Assembly of the eIF4F complex and its role in translation initiation. eIF4F-dependent translation initiation is regulated by mTOR complex 1 (mTORC1). Sequestration of eIF4E by 4E-BP1 leads to a decrease in eIF4F levels. 4E-BP1 is a direct substrate for mTORC1 and its phosphorylation causes it to dissociate from eIF4E, allowing eIF4E to assemble into the eIF4F complex. Binding of eIF4F to mRNA cap structures (via eIF4E) allows eIF4A to resolve local secondary structure in an ATP-dependent fashion and facilitates ribosome recruitment [40, 41]. The interactions between eIF4B and eIF4H are not shown.

eIF4G

eIF4G is a large scaffolding protein that mediates interactions between eIF4E, eIF4A, eIF3, and the mRNA[13]. In mammals, there are three related eIF4G proteins: eIF4G1 (aka eIF4GI), eIF4G2 (aka DAP5/p97/NAT1/eIF4GIII), and eIF4G3 (aka eIF4GII), with eIF4G1 and eIF4G3 sharing about 46% sequence similarity at the amino acid level. eIF4G1 and eIF4G3 can be divided into three main regions (N-terminal domain [NTD], middle [MIF4G], and C-terminal domain [CTD])[41]. The NTD is capable of binding to PABP and eIF4E, postulated to allow circularization

of mRNA by placing the cap structure and the poly(A) tail in close proximity to one another[42, 43]. The middle domain of eIF4G contains a HEAT domain (HEAT-1), consisting of five HEAT repeats, along with two other HEAT domains (HEAT-2 and -3) in the CTD. HEAT domains participate in eIF4A binding. Additionally, MAP-kinase interacting kinase (MNK) 1 and 2 bind eIF4G via a HEAT domain in eIF4G's CTD. MNK1 can then phosphorylate eIF4E, stimulating translation for a subset of mRNAs. This phosphorylation has been observed to increase tumorigenesis in some cases[44, 45].

eIF4A

eIF4A is a classical RNA helicase that belongs to the DEAD box family[46]. It resolves secondary structures in the mRNA 5' UTR, selectively allowing efficient translation of target mRNAs. As discussed previously, unwinding 5' UTR complexity is crucial for efficient scanning by the 43S PIC until it reaches the start codon[1]. mRNAs with minimal secondary structures still require eIF4A's helicase activity and eIF4A mediated RNA unwinding seems to be necessary for ribosome recruitment regardless of mRNA secondary structure[172]. Mammalian cells encode three eIF4A paralogs: eIF4A1 (DDX2A), eIF4A2 (DDX2B), and eIF4A3 (DDX48). eIF4A1 and eIF4A2 share ~90% identity at the amino acid level. Of the two, eIF4A1 is essential and generally more abundant than eIF4A2[1]. eIF4A3 shares ~67% identity to eIF4A1 at the amino acid level but its key molecular role is in nonsense-mediated decay as part of the exon-junction complex (EJC), rather than translation initiation[47, 48, 49]. eIF4A dependency of mRNAs for translation initiation varies based on the extent of secondary structure in the 5' UTRs and transcripts with highly structured 5' UTRs have an increased dependency on eIF4A. Interestingly, only ~5% of eIF4A is thought to reside within the eIF4F complex, while the majority exists as free form eIF4A1 in the cells. Additionally, beyond its interactions with initiation factors, eIF4A availability is regulated by the tumor suppressor programmed cell death 4 (PDCD4) as well[50, 51, 52].

1.3 Targeting eIF4F as a Therapeutic Vulnerability in Cancer

Since initiation is the rate-limiting and most regulated step in translation, there has been much interest in targeting this phase as a therapeutic target in cancer. eIF4F is a critical downstream target of the PI3K/Akt/mTOR signaling cascade. mTOR regulates the role of eIF4E by controlling its shuttling between 4E-BPs (translation initiation inhibited) and eIF4F (translation promoted)[39, 40]. In many human cancers, mTOR signaling is upregulated which means translation initiation rates are higher than normal[39]. Similarly, many cancers have elevated eIF4F and eIF4E levels, further increasing translation. Therefore, these cancer types have a higher dependency on eIF4F and eIF4E, making them therapeutic targets against cancer cell proliferation and tumorigenesis[36]. Dysregulation of eIF4A activity has also been linked to various types of cancers, including breast cancer and T-cell acute lymphoblastic leukaemia (T-ALL)[54]. Translation stimulation of oncogenes such as *c-Myc* is an important part of the mechanism of action whereby eIF4A sustains tumorigenesis[54]. It has been demonstrated that 5' UTRs with high GC content are stimulated by elevated eIF4A levels and samples from patients with lung carcinoma display higher eIF4A protein levels [53]. This reveals that as part of the eIF4F complex, eIF4A is an important druggable anti-cancer target.

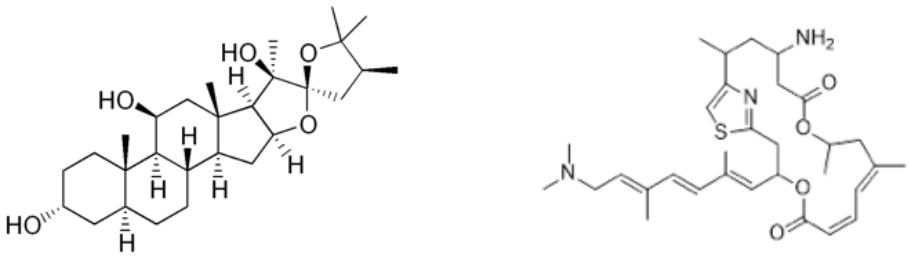
1.3.1 Identification of eIF4A inhibitors: Hippuristanol, Pateamine A, Rocaglates

The use of small molecule inhibitors has opened vast, new avenues for studying the different stages of many biological pathways, including protein synthesis. Small molecule inhibitors have the advantage of quick target engagement, allowing access to information that is harder to probe using genetic methods (where protein activity or levels are more slowly compromised). Over the years, a number of potent translation initiation inhibitors have been identified which specifically modulate eIF4A activity. Since eIF4A can exist both in its active and inactive form, directing cellular levels of eIF4A towards its inactive form would decrease its availability for ATP-mediated helicase activity, potentially offering an anti-cancer strategy. Hippuristanol is a small molecule inhibitor that abides by this mechanism of action and locks

eIF4A in an inactive form[57]. Additionally, Pateamine A is a potent inhibitor of eIF4A which does not target the protein's helicase activity but functions by causing eIF4A to clamp onto RNA[61]. Similarly, there exists a family of compounds known as rocaglates that inhibit translation initiation by also selectively clamping eIF4A onto RNA, decreasing eIF4A available for eIF4F complex formation, and inhibiting 43S PIC recruitment and scanning[68].

Hippuristanol

Hippuristanol is a polyoxygenated steroid initially isolated in 1981 from the gorgonian (soft coral), Isis hippuris[56] (Fig.6). It is a specific inhibitor of eIF4A since it binds to the CTD of eIF4A, interacting with amino acids conserved between eIF4A homologues but does not interact with other members of the DEAD-box helicase family[57]. Although hippuristanol does not inhibit eIF4A's ability to bind ATP, it inhibits the protein's helicase activity by locking it in a closed conformation[57, 58, 59].



Hippuristanol Pateamine A

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Figure 6. Structure of hippuristanol and pateamine A.

Pateamine A

Pateamine A (Pat A) was first isolated from the marine sponge Mycale sp.[60] and has been found to stimulate the binding of eIF4A to RNA in a sequence independent manner[62]. This leads to reduced levels of eIF4A available for eIF4F complex formation, resulting in inhibition of cap-dependent translation. Pat A seems to affect only free eIF4A and not when eIF4A is in complex with eIF4F, indicating that the binding site of eIF4A and Pat A is occluded when eIF4A is a part

of eIF4F[62]. It is a potent chemotherapeutic agent shown to be cytotoxic against murine leukemia cells and many other cancer cell lines. A synthetic derivative of Pat A, DMDA-Pat A is a less-cytotoxic counterpart with similar activity against eIF4A, it is well tolerated in animals and has been shown to inhibit growth of tumor xenografts[61, 65, 66].

Rocaglates

Rocaglates were found in the Aglaia genus of the angiosperm Mahogany (Meliaceae) family, and share a cyclopenta[b]benzofuran ring[67]. The first rocaglate to be characterized was rocaglamide A (RocA) and it was shown to have potent cytotoxicity against murine lymphocytic leukemia cells[67]. To date, a large collection of natural occurring members of the rocaglate family have been identified. The most well-characterized rocaglate is silvestrol[68, 69], which behaves similarly to Pat A and acts as a chemical inducer of dimerization, affecting the activity of both $eIF4A_f$ (free eIF4A) and $eIF4A_c$ (eIF4A within the eIF4F complex) [70]. The current working model for rocaglates' mechanism of action is that these compounds stimulate both $eIF4A_f$ and $eIF4A_c$ clamping onto the RNA. Clamping of $eIF4A_c$ stabilizes the eIF4F complex onto the 5' end of the mRNA, which blocks recruitment of the 43S PIC complex. $eIF4A_f$ clamping onto the RNA prevents the 43S PIC complex from being recruited to the mRNA[70, 71].

The effects of rocaglates on the translatome have been extensively investigated. Studies from 2014 indicated that mRNAs with higher structural complexity in the 5' UTR show increased sensitivity to rocaglates[72]. This explains the anti-cancer properties of rocaglates since many oncogenes have longer, more structured 5' UTRs[54]. However, it was subsequently reported that in addition to 5' leader complexity, rocaglates cause clamping to polypurine RNA and decrease the off-rate of RNA-bound eIF4A[73]. Previous mutagenesis studies by Sadlish and colleagues in yeast[74] implicated P147 (P159 in human heIF4A1), F151 (F163 in heIF4A1), Q183 (Q195 in heIF4A1), and I187 (I199 in heIF4A1) as key residues for rocaglate binding. In 2016, Chu *et al.* engineered the F163L mutation into eIF4A1 in murine cells and showed that the anti-cancer properties of these compounds is mediated by eIF4A1 engagement[75]. The molecular basis of these findings was supported by Iwasaki *et al.* in their 2019 crystal structure of eIF4A1:RocA, where F163 was found to participate in π-π stacking with the C-ring[6]. In addition, Q195 was observed to donate a crucial

hydrogen-bond to the C2 carbonyl. Rocaglates exhibit potent cytotoxicity against cancer cells and have been reported to inhibit NF-kB activation[71, 77]. Silvestrol has been shown to have anti-tumor activity *in vivo* and it inhibits tumor growth in several xenograft models including breast cancer and prostate cancer[71]. Silvestrol's clinical efficacy may be limited since it is a substrate for the ABCB1/P-Glycoprotein protein which is a transporter implicated in multi-drug resistance and is able to pump chemotherapeutic drugs out of cells into the extracellular space[78]. For this reason, there has been significant interest in modifying the rocaglate backbone in order to decrease rocaglate sensitivity to ABCB1 mediated drug-resistance[77, 79].

1.4 Targeting *c-myc* as a therapeutic vulnerability in cancer

Approximately ~80% of the human proteome is thought to be untargetable by small molecule inhibitors[80]. This is a serious impediment to the exploration and development of therapies across all disease areas. *c-myc* is one of the most commonly over-expressed and amplified oncogenes in human cancers. *c-myc* is at a pinnacle position in the oncogenic processes, playing a role in cell proliferation, apoptosis, differentiaton and metabolism[81]. It is a member of the helix-loop-helix leucine zipper family and forms heterodimers with different protein partners; the Myc:Max pair preferentially bind E-box motifs ($^{5'}$CACGTG$^{3'}$). *c-myc* has been estimated to regulate the expression of up to 15% of human genes[194]. It is able to modulate expression by a variety of mechanisms. As a transcription factor, it can recognize promoters through direct DNA binding but also indirectly through being recruited by other DNA-binding factors[82-85]. Direct targeting of c-Myc has been a challenge for decades because of its "undruggable" nature. Therefore, alternative strategies for blocking c-Myc have been widely explored, including disruption of Myc/Max heterodimers, *c-myc* transcription and translation inhibition, destabilization of c-Myc and targeting synthetic lethal partners associated with its overexpression[86-91].

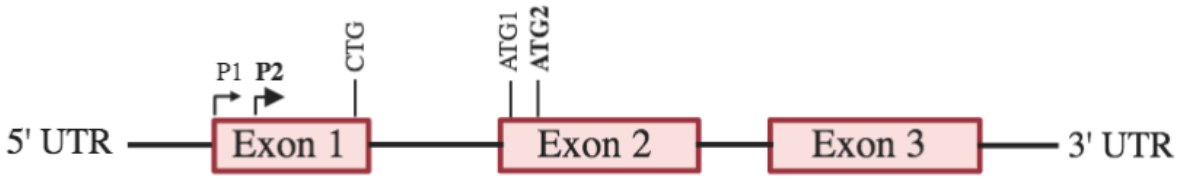


Figure 7. Schematic diagram of the *Myc* locus. The two major promoters and three translation initiation sites are denoted.

Targeting the expression of *c-myc* by blocking its transcription through inhibition of BET bromodomain proteins with compounds like JQ1 leading to potent anti-neoplastic responses[88]. The *c-myc* promoter also contains a G-quadruplex motif and small molecules that bind this structure exhibit selective activity towards tumor cells, but not against cells lacking this element. The small molecule stauprimide, a staurosporine analog, inhibits nuclear localization of the *c-myc* transcription factor NME2, leading to a reduction in *c-myc* expression and inhibition of tumor growth in rodent xenografts[89, 90]. A genome-wide shRNA screen also uncovered a heightened dependency of *c-myc* expression to spliceosome interacting factor BUD31, whose inhibition led to defective *c-myc* pre-mRNA maturation and stability[86]. Compounds from the rocaglate family of translation initiation inhibitors also show potent anti-tumor activity with a preference for inhibiting *c-myc* mRNA translation[92]. Systemic inhibition of *c-myc* through conditional expression of a dominant-negative mutant, the Omomyc allele, in tumor-bearing transgenic mice has revealed profound therapeutic effects towards diverse tumor with only mild and reversible side effects towards normal tissues observed, suggesting a therapeutic window is feasible following systemic *c-Myc* inhibition[93, 94]. Given that partial suppression of *c-myc* (~50%) is sufficient to achieve a potent anti-tumor response, there is thus considerable interest in pursuing pharmacological strategies by which to block *c-myc* expression[93-95].

