Contents

Abbreviations

Abbreviation	Term
mORF	main open reading frame
RPF	Ribosome Protected Fragment
TOP	Terminal oligopyrimidine
TE	Translation Efficiency
UTR	Untranslated region
uORF	upstream open reading frame

1. Introduction

1.1. Gene expression

1.1.1. The central dogma of gene expression The whole genetic code of an organisms i stored as deoxyribonucleic acid (DNA) molecules in a double stranded formation as chromosomes. Chromosomes hold the DNA in a condensed state using chromatin, a complex of DNA and proteins, structures. For transcription of DNA to occur chromatin is remodeled to expose promotor regions in the DNA to which fators assisting in transcription bind. One of these factors is DNA polymerase that unravels the double-stranded DNA and creates a single-stranded copy called ribonucleic acid (RNA) transcripts. This copy is less stable due to its single strandedness and therefore only temporary. The RNA transcripts undergo processing by which multiple different transcript varaints coming from the same genomic region can be produced. The protein coding portion of these transcripts are called mRNAs. Once formed mRNAs are transported into the cytoplasm where they are either degraded or associate with ribosomes. These ribosomes translate the mRNAs into proteins by which the genetic information then is expressed. Synthesised proteins, if no longer needed in the cell, can be degraded by proteosomes (**see figure 1.1**). This flow of genetic information into expressed proteins is commonly referred to as the central dogma in molecular biology (F. Crick, 1970).

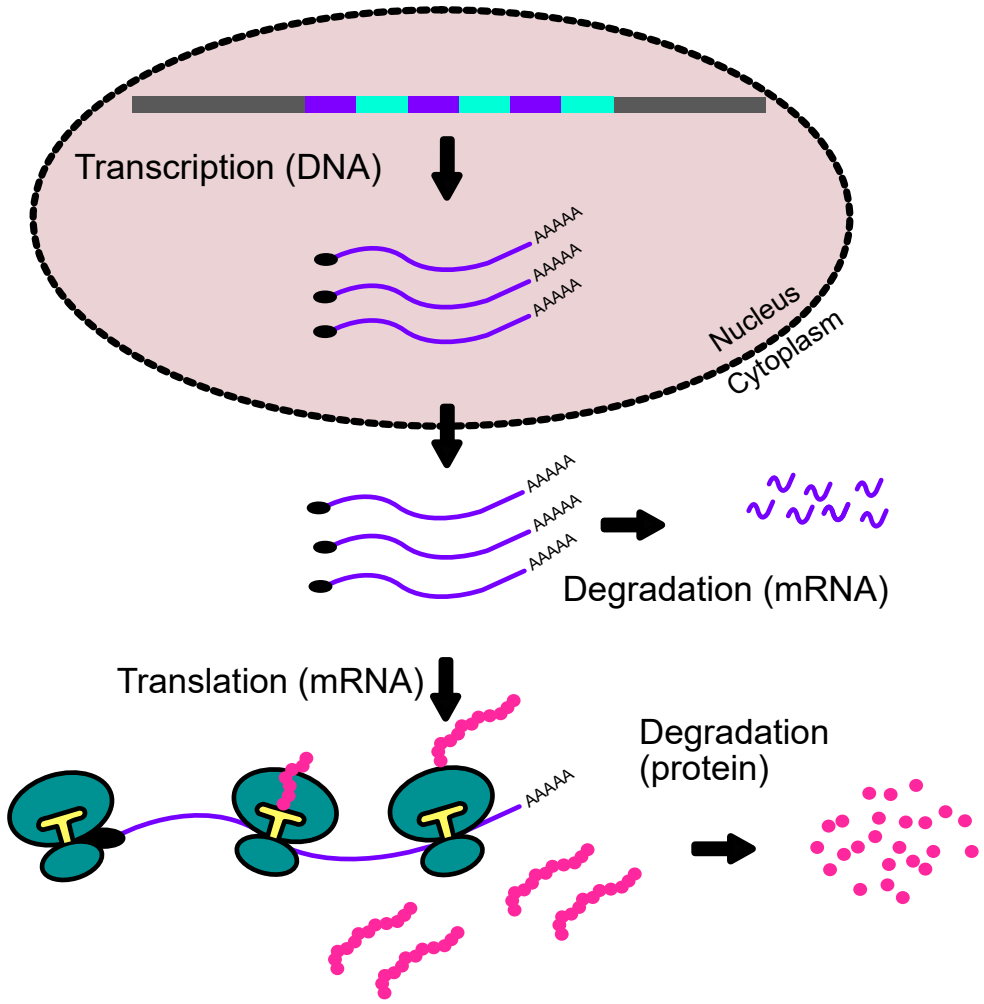

Figure 1.1: The gene expression pathway - DNA is transcribed in pre-mRNA containing a 5' cap (black oval) introns (teal boxes),exons (purple boxes) and a poly(A) tail. RNAs are processed into mRNAs consisting out ouf a 5' cap,exons and a poly(A) tail is then transported out of the cellular nucleus into the cytoplasm. Within the cytosplasm mRNAs can be degradated or translated into proteins depending on cellular demands. Synthesised proteins can be degraded by proteosomes.

1.1.2. Contribution to gene expression Proteins are the last product of the gene expression pathway and carry out the vast majority of all cellular functions. While it is apparent that modulation of protein levels will offer information on the changes in gene expression, it cannot completely answer the question as to why the levels change. In a disease context, protein levels alone might offer sufficient insight to explain phenotypic differences. However, how these differences arise machanistically, which often poses as target for therapeutic strategies in cancer, is obscured.

Experimental methods to measure gene expression at different steps are often to referred as "omics" (i.e. proteomics for protein expression, transcriptomics for mRNA expression). These methods provide snapshots of the step under scrutiny in a specific context (steady state or perturbation) for a large portion of genes or proteins. Transcriptomics studies approach gene expression with the assumption that mRNA expression results in protein expression changes and can therefore be used as a proxy. However, this view got challanged by landmark studies that observed a poor mRNA to protein correlation and indicated a larger role of post transcriptional regulation in gene expression than previously assumed (J. Lu, Tomfohr, & Kepler, 2005,Vogel & Marcotte (2012),de Sousa Abreu, Penalva, Marcotte, & Vogel (2009),Schwanhäusser et al. (2011),G. M. Silva & Vogel (2016)).

The debate on which step of the gene expression pathway contributes most is ongoing, nevertheless an understanding has been reached that the cellular context is a major determinant. At steady state mRNA levels seem to explain protein abundance best, however in perturbed systems the contribution of transcript abundance is shifted away to other steps (Y. Liu, Beyer, & Aebersold, 2016). For example in a study that challanged immune cells, protein levels were dependent on cellular transcript levels (Jovanovic et al., 2015). In contrast a study investigating cells under stress observed extensive modulation at the protein levels, whereas mRNA transcript abundance was only mildly affected (Cheng et al., 2016).

While the contribution of different steps of the gene expression is dependent on many different factors, e.g. cellular state or treatments, mRNA translation (synthesis of proteins) is an essential process of this pathway. Furthermore, dysregulation of mRNA translation has been observed in a plethora of diseases, ranging from neurological disorders to cancer which warrants for a comprehensive understaning of

this process (Kapur & Ackerman, 2018,Ruggero (2013),L. J. Lee et al. (2021),Graff et al. (2009)). This thesis will focus on the role of mRNA translation in gene expression in the context of cancer.

1.2. mRNA translation

1.2.1. Overview of an mRNA After transcription primary RNA transcripts are processed into mRNAs which is the product that will be translated into proteins. The coding region of an mRNA is flanked by untranslated regions (5' and 3' UTRs) that exert translational control over the mRNA (see 1.3). The 5' has a cap that is important for mRNA translation initiation(Grifo, Tahara, Morgan, Shatkin, & Merrick, 1983), while the 3' end has a poly-A tail protecting the mRNA against degradation (Wilusz, Wormington, & Peltz, 2001). Multiple different mRNAs (isoforms or transcript variants) from the same genomic region exist. These variants can arise due to a process called alternative splicing which alters the exon composition (i.e. coding region) of an mRNA. These variants can co-exist at the same time and have distinct properties and can perform distinct functions (Joly Anne-Laure et al., 2018).

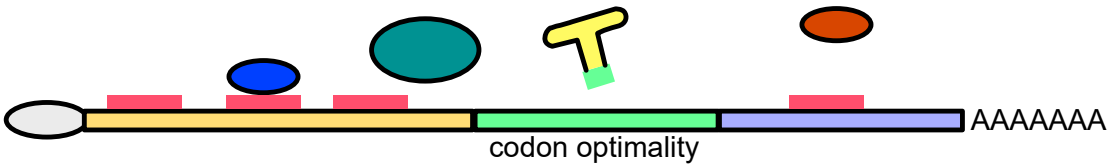

Figure 1.2: An mRNA consists of a coding sequence (green), 5' (light orange) and 3' (purple) untranslated regions flanking the coding sequence, a 5' cap and a poly-A tail. Located within the 5' and 3' untranslated region are cis elements (light red boxes) that can exert translational control by interfering with ribosomal movement along the mRNA or interact with trans factors (blue and red) or recruit the 43s ribsome (dark green) to the mRNA (see section 1.3). The codon composition of the coding sequence influences translation elongration rates (see section 1.3.3).

1.2.2. Translation of an mRNA For the vast majority of protein coding mRNAs, eukaryotic mRNA translation occurs in the cytoplasm, however a small subset of mRNAs is translated in the mitochondria. mRNA translation is a process that includes initiation, elongation, termination and ribosome recycling and is an essential process **see Figure 1.3**. During the initiation phase a ribome will associate with the mRNA and starts scanning along the mRNA for a start codon to begin synthesis of the polypeptide chain by incorporating amino acids. Amino acids are transferred to the ribosome by specialised RNAs called transfer RNAs (tRNA) that can recognise the genetic code in the mRNA. The availability of tRNAs as well as their modification can influence the rate of elongation (see 1.3.3). The order by which amino acids are incorporated is dictated by the order of the codons of the open reading frame (ORF). Redundancy in the codon availability allows that amino acids are encoded by codons, for example lysine is encoded by AAA and AAG. Once the ribosome encounters a stop codon translation will terminate and the polypeptide chain will be released. The ribosome then disassociates from the mRNA and the ribosome can be recycled to engage translation of the same or another mRNA. The following setions describe these processes in more detail.

