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DEVELOPING ANALYTICAL TOOLS TO INVESTIGATE THE ROLE OF TRANSLATION IN HOMEOSTASIS AND DISEASE

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Developing analytical tools to investigate the role of translation in homeostasis and disease

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Abstract

Transcriptome-wide studies of translation efficiencies are required to improve understanding of translational regulation and its role in homeostatic mechanisms. In **Study 1**, we developed an algorithm for analysis of translation efficiency, called anota2seq. We show that anota2seq outperforms current methodologies and, due to its unique analytical approach, it is the only method to statistically distinguish important modes for regulation of gene expression, i.e translation and translational buffering.

Pancreatic cancer is a lethal malignancy with very limited treatment options. In **Study 2**, we evaluate the impact of using an eIF4A inhibitor, CR-31, on mRNA translation in pancreatic cancer. eIF4A is a component of the eIF4F translation initiation complex. We show that inhibiting eIF4A in murine and human pancreatic ductal adenocarcinoma (PDAC) models induces an energy crisis by impacting translation of mRNAs related to oxidative phosphorylation and glycolysis. This leads to the shift of metabolic dependency of PDACs towards reductive glutamine metabolism. Exploiting this dependence using a combined treatment of eIF4A and glutaminase inhibitors revealed an exciting therapeutic treatment strategy for PDA that did not affect healthy cells.

In **Study 3**, we investigated the effecs of insulin on gene expression in malignant and non-malignant cells. This revealed that malignant cells modulate total mRNA levels differerenly in response to insulin compared to non-malignant cells, whereas in both translation was dependent on mammalian/mechanistic target of rapamycin (mTOR). However, mTOR inhibition during insulin stimulation in malignant cells lead to translational offsetting of alterations in total mRNA levels. Comparing the effects of mTOR inhibition in malignant cells to that of hypoxia in stem cells revealed that these vastly different cell types share the ability to translationally offset mRNAs.

Collectively, these studies improved analysis of translational efficiencies and

contributed to advanced understanding of the role of translational dysregulation in cancer.

List of publications

Oertlin Christian , Lorent Julie , Murie Carl , Furic Luc , Topisirovic Ivan & Larsson Ola. (2019). Generally applicable transcriptome-wide analysis of translation using anota2seq. Nucleic acids research. 47. 10.1093/nar/gkz223.

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Other publications or works

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Contents

Abbreviations

Abbreviations

Abbreviation	Term
3'	3 prime
4E-BP	4E binding protein
mcm^5s^2U	5-methoxycarbonyl-methyl-2-thiouridine
5'	5 prime
A-site	Acceptor-site
ELP3	Acetyltransferase elongator
AMP	Adenosine-mono-phosphate
AMPK	Adenosine-mono-phosphate kinase
ATP	adenosine triphosphate
APV	Analysis of partial varaince
AUC	Area under the curve
ABCE1	ATP binding cassette protein
ChIP	Chromatin immunoprecipitation
CR-31	CR-1-31-B
CHX	Cyclohexamide
CTU1/2	cytosolic thiourdylase 1/2
DNA	Deoxyribonucleic acid
dsRNA	double-stranded RNA
ER	Endoplasmatic reticulum
ERalpha	Estrogen receptor alpha
eEF	Eukaryotic elongation factor
eIF	Eukaryotic initiation facotr
eRF	Eukaryotic release factor
E-site	Exit-site
GC/MS	Gas chromatography mass spectrometry
GCN2	General control nonderepressible 2
GLM	Generalised linear model
GDP	Guanosine-di-phosphate
GTP	Guanosine triphosphate
HRI	Heme regulation eIF2alpha kinase
HuR	Human antigen R

