

Defining the translational landscape of MYC-driven cancer cells in response to therapeutic targeting of the ribosome

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Abstract

Recent studies by our group and others have demonstrated that oncogene-driven hyper-activation of ribosome synthesis and activity is a vulnerability that can be targeted for cancer treatment. Specifically, combined inhibition of ribosome synthesis using the Pol I inhibitor CX-5461 and mTORC1-dependent mRNA translation using everolimus (EV) synergistically improved survival benefit of a MYC-driven mouse model of B-lymphoma [1]. Despite this promising outcome, a few questions remain: 1) what are the molecular mechanisms that trigger the pro-death pathways in these lymphoma cells in response to CX-5461-everolimus co-treatment (CX-5461+EV); 2) what mechanisms confer resistance to this combination therapy; 3) how can we improve the efficacy of this ribosome targeting therapy. Since both inhibitors target the ribosome, this thesis applies poly(ribo)some profiling analysis as the principal technique to address these research questions.

mRNA translation is an important step in gene expression and can be examined in a genome-wide and high-throughput manner using polysome profiling analysis. Firstly, multiple aspects of this method were optimised to enable robust characterisation of the cellular translational landscape in this MYC-driven model of B-lymphoma (Chapter 3). Polysome profiling analysis was then utilised to evaluate changes in the translome of CX-5461+EV-treated MYC-driven B-lymphoma cells *in vivo* (Chapter 4). The analysis revealed that the synergistic improvement in therapeutic efficacy in response to CX-5461+EV treatment is due to selective reduction in the translation of mRNAs encoding component of translational apparatus, which is associated with decreased translation efficiency of metabolism-related mRNAs.

We have also demonstrated that the resistance to CX-5461+EV treatment was not due to the lack of on target activity of the drugs. Rather, it is due to a translation-driven activation of a metabolism-dependent, pro-survival response via the cAMP-EPAC1/2-Rap1 pathway. Importantly, the inhibition of

this pro-survival pathway by the anti-diabetic drug metformin, which inhibits energy metabolism, re-sensitise the CX-5461+EV-resistant cells to CX-5461+EV *in vitro* and *in vivo*. Together, this thesis highlights the role of translational changes in drug response and resistance development in the context of MYC-driven cancer. Furthermore, this work also identifies the potential of combined therapeutic targeting of the ribosome and metabolism for the treatment of MYC-driven haematological cancers.

Declaration

This is to certify that:

1. This thesis comprises only my original work towards the Ph.D. except where indicated in the Preface.
2. Due acknowledgement has been made in the text to all other material used.
3. The thesis is less than 100,000 words in length, exclusive of tables, maps, bibliography and appendices.

Eric Purwadinata Kusnadi

August 2018

Preface

The majority of the work described in this thesis was designed, performed and analysed by the candidate under the supervision of Prof Richard Pearson, Dr Jian Kang and Dr Katherine Hannan.

Dr Jennifer Devlin, Ms Natalie Brajanovski (Peter MacCallum Cancer Centre (PMCC), Australia), Mr David De Souza, Dr Vinod Narayana and Dr Dedreia Tull (Bio21 Institute, Australia) provided material and/or technical support. *In vivo* animal experiments were conducted with the assistance of Dr Carleen Cullinane and her team (Translational Research Laboratory, PMCC, Australia) and PMCC's Animal Facility staff members. cDNA library preparation and RNA sequencing were performed by the Molecular Genomics Core Facility (PMCC, Australia). Bioinformatics analysis was performed with the assistance of Ms Anna Trigos, Dr David Goode (PMCC, Australia) and A/Prof Ola Larsson (Karolinska Institutet, Sweden). Prof Malcolm McConville (Bio21 Institute, Australia), Prof George Thomas (IDIBELL, Spain), Prof Ross Hannan and Prof Grant McArthur (PMCC, Australia) assisted with study design.

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Kang, J., **Kusnadi, E.P.**, Ogden, A., Hall, M.N., Hicks, R.J., Hannan, R.D., Hannan, K.M., and Pearson, R.B., 2016, Nutrient-dependent regulation of rDNA transcription requires mTORC1-S6K1 and MYC signalling, *Oncotarget*, 7(31), 48887-48904.

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Wen, V.W., Somers, K.S., Middlemiss, S.M., Lim, J., Clifton, M.M., Kwek, C., **Kusnadi, E.P.**, Chudakova, D.A., Gudkov, A.V., Hannan, R.D., Pearson,

R.B., Norris, M.D., Haber, M., Henderson, M.J., A novel small molecule (CCI-006) that kills a subset of MLL-rearranged leukemia cells by inducing mitochondrial dysfunction (*under review at Experimental and Molecular Medicine*)

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No work presented in this thesis has been submitted for any other qualification or performed prior to candidature enrolment

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Chapter 1:

Introduction

Chapter 1: Introduction

Sections of this chapter were published as a review article:

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1.1 mRNA translation and cancer

mRNA translation is the process by which proteins are synthesised from mRNA via the orchestrated actions of the ribosomes, a range of auxiliary proteins termed translation factors and transfer RNAs (tRNA) [2]. Exquisite regulation of mRNA translation by a network of signalling pathways allows a normal, untransformed mammalian cell to promote protein synthesis and biomass accumulation in favourable environments. However, dysregulation of key biological processes controlling mRNA translation is often associated with the development of various pathological states including cancer [3-5]. While traditionally protein synthesis is considered as an “undruggable” housekeeping process, recent studies have demonstrated that numerous strategies that inhibit the synthesis of ribosomes, as well as its function in translating mRNAs into proteins, have a therapeutic window in the clinic for cancer treatment [1, 6-8]. This thesis will examine the hypothesis that there are coordinated changes in the cancer cells’ translome (i.e. a landscape of the entirety of mRNAs that are associated with and being translated by the ribosomes) in response to small molecule inhibitors that target ribosome synthesis and function, specifically in the context of oncogene-driven haematological malignancies.

1.1.1 Role of translational control in the regulation of gene expression in cancer

Even though cells in multicellular organisms share a common genome, they possess mechanisms to fine-tune the regulation of gene expression in order to respond appropriately to a diverse range of endogenous and exogenous stimuli and maintain cellular and therefore organismal homeostasis. In addition to the regulation at the level of gene transcription, there are also coordinated post-transcriptional regulatory mechanisms to modulate mRNA splicing, export, stability and translation.

The application of transcriptomics technologies, such as DNA microarray and mRNA sequencing (RNAseq), has facilitated extensive studies evaluating changes in the steady-state genome-wide mRNA levels in various cancer cell lines, models and patient samples, taking into account both mRNA synthesis and degradation [9-11]. However, these studies also noted that often there was a poor correlation between changes in the steady-state mRNA abundance and that of the corresponding protein [10-12]. These differences are due to a number of post-transcriptional modulations such mRNA export, mRNA splicing, mRNA translation and protein degradation, among which the regulation at the level of mRNA translation has been shown to modulate the expression levels of approximately 50% of the proteome [11-13]. These modulations can affect most mRNA transcripts in the cell (i.e. general) or only a specific subset of mRNA transcripts (i.e. selective) [14-16]. Importantly, mRNA translation, as well as other post-translational mechanism such as protein degradation, is instrumental to induce rapid changes in the cell's protein content (i.e. proteome) without the need for *de novo* DNA transcription [12, 14, 16]. Thus it is critical to evaluate alterations in cellular translational landscape in order to acquire a more complete picture of the outcomes of any changes in the gene expression program in response to the stimuli of interest.

1.1.2 Molecular mechanism of mRNA translation

The highly conserved mechanism of mRNA translation is comprised of four phases: initiation, elongation, termination and ribosome recycling [2, 17]. A salient hallmark of a vast majority of eukaryotic mRNAs is the 5' terminal 7-methylguanosine (m⁷G) cap structure, which not only protects the mRNA from 5' exonucleolytic degradation, but also more importantly facilitates “cap-dependent” mRNA translation [2, 16]. In the initiation phase of cap-dependent translation, the eukaryotic translation initiation factor (eIF) 4F complex and poly-A tail binding protein (PABP) binds to the 5' m⁷G cap and the 3' poly-A tail respectively, to “activate” and circularise the mRNA [2, 3]. The eIF4F complex is critical for directing the loading of the 43S pre-initiation complex (PIC) to the 5' cap: RNA helicase eIF4A unwinds secondary structures in the 5' untranslated region (5'UTR), the cap-binding protein eIF4E interacts with the 5' cap structure, and the scaffolding protein eIF4G facilitates the binding of a pre-assembled 43S PIC to the 5' cap [17, 18]. The 43S PIC is comprised of the 40S ribosomal subunit, the ternary complex (TC: initiator methionyl-transfer (t) RNA (Met-tRNA_i)-eIF2-GTP), eIFs 1, 1A, 3 and 5 [17, 19]. The binding of the 43S PIC to the 5' end of the circularised mRNAs forms a 48S PIC (Fig. 1.1). The 48S PIC scans the mRNA for the start codon triplet (AUG). When the AUG codon occupies the P (peptidyl) site of the 40S ribosome subunit and matches with the anticodon of met-tRNA_i, guanine triphosphate (GTP) hydrolysis occurs and results in the ejection of eIF1 and eIF2-GDP [19, 20]. The eIF5B-GTP is then recruited and facilitates the joining of the large 60S ribosomal subunit to generate the 80S ribosome [21], which then proceeds to the conserved translation elongation step. Subunit joining triggers GTP hydrolysis and the release of eIF5B-GDP and the remaining eIFs [19] (Fig. 1.1).

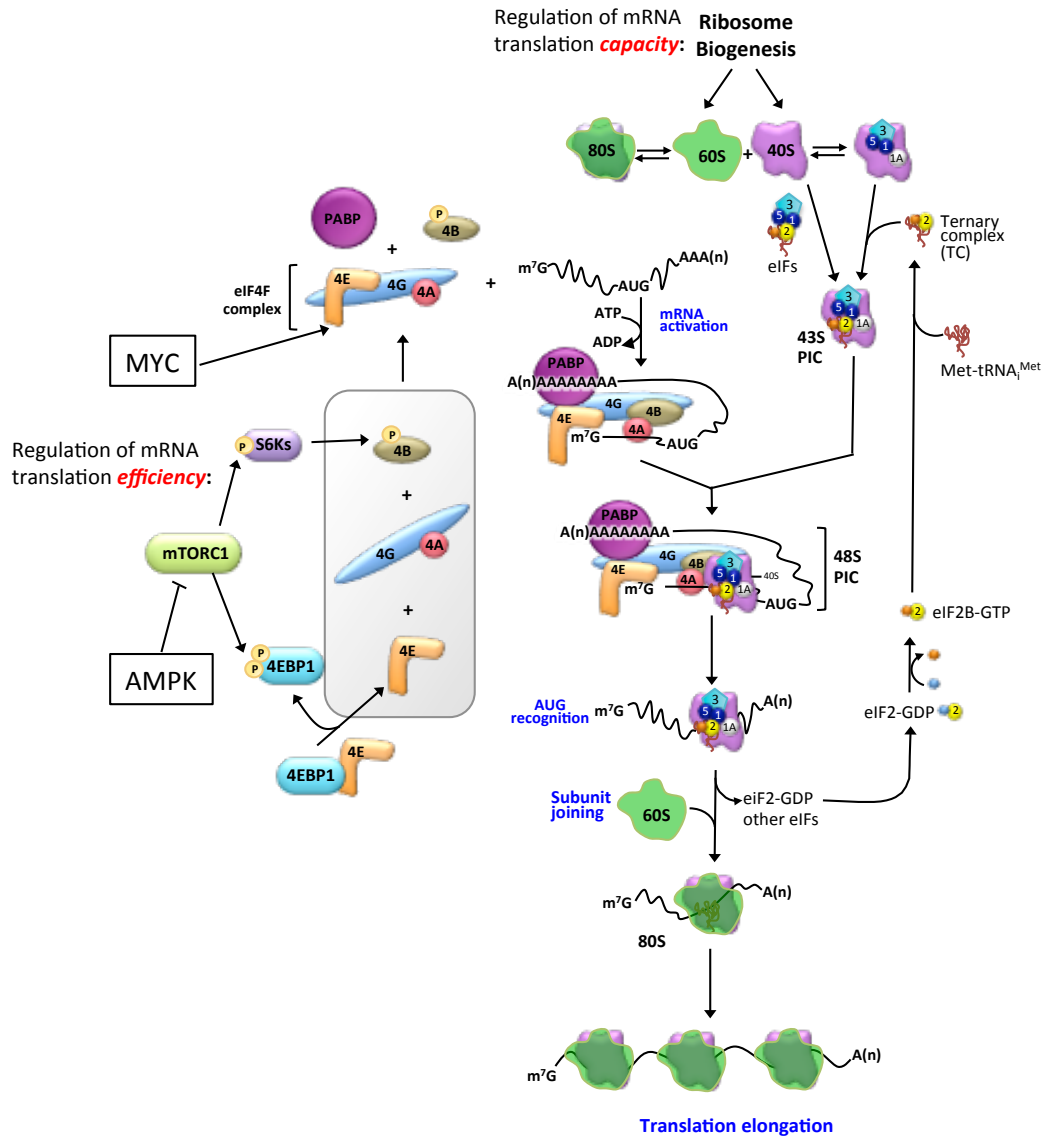


Figure 1.1: Schematic of the eukaryotic cap-dependent translation initiation

mTORC1, a master regulator of cellular growth, modulates translation efficiency via S6Ks and 4EBPs. MYC promotes the transcription of eIF4E and AMPK inhibits mTORC1 when cellular AMP:ATP ratio is high. Ribosome biogenesis influences cellular translation capacity. The 48S pre-initiation complex (PIC) comprises of the ternary complex (TC), 40S ribosomal subunit, eukaryotic translation initiation factors (eIFs) and circularised mRNAs. When the AUG codon occupies the P (peptidyl) site of the 40S ribosome subunit and matches with the anticodon of met-tRNA_i the large 60S ribosomal subunit is recruited to form the 80S ribosome, which then proceeds to the translation elongation step. In this schematic, GTP is illustrated as an orange ball and GDP as a blue ball.

At the end of the translation initiation phase, or the start of the translation elongation phase, the Met-tRNA is base-paired with the start codon in the P-site and the next codon of the open reading frame (ORF) is in the A (acceptor) site of the 80S ribosome [22] (Fig. 1.2). The GTP-bound eukaryotic elongation factor (eEF) 1A binds the cognate aminoacyl-tRNA of the next codon and directs the tRNA to the A site [23]. Correct codon recognition by the tRNA leads to GTP hydrolysis and the release of eEF1A-GDP. This is followed by the placement of the aminoacyl-tRNA into the A site and formation of a peptide bond with the P site peptidyl-tRNA (in this case methionyl-tRNA) in the peptidyl transferase centre (PTC) [23, 24]. After a peptide bond is formed, a ratcheting movement of the ribosomal subunit and recruitment of eEF2 translocates the tRNAs in the A and P sites into the P and E (exit) sites, respectively [23]. The “empty” tRNA is released from the E site and the next eEF1A-GTP-aminoacyl-tRNA is positioned in the A site [25]. The cycle continues until a stop codon (UAA, UAG or UGA) enters the A site.

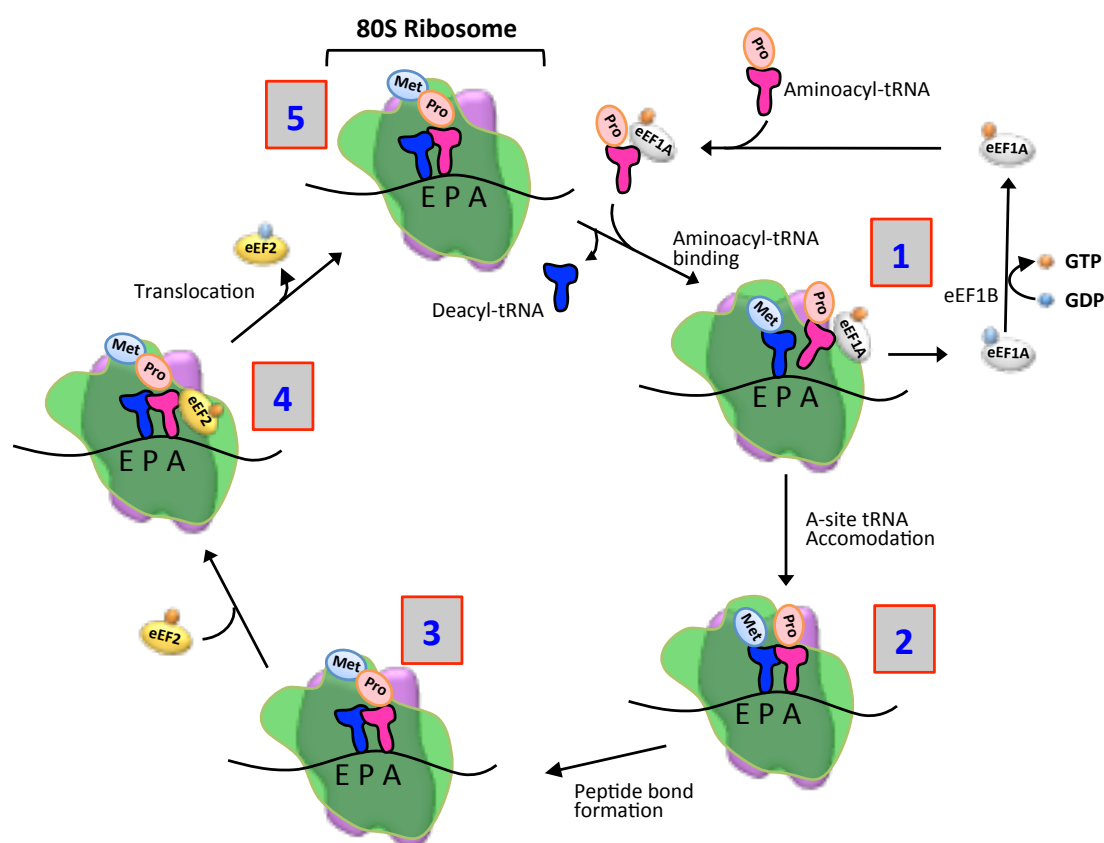


Figure 1.2: Schematic of the eukaryotic translation elongation

Starting at number 1, the Met-tRNA_i is base-paired with the start codon in the P-site and an eEF1A-GTP-aminoacyl-tRNA ternary complex binds the aminoacyl-tRNA to the 80S ribosome in the aminoacyl (A) site of the small subunit. After eEF1A-GDP is released, the aminoacyl-tRNA is accommodated into the A site (2). Following peptide bond formation (3), a ratcheting movement of the ribosomal subunit and recruitment of eEF2 translocates the tRNAs in the A and P sites into the peptidyl (P) and E (exit) sites, respectively (4). After eEF2-GDP and deacylated tRNA is released (eEF2 does not require an exchange factor), the ribosome is now ready for the next cycle of elongation (5) and binding of the appropriate eEF1A-GTP-aminoacyl-tRNA to the A site. In this schematic, GTP is illustrated as an orange ball and GDP as a blue ball.

In the termination step, the stop codon is recognised by the amino-terminal domain of the eukaryotic release factor (eRF1), which also interacts with eRF3-GTP via its carboxy-terminal domain [26, 27]. Stop codon recognition triggers GTP hydrolysis and eRF3-GDP is released, then ATPase ATP-binding cassette E1 (ABCE1) binds to eRF1 to facilitate the release of the newly synthesized polypeptide [28]. ATP hydrolysis by ABCE1 drives the dissociation of the ribosomal subunits to be recycled [29, 30]. Dissociation of the last deacetylated tRNA and mRNA is facilitated by ligatin/eIF2D and a pair of multiple copies in T-cell lymphoma/density regulated (MCT-1/DENR) proteins [23, 31]. Finally, the initiation factors eIF1, eIF1A and eIF3 again bind to the 40S subunit and after the 43S PIC is assembled, it is ready for another round of translation [23, 32].

1.1.3 Regulation of mRNA translation

Although all phases of translation described in chapter 1.1.2 are highly regulated, the majority of translational control is directed at the rate-limiting initiation step [2]. Translational control can impact on mRNAs on a global level or specifically on a particular set of mRNAs. On a global level, translation efficiency is affected by the bioavailability or phosphorylation (activity) status of certain eIFs. For instance, in response to endoplasmic reticulum (ER)

stress or nutrient deprivation, the α -subunit of eIF2 [33] is phosphorylated by PKR (interferon-induced, double-stranded RNA-activated protein kinase), PERK (PKR-like endoplasmic reticulum (ER) kinase) or GCN2 (general control non-derepressible 2) [34]. Phosphorylation of eIF2 α by these kinases on Ser51 leads to the inhibition of the guanine nucleotide exchange factor (GEF) activity of eIF2B, and thus prevents assembly of TC and global translation initiation [2, 33, 34].

Cellular signalling pathways often mediate translational control of specific pools of mRNAs. In particular, the mammalian target of rapamycin complex 1 (mTORC1) [35] and the mitogen-activated protein kinase (MAPK) [36] pathways have been extensively shown to be important pathways that regulate the translation efficiency of specific mRNAs in response to different extracellular and intracellular stimuli.

Focusing on the mTORC1 pathway, it controls the activity of the proto-oncogene eIF4E, which is rate limiting for cap-dependent translation of all mRNAs and the best characterised of all eIFs [37, 38]. mTORC1 consists of the conserved Ser/Thr kinase catalytic subunit mTOR, the scaffolding protein regulatory-associated protein of TOR (Raptor), the positive mTORC1 modulator mammalian lethal with sec-13 protein 8 (mLST8), and the two inhibitory subunits proline-rich AKT (Akt mouse thymoma) substrate 40 kDa (PRAS40) and Dep-domain mTOR-interacting protein (Deptor) [35]. Upstream of mTORC1, the phosphoinositide-3 kinase/AKT (PI3K/AKT), the Ras-related small GTP-binding proteins (RagGTPases) and adenosine monophosphate (AMP)-activated protein kinase (AMPK) pathways can modulate mTORC1 activity in response to growth factors, hormones or altered cellular energetic status [35, 39]. On activation, mTORC1 phosphorylates the 4E-binding proteins (4E-BPs: 4E-BP1, 4E-BP2 and 4E-BP3 in mammals) (Fig. 1.1), which bind to the same site as eIF4G on eIF4E [40]. The binding of 4E-BP to eIF4E prevents the assembly of the eIF4F complex and the initiation of cap-dependent mRNA translation [40] (Fig. 1.1). Phosphorylation of residues corresponding to Thr37 and Thr46 on human 4E-BP1, primes for the subsequent phosphorylation of Ser65 and Thr70, which promotes the

dissociation of the 4E-BPs from eIF4E, followed by the formation of the eIF4F complex and the initiation of mRNA translation [40, 41].

Importantly, while the depletion of 4E-BPs or expression of a non-phosphorylatable mutants of 4E-BP1 in cells only had a minimal effect on global translation [42, 43], in contrast it strongly affected the translation efficiency of specific subset of mRNAs termed “eIF4E-sensitive mRNAs” [44]. These transcripts possess a long, GC-rich and highly structured 5'-untranslated regions (5'UTR) rendering them strongly dependent on the activity of the eIF4F complex [44, 45]. mRNAs encoding proteins that are important in the regulation of survival and proliferation including MYC, VEGF, cyclin D1 and Pim kinases are some examples of eIF4E-sensitive mRNAs, whereas mRNAs encoding “housekeeping” proteins such as actins and tubulins are eIF4E-insensitive with shorter 5'UTRs and are less sensitive to changes in the levels of eIF4F [4, 38, 43, 45].

Aside from the 4E-BPs, another well-characterised downstream effector of mTORC1 is the ribosomal protein S6 kinase (S6K) [35, 46] (Fig. 1.1). There are two S6K variants in mammals, S6K1 and S6K2, and they share a high degree of homology [46, 47]. Both of them exist in two isoforms: p70 or p85 for S6K1 and p54 or p56 for S6K2 due to the presence of alternative translation initiation sites within the same mRNA [46, 47]. p70S6K1 is the more ubiquitously expressed and the only one that is predominantly cytoplasmic of the four [48]. The activation of the kinase domain of p70S6K1 requires an initial phosphorylation by mTORC1 in its hydrophobic motif (Thr389 in human p70S6K1). This allows the docking of phosphoinositide-dependent kinase 1 (PDK1), which then phosphorylates p70S6K1 in the activation loop (Thr229 in human p70S6K1) [48].

p70S6K1 regulates translation by phosphorylating proteins such as eEF2 kinase (eEF2K) [49], eIF4B [50], and the tumour suppressor programmed cell death 4 protein (PDCD4) [51]. Translation elongation is inhibited when eEF2 is phosphorylated by eEF2K, however this suppression is relieved when p70S6K1 phosphorylates eEF2K on Ser366 [49]. eIF4B is an ancillary factor that stimulates the RNA helicase activity of eIF4A, and when it is

phosphorylated by p70S6K1, this promotes its interaction with eIF3 and thus the assembly of the PIC [50] (Fig. 1.1). As for PDCD4, which competes with eIF4G for binding to eIF4A, phosphorylation by p70S6K1 on its Ser67 and Ser457 residues triggers its degradation via the action of E3-ubiquitin ligase SCF^{βTrCP1} [51]. Finally, it has been shown that p70S6K1 enhances the translation efficiency of newly spliced mRNAs when it is recruited to the exon junction complex (EJC) by its substrate and binding partner SKAR (also known as polymerase delta-interacting protein 3, POLDIP3) [52, 53].

Importantly, the proto-oncogene MYC (further details in chapter 1.2.2.2) also promotes the transcription of genes encoding eIF4E, thereby regulating the capacity of the cell to initiate mTORC1-4EBPs-dependent, eIF4F-mediated mRNA translation [54, 55]. Thus, this link between MYC and the mTORC1-4EBPs-eIF4F axis suggests that mRNA translation is a viable therapeutic target to treat MYC-driven cancers [1, 7, 54-56].

1.1.4 Techniques for genome-wide analysis of mRNA translation

Over the past two decades, there has been a rapid increase in the appreciation and evidence that translational regulation plays a pivotal role in the control of gene expression [12, 14]. Techniques that enable allow high throughput, unbiased characterisation of the translome have been developed, allowing us to compare gene expression networks in normal versus cancer cells. The most commonly used methodologies to evaluate the efficiency of the cell's translational activities on a genome-wide scale are a) polyribosome (polysome) profiling [57], the traditional “gold standard approach” developed in the 1960s, and b) ribosome profiling [58], which is also known as ribosome footprinting.

As the majority of the translational control occurs at the rate-limiting initiation step, virtually all translational responses that alter translation efficiency will therefore alter the ribosome density on a particular mRNA [2, 17]. The first step of polysome profiling analysis is to separate the polysomes, that is, actively translated mRNAs associated with several ribosomes, from free

RNAs, ribosomal subunits and single 80S ribosomes (monosomes) by sedimenting cytosolic extracts using sucrose density gradient ultracentrifugation. The ultracentrifugation separates the mRNAs based on the number of bound ribosomes, allowing the fractionation and isolation of a pool of actively translated, “heavy-polysome”-associated mRNAs that are bound by more than three ribosomes, which is the commonly used cut-off for analysis of differential translation. The mRNA composition in the pooled heavy-polysomal fractions is determined by DNA microarray or RNA-sequencing (RNA-seq). It is also essential to obtain the data on cytoplasmic (“steady-state”) RNA levels in parallel to normalise differences in the abundance of polysome-associated RNAs and identify modes of gene expression regulation (will be elaborated further in Chapter 3.1) mediating cellular response(s) in question. This approach has been applied to evaluate global changes in the translome in response to endoplasmic reticulum (ER) stress [59], hypoxia [60], apoptosis signalling [61], DNA damage [62] and drug treatment [63]. In addition, polysome profiling analysis was utilised to investigate translational reprogramming that occurs during cell differentiation [64], to investigate the impact of mutant and overexpression of key translation factors in yeast [65] and mammalian cells [66], and to study the role of non-coding RNAs in mRNA translation [67, 68].

Another commonly used translomics method is ribosome profiling, which is based on RNA-seq analysis of ribosome-protected RNA fragments (RPFs) that remains after the cell lysate is treated with RNase I [58]. The main strength of this method is that it provides information regarding the positions of the ribosomes on an mRNA species to near nucleotide resolution. Such detailed positional information allows for the discovery of novel regulatory elements along the mRNA such as upstream open reading frames (uORFs) [58, 69], alternative initiation sites and non-AUG start codons [70].

In addition to these two techniques, there are emerging translomics approaches such as ribosome-affinity purification (RAP) [71, 72] and proximity-specific ribosome profiling [73]. RAP methodology includes the expression of affinity-tagged ribosomal proteins in the 60S ribosomal subunit, which can be controlled by tissue-specific promoter [71, 72]. This allows the isolation of

tagged ribosomes that are specifically expressed in cells or tissue of interest followed by RNA extraction and RNA-seq [71, 72]. Although this technique prevents contamination from other cell types and other non-ribosomal high molecular weight complex, only a fraction of all the ribosomes in cells is captured and there is no distinction between monosomes and polysomes. In proximity-specific ribosome profiling, a spatially restricted biotin ligase called BirA is co-expressed with AviTag-containing ribosomes [73]. The biotinylation signals allows for the collection of ribosomes from defined sites to be analysed using ribosome profiling, thus providing information regarding the translational activities at specific subcellular locations [73]. Notwithstanding their advantages and drawbacks, these methods have certainly provided a more robust characterisation of cancer-associated translational reprogramming, as well as cellular responses to various stimuli including anti-cancer agents. In addition, these recent technological advances in the field of translomics highlight the importance of evaluating functional changes in the cell's translome to more accurately infer the cellular proteome.

1.2 Ribosome biogenesis and cancer

1.2.1 Efficiency and capacity of the cellular translation machinery

The 80S ribosome is a cellular machine composed of four ribosomal RNAs (rRNAs) and 80 ribosomal proteins, which carries out mRNA translation with extremely high fidelity. In 1896 Pianese [74] noted that enlarged nucleoli, the site for rRNA synthesis by RNA Polymerase I (Pol I) and ribosome assembly [75], is a common feature observed in malignant cells, indicating the abnormalities in ribosome synthesis and function [3, 76]. In order to sustain elevated protein synthesis rates and efficiency, rapidly growing and proliferating cancer cells augment their translation capacity by boosting the biogenesis of ribosomes. Ribosome biogenesis is an exquisitely regulated process that involves the synthesis of rRNAs and ribosomal protein, rRNA processing, as well as the assembly and transport of the 40S and 60S ribosomal subunits [77]. Pol I transcribes the ribosomal DNA (rDNA) gene to produce 47S rRNA, a precursor of the 18S, 5.8S and 28S mature rRNAs.

RNA Polymerase II (Pol II) facilitates the synthesis of mRNAs encoding ribosomal proteins and other transcription factors (TFs) necessary for organisation and functionality of the ribosomes. In the nucleoplasm, RNA Polymerase III synthesises the 5S rRNA and tRNAs. The 28S, 5S and 5.8S rRNAs constitute the nucleic acid backbone of the 60S ribosomal subunit, while the 18S rRNA is assembled into the 40S ribosomal subunit.

All three RNA polymerases contain similar core catalytic subunits [78], however there are a number of Pol I specific TFs including, but not limited to upstream binding factor (UBF), transcription initiation factor-1A (TIF-1A, also known as RRN3), and selectivity factor 1 (SL-1, also called TIF-1B) [79]. To form the Pol I transcription PIC at the rDNA promoter, the UBF dimer first binds to the upstream control elements (UCEs) and core promoter element (CORE) [80]. SL-1 is recruited and the resultant stable SL-1/UBF complex facilitates the recruitment of an initiation-competent, RRN3-bound Pol I to commence rDNA transcription [80].

1.2.2 Regulation of ribosome biogenesis

1.2.2.1 mTORC1 pathway and ribosome biogenesis

Pol I transcription, which is a major rate-limiting step for ribosome biogenesis, is regulated by signalling pathways such as RAS-MAPK and mTORC1 pathways, and proto-oncogenes including MYC (chapter 1.2.2.2), which can modulate the recruitment of Pol I to rDNA promoter, transcription initiation, promoter escape, elongation, termination or re-initiation [6, 81]. For example, mTORC1 via the activity of p70S6K1 drives the phosphorylation of UBF on its C-terminal domain [82], as well as putatively modulates the phosphorylation status of RRN3 on Ser44 and Ser199 to promote the Pol I transcription PIC assembly [83]. Moreover, the mTORC1 pathway regulates the translation of mRNAs encoding ribosomal proteins synthesized by Pol II [84]. Ribosomal protein mRNAs contain a 5' oligopyrimidine motif and their translation was shown to be modulated by mTORC1-regulated La-related protein 1 (LARP1) [85, 86]. mTORC1 is also capable of regulating Pol III transcription via its

interaction with Pol III auxiliary factor TFIIIC and Maf1 [87]. Raptor, a component of mTORC1, binds TFIIIC and thus recruits the whole complex and promotes phosphorylation of the Pol III transcription inhibitor Maf1 on Ser75, although it is still not clear whether this is due to direct phosphorylation by mTORC1 or via another mTORC1-associated kinase(s) [87].

1.2.2.2 MYC and ribosome biogenesis

c-MYC belongs to the MYC family of nuclear transcription factors that dimerises with MAX and binds DNA thus influencing the expression of, positively or negatively, upwards of 15% of the known mammalian genes [88, 89]. MYC is dysregulated in more than 70% of all human cancers and associated with aggressive disease and poor prognosis [90, 91]. In normal cells, the activity and abundance of MYC is tightly controlled by the activity of upstream signalling pathways and epigenetic factors such as the bromo-domain containing protein 4 (BRD4) [92]. In addition, as described in chapter 1.1.3, MYC is translationally regulated due to its highly structured UTRs in its mRNA that requires the activity of the eIF4F complex and mTORC1 signalling pathway [93]. Moreover, MYC protein has a short half-life of approximately half an hour, so its stability and activity is modulated by ubiquitination-mediated degradation [94-96].

The proto-oncogene MYC was originally identified as a homolog of a viral oncogene found in avian retrovirus called *v-myc*, which is capable of promoting the development of sarcoma and leukaemia [97, 98]. Thus it is unsurprising that ectopic expression of MYC in a number of mouse models of solid [99] and haematological cancers (such as the E μ -Myc B-lymphoma model [100, 101] utilised in this study) resulted in oncogenic transformation often associated with amplification and hyperactivation of genes that favour cellular growth, tumour initiation and progression.

As shown by a number of studies analysing gene expression programs, the transcriptional signatures of MYC is strongly associated with processes to promote biomass acquisition including ribosome biogenesis [89, 102, 103]. In

addition to the efficiency of the ribosomes to translate mRNAs to proteins, the capacity of the cells to accumulate biomass and grow is also dependent on the availability of ribosomes, which is dictated at the level of ribosome biogenesis. MYC enhances the activity of Pol I by modulating the Pol II-mediated transcription of a considerable number of factors associated with Pol I transcription termed “Pol I regulon”, which includes Pol I subunits, UBF and RRN3 [6, 102, 103]. In addition, MYC binds to the rDNA promoter directly interacting with SL-1 and remodels the rDNA chromatin structure, thus assisting the stabilisation of the SL-1/UBF complex and recruitment of Pol I [103]. Moreover, the AKT/mTORC1 pathway, which regulates the activity of some of these Pol I regulon via phosphorylation (chapter 1.2.2.1), cooperates with MYC to enhance rRNA synthesis rates by Pol I [104]. MYC also controls the Pol II-mediated transcription of genes encoding pre-rRNA processing factors, such as fibrillarin and NPM1, as well as multiple ribosomal proteins [103, 105, 106]. Furthermore, MYC binds to TFIIIB, a Pol III transcription factor, and recruits the chromatin remodelling factors GCN5 and transformation/transcription domain associated protein (TRRAP) to augment the synthesis of the 5S rRNA [107, 108]. These mechanisms therefore emphasise the influence of MYC in stimulating ribosome biogenesis by enhancing the transcriptional activities of Pol I, Pol II and Pol III.

1.2.3 Nucleolar stress response

The nucleolus is a structure within the nucleus where the 47S rRNA synthesis and assembly of the ribosomal subunits is coordinated. There are multiple nucleoli in the nucleus, and they form around rDNA repeats that are being actively transcribed, known as the nucleolus organiser regions (NORs). Although the nucleolus is not membrane-bound, it contains distinct regions: the pale-staining fibrillar centre, which is surrounded by the compact dense fibrillar component, and both are encased by the outer granular compartment [109, 110].

The dynamic nature of the nucleoli allows for the assembly of these structures when rDNA transcription rate is upregulated during the G1 phase of the cell cycle, and their disassembly at the start of mitosis when rDNA transcription is reduced [75, 111]. The nucleolus is populated by a varied collection of small nuclear RNAs (snRNAs), small nucleolar RNAs (snoRNAs) and ~4500 different proteins with known and putative nucleolar functions (according to nucleolar protein database (NOPdb); <http://lamondlab.com/NOPdb3.0/>) to carry out their main roles in the synthesis and assembly of ribosomal subunits [75, 110-112]. In addition, some of these proteins are involved in non-ribosome-related functions and have been shown to accumulate in the nucleolus when prompted by specific cellular cues, participating in the regulation of cell cycle, telomerase activity and stress response [109, 113]. Importantly, the nucleolus plays a critical role in a well-documented cellular phenomenon called “nucleolar stress response” or “nucleolar surveillance pathway” when triggered by disruption of nucleolar activities affecting ribosome biogenesis [75, 77, 109, 111].

The nucleolar stress response is largely dependent on the activity and abundance of the tumour suppressor p53, a key nuclear transcription factor in the regulation of apoptosis (programmed cell death), senescence, DNA repair and cell cycle arrest [114-117]. Under normal conditions, the abundance of cellular p53 protein is regulated by its interaction with an E3-ubiquitin ligase called mouse double minute 2 homolog (MDM2) protein [118]. MDM2 binds to p53 in the nucleus and promotes the poly-ubiquitylation and proteasome-mediated degradation of p53 [118]. Where there are perturbations in cellular conditions, for example DNA damage, the affinity of p53-MDM2 interaction is reduced and p53 accumulates, thus increases transcription of p53 target genes mediating cell cycle arrest or cell death [116, 117]. This response prevents the accumulation of cells with genetic mutations that may promote carcinogenesis and preserves the genomic integrity of the organism. In cancer, gain or loss of function mutations in the *Tp53* gene are commonly observed, and this leads to the reduction or alteration of anti-proliferative and pro-apoptotic activities of the p53 protein [119, 120].

Disruption of ribosome biogenesis, for example Pol I transcription inhibition, also leads to increased p53 stabilisation and transcriptional activity [121]. While a number of ribosomal proteins (rpS7, rpS15, rpS19, rpS20, rpL5, rpL11, rpL23, rpL37) have been shown to interact with and sequester MDM2 in response to inhibition of Pol I transcription or ribosome biogenesis, recent reports demonstrated that rpL5 and rpL11 are the key ribosomal proteins that drive the activation of p53-dependent nucleolar stress response [122-124]. In addition, activation of nucleolar stress pathway due to perturbation of rDNA chromatin structure and dynamics, triggers ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3-related protein (ATR) signalling in the absence of DNA breaks [125]. For example, inhibition of Pol I transcription by CX-5461 (chapter 1.4.2), which is thought to cause rDNA chromatin perturbations due to exposed structure and alterations in rDNA topology, is coupled to activation ATM/ATR signalling and thus induction of cell cycle arrest [125]. These nuclear response mechanisms underlie, at least in part, the therapeutic benefits of various pharmacological agents, including some chemotherapeutic compounds (chapter 1.4.2), that inhibit Pol I transcription and/or other steps in ribosome biogenesis [6, 8, 125, 126].

1.3 The interplay between cellular metabolic state and the ribosome

Ribosome biogenesis and mRNA translation are two of the most energy demanding cellular processes [127, 128] and are elevated in rapidly proliferating cancer cells. Thus, cancer cells need to accommodate elevated energy demand and they do so by altering their metabolic state. Not surprisingly then some of the common hallmarks of cancer include metabolic reprogramming, such as increasing the uptake of necessary nutrients and reshaping of the ways these are utilised by channelling them to specific metabolic pathways that overall support tumourigenic properties [129, 130].

Glucose and glutamine are regarded as the two principal nutrients required by the cell to fulfil its energy and biosynthetic demand. A cell can utilise the carbon skeletons of glucose and glutamine to generate pools of carbon intermediates required as building blocks for macromolecular biosynthesis. In

addition, glucose and glutamine are used to generate nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FADH₂) for adenosine triphosphate (ATP) production or nicotinamide adenine dinucleotide phosphate (NADPH) for the maintenance of cellular redox status. Under normal conditions, glucose is imported into the cell by glucose transporters (GLUT1-4) [131] and converted to pyruvate, generating 2 molecules of ATP per glucose molecule. Oxidative decarboxylation of pyruvate to form acetyl-Coenzyme A (CoA), followed by the tricarboxylic acid (TCA) cycle and oxidative phosphorylation (oxphos) produces a further 36 molecules of ATP. More than 90 years ago, Otto Warburg observed that in comparison to normal mammalian cells, tumour cells markedly increase their consumption of glucose, in the end converting most of it to lactate from pyruvate even in oxygen-rich conditions [132]. This led to the implementation of positron emission tomography (PET) with radioactive fluorine-labelled glucose analogue ¹⁸F-fluorodeoxyglucose (FDG) in the clinic to non-invasively image the whole-body cellular substrate utilization for the purpose of cancer diagnosis, staging and monitoring prior to and following therapy [133, 134].

Catabolism of glutamine, the most abundant amino acid in blood, provides not only carbon but also nitrogen used to synthesise nitrogen-containing compounds including but not limited to non-essential amino acids, glucosamine-6-phosphate or purine and pyrimidine nucleotides. Glutamine also plays a role in the uptake of essential amino acids. Nicklin *et al.* [135] reported that the influx of the essential amino acid leucine was coupled with efflux of glutamine via an amino acid antiporter LAT1. Glutamine is metabolised via a process called glutaminolysis. The enzyme glutaminase (GLS) catalyses the first step of glutaminolysis, which converts glutamine to glutamate. In the second step, glutamate dehydrogenase (GDH) facilitates the conversion of glutamate to α -ketoglutarate. The incorporation of α -ketoglutarate to replenish the TCA cycle is important for the generation of oxaloacetate, which then receives carbon from acetyl-coA (from glycolysis) to produce citrate required for lipid synthesis [136]. α -ketoglutarate also acts as an electron donor to promote the activity of the oxygen sensor proteins prolyl hydroxylases (PHD), which then facilitates mTORC1 activation [137].

When the cell's ability to synthesise ATP is compromised or if oxygen and nutrient resources are limited, the accumulation of adenosine monophosphate (AMP), which is generated when ATP is hydrolysed to release energy, triggers the cell's energy stress signal and activates AMP-dependent protein kinase (AMPK) [138, 139]. The heterotrimeric AMPK consists of one catalytic (α) subunit and two regulatory subunits (β and γ) [140]. The binding of AMP to the γ subunit and phosphorylation of Thr172 residue on the α subunit by the tumour suppressor liver kinase B1 (LKB1) result in the activation of AMPK [139, 141, 142]. One of the major downstream targets of the AMPK pathway is mTORC1 [143]. Importantly, AMPK-mediated inactivation of mTORC1, for example in response to treatment with the anti-diabetic drug metformin, is paralleled by a decrease in cap-dependent mRNA translation initiation in MCF-7 breast cancer cells [144]. This demonstrates the interplay between cellular energetic status and energy-consuming anabolic processes such as mRNA translation. AMPK also negatively regulates mTORC1 by directly phosphorylating tuberous sclerosis complex 2 (TSC2) [145] or Raptor [146]. TSC2 is a component of the multi-protein complex called TSC, which also contains TSC1 and Tre-2/Bub2/Cdc16 (TBC) 1 domain family member 7 (TBC1D7) [147, 148]. In this case, the GTPase-activating capability of TSC2 is inactivated when it is phosphorylated at Ser1345 by AMPK, thus preventing activation of mTORC1 by Ras homolog enriched in brain (Rheb) in response to amino acid stimulation [145]. Alternatively, AMPK inhibits mTORC1 by phosphorylating Raptor, which then preferentially binds to 14-3-3 proteins rather than mTORC1 [146, 149]. Thus, AMPK via regulation of the mTORC1 pathways acts as a link between energy metabolism and mRNA translation.

In times of energy stress, the AMPK pathway also reduces Pol I transcription and thus ribosome biogenesis in order to conserve cellular energy. It has been reported that phosphorylation of RRN3 by AMPK at Ser635 disrupts the RRN3-Pol I interaction, thereby preventing the PIC assembly [150] (Hoppe et al., 2009). In addition to the rapid AMPK-dependent response to changes in cellular energy status, Pol I transcription is also epigenetically regulated by the energy-dependent nucleolar silencing complex (eNoSC) [151]. Specifically, this silencing complex contains the NAD-dependent histone

deacetylase sirtuin 1 (SIRT1), nucleolar protein nucleomethilin (NML) and histone methyltransferase SUV39H1. eNoSC is disassembled during nutrient-sufficient conditions as elevated newly synthesized rRNA will bind NML thus preventing it from binding and recruiting SIRT1 to the NORs [152]. However, for example when glucose levels are low, it has been shown that reduced newly synthesised rRNA allows for the assembly of eNoSC and enables the formation of silenced rDNA heterochromatin [152].

On the other hand, mTORC1 can also regulate cellular metabolism and energy balance by promoting the translation of the nucleus-encoded mitochondria-related proteins such as mitochondrial transcription factor A (TFAM), components of mitochondrial electron transport chain complex I and V and mitochondrial ribosomal proteins [153, 154]. When mTORC1 signalling is inhibited, mitochondrial synthesis and activity is decreased in cells with functional 4E-BPs but remains unaffected in those without 4E-BPs, which also demonstrates the relationship between mTORC1-eIF4E-dependent mRNA translation and mitochondrial function [153]. Moreover, cells with PTEN loss and thus hyper-activated mTORC1 signalling exhibited increased mitochondrial respiratory capacity [155].

In terms of regulating the supply of building blocks required for growth and proliferation, mTORC1 also modulates the metabolism of nucleotides, lipid, glucose and glutamine. mTORC1, via S6Ks, promotes upregulation of nucleotide synthesis pathway by activating carbamoyl-phosphate synthetase 2, aspartate transcarbamylase and dihydroorotase (CAD) [156, 157], and via the 4EBPs regulates the translation of phosphoribosyl pyrophosphate synthetase 2 (PRPS2) mRNA [158]. To control lipid and sterol synthesis, mTORC1 activates sterol regulatory element-binding proteins (SREBPs), which then bind to SRE and E box sequences found in the promoter regions of genes involved in fatty acid and cholesterol biosynthesis [159, 160]. Finally, mTORC1 modulates glucose metabolism and glutaminolysis by activating hypoxia-inducible factor 1 α to upregulate glucose uptake and glycolysis [161, 162], and by promoting the synthesis of α -ketoglutarate from glutamate by GDH via repression of SIRT4 [163], respectively. Together these regulatory

mechanisms demonstrate how the synthesis and activity of the ribosome can be meticulously adjusted in order to acutely or chronically respond to changes in the cellular energetic and metabolic status, and vice versa, via hubs such as the mTORC1 and AMPK signalling networks.

1.4 Targeting the translational machinery in cancer

1.4.1 Targeting mechanisms of translational control

Dysregulation of mRNA translation is a common feature of cancer whereby many oncogenes and tumour suppressors cause aberrant activity of the ribosome in multiple types of cancer irrespective of their genetic make-up. As described in chapter 1.1.2, the majority of mRNA translation is cap-dependent and requires eIF4F. Not surprisingly, eIF4F is overexpressed and associated with poor prognosis for many cancers, including head and neck, colon, lung, prostate and breast cancers [4]. Extensive study around eIF4F has led to the design of several strategies to target this complex, resulting in inhibition of mRNA translation initiation.

Firstly, eIF4F can be inhibited by targeting its upstream regulators, for example mTORC1. mTORC1, as the name suggests, is the sole target of a naturally occurring macrolide called rapamycin. Rapamycin binds and forms a complex with FK506-binding protein (FKBP12) [164, 165], which then interacts with mTOR and inhibits its kinase activity when it is part of mTORC1 but not mTORC2. There are a number of rapamycin analogues (rapalogues), such as everolimus (which was used in chapter 3 and 4 of this thesis), temsirolimus and ridaforolimus, which have now been developed with improved pharmacodynamics properties [166]. These rapalogues are currently either in clinical trials (for example phase III trials for everolimus are underway in lymphoma, bladder cancer, hepatocellular carcinoma and gastric cancer according to *clinicaltrials.gov* database as of June 2018) or are already used in the clinic for the treatment of cancer (for example everolimus: metastatic pancreatic neuroendocrine tumours; temsirolimus: renal cell carcinoma) [167, 168]. However, their efficacy has not been as successful as

predicted due to incomplete suppression of 4EBPs phosphorylation and the presence of negative feedback loops that cause activation of PI3K/AKT signalling [167, 169]. To solve this, dual PI3K/mTORC1 inhibitors, such as NVPBEZ-235 [170], were designed and their clinical applicability is still under investigation. Small molecules that function as ATP-competitive inhibitors of mTOR's kinase activity (also known as active site mTOR inhibitors, asTORi), which can inhibit both mTORC1 and mTORC2, and thus blocks the aforementioned negative feedback loop, have recently been developed [171, 172]. These asTORi, for example PP242 and Torin1, offer a more potent anti-neoplastic effect than the rapalogues [7, 15, 171, 172]. Interestingly the anti-proliferative effect of asTORi were not altered in the absence of mTORC2, suggesting that the anti-cancer property of asTORi are attributed to the potent inhibition of rapamycin-resistant functions of mTORC1 [171, 172]. Although asTORi did show promising results in preclinical models, resistance to treatment still arose. For example in tumours with a high eIF4E/4EBPs ratio, asTORi (and in fact rapalogues) were incapable of suppressing eIF4F assembly and thus cap-dependent mRNA translation [173, 174]. Moreover, cancer cells are able to switch to cap-independent mRNA translation, or MAPK pathways to compensate for mTOR inhibition of cap-dependent translation [175].

Finally, inhibitors of MAP kinase-interacting kinases 1 and 2 (MNK1/2) signalling have also been developed. Phosphorylation of eIF4E at Ser209 by MNK1/2 after the assembly of the eIF4F is strongly associated with the cancer-promoting capacity of eIF4E, as overexpression of eIF4E^{S209A} fails to promote tumourigenesis [176-178]. Small molecule compounds such as cercosporamide [179] and eFT508 [180] have been shown to inhibit eIF4E phosphorylation and cancer cell proliferation. For example, cercosporamide inhibited the outgrowth of melanoma pulmonary metastasis *in vivo*. eFT508 was also capable of exerting anti-tumour activity *in vivo* [180], and showed synergistic effects when combined with cancer immunotherapy agents anti-PD-L1 and anti-PD-1 monoclonal antibodies [181]. In addition, other studies suggest that inhibition of MNKs can prevent resistance to pharmacological agents such as gemcitabine (a nucleoside analogue family of chemotherapy

agents) [182], trastuzumab (a monoclonal antibody targeting human epidermal growth factor receptor 2) [183] and rapamycin [184]. However, the resistance to MNK inhibitors has recently been reported. Although it has yet to be widely explored, the mechanism(s) of resistance to MNK inhibitors is thought to be related to the resistant mechanisms to MEKs (MAPK kinases) due to MNK's position downstream of MAPK [7, 185].

Since inhibiting upstream regulators of eIF4F has posed challenges due to the development of resistance, a number of other strategies have been devised that directly target the eIF4F complex and disrupt interactions necessary for the initiation of cap-dependent mRNA translation. 4Ei-1 is a pro-drug of 7-benzyl guanosine monophosphate (7Bn-GMP), a compound that is able to inhibit the interaction between eIF4E and the 5' cap structure [186]. 4Ei-1 successfully inhibited cap-dependent mRNA translation in zebrafish and more importantly, chemosensitised lung and breast cancer cells to gemcitabine treatment [186]. Alternatively, the eIF4F complex can be targeted by preventing the interaction between eIF4E and eIF4G. Compounds such as 4E1RCat [187], 4E2RCat [187] and 4EGI-1 [188, 189], which were identified using chemical library screens, inhibited the eIF4E-eIF4G interaction with have shown promise in preclinical models [190-193]. In addition, the helicase activity of eIF4A and its interaction with mRNA can be inhibited using compounds such as silvestrol [194, 195], pateamine A [196-198] or hippuristanol [199], all of which have been efficacious in a number of cell lines and mouse models.

1.4.2 Targeting ribosome biogenesis in cancer

The concept of targeting ribosome biogenesis for cancer intervention has been controversial due to the fact that it is an essential housekeeping function required to sustain growth and proliferation in any cells, normal or malignant cells. However, a number of conventional chemotherapy drugs that are currently in use in the clinic in fact affect ribosome biogenesis at multiple levels including rDNA transcription and rRNA processing [6]. Although these chemotherapy agents may not directly modulate the Pol I transcription

machinery, their efficacy can partly be attributed to their ability to directly suppress ribosome biogenesis. For instance, the ability of the chemotherapy drug actinomycin D to intercalate GC-rich region of the rDNA is highly selective for the rDNA gene which mediates inhibition of the elongation stage of Pol I transcription [200]. Another example is the platinum-based cisplatin compound, which suppress rDNA transcription due to its ability to crosslink DNA at high mobility group (HMG)-domain proteins affinity sites such as those the Pol I transcription factor UBF binds at the rDNA promoter [201, 202]. Moreover, the efficacy of targeted therapy strategies using small molecules to inhibit kinases, such as AKT and mTORC1, is also mediated in part by the subsequent inhibition of ribosome biogenesis-related processes that are regulated by these kinases (described in chapter 1.2).

Recent advances in our understanding of: (a) the importance of ribosome biogenesis in tumorigenesis, (b) the efficacy of anti-cancer drugs that are influenced by their ability to inhibit ribosome biogenesis, and (c) the activation of cellular checkpoint pathways (e.g. p53, ATM/ATR) in response to disruption of ribosome biogenesis have led to the development of several small molecules that specifically target ribosome biogenesis at the level of Pol I transcription. The first of such compound is quarfloxin, which inhibits rDNA transcription elongation by preventing the interaction between NCL and G-quadruplexes in the rRNA gene. Quarfloxin has anti-neoplastic properties in xenograft models of pancreatic and breast cancers and completed phase I clinical trial in advanced solid tumours [203-206]. There is also a group of Pol I inhibitors, such as BMH-21, ethacridine and aminacrine, which intercalate into GC-rich regions of rDNA and prevent Pol I transcription elongation [77, 207]. The next generation of Pol I transcription inhibitor, CX-5461, is the “first-in-class” oral small molecule therapeutic compound that selectively target rDNA transcription to enter the clinic [208-210]. Specifically, CX-5461 interferes with the binding of SL-1 to the rDNA promoter, thus preventing the assembly of the PIC and transcription initiation [208]. In terms of its specificity, CX-5461 is 300-400 fold more selective inhibitor for Pol I transcription than Pol II and Pol III transcription [208]. Importantly, CX-5461 exhibits remarkable anti-proliferative effects over an extensive panel of human cancer cell lines at

nanomolar (nM) concentrations with a much higher EC₅₀ values in normal and non-transformed cell lines [8, 208]. Consequently, we have shown that CX-5461 treatment selectively caused anti-neoplastic effect in haematological cancer cells due to activation of p53-dependent apoptosis via nucleolar stress response and/or induction of p53-independent CHK1/CHK2 checkpoint mechanisms [8, 211, 212]. This therefore resulted in a significant survival benefit in mouse transgenic models of human B-cell lymphoma and acute myeloid leukaemia [8, 211]. Furthermore, CX-5461 can be combined with the mTORC1 inhibitor everolimus to significantly extend the survival advantage in MYC-driven B-lymphoma to more than double of the therapeutic benefit previously achieved with CX-5461 alone [1]. This has clearly highlighted the feasibility and potential of applying coordinated inhibition of the cell's translation machinery at multiple levels as a strategy to combat cancers.