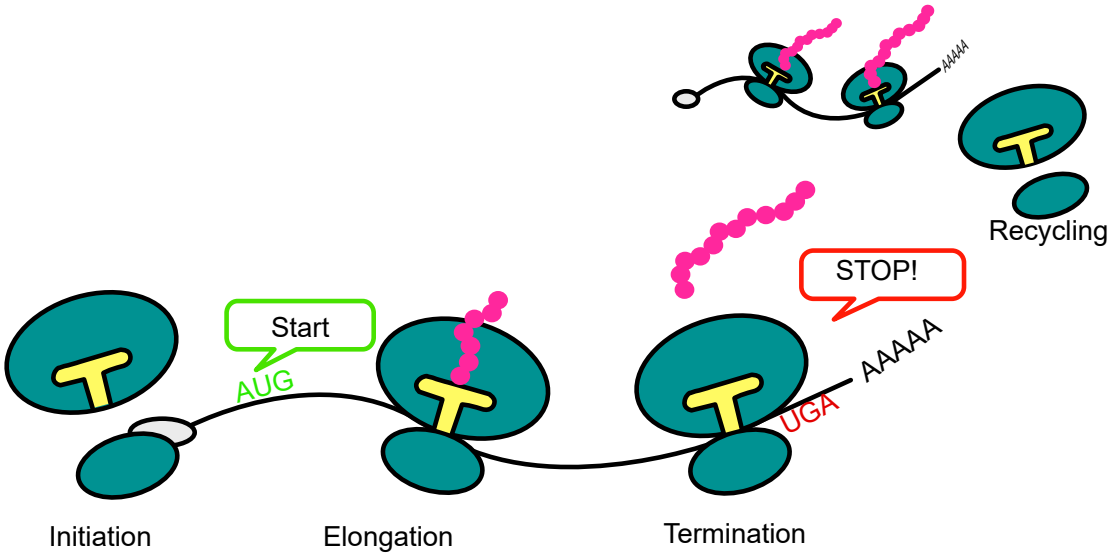

Figure 1.3: mRNA translation initiation, elongation, termination and ribosome recycling steps - The ribosome binds to the mRNA and initiates scanning for a start codon. The elongation phase incorporates amino acids into a polypeptide chain (i.e. the protein product). Once the end of the coding sequence is detected, by recognition of stop codons, the ribosome terminates translation and releases the polypeptide chain. The ribosome can then be recycled to participate in the translation of another mRNA or reinitiate.

1.2.3. Initiation For the initiation to commence, in eukaryotes, two complexes are required; the pre-initiation complex (PIC) and the eukaryotic initiation factor 4F (eIF4F) complex. Both these complexes are governed by signalling pathways that regulate their availability dependent on cellular cues (**see section 1.3**). The PIC consists of the methionyl-initiatior transfer RNA (met-tRNAi) in a ternary complex (TC) with guanosine triphosphate (GTP) bound eIF2 (Asano, Clayton, Shalev, & Hinnebusch, 2000). eIF4F is the translation initation complex containing three eIFs; eIF4E, the 5' cap binding protein, eIF4G a scaffold protein and eIF4A and RNA helicase (Grifo et al., 1983,Hinnebusch (2006)). eIF4F recruites the PIC to the 5' cap of the mRNA after which scanning for a start codon (AUG) occurs. After AUG recognition eIF2-GTP is hydrolyzed forming a stable 48S PIC. Afterrelease of eIF2-GTP the 60S ribosomal subunit joins to form the 80S ribosome and protein synthesis can start (**Figure 1.2.3**).

1.2.4. Elongation The 80S ribosome contains three sites important for decoding an mRNA; the acceptor (A), peptidyl (P) and Exit (E) sites. During elongation in eukaryotes aminoacytelated tRNAs are delivered to the A-site in a ternary complex with eukaryotic elongation factor 1A (eEF1A). When the tRNA recognises its cognate codon and pairs, a bond between the amino acid and the polypeptide chain is formed. The formation of the bonds causes the ribosomal units to rotate in relation to each other (Munro, Altman, O'Connor, & Blanchard, 2007,Moazed & Noller (1989)). The rotation causes a shift of the tRNA acceptor ends from the A and P to the P and E sites, wheras the codon end remains in the A and P site. This is the "hyrbid" state of the tRNAs in the ribosome(Dorner, Brunelle, Sharma, & Green, 2006). eEF2 then promotes the translocation by which the codon ends of the tRNA follow into the P and E sites. The deacytelated tRNA is then released from the ribosome. This process is repeated until a stop codon (UAA, UGA or UAG) is detected by the ribosome(C. U. T. Hellen, 2018)].

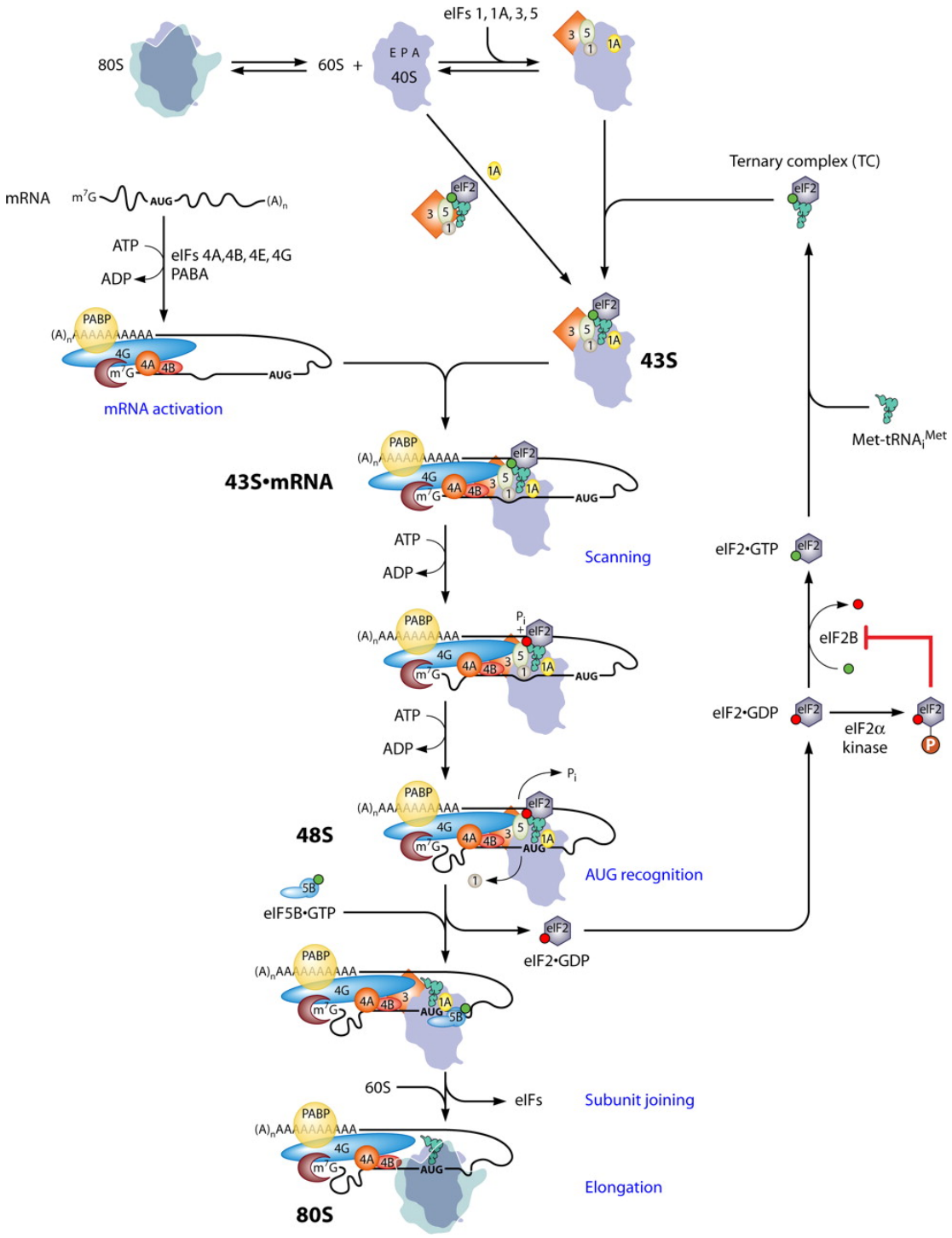

Figure 1.4: Pathway of eukaryotic translation initiation via ribosomal scanning.

1.2.5. Termination and recycling mRNa translation termination is facilitated by two eukaryotic release factors (eRF), eRF2 and eRF3-GTP(I. Stansfield et al., 1995,Alkalaeva, Pisarev, Frolova, Kisselev, & Pestova (2006)). A TC containing eRF2 and eRF3-GTP binds to the A-site of the ribosome upon recognition of a stop codon. This causes an ATP hydrolysis event resulting in a conformational change and release of the polypeptide chain. eRF1 and the ATP binding cassette protein (ABCE1) together promote the splitting of the 60S and 40S subunits after which they can be recycled(Pisarev et al., 2010,Dever & Green (2012),C. U. T. Hellen (2018)).

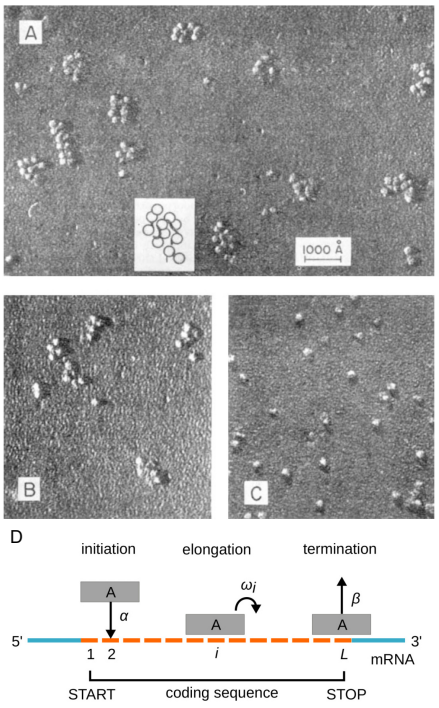

Figure 1.5: Electromicrongraph of ribosomes extracted from different positions along a sucrose gradient used for polysome fractionation (A-C). For details on polysome fractionation see section 1.4. Reprinted with permission. DR. T. STAEHELIN et. al. Nature.1963 Aug 31;199:865-70.doi: 10.1038/199865a0. Copyright © 1963, Nature Publishing Group. Schematic of the TAPES method - A ribosome (A) can initiate with rate α on an mRNA with a coding sequence with codons i= 1 ... L. The elongation rate at a specific codon is defined by ω_i and β determines the termination rate once a stop codon is encountered. TASEP has been constantly modified, e.g. to allow for correction of initiation or elongation when the following codon is already occupied. Reprinted with permission. Juraj Szavits-Nossan and Martin R. Evans. 10.1103/PhysRevE.101.062404. ©2020 American Physical Society.

1.2.6. Translation efficiency Each ribosome synthesises a single protein during translation of an mRNA assuming it is not prematurely terminated. It has been known since the '60s that translation of an mRNA occurs via multiple bound ribosomes (polysomes) simultaneaously (**see figure 1.5A-C**)(Warner, Rich, & Hall, 1962,Staehelin, Brinton, Wettstein, & Noll (1963)).

The translation efficiency of an mRNA depends on the number of ribosomes it is associated with synthesising proteins. While all steps of mRNA can affect the translation efficiency of an mRNA, it is most commonly regulated at the initiation step(Richter & Coller, 2015,Dever & Green (2012),Jackson, Hellen, & Pestova (2010)).