Expression from the *c-myc* locus is quite complex. Under most conditions, *c-myc* is transcribed from two promoters, P1 and P2, with 75% of transcripts arising from P2 (Figure 7). Reporter constructs flanked by as much as 50 kbp of natural *c-myc* sequences are unable to correctly recapitulate physiological regulation, highlighting the complexity of *c-Myc* transcriptional regulation and indicating that native chromosomal context is critical to proper *c-myc* expression and regulation[96]. c-*myc* expression is also tightly regulated at the level of

translation and mRNA/protein stability - both the mRNA and protein possessing very short half-lives (20-30 min) but this can become stabilized in disease[99, 100]. Three distinct c-Myc protein isoforms are generated from the c-*myc* locus. Myc1 is produced from a noncanonical CUG initiation codon; Myc2 from an AUG located 15 codons downstream, and MycS from an AUG located 100 codons further downstream[101]. Normally, the Myc2 isoform is predominant and its oncogenic activation is responsible for cell growth and proliferation. In the work described in this thesis, we have specifically investigated the Myc2 isoform.

1.4.1 Effect of translation inhibitors on Myc protein and mRNA levels

Increased eIF4F levels result in the stimulation of *c-myc* mRNA translation; indicating the presence of a feedforward loop involving c-Myc and eIF4F that links transcription and translation, which might rationalize the effects of c-Myc on cell proliferation and neoplastic growth[37, 98]. c-Myc is dependent on eIF4F for its translation and this makes the eIF4F complex a viable target in c-Myc-driven cancers. Inhibition of *c-myc* mRNA translation by rocaglates has been shown to exert potent cytotoxic effects in Myc-dependent tumor cells. Treatment of SW480 cells, a human colon adenocarcinoma cell line, with nanomolar concentrations of silvestrol and rocaglamide, caused a decrease in c-Myc protein expression[102]. This decrease in c-Myc levels was independent of ERK or mTOR activity. Interestingly, in this study, silvestrol did not suppress *c-myc* mRNA levels; instead, there was an increase in mRNA levels. This is postulated to be the result of a negative self-autoregulation of *c-myc*, whereby c-Myc binds to its own promoter and acts as a transcriptional repressor[103]. Silvestrol suppressed c-Myc protein levels in a number of other colon cancer cell lines including HCT116, Ls174T, SW480 and SW620[71-73].

Since c-Myc has a short half-life, the use of translation inhibitors can successfully reduce protein levels fairly quickly. Interestingly, however, it has been widely reported that protein synthesis inhibitors augment and stabilize mRNA transcript levels. For example, 36-180 μM puromycin has been found to cause increases in *c-myc* mRNA levels[105]. This phenomenon, referred to as gene super-induction, is characterized by the prolonged expression of immediate early genes that are usually only transiently induced[106]. There are several suggested mechanisms by which protein synthesis inhibitors are thought to exert this effect, including increased mRNA stability[107,

[108] increased transcription[109], decreased synthesis of labile gene repressors[110] and stimulation of nuclear signaling responses[111]. The mechanism contributing to gene super-induction may depend on the specific transcript being induced. Dramatic stabilization of *c-myc* mRNA in Hela, MCF7 and HL60 cells has been reported whereas no change in *c-myc* mRNA stability was seen in other transformed cells lines such as Daudi and normal embryonic human fibroblast[112]. This indicates that the controlling element might be a *trans*-acting labile factor that is differently affected depending on the cell type. Additionally, it was found that similarly to *c-myc*, *c-fos* and *c-jun* super-induction can also occur upon translational arrest[113]. Interestingly, it was also documented that mutation of the second initiation AUG codon to a termination codon stabilized *c-myc* mRNA, increasing its half-life and suggesting that loading with ribosomes may be required for mRNA turnover[114]. Further analysis of the mechanism by which *c-myc* transcripts appeared to be stabilized revealed two distinct regions in the mRNA mediating this effect, one in the 3' UTR harboring AU rich sequences and the other in the C terminal part of the coding region, colocalizing with sequences encoding protein-binding motifs[115]. The sequence encoding a protein-binding motif in the *c-myc* transcript was further characterized by R. Wisdom (1991)[116] in an attempt to identify the molecular basis of, *c-myc* induction. The study found that there was an induction of *c-myc* mRNA by cycloheximide (CHX) in C2 murine myoblasts and that this was due to stabilization of *c-myc* transcripts. A mutational analysis revealed that the requirement for increased levels of *c-myc* mRNA by cycloheximide resided in a sequence encoding amino acids 335-439 and that the mRNA must be competent for translation. Their results concluded that *c-myc* mRNA degradation is coupled to translation such that the sequences mediating this are contained in the protein-coding region and translation inhibitors induce expression of *c-myc* mRNA by blocking turnover mediated by this element[116]. As well, it was observed that shortening of the poly(A) tail of the c-*myc* transcript preceded degradation and this shortening was decreased when translation was blocked and the mRNA was stabilized.

Although numerous studies have attempted to discern the stabilization effect of specific transcripts with translation inhibition, the exact mechanism by which this takes place remains unclear. Further studies must be undertaken to elucidate regulatory (5' and 3' UTRs, RNA-protein interaction motifs, destabilizing features,) elements that are differentially inducing transcripts to understand the complexity of this possible feed-back regulatory mechanism.

1.5 Overview and rationale for thesis

The effect of translation inhibition on cellular protein levels and the mechanistic details by which they affect the translatome have been well-documented. What has been largely overlooked, however, is the effect of global shut-down of protein synthesis on mRNA levels. It has been observed that most mRNAs are upregulated after translation inhibition by specific compounds. Although there is evidence that suggests this phenomenon is due to increased stabilization of transcripts, the exact mechanism by which this stabilization takes place, is largely unknown. mRNA stability is a complex, multi-layered process with a variety of determinants that may differ based on the mRNA itself, cell type as well as cell cycle stage.

mRNA stability is a critical determinant that affects gene expression. Many pathways have evolved to regulate mRNA stability in response to developmental, physiological and/or environmental stimuli. Eukaryotic mRNAs have a wide range of half-lives, ranging from mins to several days. Variations in mRNA stability have been implicated in defective gene expression, such as the human β-globin mRNA, which is normally highly stable. However, many naturally occurring mutations in the globin gene can trigger accelerated mRNA degradation, affecting RNA integrity prior to translation and leading to activation of surveillance pathways (eg. NMD) that prevent translation of aberrant β-globin mRNAs; increasing the severity of the β-thalassemia phenotype[164]. Deepening our understanding of *cis* and *trans* acting mechanisms that regulate mRNA stability will allow us to develop strategies to modulate mRNA stability, creating an opportunity to design therapeutic approaches to target or augment mRNA stability where it is of corrective value.

2. MATERIALS AND METHODS

2.1 Cell Culture

JJN-3 and KMS-11 cells were maintained in RPMI-1640 with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, 2mM L-glutamine at 37 °C and 5% CO_2. Cells were kept at low passage.

2.2 Western Blotting

Protein extracts were prepared in RIPA lysis buffer (20 mmol/L Tris, pH 7.6, 100 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mmol/L PMSF, 4 mg/mL aprotinin, 2 mg/mL leupeptin, and 2 mg/mL pepstatin), Protein lysates (10 μg) were resolved by SDS-PAGE, transferred to Millipore PVDF, probed with the appropriate antibodies, and visualized using enhanced chemoluminescence detection purchased from Perkin Elmer. The antibodies used for protein expression analysis were targeted against c-Myc (Cell Signaling Technology) and GAPDH (Abcam).

2.3 In vivo ^{35}S-Methionine Labeling

JJN-3 cells were exposed to the indicated concentrations of CR-1-31-B for 1 h in methionine and cysteine-free RPMI media. During the last 15 mins of incubation, [^{35}S]-methionine/cysteine was added (50 μCi; PerkinElmer), and the labeling reactions were terminated with the addition of RIPA lysis buffer. Lysates were then spotted onto 3 MM Whatman paper (2 cm^2 squares) that had been preblocked with MEM non-essential amino acid mixture (Gibco). After drying, filters were submerged in ice-cold 10% trichloroacetic acid (TCA)/0.1% L-methionine for 20 mins, followed by boiling in 5% TCA for 15 mins. The Whatman squares were washed twice with cold 5% TCA and then twice more with 95% ethanol, with each wash lasting 1 min. Filter squares were dried, and the amount of radiolabeled precipitated protein was quantitated by scintillation counting (Beckman Coulter). Counts were standardized to total protein content that was determined using the DC protein assay (Bio-Rad).

2.4 RNA Isolation and RT-qPCR

Cellular RNA was extracted using TRIzol® (Invitrogen) as per the manufacturer's instructions. Complementary DNA (cDNA) was generated by reverse transcription using M-MuLV Reverse Transcriptase in combination with $d(T)_{23}VN$ primers according to the manufacturer's instructions (NEB). cDNA samples were diluted 1:10 and amplified in a CFX96 PCR System (Bio-Rad) using SsoFast Evagreen Supermix (Bio-Rad). Gene expression data analysis was performed using the $2^{-\Delta\Delta Ct}$ method and mRNA levels were normalized to GAPDH, unless stated otherwise. Where indicated, statistical significance was determined using one-way ANOVA on Prism GraphPad. Primer efficiency was determined and taken into account in the CT expression determinations.

The following primers were using in the study:

c-MYC_Fwd ($^{5'}$CACCACCAGCAGCGACTCT$^{3'}$)
c-MYC_Rev ($^{5'}$CTGACCTTTTGCCAGGAGC$^{3'}$)

GAPDH_Fwd ($^{5'}$GGTATCGTGGAAGGACTCAT$^{3'}$)
GAPDH_Rev ($^{5'}$GCAGGGATGATGTTCTGGAG$^{3'}$)

c-FOS_Fwd ($^{5'}$CTGGCG-TTGTGAAGACCAT$^{3'}$)
c-FOS_Rev ($^{5'}$TCCCTTCGGATTCTCCTTTT$^{3'}$)

c-JUN_Fwd ($^{5'}$ATCAAGGCGGAGAGGAAGCG$^{3'}$)
c-JUN_Rev ($^{5'}$TGAGCATGTTGGCCGTGGAC$^{3'}$)

MCL1_Fwd ($^{5'}$GGGCAGGATTGTGACTCTCATT$^{3'}$)
MCL1_Rev ($^{5'}$GATGCAGCTTTCTTGGTTTATGG$^{3'}$)

2.5 Actinomycin D inhibition assay

JJN-3 cells were exposed to vehicle or 10 μg/mL Actinomycin D in the presence or absence of 25 nM of silvestrol at the indicated time points, aliquots were taken and total RNA was extracted

using TRIzol®, following the manufacturer's recommendations. Following RT-qPCR, the expression levels of *c-myc,* c-jun, c-fos*, Mcl-1* and GAPDH were determined and the half lives were calculated using nonlinear regression on GraphPad Prism.

2.6 RNA-seq

JJN-3 cells were treated with vehicle, 50μM CHX and 25 nM Silvestrol for 2 h. Cells were then washed with PBS and RNA was extracted using the Qiagen RNeasy Mini Kit with on-column DNase I digestion. Samples were sent to the Genome Sciences Center (GSC) at the University of British Columbia for library preparation and deep sequencing. RNA integrity was first assessed using the Agilent platform after which strand-specific, barcoded libraries were prepared via cDNA production, PCR, and ribosomal RNA (rRNA) depletion. Deep sequencing was performed using the PE150 Illumina Sequencing platform with paired-end reads at roughly 50 million reads per sample. Biological triplicates for each treatment were obtained. All analysis including Gene Ontology (GO), 5' UTR, 3' UTR and CDS length, nucleotide and motif analyses were performed by Mehdi Amiri (Sonenberg lab, GCRC).

3. RESULTS

3.1 CR-1-31-B potently inhibits global translation and downregulates Myc protein levels in MM cells.

As previously described by Wiegering et al. (2015) and others[102], rocaglates strongly inhibit c-Myc protein levels in colorectal (CACO2)[105], prostate (PC-3), and breast (MDA-MB-231) cancer cells[71]. To identify the concentrations at which a potent rocaglate, CR-1-31-B (Fig. 8a), inhibits c-Myc levels, we first monitored the incorporation of ^{35}S-methionine/cysteine into protein following treatment with CR-1-31-B for 1 h in JJN-3 cells, a MM cell line. This cell line was chosen because it is well documented that multiple myeloma exhibits an addiction to c-Myc and its survival is highly susceptible to changes in c-Myc expression[170]. We observed an approximately 50% reduction of relative ^{35}S-methionine/cysteine incorporation at 10 nM treatment of the compound (Fig. 8b) and the standard error of the meam (SEM) was calculated on Prism GraphPad. We determined the effect of compound treatment on Myc protein levels by Western blot analysis and observed that the reduction in c-Myc levels was consistent with the reduction in relative ^{35}S-methionine incorporation. Since Myc is an unstable protein with a half-life of only 30-60 mins, we were able to see a large decrease in cellular c-Myc levels following 1 h of treatment (Fig. 8c).