1.5 Hypothesis and Aims

As described above, the link between MYC and mTORC1 in the regulation of the synthesis and functions of the cell's translation machinery can be harnessed in the design of therapeutic strategies to fight cancer. Strikingly, Devlin *et al.* [1] demonstrated that MYC-driven cancer cells were vulnerable to inhibitors that target ribosome synthesis and function (mRNA translation). Moreover, the study showed that the combination of the Pol I transcription inhibitor CX-5461 and mTORC1 inhibitor everolimus synergistically extended survival in the well-characterised E μ -Myc transgenic model of B-cell lymphoma. However there are two outstanding research questions that need to be addressed: (i) although our data suggests that CX-5461 and everolimus triggered cancer cell death via independent pathways, the detailed mechanisms underlying this robust synergistic effect have not been fully deciphered, and (ii) despite the more than additive extension of survival from CX-5461-everolimus combination, the E μ -Myc lymphoma-bearing mice eventually become resistant to this combination therapy and thus succumbed to disease. As both inhibitors target the cell's translation machinery, we hypothesise that there are: (i) coordinated changes in the cell's translational

landscape that underlie the aforementioned synergistic effects and (ii) translational-reprogramming alterations that allow the development of resistance.

The application of the polysome profiling analysis (chapter 1.1.4) is central to addressing the aims of this study, which are:

1. To establish a robust polysome profiling protocol for the characterisation of global translation profiles in cancer cells (Chapter 3);
2. To investigate the mechanism(s) underlying the synergy between CX-5461 and everolimus in E μ -Myc B-lymphoma model (Chapter 4).
3. To evaluate the translome of combination therapy-resistant cells and identify biological process(es) that can be targeted to overcome resistance and improve therapeutic benefit (Chapter 4).

Chapter 2:

Materials and Methods

Chapter 2: Materials and Methods

2.1 Mammalian cell culture

E μ -Myc B-lymphoma cells were cultured in Anne Kelso DMEM, supplemented with 10% heat-inactivated (h.i.) fetal bovine serum (FBS), 100 μ M L-asparagine (Merck), penicillin/streptomycin/glutamine (Thermo Fisher Scientific) and 0.5% beta-mercaptoethanol. Early-passage E μ -Myc B-lymphoma cells were cultured in Anne Kelso DMEM supplemented with 20% h.i. FBS, 100 μ M L-asparagine (Merck), penicillin/streptomycin/glutamine (Thermo Fisher Scientific) and 0.5% beta-mercaptoethanol. Human acute myeloid lymphoma cell lines (MV4-11 (MLL-AF4; p53 wild-type (WT), SKM-1 (EZH2; p53-mutant (R248Q)), SHI-1 (MLL-AF6; p53WT), THP-1 (t(9;11); p53-null R174fs)) were cultured in RPMI 1640 medium plus HEPES supplemented with 20% h.i. FBS, 4 mM Glutamax (Thermo Fisher Scientific) and 1% v/v antibiotics/antimycotics (Thermo Fisher Scientific). Cell number was determined using the Z2 Coulter Counter (Beckman Coulter 383550). For ATP assays, cells were analysed by CellTiterGlo® 2.0 (Promega G9241). 100 μ L of CellTiterGlo® was added to each well and incubated for 30 min at room temperature. After incubation, viable cells treated with CellTiterGlo® generated luminescence that could be directly measured using Cytation3 plate reader (Biotek). Absorbance of the converted dye was measured at 550nm using a Benchmark microplate reader (Biorad) and normalised to cell number.

2.2 Pharmacological compounds

Everolimus (S1120) was purchased from Selleckchem. CX-5461 was purchased from SynKinase (SYN-3031). Metformin was purchased from Merck (D150959). CEF34 was purchased from Cayman Chemicals (17767). ESI-05 and 8-pCPT-2-O-Me-cAMP were purchased from Biolog (catalogue number M092 and C041, respectively). 6-Bnz-cAMP was purchased from Tocris Bioscience (5255). H89 was purchased from Merck (B1427). For *in*

vivo experiments, everolimus was administered at 5 mg/kg via oral gavage using 20ga plastic feeding tubes (Walker Scientific FTP-20-38) in DMSO (5%) and 1% methylcellulose (95%). CX-5461 was administered at 35 mg/kg via oral gavage in 25 mM NaH₂PO₄ (pH 4.5). Metformin was administered at 500 mg/kg via oral gavage in 25 mM NaH₂PO₄ (pH 4.5).

2.3 Protein analysis and immunoblotting

Protein was extracted with sodium dodecyl sulfate (SDS) lysis buffer (Table 2.1) and protein concentrations were determined with the Bio-Rad protein assay (Bio-Rad). Protein lysates were mixed with 6x protein sample loading buffer (Table 2.1) and resolved by SDS-PAGE using the Mini-Protean Tetra Cell system (Bio-Rad) in Tris-Glycine-SDS running buffer (Table 2.1). Proteins were transferred from gels to polyvinylidene difluoride (PVDF) membranes in Tris-glycine-methanol transfer buffer (Table 2.1). Membranes were blocked with blocking buffer (Table 2.1) for 1 hour at RT and incubated overnight with primary antibodies (Table 2.2). Membranes were then washed three times with Tris buffered saline supplemented with 0.1% Tween20 (TBST) (Table 2.1) and incubated in horseradish peroxidase–conjugated secondary antibodies (Table 2.2). Protein was visualized by Amersham enhanced chemiluminescence (ECL) Western Blotting Detection Reagent (GE Healthcare Life Sciences) and X-ray film (Fujifilm SuperRX). Active Rap1 pull-down experiments were carried out using an active Rap1 pull-down and detection kit (Thermo Fisher Scientific 16120) according to manufacturer's instructions.

2.4 Quantitative real time PCR (qRT-PCR)

RNA was isolated using Qiagen RNeasy Mini Kit (Qiagen 74106) or Bioline Isolate RNA II Mini Kit (Bioline 52073) as per manufacturers' protocols. Eluted RNA concentration was quantified with a Nanodrop ND1000 spectrophotometer (Thermo Fisher Scientific). Total RNA equivalent to equal absolute amount of total RNA (1 µg) or equal cell number (25,000 cells) was treated with RNase-free DNase (Promega) at 37°C for 15 minutes followed by

15 minutes incubation at 70°C. cDNA was synthesized with 60U SuperscriptIII (Life Technologies 18080-044) and 12.25 ng random hexamer primers (Promega C1181) at room temperature (RT) for 5 minutes, 37°C for 5 minutes, 47°C for 120 minutes and 70°C for 15 minutes according to the manufacturer's instructions. Synthesized cDNA was diluted with sterile MilliQ water and stored at -20°C.

qRT-PCR reaction samples consisting of cDNA sample, 0.1 µM oligo-primers (forward-reverse mix) (in 8 µL) (Table 2.3) and Fast SYBR Green master mix (Applied Biosystem 4385612) (12 µL) were plated in triplicate in MicroAMP Optical 96-well reaction plates (Applied Biosystems N8010560). The qRT-PCR reactions were carried out by the StepOne Plus Real-Time PCR system (Applied Biosystem 4376600). Where equal RNA was used for cDNA synthesis, the expression of target gene was normalised to the expression of a housekeeping gene (mouse Eµ-Myc cells: β-2 microglobulin; *β2m*; human cell lines: β-actin; *ACTB*) and fold change was determined by $2^{-(\Delta\Delta Ct)}$. Where equal cell number was used for cDNA synthesis, fold changes in target gene expression were determined by $2^{-(\Delta\Delta Ct)}$. Statistical analysis was performed using Student's *t* test.

2.5 Pulse labelling of cells with ^{32}P -orthophosphate

Eµ-Myc B-lymphoma cells (3×10^6) were cultured in 3 ml media in the presence of 0.5 mCi of ^{32}P orthophosphate for 15 min. Cells were harvested on ice and RNA extracted using the Qiagen RNeasy Minikit (Qiagen 74106) according to the manufacturer's instructions. RNA (5 µg) was prepared in MOPS sample buffer (Table 2.1) with RNA sample loading buffer and 48 µg/mL ethidium bromide. RNA samples were separated on a 1.2% MOPS/formaldehyde agarose gel for 15 hours (45 Volts). Ethidium bromide-stained RNA (28S and 18S rRNA bands) was visualized by a Gel Doc XR+ System (Bio-Rad) and used as loading controls. The gel was dried using a Model 583 Gel Drier (Bio-Rad), exposed overnight to a phospho-imager screen (Molecular Dynamics) and scanned using a Typhoon Trio Variable Mode Imager (GE Healthcare). Band intensities were quantitated using Image

Quant TL software (GE Healthcare). Statistical analysis was performed using Student's *t* test.

2.6 Polysome Profiling

2.6.1 Cell lines

Suspension cells were harvested by removing the culture media following centrifugation (400 g, 4 minutes, 4°C). The cells were washed with ice-cold hypotonic wash buffer (5 mM Tris pH 7.5; 1.5 mM KCl; 2.5 mM MgCl₂ containing 100 µg/mL cycloheximide (Sigma); Table 2.1) and the supernatant was removed by centrifugation (400 g, 4 minutes, 4°C). A hypotonic lysis buffer (5 mM Tris pH 7.5; 1.5 mM KCl; 2.5 mM MgCl₂; 0.5% Triton-X; 0.5% sodium deoxycholate; 1X EDTA-free protease inhibitor; 2 mM DTT; 10 µL RNasin (Promega); 100 µg/mL cycloheximide; Table 2.1) was added to the cell pellet. The samples were mixed by pipetting and centrifuged (16,000 g, 7 minutes, 4°C). The supernatant was transferred to a fresh microfuge tube. 10% of the cytoplasmic lysate was transferred into a microfuge containing 500 µL TRIzol® reagent (Invitrogen 15596018). The remaining lysate was layered on top of a linear 10-40% sucrose gradient, ultracentrifuged (36,000 rpm, 2¼ hours, 4°C) and fractionated using the Foxy Jr Fraction Collector with constant monitoring of absorbance at 260 nm by an ISCO UA-6 Absorbance Detector. Cytoplasmic (input) RNAs and polysomal RNAs in fractions corresponding to four or more ribosomes were pooled, isolated using a TRIzol®-based method ((Invitrogen 15596018) according to manufacturer's instruction. RNA samples were purified by a Qiagen RNeasy Minikit (Qiagen 74106) according to manufacturer's instructions. RNA concentration was determined using a NanoDrop Spectrophotometer (Thermo Fisher Scientific) and integrity was evaluated using the RNA Nano Kit and 2100 Bioanalyzer (Agilent Technologies). cDNA libraries were generated using a ribodepletion method (TruSeq Ribo Profile Mammalian Kit, Illumina) [213]. Fragment sizes were evaluated using the DNA 1000 kit and 2100 bioanalyzer (Agilent Technologies). Libraries were subjected to single-end sequencing (SE50bp)

(HiSeq2500, Illumina) to generate ~30 million reads per sample using standard protocols. Reads were aligned with Tophat2 and reads counts were obtained with htseq with the mouse genome (Ensembl release 78) as a reference, using an in-house bioinformatics pipeline, seqliner (<http://bioinformatics.pettermac.org/seqliner/>). Differential translation was detected using anota2seq analysis [214] of polysome-associate mRNAs, using the total amount of cytosolic mRNA as a reference. Only coding genes with a count of at least 1 in any of the samples were considered. Samples were normalized using the trimmed mean of M values (TMM) normalization implemented in anota2seq. An omnibus analysis was first performed in anota2seq to enrich for genes affected by any of the treatments, using a groupRvmPAdj cut-off of 0.15. Differential translation analysis was subsequently performed using anota2seq on this reduced set. Only genes with an apvRvm adjusted p-value < 0.05 and an apvEff (fold change) greater than 1.25 were considered to be significant. To perform differential analysis of polysome data (i.e. to compare mRNAs that were differentially bound to polysomes of treated vs. control cells), we used LIMMA version 3.28.21 [215] from Bioconductor. Only coding genes with an average logCPM > 1 were considered. Genes were considered to be significant if their adjusted p-values after Benjamini & Hochberg correction were less than 0.05, and their fold change was greater than 1.5.

2.6.2 Tissue samples

Cytoplasmic lysate was obtained by pulverising snap-frozen tissue samples by grinding in a liquid nitrogen proof contained into a fine powder. A modified lysis buffer (10-fold higher concentration compared to the one used for cultured cells (1 mg/mL; RNasin was replaced with 10 mM ribonucleoside vanadyl complex) was added to the powder. The sample was transferred to an ice-cold dounce homogeniser and homogenised (30-60 strokes). The homogenate was transferred to a chilled microfuge tube and centrifuged (16,000 g, 7 minutes, 4°C). The supernatant was transferred to a fresh microfuge tube. 10% of the cytoplasmic lysate was transferred into a

microfuge containing 500 μ L TRIzol® reagent (Invitrogen 15596018). The remaining lysate was loaded onto a linear sucrose gradient (10-40%) or a step gradient [216].

2.6.3 5'UTR analysis

5'UTR sequences were obtained using the biomaRt and org.Mm.eg.db R packages from the Mouse Genome GRCm38.p6. Only 5'UTRs shorter than 500 nucleotides were considered, since others have found that longer 5'UTRs were likely to be false positives [217]. In cases where there were multiple 5'UTR for a single gene, we only considered the shortest 5'UTR. For the resistant study, only genes with fold change (FC) > 1.5 or < -1.5 were considered, with adjusted $P < 0.05$. Only expressed genes were used as background. For the acute study, metabolic downregulated genes were those with fold change (appEff) < 0 , and adjusted $P < 0.1$ (from the omnibus-sorted genes; Chapter 2.6.1). The minimum free energy of 5'UTR sequences was calculated using the ViennaRNA package version 2.4.7. In cases where there were multiple 5'UTR for a single gene, we only considered the 5'UTR with the lowest minimum free energy. One-sided Wilcoxon tests were used to determine significance.

2.7 Metabolomics

2.7.1 Cell lines

Cells were quenched by transferring cell suspensions (5 mL) into 50 mL falcon tubes containing 35 mL ice-cold saline, followed by centrifugation (500 g, 3 minutes, 4°C). Once the supernatant was removed, the cells were re-suspended in 1 mL ice-cold saline, transferred to 1.5 mL microfuge tubes, and centrifuged (10,000 g, 30 seconds, 4°C). Supernatant was removed completely and metabolites were immediately extracted in 250 μ L chloroform:methanol:water ($\text{CHCl}_3\text{:CH}_3\text{OH:H}_2\text{O}$, 1:3:1 v/v; monophasic mixture) containing 5 nM scyllo-inositol as internal standard. The extracts

were vortex mixed (~10 seconds) and incubated on ice for 15 mins with additional vortex-mixing every 5 minutes. Insoluble material was removed by centrifugation (10,000 g, 5 minutes, 4°C). H₂O was then added to the supernatant to adjust the ratio of CHCl₃:CH₃OH:H₂O to 1:3:3 (v/v; biphasic partition). The supernatant was vortex mixed and centrifuged (10,000 g, 5 minutes, 4°C) to induce phase separation.

The upper (polar) phase was transferred to a fresh microfuge and dried *in vacuo* (30°C) in 250 µL glass vial inserts. Polar metabolites were derivatized with TMS reagent (N,O-bis(trimethylsilyl) trifluoroacetamide containing 1% trimethylchlorosilane (Pierce, 20 ml, 1 hour, 25°C) and analysed by gas chromatography-mass spectrometry (GC-MS) using a DB5 capillary column (J&W Scientific, 30 m, 250 µm inner diameter, 0.25 µm film thickness), with a 10 m inert duraguard. The injector insert and GC-MS transfer line temperatures were 270 and 250°C, respectively. The oven temperature gradient was programmed as follows: 70°C (1 min); 70°C to 295 °C at 12.5°C /min; 295°C to 320°C at 25°C/min; 320°C for 2 min. The apolar (lipid) lower phase was subjected to solvolysis in methanolic HCl prior to derivatisation with N-Trimethylsilyl-N-methyl trifluoroacetamide (MSTFA) containing 1% trimethylchlorosilane (TMCS) and analysed by GC-MS as described above. Metabolites were normalised based on internal standards and identified based on GC retention time and mass spectra as compared with authentic standards and in conjunction with MSD ChemStation Data Analysis Application (Agilent) using in-house and Wiley metabolite libraries.

Liquid chromatography-mass spectrometry (LC-MS) analysis was performed using an Agilent 1200 series LC (Agilent Technologies, Santa Clara, US, USA) system equipped with two vacuum degassers, two binary pumps, a thermostat controlled autosampler and a thermostat controlled column compartment containing a 10-port 2-position valve as described in [218].

Statistical analyses were performed using Student's *t* test. Metabolites were considered to be significant if their adjusted p-values after Benjamini & Hochberg correction were less than 0.05.

2.7.2 Tissue samples

Snap frozen tissue samples were transferred into chilled cryomill tubes containing 1.4 mm ceramic beads. 600 μL of extraction solution ($\text{CH}_3\text{OH}:\text{H}_2\text{O}$, 3:1 v/v; containing 5 nM scyllo-inositol as internal standard) was added to the cryomill tubes. The samples were immediately processed using a Precellys 24/Cryolys cryomill (Bertin Technologies, France) (6,800 rpm; 3 x 30-second pulse, 45 seconds interval between pulses; 4°C). The homogenates were transferred to a fresh microfuge tube and 150 μL chloroform was added ($\text{CHCl}_3:\text{CH}_3\text{OH}:\text{H}_2\text{O}$, 1:3:1 v/v; monophasic mixture). The extracts were vortex mixed (~10 seconds) and incubated on ice for 15 mins with additional vortex-mixing every 5 minutes. Insoluble material was removed by centrifugation (10,000 g, 5 minutes, 4°C). 600 μL H_2O was then added to the supernatant to adjust the ratio of $\text{CHCl}_3:\text{CH}_3\text{OH}:\text{H}_2\text{O}$ to 1:3:3 (v/v; biphasic partition). The supernatant was vortex mixed and centrifuged (10,000 g, 5 minutes, 4°C) to induce phase separation. Upper (polar) phase and lower (apolar) phase were then processed as described above (Chapter 2.6.1)

2.8 Propidium Iodide (PI) exclusion analysis

Exponential growing cells were treated with pharmacological agents for pre-determined time periods as indicated in figure legends. Cells were stained with 1 $\mu\text{g}/\text{mL}$ PI (Merck P4170) for 15 minutes at RT, protected from light. Stained cells were analysed using a FACSVerse flow cytometer (BD Biosciences). Flow cytometry data were analysed with Flowlogic software (Inivai). Forward (FSC-A and FSC-H) and side (SSC-A) scatter readings, which evaluate cell size and external complexity, respectively, were used to gate single cells of the correct morphology (10,000 events per sample). Unstained cells were used as controls in each experiment.

2.9 *In vivo* animal (mouse) studies

All animal studies were conducted in accordance to protocols approved by the Animal Experimentation Ethics Committee at the Peter MacCallum Cancer Centre (Ethics #E462 and #E557). Mice were housed in the animal facility at the Peter MacCallum Cancer Centre.

2.9.1 Intravenous transplantation of mice with Eμ-Myc B-lymphoma cells

Eμ-Myc B-lymphoma cells (clone #4242 and #107, *Gfp*) previously generated by L. Cluse (Cancer Immunology Program, PMCC) were stored in 90% (v/v) HI-FBS and 10% (v/v) DMSO in liquid nitrogen. On the day of cell transplant, cells were thawed, washed using PBS (-/-) (Table 2.1) and resuspended in PBS (-/-). Cells were counted using a Z2 Coulter Counter (Beckman Coulter). An equal number of cells (150,000 – 200,000 cells) were injected into the tail-veins of C57BL/6 mice (6 to 8 weeks old) purchased from the Walter and Eliza Hall Institute of Medical Research, Australia.

2.9.2 Checking engraftment of the transplanted Eμ-Myc B-lymphoma cells

On Day 8-10 post-transplant, peripheral blood was taken via tail-bleed into heparinised capillary tubes. Peripheral blood samples were diluted (1:26) with PBS (-/-) and white blood cell count was performed using a Sysmex cell analyser (Sysmex). White blood cells were isolated from the remaining whole blood by adding 1 mL mouse red blood cell lysis buffer (Table 2.1) and incubated for 5 minutes at 37°C. White blood cells were collected by centrifugation (400g, 5 minutes) and resuspended in PBS (-/-) supplemented with 0.5% bovine serum albumin (BSA) (Sigma). White blood cells were stained with an APC-conjugated αB220 (CD45R) antibody (Thermo Fisher Scientific 17-0452-82) (1/400 dilution) for 20 minutes on ice. Stained cells were resuspended in PBS (-/-; 0.5% BSA) containing 1 µg/mL PI and analysed for green fluorescent protein (GFP) expression and B220 surface marker expression using a Fortessa or Cantoll flow cytometers (BD Biosciences). Flow cytometry data were analysed with Flowlogic software

(Inivai). Forward (FSC-A and FSC-H) and side (SSC-A) scatter readings, which evaluate cell size and external complexity, respectively, were used to gate single cells of the correct morphology (10,000 events per sample). Unstained cells were used as controls in each experiment.

2.9.3 Drug administration

For survival experiments, everolimus was administered daily at 5 mg/kg via oral gavage using 20ga plastic feeding tubes (Walker Scientific FTP-20-38) in DMSO (5%) and 1% methylcellulose (95%). CX-5461 was administered every Monday and Thursday at 35 mg/kg via oral gavage in 25 mM NaH₂PO₄ (pH 4.5). Metformin was administered twice daily (2 x 500 mg/kg) via oral gavage in 25 mM NaH₂PO₄ (pH 4.5). For short-term therapy experiments, single doses of CX-5461 (35 mg/kg), everolimus (5 mg/kg) and metformin (500 mg/kg; also administered as pre-treatment as indicated in figure legends) were administered and mice were sacrificed 2 or 6 hours post-dosing as indicated in figure legends.

2.9.4 Sacrifice and harvest of diseased mice

For survival studies, mice were dosed until an ethical end-point was reached (enlarged lymph nodes, inactivity, hunched posture, laboured breathing, weight loss (equal to 20% of initial body weight) or ruffled fur). Differences in Kaplan-Meier survival curves were analysed using the log-rank (Mantel-Cox) test (Prism 7). Prior to sacrifice, mice were anaesthetised with methoxyflurane (Medical Developments International Ltd). Cardiac blood was collected using 26ga heparinized needles into 1.5 mL eppendorf tubes containing 10 µL 10 mM EDTA and analysed as described in chapter 2.9.2. Mice were sacrificed (cervical dislocation) and dissected to collect tissues of interest. 1 lymph nodes (inguinal) were crushed in PBS (-/-) supplemented with 2% heat-inactivated FBS (for flow cytometry analysis (chapter 2.9.2)) or complete culture media supplemented with 10% heat-inactivated FBS (chapter 2.1; to establish banked lymph node cells for subsequent *in vivo* experiments) and filtered (0.4 µM) to generate single cell suspensions. The remaining axillary,

brachial and inguinal lymph nodes were snap-frozen in liquid nitrogen for polysome profiling, immunoblotting and RNA analysis. Spleens were weighed and snap-frozen in liquid nitrogen or crushed in PBS (-/-) supplemented with 2% heat-inactivated FBS for flow cytometry analysis (chapter 2.9.2). Snap-frozen lymph nodes or spleens were homogenized using a Precellys 24/Cryolys cryomill (Bertin Technologies, France) (6,800 rpm; 2 x 30-second pulse, 45 seconds interval between pulses; 0°C).

2.10 Bioenergetics analysis using the Seahorse XF96 Extracellular Flux Analyzer

All extracellular flux analyses were performed using the Seahorse Bioscience XF96 extracellular flux analyser (Seahorse Bioscience, Billerica, USA). Cells were washed with the assay media (unbuffered DMEM, 5 g/L glucose, 5 mM glutamine, 1 mM sodium pyruvate), before seeding in Seahorse XF96 96-well plates coated with Cell-Tek (3.5ug/cm², Corning) at 2×10^5 cells/well in 180 µl of the assay media. The plate was centrifuged at 400g for 5 minutes at room temperature to arrange the cells into a monolayer. The plate was then equilibrated in a non-CO₂ incubator for 30 minutes prior to assay. Basal energetics were established after five cycles of 3 minutes of mixing and 3 minutes of measurement periods, with oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) measured simultaneously. These initial cycles were followed by exposure to the ATP synthase inhibitor oligomycin (1 µM) for three cycles, then the proton ionophore carbonylcyanide p-trifluoromethoxy-phenylhydrazone (FCCP, 1.5 µM) for three cycles, and then the mitochondrial complex III inhibitor antimycin A (0.5 µM) and the complex I inhibitor rotenone (0.5 µM) for three cycles. At the completion of each assay, the cells were stained with Hoechst 10 µM. Images were analysed using a Cellomics Cellinsight 1 to determine the cell number per well.

Table 2.1: Buffers and reagents

Reagent/Buffer	Composition
Phosphate buffered saline (PBS)	137 mM NaCl, 2.7 mM KCl, 10mM Na ₂ HPO ₄ , 1.8mM KH ₂ PO ₄ .
Tris buffered saline (TBS)	50 mM Tris, 150 mM NaCl
Sodium dodecyl sulfate (SDS) lysis buffer	0.5 mM ethylenediaminetetraacetic acid (EDTA), 20 mM HEPES, 2% (w/v) SDS pH 7.9
6x Protein sample loading buffer	2% (w/v) SDS, 0.4 M Tris-HCl pH 6.8, 48% (v/v) Glycerol, 58 mM β-mercaptoethanol, 0.25% (w/v) Bromophenol Blue
Tris-Glycine-SDS running buffer	25 mM Tris, 192 mM Glycine, 0.1% (w/v) SDS pH 8.7
Tris-Glycine-Methanol transfer buffer	25 mM Tris, 192 mM Glycine, 15% (w/v) methanol
Blocking buffer	5% (w/v) skim milk in TBST (0.1% (v/v) Tween20 in TBS)
Hypotonic wash buffer (prepared in DEPC-treated water)	5 mM Tris-HCl pH 7.5, 1.5 mM KCl, 2.5mM MgCl ₂ , 100 µg/mL cycloheximide (Sigma)
Hypotonic lysis buffer (prepared in DEPC-treated water)	5 mM Tris-HCl pH 7.5, 1.5 mM KCl, 2.5 mM MgCl ₂ , 0.5% (v/v) Triton X-100, 0.05% (w/v) Sodium deoxycholate, 1 mM DTT, 100 µg/mL cycloheximide, 1x cOmplete EDTA-free Protease Inhibitors, (Roche 11 873 580 001), 200 U RNasin (Promega)
MOPS buffer (prepared in DEPC-treated water)	0.02 M MOPS (3-(N-morpholino) propanesulfonic acid)), 9 mM Sodium Acetate, 1 mM EDTA (prepared in DEPC-treated water)
5x RNA sample loading buffer (prepared in DEPC-treated water)	45% (v/v) glycerol, 0.25% (w/v) Xylene Cyanol, 0.25% (w/v) Bromophenol Blue in 1x TE pH 7.4 (10 mM Tris/1 mM EDTA)
Mouse red blood cell lysis buffer	144 mM NH ₄ Cl, 17 mM Tris-HCl pH 7.65

Table 2.2: Antibodies for immunoblotting and flow cytometry

Protein	Mass (kDa)	Primary Antibody Details	Primary Antibody Dilution	Secondary Antibody Details
Immunoblotting				
Actin	42 kDa	MP Biosciences 691002	1:30,000	Goat- α -rabbit HRP ¹
p53	53 kDa	Leica Biosystems	1:1,000	Goat- α -mouse HRP ²
rpL11	20 kDa	Life Technologies 37-3000	1:1,000	Goat- α -mouse HRP
rpS6	32 kDa	Cell Signalling #2317	1:2,000	Goat- α -mouse HRP
Phospho-rpS6	32 kDa	Cell Signalling #2215	1:2,000	Goat- α -rabbit HRP
4EBP1	15-20 kDa	Cell Signalling #9452	1:1,000	Goat- α -rabbit HRP
Phospho-4EBP1 (Thr37/46)	15-20 kDa	Cell Signalling # 9459	1:1,000	Goat- α -rabbit HRP
Phospho-4EBP1 (Ser65)	15-20 kDa	Cell Signalling # 9451	1:1,000	Goat- α -rabbit HRP
Rap1	21 kDa	Thermo Fisher Scientific #16120	1:1,000	Goat- α -rabbit HRP
Phospho-PKA substrate (RRXS*/T*)	N.A	Cell Signalling #9624	1:1,000	Goat- α -rabbit HRP
Notes: 1. Goat- α -rabbit horseradish peroxide (HRP) IgG (H+L) (Bio-Rad #170-6515); dilution 1:2,000 2. Goat- α -mouse horseradish peroxide (HRP) IgG (H+L) (Bio-Rad #170-1011); dilution 1:2,000				
Flow cytometry				
B220 (CD45R)-APC	N.A.	Thermo Fisher Scientific 17-0452-82	1:400	N.A.

Table 2.3 Primer sequences for qRT-PCR

Target	Species	Direction	Sequence
<i>EEF1A1</i> [219]	Human	Forward	5' TGTCGTCATTGGACACGTAGA 3'
		Reverse	5' ACGCTCAGCTTTCAGTTTATCC 3'
<i>rpS6</i> [220]	Human	Forward	5' TCTTGACCCATGGCCGTGTC 3'
		Reverse	5' GCGGCGAGGCACTGTAGTAT 3'
<i>ACTB</i> (β -actin) [221]	Human	Forward	5' GATGAGATTGGCATGGCTTT 3'
		Reverse	5' CACCTTCACCGTTCCAGTTT 3'
47S pre-rRNA 5' external transcribed sequence (5'ETS) [8]	Mouse	Forward	5' CCAAGTGTTTCATGCCACGTG 3'
		Reverse	5' CGAGCGACTGCCACAAAAA 3'
β_2 -microglobulin (β_2m) [102]	Mouse	Forward	5' TTCACCCCCACTGAGACTGAT 3'
		Reverse	5' GTCTTGGGCTCGGCCATA 3'
<i>p21</i> [8]	Mouse	Forward	5' CCAGACATTTCAGAGCCACAGG 3'
		Reverse	5' GGTCGGACATCACCAGGATT 3'

Chapter 3:
Optimisation of
polyribosome profiling
as a high-throughput
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Publications arising from this chapter:

- Devlin, J.R., Hannan, K.M., Hein, N., Cullinane, C., **Kusnadi, E.P.**, Ng, P.Y., George, A., Shortt, J., Bywater, M.J., Poortinga, G.P., Sanij, E., Kang, J., Drygin, D., O'Brien, S., Johnstone, R.W., McArthur, G.A., Hannan, R.D., and Pearson, R.B., 2016, Combination therapy targeting ribosome biogenesis and mRNA translation synergistically extends survival in MYC-driven lymphoma, *Cancer Discovery*, 6(1), 59-70.
- Kang, J., **Kusnadi, E.P.**, Ogden, A., Hall, M.N., Hicks, R.J., Hannan, R.D., Hannan, K.M., and Pearson, R.B., 2016, Nutrient-dependent regulation of rDNA transcription requires mTORC1-S6K1 and MYC signalling, *Oncotarget*, 7(31), 48887-48904.

3.1 Introduction

The regulated translation of mRNAs into proteins is a critical step in gene expression and is one of the most important post-transcriptional mechanisms affecting cellular protein abundance. As such, specific translational control, which alters only a select subset of mRNAs, can impact on many biological processes including the aetiology of cancer [4, 5, 38, 65, 178]. As such, examining cellular translomes (i.e. genome-wide pool of all mRNAs being translated) can be very informative in order to address specific questions in the field of cancer cell biology. Polysome profiling analysis is one of the most commonly used methods for evaluating genome-wide translational control [14]. Using this method, mRNAs that are associated with and being translated by “heavy” polysomes (usually defined as four or more ribosomes attached to the mRNA) are separated from those associated with “lighter” polysomes (2-3

ribosomes), a single 80S ribosome or the 40S ribosomal subunit using sucrose density gradient centrifugation, followed by RNA isolation and subsequent analysis (Fig. 3.1 A). The threshold of four or more ribosomes in defining “heavy” polysomes is based on numerous studies [12, 14, 57, 63, 153, 154, 214, 216, 222, 223] demonstrating the ability to capture the majority (~80%) of newly synthesized proteins undergoing the “heavy”-to-“light” polysomal transition, thus allowing the measurements of translation efficiency by comparing mRNA abundance on the heavy polysomes between samples.

Importantly, changes in processes upstream of translation due to transcription and other post-transcriptional regulations will influence the steady-state mRNA abundance in the cell. Thus, it is essential to obtain data on cytoplasmic steady-state mRNA abundance (input mRNA prior to sucrose density gradient centrifugation) in parallel to allow corrections and specific definition of the mRNAs that are translationally regulated independent of mRNA abundance [12, 14, 63, 222, 223]. However, the most commonly used $\log_2$ ratio approach to estimate and score translation efficiency has been shown by number of studies to be problematic and often leads to the identification of many false positives and false negatives [12, 14]. Importantly, this project applied a per-gene analysis of partial variance (APV)-based method called ANOTA (Analysis of Differential Translation), which was specifically developed by our collaborator, Dr Ola Larsson in Karolinska Institutet, Sweden, to overcome these issues [214, 223]. Based on the global cytoplasmic and polysomal data, ANOTA analyses the translome and classify whether there are changes that will impact on cellular protein abundance due to: (a) transcriptional regulation (*mRNA abundance up/down*), (b) *bona fide* adjustments in translation efficiency with no change in cellular mRNA abundance (*translation up/down*), or (c) homeostatic mechanism to sustain protein levels (*buffering up/down*) even though total mRNA levels are altered (Fig. 3.1 B) [14, 57, 223]. This chapter will focus on the optimisation of polysome profiling as a technique to investigate mechanism(s) of drug response particularly in the Eμ-Myc model of B-cell lymphoma.

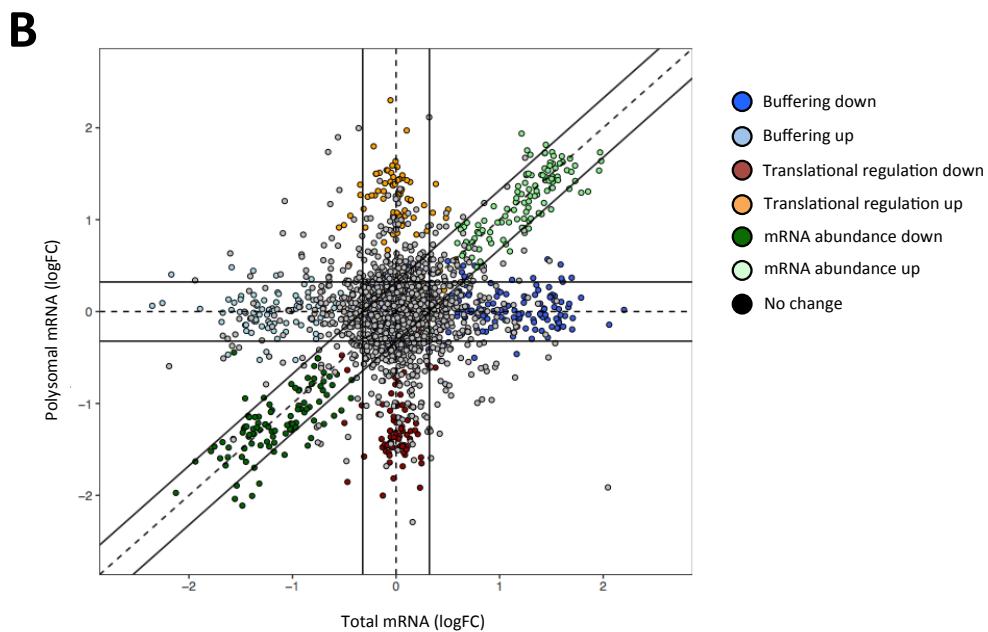
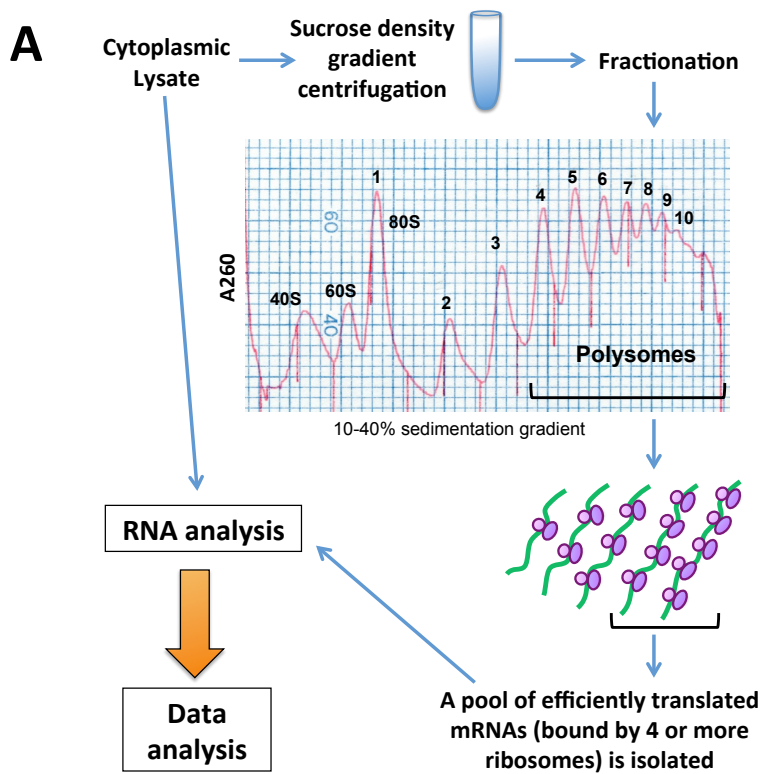


Figure 3.1: Experimental workflow for polysome profiling analysis

(A) Schematic of polysome profiling analysis: cytoplasmic lysate was layered on top of a linear 10-40% sucrose gradient, ultracentrifuged (36,000 rpm, 2¼ hours, 4°C) and

fractionated using the Foxy Jr Fraction Collector with constant monitoring of absorbance at 260 nm (A260) by an ISCO UA-6 Absorbance Detector. Fractions (one fraction per minute: 860 μ L per tube) corresponding to polysomal mRNAs that were bound by four or more ribosomes were pooled and analysed by RNA-seq followed by data analysis using ANOTA or LIMMA. For qRT-PCR analysis, fractions were kept separate or pooled as indicated in the figures. **(B)** An example of ANOTA analysis using simulated data demonstrating the possible outcomes: translational regulation, transcriptional regulation altering steady-state mRNA abundance or buffering.

3.2 Results

3.2.1 The monitoring of global and specific changes in the cellular translome using polysome profiling analysis

HeLa cells were utilised in the initial experiments as polysome profiling had been most commonly applied in the lab to characterise cellular translation profiles in adherent cells [104]. Cytoplasmic lysates from equal cell number were applied on continuous low-salt (containing 80 mM NaCl; equivalent to the concentration used in routine profiling) and high-salt (250 mM) 5-30% (w/v) sucrose gradients to distinguish the peaks in the polysomal traces. In high salt conditions, the Na⁺ ions compete with Mg²⁺ ions at the interface of the two ribosomal subunits [224, 225]. Tracings of absorbance at 260 nm (A260; which is used to measure nucleic acid abundance) and Western analysis of the fractions (Fig. 3.2 A) illustrate the dissociation of the third and beyond peaks into the first two peaks in high salt sucrose gradient, demonstrating that the peaks, going from left to right, are the 40S ribosomal subunit, 60S ribosomal subunit, 80S monosome and followed by polysomal peaks. This was confirmed by using the RNA bioanalyser for the low salt gradient fractions, which showed a transition from fractions containing only the 18S rRNA to later fractions containing both 18S and 28S rRNAs (Fig 3.2 B).

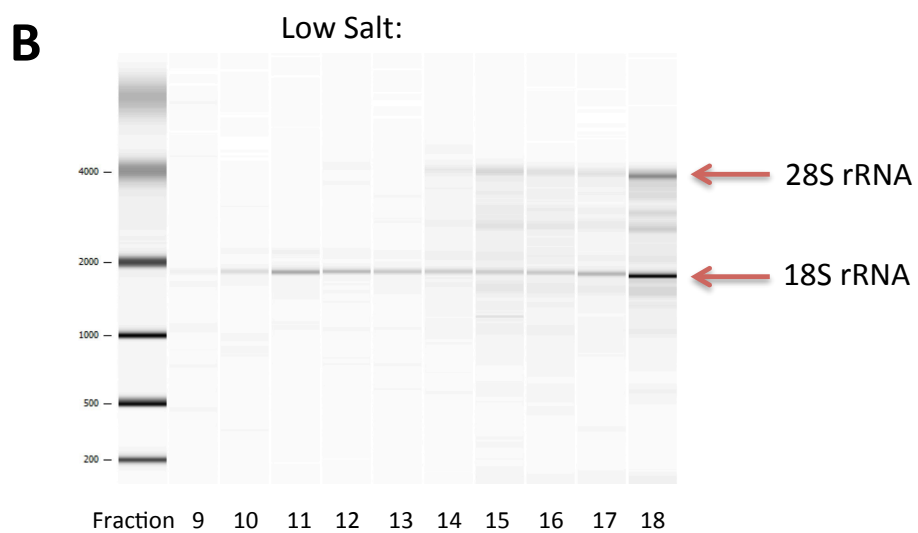
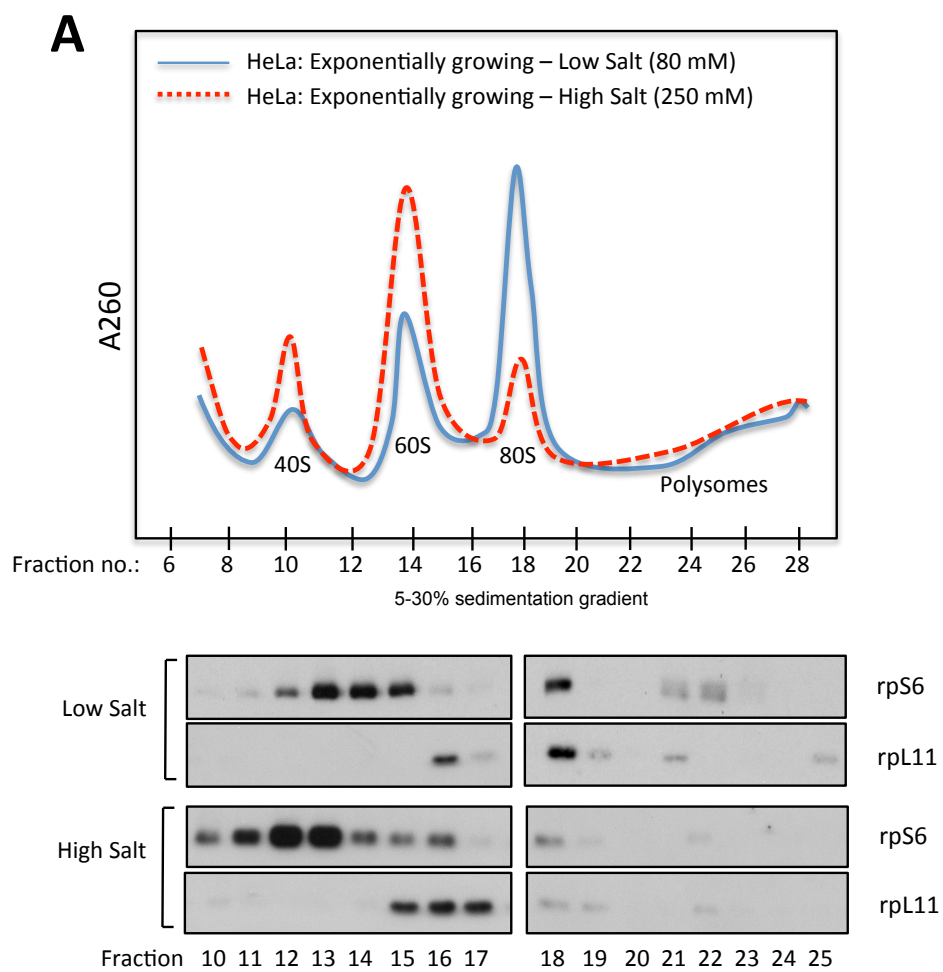


Figure 3.2: Characterisation of HeLa cells' sucrose density gradient sedimentation peaks

Cytoplasmic lysates from exponentially growing HeLa cells were separated on a low or high salt linear (5-30%) sucrose gradient by ultracentrifugation (36,000 rpm, 2¼ hours, 4°C) and fractionated using the Foxy Jr Fraction Collector with constant monitoring of A260 by ISCO UA-6 Absorbance Detector. **(A)** A260 sedimentation tracing illustrating separation of the ribosomal subunits in the high salt gradient. Equal volumes of the fractions 10-25 were analysed by Western blotting, showing successful dissociation of the 80S ribosomes into 40S and 60S ribosomal subunits (n=1). rpS6 is a component of the 40S ribosomal subunit. rpL11 is a component of the 60S ribosomal subunit. **(B)** rRNA content of fractions 9-18 were analysed by an Agilent RNA Nano Bioanalyzer chip (n=1). 18S rRNA is a component of the 40S ribosomal subunit. 28S rRNA is a component of the 60S ribosomal subunit.

In order to test the dynamic range of our method, we evaluated the capability of polysome profiling in monitoring HeLa cell's translational activity in response to altered nutrient levels. When the cells were deprived of amino acids for 2 hours, there was a robust decrease in absorbance in gradient fractions corresponding to actively translating polysomes and a concomitant increase in absorbance in sub-polysomal fractions (Fig. 3.3 A). However, amino acid stimulation of the amino acid starved cells induced the increase in the number of ribosomes engaged in polysomes (Fig. 3.3 B). We hypothesized that this effect was attributed to the roles of mTORC1 signalling pathway as a major hub that links abundance of nutrient, such as amino acids, to key anabolic processes affecting cell growth and size (Fig. 3.3 C) (Chapter 1.1.3; [226]). Thus next, to test whether our analysis of the polysomal fractions was able to detect specific changes in the translome, a doxycyclin-inducible dominant negative mutant of 4E-BP1, in which the four mTORC1 phosphorylation sites are mutated to Alanine [226, 227], was utilised. By mutating these phosphorylation sites 4E-BP1's can no longer inhibit eIF4E and thus reduces translation of mRNAs that strongly rely on eIF4E's activity, such as the 5'-terminal oligopyrimidine tract (5'TOP)-containing eEF1A and rpS6 mRNAs (Fig. 3.4 A). Overexpression of mutant 4E-BP1 in response to 1 µg/mL doxycyclin (Fig. 3.4 B) did not alter the A260

trace, thus did not globally alter mRNA translation (Fig. 3.4 C). However, mutant 4E-BP1 overexpression specifically decreased the percent of eEF1A and rpS6 mRNAs that were bound by actively translating, “heavier” polysomes thus shifted the distribution towards the “lighter” polysomes, that is a shift to the left side of the graph (Fig. 3.4 D-E). In contrast, the distribution of actin mRNA, a “house-keeping” and non-eIF4E responsive mRNA, was relatively unaltered (Fig. 3.4 F). These data established the capability of polysome profiling analysis in examining both dramatic and specific changes in cellular mRNA translation activity.

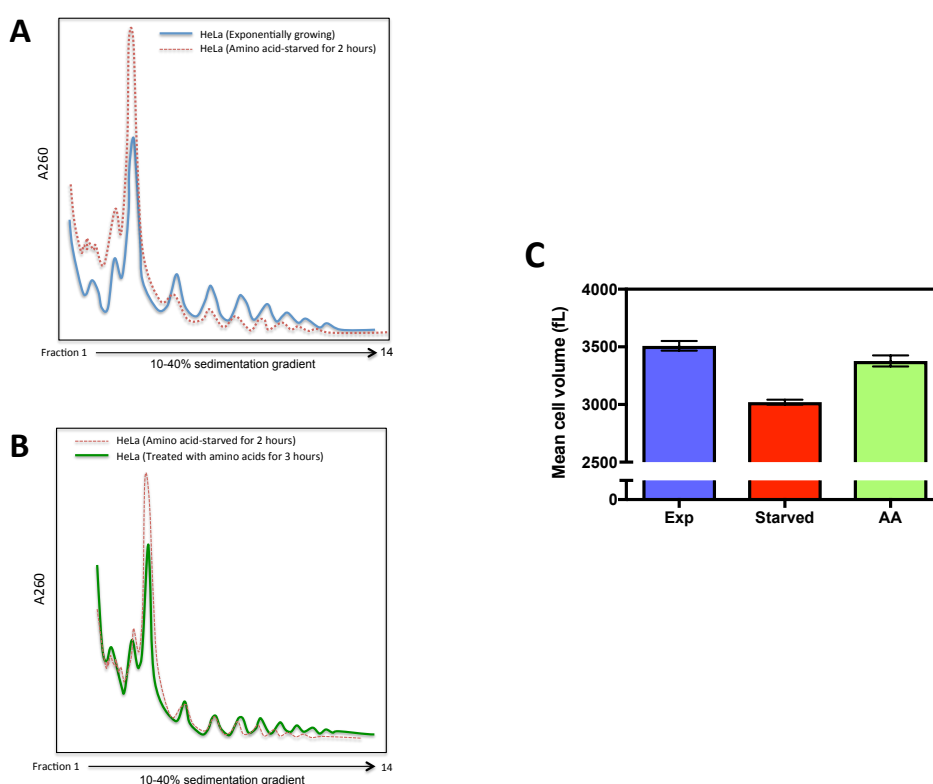


Figure 3.3: Amino acid abundance affects global mRNA translation activity

A260 sedimentation tracings (**A**) comparing translational activity of exponentially growing HeLa cells with those that were amino acid starved for 2 hours and (**B**) comparing amino acid starved cells (2 hours) with previously starved cells (2 hours) treated with 1X amino acid solution (minimal essential medium (MEM) amino acids (Gibco 11130-051) and MEM non-essential amino acids (Gibco 11140-050)). Traces are representative of n=2 and n=3 independent experiments respectively. (**C**) Mean cell volumes (MCV) of exponentially growing (Exp), amino acid-starved (Starved) and previously starved (2 hours) amino acid-treated HeLa cells (AA) were determined by Beckman Coulter Counter®. Graphs represent mean $\pm$ SD of 3 technical replicates from n=1 experiment.

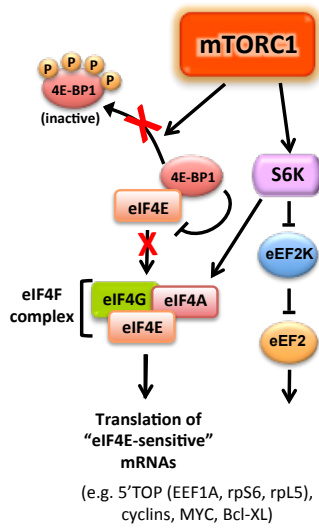
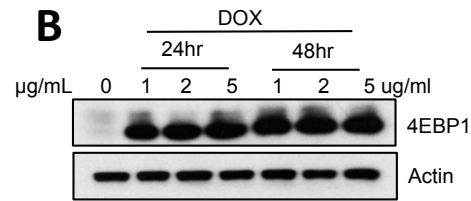
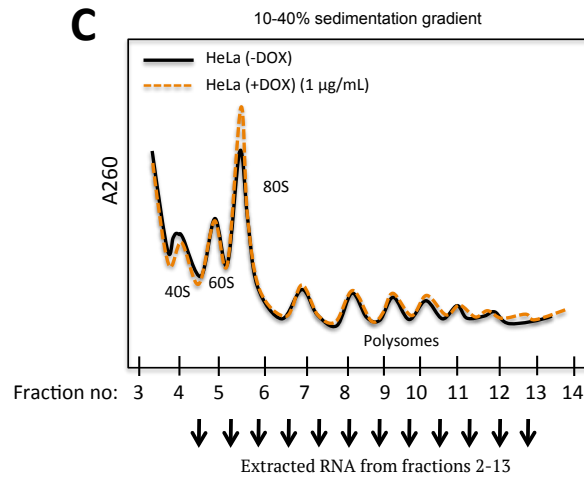
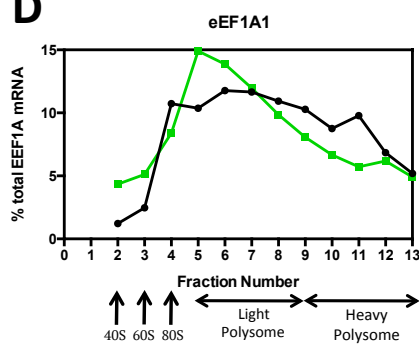
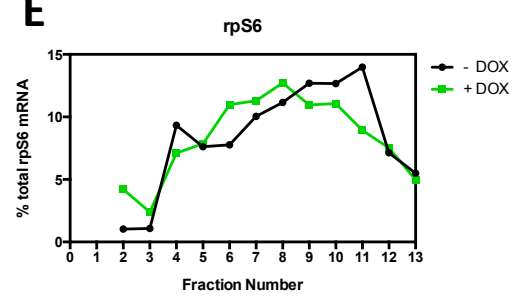
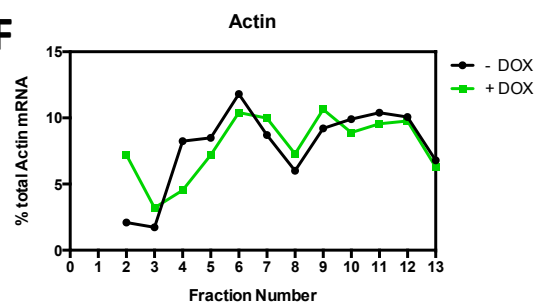
A**B****C****D****E****F**

Figure 3.4: Overexpression of a mutant 4E-BP1 protein decreases translation efficiency of eIF4E-sensitive mRNAs

(A) Schematic of the mTORC1 signalling pathway. 4E-BP1 and S6K are two of the best characterised downstream effectors of mTORC1. Four of the mTORC1 phosphorylation sites in mutant 4E-BP1 were converted to an alanine, thus preventing the activation of eIF4E and as a result decreases the translation of eIF4E-sensitive mRNAs. (B) Western blot analysis demonstrating that overexpression of mutant 4E-BP1 induced by doxycycline (DOX) treatment for 24 hours in HeLa cells stably expressing DOX-inducible 4E-BP1-4A mutant plasmid as indicated. (C) A260 sedimentations tracings comparing pre- and post-DOX induction. qRT-PCR analysis of polysome profiling fractions comparing the distribution of (D) eEF1A1, (E) rpS6 and (F) actin mRNAs in non-DOX-induced HeLa cells with DOX-induced cells. Levels of mRNA in each fraction are given as a percentage of the total mRNAs in all fractions. Sedimentations traces and mRNA distribution graphs are representative of n=2 independent experiments.