Abbreviation	Term
HIF-1	Hypoxia inducible factor 1
IGF1R	IGF1 receptor
IGF1	insulin-like growth factor 1
INSR	insulin receptor
ISR	Integrated stress response
LARP1	La ribonucleoprotein domain family member 1
mORF	Main open reading frame
mTOR	Mammalian/mechatistic target of rapamycin
mRNP	Messenger ribonucleoprotein particle
mRNA	Messenger RNA
met-tRNAi	Methionyl-initiatior transfer RNA
ALKBH8	methyltrasnferase TRM9-like domain of alkylation repair homolog 8
miRNA	microRNA
MAPK	Mitogen-activated protein kinase
nM	Nano molar
NB	Negative binomial
ncRNA	non-coding RNA
ODC1	Ornithine decarboxylase
PDA	Pancreatic ductal adenocarcinoma
P-site	Peptidyl-site
PI3K	Phosphoinositide 3-kinase
PABP	Poly A binding protein
PIC	Pre-initiation complex
PDCD4	Programmed cell death protein 4
AKT	Protein kinase A
PKR	Protein Kinase R
PERK	Protein kinase R-like endoplasmatic reticulum kinase
RVM	Random variance model
ROC	Receiver operating characteristics
RNA	Ribonucleic acid

Abbreviation	Term
rRNA	Ribosomal RNA
RPF	Ribosome protected fragment
RBP	RNA binding protein
S6K	S6 kinase
sgRNA	single guide RNA
TOP	Terminal oligopyrimidine
TC	Ternary complex
RT-qPCR	Teverse transcription quantitative polymerase chain reaction
tRNA	Transfer RNA
TE	Translation efficiency
UTR	Untranslated region
uORF	Upstream open reading frame
URM	urmylation
VEGF	Vascular endothelial growth factor

1. Introduction

1.1. Gene expression

1.1.1. The central dogma of gene expression

In eukaryotic cells, the whole genetic code is stored as deoxyribonucleic acid (DNA) molecules in the nucleus. Transcription is the process whereby temporary copies of the DNA are generated and occurs in the nucleus. These copies are called ribonucleic acid (RNA). RNAs undergo processing by which multiple different variants coming from the same genomic region are produced. A subset of protein-encoding processed RNAs is the so called messenger RNA (mRNA). mRNAs are transported from the nucleus into the cytoplasm where they can be stored,
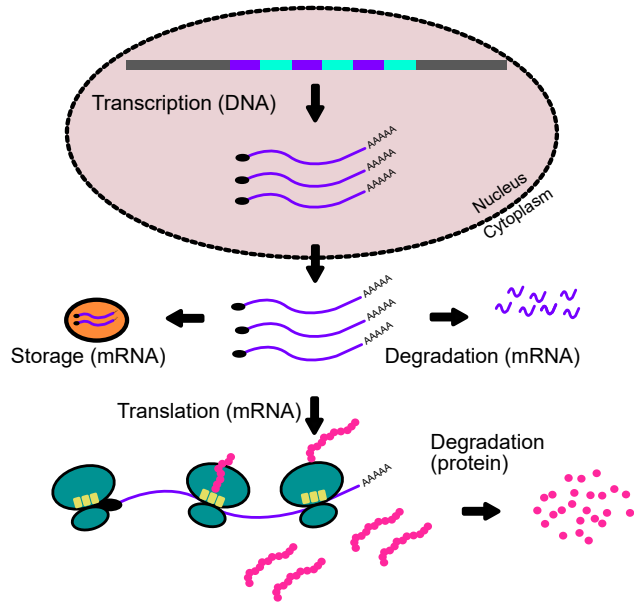
Figure 1.1: The gene expression pathway - DNA is transcribed into RNA. RNAs are processed into mRNAs that consist of a 5' cap, exons and a poly(A) tail. mRNAs can be transported out of the cellular nucleus into the cytoplasm where they can be degraded, stored or translated into proteins depending on cellular demands. Synthesised proteins can be degraded by proteosomes. Coloured boxes in the DNA refer to introns (teal) and exons (purple). Introns are non-protein-encoding parts of the genome, whereas exons encode for proteins. mRNAs are depicted as purple lines, i.e. a series of exons, with an AAAAA extension and an oval shape at its start. Proteins are depicted as a series of pink balls.

degraded or translated into proteins. Proteins themselves can also be degraded (*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).