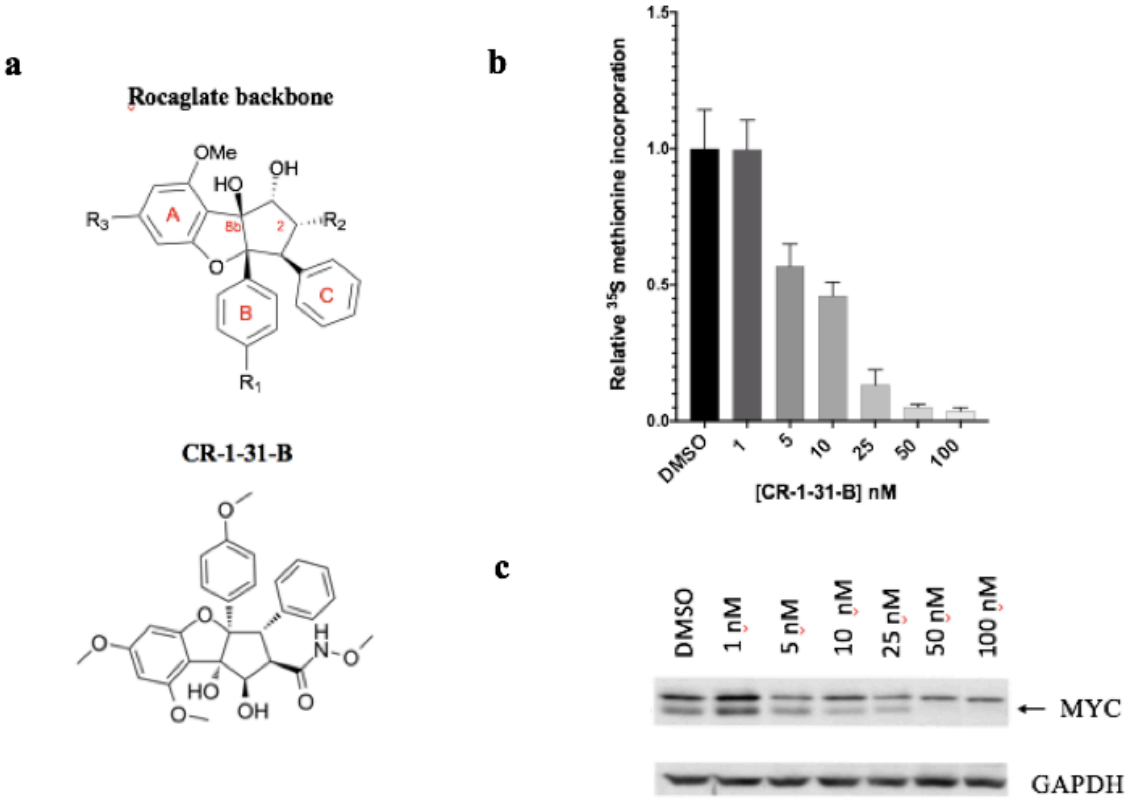


Figure 8. CR-1-31-B potently inhibits global translation and Myc protein levels. **(a)** Rocaglate cyclopenta[*b*]benzofuran backbone and structure of a potent and well-characterized rocaglate CR-1-31-B. **(b)** ^{35}S-Methionine/cysteine incorporation into TCA-insoluble protein. JJN-3 cells were treated with the indicated concentrations of CR-1-31-B and 0.5% DMSO for 1h, and labeled with 50 µCi/mL ^{35}S-methionine/cysteine for the last 15 mins. Cells were harvested, and the amount of radiolabeled protein was quantitated by TCA precipitation Values are standardized against total protein content. N = 3 ± SEM. **(c)** Western blot analysis of Myc and GAPDH levels after treatment with CR-1-31-B. JJN-3 cells treated with the indicated concentrations of CR-1-31-B and 0.5% DMSO for 1 h after which cells were lysed in RIPA buffer. Protein samples (10 µg) were fractionated on an 10% SDS-polyacrylamide gel, transferred to PVDF membrane, and probed with the indicated antibodies (Myc, GAPDH).

3.2 Translation inhibition by eIF4A inhibitors and cycloheximide leads to an increase in cellular mRNA levels.

To determine the effect of translation inhibition by CR-1-31-B on *c-myc* mRNA levels, we undertook a titration of CR-1-31-B on JJN-3 cells for 2 h and harvested RNA for RT-qPCR analysis. Interestingly, we observed that for the concentrations at which c-Myc protein levels were downregulated (Fig. 8c), *c-myc* mRNA levels were elevated 4 to 5 fold (Fig 9a). Since c-Myc protein has a short half-life, our data suggested that a global translational shut-down was associated with an increase in *c-myc* mRNA and that the latter was due to a transcriptional response or decreased mRNA turnover rates. In order to evaluate the response of other transcripts with short half-lives, we performed RT-qPCR analysis using primer pairs amplifying exon-exon junctions of *c-jun*, *c-fos* and *Mcl-1* transcripts, each of which were similarly upregulated (Fig. 9a). To further understand if translation inhibition is a pre-requisite for the strong transcriptional induction observed with CR-1-31-B, we were interested in testing other eIF4A inhibitors (silvestrol, hippuristanol, pateamine A) as well as an elongation inhibitor (cycloheximide). JJN-3 cells were treated with each of these inhibitors and *c-myc* mRNA levels were determined via RT-qPCR (Fig. 9b). Treatment with each of these translation inhibitors increasd *c-myc* mRNA levels, although to varying degrees. Silvestrol at 25 nM (shown in dark red, Fig 9b) showed the greatest increase of *c-myc* levels and we therefore used this compound to further study the effect in subsequent experiments.

This data suggests that inhibition of translation in JJN-3 cells invokes a cellular feedback response that increases cellular mRNA levels either by transcriptional induction or increased mRNA stability. The underlying mechanism needed to produce this regulatory feedback mechanism was then further investigated.

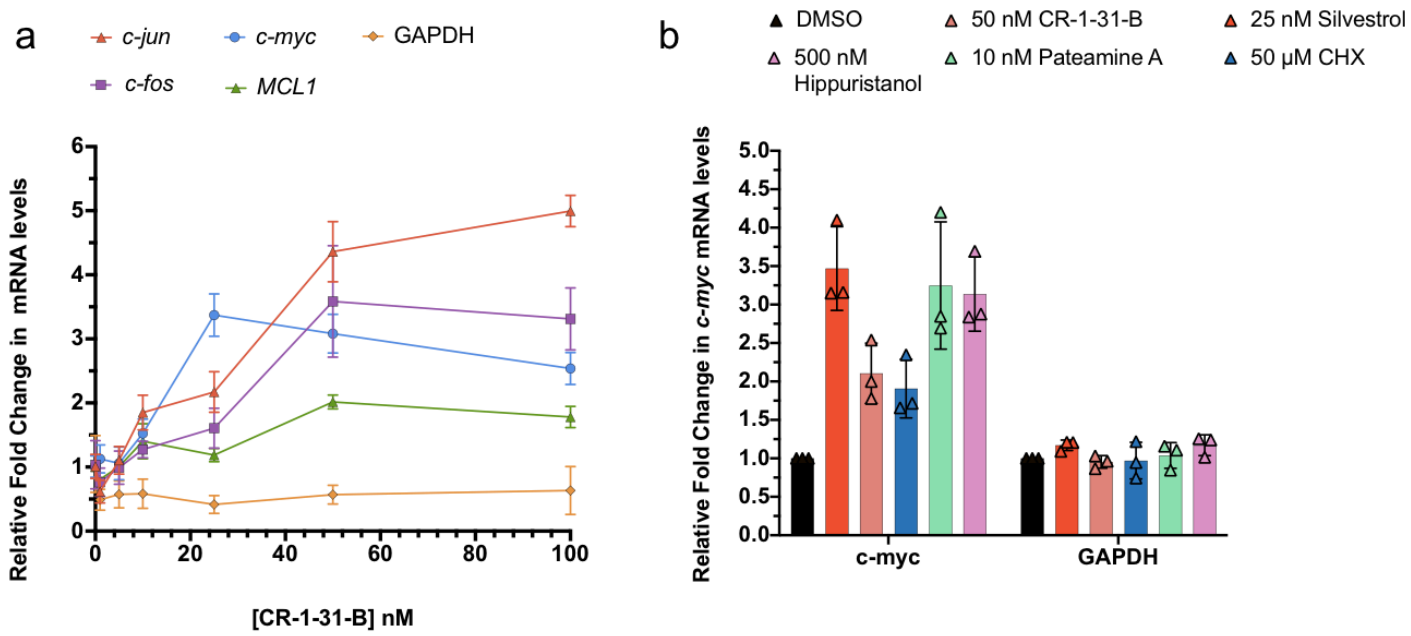
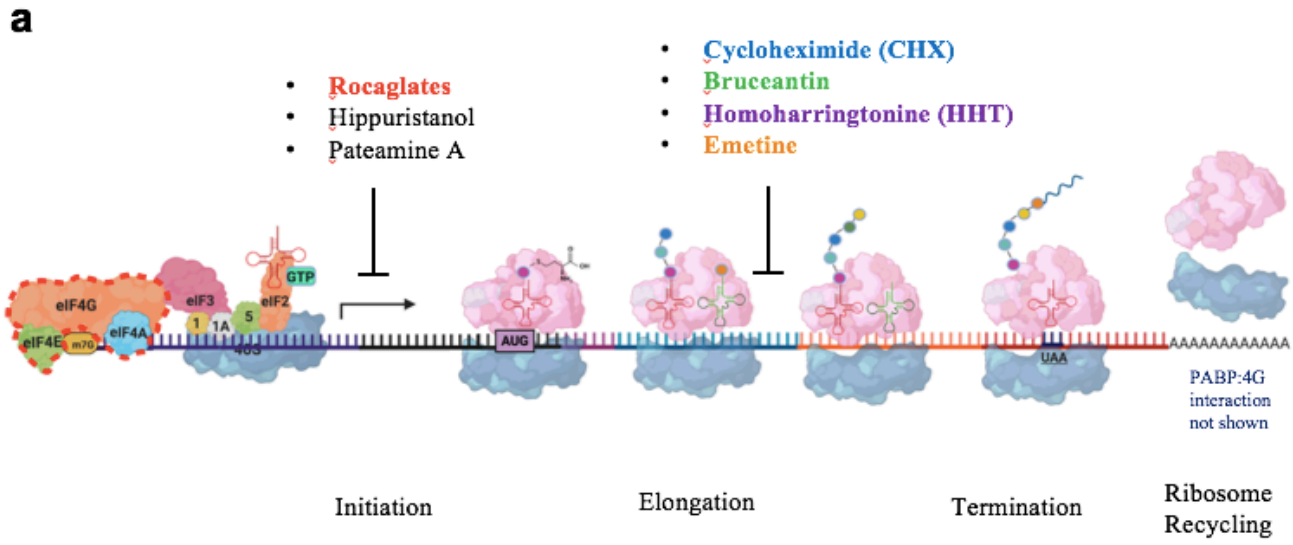


Figure 9. Translation inhibition by eIF4A inhibitors and cycloheximide leads to an increase in cellular mRNA levels. (a) Relative fold change in the indicated cellular mRNA transcript levels after 2 h treatment with CR-13-1-B. JJN-3 cells were treated with the indicated concentrations of CR-1-31-B for 1 h after which total RNA was extracted, cDNA synthesized and the relative *c-myc, c-jun, c-fos* and *Mcl-1* levels determined via RT-qPCR. Standardization was performed to GAPDH, whose levels did not change upon drug treatment. N=3 ± SEM. **(b)** *c-myc* mRNA levels increase after a 2 h treatment with the indicated translation inhibitors. RT-qPCR data shows relative fold change in *c-myc* levels in response to treatment. N=3 ± SEM.

3.3 Increased *c-myc* mRNA levels is not universally observed with all elongation inhibitors

To determine whether inhibitors of different stages of translation had a similar effect on cellular mRNA levels, we broadened our testing to include three other protein synthesis inhibitors, homoharringtonin (HHT)[117], bruceantin[118, 119], emetine[120] and CHX (Fig. 10a). HHT and bruceantin inhibits peptide chain elongation by binding the A site of the large ribosomal subunit, blocking aminoacyl-tRNA binding, and halting peptide chain elongation[117]. These two compounds both fill the A-site cleft, precluding the occupation of that same space by the amino acid side chains of A-site-bound aminoacyl-tRNA. Emetine irreversibly binds the ribosomal 40S subunit, inhibiting aminoacyl-tRNA transfer[195]. CHX binds to the E site and interferes with subsequent movement of the deacylated tRNA[121]. First, we identified the concentrations at which each of these

compounds inhibited global translation in JJN-3 cells. In order to do this, we performed an ^{35}S-methionine/cysteine *in vivo* metabolic labeling assay. The exposure time for each treatment was 1 h and we observed that 50 µM CHX, 25 µM bruceantin, 150 nM HHT and 25 µM emetine completely inhibited global translation (Fig. 10b). Since these concentrations have previously been reported to be non-toxic, we used these concentrations to inhibit translation in JJN-3 cells and monitor cellular *c-myc* mRNA levels. JJN-3 cells were treated for 2 h with each of the indicated compound concentrations, and total RNA was extracted to quantitate mRNA levels via RT-qPCR. Our results showed that other than CHX, no other elongation inhibitor increased *c-myc* mRNA levels after a 2 h treatment. This indicates that translation inhibition *per se* is not sufficient to increase *c-myc* mRNA levels, and that this effect may be coupled to each compound's specific mechanism of action.