3.2.2 Investigating the translome of Eμ-Myc B-lymphoma cells in response to inhibitors targeting ribosome synthesis and activities

Typical polysome profiling traces for Eμ-Myc B-lymphoma cell extracts (Fig. 3.5 A), showed a small peak between the first two peaks that appears to be an artefact. High-salt (250 mM NaCl) and low-salt (80 mM NaCl) sedimentations via 5-30% sucrose gradients were performed to identify the peaks in the trace. The dissociation of the 80S ribosome peak in high salt sedimentation observed in both the trace and by Western analysis (40S: rpS6; 60S: rpL11) identified the correct 40S, 60S (the “artefact”), 80S and polysomal peaks in the polysome profiling traces of Eμ-Myc B-lymphoma cells (Fig 3.5 B). Having characterised the peaks, Eμ-Myc B-lymphoma cell extracts were used to assess whether treatment with mTORC1 inhibitor everolimus (20 nM) and Pol I transcription inhibitor CX-5461 (50 nM), which are the two main pharmacological tools of interest in this study, alters the cell’s translation profile and whether these changes can be identified by polysome profiling analysis. Eμ-Myc B-lymphoma cells (clone #4242) that constitutively overexpress the anti-apoptotic protein BCL2 [8] were utilised to prevent the induction of caspase-mediated apoptosis, thus allowing the investigation of

the cell's translational activities in response to these pharmacological agents independent of the effects of cell death.

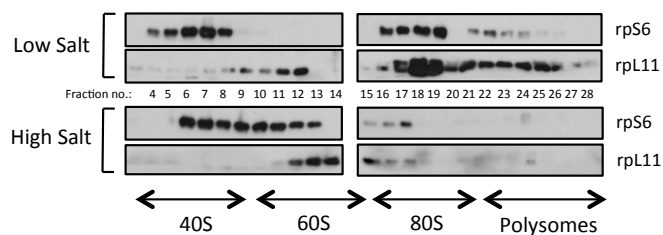
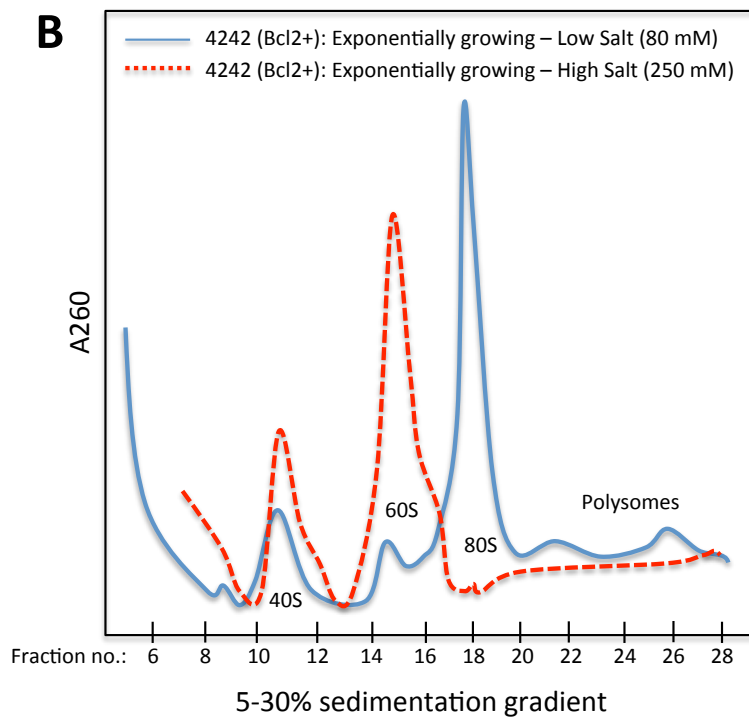
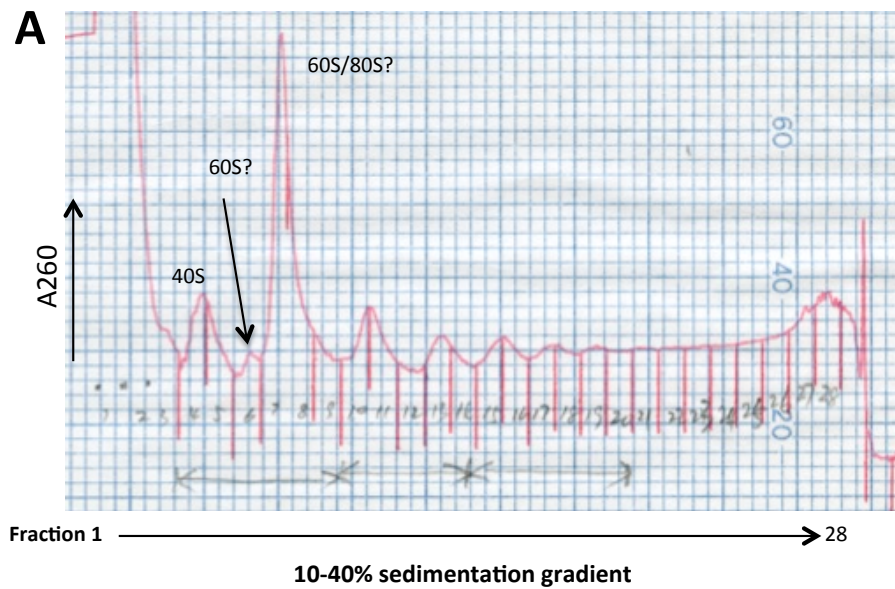


Figure 3.5: Characterisation of E μ -Myc B-lymphoma (clone #4242) cells' sucrose density sedimentation peaks.

(A) A260 sedimentation tracing showing a typical sedimentation profile of E μ -Myc B-lymphoma (clone #4242) cells. **(B)** A260 sedimentation tracings of a high salt-low salt sedimentation analysis (5-30% sucrose gradients) and Western blotting of equal volumes of fractions 2-18 to confirm dissociation of the 80S ribosomes into 40S and 60S ribosomal subunits with the high salt sucrose gradient (n=1). rpS6 is a component of the 40S ribosomal subunit. rpL11 is a component of the 60S ribosomal subunit.

Sustained repression of mTORC1 signalling was achieved following treatment with 20 nM everolimus for 3 hours and 24 hours as reflected by a robust decrease in the abundance of phosphorylated p70S6K1 (Thr389), rpS6 (Ser240/244) and 4E-BP1 (Thr37/46) (Fig. 3.6 A). Rapalogues, such as everolimus, are known to only induce partial inhibition of the 4E-BP1-eIF4E axis, thus requiring longer treatment time to significantly alter the phosphorylation status of 4E-BP1 [166-168]. Everolimus treatment reduced the number of 80S ribosomes involved in actively translating polysomes, as indicated by a decrease in the absorbance (260 nm) of fractions corresponding to “heavy” polysomes (HP fractions 11-14) and “medium” polysomes (MP fractions 8-10), plus a concomitant increase in the size of the 80S monosome peak (Fig 3.6 B). The RNA component of the fractions was isolated using Qiagen RNeasy Minikit and analysed by qRT-PCR to determine the distribution of 5'TOP-containing mRNAs across the sedimentation gradient. Everolimus-mediated inhibition of mTORC1 signalling reduced the percentage of both eEF1A1 and rpL5 mRNAs associated with heavy polysomes and shifted their distribution to the “lighter” polysomes (Fig. 3.6 C-D), with minimal changes observed in the distribution of the housekeeping, eIF4E-insensitive beta-2-microglobulin (β 2M) mRNA (Fig. 3.6 E).

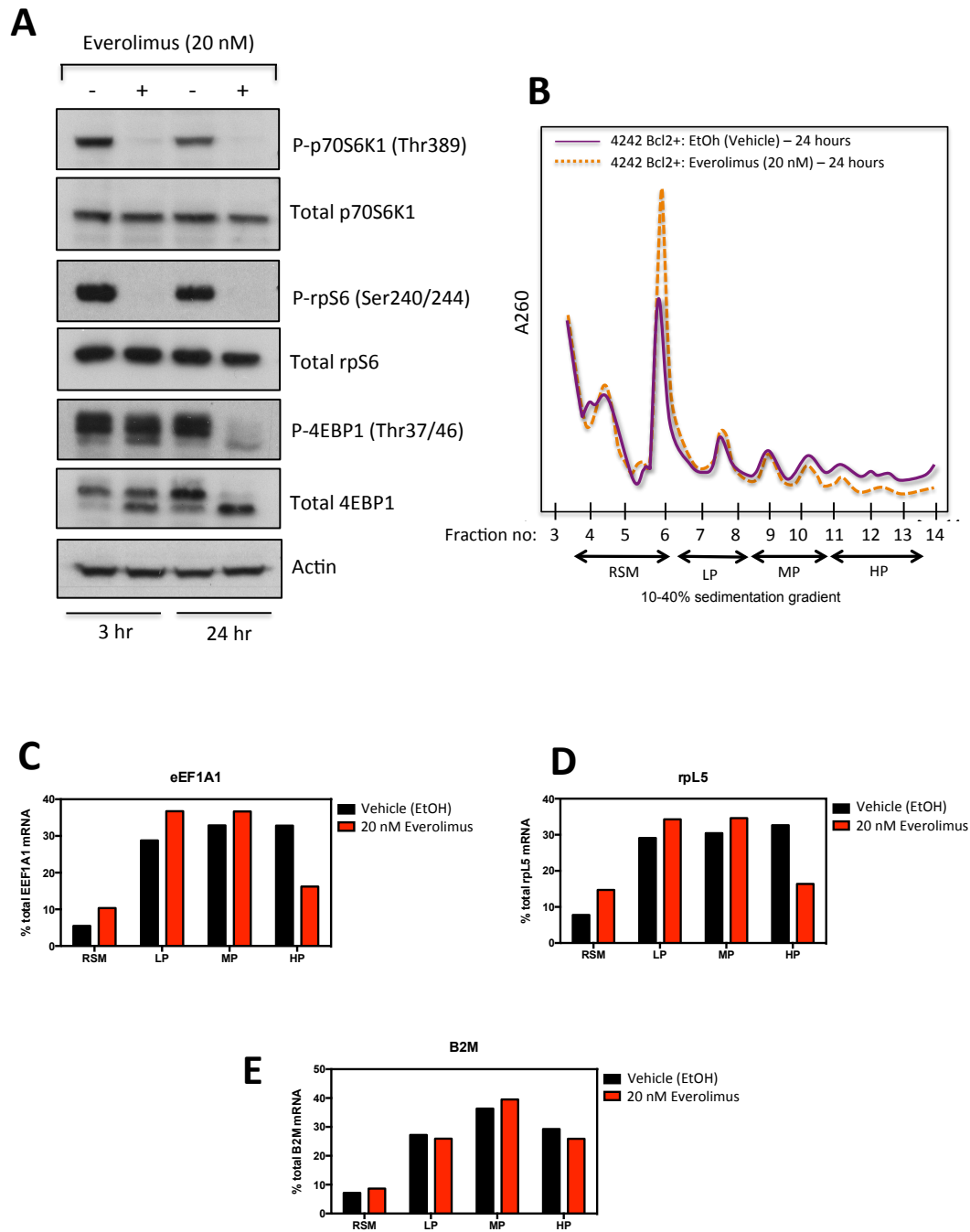


Figure 3.6: Inhibition of mTORC1 signalling by everolimus specifically reduces the translation efficiency of eIF4E-sensitive mRNAs

(A) Western blotting analysis of BCL2-overexpressing E μ -Myc B-lymphoma (clone #4242) cells treated with 20 nM everolimus for 3 or 24 hours. **(B)** 260 nm sedimentation tracings comparing BCL2-overexpressing E μ -Myc B-lymphoma (clone #4242) cells treated with everolimus as indicated for 24 hours. RSM: 40S and 60S ribosomal subunits; LP: “light”, less actively translating polysomes; MP: “medium” polysomes; HP: “heavy”, more actively translating polysomes. qRT-PCR analysis of sedimentation fractions of BCL2-overexpressing E μ -Myc B-lymphoma (clone #4242) cells treated as indicated

showing the distribution **(C)** eEF1A, **(D)** rpL5 and **(E)** β 2M mRNAs across the sucrose gradients. Levels of mRNA in each fraction are given as a percentage of the total mRNAs in all fractions. Western blots, sedimentation tracings and qRT-PCR data are representatives of n=3 independent experiment.

Treatment of E μ -Myc lymphoma cells with 50 nM RNA Pol I transcription inhibitor CX-5461 (Fig. 3.7 A) caused an accumulation of p53 protein by 3 hours which was sustained at 24 hours (Fig. 3.7 B) due to the induction of the well-characterised nucleolar stress response pathway (chapter 1.2.3). Fraction-by-fraction analysis of the sedimentation gradients (Fig. 3.7 C) by qRT-PCR indicated modest shifts in the number of ribosomes associated with the eEF1A1 and rpL5 mRNAs in response to 24-hour CX-5461 treatment. In Vehicle-treated cells, 40-50% of eEF1A1 and rpL5 mRNAs were bound by actively translating polysomes in fractions 10-14 (Fig. 3.7 D-E), while CX-5461 treatment shifted the mRNA to fractions 6-8 corresponding to 80S monosomes and disomes (Fig. 3.7 D-E). However, it's unclear whether these shifts were attributed to CX-5461-specific translational regulation of these mRNAs or as a result, for instance, of a reduced number of ribosomes in CX-5461-treated cells following 24 hour inhibition of RNA Pol I transcription. Therefore, it is necessary to examine the genome-wide translome of the E μ -Myc B-lymphoma cells in response to acute CX-5461 treatment, followed by the application of ANOTA in the analysis of the RNAseq data. This approach will not only identify whether there is a direct and CX-5461-specific translational regulation, but also evaluate the robustness of this polysome profiling experimental pipeline for future work.

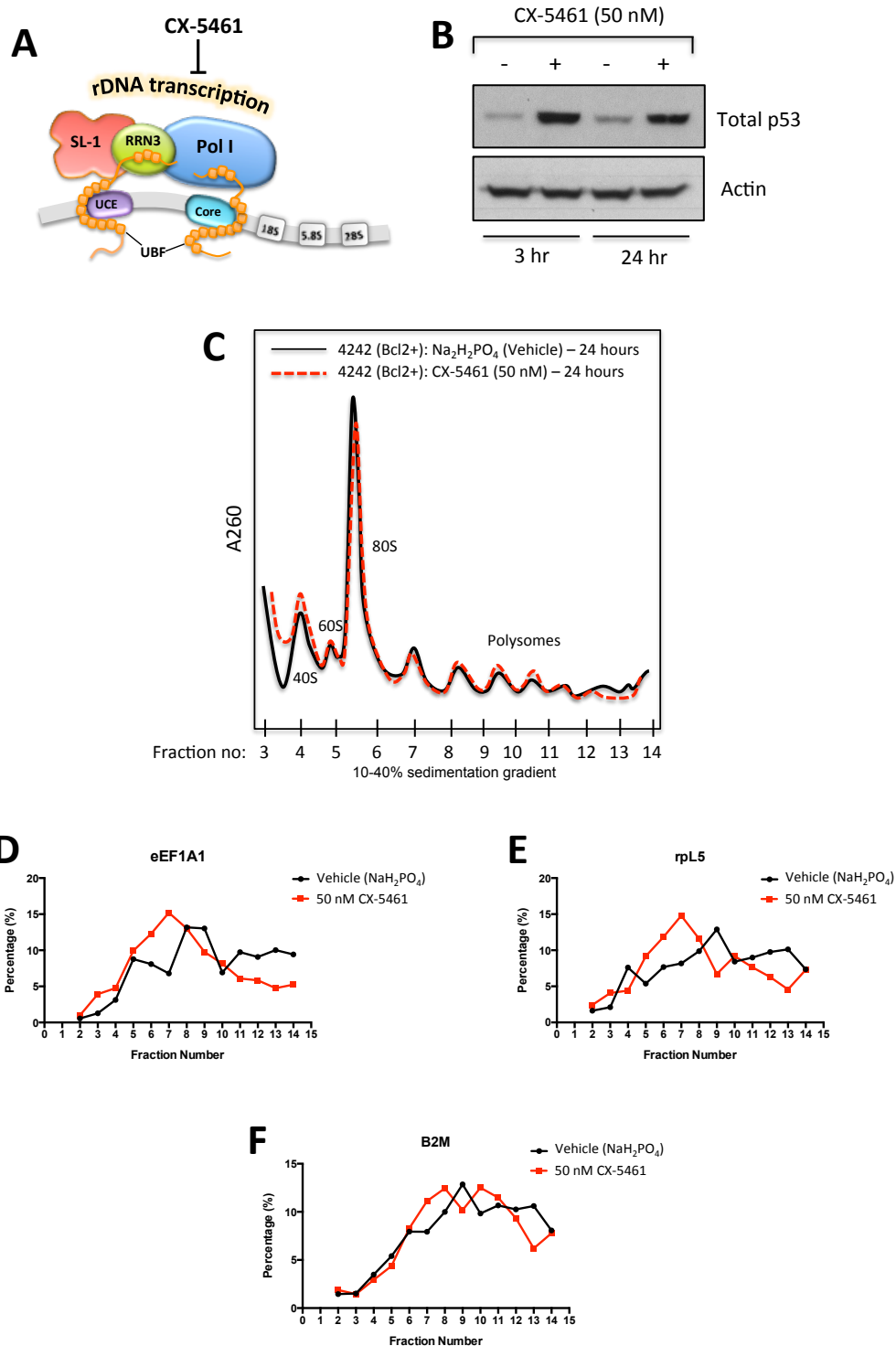


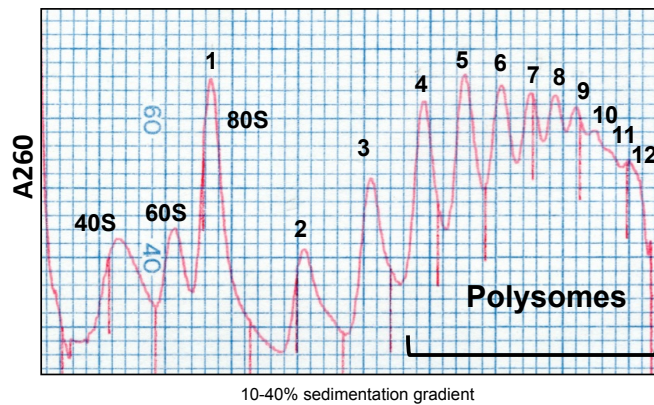
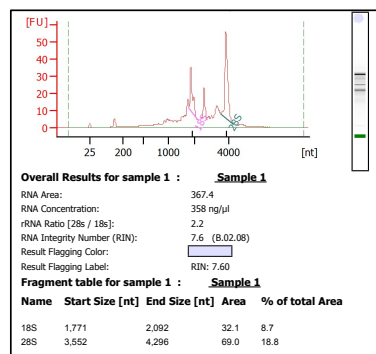
Figure 3.7: Changes in cellular translation profiles in response to prolonged CX-5461 treatment

(A) CX-5461 is a specific inhibitor of RNA Pol I-mediated transcription of the rDNA. **(B)** Western blotting analysis of BCL2-overexpressing Eμ-Myc B-lymphoma (clone #4242) cells treated with CX-5461 as indicated for 3 or 24 hours. **(C)** 260 nm sedimentation tracings comparing BCL2-overexpressing Eμ-MYC B-lymphoma (clone #4242) cells

treated as indicated for 24 hours. qRT-PCR analysis of sedimentation fractions of BCL2-overexpressing E μ -Myc B-lymphoma (clone #4242) cells treated as indicated showing the distribution **(D)** eEF1A, **(E)** rpL5 and **(F)** β 2M mRNAs across the sucrose gradients. Levels of mRNA in each fraction are given as a percentage of the total mRNAs in all fractions. Western blots, sedimentation tracings and qRT-PCR data are representatives of n=3 independent experiments.

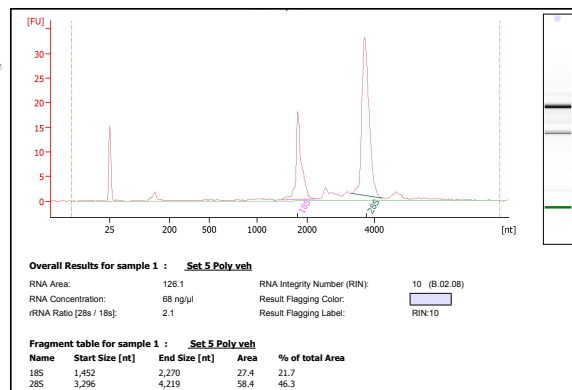
3.2.3 RNAseq-based polysome profiling analysis of CX-5461 treated E μ -Myc B-lymphoma cells

Quality RNA samples with a highly-preserved integrity are required to ensure that reliable results are obtained from a RNAseq-based, genome-wide polysome profiling analysis. Such desirable properties depend on the preservation of the polyribosome chains until the sucrose gradient has been fractionated and RNA lysis buffer has been added to the fractions (Figure 3.1 A). The protocol was optimised to improve the quality and efficiency of the polysome preparation, such as: using freshly prepared cytoplasmic lysate, reducing the cycloheximide treatment from 15 mins to 5 mins while increasing the concentration from 10 to 100 μ g/mL, optimisation of the DTT (dithiothreitol) concentration, and lastly, the implementation of a BioComp Gradient Maker that allows for the generation of 6 gradients at once in a rapid and reproducible manner. These adjustments brought about substantial improvements to the quality of polysome fractionation (comparing Fig 3.8 A to Fig 3.5 A), with complexes of more than 10 ribosomes remained intact. This, in turn, dramatically improved the integrity of the isolated polysomal RNAs as measured by RNA Integrity Number (RIN), with values of 7.6 to 10 out of 10 (Fig 3.8 B).

A**B**

Old protocol

Optimised protocol

**Figure 3.8: Optimisation of polysome profiling protocol**

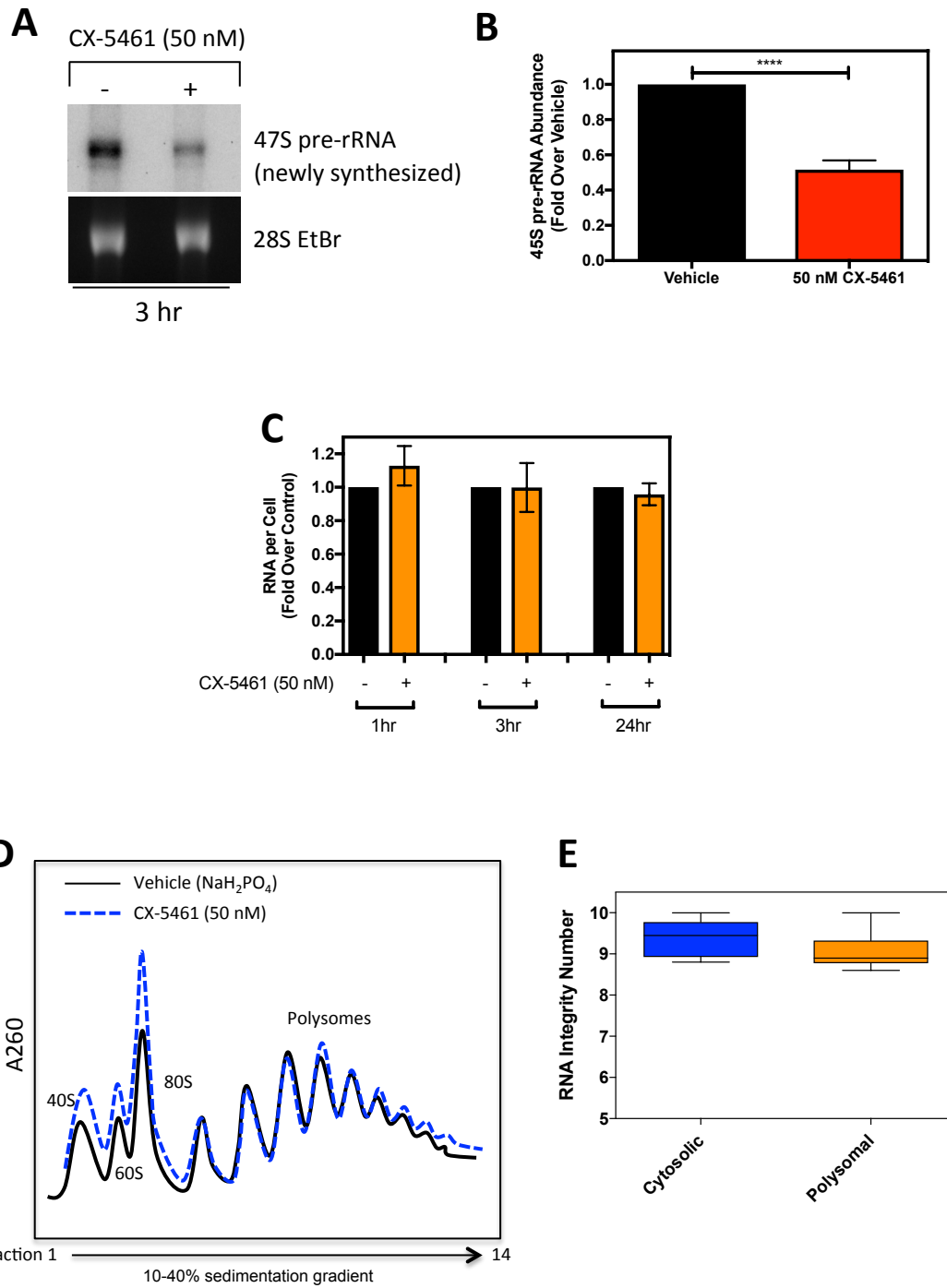
(A) A260 sedimentation tracing illustrating a typical sedimentation profile for Eμ-Myc B-lymphoma (clone #4242) cells obtained using an optimised polysome profiling protocol.

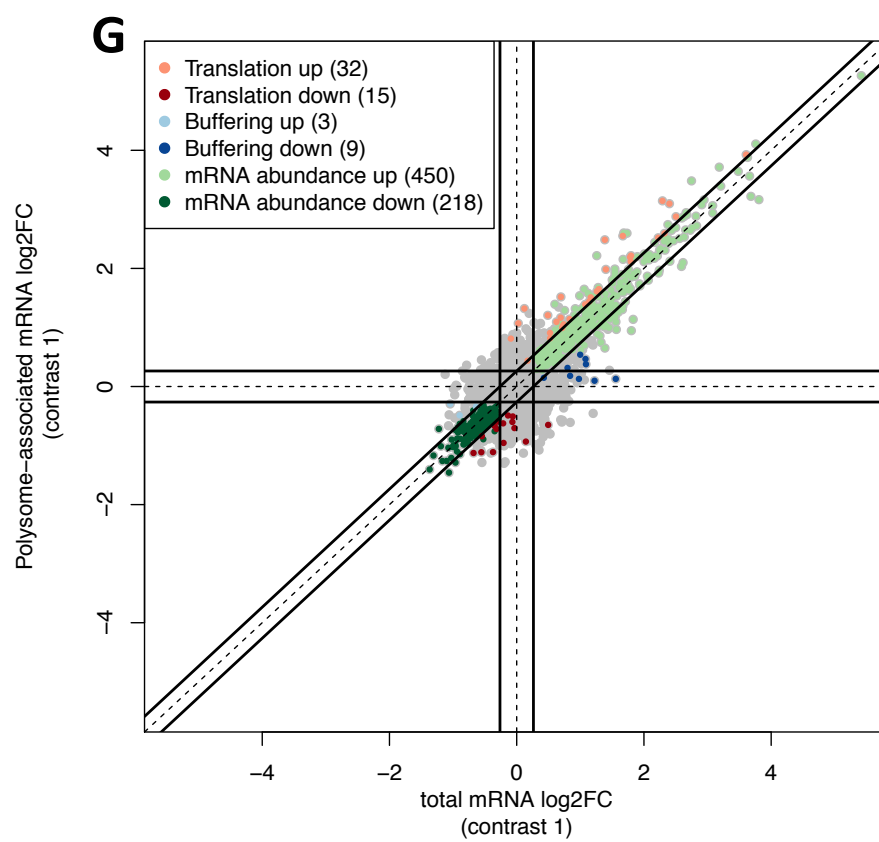
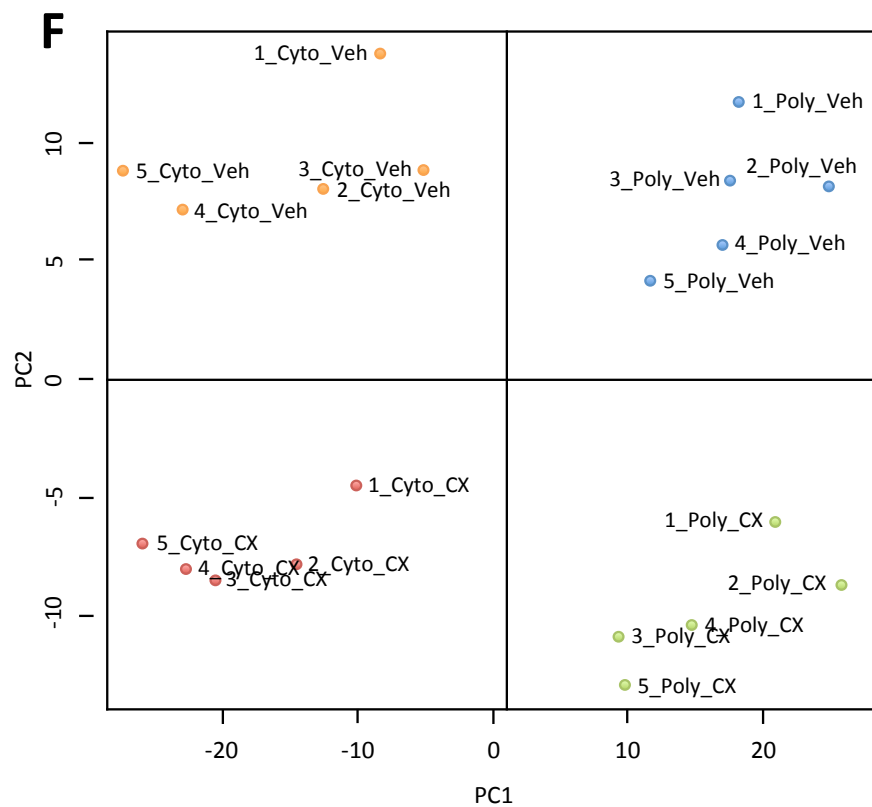
(B) Analysis of pooled sedimentation fractions containing actively translating polysomes (mRNAs bound by 4 or more ribosomes) using an Agilent RNA Nano Bioanalyzer chip illustrating an improvement of the RNA integrity number (RIN) achieved by establishing an optimised polysome profiling protocol.

Exponentially growing Eμ-Myc B-lymphoma cells (#4242, BCL2 overexpressing) in culture were treated with 50 nM CX-5461 for 3 hours (based on Fig. 3.7 and Bywater et al., [8]) and the effect of CX-5461 on the rates of *de novo* rRNA synthesis was examined by ³²P-orthophosphate labelling. At this concentration and time point, CX-5461 reduced the 47S pre-rRNA synthesis rates by 48.4% compared to control (Fig 3.9 A and B) with no change in RNA per cell (Fig 3.9 C). The 260 nm sedimentation tracing overlay (Fig 3.9 D) indicated that there were differences the cells' translational profile in response to CX-5461 treatment. These changes were then further investigated using an RNAseq-based polysome profiling analysis. Quality RNAs were isolated from the pooled polysomal fractions and the initial cytoplasmic lysate for further analysis (Fig. 3.9 E).

Principal component analysis of the RNA-seq data demonstrated a clear horizontal separation based on the sample sources ("Cyto" samples isolated from input lysates (cytosolic) from "Poly" samples isolated from pooled polysomal fractions 9-14) and vertical separation based on the treatment conditions (Fig. 3.9 F). Importantly, analysis using the ANOTA package (Fig. 3.9 G) indicated that most of the changes were due to altered mRNA abundance, that is due to transcriptional regulation (668 mRNAs), as opposed to translational regulation (47 mRNAs) or buffering (12 mRNAs). Pathway analysis of translationally regulated mRNAs unfortunately was not robust due to very few number of genes identified in this category. Thus, genes in the "mRNA abundance up" and "mRNA abundance down" (information on mRNAs that are differentially bound to polysomes, obtained by comparing polysomal vs. polysomal data) categories were evaluated using the enrichment analysis tool, Metacore® GeneGo, to identify cellular processes that were altered via transcriptional mechanisms in response to acute treatment with CX-5461 (Fig. 3.9 H). In particular, there was a strong upregulation (bright red spheres) of G1-S cell cycle checkpoint signatures ("Cell cycle_G1-S Interleukin regulation" and "Cell cycle_G1-S Growth factor regulation"; ratio >0.14; false discovery rate (FDR)/Adjusted P-value <0.05) (Fig. 3.9 H). Amongst the genes in the "Cell cycle_G1-S interleukin regulation" and "Cell cycle_G1-S Growth factor regulation" processes, p21 (*CDKN1A*)

was one of the genes with the highest fold-change, and was validated by qRT-PCR analysis (Fig. 3.9 I). Furthermore, as most of these changes were due to elevated mRNA abundances via transcriptional activation, a transcription factor enrichment analysis was also carried out using Metacore® GeneGo. The transcription factors CREB1, p53 and SP1 were determined as the most influential in mediating these cellular responses to CX-5461 (Fig. 3.9 J), with 29 genes were co-regulated by these three transcription factors (Fig. 3.9 K). Thus, these translational profiling data demonstrates the capabilities of this analysis method to not only pinpoint the key changes in the translation of specific genes in response to a particular stimulus, but also to identify the nature of regulatory mechanism underlying these responses.





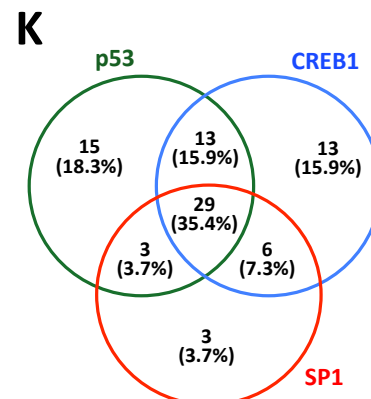
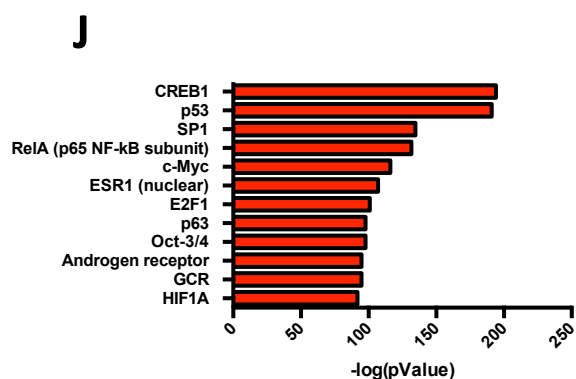
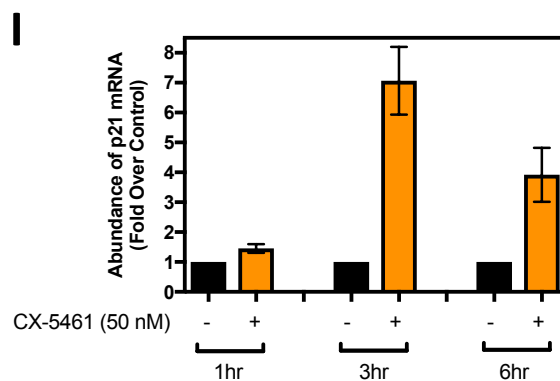
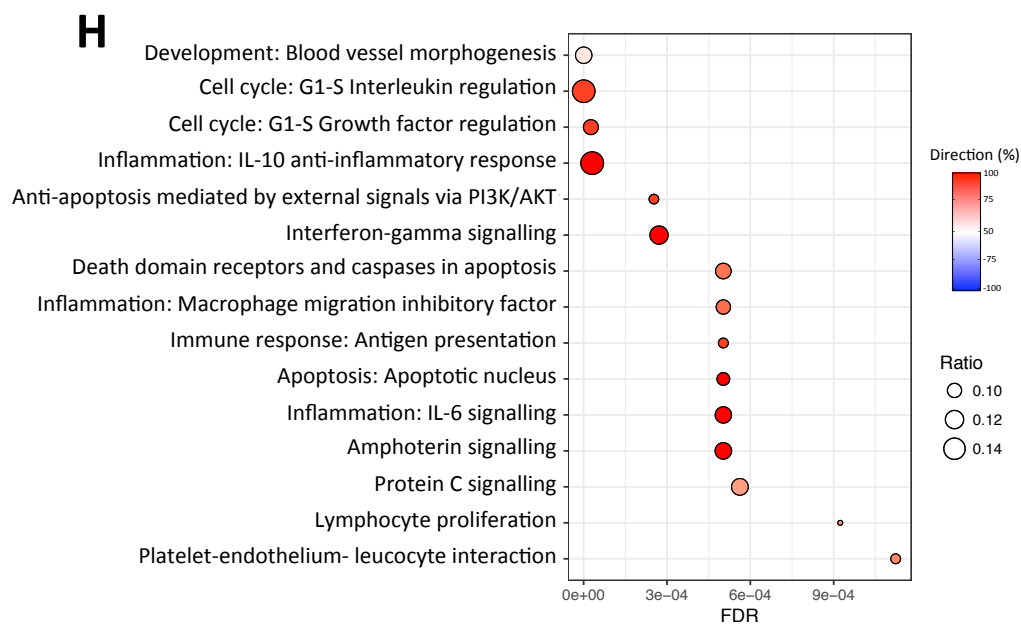


Figure 3.9: Inhibition of rDNA transcription induces alterations in cellular translation that are mediated by transcriptional changes

(A) Representative image of ^{32}P -orthophosphate labelling of BCL2-overexpressing E μ -Myc B-lymphoma (clone #4242) treated as indicated for 3 hours and (B) its densitometry analysis. Graphs represent mean $\pm$ SEM of n=4 independent experiments; ****, $p \leq 0.0001$. (C) Quantitation of total intracellular RNA using a Nanodrop spectrophotometer. Graphs represent mean $\pm$ SD of n=2 independent experiments. (D) A260 sedimentation tracings comparing BCL2-overexpressing E μ -Myc B-lymphoma (clone #4242) cells treated as indicated for 3 hours. (E) Box-and-whisker plot showing the quality of RNA samples isolated from input lysates (cytosolic) and pooled fractions 9-14 (polysomal) based on RIN analysis using an Agilent RNA Nano Bioanalyzer chip. (F) Principal component analysis (PCA) to evaluate the reproducibility of the RNA-seq data, showing the first two principal component (PC) separations which segregate the replicates based on RNA source (PC1) and biological differences (PC2). Cyto: cytosolic (input) RNA; Poly: polysomal RNA; Veh: Vehicle (NaH_2PO_4)-treated cells; CX: CX-5461-treated cells. (G) A dot-plot output from ANOTA showing the fold changes (treatment - control) of polysome-associated mRNAs against the total (input) mRNAs for all genes in the analysis. (H) Enrichment analysis by MetaCore® GeneGO of genes in “mRNA abundance up” and “mRNA abundance down” categories identified by anota2seq analysis. The colour and intensity indicate direction and percentage of genes in each gene ontology (GO) term that is up (red) or down (blue). Ratio indicates number of genes from the input list that match the GO term. FDR, false discovery rate (adjusted P). (I) qRT-PCR analysis of BCL2-overexpressing E μ -Myc B-lymphoma (clone #4242) treated as indicated. (J) Transcription factor analysis by MetaCore® GeneGO of genes in “mRNA abundance up” and “mRNA abundance down” categories identified by anota2seq analysis. (K) Venn diagram showing the number of genes that are part of the indicated transcription factor network. (D)-(K) Polysome profiling analysis was carried out using samples obtained from n=6 independent experiment

3.2.4 Optimising polysome profiling analysis for *in vivo* application

The aim of the next thesis chapter of this thesis is to investigate the mechanism(s) underlying the *in vivo* synergistic effects of CX-5461 and everolimus co-treatment. As polysome profiling is one of the main tools to answer this research question, it is important to adapt this optimised protocol

for *in vivo* applications to ensure the integrity of polysomal RNAs extracted from freshly isolated animal tissues.

A well-established and transplantable *in vivo* E μ -Myc lymphoma model was utilised in this thesis. Specifically, 6-8 weeks old C57Bl/6 mice were injected intravenously (lateral tail vein) with green fluorescent protein (GFP)-expressing (MSCV) E μ -Myc lymphoma cells. At disease onset lymph nodes were harvested and sorted by fluorescent-activated cell sorting (FACS) for GFP-expression (Fig 3.10 A). Cytoplasmic lysates of the sorted cells were sedimented, however even after combining sorted cells from 3 animals (2 axillary and 2 inguinal nodes from each mouse: total of 12 lymph nodes), the UV absorbance detector setting of the fractionator had to be adjusted from 0.5 to a lower sensitivity setting (0.2) to visualize profiles such as Figure 3.10 B, due to the low yield. In addition, the long processing times involved in tissue harvesting, live cell sorting (2-3 hours) and sucrose density gradient sedimentation also decreased cell viability and the quality of the polysome preparation.

Thus, tumour cell enrichment using a magnetic-activated cell sorting (MACS) method, rather than FACS, was then tested (Fig 3.11 A). Specifically, CD45.2-expressing E μ -Myc B-lymphoma cells generated from C57BL/6 mice, were injected into PTPRCa (Ly5.1) mice, which is a congenic strain of the C57BL/6 model that expresses CD45.1 [228]. In the first approach (Fig 3.11 B), biotin-conjugated CD45.1 antibody was added to the isolated lymph node cells (from 2 axillary and 2 inguinal nodes) from a diseased mouse. Thus, the CD45.1-expressing stromal cells were attracted to the magnetic column, while the CD45.2-expressing E μ -Myc B-lymphoma cells, together with other unbound cells, were eluted out. However using this approach, there was only a minimal improvement in the enrichment of GFP-positive tumour cells (45.1% to 67.1%) when the eluent was analysed by flow cytometry (Fig 3.11 B). In the second approach, biotin-conjugated CD45.2 antibody was added to the isolated cells, allowing for the non-CD45.2-expressing cells to be eluted first (Fig 3.11 C). The cells that were bound to the magnetic column were then released by mechanical force using a plunger. While this approach resulted in a dramatic enrichment of GFP-positive E μ -Myc B-lymphoma cells (38.5% to 79.4%), the

yield (as illustrated by the A260 sedimentation tracing) was lowered, potentially due to the reduced viability following the mechanical stress applied during the second elution (Fig. 3.11 C). Another drawback of this method is that the latest MACS separator model at the time of writing (QuadroMACS®) only allows for processing of 4 samples at once, which would in this case impede the efficiency of the harvest and sample processing.

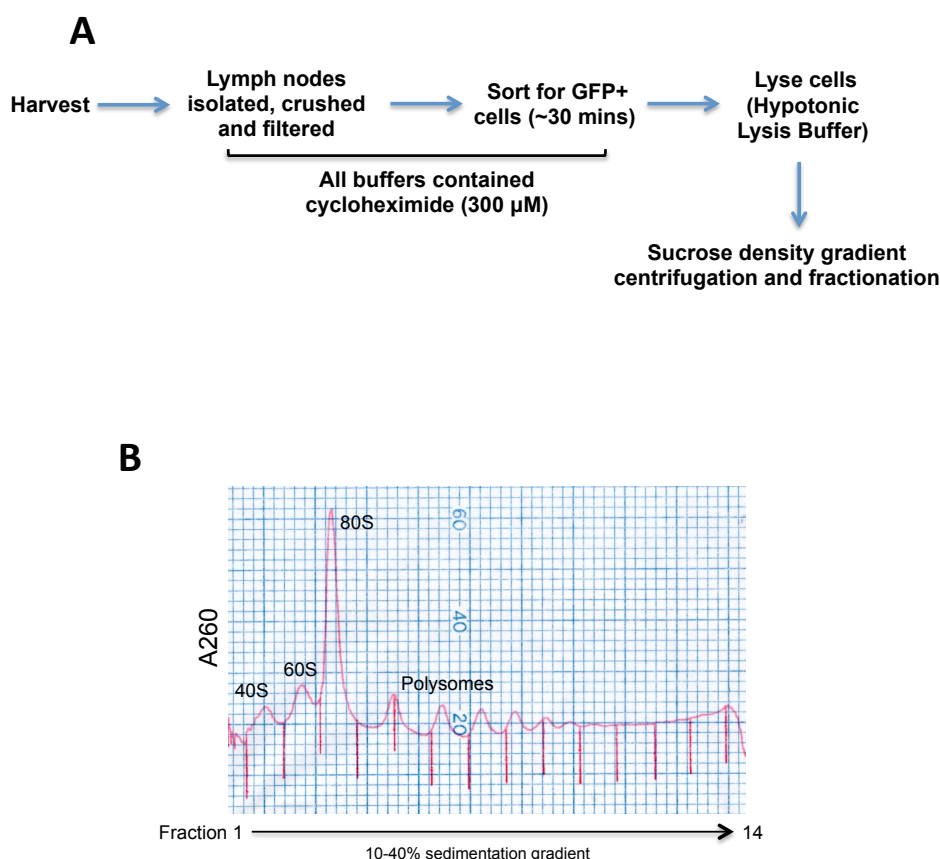
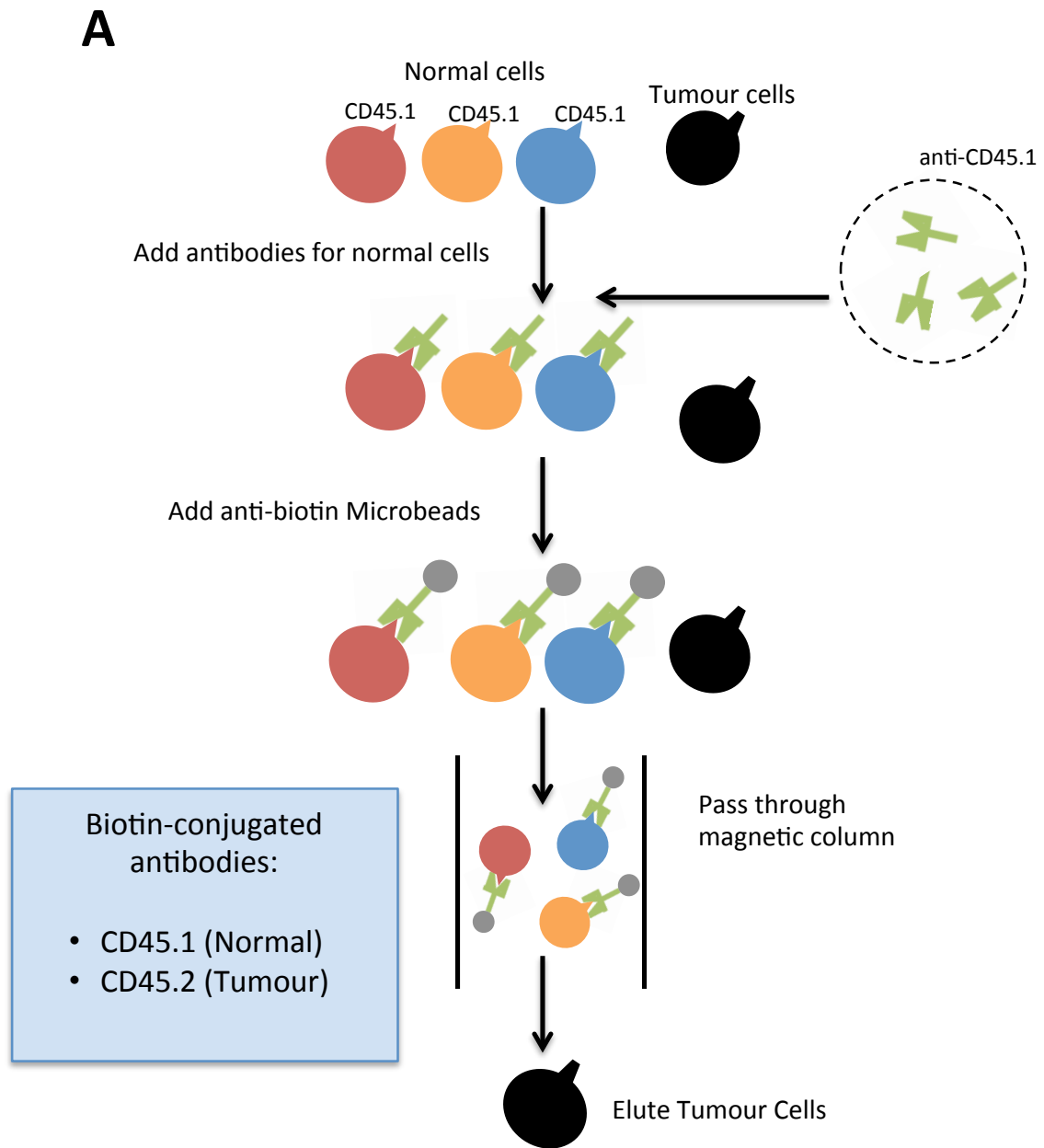
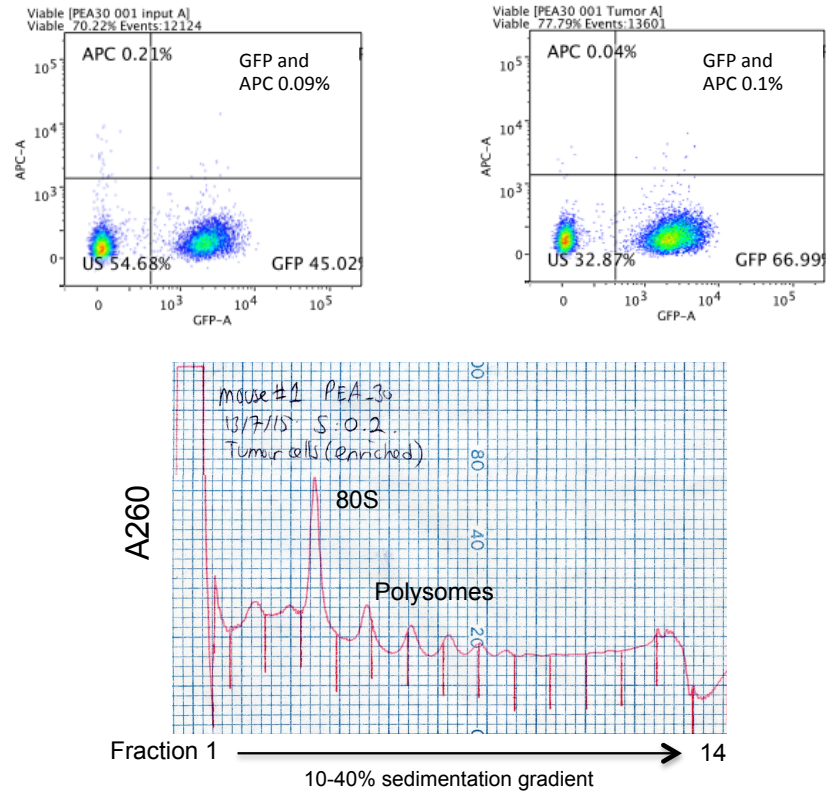


Figure 3.10: Optimisation of polysome profiling workflow to analyse mammalian tissue samples: sorting for GFP-positive cells by fluorescence-activated cell sorting (FACS)

(A) Experimental workflow for polysome profiling analysis involving FACS: freshly isolated lymph nodes were crushed, filtered and the cells sorted by FACS to enrich for GFP-positive E μ -Myc B-lymphoma cells. Cytoplasmic lysates from the sorted cells were sedimented by sucrose density gradients and then fractionated. **(B)** A260 sedimentation tracing showing a typical sedimentation profile of FACS-sorted E μ -Myc B-lymphoma cells obtained from freshly isolated lymph nodes.



B



C

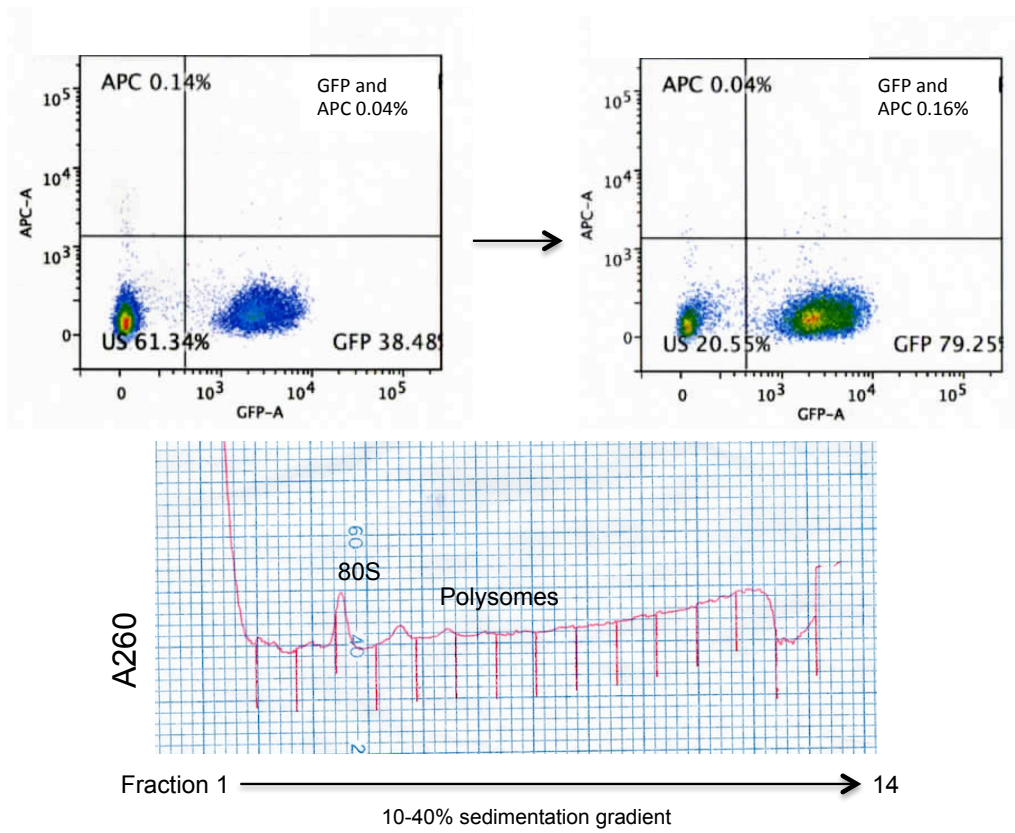
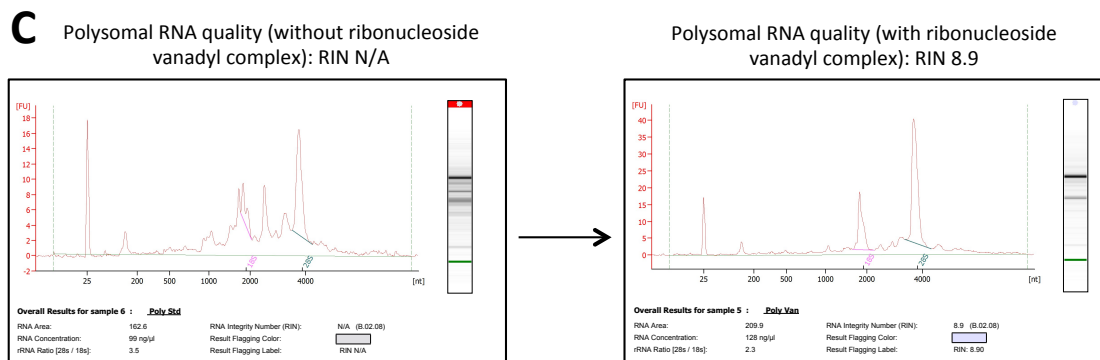
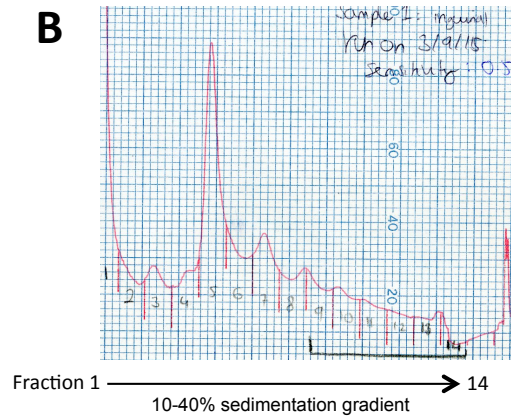
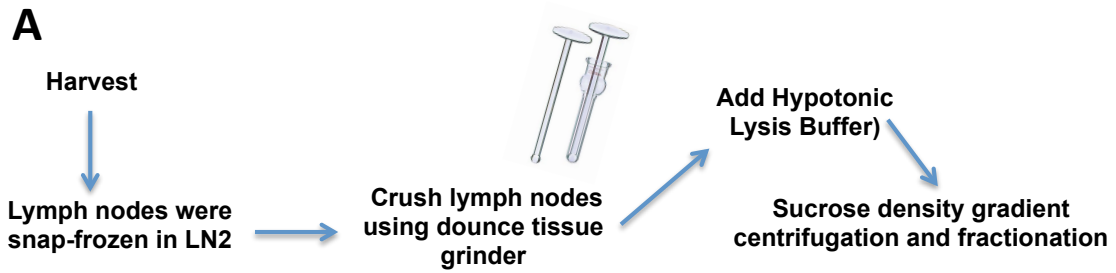


Figure 3.11: Optimisation of polysome profiling workflow for mammalian tissue samples: sorting for CD45.2-expressing E μ -Myc B-lymphoma cells by magnetic-activated cell sorting (MACS)

(A) Experimental workflow for MACS. The cells in a PTPRCa (Ly5.1) mouse model express CD45.1, whereas the transplanted E μ -Myc B-lymphoma cells express CD45.2. Representative flow cytometry dot-plots and 260 nm sedimentation tracings from sucrose density gradient centrifugation analysis of enriched GFP-positive E μ -Myc B-lymphoma cells when (B) biotin-conjugated CD45.1 antibody or (C) biotin-conjugated CD45.2 antibody was added to the starting lymph node cells mixture.