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. Studying the mechanisms that drive differences in protein expression is however required to improve understanding of biological processes and their dysregylation in disease.

Recently developed system biology methods allow investigation of gene expression at multiple levels on a genome-wide scale. Initially, transcriptomics studies were applied to study gene expression with the assumption that mRNA expression is the main determinant for protein levels and therefore could be used as a proxy for them. However, this view was challenged by several 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 (de Sousa Abreu, Penalva, Marcotte, & Vogel, 2009; J. Lu, Tomfohr, & Kepler, 2005; Schwanhäusser et al., 2011; G. M. Silva & Vogel, 2016; Vogel & Marcotte, 2012).

The debate regarding which step of the gene expression pathway contributes most to the composition of the proteome is ongoing, nevertheless an understanding has been reached that the 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 appears to have less impact relative to post-transcriptional steps in regulation of gene expression (Y. Liu, Beyer, & Aebersold, 2016). For example, in a study that stimulated bone marrow-derived dendritic cells with LPS, protein levels were dependent on cellular transcript levels (Jovanovic et al., 2015). In contrast, a study investigating cells under endoplasmatic reticulum stress observed extensive modulation of protein expression, whereas transcript abundance was only mildly affected (Cheng et al., 2016).

Thus, the contribution of different steps of the gene expression is dependent on many different factors, e.g. cellular state or treatments. mRNA translation is an essential process in determining composition of the proteome. Furthermore, dysregulation of mRNA translation has been observed in multiple diseases,

ranging from neurological disorders to cancer which warrants for a comprehensive understanding of this process (Graff et al., 2009; Kapur & Ackerman, 2018; L. J. Lee et al., 2021; Ruggero, 2013). This thesis focusses on increasing understanding of the role of mRNA translation in cancer.

1.2. mRNA translation

1.2.1. Schematic representation of mRNA mRNAs contain a protein coding region which is flanked by untranslated regions (5' and 3' UTRs). UTRs contain post-transcriptional regulatory elements that may affect localisation, stability and translation of the mRNA (Leppek, Das, & Barna, 2018; Loya et al., 2008; Mignone, Gissi, Liuni, & Pesole, 2002) (*see figure 1.2, see also section 1.3 for details*).
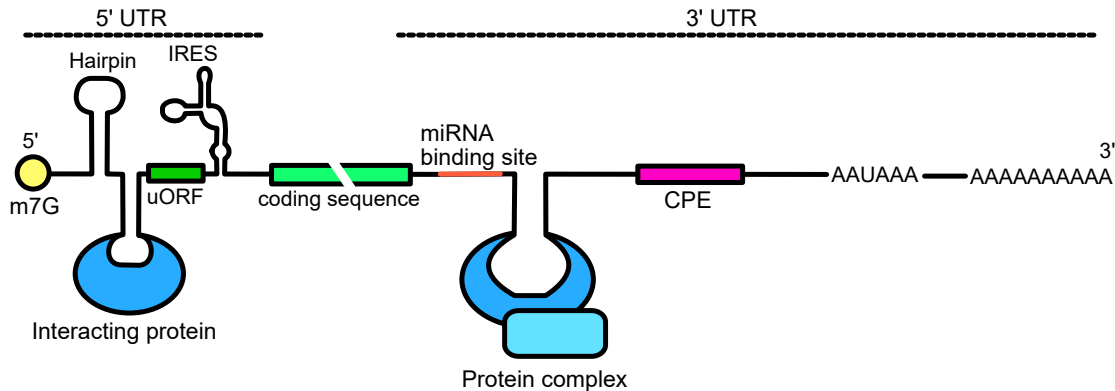
Figure 1.2: Schematic overview of mRNA - mRNA consists of a coding sequence, 5' and 3' untranslated regions flanking the coding sequence, a 5' cap and a poly-(A)-tail. Located within the 5' and 3' untranslated region are post-transcriptional regulatory elements that can influence gene expression. uORF; upstream open reading frame; IRES, internal ribosome entry site; CPE, cytoplasmic polyadenylation site; AAUAAA, polyadenylation signal. See section 1.3 for details on these elements.