Assessment which step of mRNA translation is affected is often done using experimental methods (e.g. polysome profiling or by measuring ribosome transit

time). Furthermore, there are efforts to model translation kinetics such as totally asymmetric simple exlcusion process (TASEP) to obtain a better understanding of ribosome movements along the mRNA (MacDonald, Gibbs, & Pipkin, 1968,Maniloff (1969)) (**See figure 1.5D**). A model that assessed ribosome traffic under steady state and high initiation rates indicates traffic is not only dependent on the densities of ribosomes but also condon specific elongation rates and their distribution along the mRNA (Szavits-Nossan & Evans, 2020). The next section will go furhter into detail how mRNA translation can be regulated so that intiation rates, but also elongation, are influenced thereby altering translation efficiencies of mRNAs.

1.3. Regulation of mRNA translation

mRNA translation is the most energy consuming process in the cell, for example in rat it was estimated to account for ~20% of the cellular energy consumption (Buttgereit & Brand, 1995). The role of mRNA translation in gene expression and high energy consumption therefore translational control is paramount, especially in cancer where deregulated metabolism is common(Hanahan & Weinberg, 2011).

A strong feature of translational control of initiation rates or elongation rates is that it can affect mRNA distinct populations differently. When one or more components of the translation machinery are affected (e.g. initiation factors, ribosomal proteins, tRNA availability) translation of a large set of mRNAs is affected (i.e. global regulation). Whereas translational control acting on characteristics of mRNAs, e.g. through cis elements in the UTRs or RNA binding proteins (RBP), selective regulation occurs. In some cases mRNAs escape global translational control.

Major signalling pathways, e.g. the PI3K/AKT/mTOR pathway and the integrated stress response, regulate translation at a global level. Furthermore, the codon usage of mRNAs can be used to regulate translation globally dependent on the available tRNA pool or tRNA modifications. Selective regulation of mRNA translation occurs via characteristics a limitited population of mRNA share found in the 5' and 3' UTRs of an mRNA (Leppek, Das, & Barna, 2018) (**see figure 1.2**).

1.3.1. mTOR mTOR is a conserved Ser/Thr kinase and is found in two structurally and functionally distinct complexes, mTORC1 and mTORC2 (Saxton & Sabatini, 2017,Pearce et al. (2007)). In a growth promoting environment mTOR switches cell metabolism to increased production of protein, lipids and nucleotides, while suppressing catabolic pathways. mTORC2 promotes survival via signalling through protein kinase A (AKT) (Sarbassov, Guertin, Ali, & Sabatini, 2005).

mTOR activity is modulated via growth factor signalling (i.e. insulin and insulin-like growth factor; IGF1) as well as cellular metabolism. Growth factor signalling es mediated through the phosphoinositide 3-kinase (PI3K) / AKT and Ras/mitogen-activated protein kinase (MAPK) pathways. Both these signalling pathways are involved in oncogenic signalling and under investigation as therapeutic targets in cancer(Hilger, Scheulen, & Strumberg, 2002,J. Yang et al. (2019)). In several cancers (e.g. breast, lung, prostate and colon) the gene

encoding the p110α subunit of PI3K is frequently mutated or amplified (D. A. Levine et al., 2005,Samuels et al. (2004),J. W. Lee et al. (2005)). The E545K mutation which leads to a reduced inhibition of p85 at the p110α subunit (W. Jiang et al., 2018). In **study 3** we investigate oncogenic signalling via the PI3K pathway activated by insulin and the role of mTOR in mediating the resulting effects on gene expression in the MCF7 breast cancer cell line that harbours the E545K mutation (Schneck et al., 2013). Furthermore, hyperactivity of PI3K/AKT and Ras/MAPK signalling pathways has been reported in multiple cancers and been linked anti- cancer therapy resistance (Pópulo, Lopes, & Soares, 2012,Tan & Yu (2013), Salaroglio, Mungo, Gazzano, Kopecka, & Riganti (2019)). mTOR, as a downstream actor of these pathways integrating their singals, has therefore become a focus of anti cancer therapy by either targetting mTORC1 or using dual inhibitors for PI3K and mTOR (Bhat et al., 2015).

mTOR fulfills a central role in metabolic signalling where it integrates amino acids availability, glucose and cellular oxygen levels to form an appropriate response. For example, amino acid availabilty induces relocalisation of mTOR into proximity of Rag GTPases leading to its activation (Sancak et al., 2008). Furthermore, Glucose deprivation and oxygen availability regulate mTOR activity via adenosine-mono-phosphate kinase (AMPK) signalling. AMPK is activated when a shift in the cellular AMP to adenosine-tri-phophate (ATP) ratio is sensed (Sanders,

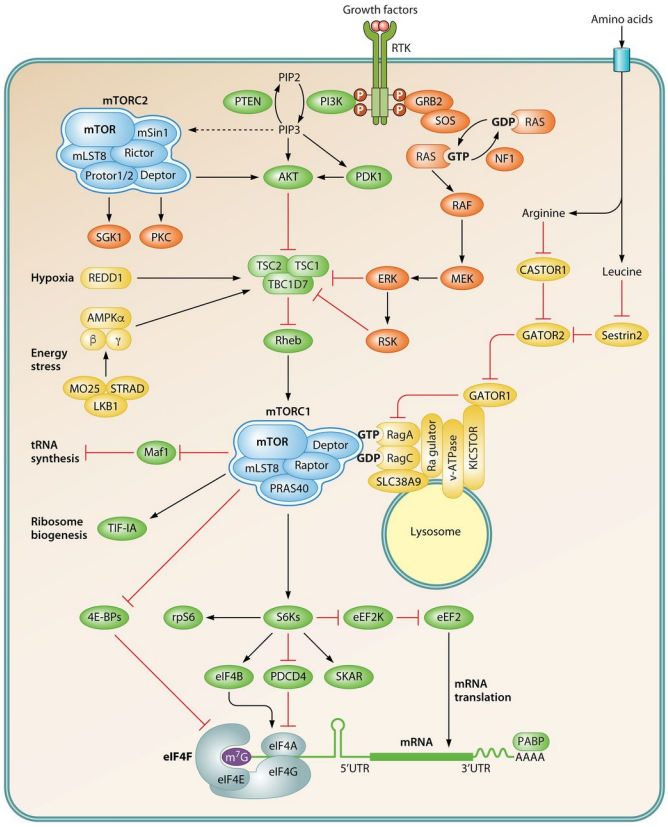

Figure 1.6: Schematic representation of mTOR signaling to the translational machinery. Philippe P. Roux, and Ivan Topisirovic Mol. Cell. Biol. 2018; doi:10.1128/MCB.00070-18. Reprinted with permission. Copyright © 2018, American Society for Microbiology

Grondin, Hegarty, Snowden, &
Carling, 2007,Kimball (2006)). While hypoxia, deprevation of oxygen, inhibits protein synthesis in normal cells, in breast cancer protein synthesis was not inhibited during hypoxia attributed to uncontrolled mTOR signalling (Connolly, Braunstein, Formenti, & Schneider, 2006).

Given the central of mTOR governing proliferation, growth and metabolism, which are often deregulated in cancer, it is vital to comprehensively understand mTORC1 signalling herein (Hanahan & Weinberg, 2011).

1.3.1.1 Global regulation of translation via mTOR

mTOR regulates mRNA translation mainly by mediating availability of eIF4E by phosphorylating 4E binding proteins (4E-BPs)(A.-C. Gingras et al., 1999). eIF4E is required for mRNA 5' cap binding and formation of the mRNA translation eIF4F complex. Therefore, inhibtion of mTOR leads to a down regulation of cap dependent mRNA translation initiation. eIF4E has overexpression is commonly found cancers and targetted using 4E-antisense oligonucleotides as anti-cancer therapy (D. S. Hong et al., 2011). Other downstream targets of mTOR such as S6k which has been shown to regulate components of the translation machinery, e.g. by activiation of programmed cell death protein 4(PDCD4) which disrupts eIF4G-eIF4A binding through competition(, Dorrello et al., 2006,A. Göke et al. (2002)).

1.3.1.2 Selective or "mTOR sensitive" regulation of translation

translation involves transcripts with a terminal oligo pyrimidine (TOP) motif consisting of a C followed by a stretch of 4-15 pyrimidines directly after the 5' cap show near complete dissociation from ribosomes under conditions when mTOR is inhibited (Meyuhas, 2000,Yamashita et al. (2008)). TOP mRNA are enriched for genes encoding for parts of the translation machinery (Meyuhas, 2000,Thoreen et al. (2012)). Recent work indicates the importance of La ribonucleoprotein domain family member 1 (LARP1) in regulation of TOPs. LARP1 is thought to bind to the 5' mRNA cap of TOP mRNAs via its DM15 domain and represses translation by obstructing eIF4E binding. mTORC1 physically interacts and phosphorylates LARP1. When phophorylation occurs close to the DM15 domain of LARP1 the

inhibitory effect on mRNA translation of TOP mRNAs is abolished (Jia et al., 2021). Other instances of selective translation are for mRNAs that lack the TOP motif, but show sensitivity to mTOR activity. These mRNAs are, in addition to mTOR, dependent on either availability of eIF4E or activity of eIF4A (**see 1.3.4**). mTOR-eIF4E sensitive mRNAs show extremely short 5' UTRs and encode for metabolic functions(Gandin et al., 2016b).

1.3.2. The integrated stress response is a signalling pathway which can be activated through kinase signalling orginating from various stress signals. These kinases include Protein kinase R-like endoplasmic reticulum kinase (PERK) which is activated by misfolded peptides in the endoplasmatic reticulum (ER), Heme regulated eIF2alpha kinase (HRI) which is activated during oxidative stress, protein kinase R (PKR) which is activated in response to certain viral infections and GCN2 which is activated when cells are deprived of amino acids(Kapur, Monaghan, & Ackerman, 2017,Guan et al. (2017),Taniuchi, Miyake, Tsugawa, Oyadomari, & Oyadomari (2016),Andreev et al. (2015)). During the integrated stress response the alpha subunit of eIF2 is phosphorylated. Upon eIF2alpha phosphorylation, eIF2 alpha directly engages the guanine nucleotide exchange factor eIF2beta and precents conversion of inactive eIF2-GDP to active eIF2-GTP needed for met-tRNAi incorpration in the TC, therefore inhibiting translation by reducing PIC availability (Sonenberg & Hinnebusch, 2009) (**see also figure 1.4**).

1.3.2.1 Global and selective regulation of translation via the ISR

is, similar to mTOR signalling, achieved at a global and selective mRNA level. Phosphorylation of eIF2 alpha limits ternary complex availability, therefore ribsome recruitment to the 5' cap is limited which results in a reduction of translation initiation. While global translation is reduced upon ISR, translation of a selective subset of mRNA with upstream open reading frames (uORFs) is increased. A uORF is a reading frame that originates in the 5' UTR of an mRNA upstream of which the AUG precedes that of the coding sequence. uORFs are out of frame with the main ORF and when translated lower the expression of the mORF(Kozak, 1984). Ribosome profiling studies indicate that 50% of mammalian mRNAs harbour uORFs including oncogenes and transcripts important in differentiation and cell cycle (Calvo, Pagliarini, & Mootha, 2009,Ingolia, Lareau, & Weissman (2011),D. R. Morris (1995)). The surrounding context of the uORF is important for its inhibitory effect through more efficient initiation, where the classical Kozak context (i.e. [A,G]..**ATG**G) is most efficient(Kozak, 1986,Calvo et al. (2009)). ATF4, a transcription factor for stress response genes, contains two uORFs of which one partially overlaps with the mORF. Under normal conditions ATF4 mRNA translation is initiated at uORF1 and reinitiation at uORF2 occurs. The close proximity of uORF2 to the mORF causes ribosomes to scan past the mORF

start thereby inhibiting the translation of the coding sequence. Limitiation of TC availability during ISR causes longer ribosome scanning times leading to that ribosomes scan past uORF2 and initiate at the mORF(Pakos-Zebrucka et al., 2016).