Subsequent trials involving pulverising snap-frozen tissue samples using glass dounce homogenizers (Fig 3.12 A, Chapter 2.6.2) resulted in a sufficient yield from two lymph nodes totalling 30-60 mg (Fig 3.12 B). The low RIN issue was improved by the addition of ribonucleoside vanadyl complex, a potent inhibitor of RNAses, to a modified hypotonic lysis buffer containing a 10-fold higher concentration of cycloheximide (1,000 μ g/mL) compared to the one used for cultured cells (Fig 3.12 C). Ribonucleoside vanadyl complex however does interfere with downstream processing, specifically reverse transcription of the RNA, including the synthesis of cDNA library for RNAseq [229]. Thus, an additional clean-up step using the Qiagen RNeasy Mini Kit was implemented to improve the outcome of the cDNA library preparation (Fig. 3.12 D). Furthermore, with regards to the efficiency of the processing workflow, tissues collected at harvest were immediately snap-frozen, and then processed and sedimented through sucrose density gradient in batches. This workflow resulted in superior and more robust outcomes compared to those in other published studies involving isolation of polysomal mRNA from mammalian tissues [216]. Therefore, this was the method applied in the next chapter to examine changes in cellular translationalome *in vivo*.



cDNA library from polysomal RNA (RIN 8.9) in Fig. 3.12 C

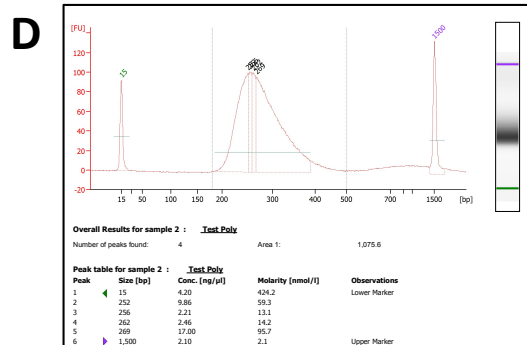


Figure 3.12: Optimisation of polysome profiling workflow to analyse mammalian tissue samples: processing snap-frozen small tissue samples using dounce homogeniser

(A) Experimental workflow and (B) 260 nm sedimentation tracing showing a typical sedimentation profile for polysome profiling analysis of pulverised snap-frozen tissue samples. (C) RIN analysis using an Agilent RNA Nano Bioanalyzer Chip demonstrating a marked improvement in the quality of polysomal RNAs isolated using hypotonic lysis buffer containing ribonucleoside vanadyl complex. (D) An electrophoresis output from an Agilent DNA Nano Bioanalyzer Chip demonstrating a typical cDNA library profile obtained using a workflow outlined in (A).

3.3 Discussion

Critical roles of mRNA translation in the modulation of gene expression have been reported in a range of physiological processes such as regulation of the immune system [14], ER stress response [34], as well as human cancers [4, 15, 178]. However, studies evaluating the translome rather than the transcriptome are less common. The data presented in this chapter demonstrated the capability of polysome profiling to monitor translation efficiency of specific mRNAs using qRT-PCR, or globally using RNAseq, in response to inhibition of the growth regulator mTORC1 pathway and/or ribosome biogenesis. As previously reported in the literature, inhibition of mTORC1 signalling mediated by overexpression of a mutant 4E-BP1 protein (Fig. 3.4) or by everolimus treatment (Fig 3.6) specifically reduced the translation efficiency of “eIF4E-sensitive” mRNAs in HeLa cells and E μ -Myc B-lymphoma cells, respectively.

On the other hand, CX-5461 suppresses the cell's translation capacity by inhibiting Pol I transcription and thus reducing the synthesis of ribosomes. qRT-PCR analysis of the polysomal fractions isolated from cells treated with CX-5461 for 24 hours indicated modest shifts in the translational efficiency of the same mRNAs analysed in the everolimus experiment. However, it was not clear whether these shifts were due to other reason(s), for example a limitation in the number of ribosomes [77, 217]. To obtain a more complete picture of the cells' translational activity in response to Pol I inhibition, global

analysis of polysome-associated mRNAs was performed (Fig. 3.9) following an acute treatment with CX-5461 to detect causative (direct) factors rather than the repercussions (indirect). The cells were treated at a concentration that inhibited *de novo* rDNA transcription by ~50% (IC₅₀) as measured by ³²P-orthophosphate labelling (Fig 3.9 A-B). The analysis of RNA-seq data from the cytoplasmic and polysome-associated mRNAs using ANOTA revealed a transcription-dependent increase in translation of mRNAs encoding proteins involved in activation of a G1-S cell cycle checkpoint as one of the main cellular responses to Pol I transcription inhibition by CX-5461. Therefore, the shifts in the qRT-PCR data (Fig. 3.7) were not due to translational regulation of these mRNAs, and unlikely due to reduced translation capacity i.e. limiting number of available ribosomes (as ribosomes are known to be stable up to 48 hours), but may be caused by a G1-S checkpoint-activated growth arrest. The results from this translational profiling analysis not only confirm published data in terms of checkpoints triggered by CX-5461 [6], but also provided a novel insight into the nature of gene expression regulation mediating these responses, the majority of which is due to transcriptional and not translational regulation. Furthermore, it provided insight on the less-investigated links between Pol I transcription and key immune responses highlighted in Fig. 3.9 H, such as Interferon gamma (IFN-γ) signalling, which could potentially be harnessed as a novel therapeutic strategy [230]. For example, we have demonstrated in previous studies that, especially in solid tumours, the efficacy of CX-5461 is associated with senescence-like phenotypes and cell cycle arrest [126, 212]. IFN-γ is one of the key cytokines secreted by senescent cells that have acquired a senescence-associated secretory phenotype (SASP) [231]. Thus IFN-γ signalling could be targeted to induce a senolytic (i.e. killing senescent cells) effect and thereby improving the efficacy of CX-5461.

The most substantial improvement to the quality of polysome fractionation was achieved by only using freshly prepared cytoplasmic lysate. While the RNA quality was minimally affected, freeze-thawing of lysate severely affected the stability of complexes of 4 or more ribosomes, thus markedly reducing the quality of polysome profiling analysis that routinely use 4 ribosomes as a cut-

off. Reducing the cycloheximide treatment from 15 mins to 5 mins while increasing the concentration from 10 to 100 µg/mL diminishes the stress experienced by the cells caused by this treatment but ensures that ribosome run-off is averted. Optimisation of the DTT (dithiothreitol) concentration allowed the identification of the most optimal reducing status of the lysis buffers, without affecting the interaction between the ribosomal subunits. Finally, BioComp Gradient Maker allows the preparation of 6 gradients at once, instead of manually preparing sucrose gradient in the previous protocol using an ISO Gradient Former Model 160 (SpectraLab Scientific). This ensures that sucrose gradients prepared within and between experiments are reproducible. Furthermore, to improve and maintain the quality of the polysome preparation, all components of the fractionator should be thoroughly cleaned using RNaseZap, followed by diethyl-pyrocabonate-treated water, 80% ethanol and once again using diethyl-pyrocabonate-treated water before and after every use to remove RNases and other contaminants that may have been lodged in any of the components, especially in the tubing system.

Polysome profiling and ribosome footprinting are the most commonly used translationalomics tools to date, each with its own strengths and weaknesses. While ribosome footprinting allows the accurate pinpointing of the ribosomes' positions on the mRNA and the identification of key ribosome binding sites, it is dependent on the abundance and the actual magnitude of the shift of the mRNAs of interests. Thus this method has a tendency to identify mRNAs that are present in high abundance and exhibit larger shifts in translation efficiency, such as those containing the 5'TOP structure, as being translationally regulated [15]. In contrast, polysome profiling allows the evaluation of mRNAs of lower abundance and less robust shifts but still possibly significant and is generally viewed as a more reliable approach to analyse translation efficiency on a global scale. In addition, the methodologies of ribosome profiling may cause some biases due to: a) the random digestion by RNase I (only degrades single-stranded RNA), b) treatment with cycloheximide often stalls ribosomes near initiation sites, and c) a number of false positive and false negative results from its "translation efficiency (TE) score". To illustrate the latter, for example there are 4 "X" mRNA molecules:

X1, X2, X3 and X4, that are bound by 4 ribosomes at position a, b, c and d. The ribosome footprinting technique and its method of data analysis via normalisation of RPFs over randomly fragmented total mRNA will not be able to distinguish between these two scenarios: (i) all 4 ribosomes are occupying X1 mRNA at 4 different positions, or (ii) X1, X2, X3 and X4 are each associated with just one ribosome at position a, b, c, and d, respectively. Such bias will generate an overestimation of the translation efficiency of X mRNA. On the other hand, polysome profiling preserves the integrity of the multi-ribosome chains, allowing a more accurate measure of changes in the translation efficiency of a particular mRNA as more ribosomes are loaded to that mRNA molecule. Therefore ideally, the application of not just a single genome-wide translational profiling approach, coupled with robust data analysis methods, are needed to comprehend the full complexities of the changes in cellular translationalome in response to stimuli in question.

Chapter 4:
Investigating
Translational
Alterations Associated
with Resistance to
Cancer Therapies
Targeting the Ribosome

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Publications arising from this chapter:

- **Kusnadi, E.P.**, Trigos, A.S., Cullinane, C., Goode, D.L., Larsson, O., Devlin, J.R., Chan, K.T., De Souza, D.P., McConville, M., McArthur, G.A., Sanij, E., Hannan, R.D., Hannan, K.M., Kang, J., and Pearson, R.B., 2018, Reprogrammed mRNA translation drives activation of the energy metabolism-dependent, pro-survival cAMP-EPAC1/2-Rap1 pathway to confer resistance to therapeutic targeting of the ribosome (*manuscript in progress*)
- Devlin, J.R., Hannan, K.M., Hein, N., Cullinane, C., **Kusnadi, E.P.**, Ng, P.Y., George, A., Shortt, J., Bywater, M.J., Poortinga, G.P., Sanij, E., Kang, J., Drygin, D., O'Brien, S., Johnstone, R.W., McArthur, G.A., Hannan, R.D., and Pearson, R.B., 2016, Combination therapy targeting ribosome biogenesis and mRNA translation synergistically extends survival in MYC-driven lymphoma, *Cancer Discovery*, 6(1), 59-70

4.1 Preface

The candidate (E.P.K) designed, performed and analysed all experiments in the chapter and wrote the original draft of the manuscript. RNAseq data analysis was assisted by A.S.T, D.L.G. and O.L. *In vivo* animal experiments were carried out with the assistance of C.C. and her team. J.R.D, K.T.C, D.P.D, G.A.M., R.D.H. and E.S. provided materials and resources (e.g. cell lines, reagents and tissue samples). K.H, J.K and R.B.P reviewed and edited the manuscript. K.M.H., J.K. and R.B.P. are the supervisors of this study.

4.2 Introduction

It has been demonstrated that inhibition of Pol I-mediated rDNA transcription, which is a key rate-limiting step for ribosome synthesis, by CX-5461 selectively targets MYC-driven B-lymphoma cells *in vivo* without killing the normal B-cells [1]. Furthermore, in Devlin *et al.* [1], we showed that the combination of CX-5461 and mTORC1 inhibitor everolimus synergistically prolonged life to more than double the survival benefit achieved by single-agent therapy in MYC-driven model of B-lymphoma.

However, despite the synergistic extension of survival, the transplanted Eμ-*Myc* cells eventually became resistant to the CX-5461-everolimus combination therapy (from here on will be referred to as “CX-5461+EV”) and the mice eventually succumbed to disease relapse. As both inhibitors target the cell’s mRNA translation machinery (chapter 1.4), we hypothesized that both the mechanism(s) of synergy and resistance will involve specific changes in the cellular translational program. Thus, polysome profiling, a high-throughput technique to evaluate cellular translatome established in Chapter 3, was applied to characterise the acute effects of CX-5461 and everolimus co-treatment *in vivo* on global mRNA translation profiles – the first aim of this chapter. To address the second aim of this chapter, which was to investigate resistance mechanism(s) to CX-5461+EV treatment, lymph nodes that were harvested at the end of a survival experiment in Devlin *et al.* [1] were collected for processing (chapter 2.9). These lymph node cells were then adapted to grow in cell culture medium (chapter 2.1) to establish early-passage cell lines that were used in the subsequent experiments and analysis including polysome profiling. Finally, based on the findings from these analyses, the third aim of this chapter was to design therapeutic approaches that could improve the efficacy of inhibitors of ribosome biogenesis and protein synthesis for treating MYC-driven haematological malignancies.

4.3 Manuscript

Reprogrammed mRNA translation drives activation of the energy metabolism-dependent, pro-survival cAMP-EPAC1/2-Rap1 pathway to confer resistance to therapeutic targeting of the ribosome

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Summary

Elevated ribosome biogenesis is a key determinant in oncogene-driven cancers, which is commonly targeted by many DNA-damaging cytotoxic drugs. New agents that specifically inhibit ribosome biogenesis and are less genotoxic are showing increasing promise in clinical trials. Here we use *in vivo*, transcriptome-wide analysis of the translational landscape to demonstrate that the efficacy of selectively inhibiting rRNA synthesis and mTORC1-dependent translation in improving survival of mice bearing MYC-driven lymphomas is associated with acute suppression of translation of mRNAs encoding enzymes regulating energy metabolism. Furthermore, we demonstrate that acquired resistance to this combination therapy is driven by translational reprogramming of mitochondrial respiration, which is mediated by activation of the cAMP-EPAC pathway that can be exploited to markedly improve the efficacy of ribosome targeted therapy. Thus compromised ribosome biogenesis and function results in a specific re-wiring of the translational machinery, which underlies the evolution of a newly-identified, targetable program of drug resistance.

Keywords: ribosome biogenesis, mRNA translation, RNA polymerase I, drug resistance metabolic rewiring

Introduction

The majority of human tumours are characterised by deregulated signaling through the PI3K/RAS/MYC network leading to increased rates of ribosome biogenesis and protein synthesis, an essential step in oncogenesis. It is now clear that many of the commonly used DNA-damaging cytotoxic drugs including 5-fluorouracil (5-FU), etoposide and oxaliplatin interfere with ribosome biogenesis and target the “addiction” of transformed cells to elevated rates of ribosome biogenesis, inducing the “impaired ribosome biogenesis checkpoint” (IRBC) (Pelletier et al., 2018). Consequently, a

number of less-genotoxic drugs that selectively target ribosome biogenesis have been developed and are showing increasing promise in clinical investigations (Pelletier et al., 2018; Quin et al., 2014; Woods et al., 2015).

The mechanism(s) underpinning the efficacy of ribosome-targeted therapies include both the p53-dependent IRBC and p53-independent nucleolar-targeted activation of the DNA-damage response that precedes genomic instability (Quin et al., 2016; Xu et al., 2017). We developed the “first in class” selective inhibitor of ribosome biogenesis, CX-5461, which targets RNA Polymerase I (Pol I), suppressing ribosomal RNA (rRNA) synthesis (Bywater et al., 2012). Moreover, we demonstrated its *in vivo* efficacy in mouse models of both blood and prostate cancer (Bywater et al., 2012; Devlin et al., 2016; Hein et al., 2017; Rebello et al., 2016). These studies served as the basis for testing CX-5461 in clinical trials in patients with solid tumours and refractory blood cancers. These dose-finding/safety trials in heavily pre-treated patients are showing promising results with evidence of therapeutic responses (Hilton et al., 2018; Khot et al., 2017). Subsequently, we reasoned that simultaneous targeting of ribosome biogenesis and protein synthesis, the critical “house-keeping” processes where oncogenic networks converge to maintain cancer cell growth and survival, would overcome many of the upstream drivers of resistance to oncogene-targeted therapies. This led to combining CX-5461 with inhibitors of mTORC1-dependent mRNA translation, which *in vivo*, markedly improved the therapeutic efficacy of either drug alone (Devlin et al., 2016; Rebello et al., 2016). However, the mechanisms underlying this synergistic effect and the development of resistance to the combination therapy are unclear and their definition will be important in optimizing the efficacy of ribosome-targeting therapies.

To interrogate the molecular basis of the response to combination therapy targeting the ribosome, we first examined the response to targeting ribosome biogenesis with the specific small molecule inhibitor CX-5461 and inhibition of mTORC1-mediated translation with everolimus. We first performed genome-wide translational profiling to characterise the acute changes in mRNA usage of MYC-driven lymphomas in mice treated with CX-5461 and everolimus. The

combination therapy specifically targeted the translation of mRNAs that encode multiple components of the translational apparatus and enzymes that regulate energy metabolism. The importance of this selective targeting of translation was emphasised by our finding that resistance eventually arose to the combination therapy, driven by reprogramming of the translation of mRNAs containing short 5'UTRs, which encode proteins that are key components of the mitochondrial respiration network and the cAMP-EPAC-Rap1 survival pathway. We confirmed the functional importance of this reprogramming by demonstrating that combination therapy-resistant cells are more metabolically active and have elevated levels of cAMP. Critically, specific inhibition of cAMP-EPAC1/2-Rap1 signalling re-sensitises resistant cells to combination therapy, demonstrating the importance of this pro-survival pathway in resistance. These studies highlight the importance of selective alterations in the translation of mRNAs encoding metabolic enzymes to the response of tumour cells to ribosome-targeting therapies. We predict our findings will be highly relevant in PI3K/AKT, MYC and RAS driven tumors targeted by selective ribosome biogenesis inhibitors as well as those treated with the classical range of cytotoxic drugs that inhibit this response.

Results

Acute inhibition of ribosome biogenesis and function selectively reduces mRNA usage of components of the translational apparatus

To examine the mechanisms of response to targeting ribosome biogenesis and protein synthesis with CX-5461 and everolimus, E μ -Myc B-lymphoma (MSCV *Gfp*) cells were transplanted into C57BL/6 mice (Bywater et al., 2012; Devlin et al., 2016). Lymphoma-bearing mice were treated on Day 10 post-transplant for 2 hours with CX-5461 (35 mg/kg), everolimus (EV; 5 mg/kg), or both (CX-5461+EV; 35 mg/kg CX-5461 and 5 mg/kg EV). We chose this early time point to exclude any confounding effects of drug-induced apoptosis on our molecular analyses. Indeed, no change in the percentage of GFP-positive

lymphoma cells was observed in lymph nodes isolated from treated animals (Figure S1A). The on-target activity of EV was confirmed by the reduction in rpS6 (Ser240/244) phosphorylation in lymph nodes treated with the EV or CX-5461+EV (Figure 1A). In addition, as expected, accumulation of p53 protein was also observed in response to CX-5461 and CX-5461+EV treatment (Figure 1A).

Given the specific alterations in translation patterns observed in response to mTOR inhibition (Hsieh et al., 2012; Morita et al., 2013) and in genetic models of compromised ribosome biogenesis (Khajuria et al., 2018), we performed polysome profiling to enable whole transcriptome analysis of mRNAs whose translation efficiency was altered by the acute ribosome-targeted combination therapy (Figure 1B). To define changes in actively-translated mRNAs (indicated by attachment of four or more ribosomes) we compared the translational signatures of lymphoma cells isolated from combination therapy-treated mice (CX-5461+EV) versus vehicle-treated mice (V/V) using anota2seq analysis (Oertlin et al., 2018). After normalization to steady-state total RNA, the polysomal RNA-seq data indicated that the only significant change was a reduction in the translation of transcripts involved in regulation of mRNA translation itself (Figure 1C). No significant effect on translation was observed upon single agent CX-5461 treatment (Figure S1B). Not surprisingly, given the role of mTORC1 in translational control (Hsieh et al., 2012; Ma and Blenis, 2009), EV treatment alone also affected the translation of mRNAs involved in mRNA translation (Figure S1C). Strikingly though, by comparing the steady-state and polysome-associated mRNA counts in the top 2 enriched processes in Figure 1C and Figure S1C (“Translation initiation” and “Translation elongation”), the increased efficacy of combining EV with CX-5461 compared with EV alone was associated with potent and specific suppression of the translation of mRNAs encoding key proteins regulating mRNA translation without altering the steady-state abundance of these mRNAs as determined by anota2seq analysis (Figure 1D). It was also clear that the suppressive effect on cellular translational activity in response to CX-5461+EV treatment was far more robust than EV alone (Figure 1D). Finally, consistent with our previous report (Devlin et al., 2016), our polysome profiling

data pointed to elevated expression of the proapoptotic protein BMF (Bcl-2-modifying factor) in response to EV and CX-5461+EV treatment (Figure S1D).

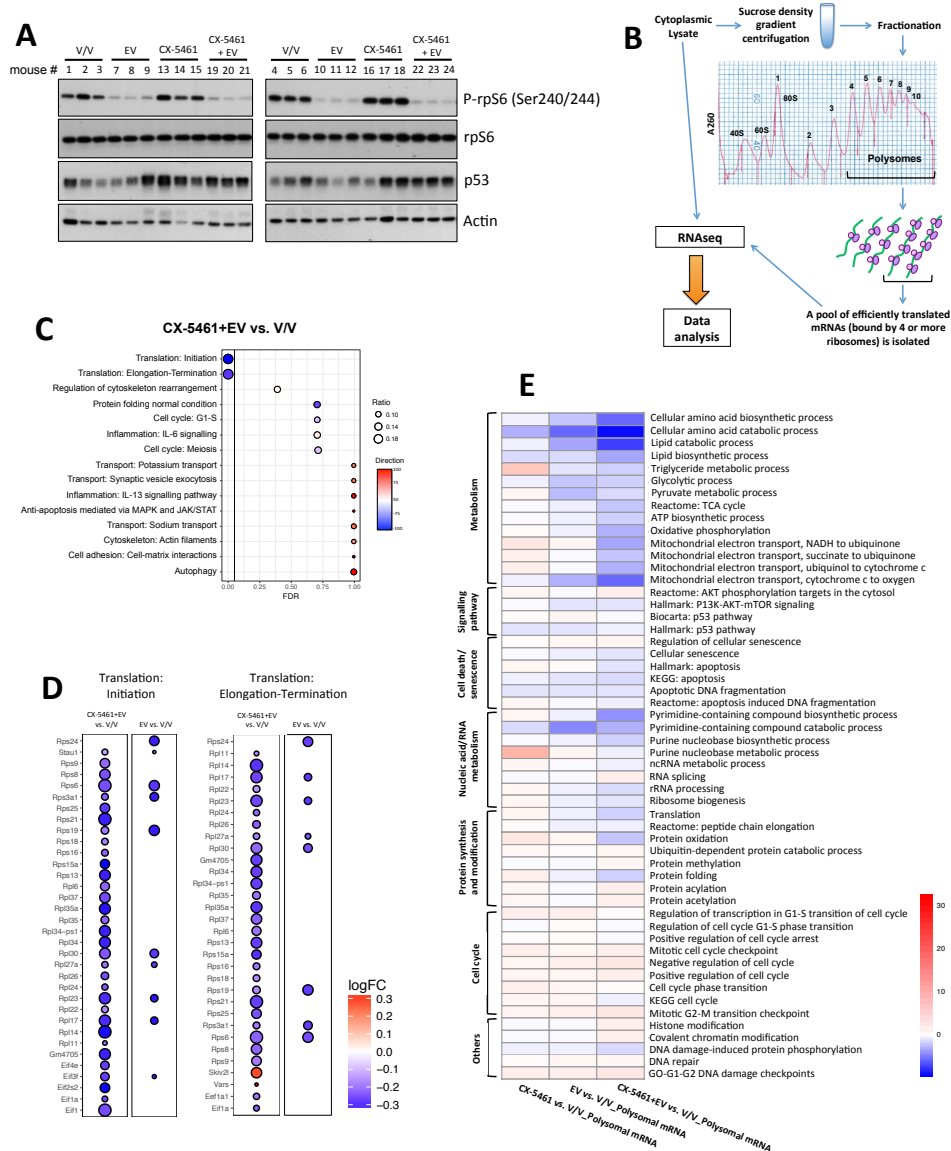


Figure 1: Acute inhibition of ribosome synthesis and function reduces translation of components of the translational machinery and decreases the abundance of polysome-associated, energy metabolism-related mRNAs

(A) Western analysis for on target effects for Everolimus (EV, p-rpS6) and CX-5461 (p53). Each lane represents proteins with equal amounts from a single mouse which received drug vehicles (everolimus vehicle: 1% methylcellulose; CX-5461 vehicle: 25 mM NaH₂PO₄) (V/V), 5 mg/kg everolimus (EV), 35 mg/kg CX-5461 or both drugs (CX-5461+EV) for 2 hours (n=6). Actin was used as a loading control. **(B)** Schematic of the polysome profiling analysis: cytoplasmic lysate was layered on top of a linear 10-40% sucrose gradient, ultracentrifuged (36,000 rpm, 2¼ hours, 4°C) and fractionated using the Foxy Jr Fraction Collector with constant monitoring of absorbance at 260 (A260) nm by an ISCO UA-6

Absorbance Detector. Fractions (one fraction per minute: 860 μ L per tube) corresponding to polysomal mRNAs that were bound by four or more ribosomes were pooled and analysed by RNA-seq followed by data analysis using ANOTA or LIMMA. **(C)** Enrichment analysis by MetaCore® GeneGO of genes in “translation up” (red) and “translation down” (blue) categories identified by anota2seq analysis comparing lymph node cells isolated from mice in CX-5461+EV group with the V/V group (n=6). “Ratio” is the value obtained by dividing the number of genes in our data assigned to a molecular process with the number of genes curated in the same process in MetaCore®’s database. **(D)** Genes implicated in “*Translation: initiation*” and “*Translation: Elongation-Termination*” processes based on Figure 1C and S1C. logFC: log fold change; FDR: false discovery rate (adjusted *P* value). **(E)** Activity levels of key cellular processes involved in cellular growth, proliferation and metabolism based on single sample gene set enrichment analysis (ssGSEA) of indicated comparisons. “Direction” visualises the percentage of genes associated with indicated pathways that are up (red) or down (blue). Data were obtained from n=6 mice per treatment group.

Acute reduction in translation of the components of the translational apparatus is associated with decreased translation of energy metabolism-related mRNAs

Critically, despite this potent inhibition of translation, cells treated with CX-5461+EV did not reduce the general translation efficiency of all mRNAs globally. In contrast to anota2seq analysis in Figure 1C, single-sample gene set enrichment analysis (ssGSEA) (Barbie et al., 2009) comparing abundance of all mRNAs associated with actively translating polysomes (without normalization to steady-state cytoplasmic mRNA abundance) revealed a specific reduction in the translation of mRNAs encoding key metabolic processes. These mRNAs included those involved in energy metabolism, particularly in glycolysis and multiple steps of mitochondrial oxidative phosphorylation (oxphos) (Figure 1E). Analysis of the 5’UTRs of the targeted metabolic mRNAs revealed that they were 2 times more likely to be long (>200 nt; 17.4% vs. 8.5%) (Figure S1E), which is consistent with the reduced translation of translation initiation factors (Figure 1D), required for efficient translation of mRNAs with long 5’UTRs (Leppek et al., 2018). Strikingly, targeting the translation of mRNAs encoding metabolic regulators was greatly enhanced in the combination therapy-treated cells, implicating this as a potential key mechanism in the synergistic prolongation of survival in response to the two drugs.

Elevated energy metabolism is associated with resistance to ribosome targeting therapy.

Despite the dramatic initial impact of the combination treatment on tumor growth, animals eventually succumb to lymphomagenesis. To allow detailed analysis of the mechanisms of acquired resistance to the combination therapy targeting ribosome biogenesis and protein synthesis, we established early-passage E μ -Myc B-lymphoma cell lines from mice that were drug-naïve (CTRL), previously treated with Everolimus (EV), CX-5461 (CX) or CX-5461+EV (CMB) (Figure 2A). To confirm that CX and CMB cells maintained their drug resistance phenotypes, they were treated with EV, CX-5461 or CX-5461+EV *in vitro*. The drug-naïve CTRL cell lines retained sensitivity to all treatments, while CX cells were resistant to CX-5461 and sensitive to CX-5461+EV and the CMB cells were unresponsive to all the treatments (Figure 2B). EV cells showed little change in sensitivity to EV but a modest resistance to CX-5461. Moreover, the CX and CMB cells maintained drug resistance *in vivo* when re-transplanted into mice and re-challenged with CX-5461 (Figure 2C) and CX-5461+EV (Figure 2D) respectively. EV and CTRL cells responded similarly to EV *in vivo* (data not shown). Furthermore, these lines maintained the appropriate on-target responses to these targeted therapies. Everolimus inhibited mTORC1 activity in all cell lines, as reflected by reduced phosphorylation of rpS6 (Ser240/244) and 4E-BP1 (Ser65) (Figure S2A). As expected, CX-5461 treatment for 3 hours robustly decreased the rate of 47S pre-rRNA synthesis in the CTRL, EV and CX cells (Figure S2B-D). Although the CX and CMB cells at 3 hours post-treatment were less sensitive to rDNA transcription inhibition (Figure S2D and S2E) compared to the CTRL and EV cells, longer term CX-5461 treatment did mediate robust on-target inhibition of Pol I transcription (Figure S2F-H). These findings were also consistent with the ability of CX-5461 to induce p53 in these cell lines (Figure S2I), confirming that loss of IRBC is not associated with the mechanism of resistance to CX-5461+EV.

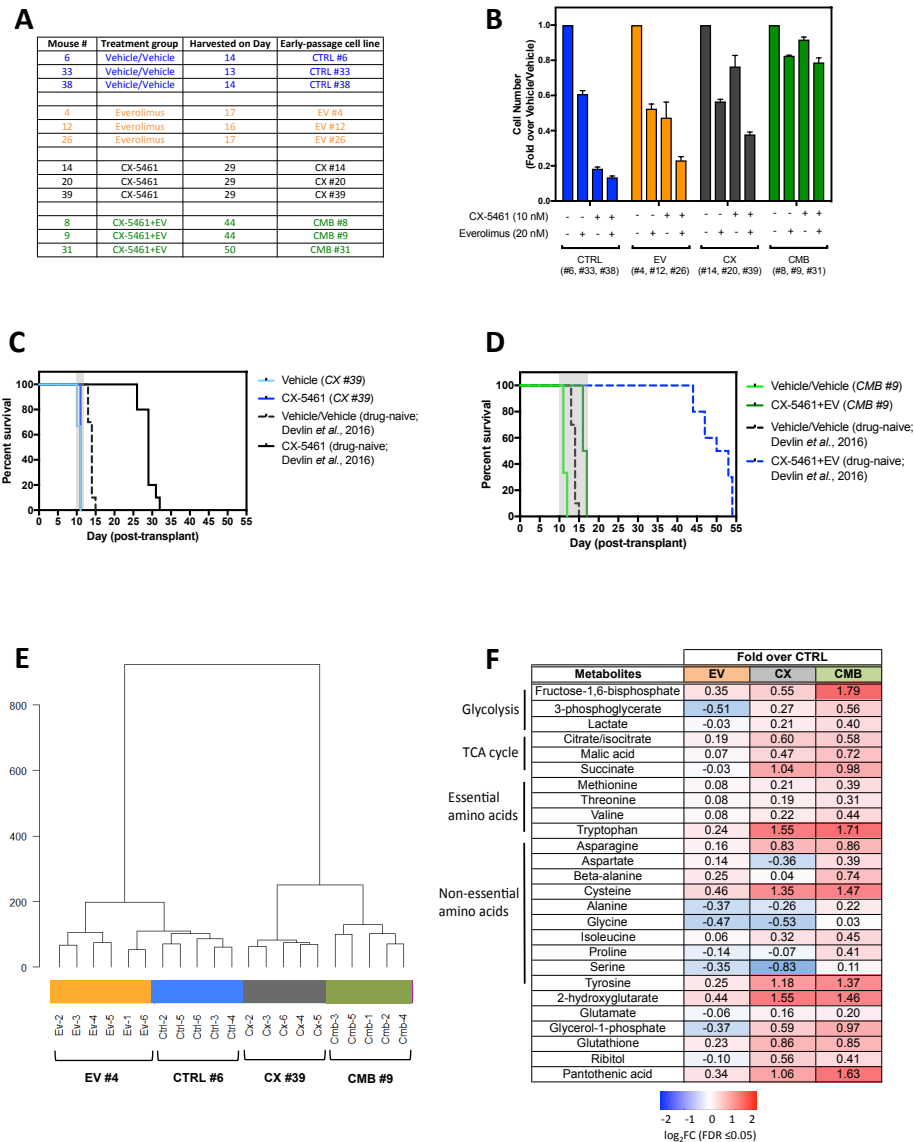


Figure 2: The early-passage Eμ-Myc B-lymphoma cells exhibit altered drug response and distinct metabolic profiles

(A) The early-passage Eμ-Myc B-lymphoma cells used in this study, which were established from mice that were drug-naïve ("CTRL" cell lines), previously treated with everolimus ("EV" cell lines) or CX-5461 ("CX" cell lines) alone, or combination of both ("CMB" cell lines). Harvest day indicates the number of days post-transplantation of lymphoma cells. **(B)** Cell viability analysis of the indicated early-passage Eμ-Myc B-lymphoma cells treated with indicated compound(s) for 24-hour as determined by Beckman® Coulter counter. Graphs represent mean ± SEM of n=3. **(C)** Kaplan-Meier curve of CX #39 cells treated with CX-5461 (35 mg/kg thrice weekly) (n=5-6). Survival curves of drug-naïve cells were actual data reproduced from Devlin et al., 2016. **(D)** Kaplan-Meier curve of CMB #9 cells treated with CX-5461 (35 mg/kg thrice weekly) and everolimus (5 mg/kg daily) (n=5-6). Survival curves of drug-naïve cells were actual data reproduced from Devlin et al., 2016. **(E)** Hierarchical clustering analysis of metabolomics data (n=5-6) obtained from gas chromatography (GC)-mass spectrometry (MS) analysis of early-passage cell lines (n=5-6). **(F)** Steady-state abundance of indicated metabolites (fold-over CTRL cells; FDR ≤ 0.05) (n=5-6).

Given translation-dependent targeting of cellular metabolism is a key mediator of the acute response to combined targeting of ribosome biogenesis and mRNA translation (Figure 1C and 1E), we hypothesized that metabolic re-wiring driven by specific changes in mRNA translation would drive acquired resistance (Bywater et al., 2012; Devlin et al., 2016). To test this hypothesis we profiled metabolite levels in CTRL, EV, CX and CMB early-passage Eμ-Myc B-lymphoma cell lines using gas chromatography-mass spectrometry (GC-MS) (Figure 2E). Multivariate analysis clearly separated the metabolic phenotype of each of the cell lines (Figure 2E). Strikingly, the CX and CMB cell lines had elevated levels of glycolytic and tricarboxylic acid (TCA) cycle intermediates as well as a number of essential and non-essential amino acids compared to the EV and CTRL cell lines (Figure 2F). This data is consistent with rewiring of the energy and amino acid metabolism of CMB cells to overcome the acute translation-dependent inhibition of metabolism observed *in vivo* when the drug-naïve/parental cells were treated with CX-5461+EV (Figure 1E). However, the changes in metabolite levels in CX cells are clearly insufficient to confer resistance to CX-5461+EV.

Given the acute targeting of translation was specific for CX-5461+EV compared with CX-5461 alone and correlated with the improved efficacy of the combination therapy, we focused on investigating the basis for acquired resistance in CMB cells. Consistent with metabolic re-wiring driving the acquired resistance to combination therapy, the CMB cells had elevated ATP content compared to the CTRL cells (Figure 3A). Seahorse XF96 analysis confirmed that CMB cells were metabolically more active, as indicated by increased glycolysis and oxidative phosphorylation (Figure 3B). To test whether the re-wired metabolism of CBM cells contributes to drug resistance, we treated CMB cells with metformin, a well-tolerated anti-diabetic drug that lowers cellular ATP levels by inhibiting its production via mitochondrial oxidative phosphorylation (Foretz et al., 2014; Pollak, 2012; Rena et al., 2017). CMB cells exhibited a greater sensitivity to metformin compared to the CTRL and CX cells (Figure 3C) and metformin re-sensitised CMB cells to CX-5461+EV treatment, increasing the percentage of cell death to 47%, a value comparable to that achieved when CTRL cells were treated with the CX-

5461+EV combination (41%) (Figure 3D). Thus, reprogramming of metformin-sensitive metabolism is specific for CMB cells and critical for acquired resistance to CX-5461+EV therapy.

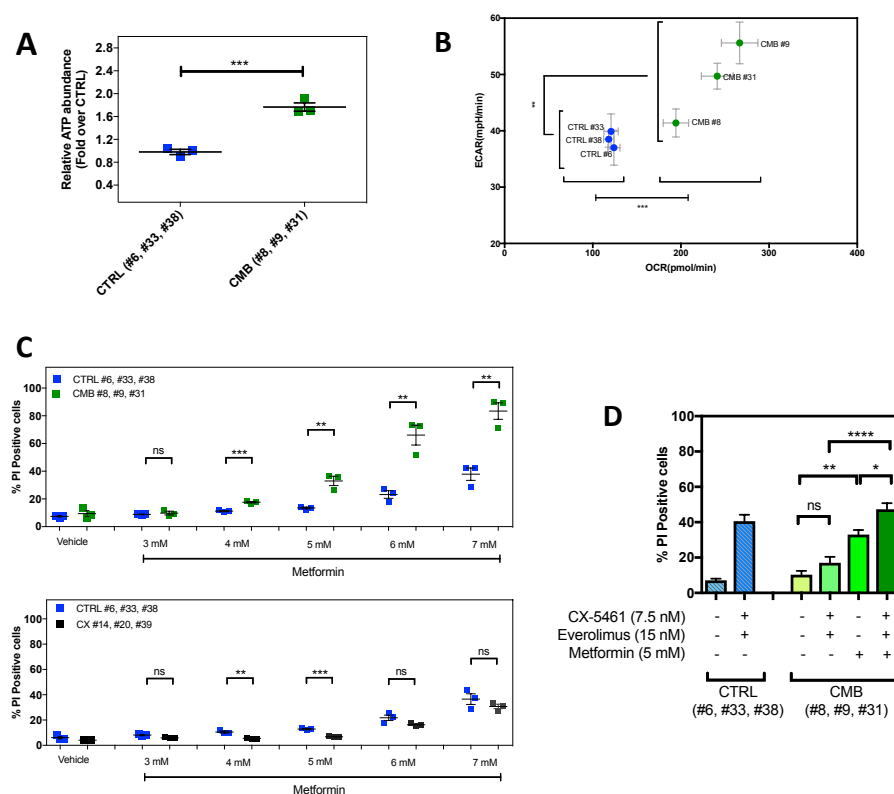


Figure 3: Metformin re-sensitises combination therapy-resistant early passage Eμ-Myc B-lymphoma cells to CX-5461-everolimus co-treatment

(A) Intracellular ATP levels determined using the CellTiterGLO® assay in drug-naïve (CTRL) and CX-5461+EV-resistant (CMB) early-passage cell lines. Graphs represent mean $\pm$ SEM of $n=3$. **(B)** Basal extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) in the early-passage cell lines determined using the Seahorse XF96 Extracellular Flux Analyzer (normalised to cell number); graphs represent mean $\pm$ SD of 6-8 technical replicates for each biological replicate ($n=3$). **(C)** Propidium iodide (PI) exclusion analysis of early-passage CX-5461-everolimus combination therapy-resistant (CMB), CX-5461-resistant (CX) and drug-naïve (CTRL) lymphoma cells treated with indicated concentrations of metformin for 48 hours. Graphs represent mean $\pm$ SEM of $n=3$. **(D)** PI analysis of CTRL and CMB cells treated with CX-5461, everolimus or metformin for 48-hour as indicated. Graphs represent mean $\pm$ SEM of $n=3$. **(A) & (C)** Data were analysed by Student's t test. **(B) & (D)** Data were analysed by one-way ANOVA. ns, not significant; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$.

Reprogramming of mRNA translation underpins the metabolic re-wiring required for resistance to ribosome targeting therapy

We next compared the polysomal RNAseq data of the CTRL and CMB cells to determine whether the metabolic changes observed in CMB cells are driven by specific reprogramming of mRNA translation. We evaluated the expression patterns of mRNAs encoding proteins that are known to be involved in the regulation of mRNA translation (genes in the “Translation” category in the Gene Ontology Consortium Pathway and Reactome databases) (Figure 4A). Importantly, the heatmap indicated that the CMB cells displayed distinct polysome-association patterns of mRNAs encoding proteins including the majority of ribosomal proteins, translation initiation (such as eIF4G1 and eIF2A) and elongation factors (such as eEF1A1 and eEF2) as compared to the CTRL and CX cells (Figure 4A). Moreover, this heatmap indicated that distinct translational reprogramming events occurred in CMB cells as compared to CX cells, which may, at least in part, explain the differences in metformin sensitivity observed in Figure 3D.

CMB cells were also characterized by significantly elevated levels of ribosomes per cell compared to CTRL and CX (Figures 4B and 4C). Characterization of the 5'UTR sequences of mRNAs whose translation was upregulated in CMB cells revealed that they are more likely to have shorter UTRs (Figure 4D). MEME motif analysis identified a uridine-rich motif similar to the pyrimidine-rich translational element (PRTE) (Figure 4E, Figure S3A) identified previously as a characteristic of mTOR targeted mRNAs (Hsieh et al., 2012), consistent with upregulation of translation of mRNAs normally targeted by mTOR inhibition as a source of resistance to ribosome targeted therapies. These data indicate that translation-driven reprogramming of cellular metabolism is driven by alterations in the way resistant cells synthesize their own translational machinery.

Comparison of the RNAseq data from polysomal mRNAs from all three cell lines also revealed that in CMB cells the mRNAs encoding multiple components of the mitochondrial electron transport chain were upregulated

(Figure S3B). This may explain the specific metformin-sensitive effect in CMB cells, which was not observed in the single-agent resistant CX cells (Figure 3C). Moreover, our polysome profiling data indicated that the expression of the proapoptotic protein BMF was not ablated in the CMB lines (Figure S3C), confirming that loss of BMF activity is not associated with the mechanism of resistance to CX-5461+EV.

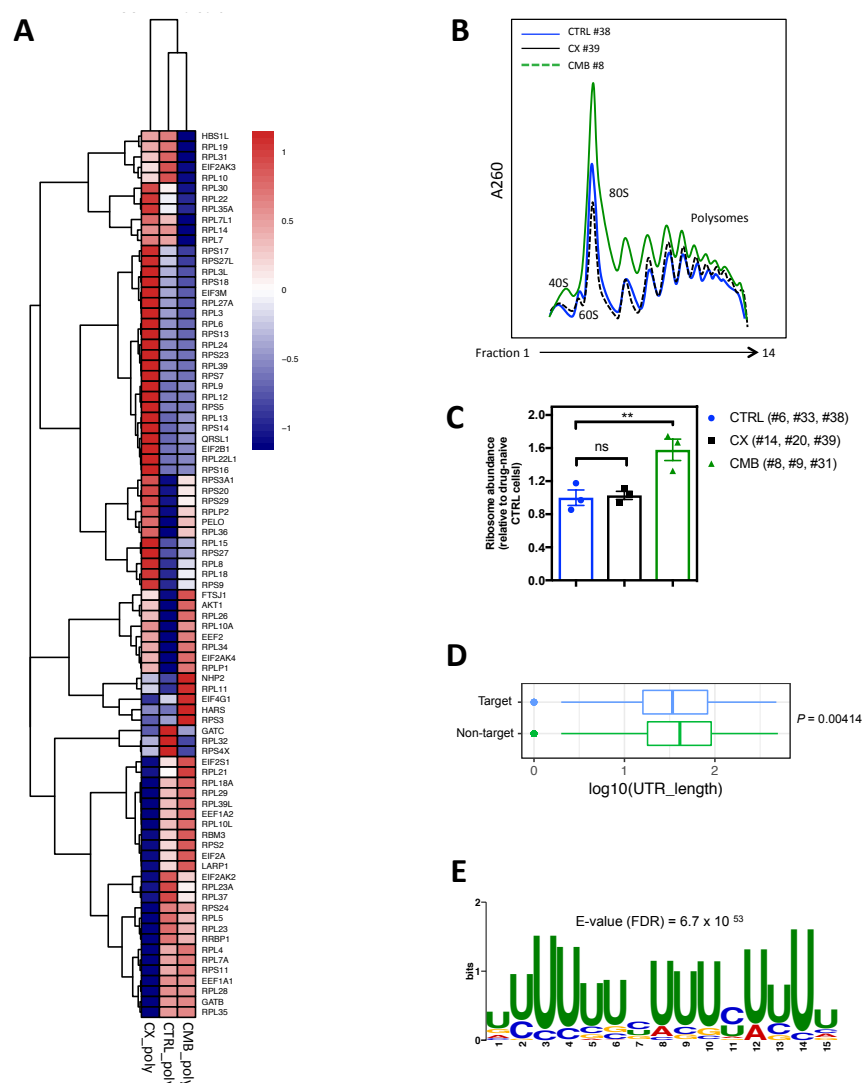


Figure 4: Polysome profiling analysis demonstrates metabolic reprogramming is associated with resistance to CX-5461-everolimus combination therapy

(A) A heatmap illustrating the abundances of polysome-associated mRNAs that are associated with the mRNA translation process based on the Reactome Pathway Database and the Gene Ontology Consortium Database in drug-naïve (CTRL) or CX-5461-everolimus combination therapy-resistant (CMB) cell lines (n=3). The colours illustrate the normalised counts of indicated genes (red: high expression; blue: low expression). **(B)** Polysome profiles demonstrating increased ribosome abundance in CMB cells as compared to CTRL and CX-5461-resistant (CX) cells (representatives of n=3). **(C)** Quantitation of ribosome abundances (monosomes plus polysomes) in the indicated early-passage Eμ-Myc B-lymphoma cells. Graph represents mean ± SEM of n=3. Data were analysed by one-way

ANOVA. ns, not significant; **, $P \leq 0.01$. **(D)** Length of the 5'UTR of mRNAs that are more actively translated by the CMB cells (as compared to CTRL cells) vs. non-target mRNAs. Boxplots indicate the range of distribution of 5'UTR lengths across genes; One-sided Wilcoxon tests were used to determine significance. **(E)** The most significantly enriched motif in the 5'UTR of mRNAs that are more actively translated in the CMB cells compared to CTRL cells based on Multiple Em for Motif Elicitation (MEME) analysis.

Increased translation of the cAMP signalling pathway is associated with resistance to ribosome targeting therapy

To investigate whether the metformin-sensitive, energy metabolism-associated changes observed in CMB cells are fundamental for resistance, we performed pathway analysis using MetaCore® GeneGO on the mRNAs enriched on the polysomes of CMB cells to identify potential driver pathways of resistance. This analysis identified the cyclic-adenosine 3',5'-monophosphate (cAMP) signalling pathway as one of the top hits (Figure 5A), with increased translation efficiency of mRNAs encoding essential components of this pathway, including adenylate cyclase and cAMP-guanine nucleotide exchange factor (cAMP-GEF), which is also known as exchange protein directly activated by cAMP (EPAC) 1 and 2 (Figure S3A). Adenylate cyclase converts ATP into cAMP, which then activates protein kinase A (PKA) or EPAC1/2, the GEFs for activation of Ras-related protein 1 (Rap1), the two best characterised direct downstream targets of cAMP (Bos, 2003, 2006; Gloerich and Bos, 2010; Holz et al., 2006; Houslay and Milligan, 1997). To confirm functional elevation of cAMP signalling, we performed liquid chromatography-MS (LC-MS) and Western analysis to demonstrate elevated cAMP abundance (Figure 5B), increased EPAC2 (Figure 5C) and polysome-association of Rap1 mRNAs (Figure S3E) in the CMB cell lines compared to CTRL.

To interrogate the role of the cAMP signalling pathway in drug resistance of the CMB cells, we utilised specific inhibitors/activators of PKA and EPAC (Brown et al., 2014; Enserink et al., 2002; Poppe et al., 2008). Inhibition PKA by H89 (Poppe et al., 2008) did not alter CMB cells' response to CX-5461+EV treatment (Figures S4A and 5D). Likewise, 6-Bnz-cAMP, which robustly

activates PKA signalling, as detected by a phospho-PKA substrate antibody, was unable to protect CTRL cells from CX-5461+EV-induced cell death (Figures S4B and 5E) (Lo et al., 2012; Poppe et al., 2008). However, inhibition of EPAC1/2 function, as measured by the reduced abundance of GTP-bound Rap1, (Figure S4C) by CE3F4 (Courilleau et al., 2012) and ESI-05 (Rehmann, 2013; Tsalkova et al., 2012) alone (Figure S4D and S4E) or together (Figure 5F) re-sensitised CMB cells to CX-5461+EV treatment. These data indicate that EPAC1/2, but not PKA, activity is required for the development of resistance to CX-5461+EV. Consistent with these findings, activation of EPAC1/2 in the CTRL cells using 8-pCPT-2-O-Me-cAMP (Enserink et al., 2002; Rehmann et al., 2003) increased Rap1-GTP abundance (Figure S4F) and reduced CX-5461+EV-mediated cell death (Figure 5G). This confirms that EPAC1/2 activity correlates with protection from CX-5461+EV-mediated cell death and therefore with the development of an energy- and cAMP-dependent resistant mechanism in the CX-5461+EV-resistant CMB cells.

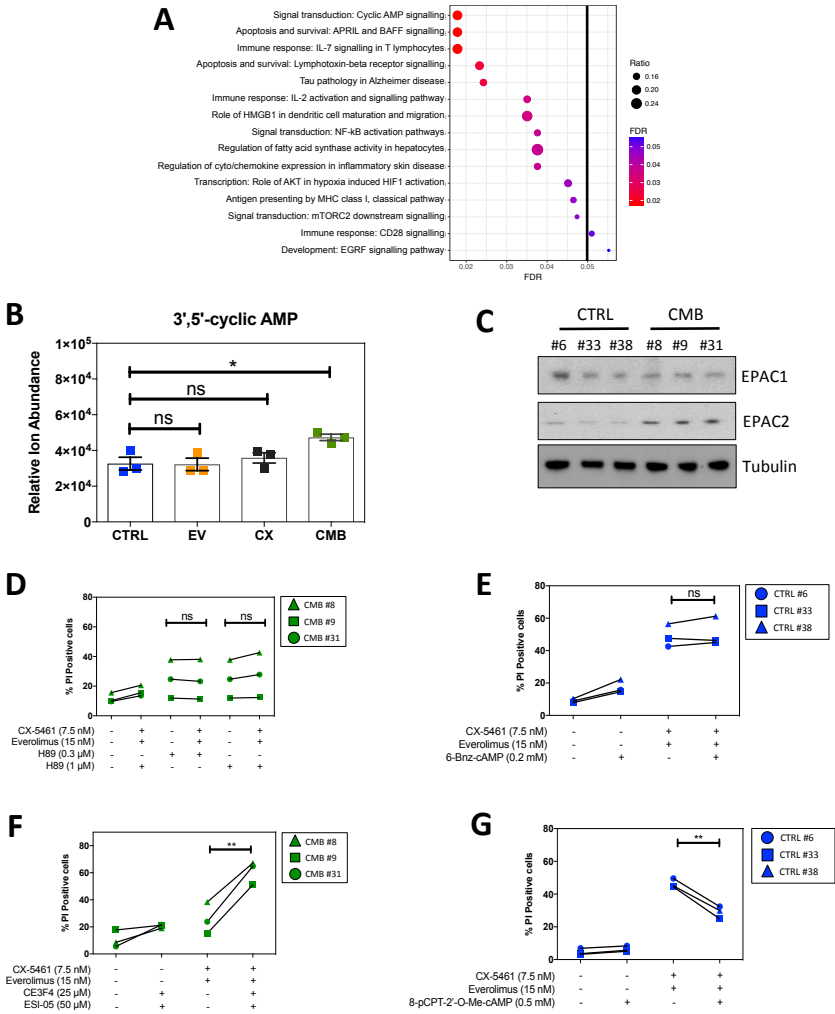


Figure 5: The cAMP-EPAC pathway mediates resistance to CX-5461-everolimus co-treatment

(A) Enrichment analysis by GeneGO MetaCore® of the polysomal RNAseq data comparing CX-5461+EV-resistant (CMB) and drug-naïve cells (CTRL) (n=3; (FDR) ≤ 0.05; fold change (FC) ≥ 1.5 or ≤ -1.5). **(B)** Abundance of intracellular 3'5'-cyclic AMP as measured by a liquid chromatography (LC)-MS analysis. Graphs represent mean ± SEM of n=3. Data were analysed by one-way ANOVA. ns, not significant; *, P ≤ 0.05. **(C)** Western analysis of EPAC1 and EPAC2 abundance in the indicated early-passage cells during their log-phase growth period (n=3). Tubulin was used as a loading control. **(D)** Propidium iodide (PI) exclusion assays of CMB cells treated with CX-5461 and everolimus as indicated in the presence or absence of a selective PKA inhibitor H89 for 24 hours. Graphs represent mean ± SEM of n=3. **(E)** PI exclusion analysis of early-passage drug-naïve (CTRL) lymphoma cells treated with CX-5461 and everolimus in the presence or absence of selective PKA activator 6-Bnz-cAMP. Graphs represent mean ± SEM of n=3. **(F)** Propidium iodide (PI) exclusion analysis of the CMB cells treated with CX-5461 and everolimus as indicated in the presence or absence of both EPAC1 and EPAC2 inhibitors for 48 hours. Graphs represent mean ± SEM of n=3. **(G)** PI exclusion analysis of early-passage drug-naïve (CTRL) cells treated with CX-5461 and everolimus in the presence or absence of the selective EPAC activator 8-pCPT-2-O-Me-cAMP. Graphs represent mean ± SEM of n=3. **(D, E, F, G)** Data were analysed by Student *t* test. Green triangle: CX-5461-everolimus combination therapy-resistant (CMB) cells clone #8; green square: CMB cells clone #9; green circle: CMB cells clone #31. Blue circle: early-passage drug-naïve (CTRL) cells clone #6; blue square: CTRL cells clone #33; blue triangle: CTRL cells clone #38. ns, not significant; *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001.