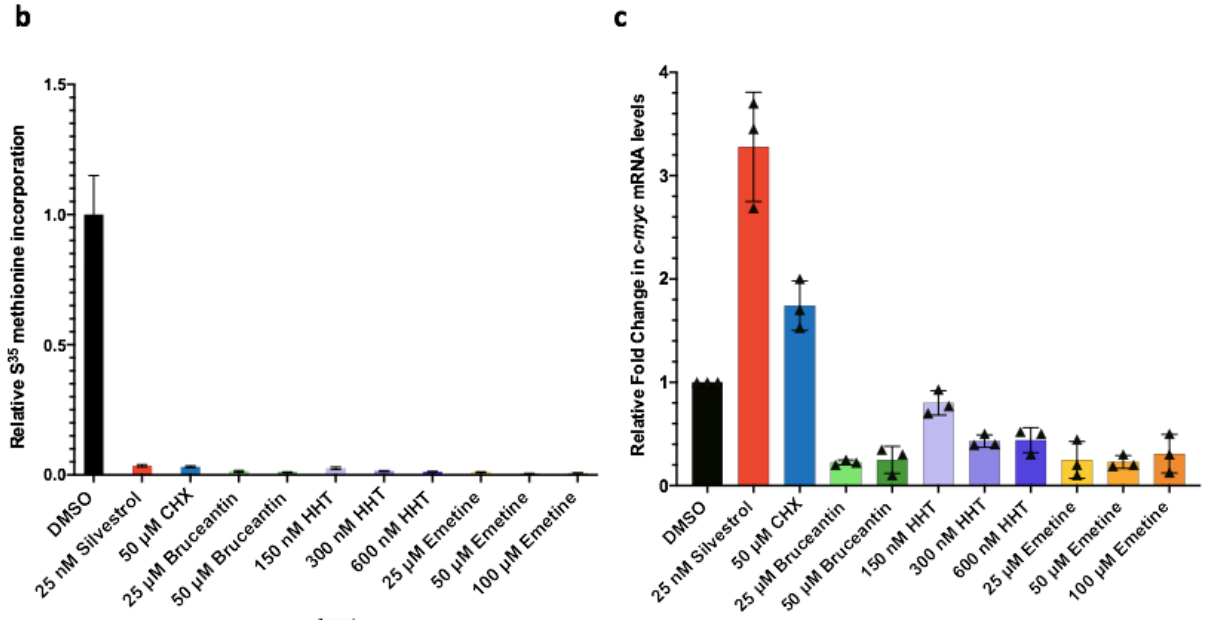


Figure 10. Effect of eIF4A and elongation inhibitors on *c-myc* mRNA induction.
(a) Protein synthesis inhibitors and the stage that they target is illustrated. **(b)** *In vivo* labeling data for protein synthesis inhibitors. JJN-3 cells were treated with the indicated concentrations of protein synthesis inhibitors and 0.5% DMSO for 1h, and labeled with 50 μCi/mL ^{35}S-methionine for the last 15 mins. Cells were harvested, and the amount of radiolabeled protein was quantitated by TCA precipitation. Values are standardized against total protein content. N = 3 ± SEM. **(c)** *c-myc* mRNA levels assessed by RT-qPCR after 2 h treatment of JJN-3 cells to indicated concentrations of compound. N = 3 ± SEM ($p_{adj} \leq 0.0206$).
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3.4 The *c-myc* mRNA induction response is not unique to JJN-3 cells.

In order to validate that the increase in *c-myc* mRNA levels observed in JJN-3 cells was not restricted to these cells, we tested silvestrol and CHX on another multiple myeloma cell line, KMS-11. We were able to reproduce our results in KMS-11 cells using RT-qPCR (Fig. 11a). In our efforts to understand how silvestrol and CHX might be mediating this effect, an extensive review of the literature revealed that CHX had been characterized by a number of studies in the 1990s' to 'stabilize' *c-myc* mRNA levels. In particular, one study[116] reported that 35 μM CHX exposure for 2 h led to an induction of *c-myc* mRNA that was due to an increased stabilization of

the labile transcripts in murine C2 myoblasts[116]. This was shown by Northern blot analysis by comparing total RNA from treated vs untreated cells and probing for *c-Myc*. In order to recapitulate this result in our system, we treated undifferentiated murine C2C12 myoblasts (derived from C2 myoblasts) with two different concentrations of silvestrol and CHX, extracted total RNA and performed RT-qPCR analysis. Our results indicate that both silvestrol and CHX induce *c-myc* levels in C2C12 myoblasts as well (Fig. 11b). Next, we aimed to distinguish the mechanism by which *c-myc* mRNA levels increased in response to silvestrol and CHX. In particular, we were curious to identify whether the increase we observed was due to a transcriptional induction of select transcripts or due to increased mRNA half-life.

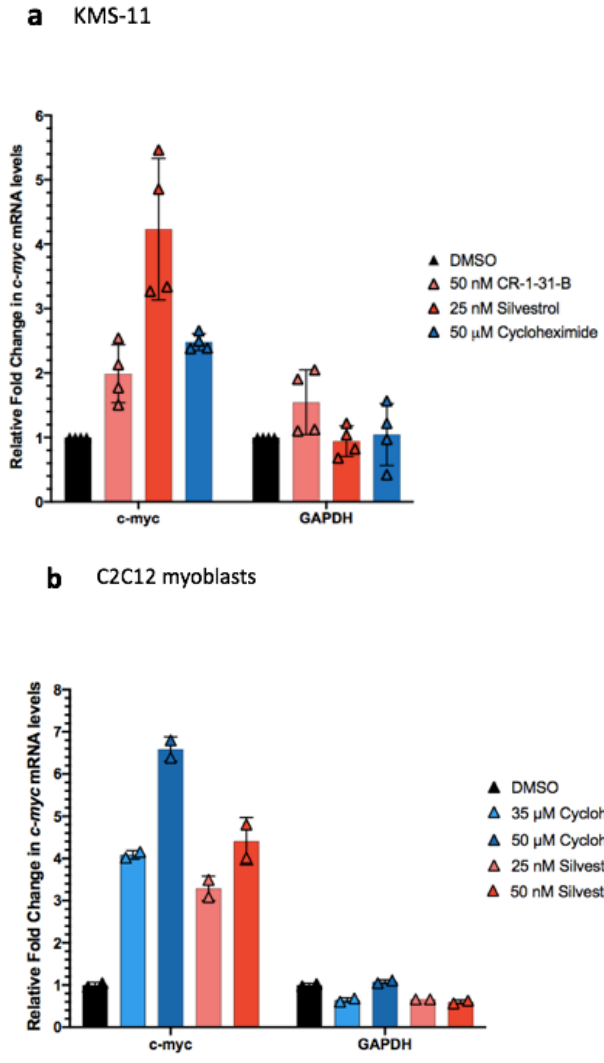
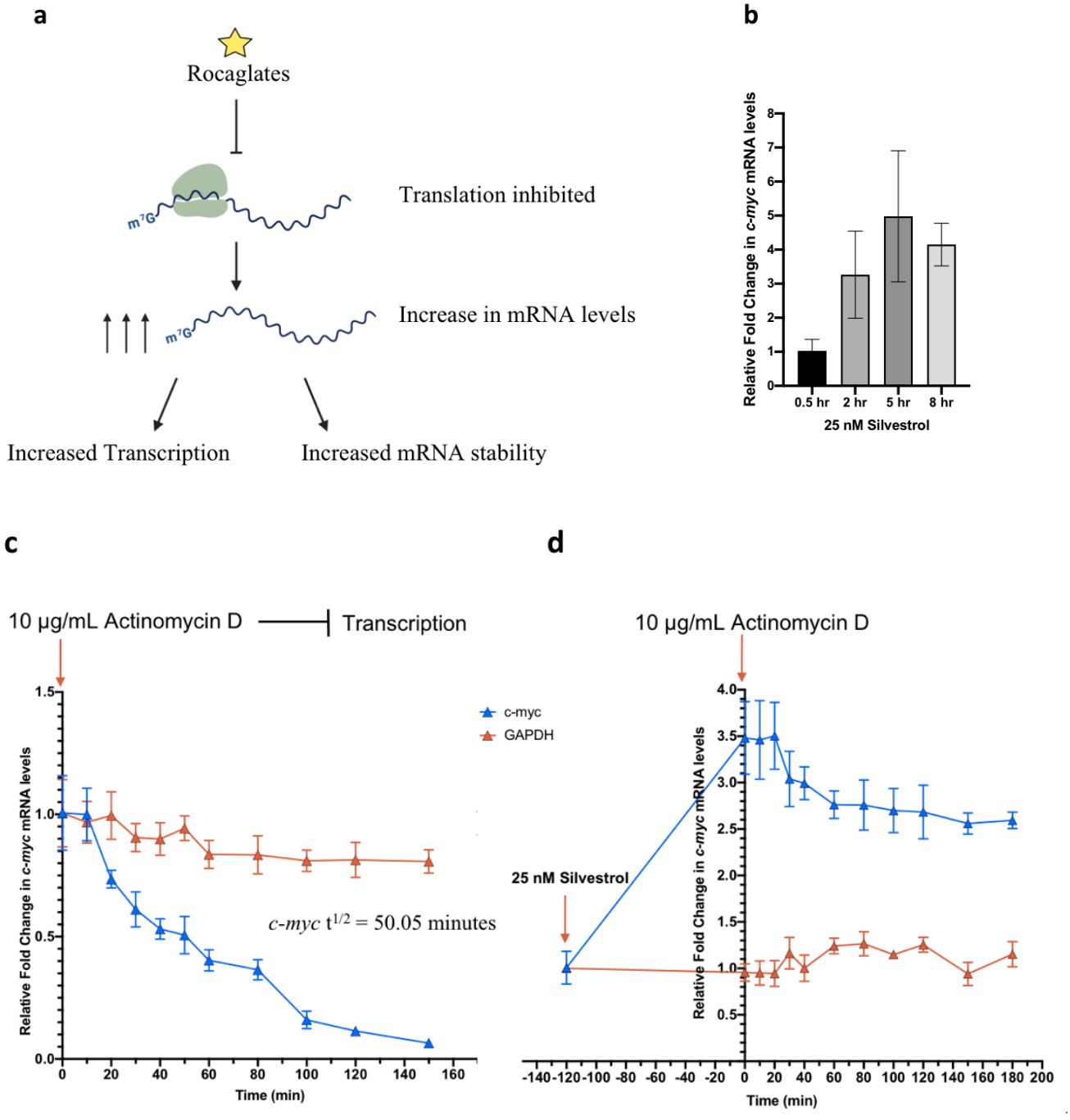


Figure 11. *c-myc* mRNA levels increase after silvestrol and CHX treatment (2h) in KMS-11 and C2C12 myoblasts. (a) *c-myc* mRNA levels increase in KMS-11 cells treated with rocaglate or CHX (2h). N = 4 ± SEM. **(b)** *c-myc* mRNA levels increase in murine C2C12 myoblasts after silvestrol and CHX treatment (2h). mRNA levels were determined via RT-qPCR. N = 2 ± SEM.

3.5 A global analysis of cellular mRNAs levels in response to silvestrol.

We aimed to understand the mechanism by which cellular mRNA levels were increasing as a result of translation inhibition by silvestrol and CHX. We hypothesized that cells were responding to shut down of protein synthesis by ramping up cellular mRNA levels as a regulatory feedback response to compensate for a reduction in protein levels. Such a strong, rapid induction could be the result of increased synthesis of nascent RNA by a transcriptional response and/or an increased stabilization of cellular mRNAs (Fig. 12a). We were curious to identify the time it takes for 25 nM of silvestrol to invoke an increase in *c-myc* mRNA levels in JJN-3 cells. To do this, we treated JJN-3 cells with 25 nM of silvestrol for 0.5, 2, 5 and 8 h, harvested RNA for each time point and compared *c-myc* mRNA levels via RT-qPCR, standardizing our results to the vehicle and GAPDH levels (Fig. 12b). Our results indicated that a 2 h exposure to silvestrol was sufficient to elevate mRNA levels to a significant extent, which was sustained up to at least 8 h. In order to uncouple increased transcription from stability, we conducted an actinomycin D block. Since actinomycin D is a fast-acting transcriptional inhibitor, it was used to block the synthesis of nascent RNA and measure the rate at which *c-myc* transcripts are degraded. Under normal conditions, we measured *c-myc* mRNA half-life to be approximately 50 mins, consistent with what has been reported in the literature[112] (Fig. 12c). Next, we monitored *c-myc* mRNA half-life following a 2 h treatment with 25 nM silvestrol. Levels of c-*myc* were dramatically stabilized upon silvestrol treatment and did not fall below 50% even after 3 h of compound exposure (Fig. 12d). This suggests that *c-myc* levels are stabilized by silvestrol treatment. We examined the half-lives of other transcripts that we had found to be elevated by silvestrol treatment (*c-jun*, *c-fos* and *Mcl-1*). In the actinomycin D block experiment, these mRNAs normally are degraded rapidly - under 30 mins, and this is consistent with their reported half-lives which are approximately 30 mins for each of the transcripts[107, 113] (Fig. 12e). However, pretreatment with silvestrol followed by an actinomycin D block lead to a significant stabilization of the RNAs, suggesting that silvestrol

increased the stability of these labile transcripts (Fig. 12f). Our findings from these actinomycin D block assays strongly suggest that mRNAs exhibit stabilization upon treatment with select protein synthesis inhibitors.