1.3.3. Regulation of mRNA translation by tRNAs As touched upon earlier, tRNAs are an essential part of the translation machinery that carry the amino acids, to be incorporated into the polypeptide chain during the elongation process, to the ribosome. tRNAs consist of a 76-90 long nucleotide sequence set into a "cloverleaf" structure forming several loops (Sharp, Schaack, Cooley, Burke, & Soil, 1985) (** see figure tRNA**). The acceptor stem binds the amino acid carried by the tRNA, while the anti-codon loop binds to the mRNA within the ribosome via classical watson-crick pairing (Watson & Crick, 1953). Multiple codons can encode for the same amino acid (synonymous codons), however the availability of the tRNAs for different codons may vary which can influence elongation rates.

This supply (i.e. tRNA availability) and demand (i.e. codon in expressed mRNA transcripts) relationship has been found to vary across different cellular states, e.g. profiferation and differentation. In this model two distinct subsets of tRNAs are oberved; A tRNA subset induced under proliferation that is otherwise repressed and a subset with similar regulation under differentiation of which the supply matches the codon demand of the transcriptome (Gingold et al., 2014). Therefore, in this model differentiation and

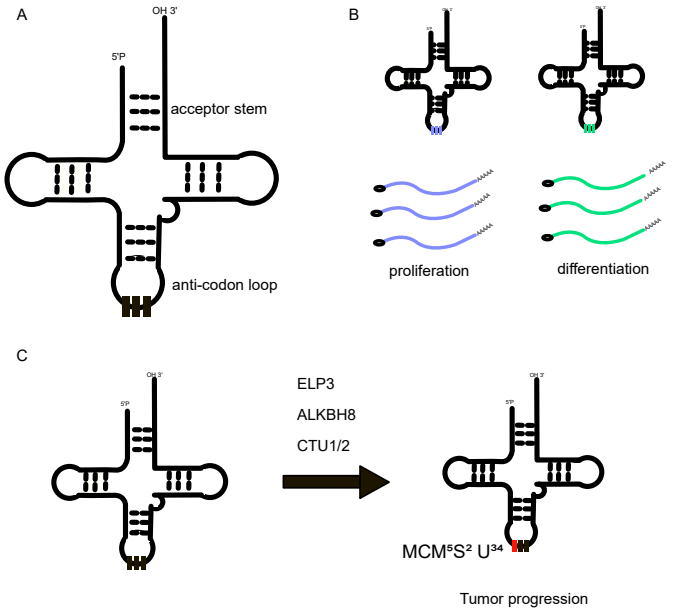

Figure 1.7: (A) Schematic representation of the tRNA cloverleaf structure with indicated anti-codon and amino acid acceptor sites. (B) Schematic representation of the proliferation and differentiation mRNAs dependent on distinct tRNA subsets. (C) Schematic representation of the U34 wobble position and the catalytic enzymes involved in this modifcation that is implied in tumor progression.

proliferation would underly two distinct translational programs dependent on tRNA expression. This model has been disputed and that the observed differences would be attributed to GC content in the mRNA (Rudolph et al., 2016). Nevertheless, abbarent tRNA expression and codon usage have been reported in cancer(Z. Zhang et al., 2018). Furthermore, a comprehensive study including small RNAseq (i.e. for identification of tRNAs) and protein samples across 17 tissues obtained from the The Cancer Genome Atlas (TCGA) reported a tRNA signature stratified by Ki67 (a proliferation marker) staining with implications for patient survival(Hernandez-Alias, Benisty, Schaefer, & Serrano, 2020). Therefore, while a consensus on proliferation specific tRNA subsets might not have been reached, emerging evidence implicates a role thereof in cancer(Hernandez-Alias et al., 2020,Gingold et al. (2014),Z. Zhang et al. (2018)).

Other reports of translational intereference by tRNAs in cacner are attributed to tRNA modifications, specifically at the U34 anti- codon (or wobble) position which is highly conserved (Rapino, Delaunay, Zhou, Chariot, & Close, 2017,El Yacoubi, Bailly, & de Crécy-Lagard (2012)). Wobbling was proposed by Francis Crick and refers to the ability of non-watson-crick base pairing of tRNA anti codons(F. H. Crick, 1966). This enables a smaller set of tRNAs (41-55 in eukaryotes) to encode for the 64 possible codon combinations (Goodenbour & Pan, 2006). In mammals, the U34 modification catalytic cascade involves the acetyltransferase Elongator (ELP3), the methyltransferase TRM9-like domain of Alkylation repair homolog 8 (ALKBH8), and the urmylation (URM) pathway, that includes the cytosolic thiouridylase homolog 1 and 2 (CTU1/CTU2)(Kalhor & Clarke, 2003,Karlsborn et al. (2014)). These enzymes ultimately modify the U34 modification into 5-methoxycarbonyl-methyl-2-thiouridine (mcm^5s^2U) which ensures cognate codon recognition. This modification is thought to occur for a small subset of tRNAs, namely $tRNA^{UUU}$, $tRNA^{UUC}$, $tRNA^{UUG}$, $tRNA^{UCC}$, and $tRNA^{UCU}$.

Loss of the ability to modify U34 has been shown to reduce translation elongation rates with varying effects on protein expression (Nedialkova & Leidel, 2015,Deng et al. (2015),Zinshteyn & Gilbert (2013)). While in some cases U34 dependent signalling led to ribosome stalling resulting in protein aggregates and increased stress (Nedialkova & Leidel, 2015,Zinshteyn & Gilbert (2013)). Other reported a subtle dowregulation of proteins encoded by mRNAs requiring U34-modified

tRNAs (Deng et al., 2015). U34 modifcation dependent tRNAs have been shown to play a role in cancer. For example, ELP3 is important in tumor initiation in the intestine and promotes breast cancer invasion as well as progresstion to metastisis(Ladang et al., 2015,Delaunay et al. (2016)). Furthermore, loss of U34 modification in a prostate cancer model lead to an adaptive transcriptional response for mRNAs whose translation was dependent on the U34 modification (Lorent et al., 2019).

1.3.4. eIF4A sensitive mRNAs Progression of the ribosome through the 5' UTR is dependent on eIF4A, the RNA helicase in the eIF4F complex, that unwinds structural elements the ribsome encounters(Wolfe et al., 2014,Rubio et al. (2014)). The importance of eIF4A's unwinding capacity was identified by treatment of KOPT-K1 cells, a lymphoma cell line, MDA-MB-231 ,a breast cancer cell line, with silvesterol of which translational control was evaluated using ribosome prolfing (**see section 1.4.2**)(Wolfe et al., 2014,Rubio et al. (2014)). These studies identified silvesterol sensitive mRNAs that are characterised by long and structured 5' UTRs. In addition eIF4A dependent mRNAs were enriched for having multiple 5' UTR variants, while independet mRNAs were not (Rubio et al., 2014). Among eIF4A dependent mRNAs are genes important for proliferation and survival which has implications for oncogenic signaling(Wolfe et al., 2014,Gandin et al. (2016b)). Among others, these studies indicate a strong therapeutic value in targetting "sensitive mRNAs" in diseases. In **study 2** we aimed to exploit eIF4A sensitve mRNA translation as a theapeutic strategy in pancreatic cancer.

1.3.5. RNA binding proteins and trans-acting factors The UTRs of an mRNA contain sequence elements to which RNA and RNA binding proteins (RBPs) bind and exert translational regulation. For instance, MicroRNAs, a small class of non coding RNA, can directly bind to other RNAs and silence them accomplished through translational repression or, more often, destabilisation [Jonas2015]. RBPs are a class of proteins involved in many regulatory steps of gene expression and account for $\sim 7.5\%$ of the protein coding genes. Poly-A-binding-protein (PABP) is thought to form a closed loop complex of the 3' end to the 5' by interacting with eIF4G. This closed loop should promote translation and prevent mRNA decay (Afonina, Myasnikov, Shirokov, Klaholz, & Spirin, 2014,Amrani, Ghosh, Mangus, & Jacobson (2008)) (**see also figure 1.4**). An RBP of particular interest in **study**

3 is Human antigen R (HuR). HuR preferentially binds to AU-rich sequences in the 3' UTR and acts as a stabilizing agent and is involved in RNA-processing (T. D. Levine, Gao, King, Andrews, & Keene, 1993,Baou, Norton, & Murphy (2011),X. C. Fan & Steitz (1998),S. S.-Y. Peng, Chen, Xu, & Shyu (1998)). Studies in breast, colon and lung cancer observed correlation between HuR and malignancy. Among HuR targets are HIF-1, VEGF (important for angiogenesis) and the oncogene Myc (Denkert et al., 2004,López de Silanes et al. (2003),López de Silanes, Lal, & Gorospe (2005)).

1.4. Expertimental methods to measure mRNA translation

Methods that measure mRNA translation try to capture the number of ribosomes an mRNA is associated with on the prinicple that this is directly correlated with their translation efficiency. There are two methods that are predominantly used for measuring mRNA translation, or changes in translation efficiencies across conditions, namely polysome profiling and ribosome profiling (**see figure 1.8**).

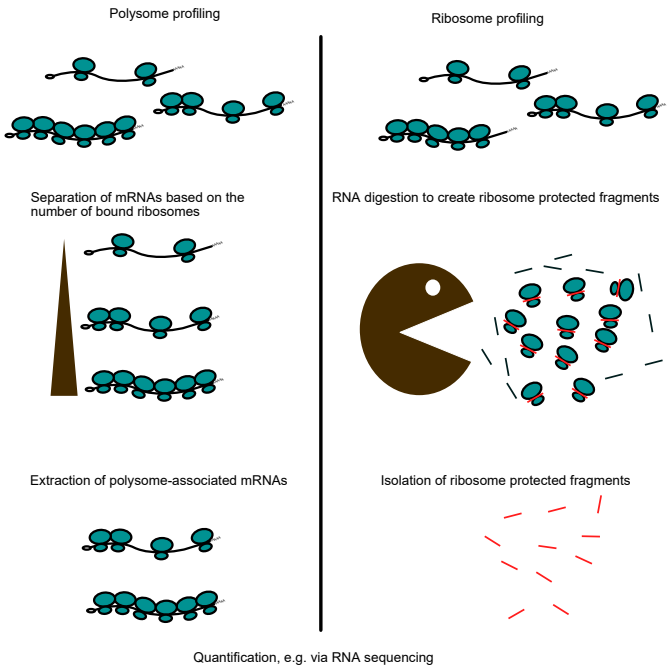

Figure 1.8: Polysome profiling and ribosome profiling workflows. In polysome profiling a fraction from whole cytoplasmic RNA is loaded onto a sucrose gradient on which they get separated by sedimentation using ultra centrifugation. Fractions corresponding to efficiently translated mRNAs are collected and can be quantified with for example RNA sequencing (left). During ribosome profiling a fraction from the whole cytoplasmic RNA is exposed to a digestion agent which disturbs the RNA. The ribosomes will protect fragments thereby creating ribosome procted fragments. These fragments are then isolated and can be sequenced.