CX-5461-EV-resistant cells are sensitised to metformin targeting of the cAMP-EPAC1/2-Rap1 pathway

The data presented above demonstrated that targeting oxidative phosphorylation or the cAMP-EPAC1/2-Rap1 signalling pathway re-sensitised the CMB cells to CX-5461+EV treatment. While there is currently no inhibitor of the cAMP-EPAC pathway in the clinic or in clinical trials, metformin is used in the clinic and can lower cellular ATP levels and reduce the intracellular abundance of cAMP (Miller et al., 2013). Although metformin's effect on the EPAC1/2-Rap1 pathway has not been reported, we hypothesized that metformin's ability to sensitise CMB cells to CX-5461+EV treatment is associated with inhibition of cAMP-EPAC1/2-Rap1 signalling. To evaluate if metformin can alter EPAC1/2 activity, Rap1-GTP pull-down assays were performed. The CMB cells, and the CX cells as controls, were treated with metformin at the GI25 concentration based on Figure 3C. This treatment reduced ATP levels in both CX and CMB cells by more than 70% (Figure 6A), and robustly lowered Rap1-GTP levels but only in the CMB cells (Figure 6B).

This data confirms that the cAMP-EPAC1/2-Rap1 mediated drug resistance, which involves a translation-dependent reprogramming of metabolism, is specific to the CMB cells.

We therefore investigated if metformin inhibition of oxidative phosphorylation and EPAC1/2 activity would re-sensitise the CMB cells to CX-5461+EV treatment *in vivo*. CMB lymphoma bearing C57BL/6 mice were pre-treated with metformin (Veiga et al., 2018) or vehicle for 3 days (600 mg/kg twice daily on the first and second day; 500 mg/kg twice daily on the third day) and then treated for 6 hr with CX-5461 and EV as single agents or in combination. As expected, CX-5461+EV did not alter the abundance of GFP-positive drug-resistant CMB cells in the lymph node (Figure 6C) or spleen (Figure 6D) compared to vehicle pre-treated mice. Importantly, the single-agent metformin had no effect on the number of GFP-positive cells, whereas they were significantly reduced in the lymph nodes (Figure 6C) and spleens (Figure 6D) of mice receiving the CX-5461+EV+Metformin treatment. Thus metformin was able to re-sensitise the CMB cells to CX-5461+EV treatment *in vivo*.

To determine whether the triple combination therapy could enhance survival of mice bearing this highly aggressive disease, C57BL/6 mice transplanted with the CMB cells were pre-treated for 5 days with metformin (500 mg/kg twice daily) then at Day 11 post-transplant, treated with either: (i) vehicle; (ii) CX-5461+EV (35 mg/kg every Mon-Wed-Fri and 5 mg/kg daily, respectively); (iii) metformin (400 mg/kg twice daily); or (iv) CX-5461+EV+metformin triple combination therapy. We demonstrated that the triple combination significantly increased the survival window compared to all the groups including the CX-5461+EV treatment (Figure 6E). Together these data show that the re-sensitisation of the aggressive, drug-resistant CMB cells to CX-5461+EV treatment in the presence of metformin *in vitro* was reproducible *in vivo*, thus providing proof-of-principle that combined inhibition of translational machinery and energy metabolism for the treatment of highly aggressive MYC-driven lymphoma can provide a therapeutic window.

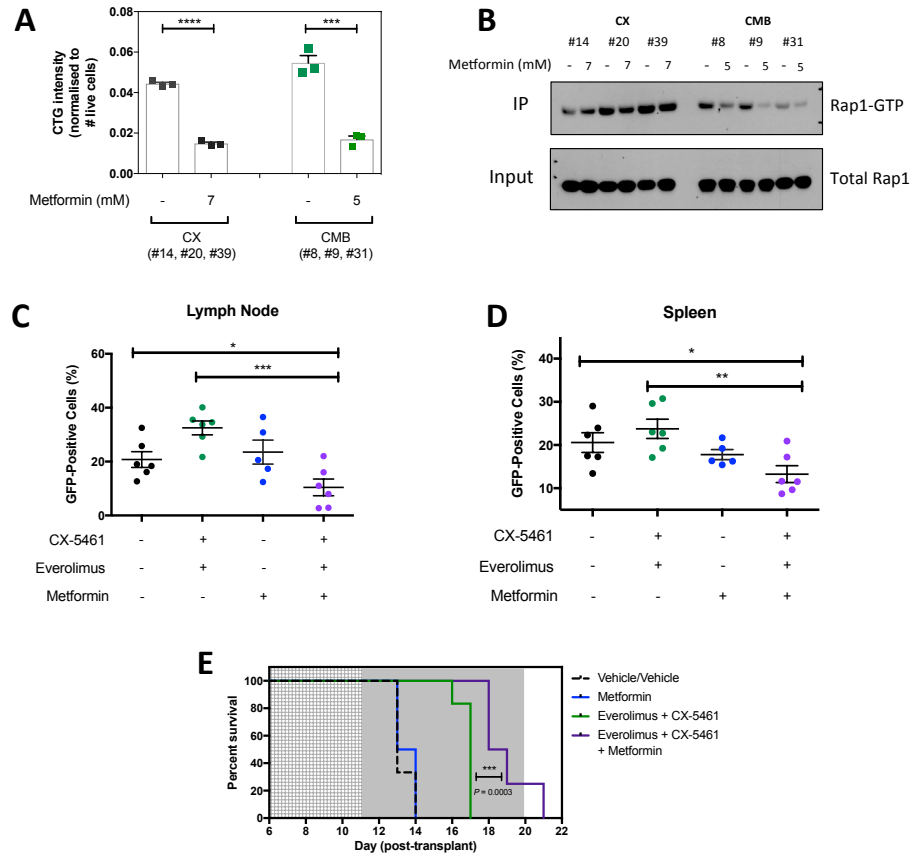


Figure 6: Metformin inhibits cAMP-EPAC signalling and re-sensitises combination therapy-resistant early passage Eμ-Myc B-lymphoma cells to CX-5461-everolimus co-treatment *in vivo*

(A) CellTiterGLO® (CTG) assay measuring cellular ATP levels of the CX-5461-resistant (CX) and CX-5461-everolimus combination therapy-resistant (CMB) cells treated with metformin as indicated for 48 hours. **(B)** Western analysis demonstrating the effects of metformin treatment for 48 hours on the levels of active GTP-bound Rap1 (n=3). Proportion of green fluorescent protein (GFP)-positive CMB (clone #8) cells in **(C)** lymph node and **(D)** spleen of transplanted C57BL/6 mice treated as indicated for 6 hours on day 12 post-transplant. Graphs represent mean ± SEM of 6 mice per group. **(E)** Kaplan-Meier curve of C57BL/6 mice transplanted with CX-5461-everolimus-resistant (CMB #8) early-passage Eμ-Myc B-lymphoma cells treated with vehicles (everolimus vehicle: 1% methylcellulose; CX-5461/metformin vehicle: 25 mM NaH₂PO₄); CX-5461 (35 mg/kg every twice weekly) and everolimus (5 mg/kg daily), metformin (400 mg/kg twice daily), or CX-5461, everolimus and metformin (35 mg/kg twice weekly, 5 mg/kg daily and 400 mg/kg twice daily, respectively). Data were analysed by a log-rank (Mantel–Cox) test. Vehicle vs. CX-5461-Everolimus: $P = 0.0006$, Vehicle vs. CX-5461-everolimus-metformin: $P = 0.0001$. CX-5461-everolimus vs. CX-5461-everolimus-metformin: $P = 0.0003$. **(A, C, D)** Graphs represent mean ± SEM of n=3. Data were analysed by Student's t test **(A)** or one-way ANOVA **(C)** and **(D)**. ns, not significant; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$.

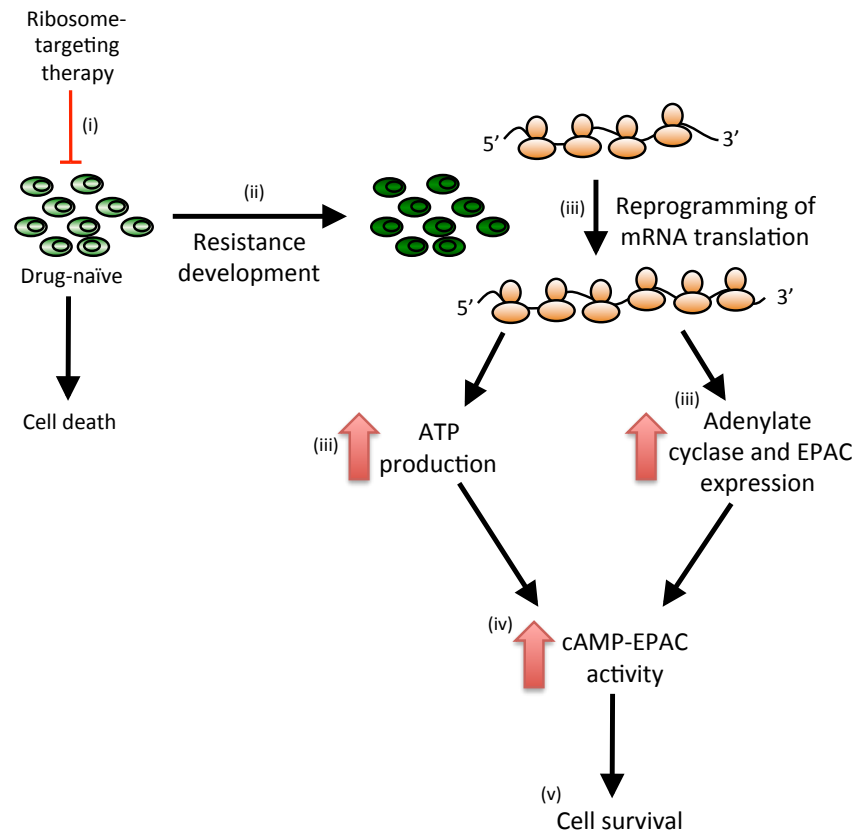


Figure 7: Model of translation-driven response to ribosome-targeting therapies

We proposed a model of translation-driven response to ribosome-targeting therapies where (i) drug therapy acutely inhibits the translation of key metabolic mRNAs (ii) continued compromised ribosome synthesis and translation allows selection of clones with elevated translation-dependent ribosome biogenesis; (iii) these clones exhibit specific upregulation of translation and synthesis of oxphos components (Figure S3B), adenylate cyclase (Figures 5A and S3D) and EPAC proteins (Figures 5A, 5C and S3D); (iv) this in turn elevates ATP production (Figure 3A) and enhances its conversion into cAMP (Figure 5B); (v) cAMP is utilised by the EPAC proteins, thus protecting the resistant cells from activation of death mechanisms (Figures 5D-G) induced by CX-5461-everolimus co-treatment.

Discussion

We have previously demonstrated that combining specific inhibition of rRNA synthesis and mTORC1-dependent translation is highly effective in treating MYC-driven lymphoma via activation of the p53-dependent IRBC and induction of the pro-survival molecule BMF (Devlin et al., 2016). Here we use a combination of polysome profiling of tumours from acutely treated mice and early passage cells from acquired resistant tumours, coupled with metabolomics analysis to interrogate the mechanisms underpinning the response to ribosome targeted therapy.

We show that the synergistic efficacy of the combination therapy is associated with dramatic suppression of the translation of mRNAs encoding key proteins controlling mRNA translation itself. This specific targeting the ribosome leads to selective inhibition of the translation of mRNAs containing longer 5'UTRs including those encoding proteins critical for the regulation of energy metabolism, with the subsequent result being increased lymphoma cell death. This is distinct from a report that mTOR regulated the translation of mRNAs containing shorter, less complex 5'UTRs (Hsieh et al., 2012), suggesting that the changes we observed in response to CX-5461+EV treatment are not driven by EV-dependent inhibition of mTOR signalling. Since p53/BMF-null E μ -Myc B-lymphomas are resistant to CX-5461+EV treatment (Devlin et al., 2016) we initially postulated that mutation or loss of p53 and/or BMF might underlie the resistance mechanism. Indeed, loss of *TP53* is observed in ~40% of human Burkitt lymphoma cases (Schmitz et al., 2014; Schmitz et al., 2012). However, we observed that the p53 and BMF responses were maintained in acquired resistant cells.

Indeed, our studies define a new model of molecular response of oncogene driven cancer to ribosome targeted therapy (Figure 7). Under the pressure of treatment with CX-5461 + EV, translational re-programing leads to increased ribosomes per cell and the translation of polysome-associated mRNAs containing short 5'UTRs that encode components of the mitochondrial

respiratory electron transport chain and the cAMP-EPAC-Rap1 pathway. The resulting increase in ATP and cAMP production further activates EPAC signaling, overcoming specific translationally driven metabolic re-wiring by the combination therapy. Functionally, specific inhibition of cAMP-EPAC1/2-Rap1 signalling re-sensitises resistant cells to combination therapy demonstrating that this pro-survival pathway is a critical mediator of resistance. Importantly, targeting the mitochondrial respiratory chain complex 1 with the anti-diabetic drug metformin reduced EPAC1/2-Rap1 signalling and markedly improved the efficacy of combination therapy in resistant disease, *in vitro* and *in vivo*. Our findings demonstrate that elevated metabolic activity and energy production is not merely a hallmark of cancer associated with uncontrolled growth, but can be harnessed to activate the pro-survival signaling through cAMP-EPAC1/2-Rap1, a new metabolic vulnerability that can be exploited to further improve the efficacy of ribosome-targeting therapy. This makes a convincing case for the importance of developing inhibitors to EPAC function with improved pharmacological properties as an additional tool for targeting oncogene-driven cancer. We believe this approach will be invaluable to improve the efficacy of both traditional chemotherapeutics that target ribosome biogenesis, as well as, the newly developed low genotoxic approaches including CX-5461 and BMH-21 (Peltonen et al., 2014; Tsai and Pederson, 2014; Woods et al., 2015).

Finally, this study reinforces the recent finding that genetically compromised ribosome biogenesis results in specific re-wiring of translation that underlies impaired erythroid differentiation (Khajuria et al., 2018). More broadly, diseases of genetically compromised ribosome biogenesis such as Diamond Blackfan anaemia, are similarly characterized by specific alterations in mRNA translation (Khajuria et al., 2018) and by significantly increased incidence of cancer, a paradox termed “Dameshek’s riddle” (De Keersmaecker et al., 2015). This riddle is re-inforced by observations of somatically acquired mutations and deletions in ribosomal proteins in T-cell acute lymphoblastic leukemia as well as solid tumors, such as gastric and ovarian cancer (De Keersmaecker et al., 2013; De Keersmaecker et al., 2015; Kandoth et al., 2013; Novetsky et al., 2013). Despite intense investigation, the mechanisms

by which genetically compromised ribosome biogenesis leads to increased cancer susceptibility in patients with ribosomopathies remain a mystery. Our findings raise the tantalising possibility that at least part of the answer to Dameshek's riddle lies in specific rewiring of translation in response to chronically compromised ribosome biogenesis, whereby the subsequent translationally-driven elevated metabolism and pro-survival mechanisms promote malignant transformation later in life.

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Author Contribution

Conceptualization, E.P.K., K.M.H. and R.B.P.; Methodology, A.S.T, C.C., D.L.G., O.L., J.R.D., K.T. D.P.D., M.J.M., G.T. and K.M.H.; Formal Analysis, E.P.K, A.S.T, O.L., D.P.D. and J.K.; Investigation, E.P.K., C.C., J.K.; Resources: J.R.D., D.P.D., M.J.M., G.P. and R.D.H.; Writing – Original Draft, E.P.K. and R.B.P.; Writing – Review and Editing, E.P.K., K.T., M.J.M., G.A.M., G.T., E.S., G.P., R.D.H., K.M.H., J.K. and R.B.P.; Supervision, K.M.H., J.K. and R.B.P.

Declaration of Interests

G.A. McArthur reports receiving commercial research grants from Celgene, Novartis, and Ventana and is a consultant/advisory board member for Provectus. R.D. Hannan is a consultant/advisory board member for Pimera, Inc. R.B. Pearson is a consultant/advisory board member for Pimera, Inc. No potential conflicts of interest were disclosed by the other authors

References

- Barbie, D.A., Tamayo, P., Boehm, J.S., Kim, S.Y., Moody, S.E., Dunn, I.F., Schinzel, A.C., Sandy, P., Meylan, E., and Scholl, C. (2009). Systematic RNA interference reveals that oncogenic KRAS-driven cancers require TBK1. *Nature* 462, 108.
- Bos, J.L. (2003). Epac: a new cAMP target and new avenues in cAMP research. *Nature reviews Molecular cell biology* 4, 733.
- Bos, J.L. (2006). Epac proteins: multi-purpose cAMP targets. *Trends in biochemical sciences* 31, 680-686.
- Brown, L.M., Rogers, K.E., Aroonsakool, N., McCammon, J.A., and Insel, P.A. (2014). Allosteric inhibition of Epac: computational modeling and experimental validation to identify allosteric sites and inhibitors. *Journal of Biological Chemistry, jbc.* M114. 569319.
- Bywater, M.J., Poortinga, G., Sanij, E., Hein, N., Peck, A., Cullinane, C., Wall, M., Cluse, L., Drygin, D., Anderes, K., *et al.* (2012). Inhibition of RNA polymerase I as a therapeutic strategy to promote cancer-specific activation of p53. *Cancer Cell* 22, 51-65.
- Courilleau, D., Bissierier, M., Jullian, J.-C., Lucas, A., Bouyssou, P., Fischmeister, R., Blondeau, J.-P., and Lezoualc'h, F. (2012). Identification of a tetrahydroquinoline analog as a pharmacological inhibitor of the cAMP-binding protein Epac. *Journal of Biological Chemistry, jbc.* M112. 422956.
- De Keersmaecker, K., Atak, Z.K., Li, N., Vicente, C., Patchett, S., Girardi, T., Gianfelici, V., Geerdens, E., Clappier, E., and Porcu, M. (2013). Exome sequencing identifies mutation in CNOT3 and ribosomal genes RPL5 and RPL10 in T-cell acute lymphoblastic leukemia. *Nature genetics* 45, 186.

- De Keersmaecker, K., Sulima, S.O., and Dinman, J.D. (2015). Ribosomopathies and the paradox of cellular hypo-to hyperproliferation. *Blood*, blood-2014-2010-569616.
- Devlin, J.R., Hannan, K.M., Hein, N., Cullinane, C., Kusnadi, E., Ng, P.Y., George, A.J., Shortt, J., Bywater, M.J., Poortinga, G., *et al.* (2016). Combination Therapy Targeting Ribosome Biogenesis and mRNA Translation Synergistically Extends Survival in MYC-Driven Lymphoma. *Cancer Discov* 6, 59-70.
- Enserink, J.M., Christensen, A.E., de Rooij, J., van Triest, M., Schwede, F., Genieser, H.G., Døskeland, S.O., Blank, J.L., and Bos, J.L. (2002). A novel Epac-specific cAMP analogue demonstrates independent regulation of Rap1 and ERK. *Nature cell biology* 4, 901.
- Fiehn, O., Robertson, D., Griffin, J., van der Werf, M., Nikolau, B., Morrison, N., Sumner, L.W., Goodacre, R., Hardy, N.W., and Taylor, C. (2007). The metabolomics standards initiative (MSI). *Metabolomics* 3, 175-178.
- Foretz, M., Guigas, B., Bertrand, L., Pollak, M., and Viollet, B. (2014). Metformin: from mechanisms of action to therapies. *Cell metabolism* 20, 953-966.
- Gloerich, M., and Bos, J.L. (2010). Epac: defining a new mechanism for cAMP action. *Annual review of pharmacology and toxicology* 50, 355-375.
- Hein, N., Cameron, D.P., Hannan, K.M., Nguyen, N.N., Fong, C.Y., Sornkom, J., Wall, M., Pavy, M., Cullinane, C., Diesch, J., *et al.* (2017). Inhibition of Pol I transcription treats murine and human AML by targeting the leukemia-initiating cell population. *Blood* 129, 2882-2895.
- Hilton, J., Cescon, D., Bedard, P., Ritter, H., Tu, D., Soong, J., Gelmon, K., Aparicio, S., and Seymour, L. (2018). 44O CCTG IND. 231: A phase 1 trial evaluating CX-5461 in patients with advanced solid tumors. *Annals of Oncology* 29, mdy048. 003.
- Holz, G.G., Kang, G., Harbeck, M., Roe, M.W., and Chepurny, O.G. (2006). Cell physiology of cAMP sensor Epac. *The Journal of physiology* 577, 5-15.
- Hortle, E., Nijagal, B., Bauer, D.C., Jensen, L.M., Ahn, S.B., Cockburn, I.A., Lampkin, S., Tull, D., McConville, M.J., and McMorran, B.J. (2016). Adenosine monophosphate deaminase 3 activation shortens erythrocyte half-life and provides malaria resistance in mice. *Blood*, blood-2015-2009-666834.
- Houslay, M.D., and Milligan, G. (1997). Tailoring cAMP-signalling responses through isoform multiplicity. *Trends in biochemical sciences* 22, 217-224.
- Hsieh, A.C., Liu, Y., Edlind, M.P., Ingolia, N.T., Janes, M.R., Sher, A., Shi, E.Y., Stumpf, C.R., Christensen, C., and Bonham, M.J. (2012). The translational landscape of mTOR signalling steers cancer initiation and metastasis. *Nature* 485, 55.

- Jeppe, K.J., Kouremenos, K.A., Townsend, K.R., MacMahon, D.F., Sharley, D., Tull, D.L., Hoffmann, A.A., Pettigrove, V., and Long, S.M. (2017). Metabolomic Profiles of a Midge (*Procladius villosimanus*, Kieffer) Are Associated with Sediment Contamination in Urban Wetlands. *Metabolites* 7, 64.
- Kandoth, C., McLellan, M.D., Vandin, F., Ye, K., Niu, B., Lu, C., Xie, M., Zhang, Q., McMichael, J.F., and Wyczalkowski, M.A. (2013). Mutational landscape and significance across 12 major cancer types. *Nature* 502, 333.
- Khajuria, R.K., Munschauer, M., Ulirsch, J.C., Fiorini, C., Ludwig, L.S., McFarland, S.K., Abdulhay, N.J., Specht, H., Keshishian, H., Mani, D.R., *et al.* (2018). Ribosome Levels Selectively Regulate Translation and Lineage Commitment in Human Hematopoiesis. *Cell* 173, 90-103.e119.
- Khot, A., Brajanovski, N., Cameron, D.P., Poortinga, G., Sanij, E., Lim, J., Soong, J., Hein, N., Link, E., and Pearson, R.B. (2017). RNA Polymerase 1 Transcription Inhibitor CX-5461 in Patients with Advanced Hematologic Malignancies: Results of a Phase I First in Human Study. (*Am Soc Hematology*).
- Leppek, K., Das, R., and Barna, M. (2018). Functional 5' UTR mRNA structures in eukaryotic translation regulation and how to find them. *Nature Reviews Molecular Cell Biology* 19, 158.
- Lo, K.W.H., Kan, H.M., Ashe, K.M., and Laurencin, C.T. (2012). The small molecule PKA-specific cyclic AMP analogue as an inducer of osteoblast-like cells differentiation and mineralization. *Journal of tissue engineering and regenerative medicine* 6, 40-48.
- Ma, X.M., and Blenis, J. (2009). Molecular mechanisms of mTOR-mediated translational control. *Nature reviews Molecular cell biology* 10, 307.
- Masukagami, Y., De Souza, D., Dayalan, S., Bowen, C., O'Callaghan, S., Kouremenos, K., Nijagal, B., Tull, D., Tivendale, K., and Markham, P. (2017). Comparative metabolomics of *Mycoplasma bovis* and *Mycoplasma gallisepticum* reveals fundamental differences in active metabolic pathways and suggests novel gene annotations. *MSystems* 2, e00055-00017.
- Miller, R.A., Chu, Q., Xie, J., Foretz, M., Viollet, B., and Birnbaum, M.J. (2013). Biguanides suppress hepatic glucagon signalling by decreasing production of cyclic AMP. *Nature* 494, 256.
- Morita, M., Gravel, S.-P., Chénard, V., Sikström, K., Zheng, L., Alain, T., Gandin, V., Avizonis, D., Arguello, M., and Zakaria, C. (2013). mTORC1 controls mitochondrial activity and biogenesis through 4E-BP-dependent translational regulation. *Cell metabolism* 18, 698-711.
- Novetsky, A.P., Zighelboim, I., Thompson Jr, D.M., Powell, M.A., Mutch, D.G., and Goodfellow, P.J. (2013). Frequent mutations in the RPL22 gene and its clinical and functional implications. *Gynecologic oncology* 128, 470-474.

Oertlin, C., Lorent, J., Gandin, V., Murie, C., Masvidal, L., Cargnello, M., Furic, L., Topisirovic, I., and Larsson, O. (2018). Generally applicable transcriptome-wide analysis of translational efficiency using anota2seq. *bioRxiv*, 106922.

Overgaard, A.J., Weir, J.M., De Souza, D.P., Tull, D., Haase, C., Meikle, P.J., and Pociot, F. (2016). Lipidomic and metabolomic characterization of a genetically modified mouse model of the early stages of human type 1 diabetes pathogenesis. *Metabolomics* 12, 13.

Pelletier, J., Thomas, G., and Volarevic, S. (2018). Ribosome biogenesis in cancer: new players and therapeutic avenues. *Nat Rev Cancer* 18, 51-63.

Peltonen, K., Colis, L., Liu, H., Trivedi, R., Moubarek, M.S., Moore, H.M., Bai, B., Rudek, M.A., Bieberich, C.J., and Laiho, M. (2014). A targeting modality for destruction of RNA polymerase I that possesses anticancer activity. *Cancer cell* 25, 77-90.

Pollak, M.N. (2012). Investigating metformin for cancer prevention and treatment: the end of the beginning. *Cancer discovery*.

Poppe, H., Rybalkin, S.D., Rehmann, H., Hinds, T.R., Tang, X.-B., Christensen, A.E., Schwede, F., Genieser, H.-G., Bos, J.L., Døskeland, S.O., *et al.* (2008). Cyclic nucleotide analogs as probes of signaling pathways. *Nature Methods* 5, 277.

Quin, J., Chan, K.T., Devlin, J.R., Cameron, D.P., Diesch, J., Cullinane, C., Ahern, J., Khot, A., Hein, N., and George, A.J. (2016). Inhibition of RNA polymerase I transcription initiation by CX-5461 activates non-canonical ATM/ATR signaling. *Oncotarget* 7, 49800.

Quin, J.E., Devlin, J.R., Cameron, D., Hannan, K.M., Pearson, R.B., and Hannan, R.D. (2014). Targeting the nucleolus for cancer intervention. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease* 1842, 802-816.

Rebello, R.J., Kusnadi, E., Cameron, D.P., Pearson, H.B., Lesmana, A., Devlin, J.R., Drygin, D., Clark, A.K., Porter, L., and Pedersen, J. (2016). The dual inhibition of RNA Pol I transcription and PIM kinase as a new therapeutic approach to treat advanced prostate cancer. *Clinical Cancer Research, clincanres*. 0124.2016.

Rehmann, H. (2013). Epac-inhibitors: facts and artefacts. *Scientific reports* 3, 3032.

Rehmann, H., Schwede, F., Døskeland, S.O., Wittinghofer, A., and Bos, J.L. (2003). Ligand-mediated activation of the cAMP-responsive guanine nucleotide exchange factor Epac. *Journal of Biological Chemistry* 278, 38548-38556.

Rena, G., Hardie, D.G., and Pearson, E.R. (2017). The mechanisms of action of metformin. *Diabetologia* 60, 1577-1585.

- Ritchie, M.E., Phipson, B., Wu, D., Hu, Y., Law, C.W., Shi, W., and Smyth, G.K. (2015). limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res* 43, e47.
- Schmitz, R., Ceribelli, M., Pittaluga, S., Wright, G., and Staudt, L.M. (2014). Oncogenic mechanisms in Burkitt lymphoma. *Cold Spring Harbor perspectives in medicine* 4, a014282.
- Schmitz, R., Young, R.M., Ceribelli, M., Jhavar, S., Xiao, W., Zhang, M., Wright, G., Shaffer, A.L., Hodson, D.J., and Buras, E. (2012). Burkitt lymphoma pathogenesis and therapeutic targets from structural and functional genomics. *Nature* 490, 116.
- Tsai, R.Y., and Pederson, T. (2014). Connecting the nucleolus to the cell cycle and human disease. *The FASEB Journal* 28, 3290-3296.
- Tsalkova, T., Mei, F.C., Li, S., Chepurny, O.G., Leech, C.A., Liu, T., Holz, G.G., Woods, V.L., and Cheng, X. (2012). Isoform-specific antagonists of exchange proteins directly activated by cAMP. *Proceedings of the National Academy of Sciences* 109, 18613-18618.
- Veiga, S.R., Ge, X., Mercer, C.A., Hernández-Alvarez, M.I., Thomas, H.E., Hernandez-Losa, J., y Cajal, S.R., Zorzano, A., Thomas, G., and Kozma, S.C. (2018). Phenformin-Induced Mitochondrial Dysfunction Sensitizes Hepatocellular Carcinoma for Dual Inhibition of mTOR. *Clinical Cancer Research*, clincanres. 0177.2018.
- Woods, S.J., Hannan, K.M., Pearson, R.B., and Hannan, R.D. (2015). The nucleolus as a fundamental regulator of the p53 response and a new target for cancer therapy. *Biochimica et Biophysica Acta (BBA)-Gene Regulatory Mechanisms* 1849, 821-829.
- Xu, H., Di Antonio, M., McKinney, S., Mathew, V., Ho, B., O'Neil, N.J., Dos Santos, N., Silvester, J., Wei, V., and Garcia, J. (2017). CX-5461 is a DNA G-quadruplex stabilizer with selective lethality in BRCA1/2 deficient tumours. *Nature communications* 8, 14432.

Materials and Methods

Cell culture and reagents

E μ -Myc B-lymphoma cells (MSCV *Gfp*) were cultured in Anne Kelso DMEM, supplemented with 10% heat-inactivated (h.i.) fetal bovine serum (FBS), 100 μ M L-asparagine (Merck), penicillin/streptomycin/glutamine (Thermo Fisher Scientific) and 0.5% beta-mercaptoethanol. Early-passage E μ -Myc B-lymphoma cells were established by processing lymph nodes isolated at the

end of a survival experiment in (Devlin et al., 2016). These early-passage Eμ-Myc B-lymphoma cells were cultured in Anne Kelso DMEM supplemented with 20% h.i. FBS, 100 μM L-asparagine (Merck), penicillin/streptomycin/glutamine (Thermo Fisher Scientific) and 0.5% beta-mercaptoethanol. Everolimus (S1120) was purchased from Selleckchem. CX-5461 was purchased from SYNkinase (SYN-3031). Metformin was purchased from Merck (D150959). CEF34 was purchased from Cayman Chemicals (17767). ESI-05 and 8-pCPT-2-O-Me-cAMP were purchased from Biolog (catalogue number M092 and C041, respectively). 6-Bnz-cAMP was purchased from Tocris Bioscience (5255). H89 was purchased from Merck (B1427).

Animal experiments

All animal experiments were performed with approval from the Animal Experimentation Ethics Committee at the Peter MacCallum Cancer Centre (Ethics number E462 and E557). For in vivo drug studies, 2×10^5 Eμ-Myc lymphoma cells were injected into the tail veins of 6-8 week old male C57BL/6 mice (Walter and Eliza Hall Institute, Australia). Disease onset/progression was monitored by peripheral blood (tail bleed) analysis for green fluorescent protein (GFP). Mice were treated with pharmacologic inhibitors from 10 days post-transplant (survival studies) or on day 12 post-transplants (acute studies). Everolimus was administered daily at 5 mg/kg via oral gavage in 5% dimethyl sulfoxide (DMSO) in 1% methylcellulose. CX-5461 was administered twice weekly (unless otherwise indicated in figure legends) at 35 mg/kg via oral gavage in 25 mM NaH₂PO₄ (pH 4.5). Metformin was administered twice daily (2 x 500 mg/kg unless otherwise indicated in figure legends) via oral gavage in 25 mM NaH₂PO₄ (pH 4.5). For survival studies, mice were dosed until an ethical end-point was reached (enlarged lymph nodes, inactivity, hunched posture, laboured breathing, weight loss (equal to 20% of initial body weight) and ruffled fur). White blood cells, lymph node cells, and spleen cells were analyzed for GFP and B220 (CD45R) (Thermo Fisher Scientific 17-0452-82) expression using BD Cantoll or BD Fortessa. Flow cytometry data was analyzed with Flowlogic software (Inivai Technologies). Snap-frozen lymph nodes or spleens were homogenized using a Precellys 24/Cryolys

cryomill (Bertin Technologies) (6,800 rpm; 2 x 30-second pulse, 45 seconds interval between pulses; 0 °C).

Protein analysis and Western blotting

Protein was extracted with SDS-lysis buffer (0.5 mM EDTA, 20 mM HEPES, 2% (w/v) SDS pH 7.9) and protein concentrations were determined with the Bio-Rad protein assay. Proteins were resolved by SDS-PAGE, transferred to PVDF membranes and immunoblotted with primary and horseradish peroxidase–conjugated secondary antibodies (Supplementary Table). Protein was visualized by Amersham enhanced chemiluminescence (ECL) Western Blotting Detection Reagent (GE Healthcare Life Sciences) and X-ray film (Fujifilm SuperRX). Active Rap1 pull-down experiments were performed using the Rap1 Activation Assay (Abcam ab212011) kit according to manufacturer's instructions.

Polysome profiling, RNA isolation and RNAseq analysis

Suspension cells were harvested by removing the culture media following centrifugation (400 g, 4 minutes, 4 °C). The cells were washed with ice-cold hypotonic wash buffer (5 mM Tris pH 7.5; 1.5 mM KCl; 2.5 mM MgCl₂ and 100 µg/mL cycloheximide (Sigma)) and the supernatant was removed by centrifugation (400 g, 4 minutes, 4 °C). A hypotonic lysis buffer (5 mM Tris pH 7.5; 1.5 mM KCl; 2.5 mM MgCl₂; 0.5% Triton-X; 0.5% sodium deoxycholate; 1X EDTA-free protease inhibitor; 2 mM DTT; 10 µL RNAsin (Promega); 100 µg/mL cycloheximide) was added to the cell pellet. The samples were mixed by pipetting and centrifuged (16,000 g, 7 minutes, 4 °C). The supernatant (cytoplasmic lysate) was transferred to a fresh microfuge tube. 10% of the cytoplasmic lysate was transferred into a microfuge tube containing 500 µL TRIzol® reagent. The remaining lysate was layered on top of a linear 10-40% sucrose gradient, ultracentrifuged (36,000 rpm, 2¼ hours, 4 °C) and fractionated using the Foxy Jr Fraction Collector with constant monitoring of absorbance at 260 nm by an ISCO UA-6 Absorbance Detector (Teledyne). Cytoplasmic (input) RNAs and polysomal RNAs from pooled fractions corresponding to four or more ribosomes were isolated using a TRIzol®-based method and purified using the Qiagen RNeasy® Minikit according to

manufacturer's instructions, and analysed by RNAseq. RNA concentration was determined using a NanoDrop Spectrophotometer (Thermo Scientific) and integrity was evaluated using the RNA Nano Kit and 2100 bioanalyzer (Agilent Technologies).

cDNA libraries were generated using a ribodepletion method (TruSeq Ribo Profile Mammalian Kit, Illumina). Fragment sizes were evaluated using the DNA 1000 kit and 2100 bioanalyzer (Agilent Technologies). Libraries were subjected to single-end sequencing (SE50bp) (HiSeq2500, Illumina) to generate ~30 million reads per sample using standard protocols. Reads were aligned with Tophat2 and reads counts were obtained with htseq with the mouse genome (Ensembl release 78) as a reference, using an in-house bioinformatics pipeline, seqliner (<http://bioinformatics.petermac.org/seqliner/>). Differential translation was detected using anota2seq analysis (Oertlin et al., 2018) of polysome-associate mRNAs, using the total amount of cytosolic mRNA as a reference. Only coding genes with a count of at least 1 in any of the samples were considered. Samples were normalized using the "voom" option in anota2seq. An omnibus analysis was first performed in anota2seq to enrich for genes affected by any of the treatments, using a groupRvmPAdj cutoff of 0.15. Differential translation analysis was subsequently performed using anota2seq on this reduced set. Only genes with an apvRvm adjusted p-value < 0.05 and an apvEff (fold change) greater than 1.25 were considered to be significant. To perform differential analysis of polysome data in resistant studies (i.e. to compare mRNAs that were differentially bound to polysomes of treated vs. control cells), we used LIMMA version 3.28.21 (Ritchie et al., 2015) from Bioconductor. Only coding genes with an average logCPM > 1 were considered. Genes were considered to be significant if their adjusted p-values after Benjamini & Hochberg correction were less than 0.05, and their fold change was greater than 1.5.

For *in vivo* translational profiling experiments, cytoplasmic lysate was obtained by grinding snap-frozen tissue samples in liquid nitrogen proof containers submerged in dry ice-100% ethanol slurry to a fine powder. A modified lysis buffer (10-fold higher concentration compared to the one used

for cultured cells; RNAsin was replaced with 10 mM ribonucleoside vanadyl complex (New England Biolabs S1402S) was added to the ground tissue. The sample was transferred to an ice-cold dounce homogeniser and homogenised (30-60 strokes). The homogenate was transferred to a chilled microfuge tube and centrifuged (16,000 g, 7 minutes, 4 °C). The supernatant (cytoplasmic lysate) was transferred to a fresh microfuge tube. 10% of the cytoplasmic lysate was transferred into a microfuge containing 500 µL TRIzol® reagent. The remaining lysate was loaded onto linear (10-40%) sucrose gradients and processed as described above.

For the 5'UTR analysis, 5'UTR sequences were obtained using the biomaRt and org.Mm.eg.db R packages from the Mouse Genome GRCm38.p6. Only 5'UTRs shorter than 500 nucleotides were considered, since others have found that longer 5'UTRs were likely to be false positives (Khajuria et al., 2018). In cases where there were multiple 5'UTR for a single gene, we only considered the shortest 5'UTR. For the resistant study, only genes with FC > 1.5 or < -1.5 were considered, with adjusted $P < 0.05$. Only expressed genes were used as background. For the acute study, metabolic downregulated genes were those with an appEff (fold change) < 0, and an apvRvmPAdj (adjusted P) < 0.1 (from the omnibus-sorted genes). The minimum free energy of 5'UTR sequences was calculated using the ViennaRNA package version 2.4.7. In cases where there were multiple 5'UTR for a single gene, we only considered the 5'UTR with the lowest minimum free energy. One-sided Wilcoxon tests were used to determine significance.

Cell viability assay

Cells were seeded in 96-well plates. 24 hours later, cells were treated with pharmacological compounds as indicated in the figure legends or text. Cells were stained with 1 µg/mL PI (Merck P4170) and analyzed using the FACSVerse (BD Biosciences). Flow cytometry data was analyzed with Flowlogic software (Inivai Technologies). Cell number was determined using the Z2 Coulter Counter (Beckman Coulter 383550).

³²P-orthophosphate labeling

E μ -Myc B-lymphoma cells (3×10^6) were cultured in 3 ml media in the presence of 0.5 mCi of ^{32}P orthophosphate for 15 min. Cells were harvested on ice and RNA extracted using the QIAGEN RNeasy Minikit according to the manufacturer's instructions. RNA (5 μg) was run overnight on a 1.2% MOPS/formaldehyde agarose gel. The gel was dried using a Model 583 Gel Drier (Bio-Rad), exposed overnight to a phospho-imager screen (Molecular Dynamics) and scanned using a Typhoon Trio Variable Mode Imager (GE Healthcare). Band intensities were quantitated using Image Quant TL software (GE Healthcare).

Bioenergetics analysis using the Seahorse XF96 Extracellular Flux Analyzer

All bioenergetics analyses were performed using the Seahorse Bioscience XF96 extracellular flux analyser (Seahorse Bioscience, Billerica, USA). Cells were washed with the assay media (unbuffered DMEM, 5 g/L glucose, 5 mM glutamine, 1mM sodium pyruvate), before seeding in Seahorse XF96 96-well plates coated with Cell-Tek (3.5 $\mu\text{g}/\text{cm}^2$, Corning) at 2×10^5 cells/well in 180 μl of the assay media. The plate was centrifuged at 400 x g for 5 minutes at room temperature to allow the cells to form a monolayer. The plate was equilibrated in a non- CO_2 incubator for 30 minutes prior to assay. The assay protocol consisted of 3 repeated cycles of 3 minutes mixing and 3 minutes of measurement periods, with oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) measured simultaneously. Basal energetics were established after three of these initial cycles, followed by exposure to the ATP synthase inhibitor, oligomycin (1 μM) for three cycles, then p-trifluoromethoxy-phenylhydrazine (FCCP, 1.5 μM), which uncouples oxygen consumption from ATP production, was added for a further three cycles. Finally, the mitochondrial complex III inhibitor antimycin A (0.5 μM) and the complex I inhibitor rotenone (0.5 μM) was added for three cycles. At the completion of each assay, the cells were stained with Hoechst 10 μM . Images were analysed using a Cellomics Cellinsight 1 to determine the cell number per well.

Gas chromatography – mass spectrometry (GC-MS)

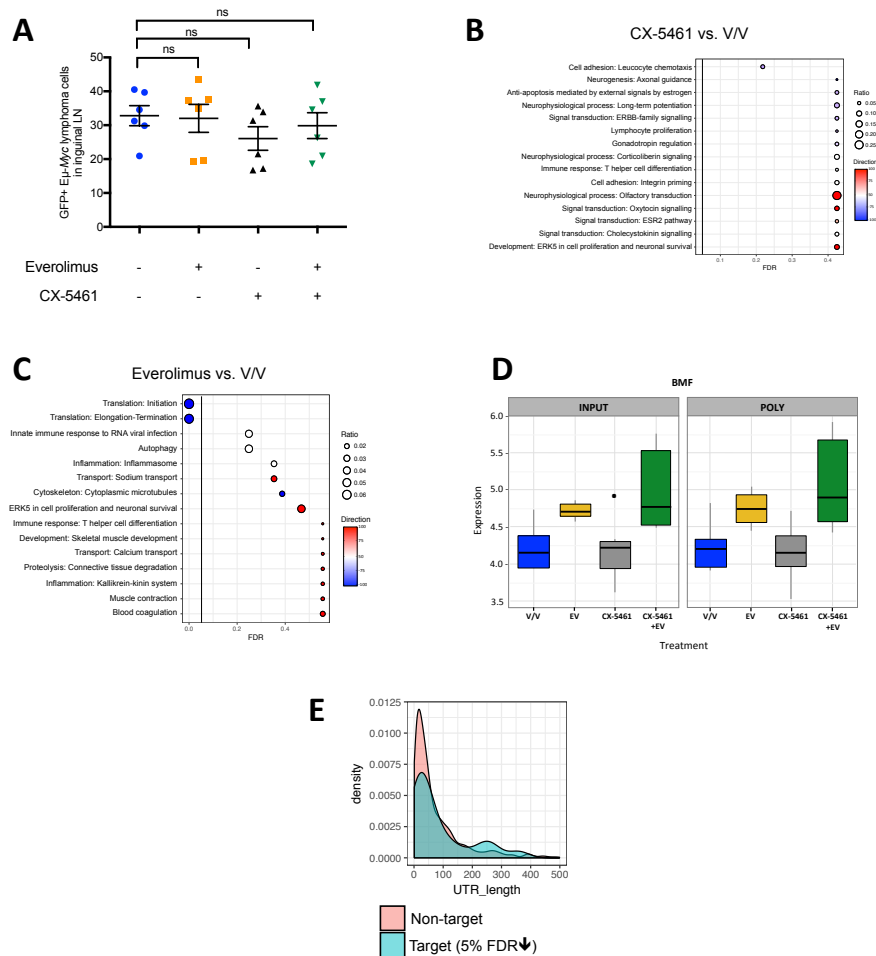
Cells were quenched by transferring cell suspensions (5 mL) into 50 mL falcon tubes containing 35 mL ice-cold saline, followed by centrifugation (500 x g, 3 minutes, 0 °C). Once the supernatant was removed, the cells were re-suspended in 1 mL ice-cold saline, transferred to 1.5 mL microfuge tubes, and centrifuged (10,000 x g, 30 sec, 0 °C). Supernatant was removed completely and metabolites were immediately extracted in 250 µL chloroform:methanol:water (CHCl₃:CH₃OH:H₂O, 1:3:1 v/v; monophasic mixture) containing 0.5 nmoles scyllo-inositol as internal standard. The extracts were vortexed (~10 sec) and incubated on ice for 15 mins with additional vortexing every 5 minutes. Insoluble material was removed by centrifugation (10,000 x g, 5 min, 0°C). H₂O was then added to the supernatant to adjust the ratio of CHCl₃:CH₃OH:H₂O to 1:3:3 (v/v; biphasic partition). The supernatant was vortexed and centrifuged (10,000 x g, 5 min, 0°C) to induce phase separation.

The upper (polar) phase was transferred to a fresh microfuge and dried *in vacuo* (30 °C) in 250 µL glass vial inserts. Samples were analysed by GC-MS as previously described (Jeppe et al., 2017; Masukagami et al., 2017; Overgaard et al., 2016). Briefly, samples were prepared for GC-MS analysis using a Gerstel MPS2 autosampler robot. Polar metabolites were first methoximated with methoxyamine hydrochloride in pyridine (Sigma, 20 µl, 30mg/ml, 2 hr, 37 °C) and then derivatized with TMS reagent (N,O-bis(trimethylsilyl) trifluoroacetamide containing 1% trimethylchlorosilane (Pierce, 20 µl, 1 hr, 37 °C) and analysed by GC-MS using a VF5 capillary column (Agilent, 30 m, 250 µm inner diameter, 0.25 µm film thickness), with a 10 m inert eziguard. The injector insert and GC-MS transfer line temperatures were 250 and 280 °C, respectively. The oven temperature gradient was programmed as follows: 35 °C (1 min); then 25 °C /min to 320 °C; and held at 320 °C for 5 min. Metabolites were detected by mass selective detector following electron ionisation (-70 eV), where the scan range was 50-600 amu at 9.2 scans/sec. Metabolites were identified and areas integrated based on GC retention time and mass spectra as compared with authentic standards and in conjunction with MSD ChemStation Data Analysis Application (Agilent) using in-house and Wiley metabolite libraries. Statistical analyses were

performed using Student *t* test following log transformation and median normalisation. Metabolites were considered to be significant if their adjusted p-values after Benjamini-Hochberg correction were less than 0.05.

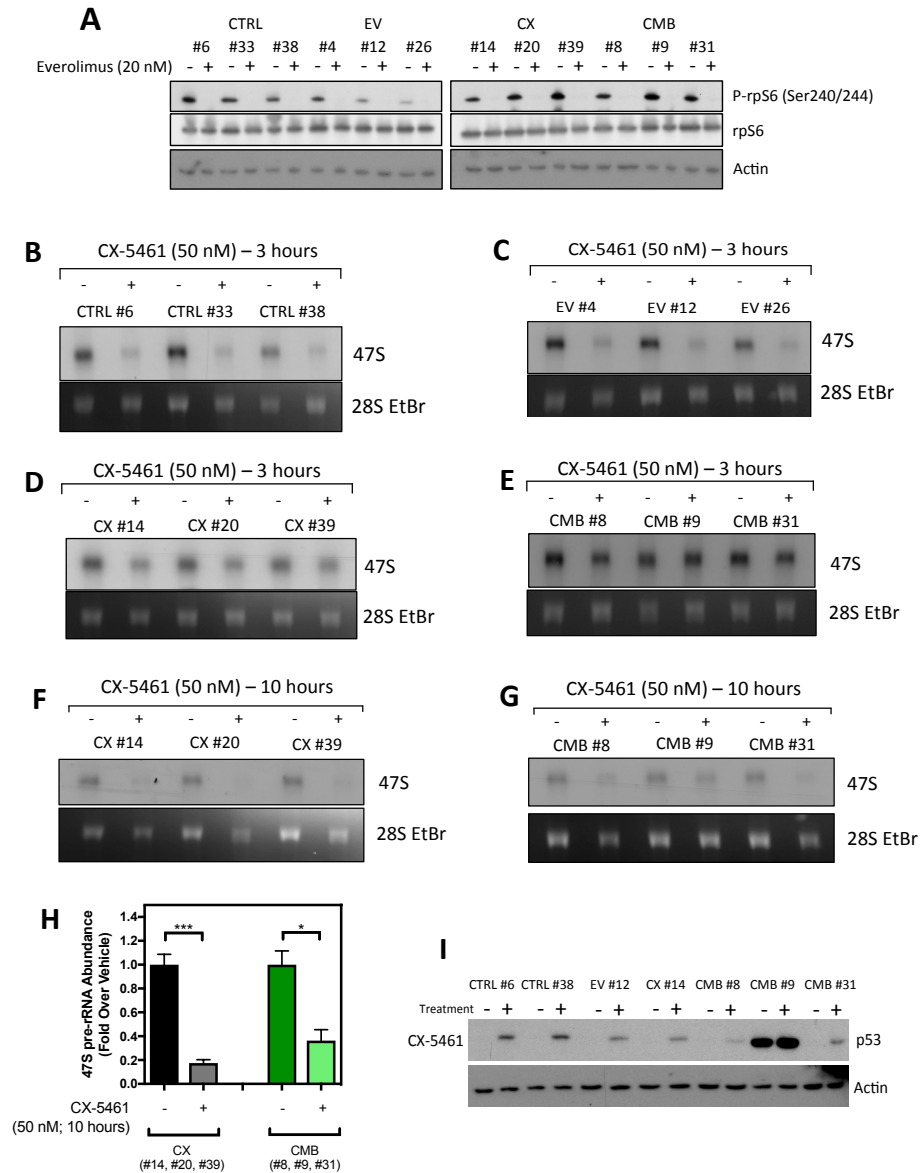
Liquid chromatography – mass spectrometry (LC-MS)

Cells were quenched by transferring cell suspensions (5 mL) into 50 mL falcon tubes containing 35 mL ice-cold saline, followed by centrifugation (500 x g, 3 min, 0 °C). Once the supernatant was removed, the cells were re-suspended in 1 mL ice-cold saline, transferred to 1.5 mL microfuge tubes, and centrifuged (10,000 x g, 30 sec, 0 °C). Supernatant was removed completely and metabolites were immediately extracted using ice-cold 80% acetonitrile:water (v/v) containing internal standards (¹³C-Sorbitol and ¹³C-Valine; final concentration 4 µM both). Metabolites in the cell extracts were analysed by high-performance liquid chromatography mass spectrometer (LC-MS) as previously described (Hortle et al., 2016; Masukagami et al., 2017). Briefly, metabolites were separated on an Agilent Technologies 1200 series HPLC system (Agilent Technologies, Santa Clara, US, USA) using a SeQuant ZIC –pHILIC (5 µm polymer) PEEK 150 x 4.6 mm metal-free HPLC column maintained at 25°C with solvent A (20 mM (NH₄)₂CO₃, pH 9.0; Sigma-Aldrich) and solvent B (100% acetonitrile) at a flow rate of 300 µl/min. Metabolites were detected by mass spectrometry on an Agilent Technologies 6545A series quadrupole time of flight mass spectrometer (QTOF MS) using an electrospray ionization source (ESI). LC-MS data was collected in negative MS mode. Data processing was performed using Agilent MassHunter workstation quantitative analysis for TOF software (Version B.07.00/Build 7.0.457.0). Metabolite identification was based on accurate mass, retention time and authentic chemical standards at level 1 confidence according to the Metabolomics Standard Initiative (Fiehn et al., 2007)



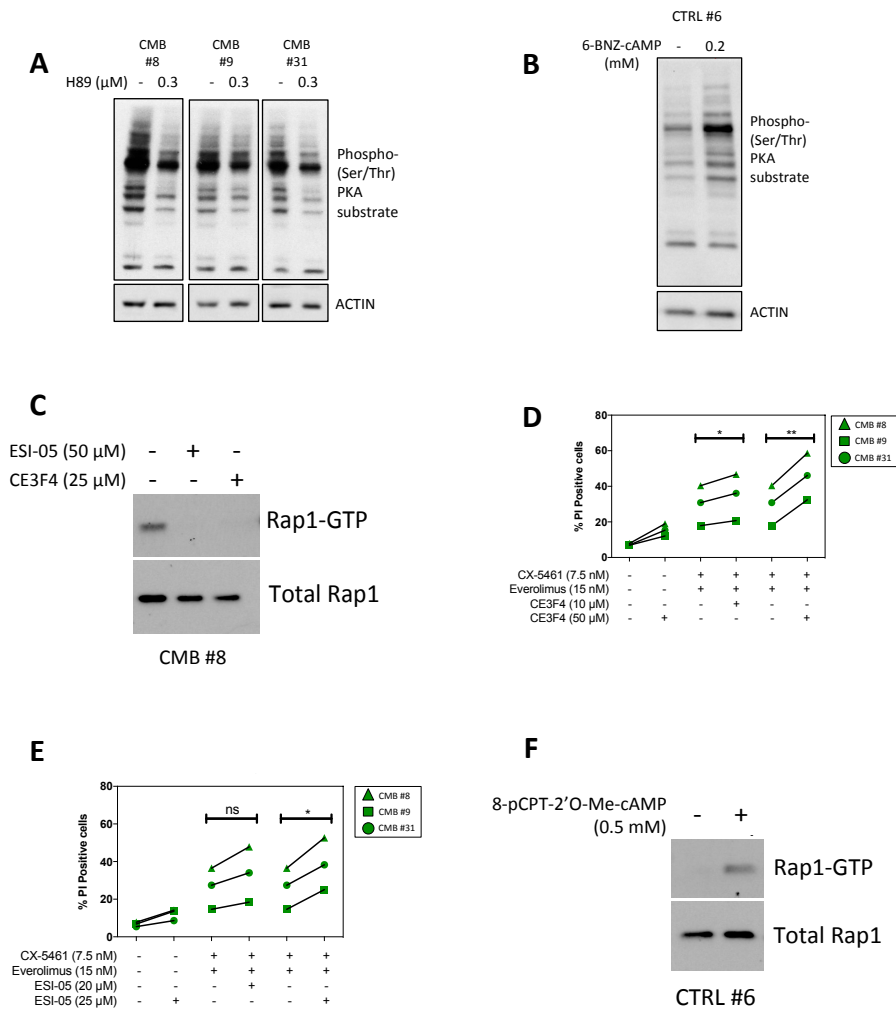
Supplemental Figure 1: *In vivo* transcriptome-wide analysis of cellular response to ribosome targeting therapy, related to Figure 1

(A) Flow cytometry analysis of inguinal lymph node cells isolated from C57BL/6 mice with transplanted Eμ-Myc B-lymphoma cells (clone #4242) treated as indicated for 2 hours. 10,000 viable cells of the correct morphology were collected for each sample. Graphs represent mean $\pm$ SEM of $n=6$. **(B and C)** Enrichment analysis by MetaCore® GeneGO of genes in “translation up” and “translation down” categories identified by anota2seq analysis comparing lymph node cells isolated from mice in **(B)** CX-5461 single-agent group or **(C)** everolimus single agent group with those isolated from mice in vehicle group ($n=6$). “Ratio” is the value obtained by dividing the number of genes in an indicated molecular process that is found in our data with the number of genes curated in MetaCore®’s database. “Direction” visualises the percentage of genes associated with indicated pathways that are up (red) or down (blue). **(D)** Steady-state abundance of mRNA encoding pro-apoptotic protein BMF in the cytoplasm (input) and on the polysomes (poly) based on polysome profiling analysis of the mouse lymph node cells ($n=6$). **(E)** Length of the 5’UTRs of metabolism-associated mRNAs that were downregulated in response to CX-5461+EV treatment *in vivo* ($n=6$).