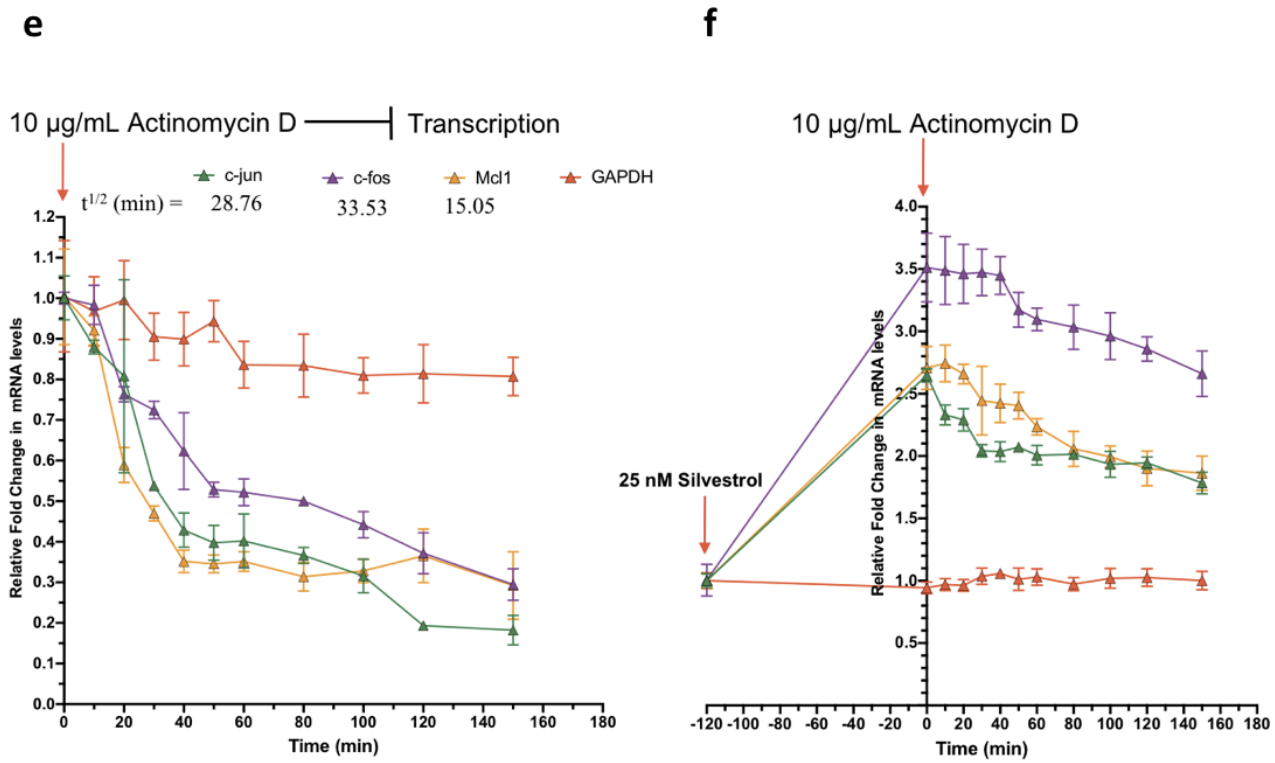


Figure 12. Silvestrol leads to increased mRNA stability. (a) Schematic showing possible mechanisms contributing to elevated mRNA levels in response to translation inhibition. **(b)** *c-myc* mRNA levels monitored during a time-course treatment with 25 nM silvestrol. mRNA levels are determined via RT-qPCR at 0.5, 2, 5 and 8h after treating with 25 nM silvestrol. N= 3 ±SEM. **(c)** *c-myc* mRNA half-life in JJN-3 cells. Cells were treated with 10 µg/mL actinomycin D and harvested at 10-20 mins increments to prepare RNA extracts for conversion to cDNA. RNA levels for *c-myc* and GAPDH quantitated using RT-qPCR and the half-life of *c-myc* is calculated using non-liner regression with Prism GraphPad. N= 3 ±SEM. **(d)** *c-myc* half-life is measured as described in (b), except that cells were treated with 25 nM of silvestrol for 2 h before the addition of actinomycin D. *c-myc* mRNA levels are measured before (-120 mins) and after (0 mins) silvestrol treatment. Actinomycin D is added to cells at t=0 (2 h after silvestrol treatment) and cells are harvested at 10-20 mins increments to quantitate changes in mRNA levels via RT-qPCR. N=3 ±SEM **(e)** The half-lives of *c-fos*, *c-jun* and *Mcl-1* mRNAs are determined by using non-linear regression with Prism GraphPad. N= 3 ±SEM. **(f)** The change in mRNA half-lives for the indicated transcripts after silvestrol treatment measured as described in (c). The half-life of GAPDH was not determined. N= 3 ±SEM

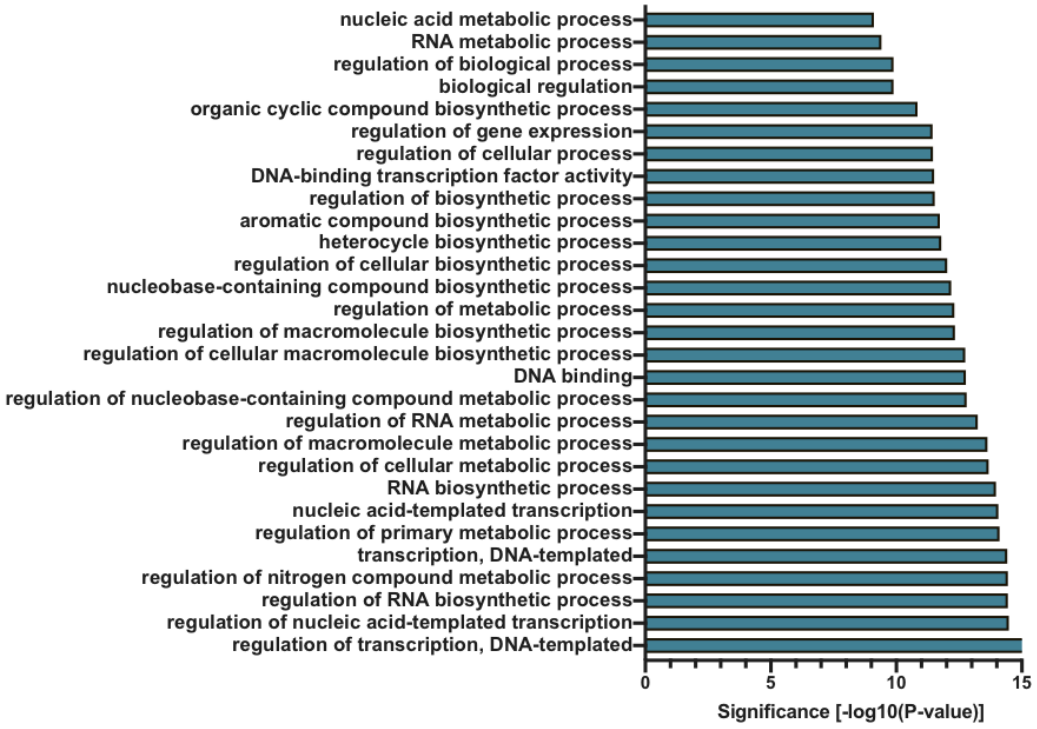
3.6 Identification of genes differentially expressed upon silvestrol/CHX treatment of JJN-3 cells.

Stabilization of mRNAs observed with silvestrol and CHX could be due to events such as *cis*-acting sequences in regulatory elements, translational suppression of short-lived mRNA nucleases, or protection by RNA-binding proteins [107-115]. Our data suggests that labile mRNAs that express immediate early genes are stabilized by silvestrol and CHX, including *c-myc*, *c-fos*, *c-jun* and *Mcl-1*. To determine if mRNA stabilization is specific to a subset of mRNAs or represents a more global phenomenon, we performed RNA-sequencing (RNA-seq) on RNA from JJN-3 cells to uncover changes in the transcriptional landscape upon a 2 h treatment with silvestrol (25 nM) or CHX (50 μM). We prepared RNA samples in triplicate from the two treatment groups and vehicle control. rRNA depleted barcoded libraries for paired-end reads were synthesized for sequencing using the PE150 Illumina platform. Approximately 50 million reads were obtained for each sample. In the initial analysis, 40% of the reads did not map to the human genome and upon further analysis, we found that these reads mapped to the Mycoplasma genome. Upon removal of these reads, we proceeded to use the remaining sequencing data to perform our analysis of interest. However, we caution that our findings may be impacted since Mycoplasma may significantly alter cellular functions, leading to changes in gene expression. Although both the vehicle and compound treated samples showed contamination, simply performing a differential gene expression analysis (DMSO vs compound) is not sufficient to distinguish between compound and Mycoplasma-mediated changes in gene expression profiles. Therefore, our results from RNA-seq data must be validated by repeating the experiments and conducting subsequent confirmatory studies.

The sequencing reads were aligned to reads to the Gencode version 32 transcriptome. A single representative transcript was chosen for each gene locus by selecting the principal isoform from the APRIS database. For genes with more than one isoform, a single transcript was selected based on the highest RNA-Seq TPM (transcripts per million). A differential gene expression analysis was performed for the silvestrol and CHX treatment groups versus DMSO using DEseq2. Differences in the number of aligned reads with an adjusted *P*-value (p_{adj}) > 0.05 were classified as non-significant and differences with an adjusted *P*-value (p_{adj}) < 0.05 were classified as

significant. The significant genes, as defined above, were classed as up/downregulated depending on whether the numbers of mapped reads were increased or decreased. The hits were defined as having a False Discovery Rate (FDR) ≤ 0.01, and a log2 fold change ≤ -0.5 or ≥ 1, respectively. These hits were then subjected to gene ontology (GO) analysis. The GO term most significantly enriched for both the silvestrol and CHX groups was: (GO:0006355) regulation of DNA-templated transcription (Fig. 13). Table 1 summarizes the top 5 most upregulated genes in both groups stratified by the adjusted *P*-value (p_{adj}) and classified according to their GO term. In each of the datasets, there is an upregulation of inflammatory response genes (DUSP-6 in silvestrol group, TNF in CHX group) which could likely be the result of mycoplasma contamination and must be validated in non-contaminated cells (Table 1). Other upregulated genes include regulators of transcription and cellular processes, which may positively modulate mRNA stability. The underlying mechanism by which these pathways play a role in mRNA turnover has yet to be determined.

a Silvestrol



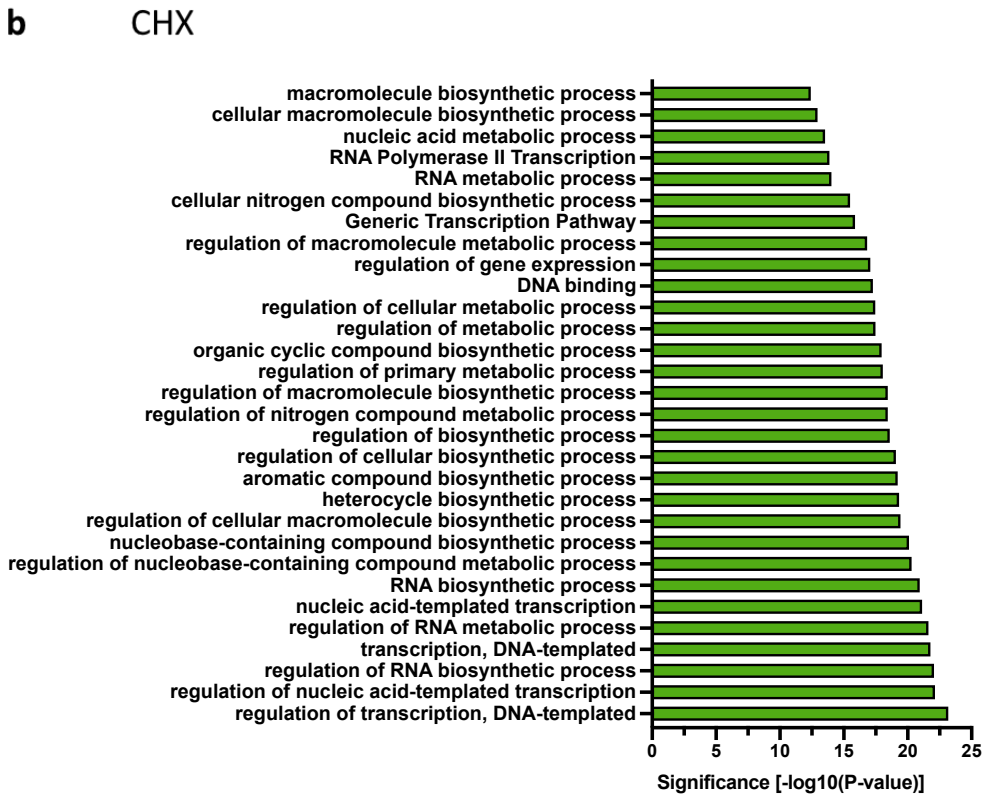


Figure 13. GO biological processes terms ranked by increasing –log10(*p*-value); significance.(a) GO terms for genes upregulated by silvestrol. **(b)** GO terms for genes upregulated by CHX. Transcripts within the FDR ≤ 0.01, and log2 fold change ≥ 1.

Upregulated Genes Silvestrol vs DMSO			**Upregulated Genes CHX vs DMSO**		
Gene	***Log2FC***	***GO Term***	***Gene***	***Log2FC***	***GO Term***
TXNIP	3.33	Regulation of transcription, DNA templated	TNF	6.33	TNF signaling pathway
DUSP6	2.52	Regulation of cellular metabolic processes	DKK1	5.67	Cellular biosynthetic processes
GPR132	2.44	Regulation of cellular processes	CD69	4.69	Metal ion binding
JUN	2.40	Regulation of transcription, DNA templated	TXNIP	4.43	Negative regulation of cellular responses
GDNF	2.22	Regulation of nucleic acid template transcription	EGR1	3.99	Regulation of transcription, DNA templated

Table 1. Top 5 most upregulated genes in silvestrol and CHX groups stratified by log2FoldChange (FC) and GO terms.

In order to correlate our RT-qPCR data with the RNA-seq data, we compared the fold change in *c-myc*, *c-jun*, *c-fos* and *Mcl-1* mRNA levels with silvestrol treatment previously determined by qPCR (Fig 12f) with that of the log2 fold change determined by RNA-seq analysis. The fold change in mRNA levels via RT-qPCR was determined using the ΔΔCT method whereas the log2 fold change from RNA-seq data was determined using DESeq2. DESeq2 performs an internal normalization where the geometric mean is calculated for each gene across all samples. It is important to note that since DESeq2 determines log2 fold change (log2FC), +1 log2FC is a 2-fold increase and -1 log2FC is a 2-fold decrease in gene expression. As summarized in Table 2, our results show that to a large extent, there is a correlation between RT-qPCR and RNA-seq data for *c-myc*, *c-fos*, *c-jun* and *Mcl-1*. Each of the transcripts is similarly upregulated in both experiments upon 2 h treatment with silvestrol.