1.4.1. Polysome profiling is a technique to measure changes in translational efficiencies of mRNAs between two or more conditions. Polysome profiling allows for separation of polysomes from monosomes, ribosomal subunits and messenger ribonucleoprotein particles (mRNPs). During the assay, ribosomes are immobilized on the mRNAs using translation elongation inhibitors (e.g. cycloheximide). A portion of cytoplasmic RNA extracts are then sedimented on a linear sucrose gradient (5-50%) using ultra centrifugation.

The resulting gradient is fractionated and mRNAs with different number of bound ribosomes can be extracted and analyzed for changes in translational efficiency (Gandin et al., 2014). An illustration of a polysome profile with peaks for the 40S, 60S subunits and 80S ribosome can be seen in (**Fig 1.9 top left**). Subsequent peaks along the frations indicate the mRNAs with 1 or more bound ribosome. mRNAs are typically normally distributed along the fractions, i.e. a pool of the same mRNA will be associated with 1- n number of ribosomes. Changes in mRNA abundance will lead to an overall increase in the amount of isolated polysome-associated mRNA without a shift of the distribution along the fractions (**Fig 1.9 top right**). This means that the translation efficiency per mRNA remains unchanged. Changes in translational efficiency can be observed by shifts of polysome association for mRNAs from the light (inefficiently translated) towards the heavy (efficiently translated) polysome fractions or vice versa (**Fig 1.9 bottom left**). Shift within the heavy polysome fractions (i.e. 3 bound ribosome to 7 bound ribosome) can also occur (**Fig 1.9 bottom right**). These shift remain undetected in cases

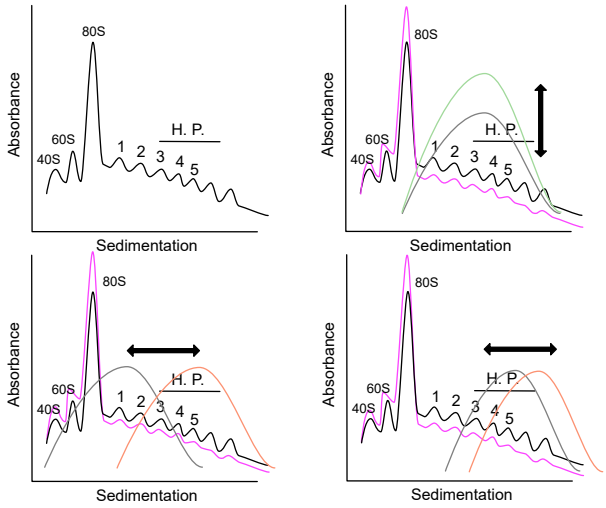

Figure 1.9: Polysome profiles - (top left) Schematic representation of a polysome profile using linear sucrose gradient fractionation. Indicated in the polysome profiles are the 40S, 60S ribosomal subunits as well as the 80S monosome. H.P. indicates heavy polysome fractions.Between conditions (i.e. black an pink lines) distribution changes for mRNA abundance (grey and green; top right), translation (grey and red; bottom left) and translation within high polysome fractions (grey and red; bottom right) are illustrated.

where the distribtion of polysome-associated mRNAs does not sufficiently shift across the fractions and is a limitation of polysome profiling. Quantification of mRNA levels within each fraction can be assessed using Northern blotting or reverse transcription quantitative polymerase chain reaction (RT-qPCR).

For transcriptome wide studies, pooling of efficiently translated mRNAs (mRNAs with >3 bound ribosomes) followed by quantification using either DNA-microarrays or RNA sequencing is common. The 3-ribosome cut off has been chosen as it is thought to capture most biologically relevant changes in translation efficiency. Pooling of mRNAs as well as collection of multiple fractions makes polysome profiling inconvenient when dealing with large samples sizes or experiments with low amounts of input RNA. Therefore, an optimized sucrose gradient was developed where most efficiently translated mRNAs are collected on a sucrose cushion and thereby can be isolated from one single fraction (Liang et al., 2018). This optimized gradient allows for application of polysome profiling in small tissue samples where RNA quantity is limiting and reduces labor intensity of the assay.

Polysome-associated mRNA levels are subject to changes in translation efficiency as well as factors contributing to cytosolic mRNA levels. Mechanisms such as transcription (i.e. in the case of mRNA abundance) or mRNA stability can affect cytosolic mRNA levels which impacts the pool of mRNAs that can be associated to polysomes. Therefore, to identify true changes in translation efficiency it is important to collect cytoplasmic mRNA levels in parallel to polysome-associated mRNA to correct for such mechanisms (e.g. transcription or mRNA stability) during downstream analysis (Gandin et al., 2014).

1.4.2. Ribosome profiling Ribosome profiling is a technique that enables sequencing of ribosome protected mRNA fragments (RPFs). In the assay ribosomes are immobilized on the mRNAs using, similar to polysome profiling, translation elongations inhibitors (e.g. cyclohexamide) (Ingolia, Ghaemmaghami, Newman, & Weissman, 2009,Ingolia (2016)). One limitation with the use of translation elongation inhibitors is the distortion of ribosome distributions especially at translation initiation sites. These introduced artefacts need to be accounted for in the downstream analysis when assessing ribosome position along the mRNA. Following the translation elongation inhibitor treatment, cells are immediately

flash frozen using liquid nitrogen. Alternatively, using only flash freezing has been seen as a robust approach in a wide range of diverse organisms (Brar & Weissman, 2015).

RPFs are obtained by RNAse treatment that breaks the links of RNA between ribosomes leaving single ribosomes with a ~28 nucleotide long RNA fragment within each ribosome. The RPFs are then isolated using ultra centrifugation through a sucrose cushion. During this step other RNA fragments such as non-coding RNAs or large ribonucleoprotein complexes can co-migrate and contaminate the sample. Typically RPFs with a size randing from 25-30 nucleotides are selected for quantification.

In parallel to RPF selection, randomly fragmented total mRNA of the same size is also retrieved. This is achieved by extraction of total mRNA from cell lysate followed by purification via recovery of polyadenylated messages or removal of ribosomal RNA. (Ingolia et al., 2009,Brar & Weissman (2015)).

1.4.3. Comparing ribosome and polysome profiling Albeit both methods generate count data after quantification with RNAsequencing, there are some key aspects that differ between the techniques. Polysome profiling separates efficiently translated mRNAs from non- efficiently translated mRNAs along a sucrose thereby creating an mRNA based perspective for analyzing changes in translational efficiencies. In contrast, ribosome profiling determines translational efficiencies by counting the number of RPFs of both efficiently and non-efficiently translated mRNAs. This gives polysome profiling the advantage in cases of transcript variants with important features in their 5´UTR. Such information, if not protected by a ribosome, would be lost in ribosome profiling.

Changes in translational efficiencies, e.g. shifts between the polysomal fractions, can be dramatic (I.e. near complete dissociation of ribosomes from an mRNA) or subtle (shifts from 2 to 4 ribosomes) (Livingstone et al., 2015). Ribosome profiling has been shown to be biased towards identification of dramatic shifts of associated ribosomes to mRNAs, whereas subtle shifts are masked which can lead to false biological conclusions. Polysome profiling is affected by this to a much lesser extent, thereby more robust in identifying such changes (Masvidal, Hulea, Furic, Topisirovic, & Larsson, 2017). Higher sensitivity in detecting changes in

translational efficiencies on a global scale makes polysome profiling more suitable for genome-wide studies (Gandin et al., 2016a).

An advantage of ribosome profiling is that it provides exact nucleotide positions occupied by ribosomes. This offers information at a single nucleotide level where the ribosome sits. Polysome profiling cannot reveal ribosome locations along the mRNA. The single nucleotide resolution of ribosome profiling is necessary in contexts studying local translation events such as ribosomal frame shifts (Rato, Amirova, Bates, Stansfield, & Wallace, 2011) or uORF translation (Andreev et al., 2015).

Both methods have their strengths and weaknesses and therefore each method should be considered depending on the underlying biological question of each experiment.

1.5. Modes for regulation of gene expression in mRNA translation

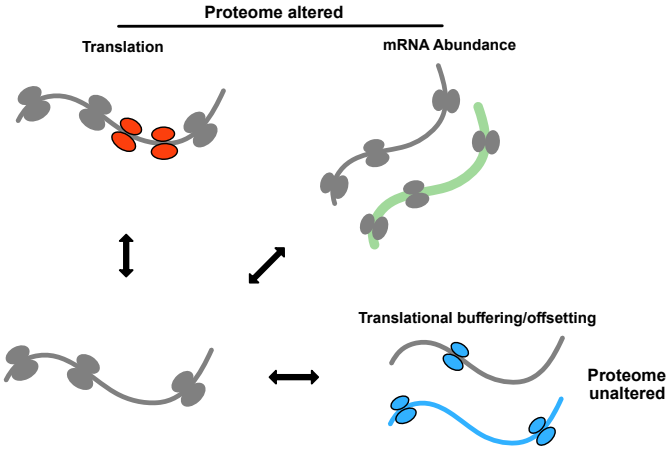

Figure 1.10: Regulatory modes of gene expression - Schematic representation of regulatory modes of translation efficiency in a fold-change scatter plot. Indicated in red are changes in translation (i.e. changes in translated mRNA but not total mRNA), in green changes in mRNA abundance (i.e. congruent changes between total mRNA and translated mRNA) and in blue translational buffering (i.e. changes in total mRNA levels but not translated mRNA levels). TE changes as the TE-score would estimate them are indicated.

As explained in the previous section, from genome-wide assessments of translation using ribosome or polysome profiling expression levels for both cytoplasmic and polysome-associated mRNAs (or RPFs). For the sake of simplicity , from now on, these RNA types will be referred to as total mRNA (i.e. cytoplasmic mRNA) and translated mRNA (i.e. polysome-associated mRNA or RPFs). The estimation of expression levels for both translated mRNA and total mRNA allows for interrogation at two steps of the gene expression pathway and their interaction. The interaction of total mRNA with translated mRNA can give valuable insights for the underlying mechanisms that govern gene expression in the studied system.

When comparing perturbed systems to their corresponding control state we typically observe three "modes" in which translated mRNA and total mRNA distinctly interact that impact gene expression (**See figure 1.5**). We refer to these modes as "translation", "mRNA abundance" and "translational buffering".