Supplemental Figure 2: Everolimus and CX-5461 maintained their appropriate on-target effects in the early-passage Eμ-Myc lymphoma cell lines, related to Figure 2

(A) Western blot analysis of markers for the on-target effect of 20 nM everolimus treatment for 3-hour, specifically phosphorylation of rpS6 at Serine 240/244 residues and 4EBP1 at Serine 65 residue (n=3). Actin was used as a loading control. Synthesis rate of 47S pre-rRNAs in (B) drug-naïve (CTRL), (C) Everolimus-resistant (EV), (D) CX-5461-resistant (CX) and (E) CX-5461+EV-resistant (CMB) cell lines in response to 3-hour 50nM CX-5461, or (F) CX and (G) CMB cell lines in response to 10-hour 50nM CX-5461 were determined by ³²P-orthophosphate “pulse” labelling and (H) its quantitation (n=3). 28S rRNA abundance was used as a loading control. EtBr: ethidium bromide. Data were analysed by Student's *t* test. Graphs represent mean ± SEM of n=3. *, *P* ≤ 0.05. (I) Western blot analysis for p53 abundance in response to 50 nM CX-5461 treatment for 3 hours (n=3). Actin was used as a loading control.



Supplemental Figure 4: The effects of specific modulators of cAMP pathway on the early-passage E μ -Myc lymphoma cell lines, related to Figure 5

Western analysis of PKA activity using the phosphorylated PKA substrate antibody on whole cell extracts isolated from the indicated early-passage cell lines treated for 24 hours with **(A)** H89 or **(B)** 6-BNZ-cAMP (representative of $n=3$). Actin was used as a loading control. **(C)** Western analysis of the Rap1-GTP pull-down assays of the early-passage CX-5461+EV-resistant (CMB) cells extracts isolated following treatments with indicated EPAC inhibitors for 24 hours ($n=1$). Propidium iodide (PI) exclusion analysis of the CMB cells treated with CX-5461 and everolimus as indicated in the presence or absence of the selective **(D)** EPAC1 inhibitor CE3F4 or **(E)** EPAC2 inhibitor ESI-05 for 48 hours. Graphs represent mean $\pm$ SEM of $n=3$. Data were analysed by paired Student's t test. Green triangle: CX-5461-everolimus combination therapy-resistant (CMB) cells clone #8; green square: CMB cells clone #9; green circle: CMB cells clone #31. ns, $P \geq 0.05$; *, $P \leq 0.05$; **, $P \leq 0.01$. **(F)** Western analysis of Rap1-GTP pull-down assays using early-passage drug-naive (CTRL) E μ -Myc B-lymphoma cell extracts isolated following treatments with the selective EPAC activator 8-pCPT-2'-O-Me-cAMP for 24 hours ($n=1$).

Supplementary Table

Protein	Mass (kDa)	Primary Antibody Details	Primary Antibody Dilution	Secondary Antibody Details
Immunoblotting				
Actin	42 kDa	MP Biosciences 691002	1:30,000	Goat- α -rabbit HRP ¹
p53	53 kDa	Leica Biosystems	1:1,000	Goat- α -mouse HRP ²
rpS6	32 kDa	Cell Signalling #2317	1:2,000	Goat- α -mouse HRP ²
Phospho-rpS6	32 kDa	Cell Signalling #2215	1:2,000	Goat- α -rabbit HRP ¹
EPAC1	100 kDa	Cell Signalling #4155	1:1,000	Goat- α -mouse HRP ²
EPAC2	110 kDa	Abcam #193665	1:2,000	Goat- α -rabbit HRP ¹
Rap1	21 kDa	Thermo Fisher Scientific #16120	1:1,000	Goat- α -rabbit HRP ¹
Phospho-PKA substrate (RRXS*/T*)	N.A	Cell Signalling #9624	1:1,000	Goat- α -rabbit HRP ¹
Notes: 1. Goat- α -rabbit horseradish peroxide (HRP) IgG (H+L) (Bio-Rad #170-6515); dilution 1:2,000 2. Goat- α -mouse horseradish peroxide (HRP) IgG (H+L) (Bio-Rad #170-1011); dilution 1:2,000				
Flow cytometry				
B220 (CD45R)-APC	N.A.	Thermo Fisher Scientific 17-0452-82	1:400	N.A.

4.4 Data not included in the manuscript

Concurrent inhibition of energy metabolism as a new avenue to improve the efficacy of ribosome targeting therapy in MYC-driven haematological malignancies

The data presented in Chapter 4.2 demonstrated that CX-5461+EV treatment *in vivo* decreased the abundance of polysome-associated energy metabolism-related mRNAs. Therefore we hypothesized that metformin can be used to enhance the therapeutic efficacy of CX-5461+EV in drug-naïve Eμ-Myc B-lymphoma cells by exacerbating this translation-induced inhibition of energy metabolism. We tested the efficacy of CX-5461+EV+metformin triple combination treatment *in vitro* using the drug naïve CTRL cells. Strikingly, metformin (at 4, 5 and 6 mM) cooperated with CX-5461+EV treatment, producing an improved therapeutic efficacy that was greater than additive as determined by a PI exclusion assay (Fig. 4.1).

To determine whether simultaneous targeting of the ribosome and cellular energy metabolism can be applied more broadly to other MYC-driven blood cancers, the CX-5461+EV plus metformin triple combination was trialled in human acute myeloid leukaemia (AML) cell lines. While the efficacy of CX-5461 as a single agent therapy has been established in AML models [211], the efficacy of CX-5461+EV plus metformin has not been tested. Four human AML cell lines were used, all with elevated levels of MYC but representing a range of oncogenic drivers, p53 status and sensitivity to CX-5461: MV4-11 (MLL-AF4; p53 wild-type (WT)), SKM-1 (EZH2; p53-mutant (R248Q: arginine (R) replaced by glutamine (Q) on position 248)), SHI-1 (MLL-AF6; p53WT) and THP-1 (t(9;11); p53-null due to an R174 frameshift deletion).

Since it was previously demonstrated that p53 was required in mediating the efficacy of CX-5461+EV treatment in the Eμ-Myc lymphoma model [1], we proceeded to test the CX-5461-everolimus-metformin triple combination therapy using the p53WT AML cell lines, MV4-11 and SHI-1. Significant

improvement of efficacy was observed when the p53WT AML cell lines MV4-11 and SHI-1 were treated with the triple combination, as demonstrated by the robust decrease in cell number compared to the CX-5461+EV double combination (Fig. 4.2 A and 4.2 B). Consistent with this result, PI exclusion assay demonstrated a greater than additive increase in the PI-positive cell populations in response to the triple combination compared to CX-5461+EV (Fig. 4.2 C and 4.2 D). Additionally, while the triple combination treatment (CX-5461+EV+metformin) did not improve CX-5451+EV treatment in p53-mutant SKM-1 and p53-null THP-1 cells (data not shown), the combination of CX-5461 plus metformin was better than the single agent treatment as demonstrated by PI exclusion assays (Fig 4.3 A and 4.3 B). Together, these data demonstrated the potential of co-targeting energy metabolism and the cell's translational machinery to treat MYC-driven haematological malignancies.

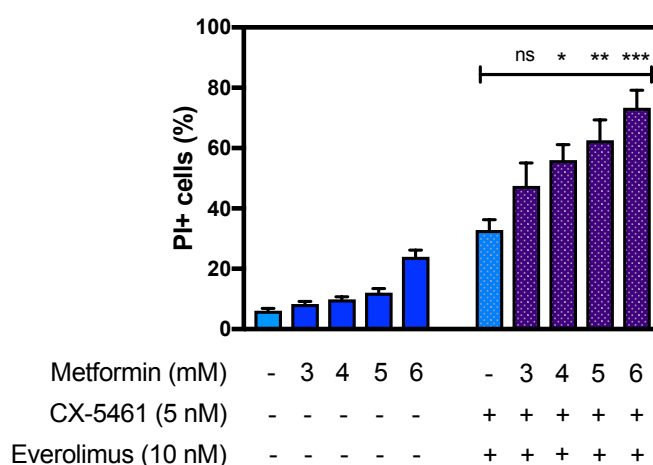


Figure 4.1: Metformin improves the efficacy of CX-5461-everolimus co-treatment in drug-naïve Eμ-Myc B-lymphoma cells.

Propidium iodide exclusion analysis of early-passage drug naïve (CTRL) Eμ-Myc B-lymphoma cells treated as indicated for 48 hours. Graphs represent mean $\pm$ SEM of n=3 independent experiments. Data were analysed by one-way ANOVA. ns, $P \geq 0.05$; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

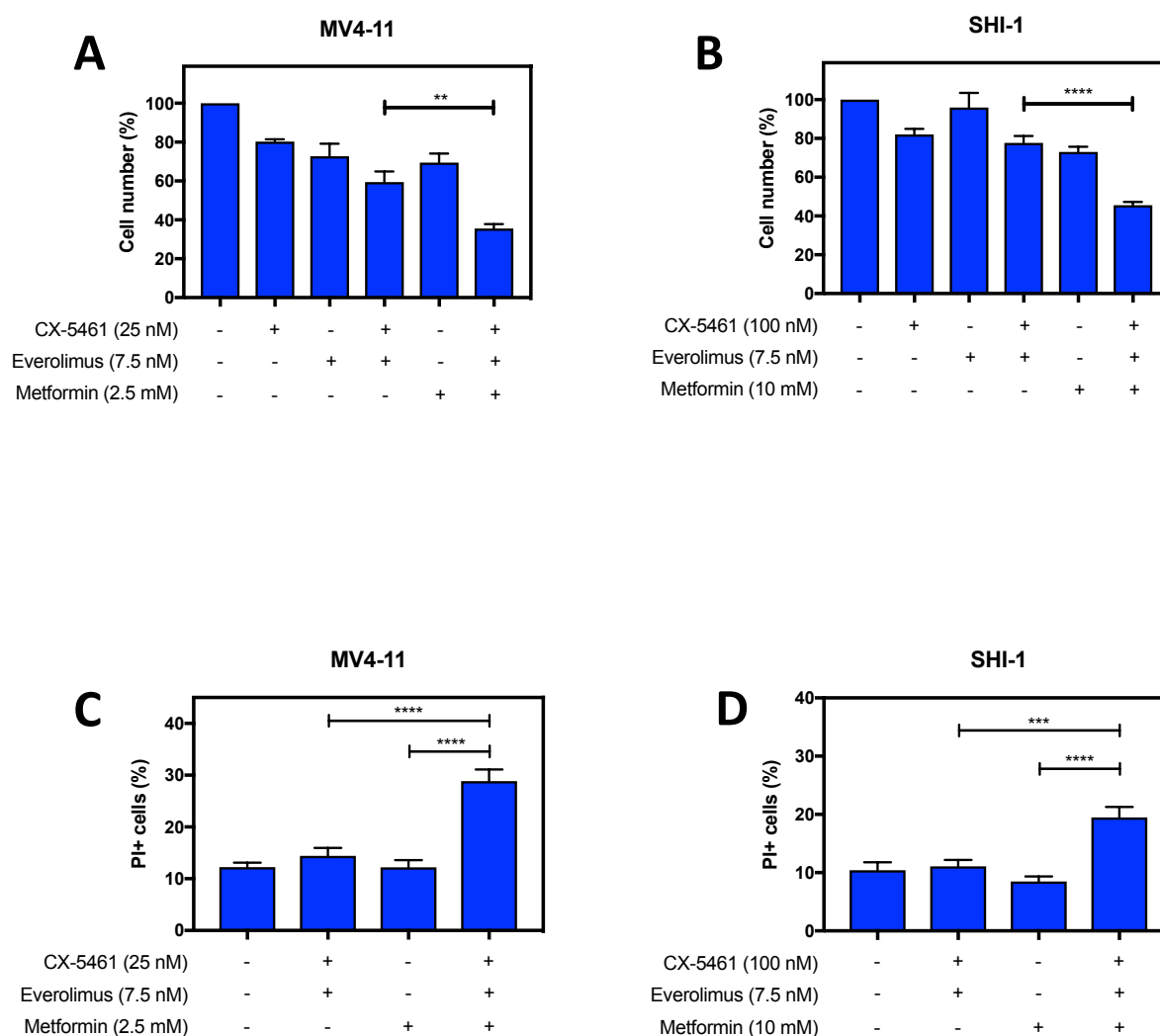


Figure 4.2: Metformin improves the efficacy of CX-5461-everolimus co-treatment in p53WT human acute myeloid lymphoma (AML) cell lines.

Changes in the number of (A,C) MV4-11 and (B,D) SHI-1 cells treated as indicated for 5 days as determined by Beckman® Coulter Counter or propidium iodide exclusion analysis, respectively. Graphs represent mean $\pm$ SEM of n=3 independent experiments. Data were analysed by Student *t* test. **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$.

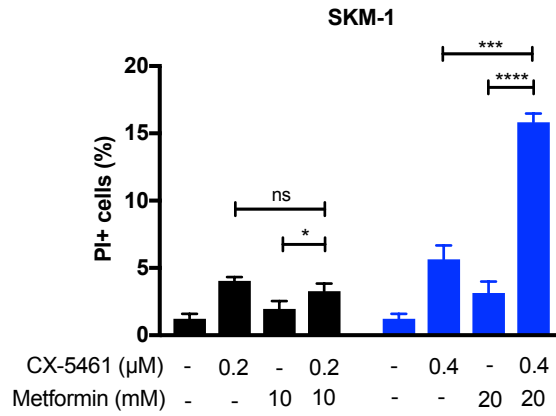
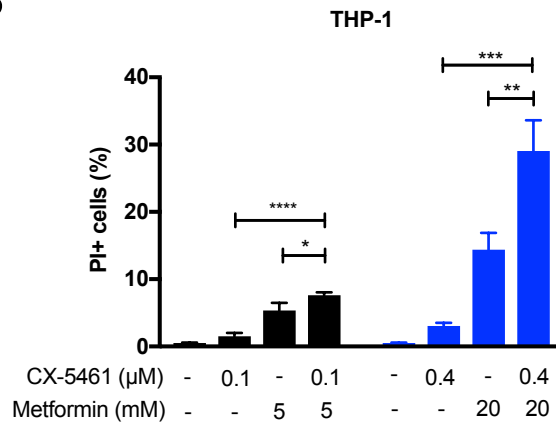
A**B**

Figure 4.3: Metformin improves the efficacy of CX-5461 single-agent treatment in human acute myeloid lymphoma (AML) cell lines SKM-1 and THP-1.

PI exclusion analysis of human AML cell lines **(A)** SKM-1 or **(B)** THP-1 treated as indicated for 48 hours. Graphs represent mean $\pm$ SEM of $n=3$ independent experiments. Data were analysed by Student t test. ns, $P \geq 0.05$; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$.

Chapter 5:

Discussion

Chapter 5: Discussion

5.1 Introduction

Dysregulation of multiple steps governing the synthesis of ribosomes, and their activities in translating mRNAs into proteins contributes immensely to the rapid biomass accumulation in cancer cells. This often occurs as a result of aberrations in the oncogenic signalling pathways regulating these processes, such as the PI3K/AKT/mTORC1 pathway, or in the activity of transcriptional regulators such as MYC (Chapter 1.1.3 and 1.2.2). As cancer cells driven by these oncogenic networks are often “addicted” to elevated ribosome biogenesis and mRNA translation activities [5], it is logical to target these processes to preferentially kill cancer cells and inhibit neoplastic growth (Chapter 1.4). In particular, proof of concept has come from targeting rDNA transcription, a key rate-limiting step in ribosome synthesis, with a small-molecule inhibitor CX-5461, which has been shown to be highly effective in animal models [8, 211] and has progressed to phase I/II clinical trials in solid and blood cancers (NCT02719977) [209, 210]. Moreover, promising outcomes have been demonstrated from combined targeting of ribosome biogenesis and mRNA translation, which resulted in improved treatment efficacy in malignancies driven by high MYC and elevated rates of eIF4E-dependent mRNA translation such as lymphoma [1] and prostate cancer [232]. This thesis focuses on characterising the cellular response to combined inhibition of ribosome biogenesis and activity using CX-5461 and mTORC1 inhibitor everolimus in a MYC-driven B-lymphoma setting. By applying high-throughput translomics approaches (established in Chapter 3), two critical aims were addressed in Chapter 4: (1) to determine the mechanisms underlying the synergy between CX-5461 and everolimus; and (2) to evaluate the mechanism of resistance to CX-5461-everolimus combination therapy.

5.2 Targeting the translational machinery in MYC-driven haematological cancers: challenges and perspectives

Amplification of MYC (abundance and/or activity) is reported for more than 70% of all human malignancies, and is often associated with aggressive disease and poor prognosis [88, 90, 91, 233, 234]. MYC has a well-recognised role as a master controller of cellular growth. It is capable of driving the activities of all three RNA polymerases, which are required to synthesise ribosomes, and activate mRNA translation. Extensive research driven by this urgent need to combat MYC-driven cancers has resulted in several therapeutic strategies to target MYC, for example using small molecules to inhibit MYC-MAX dimerisation [235, 236], epigenetic modulators to inhibit the *MYC* transcription [237] or strategies to inhibit MYC target genes (e.g. ornithine decarboxylate or casein kinase 1 epsilon) [238, 239]. However, these approaches have limited success due to a wide range of MYC-associated transcriptional mechanisms that regulate its influence on Pol I, Pol II and Pol III [6, 90, 105], thereby allowing activation of compensatory mechanisms. Moreover, the heterogeneity of MYC signatures and penetrance vary depending on the disease and cell types, as well as stage of development, rendering the inhibition a single MYC target gene difficult to be applied broadly [90, 102, 103].

In 2012, a seminal paper from our research group showed that the Pol I-transcription inhibitor CX-5461 selectively kills MYC-driven B-lymphoma cells and not the normal B-cell population [8]. Since then, there has been the emergence of other studies supporting the efficacy of CX-5461 as single agent therapy in AML [211], osteosarcoma [240] and multiple myeloma [208, 241]. Furthermore, improved efficacy has been achieved for CX-5461 if used in combination with the mTORC1 inhibitor everolimus in lymphoma [1], with Pim Kinase inhibitor CX-6258 [232] in prostate cancer and with bromo- and extra-terminal domain (BET) protein inhibitor iBET-151 (Sornkom, Poortinga, Hannan, unpublished data), all using mouse cancer models characterised by elevated MYC activity. In the past, ribosome biogenesis has been viewed as a “housekeeping process” that was previously deemed to be undruggable with the fear of adverse effects on the host’s normal cells. However, these recent

research efforts have demonstrated that, since elevated ribosome biogenesis is a ubiquitous signature in virtually all cancers with elevated MYC activity, the inhibition Pol I transcription using CX-5461 is a promising non-genotoxic approach for the treatment of MYC-driven cancers.

Nevertheless just like the majority of all cancer therapeutics [230, 242-244], these studies have also demonstrated the development of resistance to CX-5461 in these cancer models. As such, by understanding differences between those cancer cells that are and are not resistant to CX-5461, we aimed to provide novel insights into potential combination therapies that can improve the efficacy of CX-5461.

Interestingly single-agent CX-5461-resistant cells exhibited a distinct expression pattern with respect to their translational regulator proteins (Chapter 4 Fig. 4A). This raises the possibility that these changes might play a role in the development of resistant to CX-5461 treatment. In addition, based on exome sequencing data, an ongoing study in our group identified DNA topoisomerase II alpha (TOP2A) as a potential mediator for resistance to CX-5461 single agent therapy in *Eμ-Myc* cells (Cameron, Poortinga, Hannan, unpublished data). One of the main functions of TOP2A is to ensure that the correct DNA topology is maintained, which it does so by creating DNA double-strand breaks (DSBs), thereby relieving the tension created by processes such as transcription and DNA replication [245, 246]. This supports our group's recent findings that CX-5461 can induce a p53-independent checkpoint kinase (CHK)1/CHK2-mediated DNA damage response (DDR) in the absence of DNA damage in BJ cell lines [125]. While the p53-mediated nucleolar stress response was demonstrated to be critical for the anti-tumour activity of CX-5461 in p53WT cell lines, this DDR response underpins the efficacy of CX-5461 single-agent therapy in acute lymphoblastic leukemia [247] and in murine models of highly aggressive p53-null AML [211]. These reports support the design of several novel and effective therapeutic combination strategies with CX-5461: a) with an inhibitor of Poly(ADP-ribose) polymerase 1 (PARP1), a crucial enzyme in the repair of DSBs, for the treatment of high grade serous ovarian cancer (Sanij, Hannan, Sheppard, Pearson, unpublished data); and b) with iBET-151 in AML whereby enhanced

DNA damage was proposed as the underlying mechanism of the cooperativity between these two compounds. (Sornkom, Poortinga, Hannan, unpublished data).

While the combination of CX-5461 and everolimus (CX-5461+EV) successfully delayed the onset of resistant disease *in vivo* as was described previously, the animals eventually succumbed to disease relapse due to accumulation of Eμ-Myc B-lymphoma cells that are resistant to CX-5461+EV co-treatment. Using early-passage cells established from this experiment, we first demonstrated that the resistant phenotype not simply caused by the inability of the drugs to bind to their molecular targets, for example due to the uncontrolled activity of ATP-dependent xenobiotic pumps or mutation in the drug targets' binding sites, both of which are commonly observed in a number of targeted cancer therapeutic cases [248-250] (Chapter 4 Supp. Fig. 2B-D). Initial clues about the resistant mechanism were first obtained from the metabolic analysis of these early-passage Eμ-Myc B-lymphoma cells, which showed that the CX-5461+EV-resistant cells are producing more ATP (Chapter 4 Fig. 3A-B). Subsequent polysome profiling analysis uncovered a "translational orchestration" which increased translation efficiency of mRNAs encoding components of mitochondrial oxidative phosphorylation and cAMP signalling pathway (Chapter 4 Supp. Fig. 3). This in turn allows more efficient ATP production (Chapter 4 Supp. Fig. 3A) and utilisation via the cAMP-EPAC1/2-Rap1 pathway (Chapter 4 Supp. Fig. 5B), whose role in mediating a death evasion response was confirmed (Chapter 4 Supp. Fig. 5D-G) using specific modulators of this pathway. In addition, it is also possible that elevated ATP levels are mostly directed towards increased energy requirement for elevated translational activity in the CX-5461+EV-resistant cells, thus creating a feed-forward mechanism that promote malignant and accelerated cell growth proliferation.

Moreover, the mRNA translation reprogramming phenomenon highlighted in Chapter 4 provided some insights into an intriguing paradox known as "Dameshek's riddle". In 1967, William Dameshek, the founding editor-in-chief of *Blood* journal, stated the following riddle: "What do aplastic anemia, paroxysmal nocturnal hemoglobinuria (PNH) and 'hypoplastic' leukemia have

in common?” based on the observation that these diseases were hypo-proliferative in their early stages, but in the later stages develop into hyper-proliferative diseases such as acute myeloid leukemia [251]. Interestingly, a class of diseases associated with dysregulated ribosome biogenesis, which is termed ribosomopathies, is also characterised by early stages of hypo-proliferativity, and ribosomopathy patients have a propensity to be diagnosed with acute myeloid leukemia later in life [252-256]. Ribosomopathies are associated with loss of function mutations in ribosomal proteins (such as Diamond Blackfan anemia (DBA) [257] and 5q- syndrome [258]), factors associated with Pol I transcription and processing (such as Treacher Collins Syndrome [259, 260]), mutations affecting rRNA processing and modification such as Dyskeratosis Congenita [261] and Shwachman–Diamond Syndrome [262]. Indeed, somatic mutations and deletions in ribosomal protein genes were observed in T-cell acute lymphoblastic leukemia and solid tumours such as gastric and ovarian cancer [254, 263-265]. A recent study using a model of DBA reported that genetic mutation-associated impairment of ribosome biogenesis caused translation reprogramming that affects erythroid differentiation. This thesis demonstrated that using small molecule inhibitor to compromise ribosome biogenesis resulted in selection for highly aggressive clones with reprogrammed translational profiles that drives elevated metabolism activity to promote pro-survival mechanisms, suggesting that part of the answer to “Dameshek’s riddle” may be attributed to specific translational rewiring during disease progression.

Importantly, we demonstrated that this translational orchestration of metabolism-dependent death evasion mechanism could be targeted using the antibiotic drug metformin (Chapter 4 Fig. 3D and 6). Specifically, metformin inhibited ATP production by oxphos and increased the levels of AMP, which in turn inhibits adenylate cyclase and the activation cAMP-dependent pathway including EPAC. We demonstrated that in the presence of metformin, the CMB cells are re-sensitised to CX-5461 plus everolimus co-treatment (Chapter 4 Fig. 3D). Given that MYC plays an important role in the regulation of metabolism, the data in this thesis therefore conceptualise a novel combination therapy strategy for the treatment of MYC-driven cancers

involving simultaneous targeting of two housekeeping processes, namely translation machinery and energy metabolism, which will be further discussed in the following section.

5.3 Dual targeting of cellular translation machinery and energy metabolism as a novel therapeutic approach to treat MYC-driven cancer

The role of MYC in upregulating cellular metabolic activity by amplifying the expression of metabolic genes has been well-documented [91]. For example, MYC regulates glycolysis by stimulating the expression of glucose transporter 1 and glycolytic enzymes such as phosphofructokinase, glyceraldehyde-3-phosphate dehydrogenase and enolase [266]. In addition, MYC also promotes mitochondrial biogenesis and function by inducing the transcription of genes encoding mitochondrial DNA polymerase gamma, mitochondrial glutaminase and structural components of the mitochondria [267-270]. Importantly, our Seahorse XF96 data indicated that both processes were upregulated in the CX-5461+EV-resistant early-passage cells compared to drug-naïve cells as determined by the measurement of glycolysis and oxphos rates (Chapter 4 Fig. 3B). Hence, we tested metformin in these early-passage cells because it targets two of the most striking changes in CMB cells: translational-dependent increase in mitochondrial oxidative phosphorylation and cAMP signalling.

Although metformin is universally recognised as an anti-diabetic drug, numerous studies have shown its efficacy for the treatment of human cancers including hepatocellular carcinoma [271], breast cancers [272, 273], pancreatic cancer [274], colorectal cancer [275] and prostate cancer [276]. Moreover, based on the clinicaltrials.gov database (as of June 2018), more than 30 phase II and III clinical trials using metformin are in progress, some are in combination with other targeted therapeutic and chemotherapeutic agents. It is widely accepted that metformin's fundamental mechanism of action involves the suppression of mitochondrial oxidative phosphorylation, however some mechanistic details are still unclear. A number of reports support the concept that metformin directly binds to mitochondrial electron transport chain complex I components [277, 278], however this is yet to be

confirmed [279]. Other more indirect mechanisms of action for metformin, which include mTOR as an integral module, have recently been proposed [280, 281]. Either way, the energy sensor protein AMPK is involved in relaying energy cues to mTOR and its downstream anabolic processes including ribosome biogenesis (reviewed in Chapter 1.3). As discussed above, targeting ribosome biogenesis is an attractive idea as it is a universal signature of MYC-driven cancers. Consequently, since there is a cellular demand for energetic and building blocks required by this resource-consuming process, therapeutic targeting of the associated MYC-driven upregulation of metabolic processes has received some attention. Therefore, due to these reasons and the possibility that this will allow rapid translation to the clinic, we tested metformin in this study.

One of the earliest recorded clinical successes from targeting MYC-driven metabolism was in 1948 using a folate antagonist called aminopterin, which was used to treat 16 children with ALL [282]. Later it was discovered that one of the patients who was cured, lived until 2001, in fact had MYC-driven Burkitt lymphoma [282]. Derivatives of aminopterin are still used in the clinic with recent studies demonstrating that the members of the serine-one carbon folate-glycine cleavage system (SOG) pathway are MYC target genes [283, 284]. In addition, a study reported that the loss of one copy of glutaminase (GLS) gene suppressed disease progression of a mouse model of MYC-driven hepatocellular carcinoma [285]. A phase 2 clinical trial is investigating a small molecule inhibitor of GLS called CB-839 [285] in combination with nivolumab in patients with non-small cell lung cancer, melanoma and renal cell carcinoma (NCT02771626). Interestingly, an ongoing study in our group also demonstrated the synergistic killing of E μ -Myc B-lymphoma cells (clone #4242) in response to the combination of CX-5461 and CB-839 (J. Kang, Hannan, Pearson, unpublished data), which highlights the prospect of co-targeting the ribosome and energy metabolism.

In this thesis, we first tested the CX-5461-everolimus-metformin combination on the CX-5461+EV-resistant E μ -Myc B-lymphoma cells with our *in vitro* and acute *in vivo* data demonstrating the efficacy of this triple combination (Chapter 4 Fig. 6). However, due to the aggressive nature of these

translationally and metabolically active CMB cells, the extension in the survival window was not as robust as expected (Chapter 4 Fig. 6E). Thus, we introduced this triple drug combination before drug resistance developed in these MYC-driven lymphoma cells, that is, by testing it on drug naïve cells. Moreover, we utilised an *in vivo* genome-wide translational profiling approach to elucidate the mechanism of cooperativity between CX-5461 and everolimus. As previously described, our translomics data indicated that the enhanced efficacy in response to CX-5461+EV-treatment is due to a robust inhibition of cellular translational activity (Chapter 4 Fig. 1C). Interestingly, rather than decreasing the translation of all mRNAs globally, our data indicate that the CX-5461+EV-treated cells specifically reduced the translation of mRNAs encoding proteins involved in multiple metabolic processes, especially those with roles in energy metabolism (Chapter 4 Fig. 1E). These data therefore provide further rationale for the combination of metformin with CX-5461 and everolimus in order to further suppress energy metabolism and improve the efficacy of CX-5461+EV treatment in drug-naïve Eμ-Myc B-lymphoma cells.

The possibility of a broader application of this combination therapy strategy was also studied in human AML cell lines with elevated MYC activities. Improvement of efficacy was observed in p53WT AML cell lines MV4-11 and SHI-1, both of which display abnormally high MYC activity [286, 287] (Fig. 4.2). Interestingly, EZH2, which is an important oncogenic driver of SHI-1 cell line, has been shown to promote metabolic rewiring in glioblastoma via epigenetic regulation of hypoxia-inducible factor (HIF) 1α [288]. Thus, this may influence the sensitivity of this particular cell line to the triple combination therapy. Nevertheless, future *in vivo* studies will be necessary to test this triple combination therapy in both Eμ-Myc and AML models *in vivo*. Together, these data provided crucial pre-clinical evidences that simultaneous targeting of cellular translation machinery and energy metabolism has an exciting potential to be applied broadly to treat MYC-driven haematological malignancies.

5.4 Future Directions

Despite the promising results from early implementation of the CX-5461-everolimus-metformin combination in drug naïve E μ -Myc B-lymphoma cells, it remains to be determined whether this triple combination therapy will significantly prolong the survival benefit *in vivo*. Our pilot experiment (Appendix 2) demonstrated that the triple combination therapy, which consists of daily dosing of 500 mg/kg metformin (compared to 800 mg/kg delivered as 400 mg/kg twice daily in this study) in combination with the well established dosing regime of CX-5461 (35 mg/kg on Monday, Wednesday and Friday) and everolimus (5 mg/kg daily), was well tolerated by the C57BL/6 for up to 60 days. However, since the survival benefit also depends on other factors, such as the extent of the initial tumour cell clearance, further testings involving a more aggressive dosing regime towards the start of the treatment period are certainly required.

Analysis of the polysome-association of mRNAs encoding key components and regulators of mRNA translation (Chapter 4 Figure 4A) revealed distinct translational landscape between the drug-naïve, CX-5461-resistant and CX-5461+EV-resistant cells. This indicates that the efficacy of CX-5461+EV treatment may also involve alterations in the ribosome composition in the drug-naïve cells in response to this combination treatment as previously reported [217]. To evaluate ribosome composition, polysomal fractions may be pooled into sub-polysomal, light polysomal and heavy polysomal fractions, followed by protein precipitation (e.g. trichloroacetic acid precipitation) and mass spectrometry analysis to evaluate ribosomal protein composition in each pooled fractions.

Although it was hypothesised that the efficacy of the triple combination therapy was due to further metabolic inhibition by metformin on top of the CX-5461+EV-induced metabolic stress, some gaps in knowledge remained. As previously mentioned, disruption of the ATP:AMP ratio by metformin promotes activation of the energy sensor protein AMPK, which in turn inhibits mTORC1. Thus, it was possible that everolimus and metformin exerted a more potent

inhibition of mTORC1-dependent mRNA translation activities via AMPK, which in turn resulted in an improved cooperativity with CX-5461 with respect to suppressing both ribosome synthesis and function. This can be investigated by monitoring the phosphorylation status of AMPK, especially on the Thr172 residue in its catalytic domain required for its activation, and 4EBP1 in response to acute treatment of tumour-bearing mice with the triple combination therapy at multiple time points *in vivo*.

In addition, the role of immune system in the tumour microenvironment, especially at the locations where E μ -Myc B-lymphoma cells engraft *in vivo* (e.g. lymph nodes, spleen and bone marrow), should be considered with respect to the efficacy of this triple combination therapy. For instance, metformin via an AMPK dependent mechanism is reported to increase the number of CD8⁺ tumour infiltrating lymphocytes and protected them from apoptosis and “immune exhaustion” [289]. This resulted in a decrease in tumour burden and an extension of survival window in a transgenic mouse breast cancer model [289]. In another example, metformin-based combination therapy was shown to block tumour growth by inhibiting the activation of nuclear factor (NF)- κ B-dependent inflammatory pathways [290]. Furthermore, since NF- κ B activity is required to promote the survival of CD8⁺ cells [291], perhaps this pathway play a role in the mechanism of the efficacy of the combination therapy. Thus, it would be interesting to test the ability of metformin to induce clearance of E μ -Myc lymphoma cells in a transgenic mouse model that expresses a constitutively active form of the NF- κ B inhibitor I κ B-kinase, which is a commonly used approach to investigate the role of NF- κ B *in vivo* [292, 293].

While metformin is considered to be the best biguanide (guanidine derivatives) in terms of therapeutic index, safety and efficacy in humans and animal models, another biguanide called phenformin ranks second among these family of compounds. The higher potency of phenformin is often attributed to its ability to enter the mitochondria more freely, unlike metformin which requires organic cation transporters (OCT) for its transport. This in turn results in increased severity of side effects such as lactic acidosis, and thus it was withdrawn from the clinic in the 1970s. However, phenformin has recently

resurfaced and is currently being investigated in a clinical trial (NCT03026517; started in January 2017) in combination with the BRAF-inhibitor dabrafenib and the MAPK kinase (MAPKK) (chapter 1.1.3) inhibitor trametinib for the treatment of melanoma. Thus it will be interesting to see if like metformin, phenformin can also cooperate with CX-5461 or CX-5461 plus everolimus *in vitro* and *in vivo* to selectively kill Eμ-Myc B-lymphoma cells.

As described above, therapeutic efficacy from the combined inhibition of ribosome synthesis and function was also achieved by the combination of CX-5461 and a pan-PIM Kinase inhibitor CX-6258 in a Hi-Myc mouse model of castrate-resistant prostate cancer [232]. PIM kinases elevate MYC transcriptional activity [294], promote eIF4E-dependent mRNA translation [54] and were demonstrated to be simultaneously overexpressed with MYC in prostate cancer [295, 296]. Rebello *et al.* [232] demonstrated that the CX-5461-CX-6258 combination therapy successfully reverted an aggressive adenocarcinoma back to a benign high-grade prostatic intraepithelial neoplasia. It remains to be determined, perhaps using polysome profiling analysis, whether similar translational and metabolic alterations play a role in mediating drug response in this MYC-driven prostate cancer model.

In Chapter 4, we identified the cAMP-EPAC1/2-Rap1 pathway as a key player in mediating a pro-survival response in the CX-5461+EV-resistant Eμ-Myc B-lymphoma cells. However, the exact mechanism(s) by which this pathway regulates cell death response(s) in this cancer model is still undefined. The role of cAMP-EPAC1/2 pathway in preventing both tumour necrosis factor alpha (TNFα) and caspase-mediated apoptosis have been previously shown in leukocytes [297, 298] and in hepatocytes [299]. Interestingly, cAMP can have a diverging effects on cell survival depending on the cell types, and in some cases the PKA arm downstream of cAMP plays a more dominant role (reviewed in [300]). As a continuation of this project, the roles of cAMP-EPAC1/2-Rap1 pathway in response to CX-5461+EV treatment would be assessed by evaluating the mRNA and protein expression levels, using qRT-PCR and immunoblotting respectively, of BCL2-family members that are known to be involved in regulating death responses in lymphoma cells, both pro-apoptotic, such as BAX and BAK, and anti-apoptotic such as BCL2 and

MCL1 [301]. Importantly, similar to the experiments presented in Chapter 4 Fig. 5D-G, the aforementioned experiment should be carried out in the presence and absence of specific modulators of EPAC1/2 activity or PKA activity in order to evaluate whether EPAC1/2 activity is associated with BCL2-family associated pro-survival response. In addition, the levels of cleaved caspase proteins such as caspase-3, as well as cleaved PARP proteins associated with TNF- α -mediated apoptosis, should also be evaluated in order to define the mechanism by which cell death response is evaded.

Moreover, EPAC1/2-Rap1 has been shown to operate upstream of PI3K/AKT/mTOR signalling pathway [302, 303]. Our preliminary data indicated that the CX-5461+EV-resistant cells, which express higher levels of EPAC2 proteins as determined by Western analysis (Chapter 4 Fig. 5C), also have increased abundance of phosphorylated AKT (Ser473) (data not shown). Thus, since MYC has been shown to cooperate with the PI3K/AKT/mTORC1 pathway to drive ribosome biogenesis [104] and cell cycle progression [304], the possibility that MYC via the cAMP-EPAC1/2-Rap1 pathway cooperate with the PI3K/AKT/mTORC1 pathway to mediate pro-survival responses should also be considered. All in all, these potential approaches aim to identify whether cAMP-EPAC1/2-Rap1-mediated death evasion response, which is identified in this thesis, is common pro-survival response in MYC-driven cancer to evade stress- or drug-induced death response, as well as to validate whether it is a vulnerability that can be exploited to kill a broad range of MYC-driven cancer

Second generation of Pol I inhibitors have recently been developed. One of such compounds is PMR-116 (Pimera, Inc.), and our research group is currently evaluating its efficacy in normal and cancer cells as well as its mechanisms of action in comparison to CX-5461. Thus far, our preliminary data indicated that PMR-116 inhibits Pol I transcription by preventing its association with the rDNA, thereby promoting p53 accumulation. Interestingly, PMR-116 can cross the blood-brain barriers, which is a property that is not possessed by CX-5461. This opens up new possibilities for using PMR-116 to treat brain cancers. Nevertheless, it remains to be determined whether PMR-116, like CX-5461, is able to induce DNA damage responses, or whether it

can be combined with inhibitors of ribosome activity such as everolimus or CX-6258 for the treatment of MYC-driven cancers.

5.5 Conclusions

This thesis has demonstrated that combined inhibition of ribosome synthesis and activities using CX-5461 and everolimus, respectively, in MYC-driven lymphoma cells resulted in a mRNA translation-dependent metabolic repression. Furthermore, cells that are resistant to this combination therapy overcome that repression via a translation-dependent orchestration of cellular metabolic activity. Specifically by mediating a reprogramming of the cellular translational landscape, the CX-5461+EV-resistant cells increase the translation of mRNAs encoding the necessary proteins to fuel and activate an energy-dependent pathway required to evade cell death response induced by the ribosome-targeting therapy. Finally, this thesis demonstrates the potential of simultaneous targeting of cellular translational machinery and energy metabolism as a new paradigm for the treatment of cancers that rely on elevated oncogenic MYC activity for their growth and metabolic plasticity.

Bibliography

1. Devlin, J.R., et al., *Combination Therapy Targeting Ribosome Biogenesis and mRNA Translation Synergistically Extends Survival in MYC-Driven Lymphoma*. *Cancer Discov*, 2016. **6**(1): p. 59-70.
2. Sonenberg, N. and A.G. Hinnebusch, *Regulation of Translation Initiation in Eukaryotes: Mechanisms and Biological Targets*. *Cell*, 2009. **136**(4): p. 731-745.
3. Ruggero, D. and P.P. Pandolfi, *Does the ribosome translate cancer?* *Nature Reviews Cancer*, 2003. **3**: p. 179.
4. Silvera, D., S.C. Formenti, and R.J. Schneider, *Translational control in cancer*. *Nature Reviews Cancer*, 2010. **10**(4): p. 254-266.
5. Ruggero, D., *Translational control in cancer etiology*. Cold Spring Harbor perspectives in biology, 2012: p. a012336.
6. Bywater, M.J., et al., *Dysregulation of the basal RNA polymerase transcription apparatus in cancer*. *Nature Reviews Cancer*, 2013. **13**(5): p. 299.
7. Bhat, M., et al., *Targeting the translation machinery in cancer*. *Nat Rev Drug Discov*, 2015. **14**(4): p. 261-78.
8. Bywater, M.J., et al., *Inhibition of RNA polymerase I as a therapeutic strategy to promote cancer-specific activation of p53*. *Cancer Cell*, 2012. **22**(1): p. 51-65.
9. Schena, M., et al., *Parallel human genome analysis: microarray-based expression monitoring of 1000 genes*. *Proceedings of the National Academy of Sciences*, 1996. **93**(20): p. 10614-10619.
10. Vogel, C. and E.M. Marcotte, *Insights into the regulation of protein abundance from proteomic and transcriptomic analyses*. *Nature Reviews Genetics*, 2012. **13**(4): p. 227.
11. Schwanhäusser, B., et al., *Global quantification of mammalian gene expression control*. *Nature*, 2011. **473**(7347): p. 337.
12. Larsson, O., B. Tian, and N. Sonenberg, *Toward a genome-wide landscape of translational control*. Cold Spring Harbor perspectives in biology, 2013. **5**(1): p. a012302.
13. Kristensen, A.R., J. Gsponer, and L.J. Foster, *Protein synthesis rate is the predominant regulator of protein expression during differentiation*. *Molecular systems biology*, 2013. **9**(1): p. 689.
14. Piccirillo, C.A., et al., *Translational control of immune responses: from transcripts to translomes*. *Nature immunology*, 2014. **15**(6): p. 503.
15. Hsieh, A.C., et al., *The translational landscape of mTOR signalling steers cancer initiation and metastasis*. *Nature*, 2012. **485**(7396): p. 55.
16. Topisirovic, I., et al., *Cap and cap-binding proteins in the control of gene expression*. *Wiley Interdisciplinary Reviews: RNA*, 2011. **2**(2): p. 277-298.
17. Hinnebusch, A.G. and J.R. Lorsch, *The mechanism of eukaryotic translation initiation: new insights and challenges*. Cold Spring Harbor perspectives in biology, 2012: p. a011544.

18. Pestova, T.V., et al., *Molecular mechanisms of translation initiation in eukaryotes*. Proceedings of the National Academy of Sciences, 2001. **98**(13): p. 7029-7036.
19. Holcik, M. and T.V. Pestova, *Translation mechanism and regulation: old players, new concepts: Meeting on Translational Control and Non-Coding RNA*. EMBO reports, 2007. **8**(7): p. 639-643.
20. Cheung, Y.-N., et al., *Dissociation of eIF1 from the 40S ribosomal subunit is a key step in start codon selection in vivo*. Genes & development, 2007. **21**(10): p. 1217-1230.
21. Pestova, T.V., et al., *The joining of ribosomal subunits in eukaryotes requires eIF5B*. Nature, 2000. **403**(6767): p. 332.
22. Pisarev, A.V., et al., *Ribosomal position and contacts of mRNA in eukaryotic translation initiation complexes*. The EMBO journal, 2008. **27**(11): p. 1609-1621.
23. Dever, T.E. and R. Green, *The elongation, termination, and recycling phases of translation in eukaryotes*. Cold Spring Harbor perspectives in biology, 2012. **4**(7): p. a013706.
24. Ben-Shem, A., et al., *The structure of the eukaryotic ribosome at 3.0 Å resolution*. Science, 2011. **334**(6062): p. 1524-1529.
25. Chen, C., et al., *Allosteric vs. spontaneous exit-site (E-site) tRNA dissociation early in protein synthesis*. Proceedings of the National Academy of Sciences, 2011.
26. Stansfield, I., et al., *The products of the SUP45 (eRF1) and SUP35 genes interact to mediate translation termination in Saccharomyces cerevisiae*. The EMBO journal, 1995. **14**(17): p. 4365-4373.
27. Cheng, Z., et al., *Structural insights into eRF3 and stop codon recognition by eRF1*. Genes & development, 2009. **23**(9): p. 1106-1118.
28. Alkalaeva, E.Z., et al., *In vitro reconstitution of eukaryotic translation reveals cooperativity between release factors eRF1 and eRF3*. Cell, 2006. **125**(6): p. 1125-1136.
29. Pisarev, A.V., et al., *The role of ABCE1 in eukaryotic posttermination ribosomal recycling*. Molecular cell, 2010. **37**(2): p. 196-210.
30. Barthelme, D., et al., *Ribosome recycling depends on a mechanistic link between the FeS cluster domain and a conformational switch of the twin-ATPase ABCE1*. Proceedings of the National Academy of Sciences, 2011. **108**(8): p. 3228-3233.
31. Skabkin, M.A., et al., *Activities of Ligatin and MCT-1/DENR in eukaryotic translation initiation and ribosomal recycling*. Genes & development, 2010. **24**(16): p. 1787-1801.
32. Pisarev, A.V., C.U. Hellen, and T.V. Pestova, *Recycling of eukaryotic posttermination ribosomal complexes*. Cell, 2007. **131**(2): p. 286-299.
33. Donnelly, N., et al., *The eIF2 α kinases: their structures and functions*. Cellular and Molecular Life Sciences, 2013. **70**(19): p. 3493-3511.
34. Pavitt, G.D. and D. Ron, *New insights into translational regulation in the endoplasmic reticulum unfolded protein response*. Cold Spring Harbor perspectives in biology, 2012: p. a012278.
35. Wullschleger, S., R. Loewith, and M.N. Hall, *TOR signaling in growth and metabolism*. Cell, 2006. **124**(3): p. 471-484.

36. Cargnello, M. and P.P. Roux, *Activation and function of the MAPKs and their substrates, the MAPK-activated protein kinases*. Microbiology and molecular biology reviews, 2011. **75**(1): p. 50-83.
37. Lazaris-Karatzas, A., K.S. Montine, and N. Sonenberg, *Malignant transformation by a eukaryotic initiation factor subunit that binds to mRNA 5'cap*. Nature, 1990. **345**(6275): p. 544.
38. Mamane, Y., et al., *eIF4E—from translation to transformation*. Oncogene, 2004. **23**(18): p. 3172.
39. Mendoza, M.C., E.E. Er, and J. Blenis, *The Ras-ERK and PI3K-mTOR pathways: cross-talk and compensation*. Trends in biochemical sciences, 2011. **36**(6): p. 320-328.
40. Richter, J.D. and N. Sonenberg, *Regulation of cap-dependent translation by eIF4E inhibitory proteins*. Nature, 2005. **433**(7025): p. 477.
41. Schalm, S.S., et al., *TOS motif-mediated raptor binding regulates 4E-BP1 multisite phosphorylation and function*. Current biology, 2003. **13**(10): p. 797-806.
42. Hsieh, A.C., et al., *Genetic dissection of the oncogenic mTOR pathway reveals druggable addiction to translational control via 4EBP-eIF4E*. Cancer cell, 2010. **17**(3): p. 249-261.
43. Dowling, R.J., et al., *mTORC1-mediated cell proliferation, but not cell growth, controlled by the 4E-BPs*. Science, 2010. **328**(5982): p. 1172-1176.
44. Koromilas, A.E., A. Lazaris-Karatzas, and N. Sonenberg, *mRNAs containing extensive secondary structure in their 5' non-coding region translate efficiently in cells overexpressing initiation factor eIF-4E*. The EMBO journal, 1992. **11**(11): p. 4153-4158.
45. Mamane, Y., et al., *mTOR, translation initiation and cancer*. Oncogene, 2006. **25**: p. 6416.
46. Grove, J., et al., *Cloning and expression of two human p70 S6 kinase polypeptides differing only at their amino termini*. Molecular and cellular biology, 1991. **11**(11): p. 5541-5550.
47. Gout, I., et al., *Molecular cloning and characterization of a novel p70 S6 kinase, p70 S6 kinase β containing a proline-rich region*. Journal of Biological Chemistry, 1998. **273**(46): p. 30061-30064.
48. Fenton, T.R. and I.T. Gout, *Functions and regulation of the 70 kDa ribosomal S6 kinases*. The international journal of biochemistry & cell biology, 2011. **43**(1): p. 47-59.
49. Wang, X., et al., *Regulation of elongation factor 2 kinase by p90RSK1 and p70 S6 kinase*. The EMBO journal, 2001. **20**(16): p. 4370-4379.
50. Shahbazian, D., et al., *The mTOR/PI3K and MAPK pathways converge on eIF4B to control its phosphorylation and activity*. The EMBO journal, 2006. **25**(12): p. 2781-2791.
51. Dorrello, N.V., et al., *S6K1-and β TRCP-mediated degradation of PDCD4 promotes protein translation and cell growth*. Science, 2006. **314**(5798): p. 467-471.
52. Richardson, C.J., et al., *SKAR is a specific target of S6 kinase 1 in cell growth control*. Current Biology, 2004. **14**(17): p. 1540-1549.

53. Ma, X.M., et al., *SKAR links pre-mRNA splicing to mTOR/S6K1-mediated enhanced translation efficiency of spliced mRNAs*. Cell, 2008. **133**(2): p. 303-313.
54. Pourdehnad, M., et al., *Myc and mTOR converge on a common node in protein synthesis control that confers synthetic lethality in Myc-driven cancers*. Proceedings of the National Academy of Sciences, 2013. **110**(29): p. 11988-11993.
55. Lin, C.-J., A. Malina, and J. Pelletier, *c-Myc and eIF4F constitute a feedforward loop that regulates cell growth: implications for anticancer therapy*. Cancer research, 2009. **69**(19): p. 7491-7494.
56. Lin, C.-J., et al., *Targeting synthetic lethal interactions between Myc and the eIF4F complex impedes tumorigenesis*. Cell reports, 2012. **1**(4): p. 325-333.
57. Gandin, V., et al., *Polysome fractionation and analysis of mammalian translatomes on a genome-wide scale*. Journal of visualized experiments: JoVE, 2014(87).
58. Ingolia, N.T., et al., *Genome-wide analysis in vivo of translation with nucleotide resolution using ribosome profiling*. science, 2009. **324**(5924): p. 218-223.
59. Kawai, T., et al., *Global mRNA stabilization preferentially linked to translational repression during the endoplasmic reticulum stress response*. Molecular and cellular biology, 2004. **24**(15): p. 6773-6787.
60. Thomas, J.D. and G.J. Johannes, *Identification of mRNAs that continue to associate with polysomes during hypoxia*. Rna, 2007.
61. Bushell, M., et al., *Polypyrimidine tract binding protein regulates IRES-mediated gene expression during apoptosis*. Molecular cell, 2006. **23**(3): p. 401-412.
62. Powley, I.R., et al., *Translational reprogramming following UVB irradiation is mediated by DNA-PKcs and allows selective recruitment to the polysomes of mRNAs encoding DNA repair enzymes*. Genes & development, 2009. **23**(10): p. 1207-1220.
63. Larsson, O., et al., *Distinct perturbation of the translatome by the antidiabetic drug metformin*. Proceedings of the National Academy of Sciences, 2012. **109**(23): p. 8977-8982.
64. Spangenberg, L., et al., *Polysome profiling shows extensive posttranscriptional regulation during human adipocyte stem cell differentiation into adipocytes*. Stem cell research, 2013. **11**(2): p. 902-912.
65. Cridge, A.G., et al., *Identifying eIF4E-binding protein translationally-controlled transcripts reveals links to mRNAs bound by specific PUF proteins*. Nucleic acids research, 2010. **38**(22): p. 8039-8050.
66. Carrascoso, I., C. Sánchez-Jiménez, and J.M. Izquierdo, *Genome-wide profiling reveals a role for T-cell intracellular antigens TIA1 and TIAR in the control of translational specificity in HeLa cells*. Biochemical Journal, 2014. **461**(1): p. 43-50.
67. Janas, M.M., et al., *Reduced expression of ribosomal proteins relieves microRNA-mediated repression*. Molecular cell, 2012. **46**(2): p. 171-186.