Transcript	Fold Change (RT-qPCR)	Log2 Fold Change (DEseq2)
c-myc	3.51	1.25
c-jun	2.63	2.33
c-fos	3.53	0.97
Mcl-1	2.70	0.56

Table 2. Comparison of fold change in mRNA levels determined by RT-qPCR and RNA-seq (DESeq2). In the DEseq2 analysis, *c-myc*, *c-fos*, *c-jun* and *Mcl-1* each had a p-value lower than 0.05.

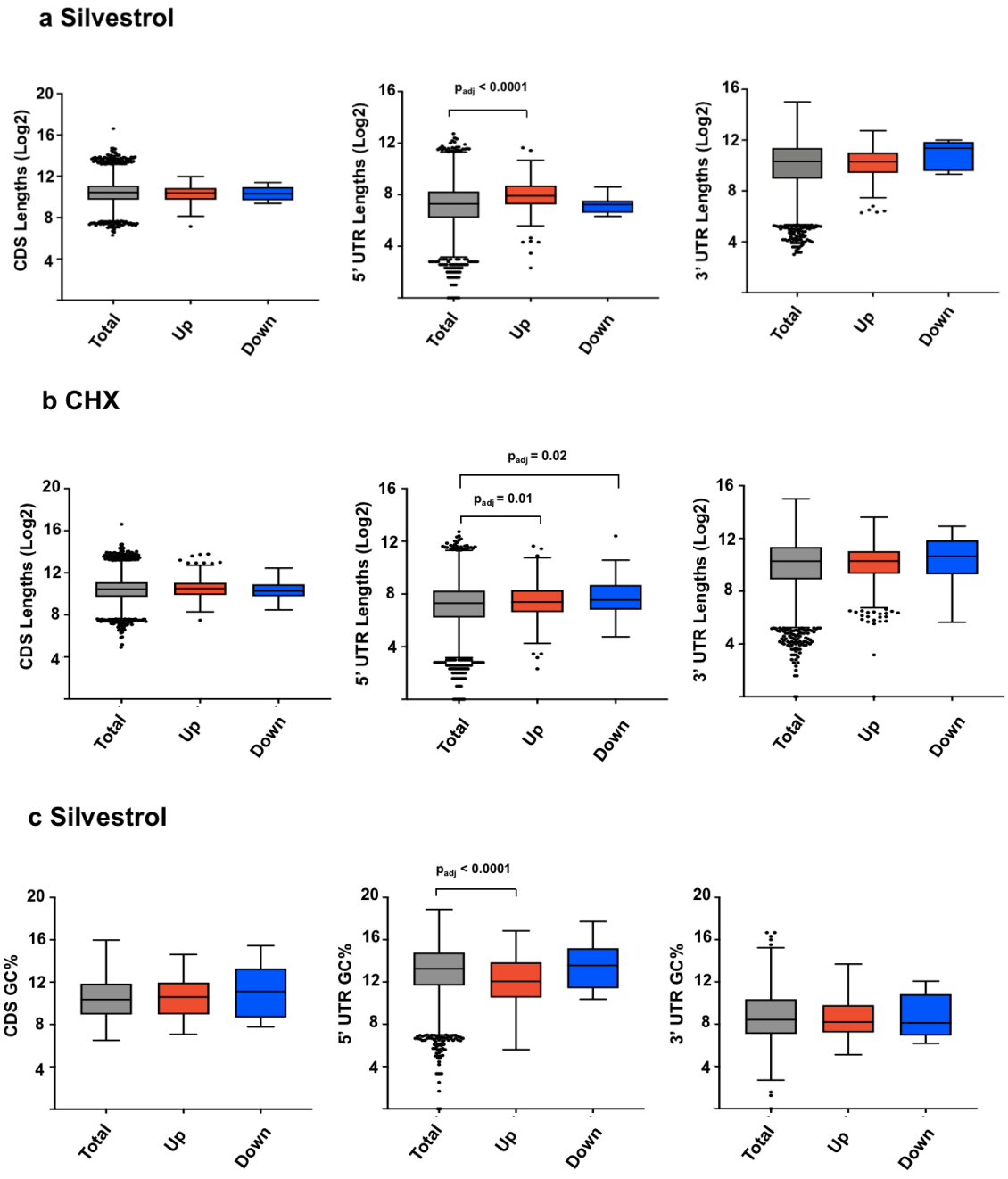
3.8 Genes upregulated by silvestrol/CHX treatment have longer 5' UTR with low GC content.

In order to identify elements that functionally modulate mRNA stability, we looked at the 5' UTR, 3' UTR and coding sequence (CDS) lengths and nucleotide composition. Although many studies identify variations within UTR lengths and composition that regulate mRNA stability, a systematic approach to predict this is still incomplete. It has been previously reported that long, structured 5' UTRs are typically more unstable and that *cis*-regulatory elements in the 3' UTR can either stabilize or de-stabilize transcripts[2]. Much of the work addressing mRNA stability determinants deal with mRNA decay signals in the 3' UTR, suggesting that the half-lives of most mRNAs are influenced by the region[133]. A few of these well characterized 3' UTR determinants

include protein-binding sites, which will be further discussed in Section 3.9. The correlation between CDS length and mRNA stability is less thoroughly established. Previous studies suggest that CDS length is not a significant determinant of mRNA stability in eukaryotes[172, 173, 174]. Subsequent studies, however, have reported a strong negative correlation between CDS (and ORF) length and mRNA stability in yeast[175] and human[176] cells. Additionally, the distinct fate of GC-rich and AU-rich mRNAs has been shown to correlate with a difference in protein yield resulting from both varying codon usage and CDS length[177]. AU-rich and GC-rich transcripts tend to follow distinct decay pathways and the targets of sequence-specific RBPs are also biased in terms of GC content[177]. Altogether, these results suggest an integrated view of post-transcriptional control in human cells where most regulation of inefficiently translated AU-rich mRNAs is mediated by RNA localization mechanisms, whereas control at the level of 5' decay applies to optimally translated GC-rich mRNAs[177].In order to determine if there is a significant correlation between length/GC content (in the 5' UTR, 3'UTR and CDS) of differentially expressed genes, we extracted the untranslated regions and coding sequences from up/downregulated genes ($p_{adj} < 0.05$) and compared their sequence lengths and GC content. Lengths of the CDS, 5' leader and 3' UTR regions for the silvestrol and CHX groups are each shown in Figs. 14a and b, respectively: total mRNAs, upregulated mRNAs (log2 fold change ≥ 1), and downregulated mRNAs (log2 fold change ≤ -0.5) are shown. In the silvestrol treatment group (Fig. 14a) the 5' UTR length is higher in upregulated genes ($p_{adj} < 0.0001$), with no significant difference in 3' UTR and CDS lengths compared to the total mRNA pool ($p_{adj} > 0.05$). Similarly, in the CHX treatment group (Fig. 14b), the 5' UTR length is higher in upregulated genes ($p_{adj} = 0.01$) and lower for downregulated genes ($p_{adj} = 0.02$), with no significant differences in the 3' UTR and CDS lengths.

Similarly, the %GC composition of the CDS, 5' leader and 3' UTR for the silvestrol and CHX group are each shown in Figs. 14c and d, respectively. Nucleotide base compositions of each transcript region for each treatment group are represented as percentages. In the silvestrol treatment group (Fig. 14c), upregulated mRNAs have a lower %GC in their 5' UTR ($p_{adj} < 0.0001$), with no significant difference in %GC in the 3' UTR and CDS. In the CHX treatment group (Fig. 14d), downregulated mRNAs had a higher %GC in their CDS ($p_{adj} = 0.0002$) and upregulated mRNAs had a lower %GC in their 5' UTR ($p_{adj} < 0.0002$). The %GC analysis in the 3' UTR was lower for upregulated mRNAs ($p_{adj} = 0.01$) and higher for downregulated mRNAs ($p_{adj} = 0.01$).

Overall, our results indicate that mRNAs upregulated by silvestrol treatment had longer 5' UTRs with lower %GC and mRNAs upregulated by CHX treatment had longer 5' UTRs and lower %GC in both their 5' and 3' UTRs.



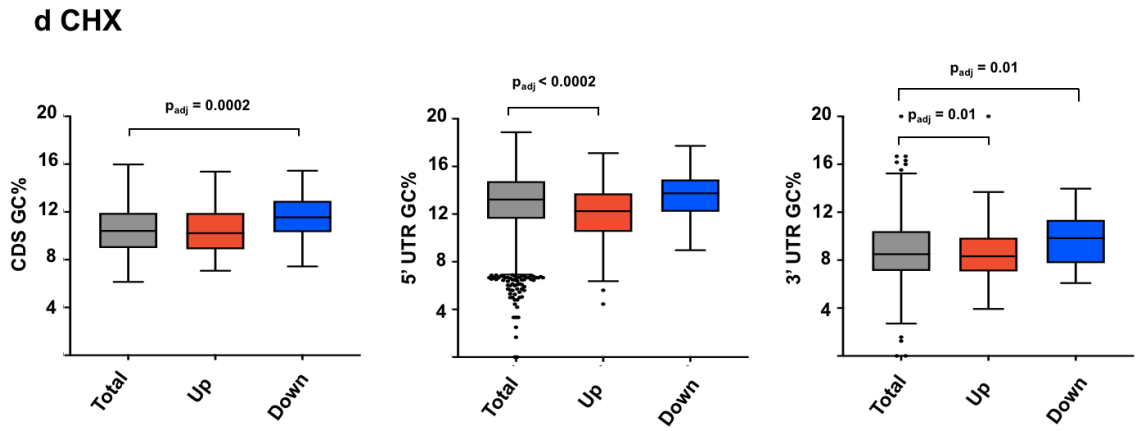


Figure 14. Length and nucleotide analysis of genes upregulated by silvestrol and CHX treatment. (a), (b) Lengths of CDS, 5' UTR and 3' UTR of differentially expressed genes with silvestrol and CHX treatment. Total indicates all mRNAs, up indicates upregulated mRNAs (log2 fold change ≥ 1) and down indicates downregulated mRNAs (log2 fold change ≤ -0.5). **(c), (d)** The GC% of CDS, 5' UTR and 3' UTR of genes up and downregulated by silvestrol and CHX treatment are shown. For length and GC% analysis, a one-way ANOVA with Tukey's multiple comparison test was used to calculate the adjusted *P*-values shown (p_{adj}).

3.9 mRNA stability determinants known for protein-binding are enriched in genes upregulated by silvestrol/CHX

In addition to *cis*-acting elements within transcript sequences, *trans*-acting factors (other than RNases themselves) also seem to influence mRNA decay[113]. Regulatory factors, that have a distinctive role, may mediate mRNA stability by virtue of function and not by simply acting as constitutive stabilizers or destabilizers. For example, if PABP were bound to all mRNAs with the same avidity, protecting them from degradation, and if the rate-limiting step in the decay of the PABP-bound mRNAs was deadenylation, the half-lives of these mRNAs would be identical, which is definitely not the case. It is therefore intuitive that the binding of one and more proteins likely varies between mRNAs, and is somehow regulated. It is possible that an mRNA is stabilized or destabilized as a result of the combined actions of more than one regulatory factor. There are known RNA-binding proteins that can be classed as *trans*-acting factors that influence mRNA stability, such as PABP which binds to the poly(A) tail and prevents deadenylation and mRNA degradation[130], AU-binding proteins[136] such as HuR (stabilizing) and AUF1 (destabilizing) which

bind to AU-rich regions in the 3' UTR, and ribonucelotide reductase (RR) mRNA binding proteins which bind to the 3' UTR of RR1 and RR2 mRNAs to increase their stability[178]. Given the central role of RBPs in regulating mRNA stability, we were interested in looking for RNA-binding motifs in our genes upregulated by silvestrol and CHX. This would allow us to predict the RNA-binding proteins likely to be associated with those motifs, and given the regulatory role of enriched RBPs, we would be able to correlate the upregulation of mRNAs with stabilizing RNA-binding proteins.

To determine if genes upregulated by silvestrol and CHX treatment shared known RNA binding motifs that may modulate mRNA stability, we performed a motif analysis to search for sequences in the 5' UTR, 3' UTR and CDS known to be recognized by RNA-binding proteins. We used the HOMER Motif Analysis software to screen for enrichment of known motifs (homer2) in the compound treated vs DMSO treated samples. We identified known motifs enriched in the CDS, 5' UTR and 3' UTR of upregulated mRNAs from both treatment groups and classified them as significant ($p < 0.01$) or insignificant ($p > 0.01$) with an FDR ≤ 0.01. The enrichment *P*-values were determined using a cumulative binomial distribution. For both treatment groups, the top 10 enriched motifs predominantly contained A-rich and U-rich elements in the 3' UTR, U-rich elements in the 5' UTR and no specific nucleotide enrichment was seen in motifs found in the CDS. Next, we used Tomtom (motif comparison tool) to analyze the top 10 enriched motifs in the CDS, 5' UTR and 3' UTR and identify binding sites for RNA-binding proteins. Interestingly, we found that more than one of the top 10 enriched motifs was a binding site for the same RNA-binding protein. For example, in the 3' UTR of upregulated mRNAs from the silvestrol treatment group, three different U-rich motifs were strong binding sites for HuR ($p = <0.0001$). We have summarized the top 5 enriched motifs with their most significant RNA-binding partner (based on p value) in Table 3 (silvestrol) and Table 4 (CHX). The tables show the top 5 enriched motifs for each transcript region and treatment, as well as its most significant RNA-binding protein (above of each motif logo). Interestingly, the silvestrol and CHX groups have shared motifs in their 5' UTR and 3' UTR, including a binding site for HuR (binds to ARE elements in the 3' UTR, stabilizes transcripts and actively recruits mRNAs to SGs and PBs)[127] and PABPC1 (also known to recruit mRNAs to RNP granules)[128]. In the CDS of enriched genes in both the silvestrol and CHX treatment groups, we identified a binding motif for YBX1, which has been implicated in cellular functions such as mRNA translation, localization and stability, transcriptional control, and

cell cycle modulation[129-132]. Cloning these motifs into reporter mRNAs and assessing their effects on turnover in the presence of translation initiation inhibitors is the important next step for validation.