1.5.1. Translation A change in "translation" occurs when total mRNA levels remain unaltered, however translated mRNA levels either increase or decrease resulting in a change of their translation efficiency. A prominent example of this mode can be observed to TOP mRNAs. These mRNAs, under conditions when mTOR is inhibited, show a near complete dissassociated from ribosomes(Gandin et al., 2016b). mRNAs under the translation mode are expected to reshape the proteome (**See figure 1.5**).

1.5.2. mRNA Abundance Another mode that impacts the proteome is a change in mRNA abundance which its concept is straight forward, here the translated mRNA level changes to a similar magnitude as the total mRNA level. For these mRNAs the translation efficiency is unaltered, as the change in total mRNA levels explains the change in translated mRNA levels. Nevertheless, since there are more (or less) mRNAs that are being translated an effect the proteome is expected. The underlying biological implication for this mode mRNAs is often related to transcription (**See figure 1.5**).

1.5.3. Translational buffering In recent years, evidence emerged where translation efficiencies of mRNAs can be altered to compensate for changes in total mRNA levels. This mode is characterised by changes in total mRNA levels that are not matched by translated mRNA levels. The expected result on the proteome is that it maintaining (i.e. keeping levels constant) rather than reshaping it (McManus, May, Spealman, & Shteyman, 2014,Lorent et al. (2019)) (**See figure 1.5**).

This mode was named "Translational buffering" and currently the literature supports multiple different instances where translation "buffers" changes in transcription to retain a constant proteome. At steady state, translation compensates for inter-indivual, inter-species or inter-tissue differences

At steady state a compensatory aspect of translational buffering is observed when corrections are made, for e.g. gene dosage or transcriptional noise, at an inter-species or inter-individual level (Artieri & Fraser, 2014,C. Cenik et al. (2015),Perl et al. (2017),Z.-Y. Wang et al. (2020),G.-W. Li, Burkhardt, Gross, & Weissman (2014),Lalanne et al. (2018)). Which is consistent that protein abundance is overall more conserved across species (Laurent et al., 2010).

An example of such compensation was observed when comparing evolutionary distant bacteria species, i.e. B. subtilis, E. coli and S. cerevisiae. It was found that while extensive remodeling of promotors and terminators diverged transcript abundance, post-transcriptional regulation was altered to maintain a preferred stoichiometry of pathways(Lalanne et al., 2018). In B. subtilis translation related factors rpsP and rplS are transcriptionally fine tuned, whereas in E. coli they lie within an operon together with rimM and trmD which are only required in low

abundance at the protein level. Therefore, E. coli compensates the transcriptional input at the translational level(Lalanne et al., 2018).

A different form of translational buffering can be observed at in perturbed systems. For example, in prostate cancer cells a transcriptional program was induced under estrogen receptorα (ERα) depletion that was offset at the level of translation. mRNAs whose transcription was induced required the tRNA u34 modification, of which the catalytic enzymes were dependent on ERα, for efficient translation (Lorent et al., 2019). In **study 3** we discuss the occurence of translational buffering in its "offset" context.

1.6. Algorithms for analysis of changes in translation efficiencies

Given these multiple roles of mRNA translation to regulate the proteome it is critical to distinguish them as their underlying mechanisms can have different biological implications. In this section we will discuss methods that analyse polysome-profiling and ribosome profiling data to estimate changes in translation efficiencies across 2 or more conditions and how these methods identify different modes of gene expression.

Initially analysis of transcriptome-wide translation studies used an approach called the translation efficiency (TE-score) that uses the following equation:

$$\Delta TE = \frac{\frac{P_{c2}}{T_{c2}}}{\frac{P_{c1}}{T_{c1}}}$$

This score calculates the ratio of the ratios between polysome-associated mRNA levels (P) divided by total mRNA levels (T) within each condition (i.e. C1 and C2). The TE- score approach has been shown to be prone to spurious correlations (Larsson, Sonenberg, & Nadon, 2010). Spurious correlations arise due to that the ratio of polysome-associated mRNA and total mRNA can systematically correlate with total mRNA levels which is not corrected for in this equation and leads to an elevated type-1 error.

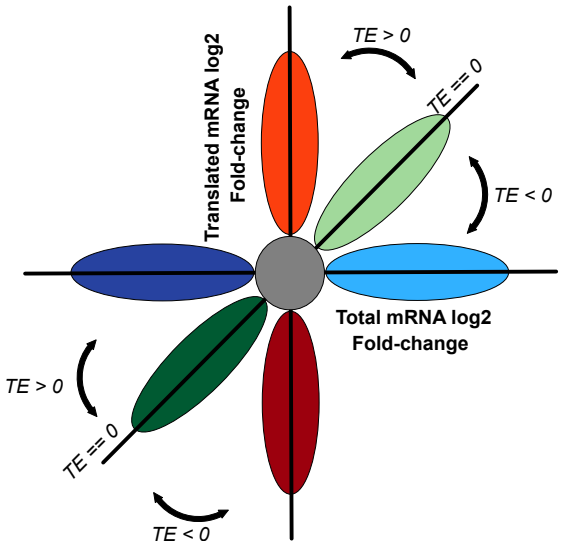

Figure 1.11: TE scores for regulatory modes of gene expression - Schematic representation of regulatory modes of translation efficiency in a fold-change scatter plot. Indicated in red are changes in translation efficiency altering protein levels, in green changes in mRNA abundance and in blue changes in translation efficiency leading to translational buffering/offsetting. The shifts for the translation efficiency (TE) score are indicated.

Figure 1.11 gives an overview of the relationship between a change in TE and each regulatory mode of gene expression (**see also figure 1.10**). Changes in mRNA abundance will lead to a ΔTE close to 0 in log space (i.e. no change) as total mRNA and translated mRNA change with a similar magnitude. However, in the case of both translation and translational buffering, terms in the TE-score equation change leading to a ΔTE (TE $<$ 0 or TE $>$ 0) and thereby identification of both changes in translation and translational buffering simultaneously. Therefore, the TE-score method fails to differentiate between changes in translation and translational buffering which can have drastic consequences for the biological interpretation of the results (Oertlin et al., 2019) (**see also section 1.5**).

The TE-score approach was challenged by the Analysis of Translation Activity (anota) algorithm which was developed for DNA-microarray data (Larsson, Sonenberg, & Nadon, 2011). anota combines analysis of partial variance (APV)(Schleifer, Eckholdt, Cohen, & Keller, 1993) with a random variance model (RVM)(G. W. Wright & Simon, 2003). RVM estimates gene variance using shared information across all genes to increase power for detection of differential expression(G. W. Wright & Simon, 2003). anota uses a two-step process that firstly assesses the model assumptions for (i) absence of highly influential data points, (ii) common slopes of sample classes, (iii) homoscedasticity of residuals and (iv) normal distribution of per gene residuals. In the second step then performs analysis of changes in translational activity using the following model:

$$log(y_{gi}) = \beta_g^{RNA} \; X_i^{RNA} + \beta_g^{cond} \; X_i^{cond} + \varepsilon_{gi}$$

here β_g^{RNA} described the relationship to total RNA gth gene ith sample of model matrix X; β_g^{cond} represent the log2 fold change for treatment classes and ε_{gi} denotes the residual error.

Within anota a common slope for the treatment classes that describes the translated mRNA to total mRNA relationship is calculated. The difference between the slope intercepts is then interpreted as the Δ TE. A simplified view of this model can be seen in (**Figure 1.12 top left**). Here expression for translated mRNA and total mRNA are modeled over two sample classes with each 4 replicates. Furthermore, changes in translation efficiencies can also be observed when translated mRNAs shift to a larger extent than the total mRNA levels (**Figure 1.12 top right**). Identification of genes in this categorie can be a challenge, especially in highyl variable data set, as they resemble mRNA abundance genes (**Figure 1.12 bottom left**). Nevertheless, Using the linear regression analysis anota accurately corrects changes in translated mRNA as can be seen in (**Figure 1.12 bottom right**) where a change in total mRNA but not translated mRNA levels is observed. For this gene the difference in slope intercepts is small and will not be identified as difference in translation as would be the case in the TE-score approach. anota was developed at a time where translational buffering was uncommonly seen in data sets. Naturally, the methods lacks a setting to analyse translational buffering. This was addressed in anota's

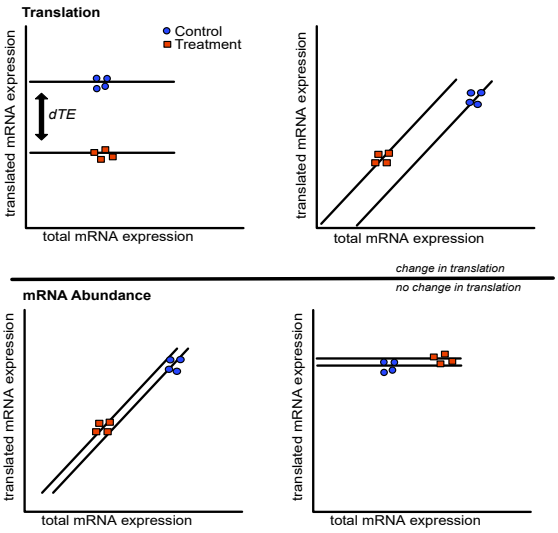

Figure 1.12: anota gene models - Schematic representation of the anota analysis models. Translation mRNA expression is set out against total mRNA expression for each biological replicate and treatment condition. Top left shows the model of a gene that is differentially translated (i.e. change in translated but not total mRNA). The difference in the slope intercepts are used to estimate changes in translation efficiencies between conditions i.e. dTE. Other gene models are shown; change in translation efficiency with varying total mRNA levels (top right); change in mRNA abundance (bottom left) and translational buffering (bottom right).

successor, anota2seq, and will be discussed in **Study 1**.

Advances in experimental methods warrant for appropriate statistical methods to analyse data resulting from them. DNA- microarray was the dominant platform to assess genome-wide changes before the advent of RNA sequencing. In DNA-microarray RNA hybridizes probes on a chip and generate a signal of which the measured intensity is an indicator of expression, whereas in RNA sequencing reads from RNAs are counted. Intensity data from DNA microarray can be normalised and transformed (i.e. log transformation) to fulfill the requirements for application of linear models, whereas RNA sequencing harbours additional characteristics that need to be accounted for. Therefore, algorithms developed for analysis of DNA-microarray are not directly applicable to RNA sequencing data as is the case for the anota algorithm.