68. Archer, S.K., et al., *Dynamics of ribosome scanning and recycling revealed by translation complex profiling*. Nature, 2016. **535**(7613): p. 570.
69. Fritsch, C., et al., *Genome-wide search for novel human uORFs and N-terminal protein extensions using ribosomal footprinting*. Genome research, 2012: p. gr. 139568.112.
70. Lee, S., et al., *Global mapping of translation initiation sites in mammalian cells at single-nucleotide resolution*. Proceedings of the National Academy of Sciences, 2012. **109**(37): p. E2424-E2432.
71. Halbeisen, R.E., T. Scherrer, and A.P. Gerber, *Affinity purification of ribosomes to access the translatome*. Methods, 2009. **48**(3): p. 306-310.
72. Heiman, M., et al., *Cell type-specific mRNA purification by translating ribosome affinity purification (TRAP)*. Nature protocols, 2014. **9**(6): p. 1282.
73. Jan, C.H., C.C. Williams, and J.S. Weissman, *Principles of ER cotranslational translocation revealed by proximity-specific ribosome profiling*. Science, 2014. **346**(6210): p. 1257521.
74. Pianese, G., *Beitrag zur Histologie und Aetiologie des Carcinoms*. Vol. 1. 1896: G. Fischer.
75. Hein, N., et al., *The nucleolus: an emerging target for cancer therapy*. Trends in molecular medicine, 2013. **19**(11): p. 643-654.
76. Hannan, K., et al., *Dysregulation of RNA polymerase I transcription during disease*. Biochimica et Biophysica Acta (BBA)-Gene Regulatory Mechanisms, 2013. **1829**(3): p. 342-360.
77. Pelletier, J., G. Thomas, and S. Volarevic, *Ribosome biogenesis in cancer: new players and therapeutic avenues*. Nat Rev Cancer, 2018. **18**(1): p. 51-63.
78. Roeder, R.G. and W.J. Rutter, *Multiple forms of DNA-dependent RNA polymerase in eukaryotic organisms*. Nature, 1969. **224**(5216): p. 234.
79. Roeder, R.G., *The eukaryotic transcriptional machinery: complexities and mechanisms unforeseen*. Nature medicine, 2003. **9**(10): p. 1239.
80. Drygin, D., W.G. Rice, and I. Grummt, *The RNA polymerase I transcription machinery: an emerging target for the treatment of cancer*. Annual review of pharmacology and toxicology, 2010. **50**: p. 131-156.
81. Kusnadi, E.P., et al., *Regulation of rDNA transcription in response to growth factors, nutrients and energy*. Gene, 2015. **556**(1): p. 27-34.
82. Hannan, K.M., et al., *mTOR-dependent regulation of ribosomal gene transcription requires S6K1 and is mediated by phosphorylation of the carboxy-terminal activation domain of the nucleolar transcription factor UBF*. Molecular and cellular biology, 2003. **23**(23): p. 8862-8877.
83. Mayer, C., et al., *mTOR-dependent activation of the transcription factor TIF-IA links rRNA synthesis to nutrient availability*. Genes & development, 2004. **18**(4): p. 423-434.
84. Ma, X.M. and J. Blenis, *Molecular mechanisms of mTOR-mediated translational control*. Nature reviews Molecular cell biology, 2009. **10**(5): p. 307.
85. Fonseca, B.D., et al., *La-related protein 1 (LARP1) represses terminal oligopyrimidine (TOP) mRNA translation downstream of mTOR*

- complex 1 (mTORC1)*. Journal of Biological Chemistry, 2015: p. jbc.M114. 621730.
86. Tcherkezian, J., et al., *Proteomic analysis of cap-dependent translation identifies LARP1 as a key regulator of 5' TOP mRNA translation*. Genes & development, 2014. **28**(4): p. 357-371.
 87. Kantidakis, T., et al., *mTOR associates with TFIIIC, is found at tRNA and 5S rRNA genes, and targets their repressor Maf1*. Proceedings of the National Academy of Sciences, 2010. **107**(26): p. 11823-11828.
 88. Albiñ, A., J.I. Johnsen, and M.A. Henriksson, *MYC in oncogenesis and as a target for cancer therapies*, in *Advances in cancer research*. 2010, Elsevier. p. 163-224.
 89. Conacci-Sorrell, M., L. McFerrin, and R.N. Eisenman, *An overview of MYC and its interactome*. Cold Spring Harbor perspectives in medicine, 2014. **4**(1): p. a014357.
 90. Poortinga, G., L. Quinn, and R. Hannan, *Targeting RNA polymerase I to treat MYC-driven cancer*. Oncogene, 2015. **34**(4): p. 403.
 91. Stine, Z.E., et al., *MYC, metabolism, and cancer*. Cancer discovery, 2015.
 92. Venkataraman, S., et al., *Inhibition of BRD4 attenuates tumor cell self-renewal and suppresses stem cell signaling in MYC driven medulloblastoma*. Oncotarget, 2014. **5**(9): p. 2355.
 93. West, M.J., M. Stoneley, and A.E. Willis, *Translational induction of the c-myc oncogene via activation of the FRAP/TOR signalling pathway*. Oncogene, 1998. **17**(6): p. 769.
 94. Salghetti, S.E., S.Y. Kim, and W.P. Tansey, *Destruction of Myc by ubiquitin-mediated proteolysis: cancer-associated and transforming mutations stabilize Myc*. The EMBO journal, 1999. **18**(3): p. 717-726.
 95. Welcker, M., et al., *A nucleolar isoform of the Fbw7 ubiquitin ligase regulates c-Myc and cell size*. Current Biology, 2004. **14**(20): p. 1852-1857.
 96. Gregory, M.A. and S.R. Hann, *c-Myc proteolysis by the ubiquitin-proteasome pathway: stabilization of c-Myc in Burkitt's lymphoma cells*. Molecular and cellular biology, 2000. **20**(7): p. 2423-2435.
 97. Duesberg, P.H., K. Bister, and P.K. Vogt, *The RNA of avian acute leukemia virus MC29*. Proceedings of the National Academy of Sciences, 1977. **74**(10): p. 4320-4324.
 98. Sheiness, D., L. Fanshier, and J.M. Bishop, *Identification of nucleotide sequences which may encode the oncogenic capacity of avian retrovirus MC29*. Journal of virology, 1978. **28**(2): p. 600-610.
 99. Ellwood-Yen, K., et al., *Myc-driven murine prostate cancer shares molecular features with human prostate tumors*. Cancer cell, 2003. **4**(3): p. 223-238.
 100. Langdon, W.Y., et al., *The c-myc oncogene perturbs B lymphocyte development in Eμ-myc transgenic mice*. Cell, 1986. **47**(1): p. 11-18.
 101. Harris, A.W., et al., *The E μ-myc transgenic mouse. A model for high-incidence spontaneous lymphoma and leukemia of early B cells*. Journal of Experimental Medicine, 1988. **167**(2): p. 353-371.

102. Poortinga, G., et al., *MAD1 and c-MYC regulate UBF and rDNA transcription during granulocyte differentiation*. The EMBO journal, 2004. **23**(16): p. 3325-3335.
103. Poortinga, G., et al., *c-MYC coordinately regulates ribosomal gene chromatin remodeling and Pol I availability during granulocyte differentiation*. Nucleic acids research, 2010. **39**(8): p. 3267-3281.
104. Chan, J.C., et al., *AKT promotes rRNA synthesis and cooperates with c-MYC to stimulate ribosome biogenesis in cancer*. Sci. Signal., 2011. **4**(188): p. ra56-ra56.
105. Gomez-Roman, N., et al., *Direct activation of RNA polymerase III transcription by c-Myc*. Nature, 2003. **421**(6920): p. 290.
106. Zeller, K.I., et al., *Characterization of nucleophosmin (B23) as a Myc target by scanning chromatin immunoprecipitation*. Journal of Biological Chemistry, 2001. **276**(51): p. 48285-48291.
107. Felton-Edkins, Z.A., et al., *Direct regulation of RNA polymerase III transcription by RB, p53 and c-Myc*. Cell Cycle, 2003. **2**(3): p. 180-183.
108. Kenneth, N.S., et al., *TRRAP and GCN5 are used by c-Myc to activate RNA polymerase III transcription*. Proceedings of the National Academy of Sciences, 2007. **104**(38): p. 14917-14922.
109. Boisvert, F.-M., et al., *The multifunctional nucleolus*. Nature reviews Molecular cell biology, 2007. **8**(7): p. 574.
110. Sirri, V., et al., *Nucleolus: the fascinating nuclear body*. Histochemistry and cell biology, 2008. **129**(1): p. 13-31.
111. Hernandez-Verdun, D., et al., *The nucleolus: structure/function relationship in RNA metabolism*. Wiley Interdisciplinary Reviews: RNA, 2010. **1**(3): p. 415-431.
112. Leary, D.J. and S. Huang, *Regulation of ribosome biogenesis within the nucleolus*. FEBS letters, 2001. **509**(2): p. 145-150.
113. Derenzini, M., et al., *Distribution of silver-stained interphase nucleolar organizer regions as a parameter to distinguish neoplastic from nonneoplastic reactive cells in human effusions*. Acta cytologica, 1989. **33**(4): p. 491-498.
114. Levine, A.J., J. Momand, and C.A. Finlay, *The p53 tumour suppressor gene*. Nature, 1991. **351**(6326): p. 453.
115. Zuckerman, V., et al., *Tumour suppression by p53: the importance of apoptosis and cellular senescence*. The Journal of Pathology: A Journal of the Pathological Society of Great Britain and Ireland, 2009. **219**(1): p. 3-15.
116. Shieh, S.-Y., et al., *The human homologs of checkpoint kinases Chk1 and Cds1 (Chk2) phosphorylate p53 at multiple DNA damage-inducible sites*. Genes & development, 2000. **14**(3): p. 289-300.
117. Tibbetts, R.S., et al., *A role for ATR in the DNA damage-induced phosphorylation of p53*. Genes & development, 1999. **13**(2): p. 152-157.
118. Prives, C., *Signaling to p53: breaking the MDM2-p53 circuit*. Cell, 1998. **95**(1): p. 5-8.
119. Hollstein, M., et al., *p53 mutations in human cancers*. Science, 1991. **253**(5015): p. 49-53.

120. Muller, P.A. and K.H. Vousden, *Mutant p53 in cancer: new functions and therapeutic opportunities*. Cancer cell, 2014. **25**(3): p. 304-317.
121. Woods, S.J., et al., *The nucleolus as a fundamental regulator of the p53 response and a new target for cancer therapy*. Biochimica et Biophysica Acta (BBA)-Gene Regulatory Mechanisms, 2015. **1849**(7): p. 821-829.
122. Zhang, Y., et al., *Ribosomal protein L11 negatively regulates oncoprotein MDM2 and mediates a p53-dependent ribosomal-stress checkpoint pathway*. Molecular and cellular biology, 2003. **23**(23): p. 8902-8912.
123. Dai, M.-S. and H. Lu, *Inhibition of MDM2-mediated p53 ubiquitination and degradation by ribosomal protein L5*. Journal of Biological Chemistry, 2004. **279**(43): p. 44475-44482.
124. Dai, M.-S., et al., *Regulation of the MDM2-p53 pathway by ribosomal protein L11 involves a post-ubiquitination mechanism*. Journal of Biological Chemistry, 2006.
125. Quin, J., et al., *Inhibition of RNA polymerase I transcription initiation by CX-5461 activates non-canonical ATM/ATR signaling*. Oncotarget, 2016. **7**(31): p. 49800-49818.
126. Quin, J.E., et al., *Targeting the nucleolus for cancer intervention*. Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease, 2014. **1842**(6): p. 802-816.
127. Buttgereit, F. and M.D. Brand, *A hierarchy of ATP-consuming processes in mammalian cells*. Biochemical Journal, 1995. **312**(Pt 1): p. 163.
128. Rolfe, D. and G.C. Brown, *Cellular energy utilization and molecular origin of standard metabolic rate in mammals*. Physiological reviews, 1997. **77**(3): p. 731-758.
129. Hanahan, D. and R.A. Weinberg, *Hallmarks of cancer: the next generation*. cell, 2011. **144**(5): p. 646-674.
130. Altman, B.J. and C.V. Dang, *Normal and cancer cell metabolism: lymphocytes and lymphoma*. The FEBS journal, 2012. **279**(15): p. 2598-2609.
131. Thorens, B. and M. Mueckler, *Glucose transporters in the 21st Century*. American Journal of Physiology-Endocrinology and Metabolism, 2009. **298**(2): p. E141-E145.
132. Warburg, O., F. Wind, and E. Negelein, *The metabolism of tumors in the body*. The Journal of general physiology, 1927. **8**(6): p. 519.
133. Hicks, R.J., *Role of 18F-FDG PET in assessment of response in non-small cell lung cancer*. Journal of Nuclear Medicine, 2009.
134. MacManus, M.P., J.F. Seymour, and R.J. Hicks, *Overview of early response assessment in lymphoma with FDG-PET*. Cancer Imaging, 2007. **7**(1): p. 10-18.
135. Nicklin, P., et al., *Bidirectional transport of amino acids regulates mTOR and autophagy*. Cell, 2009. **136**(3): p. 521-534.
136. Raimundo, N., B.E. Baysal, and G.S. Shadel, *Revisiting the TCA cycle: signaling to tumor formation*. Trends in molecular medicine, 2011. **17**(11): p. 641-649.
137. Durán, R., et al., *HIF-independent role of prolyl hydroxylases in the cellular response to amino acids*. Oncogene, 2013. **32**(38): p. 4549.

138. Hardie, D.G., S.A. Hawley, and J.W. Scott, *AMP-activated protein kinase—development of the energy sensor concept*. The Journal of physiology, 2006. **574**(1): p. 7-15.
139. Hardie, D.G., F.A. Ross, and S.A. Hawley, *AMPK: a nutrient and energy sensor that maintains energy homeostasis*. Nature reviews Molecular cell biology, 2012. **13**(4): p. 251.
140. Kahn, B.B., et al., *AMP-activated protein kinase: ancient energy gauge provides clues to modern understanding of metabolism*. Cell metabolism, 2005. **1**(1): p. 15-25.
141. Shaw, R.J., et al., *The tumor suppressor LKB1 kinase directly activates AMP-activated kinase and regulates apoptosis in response to energy stress*. Proceedings of the National Academy of Sciences, 2004. **101**(10): p. 3329-3335.
142. Hardie, D., *AMPK: a key regulator of energy balance in the single cell and the whole organism*. International journal of obesity, 2008. **32**(S4): p. S7.
143. Alers, S., et al., *AMPK–mTOR–Ulk1/2 in the regulation of autophagy: crosstalk, shortcuts and feedbacks*. Molecular and cellular biology, 2011: p. MCB. 06159-11.
144. Dowling, R.J., et al., *Metformin inhibits mammalian target of rapamycin–dependent translation initiation in breast cancer cells*. Cancer research, 2007. **67**(22): p. 10804-10812.
145. Inoki, K., et al., *TSC2 integrates Wnt and energy signals via a coordinated phosphorylation by AMPK and GSK3 to regulate cell growth*. Cell, 2006. **126**(5): p. 955-968.
146. Gwinn, D.M., et al., *AMPK phosphorylation of raptor mediates a metabolic checkpoint*. Molecular cell, 2008. **30**(2): p. 214-226.
147. Li, Y., et al., *TSC2: filling the GAP in the mTOR signaling pathway*. Trends in biochemical sciences, 2004. **29**(1): p. 32-38.
148. Inoki, K., M.N. Corradetti, and K.-L. Guan, *Dysregulation of the TSC-mTOR pathway in human disease*. Nature genetics, 2005. **37**(1): p. 19.
149. Morrison, D.K., *The 14-3-3 proteins: integrators of diverse signaling cues that impact cell fate and cancer development*. Trends in cell biology, 2009. **19**(1): p. 16-23.
150. Hoppe, S., et al., *AMP-activated protein kinase adapts rRNA synthesis to cellular energy supply*. Proceedings of the National Academy of Sciences, 2009: p. pnas. 0909873106.
151. Murayama, A., et al., *Epigenetic control of rDNA loci in response to intracellular energy status*. Cell, 2008. **133**(4): p. 627-639.
152. Yang, L., et al., *Regulation of SirT1-NML binding by rRNA coordinates ribosome biogenesis with nutrient availability*. Molecular and cellular biology, 2013: p. MCB. 00476-13.
153. Morita, M., et al., *mTORC1 controls mitochondrial activity and biogenesis through 4E-BP-dependent translational regulation*. Cell metabolism, 2013. **18**(5): p. 698-711.
154. Morita, M., et al., *mTOR controls mitochondrial dynamics and cell survival via MTFP1*. Molecular cell, 2017. **67**(6): p. 922-935. e5.
155. Goo, C.K., et al., *PTEN/Akt signaling controls mitochondrial respiratory capacity through 4E-BP1*. PloS one, 2012. **7**(9): p. e45806.

156. Ben-Sahra, I., et al., *Stimulation of de novo pyrimidine synthesis by growth signaling through mTOR and S6K1*. Science, 2013: p. 1228792.
157. Robitaille, A.M., et al., *Quantitative phosphoproteomics reveal mTORC1 activates de novo pyrimidine synthesis*. Science, 2013. **339**(6125): p. 1320-1323.
158. Cunningham, J.T., et al., *Protein and nucleotide biosynthesis are coupled by a single rate-limiting enzyme, PRPS2, to drive cancer*. Cell, 2014. **157**(5): p. 1088-1103.
159. Porstmann, T., et al., *SREBP activity is regulated by mTORC1 and contributes to Akt-dependent cell growth*. Cell metabolism, 2008. **8**(3): p. 224-236.
160. Porstmann, T., et al., *A new player in the orchestra of cell growth: SREBP activity is regulated by mTORC1 and contributes to the regulation of cell and organ size*. 2009, Portland Press Limited.
161. Düvel, K., et al., *Activation of a metabolic gene regulatory network downstream of mTOR complex 1*. Molecular cell, 2010. **39**(2): p. 171-183.
162. Semenza, G.L., *HIF-1: upstream and downstream of cancer metabolism*. Current opinion in genetics & development, 2010. **20**(1): p. 51-56.
163. Csibi, A., et al., *The mTORC1 pathway stimulates glutamine metabolism and cell proliferation by repressing SIRT4*. Cell, 2013. **153**(4): p. 840-854.
164. Brown, E.J., et al., *A mammalian protein targeted by G1-arresting rapamycin–receptor complex*. Nature, 1994. **369**(6483): p. 756.
165. Sabatini, D.M., et al., *RAFT1: a mammalian protein that binds to FKBP12 in a rapamycin-dependent fashion and is homologous to yeast TORs*. Cell, 1994. **78**(1): p. 35-43.
166. Lamming, D.W., et al., *Rapalogs and mTOR inhibitors as anti-aging therapeutics*. The Journal of clinical investigation, 2013. **123**(3): p. 980-989.
167. Markman, B., V. Serra, and J. Tabernero, *mTOR Inhibition Beyond Rapalogs*, in *mTOR Inhibition for Cancer Therapy: Past, Present and Future*. 2016, Springer. p. 251-275.
168. Roldan-Romero, J.M., et al., *mTOR Pathway Mutations and Response to Rapalogs in RCC*. Clinical Cancer Research, 2017. **23**(17): p. 5320-5320.
169. Saxton, R.A. and D.M. Sabatini, *mTOR signaling in growth, metabolism, and disease*. Cell, 2017. **168**(6): p. 960-976.
170. Seront, E., et al., *Phase II study of dual phosphoinositol-3-kinase (PI 3K) and mammalian target of rapamycin (mTOR) inhibitor BEZ 235 in patients with locally advanced or metastatic transitional cell carcinoma*. BJU international, 2016. **118**(3): p. 408-415.
171. Thoreen, C.C., et al., *An ATP-competitive mTOR inhibitor reveals rapamycin-insensitive functions of mTORC1*. Journal of Biological Chemistry, 2009.
172. Feldman, M.E., et al., *Active-site inhibitors of mTOR target rapamycin-resistant outputs of mTORC1 and mTORC2*. PLoS biology, 2009. **7**(2): p. e1000038.

173. Alain, T., et al., *eIF4E/4E-BP ratio predicts the efficacy of mTOR targeted therapies*. Cancer research, 2012: p. canres. 2395.2012.
174. Alain, T., N. Sonenberg, and I. Topisirovic, *mTOR inhibitor efficacy is determined by the eIF4E/4E-BP ratio*. Oncotarget, 2012. **3**(12): p. 1491.
175. Carracedo, A., et al., *Inhibition of mTORC1 leads to MAPK pathway activation through a PI3K-dependent feedback loop in human cancer*. The Journal of clinical investigation, 2008. **118**(9): p. 3065-3074.
176. Ueda, T., et al., *Mnk2 and Mnk1 are essential for constitutive and inducible phosphorylation of eukaryotic initiation factor 4E but not for cell growth or development*. Molecular and cellular biology, 2004. **24**(15): p. 6539-6549.
177. Topisirovic, I., M. Ruiz-Gutierrez, and K.L. Borden, *Phosphorylation of the eukaryotic translation initiation factor eIF4E contributes to its transformation and mRNA transport activities*. Cancer research, 2004. **64**(23): p. 8639-8642.
178. Furic, L., et al., *eIF4E phosphorylation promotes tumorigenesis and is associated with prostate cancer progression*. Proceedings of the National Academy of Sciences, 2010. **107**(32): p. 14134-14139.
179. Konicek, B.W., et al., *Therapeutic inhibition of MAP kinase interacting kinase blocks eukaryotic initiation factor 4E phosphorylation and suppresses outgrowth of experimental lung metastases*. Cancer research, 2011: p. canres. 3298.2010.
180. Webster, K.R., et al., *eFT508, a potent and selective mitogen-activated protein kinase interacting kinase (MNK) 1 and 2 inhibitor, is efficacious in preclinical models of diffuse large B-cell lymphoma (DLBCL)*. Blood, 2015. **126**(23): p. 1554-1554.
181. Sharma, R.K., et al., *eFT508, a potent and highly selective inhibitor of MNK1 and MNK2, regulates T-cell differentiation promoting an antitumor immune response*. 2018, AACR.
182. Adesso, L., et al., *Gemcitabine triggers a pro-survival response in pancreatic cancer cells through activation of the MNK2/eIF4E pathway*. Oncogene, 2013. **32**(23): p. 2848.
183. Astanehe, A., et al., *MKNK1 is a YB-1 target gene responsible for imparting trastuzumab resistance and can be blocked by RSK inhibition*. Oncogene, 2012. **31**(41): p. 4434.
184. Sun, S.-Y., et al., *Activation of Akt and eIF4E survival pathways by rapamycin-mediated mammalian target of rapamycin inhibition*. Cancer research, 2005. **65**(16): p. 7052-7058.
185. Sale, M.J. and S.J. Cook, *Intrinsic and acquired resistance to MEK1/2 inhibitors in cancer*. 2014, Portland Press Limited.
186. Li, S., et al., *Treatment of breast and lung cancer cells with a N-7 benzyl guanosine monophosphate tryptamine phosphoramidate pronucleotide (4Ei-1) results in chemosensitization to gemcitabine and induced eIF4E proteasomal degradation*. Molecular pharmaceutics, 2013. **10**(2): p. 523-531.
187. Cencic, R., et al., *Blocking eIF4E: eIF4G interaction as a strategy to impair coronavirus replication*. Journal of virology, 2011: p. JVI. 00078-11.

188. Moerke, N.J., et al., *Small-molecule inhibition of the interaction between the translation initiation factors eIF4E and eIF4G*. Cell, 2007. **128**(2): p. 257-267.
189. Papadopoulos, E., et al., *Structure of the eukaryotic translation initiation factor eIF4E in complex with 4EGI-1 reveals an allosteric mechanism for dissociating eIF4G*. Proceedings of the National Academy of Sciences, 2014. **111**(31): p. E3187-E3195.
190. Descamps, G., et al., *The cap-translation inhibitor 4EGI-1 induces apoptosis in multiple myeloma through Noxa induction*. British journal of cancer, 2012. **106**(10): p. 1660.
191. Yi, T., et al., *4EGI-1 targets breast cancer stem cells by selective inhibition of translation that persists in CSC maintenance, proliferation and metastasis*. Oncotarget, 2014. **5**(15): p. 6028.
192. Willimott, S., et al., *Cap-translation inhibitor, 4EGI-1 restores sensitivity to ABT-737 apoptosis through cap-dependent and independent mechanisms in chronic lymphocytic leukaemia*. Clinical Cancer Research, 2013: p. clincanres. 2185.2012.
193. Chen, L., et al., *Tumor suppression by small molecule inhibitors of translation initiation*. Oncotarget, 2012. **3**(8): p. 869.
194. Cencic, R., et al., *Antitumor activity and mechanism of action of the cyclopenta [b] benzofuran, silvestrol*. PloS one, 2009. **4**(4): p. e5223.
195. Liu, T., et al., *Synthetic silvestrol analogues as potent and selective protein synthesis inhibitors*. Journal of medicinal chemistry, 2012. **55**(20): p. 8859-8878.
196. Bordeleau, M.-E., et al., *Stimulation of mammalian translation initiation factor eIF4A activity by a small molecule inhibitor of eukaryotic translation*. Proceedings of the National Academy of Sciences, 2005. **102**(30): p. 10460-10465.
197. Low, W.-K., et al., *Second-generation derivatives of the eukaryotic translation initiation inhibitor pateamine A targeting eIF4A as potential anticancer agents*. Bioorganic & medicinal chemistry, 2014. **22**(1): p. 116-125.
198. Kuznetsov, G., et al., *Potent in vitro and in vivo anticancer activities of des-methyl, des-amino pateamine A, a synthetic analogue of marine natural product pateamine A*. Molecular cancer therapeutics, 2009: p. 1535-7163. MCT-08-1026.
199. Tsumuraya, T., et al., *Effects of hippuristanol, an inhibitor of eIF4A, on adult T-cell leukemia*. Biochemical pharmacology, 2011. **81**(6): p. 713-722.
200. Iapalucci-Espinoza, S. and M.T. Franze-Fernández, *Effect of protein synthesis inhibitors and low concentrations of actinomycin D on ribosomal RNA synthesis*. FEBS letters, 1979. **107**(2): p. 281-284.
201. Huang, R., et al., *Upstream binding factor up-regulated in hepatocellular carcinoma is related to the survival and cisplatin-sensitivity of cancer cells*. The FASEB journal, 2002. **16**(3): p. 293-301.
202. Siddik, Z.H., *Cisplatin: mode of cytotoxic action and molecular basis of resistance*. Oncogene, 2003. **22**(47): p. 7265.
203. Papadopoulos, K., et al., *Pharmacokinetic findings from the phase I study of Quarfloxin (CX-3543): a protein-rDNA quadruplex inhibitor, in patients with advanced solid tumors*. 2007, AACR.

204. Drygin, D., et al., *Quarfloxin (CX-3543) disrupts the Nucleolin/rDNA quadruplex complexes, inhibits the elongation by RNA Polymerase I and exhibits potent antitumor activity in models of cancer*. 2008, AACR.
205. Lim, J., et al., *Abstract# 3599: Quarfloxin phase I clinical data and scientific findings supporting the selection of carcinoid/neuroendocrine tumors as the phase II indication*. 2009, AACR.
206. Drygin, D., et al., *Anticancer activity of CX-3543: a direct inhibitor of rRNA biogenesis*. *Cancer research*, 2009. **69**(19): p. 7653-7661.
207. Colis, L., et al., *DNA intercalator BMH-21 inhibits RNA polymerase I independent of DNA damage response*. *Oncotarget*, 2014. **5**(12): p. 4361.
208. Drygin, D., et al., *Targeting RNA polymerase I with an oral small molecule CX-5461 inhibits ribosomal RNA synthesis and solid tumor growth*. *Cancer research*, 2010: p. canres. 1728.2010.
209. Hilton, J., et al., *44O CCTG IND. 231: A phase 1 trial evaluating CX-5461 in patients with advanced solid tumors*. *Annals of Oncology*, 2018. **29**(suppl_3): p. mdy048. 003.
210. Khot, A., et al., *RNA Polymerase 1 Transcription Inhibitor CX-5461 in Patients with Advanced Hematologic Malignancies: Results of a Phase I First in Human Study*. 2017, Am Soc Hematology.
211. Hein, N., et al., *Inhibition of Pol I transcription treats murine and human AML by targeting the leukemia-initiating cell population*. *Blood*, 2017. **129**(21): p. 2882-2895.
212. Quin, J., et al., *Inhibition of RNA polymerase I transcription initiation by CX-5461 activates non-canonical ATM/ATR signaling*. *Oncotarget*, 2016. **7**(31): p. 49800.
213. Zhao, W., et al., *Comparison of RNA-Seq by poly (A) capture, ribosomal RNA depletion, and DNA microarray for expression profiling*. *BMC genomics*, 2014. **15**(1): p. 419.
214. Oertlin, C., et al., *Generally applicable transcriptome-wide analysis of translational efficiency using anota2seq*. *bioRxiv*, 2018: p. 106922.
215. Ritchie, M.E., et al., *limma powers differential expression analyses for RNA-sequencing and microarray studies*. *Nucleic Acids Res*, 2015. **43**(7): p. e47.
216. Liang, S., et al., *Polysome-profiling in small tissue samples*. *Nucleic acids research*, 2017. **46**(1): p. e3-e3.
217. Khajuria, R.K., et al., *Ribosome Levels Selectively Regulate Translation and Lineage Commitment in Human Hematopoiesis*. *Cell*, 2018. **173**(1): p. 90-103.e19.
218. Pyke, J.S., et al., *A tandem liquid chromatography–mass spectrometry (LC–MS) method for profiling small molecules in complex samples*. *Metabolomics*, 2015. **11**(6): p. 1552-1562.
219. Spandidos, A., et al., *PrimerBank: a resource of human and mouse PCR primer pairs for gene expression detection and quantification*. *Nucleic acids research*, 2009. **38**(suppl_1): p. D792-D799.
220. Fumagalli, S., et al., *Absence of nucleolar disruption after impairment of 40S ribosome biogenesis reveals an rpL11-translation-dependent mechanism of p53 induction*. *Nature cell biology*, 2009. **11**(4): p. 501.

221. Felfly, H. and O.D. Klein, *Sprouty genes regulate proliferation and survival of human embryonic stem cells*. Scientific reports, 2013. **3**: p. 2277.
222. Gandin, V., et al., *nanoCAGE reveals 5'UTR features that define specific modes of translation of functionally related MTOR-sensitive mRNAs*. Genome research, 2016: p. gr. 197566.115.
223. Larsson, O., N. Sonenberg, and R. Nadon, *anota: Analysis of differential translation in genome-wide studies*. Bioinformatics, 2011. **27**(10): p. 1440-1441.
224. Klein, D.J., P.B. Moore, and T.A. Steitz, *The contribution of metal ions to the structural stability of the large ribosomal subunit*. Rna, 2004. **10**(9): p. 1366-1379.
225. Nierhaus, K.H., *Mg²⁺, K⁺, and the ribosome*. Journal of bacteriology, 2014. **196**(22): p. 3817-3819.
226. Kang, J., et al., *Amino acid-dependent signaling via S6K1 and MYC is essential for regulation of rDNA transcription*. Oncotarget, 2016. **7**(31): p. 48887.
227. Gingras, A.-C., et al., *Regulation of 4E-BP1 phosphorylation: a novel two-step mechanism*. Genes & development, 1999. **13**(11): p. 1422-1437.
228. Charbonneau, H., et al., *The leukocyte common antigen (CD45): a putative receptor-linked protein tyrosine phosphatase*. Proceedings of the National Academy of Sciences, 1988. **85**(19): p. 7182-7186.
229. Berger, S., et al., *Characterization of interferon messenger RNA synthesis in Namalva cells*. Journal of Biological Chemistry, 1980. **255**(7): p. 2955-2961.
230. Martin, C.A., et al., *Palbociclib synergizes with BRAF and MEK inhibitors in treatment naïve melanoma but not after the development of BRAF inhibitor resistance*. International Journal of Cancer, 2018. **142**(10): p. 2139-2152.
231. Crescenzi, E., et al., *NF-κB-dependent cytokine secretion controls Fas expression on chemotherapy-induced premature senescent tumor cells*. Oncogene, 2011. **30**(24): p. 2707.
232. Rebello, R.J., et al., *The dual inhibition of RNA Pol I transcription and PIM kinase as a new therapeutic approach to treat advanced prostate cancer*. Clinical Cancer Research, 2016: p. clincanres. 0124.2016.
233. Field, J., et al., *Elevated expression of the c-myc oncoprotein correlates with poor prognosis in head and neck squamous cell carcinoma*. Oncogene, 1989. **4**(12): p. 1463-1468.
234. Pagnano, K.B., et al., *p53, Mdm2, and c-Myc overexpression is associated with a poor prognosis in aggressive non-Hodgkin's lymphomas*. American journal of hematology, 2001. **67**(2): p. 84-92.
235. Berg, T., et al., *Small-molecule antagonists of Myc/Max dimerization inhibit Myc-induced transformation of chicken embryo fibroblasts*. Proceedings of the National Academy of Sciences, 2002. **99**(6): p. 3830-3835.
236. Kiessling, A., et al., *Selective inhibition of c-Myc/Max dimerization and DNA binding by small molecules*. Chemistry & biology, 2006. **13**(7): p. 745-751.

237. Wang, L., et al., *BRD4 inhibitor IBET upregulates p27kip/cip protein stability in neuroendocrine tumor cells*. Cancer biology & therapy, 2017. **18**(4): p. 229-236.
238. Nilsson, J.A., et al., *Targeting ornithine decarboxylase in Myc-induced lymphomagenesis prevents tumor formation*. Cancer cell, 2005. **7**(5): p. 433-444.
239. Toyoshima, M., et al., *Functional genomics identifies therapeutic targets for MYC-driven cancer*. Proceedings of the National Academy of Sciences, 2012. **109**(24): p. 9545-9550.
240. Li, L., et al., *CX-5461 induces autophagy and inhibits tumor growth via mammalian target of rapamycin-related signaling pathways in osteosarcoma*. OncoTargets and therapy, 2016. **9**: p. 5985.
241. Lee, H.C., et al., *RNA polymerase I inhibition with CX-5461 as a novel therapeutic strategy to target MYC in multiple myeloma*. British journal of haematology, 2017. **177**(1): p. 80-94.
242. Emery, C.M., et al., *MEK1 mutations confer resistance to MEK and B-RAF inhibition*. Proceedings of the National Academy of Sciences, 2009. **106**(48): p. 20411-20416.
243. Nahta, R., et al., *Mechanisms of disease: understanding resistance to HER2-targeted therapy in human breast cancer*. Nature Reviews Clinical Oncology, 2006. **3**(5): p. 269.
244. Holohan, C., et al., *Cancer drug resistance: an evolving paradigm*. Nature Reviews Cancer, 2013. **13**(10): p. 714.
245. Li, T.-K. and L.F. Liu, *Tumor cell death induced by topoisomerase-targeting drugs*. Annual review of pharmacology and toxicology, 2001. **41**(1): p. 53-77.
246. Chhatiwala, H., N. Jafri, and R. Salgia, *A review of topoisomerase inhibition in lung cancer*. Cancer biology & therapy, 2006. **5**(12): p. 1600-1607.
247. Negi, S.S. and P. Brown, *rRNA synthesis inhibitor, CX-5461, activates ATM/ATR pathway in acute lymphoblastic leukemia, arrests cells in G2 phase and induces apoptosis*. Oncotarget, 2015. **6**(20): p. 18094.
248. König, J., et al., *Conjugate export pumps of the multidrug resistance protein (MRP) family: localization, substrate specificity, and MRP2-mediated drug resistance*. Biochimica et Biophysica Acta (BBA)-Biomembranes, 1999. **1461**(2): p. 377-394.
249. Cui, Y., et al., *Drug resistance and ATP-dependent conjugate transport mediated by the apical multidrug resistance protein, MRP2, permanently expressed in human and canine cells*. Molecular pharmacology, 1999. **55**(5): p. 929-937.
250. Glavinas, H., et al., *The role of ABC transporters in drug resistance, metabolism and toxicity*. Current drug delivery, 2004. **1**(1): p. 27-42.
251. DAMESHEK, W., *Riddle: what do aplastic anemia, paroxysmal nocturnal hemoglobinuria (PNH) and "hypoplastic" leukemia have in common?* Blood, 1967. **30**(2): p. 251-254.
252. Barlow, J.L., et al., *New insights into 5q-syndrome as a ribosomopathy*. Cell Cycle, 2010. **9**(21): p. 4286-4293.
253. De Keersmaecker, K., *A novel mouse model provides insights into the neutropenia associated with the ribosomopathy Shwachman-Diamond syndrome*. 2015, Haematologica.

254. De Keersmaecker, K., S.O. Sulima, and J.D. Dinman, *Ribosomopathies and the paradox of cellular hypo-to hyperproliferation*. Blood, 2015: p. blood-2014-10-569616.
255. Garcia, M. and C.-C. Chen, *The bone marrow microenvironment—driver of leukemia evolution?* Stem cell investigation, 2017. **4**.
256. Vlachos, A., et al., *The incidence of neoplasia in Diamond-Blackfan anemia: a report from the Diamond-Blackfan Anemia Registry*. Blood, 2012: p. blood-2011-08-375972.
257. Chiabrando, D. and E. Tolosano, *Diamond blackfan anemia at the crossroad between ribosome biogenesis and heme metabolism*. Advances in hematology, 2010. **2010**.
258. Pellagatti, A., et al., *Haploinsufficiency of RPS14 in 5q- syndrome is associated with deregulation of ribosomal-and translation-related genes*. British journal of haematology, 2008. **142**(1): p. 57-64.
259. Trainor, P.A., J. Dixon, and M.J. Dixon, *Treacher Collins syndrome: etiology, pathogenesis and prevention*. European Journal of human genetics, 2009. **17**(3): p. 275.
260. Valdez, B.C., et al., *The Treacher Collins syndrome (TCOF1) gene product is involved in ribosomal DNA gene transcription by interacting with upstream binding factor*. Proceedings of the National Academy of Sciences, 2004. **101**(29): p. 10709-10714.
261. Yoon, A., et al., *Impaired control of IRES-mediated translation in X-linked dyskeratosis congenita*. Science, 2006. **312**(5775): p. 902-906.
262. Ganapathi, K.A., et al., *The human Shwachman-Diamond syndrome protein, SBDS, associates with ribosomal RNA*. Blood, 2007. **110**(5): p. 1458-1465.
263. Kandoth, C., et al., *Mutational landscape and significance across 12 major cancer types*. Nature, 2013. **502**(7471): p. 333.
264. Novetsky, A.P., et al., *Frequent mutations in the RPL22 gene and its clinical and functional implications*. Gynecologic oncology, 2013. **128**(3): p. 470-474.
265. De Keersmaecker, K., et al., *Exome sequencing identifies mutation in CNOT3 and ribosomal genes RPL5 and RPL10 in T-cell acute lymphoblastic leukemia*. Nature genetics, 2013. **45**(2): p. 186.
266. Osthus, R.C., et al., *Deregulation of glucose transporter 1 and glycolytic gene expression by c-Myc*. Journal of Biological Chemistry, 2000. **275**(29): p. 21797-21800.
267. Ahuja, P., et al., *Myc controls transcriptional regulation of cardiac metabolism and mitochondrial biogenesis in response to pathological stress in mice*. The Journal of clinical investigation, 2010. **120**(5): p. 1494-1505.
268. Gao, P., et al., *c-Myc suppression of miR-23a/b enhances mitochondrial glutaminase expression and glutamine metabolism*. Nature, 2009. **458**(7239): p. 762.
269. Kim, J., J.-h. Lee, and V.R. Iyer, *Global identification of Myc target genes reveals its direct role in mitochondrial biogenesis and its E-box usage in vivo*. PloS one, 2008. **3**(3): p. e1798.
270. Li, F., et al., *Myc stimulates nuclearly encoded mitochondrial genes and mitochondrial biogenesis*. Molecular and cellular biology, 2005. **25**(14): p. 6225-6234.

271. Lai, S.-W., et al., *Risk of hepatocellular carcinoma in diabetic patients and risk reduction associated with anti-diabetic therapy: a population-based cohort study*. The American journal of gastroenterology, 2012. **107**(1): p. 46.
272. Jiralerspong, S., et al., *Metformin and pathologic complete responses to neoadjuvant chemotherapy in diabetic patients with breast cancer*. Journal of clinical oncology, 2009. **27**(20): p. 3297.
273. Chlebowski, R.T., et al., *Diabetes, metformin, and breast cancer in postmenopausal women*. Journal of Clinical Oncology, 2012. **30**(23): p. 2844.
274. Sadeghi, N., et al., *Metformin use is associated with better survival of diabetic patients with pancreatic cancer*. Clinical cancer research, 2012.
275. Lee, G.E., et al., *Examining the effects of metformin on survival outcome in stage II/III colorectal cancer patients with diabetes mellitus*. 2012, American Society of Clinical Oncology.
276. Dell'Aniello, S., et al., *Metformin and the incidence of prostate cancer in patients with type 2 diabetes*. Cancer Epidemiology and Prevention Biomarkers, 2010: p. cebp. 0940.2010.
277. Wheaton, W.W., et al., *Metformin inhibits mitochondrial complex I of cancer cells to reduce tumorigenesis*. Elife, 2014. **3**: p. e02242.
278. Luengo, A., L.B. Sullivan, and M.G. Vander Heiden, *Understanding the complex-I-ty of metformin action: limiting mitochondrial respiration to improve cancer therapy*. BMC biology, 2014. **12**(1): p. 82.
279. Foretz, M., et al., *Metformin: from mechanisms of action to therapies*. Cell metabolism, 2014. **20**(6): p. 953-966.
280. Wu, L., et al., *An ancient, unified mechanism for metformin growth inhibition in C. elegans and cancer*. Cell, 2016. **167**(7): p. 1705-1718. e13.
281. Howell, J.J., et al., *Metformin inhibits hepatic mTORC1 signaling via dose-dependent mechanisms involving AMPK and the TSC complex*. Cell metabolism, 2017. **25**(2): p. 463-471.
282. Miller, D.R., *A tribute to Sidney Farber—the father of modern chemotherapy*. British journal of haematology, 2006. **134**(1): p. 20-26.
283. Tedeschi, P.M., et al., *Contribution of serine, folate and glycine metabolism to the ATP, NADPH and purine requirements of cancer cells*. Cell death & disease, 2013. **4**(10): p. e877.
284. Vazquez, A., P.M. Tedeschi, and J.R. Bertino, *Overexpression of the mitochondrial folate and glycine–serine pathway: a new determinant of methotrexate selectivity in tumors*. Cancer research, 2012.
285. Xiang, Y., et al., *Targeted inhibition of tumor-specific glutaminase diminishes cell-autonomous tumorigenesis*. The Journal of clinical investigation, 2015. **125**(6): p. 2293-2306.
286. Brondfield, S., et al., *Direct and indirect targeting of MYC to treat acute myeloid leukemia*. Cancer chemotherapy and pharmacology, 2015. **76**(1): p. 35-46.
287. Wu, C., et al., *BioGPS: building your own mash-up of gene annotations and expression profiles*. Nucleic acids research, 2015. **44**(D1): p. D313-D316.

288. Pang, B., et al., *EZH2 promotes metabolic reprogramming in glioblastomas through epigenetic repression of EAF2-HIF1 α signaling*. *Oncotarget*, 2016. **7**(29): p. 45134.
289. Eikawa, S., et al., *Immune-mediated antitumor effect by type 2 diabetes drug, metformin*. *Proceedings of the National Academy of Sciences*, 2015. **112**(6): p. 1809-1814.
290. Hirsch, H.A., D. Iliopoulos, and K. Struhl, *Metformin inhibits the inflammatory response associated with cellular transformation and cancer stem cell growth*. *Proceedings of the National Academy of Sciences*, 2013. **110**(3): p. 972-977.
291. Hettmann, T. and J.M. Leiden, *NF- κ B is required for the positive selection of CD8 $^{+}$ thymocytes*. *The Journal of Immunology*, 2000. **165**(9): p. 5004-5010.
292. Jimi, E., et al., *Differential role of the transcription factor NF- κ B in selection and survival of CD4 $^{+}$ and CD8 $^{+}$ thymocytes*. *Immunity*, 2008. **29**(4): p. 523-537.
293. Shaked, H., M. Guma, and M. Karin, *Analysis of NF- κ B activation in mouse intestinal epithelial cells*, in *NF-kappa B*. 2015, Springer. p. 593-606.
294. Zippo, A., et al., *PIM1-dependent phosphorylation of histone H3 at serine 10 is required for MYC-dependent transcriptional activation and oncogenic transformation*. *Nature cell biology*, 2007. **9**(8): p. 932.
295. Cibull, T., et al., *Overexpression of Pim-1 during progression of prostatic adenocarcinoma*. *Journal of clinical pathology*, 2006. **59**(3): p. 285-288.
296. Wang, J., et al., *Pim1 kinase synergizes with c-MYC to induce advanced prostate carcinoma*. *Oncogene*, 2010. **29**(17): p. 2477.
297. Grandoch, M., et al., *Epac inhibits apoptosis of human leukocytes*. *Journal of leukocyte biology*, 2009. **86**(4): p. 847-849.
298. Krakstad, C., A.E. Christensen, and S.O. Døskeland, *cAMP protects neutrophils against TNF- α -induced apoptosis by activation of cAMP-dependent protein kinase, independently of exchange protein directly activated by cAMP (Epac)*. *Journal of leukocyte biology*, 2004. **76**(3): p. 641-647.
299. Sinclair, E.M., et al., *Glucagon receptor signaling is essential for control of murine hepatocyte survival*. *Gastroenterology*, 2008. **135**(6): p. 2096-2106.
300. Insel, P.A., et al., *Cyclic AMP is both a pro-apoptotic and anti-apoptotic second messenger*. *Acta physiologica*, 2012. **204**(2): p. 277-287.
301. Youle, R.J. and A. Strasser, *The BCL-2 protein family: opposing activities that mediate cell death*. *Nature reviews Molecular cell biology*, 2008. **9**(1): p. 47.
302. Bos, J.L., J. de Rooij, and K.A. Reedquist, *Rap1 signalling: adhering to new models*. *Nature reviews Molecular cell biology*, 2001. **2**(5): p. 369.
303. Cahill, M.E., et al., *Bidirectional synaptic structural plasticity after chronic cocaine administration occurs through Rap1 small GTPase signaling*. *Neuron*, 2016. **89**(3): p. 566-582.

304. Bouchard, C., et al., *Myc-induced proliferation and transformation require Akt-mediated phosphorylation of FoxO proteins*. The EMBO journal, 2004. **23**(14): p. 2830-2840.

Appendices

Appendix 1:

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Review

Regulation of rDNA transcription in response to growth factors, nutrients and energy



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ABSTRACT

Exquisite control of ribosome biogenesis is fundamental for the maintenance of cellular growth and proliferation. Importantly, synthesis of ribosomal RNA by RNA polymerase I is a key regulatory step in ribosome biogenesis and a major biosynthetic and energy consuming process. Consequently, ribosomal RNA gene transcription is tightly coupled to the availability of growth factors, nutrients and energy. Thus cells have developed an intricate sensing network to monitor the cellular environment and modulate ribosomal DNA transcription accordingly. Critical controllers in these sensing networks, which mediate growth factor activation of ribosomal DNA transcription, include the PI3K/AKT/mTORC1, RAS/RAF/ERK pathways and MYC transcription factor. mTORC1 also responds to amino acids and energy status, making it a key hub linking all three stimuli to the regulation of ribosomal DNA transcription, although this is achieved via overlapping and distinct mechanisms. This review outlines the current knowledge of how cells respond to environmental cues to control ribosomal RNA synthesis. We also highlight the critical points within this network that are providing new therapeutic opportunities for treating cancers through modulation of RNA polymerase I activity and potential novel imaging strategies.

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Abbreviations: 4E-BP1, eukaryotic translation initiation factor 4E-binding protein 1; ADP, adenosine diphosphate; AMP, adenosine monophosphate; AMPK, AMP-dependent protein kinase; ATP, adenosine triphosphate; Deptor, Dep-domain mTOR interacting protein; DON, 6-diazo-5-oxo-L-norleucine; eNoSC, energy-dependent nucleolar silencing complex; ERK, extracellular signal-regulated kinase; GAP, GTPase activating protein; GDH, glutamate dehydrogenase; GDP, guanosine diphosphate; GLS, glutaminase; GTP, guanosine-5'-triphosphate; LeuRS, leucyl-tRNA synthetase; mLS8, mammalian lethal with sec-13 protein 8; mTORC1, mammalian target of rapamycin complex 1; PI3K, phosphoinositide 3-kinase; PIC, pre-initiation complex; Pol I, RNA polymerase I; Pol II, RNA polymerase II; Pol III, RNA polymerase III; PRAS40, proline-rich AKT substrate 40 kDa; Raptor, regulatory-associated protein of mTOR; Rheb, Ras homolog enriched in brain; RPs, ribosomal proteins; rRNA, ribosomal RNA; S6K1, ribosomal protein S6 kinase 1; Ser, serine; SIRT1, sirtuin 1; SL-1, selectivity factor 1; SLC, solute carrier family; TCA, tricarboxylic acid; tRNA, transfer RNA; TSC, tuberous sclerosis complex; TTT-RUVBL1/2, Tel2-Tti1-Tti2-RuvB-like 1 and 2; UBF, upstream binding factor; v-ATPase, vacuolar H⁺-ATPase; αKG, α-ketoglutarate.

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1. Introduction

Mammalian cells modify their growth and proliferation rate in response to a diverse range of environmental cues, including the availability of growth factors, nutrients and energy. This response is critical for the maintenance of intracellular and organismal homeostasis, and is achieved by an integrated network of signaling pathways (reviewed in Zinzalla and Hall, 2008; Howell and Manning, 2011; Kim et al., 2013a; Shimobayashi and Hall, 2014).

Cellular proliferation is critically dependent on the synthesis of macromolecules including proteins, nucleotides and lipids, which are required for all aspects of cellular growth. As such, cell growth and proliferation are tightly coupled to the rate of ribosome production and thus protein synthesis (reviewed in Grummt, 2003). Ribosome biogenesis is a complex process that requires the synthesis and processing of ribosomal RNA (rRNA), synthesis of ribosomal proteins (RPs) and their subsequent assembly into ribosomal subunits; which can account for up to 80% of the energy consumption of rapidly proliferating cells (Schmidt, 1999) and as such requires exquisite regulation. Furthermore, increased ribosome biogenesis is an invariant characteristic of transformed cells (reviewed in Ruggero and Pandolfi, 2003; Bywater et al., 2013) and is achieved by increased signaling through pathways

downstream of growth factors, nutrients (amino acids) and energy (glucose), as well as oncogenes and tumor suppressors. Targeting ribosome biogenesis directly at the level of rRNA synthesis (Drygin et al., 2011; Bywater et al., 2012; Peltonen et al., 2014a,2014b) or by inhibiting upstream pathways (Chan et al., 2011; Devlin et al., 2013; Wall et al., 2013) are promising new approaches for treating cancer (reviewed in Bywater et al., 2013; Hein et al., 2013; Drygin et al., 2014; Poortinga et al., 2014; Quin et al., 2014).

This review will focus on the current knowledge of how both normal and cancer cells respond to changes in the abundance of growth factors, nutrients and energy to control rRNA synthesis by RNA polymerase I (Pol I). We will also highlight the critical points within this regulatory network that are providing new therapeutic opportunities for treating cancers characterized by dysregulated cell growth and potential novel imaging strategies.

2. Ribosome biogenesis

There are approximately 300 copies of the rRNA gene per haploid genome distributed in repeated arrays among 5 acrocentric human chromosomes (Henderson et al., 1972; Stults et al., 2008). Transcription of ribosomal DNA (rDNA) by Pol I in the nucleolus generates the 47S pre-rRNA that is rapidly processed to the 18S, 5.8S and 28S mature rRNAs (reviewed in Moss et al., 2007). RNA polymerase III (Pol III) synthesizes small structural RNAs such as 5S rRNA and transfer RNA (tRNA) in the nucleoplasm (reviewed in Moir and Willis, 2013). The RPs and numerous other proteins required for transcription, processing, modification and organization of the ribosome and its components are all transcribed by RNA polymerase II (Pol II) in the nucleoplasm. These proteins are synthesized in the cytoplasm, then exported to the nucleolus and assembled with the rRNAs to form the 40S and 60S ribosomal subunits (reviewed in Panse and Johnson, 2010). Thus successful ribosome biogenesis requires the coordinated effort of all three RNA polymerases and is regulated at many levels. A major regulatory point in this process is rDNA transcription by Pol I, which comprises a series of coordinated steps including transcription initiation, promoter escape, elongation and termination (reviewed in Russell and Zomerijk, 2005; Schneider, 2012) and forms the focus of this review. To a large extent the regulation of rDNA transcription is achieved via signaling pathways modulating the expression and activities of Pol I-specific transcription factors, particularly RRN3 (also known as transcription initiation factor 1A, TIF-1A), selectivity factor 1 (SL-1; also known as TIF-1B) and upstream binding factor (UBF) (reviewed in Grummt, 2010; Bywater et al., 2013). In the evolving model of Pol I pre-initiation complex (PIC) assembly, the initial binding of UBF to the rDNA promoter leads to recruitment of the SL-1 complex. The resultant UBF/SL-1 complex facilitates binding of an initiation-competent subpopulation of Pol I which is associated with RRN3 and RRN3 mediates the interaction between Pol I and SL-1, to form the PIC at the rDNA promoter (reviewed in Diesch et al., 2014). In addition to transcription initiation, UBF also plays a multifunctional role in rDNA transcription elongation and chromatin remodeling (V. Stefanovsky et al., 2006; Sanij et al., 2008).

3. Regulation of rDNA transcription by growth factors

It is well established, in numerous model systems, that the availability of growth factors acutely regulates Pol I transcription and thus ribosome synthesis. This regulation is, in a large part, due to growth factor-dependent signaling via the phosphoinositide 3-kinase (PI3K)/AKT/mammalian target of rapamycin complex 1 (mTORC1) or RAS/RAF/extracellular signal-regulated kinase (ERK) pathways (Fig. 1). These pathways also form an intricate control network with the transcription factor, MYC, to impact on the functions of Pol I and its specific transcription factors, which has been the subject of numerous recent reviews (reviewed in Grummt, 2010; Hannan et al., 2011; Iadevaia et al., 2014; Poortinga et al., 2014).

A critical role of RAS/RAF/ERK signaling in regulating rDNA transcription has been established via ERK phosphorylation of RRN3 and UBF. Specifically, ERK phosphorylation of RRN3 at Serine (Ser)649 and Ser633 on the C-terminus promoted transcription initiation (Zhao, 2003), and phosphorylation of UBF in the two N-terminal high mobility group (HMG) boxes increased the Pol I transcription elongation rate by unfolding the enhancosome in the rDNA, a nucleosome-like structure associated with UBF (Stefanovsky et al., 2001; V. Stefanovsky et al., 2006; V.Y. Stefanovsky et al., 2006; Stefanovsky and Moss, 2008).

Growth factor dependent activation of PI3K/AKT/mTORC1 also regulates rDNA transcription via RRN3 and UBF. mTORC1 contains the evolutionary conserved serine/threonine kinase mTOR, the scaffolding protein regulatory-associated protein of mTOR (Raptor), a positive regulator mammalian lethal with sec-13 protein 8 (mLST8), and the inhibitory subunits Dep-domain mTOR interacting protein (Deptor) plus proline-rich AKT substrate 40 kDa (PRAS40) (reviewed in Shimobayashi and Hall, 2014). Activation of mTORC1 by growth factors is transmitted through the tuberous sclerosis complex (TSC)–Ras homolog enriched in brain (Rheb) axis. Growth factors promote phosphorylation and inactivation of TSC2, which exists in a complex with TSC1 and Tre2–Bub2–Cdc16 (TBC) 1 domain family member 7 (TBC1D7) (Dibble et al., 2012). The TSC complex inhibits mTORC1 through its GTPase activating protein (GAP) activity on Rheb, a potent activator of mTORC1 (Inoki et al., 2003a; Tee et al., 2003). Inhibitory phosphorylation of TSC2 upon growth factor stimulation promotes guanosine-5'-triphosphate (GTP)-loading of Rheb, consequently activating mTORC1. mTORC1 putatively activates RRN3 by increasing phosphorylation at Ser44 and reducing phosphorylation at Ser199, which promotes formation of the Pol I PIC at the promoter of rDNA (Mayer et al., 2004). Moreover, mTORC1 via ribosomal protein S6 kinase 1 (S6K1) regulates the activity of UBF by promoting phosphorylation of its C-terminal activation domain (Hannan et al., 2003). Inhibition of mTORC1 with rapamycin, leads to a reduced interaction between UBF and SL-1 and thus decreased Pol I activity (Hannan et al., 2003). The serine/threonine kinase, AKT upregulates rDNA transcription at multiple steps, including Pol I loading on the rDNA promoter, transcription elongation, and rRNA processing, through both mTORC1-dependent and independent mechanisms (Chan et al., 2011).

The other key regulator of ribosome biogenesis and cell growth is the transcription factor MYC. Elevated expression of MYC is sufficient to up-regulate Pol I transcription by a number of mechanisms. MYC stimulates the Pol II-dependent transcription of a cohort of factors associated with Pol I transcription (termed “Pol I regulon”), which includes the core factors required for Pol I PIC formation and transcription, for example, UBF, RRN3 and Pol I subunits (Poortinga et al., 2004; Grewal et al., 2005; Poortinga et al., 2011; reviewed in Poortinga et al., 2014). Moreover, MYC binds to the rDNA promoter region and activates Pol I transcription through remodeling rDNA chromatin structure, and/or directly interacting with SL-1, ultimately promoting the stabilization of the UBF/SL-1 complex and thus recruitment of Pol I (Arabi et al., 2005; Grandori et al., 2005; Shiue et al., 2009). In addition, ERK phosphorylation of MYC increases MYC stability (Sears et al., 1999), while mTORC1 signaling, via the eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1), promotes MYC translation (De Benedetti et al., 1994; West et al., 1998; Wall et al., 2008; Csibi et al., 2014) thus enabling further upregulation of Pol I transcription.