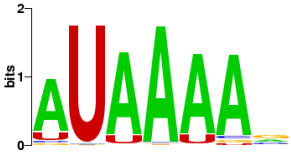
ENRICHMENT OF MOTIFS AND THEIR RBPs FOR UPREGULATED GENES		
SILVESTROL		
5' UTR	3' UTR	CDS
RBMS3 	PABPC1 	LIN28A 
SRSF1 	SART3 	SRSF1 
KHDRBS1 	HuR 	SRSF2 
PTBP1 	CPEB4 	YBX1 
RBMS3 	TIA1 	SRSF10A 

Table 3. Motif enrichment analysis reveals sites for RNA-binding proteins in genes upregulated by silvestrol treatment. Logos of motifs enriched in the CDS, 5' UTR and 3' UTR of upregulated mRNAs are displayed ($p < 0.01$) with an FDR ≤ 0.01. The enrichment *P*-values were determined using a cumulative binomial distribution. Tomtom (motif comparison tool) was used to analyze the enriched motifs in the CDS, 5' UTR and 3' UTR and identify binding sites for

RNA-binding proteins. The top 5 enriched motifs with their most significant RNA-binding partner ($p < 0.0001$) are shown.

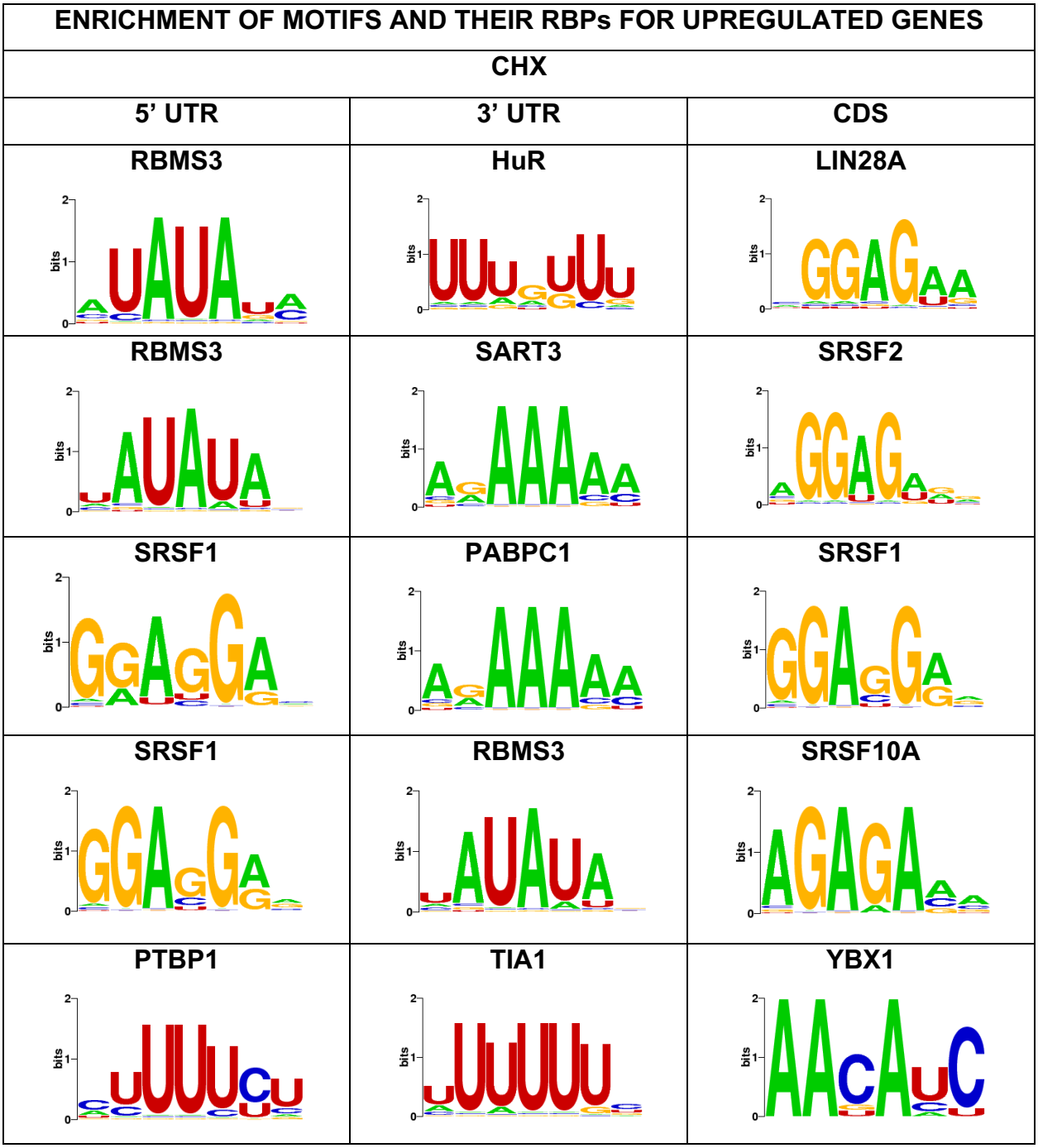


Table 4. Motif enrichment analysis reveals sites for RNA-binding proteins in genes upregulated by CHX treatment. Logos of motifs enriched in the CDS, 5' UTR and 3' UTR of upregulated mRNAs are displayed ($p < 0.01$) with an FDR ≤ 0.01. The enrichment *P*-values were

determined using a cumulative binomial distribution. Tomtom (motif comparison tool) was used to analyze the enriched motifs in the CDS, 5' UTR and 3' UTR and identify binding sites for RNA-binding proteins. The top 5 enriched motifs with their most significant RNA-binding partner ($p < 0.0001$) are shown.

DISCUSSION

The use of small molecule inhibitors to target protein synthesis is a powerful approach by which to dissect the process and identify potential feedback regulatory mechanisms. Over the years, the discovery and characterization of translation inhibitors has seen major advancements, shifting from targeting the elongation step (CHX, HHT, bruceantin, emetine) to the identification of potent selective compounds that target the initiation step (rocaglates, hippuristanol, pateamine A). The effect of some of these inhibitors on the translatome has been widely characterized by polysome and ribosome profiling, identifying the extent of mRNA translation as well as a "global snapshot" of all the ribosomes active in a cell at a particular moment. Although the effect of these compounds on cellular protein levels is well understood, there is little consensus on how they affect mRNA levels, which we uncovered in the work described in this thesis.

Regulation of mammalian mRNA stability is multi-faceted[134-141]. It plays a major role in gene expression in mammalian cells, affecting the rates at which mRNAs disappear following transcriptional repression and accumulate following transcriptional induction, as well as following their translation in the cytoplasm. mRNA stability depends on the activation or deactivation of pathways that are responsible for regulating mRNA decay. The majority of eukaryotic mRNA degradation pathways start with shortening of the poly(A) tail by 3'-5' exonucleases[185]. CCR4-NOT complex is a major deadenylase, and deadenylation is the first and hence, rate-limiting step of mRNA degradation. Following deadenylation, the 5' cap may be removed by decapping enzymes Dcp1-Dcp2 complex and the mRNA may be subsequently subjected to degradation by 5'-3' exonuclease Xrn1[185]. Alternatively, the exosome complex may degrade the mRNA in the 3'-5'direction. Additionally, there are several cytoplasmic RNA quality-control mechanisms that prevent the formation of aberrantly synthesized and potentially toxic proteins[185]. According to the error present in the mRNA, NMD, no-go decay (NGD) or non-stop decay (NSD) can be triggered.

NMD eliminates transcripts containing a premature termination codon, no-go decay (NGD) takes place when the elongation complex is blocked during translation (e.g., mRNA secondary structures), and transcripts lacking an in-frame stop codon trigger non-stop decay (NSD) (the latter can be generated by cryptic polyadenylation signals[185]). These mRNA degradation pathways play a central role in regulating mRNA stability, and must be considered when evaluating factors influencing stability.

In addition to mRNA degradation pathways, a multitude of exogenous factors, such as hormones, viruses and ion flux can influence mRNA stability. Sequence determinants of mRNA stability include *cis* determinants, for example the poly(A) tail[43, 151], 3' UTR elements (eg, histone mRNA 3' terminal stem loop[138], AU-rich elements[144, 146, 148], iron-responsive element[153], IGFII[133]), mRNA coding region, 5' UTR, and the presence and nature of the mRNA cap[154]. There exist *trans*-acting regulatory factors such as RNA-binding proteins that protect mRNAs from degradation, including poly(A)-binding protein (PABP)[130, 143], AU-rich binding proteins (AUBPs)[146], iron regulatory protein (IRP)[153] and coding region determinant binding proteins (*c-fos*[155, 156], *c-myc*[116]). In addition, there are *trans*-activating factors that regulate stability but are not known to bind to specific sequences within mRNAs, examples of which are beta-tubulin[157], histones[158], heat shock proteins[159]; virion host shut off protein of herpes simplex virus[142], and p27^{rex} of human T-cell leukemia virus[133].

Studies have suggested that external stimuli such as protein synthesis inhibitors can stimulate and stabilize mRNA levels by a process referred to as gene super-induction[105], which is postulated to be the result of prolonged expression of immediate early genes, which are transcriptionally regulated and respond rapidly to cellular stimuli[106]. In our study, we initially observed that upon translation inhibition by CR-1-31-B, *c-myc* mRNA levels were significantly upregulated (Fig. 9a). We examined other transcripts with short half-lives of ~30 mins and our results supported the notion that immediate early genes (for example; *c-Myc*, *c-fos*, c-*jun*, *Mcl-1*) were specifically 'super-induced' by silvestrol (Table 2) and CHX. Since the super-induction of immediate early genes by CHX has previously been attributed to increased mRNA stability[107, 108, 113, 115], we compared the half-lives of these transcripts in the presence and absence of silvestrol and found that the half-lives of the tested mRNAs were increased in the presence of compound (Fig. 12c, d). Although our results do not eliminate the possible contribution of increased transcriptional

output to the super-induction phenomenon we uncovered with rocaglates, they do define a major contribution of altered mRNA stability to the phenotype [133].

Although it has been established that there is a link between mRNA stability and translation, it is not yet fully understood. One of the general observations that demonstrates this connection is that most mRNAs are stabilized in cells exposed to translation inhibition, but different mRNAs might be stabilized to varying degrees for different reasons. A handful of mechanistic details for this phenomenon are known. For example, CHX prolongs *c-myc* mRNA half-life by decreasing the rate of deadenylation but does not affect degradation of the mRNA body[160]. In yeast cells, CHX has been shown to block decapping, which is an important initial step for mRNA decay[161]. Another key link between mRNA decay and translation is that mRNA half-lives are influenced by changes in mRNA architecture that affect translation. For example, changes in 5' UTR structure can affect translation initiation and alter the number of ribosomes migrating along the CDS, both of which influence mRNA half-life[133]. Continued translation is required to maintain the levels and/or activity of a *trans*-acting factor. For some mRNAs, translation elongation is an important factor influencing turnover. For example, beta-tubulin mRNA, whose half-life is regulated by the levels of tubulin monomers, is not destabilized by said monomers when elongation is inhibited[179]. Other mRNAs, however, may require ribosomes to translate a specific segment of the coding region for normal rates of decay. Full length *c-myc* transcript is stabilized 3-4 fold by elongation inhibitors[112] (eg. CHX) but *c-myc* mRNA lacking the C-terminal segment of the coding sequence is not stabilized by CHX[116]. This could possibly be due to the presence of a cleavage site for a ribosome-associated RNase, for which ribosomes must enter the region to juxtapose the RNase and its substrate.

Additionally, polysome distribution is likely an important determinant of mRNA stability which is why a translation inhibitor, by blocking initiation or elongation, could affect mRNA turnover by changing the polysome conformation. For example, histone mRNA levels are regulated by the cell cycle and mRNA levels peak only during DNA synthesis, and are destabilized after DNA synthesis is inhibited[180]. A 39 nt terminal stem loop located 40-50 nt downstream from the stop codon is necessary for proper mRNA regulation. If a large segment of about 500 nts is inserted between the stop codon and the stem loop, the mRNA is not properly regulated. Still, the unregulated mRNA retains the usual histone mRNA coding region with a 39 nt terminal stem-

loop, and could still generate protein[180]. It is plausible that the sequence inserted alters the distribution of the polysomes by increasing the distance between the translation stop codon and the stem-loop, that the normal regulatory signals are disabled. Similarly, when a mutation of the stop codon allows translation of the mRNA past the regular stop codon and into the 39nt terminal stem loop, the mRNAs are destabilized or are not properly regulated[181]. Perhaps ribosomes translating within what should be an untranslated region disrupt interactions between the mRNA and polysome-associated mRNA-binding proteins[181]. Our current understanding of the relationship between translation inhibition and mRNA stability is limited to the association between mRNA decay and deadenylation, *trans*-acting factors and polysome distribution.