RNA sequencing data shows variance that is greater than the mean which is commonly referred to as overdispersion. Count data from RNA sequencing have been initially approached using Poisson distributions which assumes that the variance is equal to the mean(J. Lu et al., 2005). Now established RNA sequencing analysis frameworks such as edgeR and DESeq2 use negative binomial distributions in combination with generalized linear models (GLMs) (Robinson, McCarthy, & Smyth, 2010, Love, Huber, & Anders (2014)). The negative binomial distribution uses a dispersion parameter to account for differences in the mean-variance relationship across the expression range (McCarthy, Chen, & Smyth, 2012). While analysis principles of DESeq2 and edgeR are similar they differ in their normalisation method, dispersion estimation and information sharing across genes. In a simple differential expression analysis between two conditions with one RNA type the GLM model would be as in the following equation:

$$log(y_{gi}) = \beta_g^{cond} \, X_i^{cond} + \varepsilon_{gi}$$

here $\beta_g^{cond} \, X_i^{cond}$ represent the condition (i.e. control and treatment) log2 fold change for the gth gene ith sample of the model matrix X and ε_{gi} denotes the residual error. When analysing changes in translation effiencies additional parameter for RNA type (i.e. total mRNA or translated mRNA) and the interaction between the RNA type and condition are added so that:

$$log(y_{gi}) = \beta_g^{RNA}\ X_i^{RNA} + \beta_g^{cond}\ X_i^{cond} + \beta_g^{RNA:cond}\ X_i^{interaction} + \varepsilon_g i$$

In this model the interaction term is interpreted as the change in translation effiencies (Chothani et al., 2019). Other methods (i.e. Ribodiff(Zhong et al., 2017), Riborex(W. Li, Wang, Uren, Penalva, & Smith, 2017) and deltaTE (Chothani et al., 2019)) borrow this analysis principle of an GLM with an interaction term by often applying this exact model. A noteable difference is that Ribodiff allows dispersion estimation for translated mRNA and total mRNA separetly as variance differences between the RNA types can be expected due to varying experimental protocols (Zhong et al., 2017, Liang et al. (2018)). While the flexibility of GLMs allows for complex study designs involving 2 or more treatment conditions, Riborex and Ribodiff limit the study design to only two conditions. DeltaTE gives their users full flexibility of the DESeq2 GLM model. Xtail is a method developed for ribosome profiling that makes use of DESeq2 for RNAseq count normalisation (Z. Xiao, Zou, Liu, & Yang, 2016). Their assessment of differences in translation efficiencies relies on probability matrices for the ratio of translated mRNA over total mRNA within condition and a between condition ratio of these ratios. Babel was the first algorithm designed solely for analysis of differential translation and uses an error-in-varaibles regression analysis (A. B. Olshen et al., 2013). The error-in- variables regression allows accounting for variable total mRNA levels when assessing changes in translation. Although these methods have distinct approaches to identify changes in translation efficiencies, their principle of analysis is similar to comparing a ratio of ratios. Therefore these methods suffer from similar issues as the TE-score which will be discussed in **Study 1**.

2. Aims of this thesis

The aims of this thesis are to expand current methodologies for analysis of translation efficiency data and explore the regulation of gene expression in cancer.

In **Study I** we adapted an algorithm for ANalysis Of Translation Activity data (anota) so that it could be applied to next generation sequencing data. Furthermore, we implemented the analysis of translational buffering a recently described regulatory mode of gene expression. The resulting algorithm was named anota2seq.

We then applied the anota2seq algorithm to investigate changes in translation efficiencies in two cancer models:

In **Study II** we unravelled the effects of eIF4A, an RNA helicase, inhibition using a synthetic rocaglate CR-1-31-B (CR-31) in pancreatic ductal adenocarcinoma.

In **Study III** we explored the effects of insulin on gene expression in multiple cell lines.

3. Results and discussion

3.1. Study 1 - Generally applicable transcriptome-wide analysis of translation using anota2seq

Initially changes in translation efficiencies were estimated using the TE-score approach as outlined in section 1.6. However, this method was being shown to be prone to identification of spurious correlations leading to elevated false positive identification that can result in false biological conclusions (Larsson et al., 2010). The identification of spurious correlations, when using the TE-score, can be attributed the inadequate correction for changes in total mRNA levels when estimating translation efficiencies (Larsson et al., 2010,Larsson et al. (2011)). The Analysis of Translation Activity (anota) algorithm facilitates analysis of translational efficiencies that are corrected for changes in total mRNA levels and therefore is not prone to spurious correlations(Larsson et al., 2011).

anota was developed for analysis of transcriptome-wide analysis for data quantified by DNA- microarrays (Larsson et al., 2010). However, advances in experimental methodologies lead to the development in RNA sequencing. RNA sequencing and DNA microarray data have distinct characteristics that need to be accounted for before analysis (**see section 1.6**). Therefore, while the statistical framework of anota had been shown as an adequate approach for analysis of translational efficiencies for data from DNA microarrays it was not directly applicable to RNA sequencing data. The characteristic of RNA sequencing data, that makes applying anota directly not possible, is the mean variance relationship. This encompasses that the counts for lower expressed genes show higher variability than counts for higher expressed genes even after log transformation. Efforts have been made to make RNA sequencing data more DNA- microarray like so that algorithms developed for intensity based microarray data can be applied to count based RNA sequencing data (Law, Chen, Shi, & Smyth, 2014,Love et al. (2014)). Anota2seq, the algorithm developed in this study, allows for transformation and normalisation of RNA sequencing data so that the anota statistical frame work can be applied for analysis of count data.

Another feature of anota2seq is that it allows for statistical analysis of translational buffering. The need for the analysis of translational buffering, or the uncoupling of transcription from translation, has been noted before anota2seq's development by comparing 20 translatomes and transcriptomes with different underlying stimuli in

mammalian cells (Tebaldi et al., 2012). The same authors proposed a framework, called tRanslatome, that combines several methodologies for analysis of differential transcription and translation efficiencies, including anota, for a comprehensive analysis of transcription and translation as well as their underlying mechanisms (Tebaldi, Dassi, Kostoska, Viero, & Quattrone, 2014).

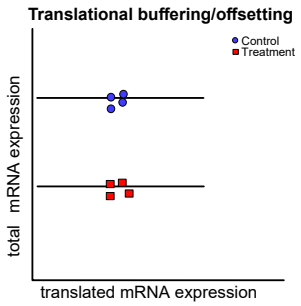

Figure 3.1: anota2seq gene model for analysis of translational buffering /offsetting - Total mRNA expression is set out against translated mRNA expression for each biological replicate and treatment condition. The model shows total mRNA changes that are independent of translated mRNA changes which is classified as translational buffering. It is important to distinguish between the gene modes as their regulation could be due to different underlying biological mechanisms (see section 1.5).

Nevertheless, commonly observed in polysome and ribosome profiling data sets are three gene expression modes, translation, translational buffering and mRNA abundance. While anota can be used to identify genes among the translation and mRNA abundance mode, analysis for translational buffering was not implemented therein (*See Figure 1.12*). Therefore, one would need to rely on the integration of several methods to efficiently analyse transcriptome-wide studies of translation efficiences. Anota2seq addresses this issue by changing the analysis model as described in section 1.6 to analyse changes in total mRNA levels corrected for changes in translated mRNA levels (i.e. translational buffering, **see figure 3.1**).

Appliance of anota2seq has successfuly identified translational buffering to which biological mechanisms could be linked,e.g. as mentioned earlier translationally bufferring under ERα depletion in prostate cancer (**see section 1.5.3**) (Lorent et al., 2019). Furthermore, in **study 2** translational buffering can be observed as a compensating mechanisms in "healthy" cells upon treatment with an eIF4A inhibitor and in **study 3** we identify mTOR dependent translational buffering for mRNAs with certain 3' UTR charactersitics.

The aim of this study is to compare anota2seq's performance to other established algorithms (i.e. DESeq2, RiboDiff, babel, TE-score and Xtail) for analysis of

translation efficiencies, specifically their ability to distinguish the three prominent modes of gene expression. To achieve we used a simulated data based approach. While it is arguable to what extent conclusion drawn from simulated data can be extended towards empircal data it allows for a controlled environment where true positive changes are known in advance. Furthermore, the mean-variance relationship in the simulated data is based on a real polysome profiling data set to increase confidence that drawn conclusions are also applicable to emprical data (Guan et al., 2017).

The simulated data consisted of four replicates for translated mRNA and total mRNA with a "control" and a "treatment" condition. Furthermore, the data sets contained a combination of the following gene sets:

"Unchanged": For this simulation category we drew reads from the same NB distrubution for both the control and treatment conditions in both the translated and total mRNA. This catergory reprents genes that would be unaffected by e.g. a stimulus of between cellular states.

"mRNA abundance": For this category the control condition for both the translated mRNA and total mRNA were drawn from the same NB distribution. The NB distribution for *both translated mRNA and total mRNA* of the treatment condition was altered so that values would be drawn corresponding to a fold change (negative or positive) ranging between 1.5 to 3.0. The directionality of the fold changes (i.e. up or down regulation) was the same for translated mRNA and total mRNA.

"translation": For this category the control condition for both the translated mRNA and total mRNA were drawn from the same NB distribution. The NB distribution for *translated mRNA only* of the treatment condition was altered so that values would be drawn corresponding to a fold change (negative or positive) ranging between 1.5 to 3.0.

"buffering": For this category the control condition for both the translated mRNA and total mRNA were drawn from the same NB distribution. The NB distribution for *total mRNA only* of the treatment condition was altered so that values would be drawn corresponding to a fold change (negative or positive) ranging between 1.5 to 3.0.

As a first step we tested whether the methods could properly control for type-1 errors (i.e. false positive identifcation). For this we simulated a data set with genes belonging only to the "unchanged" category. This revealed that babel, but to an even greater extent Xtail, were unable to control their type-1 error as these methods assigned low p-values and FDRs when no real changes were present. This indicated a limited applicability of Xtail and babel for statistical analysis of translatomes.

From the comparative analysis of the analysis for changes in translation efficiencies affecting protein levels we concluded that anota2seq outperforms all other methods. This was assessed by comparing the area under the curve from Receiver operating characteristics (ROC) and precision recall curves. The ROC curves showed a, albeit slightly, better performance for detecting changes in translation. However, the precision recall was much higher for anota2seq which can be accredited to that the analysis principle of the other methods is based on identifying changes regardless of whether the change is in the translated mRNA or total mRNA (*as explained in section 1.6*). Nevertheless, when comparing the performance using simulatd data in the absence of genes belonging to the "buffering" category anota2seq still showed superior performance.

Next to the a prior knowledge of the introduced changes in the simulation data, it also allowed us to modify parameters to investigate the robustness of the methods to increased variance, overall sequencing depth and differing sequencing depth between samples. Here, all methods showed robustness against variance and sequencing depth differences between samples as long as a minimum of 5 million counts per sample was reached.

A short coming in the simulation study is that we did not assess the effects of systematic batch effects. Batch effects can be introduced e.g. during experimental design and there are many methods that try to correct for these(W. E. Johnson, Li, & Rabinovic, 2007,Leek (2014),Y. Zhang, Parmigiani, & Johnson (2020)). Other ways to correct for batch effects is their inclusion in the analysis model, which can be supplied to the analysis model in DESeq2, edgeR as well as anota2seq. Indeed analysis of a dataset with prominent batch effects showed that batch effects can dampen the efficiency of the anota2seq algorithm to identify changes but can be effectively corrected for in the algorithm.

In this study we developed an analysis algorithm for efficient transcriptome-wide analysis of translation efficiencies applicable to DNA-microarrays and RNA seq. Furthermore, anota2seq has been successfully applied to broaden the knowledge around mRNA translation in various different contexts (Lorent et al., 2019,Chan et al. (2019),Hipolito et al. (2019),Chaparro et al. (2020)).