It has become apparent that the PI3K/AKT/mTORC1 and RAS/RAF/ERK pathways, in association with MYC, form a super-control network for ribosome biogenesis. As described above, multiple members of the MYC-regulated Pol I regulon are phosphorylated/activated downstream of PI3K and RAS signaling resulting in stimulation of rDNA transcription. Furthermore, mTORC1 signaling and MYC can impact on the expression of the other ribosomal component, the RPs (Kim et al., 2000; Thoreen et al., 2012). Moreover, S6K1 was shown to regulate the transcription of many key ribosome biogenesis factors (Chauvin et al., 2013). Critically, AKT and MYC cooperate to drive higher rates of rRNA synthesis,

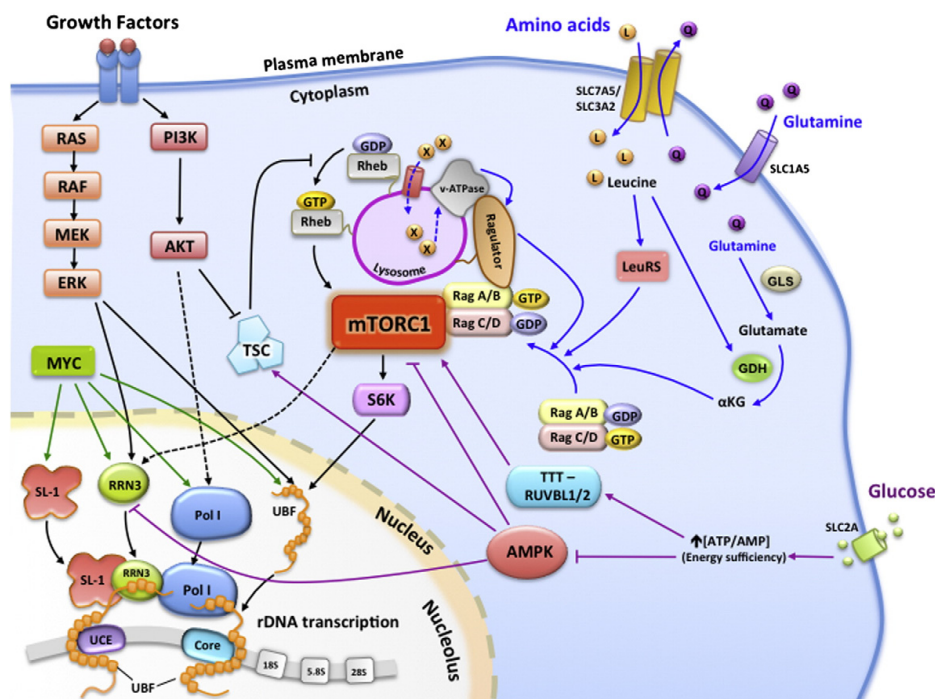


Fig. 1. mTORC1 links the availability of growth factors, amino acids and glucose to rDNA transcription. Growth factor-dependent signaling via the PI3K/AKT/mTORC1 and RAS/RAF/ERK pathways forms an intricate control network with the transcription factor MYC to regulate the rate of rDNA transcription. Amino acids enter the cell through members of solute carrier family (SLC7A5/SLC3A2; SLC1A5) and activate mTORC1 either via: i) the vacuolar H^+ -ATPase (v-ATPase) activation of Ragulator; ii) activation of leucyl-tRNA synthetase (LeuRS); or iii) the glutaminolysis pathway. All of these processes promote conversion of the heterodimer Rag A/B-GDP:Rag C/D-GDP to Rag A/B-GTP:Rag C/D-GTP which mediate subsequent activation of mTORC1. Glucose also enters the cell via SLC2A1–5 and is then processed to generate ATP, an increase of which inhibits AMP-dependent protein kinase (AMPK) activity or stabilizes Ttt1–Ttt2–RuvB-like 1 and 2 (TTT–RUVBL1/2) complex resulting in the activation of mTORC1. The signaling pathways regulated by growth factors, amino acids, glucose or MYC are connected by the black, blue, violet or green lines, respectively. The dashed lines represent undefined mechanisms. α KG, α -ketoglutarate; GDH, glutamate dehydrogenase; GLS, glutaminase; L, leucine; Pol I, RNA polymerase I; Q, glutamine; Rheb, Ras homolog enriched in brain; rDNA, ribosomal DNA; SL-1, selectivity factor 1; TSC, tuberous sclerosis complex; UBF, upstream binding factor; X, unspecified amino acid.

while inhibition of AKT reduces Pol I transcription, promoting apoptosis in a MYC-driven model of mouse B-lymphoma cells (Chan et al., 2011; Devlin et al., 2013). Thus growth factor activation of the PI3K-RAS-MYC super control network provides numerous potential targets for the development of therapies in cancers where these pathways are often dysregulated and tightly linked to increased ribosome biogenesis.

4. Regulation of rDNA transcription by nutrient availability

In addition to responding to growth factor cues, cells also monitor and respond to nutrient availability, specifically amino acid levels, by actively regulating the rate of rDNA transcription (Grummt et al., 1976; Yuan et al., 2002). The mechanism for sensing and reacting to changes in amino acid availability differs from growth factors. For example activation of mTORC1 by amino acids is independent of AKT and TSC (Fig. 1). Furthermore, growth factors are incapable of stimulating mTORC1 efficiently in amino acid-deficient conditions (Hara et al., 1998; Wang et al., 1998; Campbell et al., 1999; Kleijn and Proud, 2000), consistent with mTORC1 acting as a hub coordinating the cellular responses to distinct pathways downstream of amino acids and growth factors.

Amino acids are transported into the cell via dedicated membrane transport proteins, typically members of the solute carrier family (SLC), which couple this process with the movement of ions, for instance, the uptake of glutamine via the Na^+ -dependent SLC1A5

transporter (reviewed in Taylor, 2014). In some cell systems, the uptake of certain amino acids is dependent on the transport of other amino acids across the plasma membrane. For example, the efflux of intracellular glutamine leads to the influx of leucine and other essential amino acids via the SLC7A5/SLC3A2 bidirectional transporter (Nicklin et al., 2009).

The mechanism by which cells monitor the availability of amino acids is not fully understood. At present there are two proposed mechanisms based on the involvement of the vacuolar H^+ -ATPase (v-ATPase) in the “inside-out” model and leucyl-tRNA synthetase (LeuRS) in the “cytoplasmic sensing” model (Fig. 1). Central to both models is the Rag-GTPases family (RagA, RagB, RagC and RagD). RagA and RagB have similar amino acid sequences and are functionally equivalent, as are RagC and RagD. Amino acids promote GTP-loading of RagA or RagB and the formation of a heterodimer with guanosine diphosphate (GDP)-bound RagC or D (Sekiguchi et al., 2001; Sancak et al., 2008).

In the “inside-out” model, the v-ATPase senses accumulation of amino acids in the lysosome through an unknown mechanism, resulting in signal propagation to a pentameric complex termed Ragulator with which v-ATPase directly interacts (Zoncu et al., 2011). The Ragulator complex consists of: p18 (late-endosomal/lysosomal adaptor, MAPK and mTOR activator 1 (LAMTOR1)), p14 (LAMTOR2), MAPK-scaffold protein 1 (MP1; LAMTOR3), C7orf59 (LAMTOR4) and hepatitis B virus X-interacting protein (HBXIP; LAMTOR5). The Ragulator interacts with

the Rag-GTPase heterodimer and tethers it to the lysosome, mediated specifically by myristoylated and palmitoylated residues on p18 (Sancak et al., 2010; Bar-Peled et al., 2012). Ragulator also plays a role as a guanine nucleotide exchange factor for RagA/B, promoting the exchange of GDP to GTP (Bar-Peled et al., 2012). As for the cytoplasm-sensing model, LeuRS, the enzyme responsible for catalyzing the ligation of leucine to its cognate tRNA, in the presence of high levels of leucine, acts as a GAP for RagD (Han et al., 2012). In both cases a heterodimer of RagA/B-GTP and Rag C/D-GDP is generated thus mediating activation of mTORC1 signaling.

As might be predicted, mTORC1 activity is inhibited in nutrient-deprived conditions and its reactivation is triggered by re-addition of amino acids (Hara et al., 1998; Kim et al., 2002). Activation of mTORC1 by amino acids however occurs in a TSC-independent and Rheb-dependent manner, with the Rag-GTPases as key mediators as described above (Long et al., 2005; Smith et al., 2005; Sancak et al., 2008). Specifically, the RagA/B-GTP-RagC/D-GDP heterodimer binds the mTORC1 member Raptor to facilitate its translocation to the lysosome, where mTORC1 interacts with the growth factor-activated GTPase Rheb (Sancak et al., 2008, 2010). Knockdown of Rag-GTPases abrogates mTORC1 relocation even in the presence of amino acids, causing its dispersal through the cytoplasm, the same phenomenon observed when normal cells are amino acid starved (Sancak et al., 2010). The requirement for Rheb provides an important link mechanistically explaining why full activation of mTORC1 in higher eukaryotes requires the presence of both growth factors and amino acids. Thus Rheb is a key intersection point for the growth factor and amino acid-mediated regulation of mTORC1.

Both models for sensing amino acid levels link nutrient status to activation of mTORC1 and cell growth. As described above, basal levels of amino acids are required for growth factor-driven activation of mTORC1. Moreover, growth factor induced rDNA transcription is impaired in the absence of amino acids (James and Zomerdijk, 2004) suggesting that the availability of amino acids affects the response of rDNA transcription to growth factors. Taken together, these findings support a critical role of signaling via the amino acid sensing network in the control of rDNA transcription and ribosome biogenesis. However, further studies are required to define the mechanisms by which regulation of ribosome biogenesis in response to changes in amino acid availability is mediated.

Individual amino acids have also been shown to modulate mTORC1 activity, including leucine (Hara et al., 1998), glutamine (Durán et al., 2012) and arginine (Bauchart-Thevet et al., 2010). Glutaminolysis, the metabolism of glutamine, is an important regulator of mTORC1 activity and it is well established that most tumor cells are dependent on rapid glutamine uptake and metabolism (reviewed in Wise and Thompson, 2010; Daye and Wellen, 2012; Katt and Cerione, 2014). Glutamine is metabolized to α -ketoglutarate (α KG) via a two-step reaction: glutamine is first deaminated to glutamate by glutaminase (GLS), followed by a second deamination reaction catalyzed by glutamate dehydrogenase (GDH) to generate α KG which enters and replenishes the tricarboxylic acid (TCA) cycle. Glutamine flux facilitates the uptake of leucine via the antiporter SLC7A5/SLC3A2 (Fuchs and Bode, 2005). A recent study demonstrated that both leucine and glutamine stimulate glutaminolysis, increasing α KG production and promoting GTP-loading of RagB, activation of mTORC1 signaling and cell growth via an unknown mechanism (Durán et al., 2012). Conversely, inhibition of glutaminolysis by a glutaminase inhibitor 6-diazo-5-oxo-L-norleucine (DON) prevented lysosomal translocation and activation of mTORC1, as well as increased cell size in the presence of leucine and glutamine (Durán et al., 2012). Additionally, the introduction of a cell-permeable α KG analogue to amino acid starved cells restored mTORC1 activity (Durán et al., 2012). Thus cells sense and respond to intracellular levels of leucine and glutamine to regulate mTORC1 activation. Notably, recent studies from the Blenis group demonstrated that mTORC1 activation stimulates glutamine metabolism by promoting the activity of GDH

and GLS, providing a key mechanism linking aberrant mTORC1 signaling to abnormal cellular metabolism in cancer (Csibi et al., 2013, 2014).

The above studies suggest that mammalian cells monitor both the total amino acid flux as well as the individual amino acid concentrations and then transmit these nutrient signals to mTORC1, rDNA transcription and ribosome biogenesis. Although inhibition of mTORC1 activity with rapamycin reduced rDNA transcription (Mahajan, 1994; Hannan et al., 2003), the contribution of amino acid levels to this regulation has not been well explored. We observed that amino acid stimulation of rDNA transcription is potently inhibited by the glutaminase inhibitor DON (Kang, Kusnadi, Hannan and Pearson unpublished data), indicating that, mTORC1-dependent regulation of rDNA transcription involves glutamine metabolism in addition to a sufficient level of the other amino acids. Targeting rDNA transcription has already shown great potential as a novel approach to treating cancer (Drygin et al., 2011; Bywater et al., 2012; Peltonen et al., 2014a, 2014b). Deciphering the mechanism(s) by which amino acids modulate mTORC1 activity and rDNA transcription may provide important additional targets to enhance the efficacy of this approach, either for use as single agents or in combination with Pol I inhibitors now in clinical trial.

5. Regulation of rDNA transcription by energy

In proliferating and transformed cells, glucose is considered as the primary energy source (Altman and Dang, 2012). Glucose is imported into the cell by the SLC2 (facilitated glucose transporter), member 1 to 5 (SLC2A1 to 5) (reviewed in Thorens and Mueckler, 2010) (Fig. 1). Once in the cell, the glycolytic pathway converts glucose into pyruvate, producing two molecules of adenosine triphosphate (ATP) while oxidative decarboxylation of pyruvate, the TCA cycle and oxidative phosphorylation generates a further 36 molecules of ATP. Accordingly, inhibition of glucose metabolism causes depletion of ATP and accumulation of adenosine diphosphate (ADP) and adenosine monophosphate (AMP), thus increasing ADP/ATP and AMP/ATP ratios. An abnormal dependence on aerobic glycolysis for energy production and biosynthesis in cancer cells provides new opportunities for cancer therapy. Indeed, inhibitors to components of the energy production pathway including 2-deoxy-glucose, lonidamine, 3-bromopyruvate, koniginic acid and dichloroacetate have been trialed with mixed success in a number of diseases including cancer (reviewed in Ganapathy-Kanniappan et al., 2012; Zhao et al., 2013).

The established crucial energy sensor in the cell is the AMP-dependent protein kinase (AMPK), which is a heterotrimeric complex consisting of one catalytic (α) and two regulatory (β and γ) subunits. AMPK responds to increased AMP to ATP ratio by negatively regulating mTORC1 activity. AMPK is activated by an allosteric change when AMP binds to the γ subunit and the α -subunit is phosphorylated on threonine 172 by one of its three upstream kinases: the tumor suppressor liver kinase B1 (Woods et al., 2003; Lizcano et al., 2004), the calcium/calmodulin-dependent protein kinase β (Hawley et al., 2005; Woods et al., 2005) or transforming growth factor β -activated kinase-1 (Momcilovic et al., 2006). More recently glycogen synthase kinase 3, which is downregulated by PI3K/AKT signaling, was reported to inhibit AMPK function by forming a stable complex with its β regulatory subunit and also phosphorylating the α catalytic subunit (Suzuki et al., 2013). Once AMPK is activated it transmits the energetic stress signals to mTORC1 through direct phosphorylation of TSC2 or Raptor (Fig. 1). Specifically AMPK phosphorylates TSC2 at Ser1345 elevating its GAP activity which mediates inactivation of Rheb (Inoki et al., 2003b). AMPK also prevents mTORC1 activation via a TSC-independent mechanism, by directly phosphorylating Raptor which then preferentially associates with 14-3-3 proteins rather than mTORC1 (Gwinn et al., 2008). This AMPK-mediated inactivation of mTORC1, in times of energy stress, is predicted to reduce rDNA transcription and hence ribosome biogenesis, conserving energy expenditure. Interestingly, AMPK has been reported to directly inactivate RRN3 by phosphorylation at Ser635, a unique

phosphorylation site unaffected by mTORC1 signaling. Phosphorylation of RRN3 at this site disrupts the interaction of the RRN3/Pol I complex with the promoter bound SL-1, thereby inhibiting Pol I PIC assembly (Hoppe et al., 2009).

An alternative mechanism, independent of AMPK, of mTORC1 pathway inhibition was reported recently involving the Tel2–Tti1–Tti2 (TTT)–RuvB-like 1 and 2 (RUVBL1/2) complex (Kim et al., 2013b) (Fig. 1). This ATPase containing complex is able to regulate the assembly and stability of the phosphoinositide-3-kinase-related protein kinases family, which includes mTORC1 (Takai et al., 2007; Hořejší et al., 2010; Izumi et al., 2010). Under energetic stress the interaction between the TTT components and RUVBL proteins is disrupted thereby suppressing mTORC1 activity by preventing its dimerization and lysosomal localization (Kim et al., 2013b). The dependence of rDNA transcription on the activity of the TTT–RUVBL1/2 complex is yet to be reported.

rDNA transcription is also modulated at an epigenetic level by altered cellular energy status. Heterochromatin modification of the rRNA genes is mediated by the energy-dependent nucleolar silencing complex (eNoSC), which contains the nicotinamide adenine dinucleotide (NAD)-dependent histone deacetylase sirtuin 1 (SIRT1), the heterochromatic histone methyltransferase SUV39H1 and nucleomethylin (NML), which is a nucleolar protein that specifically binds to the heterochromatic histone mark H3K9me2 (dimethylation of Histone H3 at lysine 9) (Murayama et al., 2008). A recent study proposed a positive feedback model linking SIRT1 nucleolar function with nutrient signaling: under nutrient sufficient conditions, activation of mTORC1 and other nutrient responsive signaling pathways promoted the synthesis of nascent rRNA and this mediated disassembly of the eNoSC since the excess rRNA preferentially bound NML thus preventing it from binding and recruiting SIRT1 to the nucleolus (Yang et al., 2013). Alternatively, upon glucose deprivation, reduced levels of nascent rRNA enabled eNoSC assembly and promoted rDNA heterochromatin formation and silencing (Yang et al., 2013). Thus it appears that cells are able to utilize at least two mechanisms of modulating rDNA transcription according to the overall cellular energy level; a fast response mediated by the AMPK/mTORC1-nutrient signaling pathways which impact on Pol I functions and a more chronic or delayed response through epigenetic regulation of rDNA silencing. In either case, as reported for growth factor and amino acid availability, mTORC1 emerges as the hub adjusting the rate of rDNA transcription to match changes in the cellular environment, a regulation that is essential for both the proliferative normal and transformed cells.

6. Metabolic regulation of rDNA transcription in cancer

While there is no direct evidence to demonstrate that up-regulation of rDNA transcription is sufficient to drive malignant transformation, elevated rDNA transcription, characterized by enlarged and/or dysregulated nucleoli, is regarded as a feature of many cancers with potential prognostic value (Williamson et al., 2006; Derenzini et al., 2009; Uemura et al., 2011). Hyperactivation of Pol I transcription is required to mediate increased ribosome synthesis, thereby enhancing cellular protein translation capacity and growth, characteristic of many tumor cells (reviewed in Bywater et al., 2013; Hannan et al., 2013; Hein et al., 2013; Poortinga et al., 2014; Quin et al., 2014). Typically, accelerated rDNA transcription in cancer cells is mediated through activation of oncogenic signaling and/or repression of tumor suppressive pathways rather than mutations in the Pol I apparatus (reviewed in Bywater et al., 2013; Hein et al., 2013; Quin et al., 2014). While accelerated rDNA transcription may not be sufficient to drive the malignant process, recent studies have unequivocally shown that accelerated rDNA transcription is necessary for the survival of certain tumors (Drygin et al., 2011; Bywater et al., 2012; Peltonen et al., 2014a, 2014b). Specifically it has been shown that selective inhibition of Pol I transcription *in vivo* therapeutically treated a hematological malignancy through the activation of p53-dependent apoptosis, while sparing normal cells (Bywater

et al., 2012). One such compound, CX-5461, which prevents Pol I PIC formation by interfering with SL-1 binding to the rDNA promoter and thus suppresses transcription initiation (Drygin et al., 2011; Bywater et al., 2012), has entered a phase I clinical trial at the Peter MacCallum Cancer Centre, Australia. Very recently, a structurally unrelated small molecule named BMH-21 has been reported to represses Pol I transcription via an as yet uncharacterized mechanism, resulting in degradation of the Pol I catalytic subunit RPA194 (Peltonen et al., 2014b). Like CX-5461, BMH-21 inhibits tumor growth *in vivo*, further emphasizing the therapeutic potential for targeting Pol I transcription to treat cancer.

In addition to deregulated ribosome biogenesis, cancer cells adapt their cellular metabolism to support tumor growth and survival. Reprogramming cellular metabolism has recently been included as a hallmark of cancer in Hanahan and Weinberg's updated review (reviewed in Hanahan and Weinberg, 2011). This inclusion was based on the following observations: a) tumor cells exhibit enhanced glucose uptake and sustained high rates of glycolysis irrespective of oxygen availability (termed the Warburg effect); and b) tumor cells demonstrate increased glutamine uptake and glutaminolysis (termed glutamine addiction) (reviewed in Cairns et al., 2011; Schulze and Harris, 2012). It is becoming clear that these metabolic adaptations mediate increased energy production, sustain sufficient macromolecule biosynthesis and maintain redox homeostasis in order to support the accelerated growth and thus survival of cells in the stressful and heterogeneous tumor microenvironment. Multiple oncogenic signaling pathways, such as PI3K/AKT/mTORC1, RAS/RAF/ERK and AMPK pathways, have key roles in the regulation of cancer cell metabolism and in turn, the metabolic changes further enhance the activities of these pathways (reviewed in Cairns et al., 2011; Chen and Russo, 2012). Altering the metabolic phenotype of tumor cells may potentially affect tumor progression, response to therapy and patient outcome (Butler et al., 2013; Parmenter et al., 2014). Of a practical clinical interest, the ability to image cellular substrate utilization on a whole-body scale using positron emission tomography (PET) provides the potential for a non-invasive biomarker of altered metabolism both preceding and following therapy. The glucose analogue ¹⁸F-fluorodeoxyglucose (FDG) is the most widely available tracer, but there are several others that can interrogate amino acid transport and more recent agents that assess glutaminolysis. In particular, novel glutamine analogues would provide an interesting approach to the assessment of a cancer cell-dependence on glutaminolysis *in vivo* (Krasikova et al., 2011; Wu et al., 2014).

More far reaching is the idea that metabolic reprogramming is coupled to accelerated ribosome biogenesis and protein synthesis which are required for increased proliferative growth of cancer cells. Take for example MYC-driven oncogenesis in which elevated rDNA transcription has been implicated to play a key role (reviewed in Poortinga et al., 2014). MYC-driven lymphoma is highly sensitive to either Pol I inhibition (Bywater et al., 2012) or the inhibition of eIF4E-dependent protein synthesis (Pourdehnad et al., 2013). The mechanisms underlying the apparent addiction to rDNA transcription are still to be defined, however it is evident that activation of the nucleolar stress/surveillance pathway, resulting in p53 accumulation and subsequent tumor cell apoptosis plays a critical role (Bywater et al., 2012). Oncogenic MYC has also been linked to up-regulated glutaminolysis through a coordinated transcriptional program (Yuneva et al., 2007; Wise et al., 2008; Gao et al., 2009). In addition, MYC-driven lymphoma cell lines are sensitive to the glutaminase selective inhibitor bis-2-(5-phenylacetamido-1,2,4-thiadiazol-2-yl)ethyl sulfide (BPTES) *in vitro* and in xenograft models (Le et al., 2012; Yuneva et al., 2012). Thus, given the tight link between rDNA transcription and cellular metabolic status discussed in this review, it is tempting to speculate that the apparent addiction of MYC-driven malignancy to glutaminolysis is, at least in part, mediated via a reliance on nutrient-dependent ribosome biogenesis. If MYC-driven glutamine metabolic re-direction does converge on ribosome biogenesis, inhibition of glutaminolysis in combination with Pol I transcription-based therapy may achieve a synergistic

therapeutic effect in MYC-driven malignancies. Nutrient-dependent rDNA transcription also raises the possibility that malignant disease characterized by hyper-activation of metabolism may be vulnerable to therapeutic strategies that target rDNA transcription. Thus developing and applying molecular imaging PET technology to monitor rDNA transcription in parallel with metabolic activity may facilitate patient selection for Pol I transcription-based therapy.

7. Conclusions

Synthesis of rRNA by Pol I is a critical step in ribosome biogenesis, and a major biosynthetic and energy consuming process in proliferating cells. Consequently cells have developed an intricate nutrient and energy-sensing network to modulate rDNA transcription and tightly couple it to growth factor, nutrient, and energy levels.

Extensive research has defined the PI3K/AKT/mTORC1, RAS/RAF/ERK and MYC pathways as critical controllers in the coupling of rDNA transcription to growth factor stimulation. Some components of these growth factor-responsive pathways have also been implicated in controlling rDNA transcription in response to nutrient and energy status, through overlapping and distinct mechanisms. We are now gaining a clearer understanding of the crosstalk between the different signaling networks downstream of these environmental cues with mTORC1 clearly providing one critical nexus between these mechanisms. Importantly, the dysregulation of these signaling pathways in transformed cells and accompanying re-programming of cancer cell responses to altered nutrient and energy requirements provide new opportunities for targeting cancer. We propose that targeting multiple components in the growth factor, nutrient and energy control network will increase the potency of therapeutic approaches targeting ribosome biogenesis in the ~90% of cancers characterized by dysregulated MYC, PI3K or RAS signaling.

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References

- Altman, B.J., Dang, C.V., 2012. Normal and cancer cell metabolism: lymphocytes and lymphoma. *FEBS J.* 279, 2598–2609.
- Arabi, A., Wu, S., Ridderstrale, K., Bierhoff, H., Shiue, C., Fathyol, K., Fahlen, S., Hydbring, P., Soderberg, O., Grummt, I., Larsson, L.G., Wright, A.P., 2005. c-Myc associates with ribosomal DNA and activates RNA polymerase I transcription. *Nat. Cell Biol.* 7, 303–310.
- Bar-Peled, L., Schweitzer, L.D., Zoncu, R., Sabatini, D.M., 2012. Ragulator is a GEF for the rag GTPases that signal amino acid levels to mTORC1. *Cell* 150, 1196–1208.
- Bauchart-Thévret, C., Cui, L., Wu, G., Burrin, D.G., 2010. Arginine-induced stimulation of protein synthesis and survival in IPEC-J2 cells is mediated by mTOR but not nitric oxide. *Am. J. Physiol. Endocrinol. Metab.* 299, E899–E909.
- Butler, E.B., Zhao, Y., Muñoz-Pinedo, C., Lu, J., Tan, M., 2013. Stalling the engine of resistance: targeting cancer metabolism to overcome therapeutic resistance. *Cancer Res.* 73, 2709–2717.
- Bywater, M.J., Poortinga, G., Sanij, E., Hein, N., Peck, A., Cullinane, C., Wall, M., Cluse, L., Drygin, D., Anderes, K., Huser, N., Proffitt, C., Bliesath, J., Haddach, M., Schwaeb, M.K., Ryckman, D.M., Rice, W.G., Schmitt, C., Lowe, S.W., Johnstone, R.W., Pearson, R.B., McArthur, G.A., Hannan, R.D., 2012. Inhibition of RNA polymerase I as a therapeutic strategy to promote cancer-specific activation of p53. *Cancer Cell* 22, 51–65.
- Bywater, M.J., Pearson, R.B., McArthur, G.A., Hannan, R.D., 2013. Dysregulation of the basal RNA polymerase transcription apparatus in cancer. *Nat. Rev. Cancer* 13, 299–314.
- Cairns, R.A., Harris, I.S., Mak, T.W., 2011. Regulation of cancer cell metabolism. *Nat. Rev. Cancer* 11, 85–95.
- Campbell, L.E., Wang, X., Proud, C.G., 1999. Nutrients differentially regulate multiple translation factors and their control by insulin. *Biochem. J.* 344 (Pt 2), 433–441.
- Chan, J.C., Hannan, K.M., Riddell, K., Ng, P.Y., Peck, A., Lee, R.S., Hung, S., Astle, M.V., Bywater, M., Wall, M., 2011. AKT promotes rRNA synthesis and cooperates with c-MYC to stimulate ribosome biogenesis in cancer. *Sci. Signal.* 4, ra56.
- Chauvin, C., Koka, V., Nouschi, A., Mieulet, V., Hoareau-Aveilla, C., Dreazen, A., Cagnard, N., Carpentier, W., Kiss, T., Meyuhas, O., 2013. Ribosomal protein S6 kinase activity controls the ribosome biogenesis transcriptional program. *Oncogene* 33, 474–483.
- Chen, J.-Q., Russo, J., 2012. Dysregulation of glucose transport, glycolysis, TCA cycle and glutaminolysis by oncogenes and tumor suppressors in cancer cells. *Biochimica et Biophysica Acta (BBA)—Reviews on Cancer* 1826, 370–384.
- Csibi, A., Fendt, S.M., Li, C., Poulgiannis, G., Choo, A.Y., Chapski, D.J., Jeong, S.M., Dempsey, J.M., Parkhitko, A., Morrison, T., Henske, E.P., Haigis, M.C., Cantley, L.C., Stephanopoulos, G., Yu, J., Blenis, J., 2013. The mTORC1 pathway stimulates glutamine metabolism and cell proliferation by repressing SIRT4. *Cell* 153, 840–854.
- Csibi, A., Lee, G., Yoon, S.O., Tong, H., Ilter, D., Elia, I., Fendt, S.M., Roberts, T.M., Blenis, J., 2014. The mTORC1/S6K1 pathway regulates glutamine metabolism through the eIF4B-dependent control of c-Myc translation. *Curr. Biol.* 24, 2274–2280.
- Daye, D., Wellen, K.E., 2012. Metabolic reprogramming in cancer: unraveling the role of glutamine in tumorigenesis. *Semin. Cell Dev. Biol.* 23, 362–369.
- De Benedetti, A., Joshi, B., Graff, J., Zimmer, S., 1994. CHO cells transformed by the translation factor eIF-4E display increased c-myc expression, but require overexpression of Max for tumorigenicity. *Mol. Cell. Differ.* 2, 347–371.
- Derenzi, M., Montanaro, L., Treré, D., 2009. What the nucleolus says to a tumour pathologist. *Histopathology* 54, 753–762.
- Devlin, J.R., Hannan, K.M., Ng, P.Y., Bywater, M.J., Shortt, J., Cullinane, C., McArthur, G.A., Johnstone, R.W., Hannan, R.D., Pearson, R.B., 2013. AKT signalling is required for ribosomal RNA synthesis and progression of Eμ-Myc B-cell lymphoma in vivo. *FEBS J.* 280, 5307–5316.
- Dibble, C.C., Elis, W., Menon, S., Qin, W., Klekota, J., Asara, J.M., Finan, P.M., Kwiatkowski, D.J., Murphy, L.O., Manning, B.D., 2012. TBC1D7 is a third subunit of the TSC1–TSC2 complex upstream of mTORC1. *Mol. Cell* 47, 535–546.
- Diesch, J., Hannan, R., Sanij, E., 2014. Perturbations at the ribosomal genes loci are at the centre of cellular dysfunction and human disease. *Cell Biosci.* 4, 43.
- Drygin, D., Lin, A., Bliesath, J., Ho, C.B., O'Brien, S.E., Proffitt, C., Omori, M., Haddach, M., Schwaeb, M.K., Siddiqui-Jain, A., 2011. Targeting RNA polymerase I with an oral small molecule CX-5461 inhibits ribosomal RNA synthesis and solid tumor growth. *Cancer Res.* 71, 1418–1430.
- Drygin, D., O'Brien, S.E., Hannan, R.D., McArthur, G.A., Von Hoff, D.D., 2014. Targeting the nucleolus for cancer-specific activation of p53. *Drug Discov. Today* 19, 259–265.
- Durán, R.V., Opplinger, W., Robitaille, A.M., Heiserich, L., Skendaj, R., Gottlieb, E., Hall, M.N., 2012. Glutaminolysis activates Rag-mTORC1 signaling. *Mol. Cell* 47, 349–358.
- Fuchs, B.C., Bode, B.P., 2005. Amino acid transporters ASC2 and LAT1 in cancer: partners in crime? *Semin. Cancer Biol.* 15, 254–266.
- Ganapathy-Kanniappan, S., Kunjithapatham, R., Geschwind, J.-F., 2012. Glyceraldehyde-3-phosphate dehydrogenase: a promising target for molecular therapy in hepatocellular carcinoma. *Oncotarget* 3, 940.
- Gao, P., Tchernyshyov, I., Chang, T.-C., Lee, Y.-S., Kita, K., Ochi, T., Zeller, K.I., De Marzo, A.M., Van Eyk, J.E., Mendell, J.T., 2009. c-Myc suppression of miR-23a/b enhances mitochondrial glutaminase expression and glutamine metabolism. *Nature* 458, 762–765.
- Grandori, C., Gomez-Roman, N., Felton-Edkins, Z.A., Ngouenet, C., Galloway, D.A., Eisenman, R.N., White, R.J., 2005. c-Myc binds to human ribosomal DNA and stimulates transcription of rRNA genes by RNA polymerase I. *Nat. Cell Biol.* 7, 311–318.
- Grewal, S.S., Li, L., Orian, A., Eisenman, R.N., Edgar, B.A., 2005. Myc-dependent regulation of ribosomal RNA synthesis during Drosophila development. *Nat. Cell Biol.* 7, 295–302.
- Grummt, I., 2003. Life on a planet of its own: regulation of RNA polymerase I transcription in the nucleolus. *Genes Dev.* 17, 1691–1702.
- Grummt, I., 2010. Wisely chosen paths—regulation of rRNA synthesis. *FEBS J.* 277, 4626–4639.
- Grummt, I., Smith, V.A., Grummt, F., 1976. Amino acid starvation affects the initiation frequency of nucleolar RNA polymerase. *Cell* 7, 439–445.
- Gwinn, D.M., Shackelford, D.B., Egan, D.F., Mihaylova, M.M., Mery, A., Vasquez, D.S., Turk, B.E., Shaw, R.J., 2008. AMPK phosphorylation of raptor mediates a metabolic checkpoint. *Mol. Cell* 30, 214–226.
- Han, J.M., Jeong, S.J., Park, M.C., Kim, G., Kwon, N.H., Kim, H.K., Ha, S.H., Ryu, S.H., Kim, S., 2012. Leucyl-tRNA synthetase is an intracellular leucine sensor for the mTORC1-signaling pathway. *Cell* 149, 410–424.
- Hanahan, D., Weinberg, R.A., 2011. Hallmarks of cancer: the next generation. *Cell* 144, 646–674.
- Hannan, K.M., Brandenburger, Y., Jenkins, A., Sharkey, K., Cavanaugh, A., Rothblum, L., Moss, T., Poortinga, G., McArthur, G.A., Pearson, R.B., 2003. mTOR-dependent regulation of ribosomal gene transcription requires S6K1 and is mediated by phosphorylation of the carboxy-terminal activation domain of the nucleolar transcription factor UBF. *Mol. Cell Biol.* 23, 8862–8877.
- Hannan, K.M., Sanij, E., Hein, N., Hannan, R.D., Pearson, R.B., 2011. Signaling to the ribosome in cancer—it is more than just mTORC1. *IUBMB Life* 63, 79–85.
- Hannan, R.D., Drygin, D., Pearson, R.B., 2013. Targeting RNA polymerase I transcription and the nucleolus for cancer therapy. *Expert Opin. Ther. Targets* 17, 873–878.
- Hara, K., Yonezawa, K., Weng, Q.-P., Kozlowski, M.T., Belham, C., Avruch, J., 1998. Amino acid sufficiency and mTOR regulate p70 S6 kinase and eIF-4E BP1 through a common effector mechanism. *J. Biol. Chem.* 273, 14484–14494.
- Hawley, S.A., Pan, D.A., Mustard, K.J., Ross, L., Bain, J., Edelman, A.M., Frenguelli, B.G., Hardie, D.G., 2005. Calmodulin-dependent protein kinase kinase-β is an alternative upstream kinase for AMP-activated protein kinase. *Cell Metab.* 2, 9–19.
- Hein, N., Hannan, K.M., George, A.J., Sanij, E., Hannan, R.D., 2013. The nucleolus: an emerging target for cancer therapy. *Trends Mol. Med.* 19, 643–654.

- Henderson, A.S., Warburton, D., Atwood, K.C., 1972. Location of ribosomal DNA in the human chromosome complement. *Proc. Natl. Acad. Sci. U. S. A.* 69, 3394–3398.
- Hoppe, S., Bierhoff, H., Cado, I., Weber, A., Tiebe, M., Grummt, I., Voit, R., 2009. AMP-activated protein kinase adapts rRNA synthesis to cellular energy supply. *Proc. Natl. Acad. Sci. U. S. A.* 106, 17781–17786.
- Hofeš, Z., Takai, H., Adelman, C.A., Collis, S.J., Flynn, H., Maslen, S., Skehel, J.M., de Lange, T., Boulton, S.J., 2010. CK2 phospho-dependent binding of R2TP complex to TEL2 is essential for mTOR and SMG1 stability. *Mol. Cell* 39, 839–850.
- Howell, J.J., Manning, B.D., 2011. mTOR couples cellular nutrient sensing to organismal metabolic homeostasis. *Trends Endocrinol. Metab.* 22, 94–102.
- Iadevaia, V., Liu, R., Proud, C.G., 2014. mTORC1 signaling controls multiple steps in ribosome biogenesis. *Semin. Cell Dev. Biol.* <http://dx.doi.org/10.1016/j.semcdb.2014.08.004>.
- Inoki, K., Li, Y., Xu, T., Guan, K.-L., 2003a. Rheb GTPase is a direct target of TSC2 GAP activity and regulates mTOR signaling. *Genes Dev.* 17, 1829–1834.
- Inoki, K., Zhu, T., Guan, K.-L., 2003b. TSC2 mediates cellular energy response to control cell growth and survival. *Cell* 115, 577–590.
- Izumi, N., Yamashita, A., Iwamatsu, A., Kurata, R., Nakamura, H., Saari, B., Hirano, H., Anderson, P., Ohno, S., 2010. AAA+ proteins RUVBL1 and RUVBL2 coordinate PI3K activity and function in nonsense-mediated mRNA decay. *Sci. Signal.* 3, ra27.
- James, M.J., Zomerijk, J.C., 2004. Phosphatidylinositol 3-kinase and mTOR signaling pathways regulate RNA polymerase I transcription in response to IGF-1 and nutrients. *J. Biol. Chem.* 279, 8911–8918.
- Katt, W.P., Cerione, A.A., 2014. Glutaminase regulation in cancer cells: a druggable chain of events. *Drug Discov. Today* 19, 450–457.
- Kim, S., Li, Q., Dang, C.V., Lee, L.A., 2000. Induction of ribosomal genes and hepatocyte hypertrophy by adenovirus-mediated expression of c-Myc in vivo. *Proc. Natl. Acad. Sci.* 97, 11198–11202.
- Kim, D.-H., Sarbassov, D.D., Ali, S.M., King, J.E., Latek, R.R., Erdjument-Bromage, H., Tempst, P., Sabatini, D.M., 2002. mTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery. *Cell* 110, 163–175.
- Kim, S.G., Buel, G.R., Blenis, J., 2013a. Nutrient regulation of the mTOR complex 1 signaling pathway. *Mol. Cells* 35, 463–473.
- Kim, S.G., Hoffman, G.R., Pouloukianis, G., Buel, G.R., Jang, Y.J., Lee, K.W., Kim, B.-Y., Erikson, R.L., Cantley, L.C., Choo, A.Y., Blenis, J., 2013b. Metabolic stress controls mTORC1 lysosomal localization and dimerization by regulating the TTT-RUVBL1/2 complex. *Mol. Cell* 49, 172–185.
- Kleijn, M., Proud, C.G., 2000. Glucose and amino acids modulate translation factor activation by growth factors in PC12 cells. *Biochem. J.* 347, 399–406.
- Krasikova, R.N., Kuznetsova, O.F., Fedorova, O.S., Belokon, Y.N., Maleev, V.I., Mu, L., Ametamey, S., Schubiger, P.A., Friebe, M., Berndt, M., Koglin, N., Mueller, A., Graham, K., Lehmann, L., Dinkelborg, L.M., 2011. 4-[¹⁸F]fluoroglutamic acid (BAY 85-8050), a new amino acid radiotracer for PET imaging of tumors: synthesis and in vitro characterization. *J. Med. Chem.* 54, 406–410.
- Le, A., Lane, A.N., Hamaker, M., Bose, S., Gouw, A., Barbi, J., Tsukamoto, T., Rojas, C.J., Slusher, B.S., Zhang, H., 2012. Glucose-independent glutamine metabolism via TCA cycling for proliferation and survival in B cells. *Cell Metab.* 15, 110–121.
- Lizcano, J.M., Göransson, O., Toth, R., Deak, M., Morrice, N.A., Boudeau, J., Hawley, S.A., Udd, L., Mäkelä, T.P., Hardie, D.G., 2004. LKB1 is a master kinase that activates 13 kinases of the AMPK subfamily, including MARK/PAR-1. *EMBO J.* 23, 833–843.
- Long, X., Ortiz-Vega, S., Lin, Y., Avruch, J., 2005. Rheb binding to mammalian target of rapamycin (mTOR) is regulated by amino acid sufficiency. *J. Biol. Chem.* 280, 23433–23436.
- Mahajan, P.B., 1994. Modulation of transcription of rRNA genes by rapamycin. *Int. J. Immunopharmacol.* 16, 711–721.
- Mayer, C., Zhao, J., Yuan, X., Grummt, I., 2004. mTOR-dependent activation of the transcription factor TIF-IA links rRNA synthesis to nutrient availability. *Genes Dev.* 18, 423–434.
- Moir, R.D., Willis, I.M., 2013. Regulation of Pol III transcription by nutrient and stress signaling pathways. *Biochim. Biophys. Acta* 1829, 361–375.
- Momcilovic, M., Hong, S.-P., Carlson, M., 2006. Mammalian TAK1 activates Snf1 protein kinase in yeast and phosphorylates AMP-activated protein kinase in vitro. *J. Biol. Chem.* 281, 25336–25343.
- Moss, T., Langlois, F., Gagnon-Kugler, T., Stefanovsky, V., 2007. A housekeeper with power of attorney: the rRNA genes in ribosome biogenesis. *Cell. Mol. Life Sci.* 64, 29–49.
- Murayama, A., Ohmori, K., Fujimura, A., Minami, H., Yasuzawa-Tanaka, K., Kuroda, T., Oie, S., Daitoku, H., Okuwaki, M., Nagata, K., 2008. Epigenetic control of rDNA loci in response to intracellular energy status. *Cell* 133, 627–639.
- Nicklin, P., Bergman, P., Zhang, B., Triantafellow, E., Wang, H., Nyfeler, B., Yang, H., Hild, M., Kung, C., Wilson, C., 2009. Bidirectional transport of amino acids regulates mTOR and autophagy. *Cell* 136, 521–534.
- Panse, V.G., Johnson, A.W., 2010. Maturation of eukaryotic ribosomes: acquisition of functionality. *Trends Biochem. Sci.* 35, 260–266.
- Parmenter, T.J., Kleinschmidt, M., Kinross, K.M., Bond, S.T., Li, J., Kaadige, M.R., Rao, A., Sheppard, K.E., Hugo, W., Pupo, G.M., 2014. Response of BRAF-mutant melanoma to BRAF inhibition is mediated by a network of transcriptional regulators of glycolysis. *Cancer Discov.* 4, 423–433.
- Peltonen, K., Colis, L., Liu, H., Jaamaa, S., Zhang, Z., Af Hallstrom, T., Moore, H.M., Sirajuddin, P., Laiho, M., 2014a. Small molecule BMH-compounds that inhibit RNA polymerase I and cause nucleolar stress. *Mol. Cancer Ther.* <http://dx.doi.org/10.1158/1535-7163.MCT-14-0256>.
- Peltonen, K., Colis, L., Liu, H., Trivedi, R., Moubarek, M.S., Moore, H.M., Bai, B., Rudek, M.A., Bieberich, C.J., Laiho, M., 2014b. A targeting modality for destruction of RNA polymerase I that possesses anticancer activity. *Cancer Cell* 25, 77–90.
- Poortinga, G., Hannan, K.M., Snelling, H., Walkley, C.R., Jenkins, A., Sharkey, K., Wall, M., Brandenburger, Y., Palatsides, M., Pearson, R.B., 2004. MAD1 and c-MYC regulate UBF and rDNA transcription during granulocyte differentiation. *EMBO J.* 23, 3325–3335.
- Poortinga, G., Wall, M., Sanij, E., Siwicki, K., Ellul, J., Brown, D., Holloway, T.P., Hannan, R.D., McArthur, G.A., 2011. c-MYC coordinately regulates ribosomal gene chromatin remodeling and Pol I availability during granulocyte differentiation. *Nucleic Acids Res.* 39, 3267–3281.
- Poortinga, G., Quinn, L., Hannan, R., 2014. Targeting RNA polymerase I to treat MYC-driven cancer. *Oncogene* <http://dx.doi.org/10.1038/onc.2014.13>.
- Pourdehnan, M., Truitt, M.L., Siddiqi, I.N., Ducker, G.S., Shokat, K.M., Ruggero, D., 2013. Myc and mTOR converge on a common node in protein synthesis control that confers synthetic lethality in Myc-driven cancers. *Proc. Natl. Acad. Sci. U. S. A.* 110, 11988–11993.
- Quin, J.E., Devlin, J.R., Cameron, D., Hannan, K.M., Pearson, R.B., Hannan, R.D., 2014. Targeting the nucleolus for cancer intervention. *Biochim. Biophys. Acta (BBA) - Mol. Basis Dis.* 1842, 802–816.
- Ruggero, D., Pandolfi, P.P., 2003. Does the ribosome translate cancer? *Nat. Rev. Cancer* 3, 179–192.
- Russell, J., Zomerijk, J.C., 2005. RNA-polymerase-I-directed rDNA transcription, life and works. *Trends Biochem. Sci.* 30, 87–96.
- Sancak, Y., Peterson, T.R., Shaul, Y.D., Lindquist, R.A., Thoreen, C.C., Bar-Peled, L., Sabatini, D.M., 2008. The Rag GTPases bind raptor and mediate amino acid signaling to mTORC1. *Science* 320, 1496–1501.
- Sancak, Y., Bar-Peled, L., Zoncu, R., Markhard, A.L., Nada, S., Sabatini, D.M., 2010. Ragulator–Rag complex targets mTORC1 to the lysosomal surface and is necessary for its activation by amino acids. *Cell* 141, 290–303.
- Sanij, E., Poortinga, G., Sharkey, K., Hung, S., Holloway, T.P., Quin, J., Robb, E., Wong, L.H., Thomas, W.G., Stefanovsky, V., Moss, T., Rothblum, L.I., Hannan, K.M., McArthur, G.A., Pearson, R.B., Hannan, R.D., 2008. UBF levels determine the number of active ribosomal RNA genes in mammals. *J. Cell Biol.* 183, 1259–1274.
- Schmidt, E.V., 1999. The role of c-myc in cellular growth control. *Oncogene* 18, 2988–2996.
- Schneider, D.A., 2012. RNA polymerase I activity is regulated at multiple steps in the transcription cycle: recent insights into factors that influence transcription elongation. *Gene* 493, 176–184.
- Schulze, A., Harris, A.L., 2012. How cancer metabolism is tuned for proliferation and vulnerable to disruption. *Nature* 491, 364–373.
- Sears, R., Leone, G., DeGregori, J., Nevins, J.R., 1999. Ras enhances Myc protein stability. *Mol. Cell* 3, 169–179.
- Sekiguchi, T., Hirose, E., Nakashima, N., Ii, M., Nishimoto, T., 2001. Novel G proteins, Rag C and Rag D, interact with GTP-binding proteins, Rag A and Rag B. *J. Biol. Chem.* 276, 7246–7257.
- Shimobayashi, M., Hall, M.N., 2014. Making new contacts: the mTOR network in metabolism and signalling crosstalk. *Nat. Rev. Mol. Cell Biol.* 15, 155–162.
- Shiue, C.N., Berkson, R.G., Wright, A.P., 2009. c-Myc induces changes in higher order rDNA structure on stimulation of quiescent cells. *Oncogene* 28, 1833–1842.
- Smith, E.M., Finn, S.G., Tee, A.R., Browne, G.J., Proud, C.G., 2005. The tuberous sclerosis protein TSC2 is not required for the regulation of the mammalian target of rapamycin by amino acids and certain cellular stresses. *J. Biol. Chem.* 280, 18717–18727.
- Stefanovsky, V.Y., Moss, T., 2008. The splice variants of UBF differentially regulate RNA polymerase I transcription elongation in response to ERK phosphorylation. *Nucleic Acids Res.* 36, 5093–5101.
- Stefanovsky, V.Y., Pelletier, G., Hannan, R., Gagnon-Kugler, T., Rothblum, L.I., Moss, T., 2001. An immediate response of ribosomal transcription to growth factor stimulation in mammals is mediated by ERK phosphorylation of UBF. *Mol. Cell* 8, 1063–1073.
- Stefanovsky, V., Langlois, F., Gagnon-Kugler, T., Rothblum, L.I., Moss, T., 2006a. Growth factor signaling regulates elongation of RNA polymerase I transcription in mammals via UBF phosphorylation and r-chromatin remodeling. *Mol. Cell* 21, 629–639.
- Stefanovsky, V.Y., Langlois, F., Bazett-Jones, D., Pelletier, G., Moss, T., 2006b. ERK modulates DNA bending and enhancesome structure by phosphorylating HMG1-boxes 1 and 2 of the RNA polymerase I transcription factor UBF. *Biochemistry* 45, 3626–3634.
- Stults, D.M., Killen, M.W., Pierce, H.H., Pierce, A.J., 2008. Genomic architecture and inheritance of human ribosomal RNA gene clusters. *Genome Res.* 18, 13–18.
- Suzuki, T., Bridges, D., Nakada, D., Skiniotis, G., Morrison, S.J., Lin, J.D., Saltiel, A.R., Inoki, K., 2013. Inhibition of AMPK catabolic action by GSK3. *Mol. Cell* 50, 407–419.
- Takai, H., Wang, R.C., Takai, K.K., Yang, H., de Lange, T., 2007. Tel2 regulates the stability of PI3K-related protein kinases. *Cell* 131, 1248–1259.
- Taylor, P.M., 2014. Role of amino acid transporters in amino acid sensing. *Am. J. Clin. Nutr.* 99, 223S–230S.
- Tee, A.R., Manning, B.D., Roux, P.P., Cantley, L.C., Blenis, J., 2003. Tuberous sclerosis complex gene products, Tuber and Hamartin, control mTOR signaling by acting as a GTPase-activating protein complex toward Rheb. *Curr. Biol.* 13, 1259–1268.
- Thoreen, C.C., Chantranupong, L., Keys, H.R., Wang, T., Gray, N.S., Sabatini, D.M., 2012. A unifying model for mTORC1-mediated regulation of mRNA translation. *Nature* 485, 109–113.
- Thorens, B., Mueckler, M., 2010. Glucose transporters in the 21st century. *Am. J. Physiol. Endocrinol. Metab.* 298, E141–E145.
- Uemura, M., Zheng, Q., Koh, C., Nelson, W., Yegnasubramanian, S., De Marzo, A., 2011. Overexpression of ribosomal RNA in prostate cancer is common but not linked to rDNA promoter hypomethylation. *Oncogene* 31, 1254–1263.
- Wall, M., Poortinga, G., Hannan, K.M., Pearson, R.B., Hannan, R.D., McArthur, G.A., 2008. Translational control of c-MYC by rapamycin promotes terminal myeloid differentiation. *Blood* 112, 2305–2317.
- Wall, M., Poortinga, G., Stanley, K.L., Lindemann, R.K., Bots, M., Chan, C.J., Bywater, M.J., Kinross, K.M., Astle, M.V., Waldeck, K., Hannan, K.M., Shortt, J., Smyth, M.J., Lowe, Scott W., Hannan, R.D., Pearson, R.B., Johnston, R.W., McArthur, G.A., 2013. The mTORC1 inhibitor everolimus prevents and treats Eμ-Myc lymphoma by restoring oncogene-induced senescence. *Cancer Discov.* 3, 82–95.

- Wang, X., Campbell, L., Miller, C., Proud, C., 1998. Amino acid availability regulates p70 S6 kinase and multiple translation factors. *Biochem. J.* 334, 261–267.
- West, M.J., Stoneley, M., Willis, A.E., 1998. Translational induction of the c-myc oncogene via activation of the FRAP/TOR signalling pathway. *Oncogene* 17, 769–780.
- Williamson, D., Lu, Y.J., Fang, C., Pritchard-Jones, K., Shipley, J., 2006. Nascent pre-rRNA overexpression correlates with an adverse prognosis in alveolar rhabdomyosarcoma. *Genes Chromosom. Cancer* 45, 839–845.
- Wise, D.R., Thompson, C.B., 2010. Glutamine addiction: a new therapeutic target in cancer. *Trends Biochem. Sci.* 35, 427–433.
- Wise, D.R., DeBerardinis, R.J., Mancuso, A., Sayed, N., Zhang, X.-Y., Pfeiffer, H.K., Nissim, I., Daikhin, E., Yudkoff, M., McMahon, S.B., 2008. Myc regulates a transcriptional program that stimulates mitochondrial glutaminolysis and leads to glutamine addiction. *Proc. Natl. Acad. Sci.* 105, 18782–18787.
- Woods, A., Johnstone, S.R., Dickerson, K., Leiper, F.C., Fryer, L.G., Neumann, D., Schlattner, U., Wallimann, T., Carlson, M., Carling, D., 2003. LKB1 is the upstream kinase in the AMP-activated protein kinase cascade. *Curr. Biol.* 13, 2004–2008.
- Woods, A., Dickerson, K., Heath, R., Hong, S.-P., Momcilovic, M., Johnstone, S.R., Carlson, M., Carling, D., 2005. Ca^{2+} /calmodulin-dependent protein kinase kinase- β acts upstream of AMP-activated protein kinase in mammalian cells. *Cell Metab.* 2, 21–33.
- Wu, Z., Zha, Z., Li, G., Lieberman, B.P., Choi, S.R., Ploessl, K., Kung, H.F., 2014. [(18)F](2S,4S)-4-(3-fluoropropyl)glutamine as a tumor imaging agent. *Mol. Pharm.* <http://dx.doi.org/10.1021/mp500236y>.
- Yang, L., Song, T., Chen, L., Kabra, N., Zheng, H., Koomen, J., Seto, E., Chen, J., 2013. Regulation of SirT1-nucleomethylin binding by rRNA coordinates ribosome biogenesis with nutrient availability. *Mol. Cell. Biol.* 33, 3835–3848.
- Yuan, X., Zhao, J., Zentgraf, H., Hoffmann-Rohrer, U., Grummt, I., 2002. Multiple interactions between RNA polymerase I, TIF-IA and TAF(I) subunits regulate preinitiation complex assembly at the ribosomal gene promoter. *EMBO Rep.* 3, 1082–1087.
- Yuneva, M., Zamboni, N., Oefner, P., Sachidanandam, R., Lazebnik, Y., 2007. Deficiency in glutamine but not glucose induces MYC-dependent apoptosis in human cells. *J. Cell Biol.* 178, 93–105.
- Yuneva, M.O., Fan, T.W., Allen, T.D., Higashi, R.M., Ferraris, D.V., Tsukamoto, T., Mates, J.M., Alonso, F.J., Wang, C., Seo, Y., Chen, X., Bishop, J.M., 2012. The metabolic profile of tumors depends on both the responsible genetic lesion and tissue type. *Cell Metab.* 15, 157–170.
- Zhao, J., Yuan, X., Frödin, M., Grummt, I., 2003. ERK-dependent phosphorylation of the transcription initiation factor TIF-IA is required for RNA polymerase I transcription and cell growth. *Mol. Cell* 11, 405–413.
- Zhao, Y., Butler, E., Tan, M., 2013. Targeting cellular metabolism to improve cancer therapeutics. *Cell Death Dis.* 4, e532.
- Zinzalla, V., Hall, M.N., 2008. Signal transduction: linking nutrients to growth. *Nature* 454, 287–288.
- Zoncu, R., Bar-Peled, L., Efeyan, A., Wang, S., Sancak, Y., Sabatini, D.M., 2011. mTORC1 senses lysosomal amino acids through an inside-out mechanism that requires the vacuolar H^{+} -ATPase. *Science* 334, 678–683.

Appendix 2:

Kaplan-Meier curve of C57BL/6 mice transplanted with drug-naïve Eμ-Myc B-lymphoma cells (clone #4242) treated with vehicles (everolimus vehicle: 1% methylcellulose; CX-5461/metformin vehicle: 25 mM NaH₂PO₄); CX-5461 (35 mg/kg every Monday, Wednesday and Friday) and everolimus (5 mg/kg daily), metformin (500 mg/kg once daily), or CX-5461, everolimus and metformin (35 mg/kg every Monday, Wednesday and Friday, 5 mg/kg daily and 500 mg/kg daily, respectively)