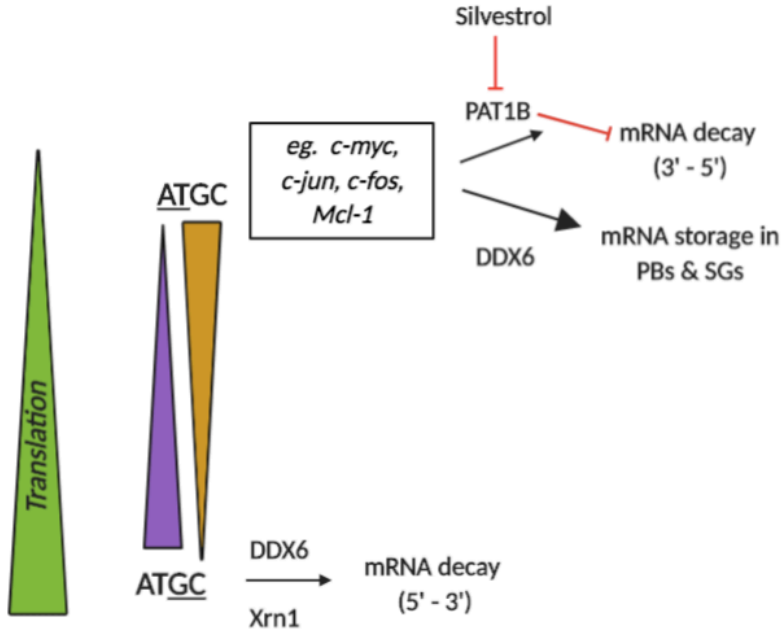
Given the intricacy of mRNA stability and the *cis*- and *trans*- acting regulatory factors influencing degradation, we were interested in identifying sequence elements and motifs within transcripts that are stabilized by translation inhibition by silvestrol and CHX. In an effort to obtain gene expression profiles of JJN-3 cells after a 2 h treatment with silvestrol and CHX, we performed RNA-seq but unfortunately, the sequencing revealed Mycoplasma contamination in our cell culture, adding a layer of complexity to the interpretation of data. Although all hits from sequencing data must be validated by repeating the experiment with an uncontaminated batch of cells, we proceeded to perform preliminary analyses. A differential gene expression analysis revealed that in samples that were treated with silvestrol and CHX, there is an enrichment of mRNAs with longer 5' UTRs (Figs. 14b, c). This is particularly interesting, since the link between 5' UTR length and mRNA stability is not well-defined. Although it is not fully clear whether 5' UTRs are major determinants of mRNA stability, sometimes they can dramatically affect mRNA half-life. In theory, the half-life of every mRNA can be affected by how its 5' UTR affects translational efficiency, and introducing secondary structure in the 5' leader region can influence this[181]. *c-myc*, yet again a classic example reflecting the complexity of mRNA regulation, is a transcript whose half-life depends on 5' UTR length in a translation independent fashion[147]. Mutational studies suggest that longer 5' UTR containing *c-myc* transcripts are 3 - 8 fold more stable than wild-type transcripts. Even more interestingly, this holds true only if the 5' UTR length is increased by the addition of immunoglobulin intron-derived sequences about 600 nts long[181, 147]. Since both the wild-type and immunoglobulin-intron sequence containing *c-myc* mRNAs have comparable translational efficiency, it indicates that the immunoglobulin intron sequences play a

direct role in stabilization[182]. These results are important since immunoglobulin rearrangements frequently occur at the *c-myc* locus and are drivers of over-expression and lymphomagenesis[182]. Although our results do not offer a complete understanding of the link between 5'UTR length and mRNA stability, they identify a significant correlation.

Determining length variance in genes differentially expressed by translation inhibitors is the tip of the iceberg. Given that our data has identified that upregulated genes have longer 5' UTRs, an important next step is to determine the Minimum Free Energy (MFE) of these 5' UTRs and predicted secondary structures. Previous studies in *S. cerevisiae* have suggested that mRNA secondary structures of endogenous 5′-UTRs have small but significant inverse correlation to protein abundance and ribosomal density[182]. These findings identify a clear association between thermodynamically stable secondary structures (lower MFE) and reduced protein levels for all folding segments, strongly suggesting that mRNAs with more structured 5' UTRs are translationally repressed[182]. As previously discussed, given the link between translation and mRNA stability, structured 5' UTRs may very well be implicated in the regulation of stability.

In addition to length analysis, we assessed GC content in the CDS, 5' UTR and 3' UTR of upregulated genes. The 5' UTR of genes upregulated by silvestrol and CHX had lower GC% compared to the DMSO group. Additionally, for the CHX treated sample, the CDS and 3' UTR of downregulated genes also had a higher GC% (Fig. 14c, d). It has recently been shown that GC-rich and AU-rich mRNAs have distinct fates, as a result of different protein yields and different codon usage and CDS length[177]. Thus, 5' mRNA decay appears to preferentially regulate mRNAs with optimal translation (mostly GC-rich), whereas mRNAs with lower translational efficiency (mostly AU-rich) are regulated by mRNA storage[177]. Interestingly, GC rich mRNAs are excluded from PBs and mostly controlled by a helicase DDX6 and the 5'-3' exonuclease Xrn1. In contrast, AU-rich mRNAs are enriched in PBs and SGs, controlled at the level of translation and their accumulation depends on DDX6 and its partner PAT1B[177]. DDX6 acts as an enhancer of decapping to stimulate mRNA decay, upstream of RNA degradation by the Xrn1 5'−3' exonuclease and PAT1B is a well-characterized direct DDX6 partner known for its involvement in mRNA decay[177]. Courel *et al.* (2019)[177] showed that GC-rich mRNAs are preferentially degraded by DDX6 and Xrn1. In addition to giving Xrn1 access to the 5' end, DDX6 unwinds GC-rich double-stranded regions over the entire length of the mRNA, facilitating Xrn1

progression. This model for post-transcriptional regulation by DDX6 and PAT1B in determining mRNA fate is summarized in a schematic overview in Figure 15. UPF1, another RNA helicase involved in mRNA decay, has also been shown to preferentially affect the decay of GC-rich mRNAs[184]. PAT1B showed a strong preference for AU-rich targets, including those containing AREs (AU-rich elements)[177]. Most ARE-BPs (AU-rich binding proteins) promote mRNA destabilization while some ARE-BPs, such as HuR[128] and AUF1,[185] for a subset of mRNAs, can be stabilizing. Altogether, these observations raise the possibility that ARE-BPs behave either as enhancers or inhibitors of PAT1B activity in mRNA decay. Moreover, Courel *et al.* (2019)[177] report that PAT1B's effect on mRNA stability in human cells relies prominently on 3' to 5' decay[177]. Given what is in the literature together with our finding that 5' UTRs of genes upregulated by silvestrol and CHX have low GC%, it is plausible that GC content plays a role in mediating mRNA decay rates in a manner that is PAT1B and DDX6 dependent.



15. Schematic representation recapitulating the features of mRNA post-transcriptional regulation depending on GC content. mRNAs with higher GC% yield higher protein levels due to codon optimization in their CDS, and are degraded 5'-3' by the combined efforts of DDX6 and Xrn1. mRNAs with low GC%, are enriched in PBs, as a translational regulation to store mRNAs with suboptimal codon usage in their CDS, they are degraded 3'-5' by a PAT1B dependent mechanism[177]. A possible mechanism by which AU-rich mRNAs as stabilized by silvestrol is the

inhibition of PAT1B, resulting in decreased 3'-5' mRNA decay and increased storage of mRNAs in RNP granules.

We were interested in exploring whether ARE-BPs act as enhancers or inhibitors of PAT1B mediated mRNA decay activity, leading to stabilization of AU-rich mRNAs in cells exposed to silvestrol or CHX. To achieve this, we extracted the 5' UTR, 3' UTR and CDS of upregulated genes and performed a known motif enrichment analysis using HOMER. This revealed an enrichment of known motifs, which we further analyzed to identify binding sites for known RNA-binding proteins. This approach allowed us to identify binding domains for RNA-binding proteins that could potentially contribute to RNA stability and colocalization (Table 3 and 4). There was a significant enrichment of AU-rich elements in the 3' UTR of upregulated genes, to which a number of proteins known to impact RNA localization to RNP granules were identified (HuR[128, 187] and TIA1[124]). As discussed earlier, PAT1B sensitive mRNAs have been shown to be AU-rich. Studies have investigated the correlation between the stability of mRNAs containing AREs and PAT1B[186, 187]. Upon siRNA mediated knockdown of PAT1B, a differential gene expression analysis using RNA-seq showed that ARE-containing elements were more frequent in upregulated mRNAs than in downregulated mRNAs[186]. Moreover, Courel *et al.* (2019)[177] used cross linking immunoprecipitation (CLIP) to document that 53% of upregulated mRNAs were targets of the AU-rich binding protein HuR. This indicates that PAT1B-dependent decay preferentially affects mRNAs containing AU-rich elements and HuR targets[186, 187], suggesting that HuR binding plays a role in stabilizing these transcripts. Our data shows that genes upregulated by translation inhibition have low GC%, and enrichment of AREs in the 3' UTR, and are significant targets of HuR. In theory, they should undergo PAT1B-mediated decay, unless PAT1B is a labile protein highly sensitive to translation inhibition. This raises the possibility that the upregulation of AU-rich mRNAs is due to inhibition of PAT1B, allowing them to escape mRNA decay pathways and become dramatically stabilized. Although this is speculative, it can be further investigated using Western blot analysis by probing for PAT1B following treatment with silvestrol/CHX for 2 h (JJN-3 cells).

Furthermore, an important regulatory role of HuR and TIA1(AU-BPs) is the recruitment of mRNAs to membrane-free loci called stress granules (SGs) that quickly assemble as a result of translation inhibition[128, 187, 124], and can be independent of eIF2α phosphorylation[193]. In fact,

isolation and characterization of SGs from mammalian kidney cells revealed that these RNP granules were enriched with HuR and TIA1 proteins, in addition to other RNA-binding proteins[192]. Interestingly, a significant link between SG formation and eIF4A inhibitors has previously been characterized[123]. In 2017, Slaine *et al*[123] reported that treatment with silvestrol causes SG formation in HeLa and Vero cells. Given that silvestrol induces SG formation, and our data suggests that mRNAs upregulated by silvestrol are enriched for HuR and TIA1 binding motifs, perhaps there is a correlation between mRNA stability and SG formation. In theory, it may be possible that upon translation inhibition, a subset of mRNAs is recruited to SGs for storage where it is protected from degradation. Although an attractive postulation, this does not offer any explanation for the stabilization of mRNAs by CHX, since CHX does *not* induce SGs[124]. To explore this further, single molecule fluorescence *in situ* hybridization (smFISH) can be employed to visualize the co-localization of *c-myc* mRNA with markers for SG such as G3BP1 or TIA1. If *c-myc* levels do indeed co-localize with SGs, siRNA mediated knockdown of G3BP1/TIA1 followed by a rescue experiment with cDNA overexpression may provide a direct correlation.

Our motif analysis also showed an enrichment of the RNA-binding motif for PTB1 (polypyrimidine tract binding protein 1) in the 5' UTR and YBX1 (Y-box binding protein 1) in the CDS of upregulated genes. These two RNA-binding proteins have been shown to function as *c-myc* IRES trans-acting factors (ITAFs) in cell lines derived from patients in MM[189, 190]. Although the existence of an IRES in the 5' UTR of *c-myc* is relatively controversial, studies have shown that *c-myc* can be translated in both a cap-dependent and cap-independent fashion[188]. It has been shown previously that in patients with multiple myeloma (MM) and in MM-derived cell lines, there is a C to T mutation in the c-*myc* IRES that increases IRES activity and the corresponding synthesis of c-Myc protein although it is not fully understood how this occurs[189]. Subsequent studies identified PTB1 and YBX1 as important ITAFs for *c-myc*'s IRES activity, and these proteins exhibited stronger (approximately 3.5- and 2-fold respectively) binding to the mutated version of the c-*myc* IRES (in vitro), exerting their effect synergistically to stimulate IRES activity of the mutant IRES 4.5-fold more than the wild-type version[190]. Importantly, it was also shown that there is a strong correlation between the expression of PTB1, YBX1 and *c-myc* in MM-derived cell lines[189, 190], suggesting that by reducing either PTB-1 or YBX1 protein levels it is possible to decrease *c-myc* expression and inhibit cell proliferation of MM-derived cell lines[190]. This allows

the possibility that elevated levels of *c-myc* in response to translation inhibition, are due to a stabilizing affect by the binding of PTB1 and YBX1 to the mRNA, protecting it from 5'-3' exonuclease mediated degradation and/or endonucleolytic attack. The validity of this theory depends on the stability of PTB1 and YBX1 proteins, and how long they persist in the cytoplasm after inhibition of protein synthesis. The first step to investigating this potential association is to perform a Western Blot analysis of PTB1 and YBX1 after a 2 h treatment with silvestrol and CHX (JJN-3).

Additionally, we also observed an enrichment of binding sites for PABPC1 which binds to the poly(A) tail while simultaneously interacting with eIF4G, circularizing the mRNA and potentially stimulating translation[42, 43]. Additionally, we found an enrichment of CPE motifs in the 3' UTRs of upregulated genes, which are preferentially bound by CPEB1 which recruits poly(A) polymerase to the mRNA, and thus triggers polyadenylation[191]. Perhaps upregulated mRNAs are subjected to delayed deadenylation after translation inhibition, and are therefore less susceptible to 3'-5' mediated decay. The mechanism by which these transcripts may escape deadenylation to become stabilized, remains to be explored.

In our work described here, we employed a chemical biology approach to identify a link between mRNA stability and translation in eukaryotic cells. We identified potential *cis*- and *trans*-acting factors involved in the of regulation mRNA stability, recognizing the degree of cross-talk between these mechanisms that contributes to its overall complexity. However, the exact mechanism by which mRNA stability and translation are connected, is far from elucidated. The next step towards deepening our understanding of this feedback regulatory-loop would be to identify the biomolecules that function as major players and link these two gene expression pathways.

CONCLUSION

Through mRNA stability assays, we identified that inhibition of translation by either silvestrol or cycloheximide is able to stabilize certain mRNA transcripts. Through a transcriptome-wide approach, we uncovered that the 'induction' or stabilization of transcripts was not specific to a certain class of mRNAs, but extended to a wide variety of transcripts. Upon a deeper look at the mRNA architecture of enriched genes, we identified that seemingly more stable mRNAs had longer 5' UTRs and overall low GC%. Furthermore, we tentatively identified common RNA-binding domains amongst upregulated genes, providing insight into the cellular arrangement of specific mRNAs in mRNP complexes. Although our transcriptome data must be validated, it directs attention towards a set of factors that are implicated in regulating mRNA stability. Our study attempts a systematic approach at deepening our understanding of the pathways regulating mRNA stability and probing the link between mRNA stability and translation.

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