3.2. Study 2 - eIF4A supports an oncogenic translation program in pancreatic ductal adenocarcinoma

Pancreatic cancer is considered a lethal malignancy and has limitited treatment options. A study on predicitons for european cancer mortality rates for 2021 concluded that health efforts should focus on pancreatic cancer. While other cancers (e.g. ovary, breast and stomach) showed a decline in mortality rates, no major overall decline was observed for pancreatic cancer in the period of 1970-2021 (Carioli et al., 2021).

Pancreatic ductal adeno carcinoma (PDAC) accounts for over 90% of exocrine pancreatic cancer, whereas non ductal pancreatic cancers e.g. acinar cell carcinomas are uncommon (Feldmann, Beaty, Hruban, & Maitra, 2007,Jun & Hong (2016)). It is estimated the 60-70% of the PDACs arise in the head (Luchini, Capelli, & Scarpa, 2016). So far treatment options are mostly limited to surgical removal, which can be complicated due to the anatomical location of the pancreas head.

With the increasing understanding of tumor heterogeneity, i.e. tumors with similar tissue origin are not necessarily identical at a molecular level, anti cancer therapy improved (Biankin & Hudson, 2011). In breast cancer stratification by histological,molecular and gene expression features lead to identification of several breast cancer subtypes for which different treatment options exist, e.g. ER^+ breast cancer subtypes respond to endocrine therapy whereas ER^- do not(Andre & Pusztai, 2006,Parker et al. (2009)). While breast canacer treatment strategies benefit from a rather well established understanding of the molecular subtypes, in pancreatic cancer trascriptomic based subtyping is still ongoing (P. Bailey et al., 2016,Puleo et al. (2018),Collisson et al. (2011),Moffitt et al. (2015),Collisson, Bailey, Chang, & Biankin (2019)). Therefore, it is warranted to extend current knowledge around pancreatic cancer to advance therapeutic strategies in this lethal disease.

Nevertheless, commonly shared among PDAs are oncogenic mutations in KRAS as well as inactivation of tumor supressors e.g. TP53 (S. Jones et al., 2008). Furthermore, PDAs have been shown to be dependent on increased protein synthesis mediated by Kras (Chio et al., 2016). This indicates an important role of mRNA translation in PDA.

The aim of *study 2* was to investigate the therapeutic effects of targetting eIF4A in a three dimensional PDA organoid cell culture with mutations in the $Kras^{LSL-G12D}$, $Trp53^{LSL-R172H}$ and Pdx1-cre alleles that has been shown to recapitulate PDA tumor progression (Boj et al., 2015). The inhibition of eIF4A was done using a synthetic rocaglate, CR-1-31B (CR-31). Rocaglates have been shown to inhibit eIF4A helicase activity and displayed anti tumor activity (Cencic et al., 2009).

We first wanted to establish the therapeutic validity of targetting eIF4A in PDA. In vitro experiments comparing treated PDA organoids (KP) to their normal (N) counter parts revealed hightened sensitivity of PDA organoids to CR-31 treatment. OP-puromycin incorporation showed reduced protein synthesis in KP, whereas N were affected to a lesser extent. Furthermore, similar effects were found in vivo for PDA tumours where also CR-31 reduced protein synthesis (assessed by SUnSET assay), tumor growth (assessed by ultra sound imaging) and increased survival. The effect on protein synthesis was not due to inhibition of oncogenic signalling pathways which was evaluated western blot assessing the phosphorylation of e.g. AKT, mTOR and 4E-BP1. From these findings we concluded that there is therapeutic validity in targetting eIF4A in PDA that acts downsteam of oncogenic signalling pathways.

Using polysome profiling we then sought to decipher the mechanisms explaining the increased sensivity to CR-31 in KP organoids. First we investigated the differences in gene expression between untreated KP and N organoids. Analysis of changes in translation efficiencies using anota2seq revealed massive modulation at both transcription and translation indicative of the underlying differences in e.g. genomic stability and enhanced oncogenic signalling impinging on protein synthesis. Consistent with the in vitro OP-puromycin incorporation and in vivo SUNsET experiments, CR-31 had only little effect on translation in N organoids, whereas in KP organoids exclusively changes in translation were detected.

We then compared the mRNAs affected by CR-31 in KP to the acquired translational profile of untreated KP and N organoids. In order to achieve this we visualised the mRNAs affected by CR-31 in KP onto the data of the untreated KP vs N organoids comparison. This revealed that the acquired translational program of KP organoids is reversed when treated with CR-31.

Performing a similar analysis, we visualised mRNAs sensitive to CR-31 in KP onto the data of the CR-31 treated N organoids. mRNAs affected by CR-31 in KP were translationally buffered in CR-31 treated N organoids. Translational buffering, as an adaptive response to treatment, has been shown to maintain protein homeostasis by inducing a transcriptional response increasing the pool of mRNAs that can be translated (Lorent et al., 2019). The ability for N organoids in increase transcription for mRNAs affected by CR-31, whereas KP cannot, could partially explain as to why protein synthesis is not reduced to a similar extent in N as in KP.

We then assessed 5' UTR characteristics of the translationally regulated mRNAs upon CR-31 treatment in KP organoids. It was reported that eIF4A-senstive mRNAs showed overall and more structured 5' UTRs (e.g. containing G-quadruplexes) (Rubio et al., 2014,Wolfe et al. (2014),Gandin et al. (2016b)). Furthermore, a mechanisms by which rocaglates would clamp eIF4A to mRNAs with [A,G] repeats in their 5 UTR was described (Iwasaki, Floor, & Ingolia, 2016). However, mRNAs sensitive to CR-31 treatment herien showed overall shorter 5' UTRs that were more structured when corrected for their length without enrichment for 4G-quadruplexes or [A,G] repeats. From the polysome profiling and UTR analysis we concluded that eIF4A supports an oncogenic translation program in PDA cells for mRNAs with shorter but structured 5' UTRs.

Shorter 5' UTRs have been shown to be sensitive to eIF4E expression and enconde for metabolic functions (see section 1.3). When we compared an eIF4E overexpression signature in the KP vs N and CR-31 treated KP we observed that in KP organoids translationally regulated mRNAs under eIF4E overexpression were also translationally activated. This observation is consistent with reports of 4E-BP1 loss in pancreatic cancer (Y. Martineau et al., 2014). CR-31 treatment in KP reversed the translational profile for these mRNAs. Therefore, while rocaglates have been shown to directly target eIF4A (Cencic et al., 2009). eIF4A inhibition in tumors resistant to mTOR inhibiton by loss of 4E-BP1 has been shown to circumvent this resistance (D. Müller et al., 2019). Therefore, eIF4F complex formation could be disrupted by reduced eIF4A availability to an extent that also cap dependent translation is affected in PDA organoids herein.

When further inspecting the regulated gene sets in treated and untreated KP

compared to their normal counterparts we could see an enrichtment in metabolic pathways, e.g. Oxidative phosphorylation. This pathway was upregulated in untreated KP compared to N, whereas in KP CR-31 treatment reversed the translational profile of this pathway. These findings were experimentally validated and shown to disrupt the mitochondrial respiration (measured by oxygen consumption rates).

A way to counter loss of energy production through oxidative phosphorylation is to increase activity of other anaerobic metabolic pathways, i.e. glycolysis. However, in CR-31 treated KP we could not detect a upregulation of glycolysis measured by $U - C^{13}$ glucose labeling and extra cellular acidification rates nor did CR-31 treatement affect expression of glycolytic enzymes (e.g. HK1,HK2, LDHA, SLCA1, SLCA3). Furthermore, glucose deprivation did not further sensitise to CR-31 treatment. This was rather unexpected, given that increased glycolysis is characteristic for cancers as described by Otto walburg (Warburg, Wind, & Negelein, 1927). However, the polysome prolfing data revealed translational downregulation and subsequent reduction of protein expression for the glucose transporter Slc2a6. Indeed, perturbation of Slc2a6 using $sgRNA^{Slc2a6}$ in N and KP organoids revealed a decrease in glucose uptake. From this we concluded that glycolytic compensation of KP is diminished by translational regulation of the glucose transporter Slc2a6 upon CR-31 treatment.

Among the translationally activated genes in the CR-31 treated KP organoids where mRNAs involved in the glutamine metabolism (i.e. Slc1a5 and Gls1). Furthermore, glutamine levels were elevated in patient derived PDA cell lines treated with CR-31. Glutamine can be converted into α-ketoglutarate and funneled into the krebs cycle and therefore can serve to increase energy production (D. Xiao et al., 2016). Indeed, using gas chromatography mass spectrometry (GC/MS) to quantify downstream metabolites after culturing PDA cells in C_5^{13}- glutamine, we identified a shift towards reductive carboxylation of α-ketoglutarate obtained from C_5^{13}- glutamine to produce acetyl-CoA. Notably, the glutamine metabolism was not elevated in N organoids.

A combined treatment of CR-31 with glutaminase inhibitors (BPTES or CB839) could sensitise to CR-31 treatment patient-derived PDA cells to CR-31 treatment. Therefore, our study suggests an eIF4A dependent translational program in PDA

that can act as a theurapeutic target in PDA. Furthermore, a recently published ribosome profiling study of a CR-31 treated human pancreatic cancer cell line (PANC1) observed the same therapeutic effect of CR-31 treatment in vivo on survival and tumor volume (Singh et al., 2021). This underlines the significance of our study in identifying eIF4A as therapeutic target in PDA.

Nevertheless, the same study indicated differences on the underlying regulated mRNA subsets. They report, in line with the literature, that eIF4A depdent mRNAs show long and structured 5' UTRs containing G-quadruplexes (Singh et al., 2021,Wolfe et al. (2014)). While the 5' UTRs of the mRNAs identified herein where overall shorter, they were overall more structured. Furthermore, the mRNAs identifed in Singh et. al. were involved in KRAS signalling, which was unaltered in our study.

This raises some questions about the differences between experimental setups and their potential influence on biological outcomes. For instance, Singh et. al. performed ribosome profiling on a PANC1 cell line culture treated with 25nM CR-31, wheres herein we performed polysome profiling on a 3D-organoid culture treated with 10nM CR-31. The differences between ribosome and polysome profiling have been discussed extensively (see section 1.4). However, by measuring IC50 concentrations for CR-31 in a panel of pancreatic cancer cell lines, the autors show a ~6-fold difference in susceptability to CR-31. PANC1 cells were most affected by CR-31. Dosage dependent viability experiments of patient derived PDA cells in our study revealed that at 10nM viability was reduced by ~30%, whereas treatment with 25nM reduced viability by $> 50\%$. Furthermore, a combination treatment of CR-31 and CB839 showed no difference at 25nM CR-31, whereas at 12.50nM a difference was observed. Therefore, combining the findings of these two studies indicate that CR-31 treatment in PDA indeed has a therapetuic effect, however the underlying mechanisms that are observed in the transcriptome wide analysis of translation efficiencies are likeley dependent on the cell culture and treatment dosages.

3.3. Study 3 - mTOR-dependent translational buffering overrides transcriptome alterations leading to maintained proteome composition

4. Conclusions

Acknowledgments

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