The 5' end has a 7-methyl-guanylate (m7G) cap that is important for translation initiation, while the 3' end has a poly-A tail protecting the mRNA against degradation (Grifo, Tahara, Morgan, Shatkin, & Merrick, 1983; Wilusz, Wormington, & Peltz, 2001). Multiple different mRNAs variants (also called isoforms) from the same genomic region can exist. Isoforms may arise due to alternave transcription start site usage or a process called alternative splicing. The processes alter the exon composition of mRNA. Isoforms can co-exist at the same time and have differing properties that can perform distinct functions (Joly Anne-Laure et al., 2018). Furthermore, alternative splicing resulting in isoforms

with different 5' UTRs can alter translation for mRNAs encoding for the same protein (S. N. Floor & Doudna, 2016; Jewer et al., 2020).

1.2.2. mRNA translation In eukryotes, mRNA translation occurs in the cytoplasm for the vast majority of protein coding mRNAs. However a small subset of not nuclear-encoded mRNAs is translated in the mitochondria (D'Souza & Minczuk, 2018). mRNA translation is a process that consits of several steps: initiation, elongation, termination and ribosome recycling (*see figure 1.3*). These steps will be discussed in detail below.
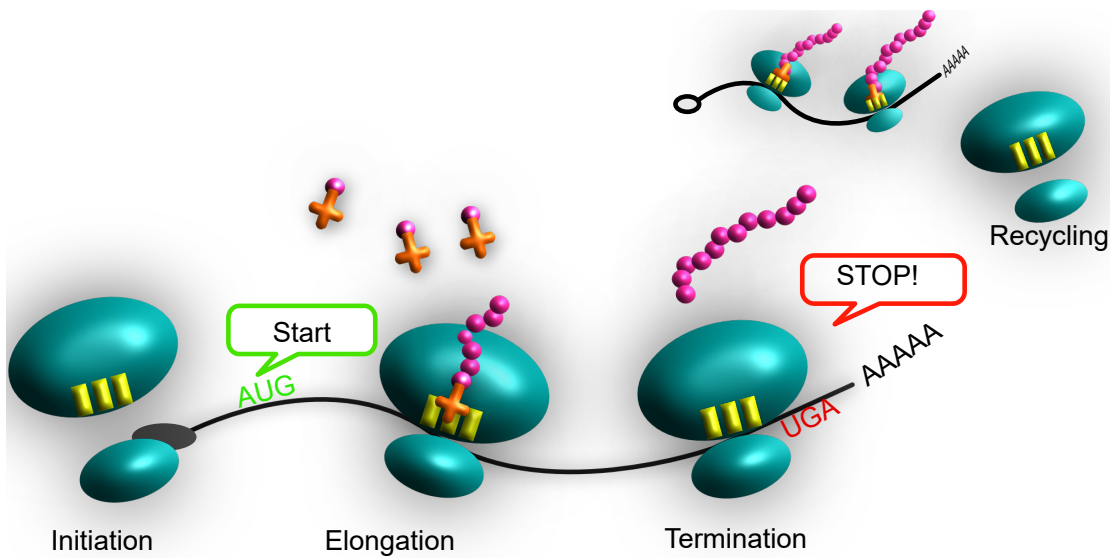
Figure 1.3: mRNA translation initiation, elongation, termination and ribosome recycling steps - The ribosome binds to the mRNA and initiates scanning for a start codon (AUG). The elongation phase incorporates amino acids into a polypeptide chain (i.e. the protein product). Once a stop codon (e.g. UGA) is detected 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 on the same mRNA. Green ovals represent the 80s ribosome. Within the ribosome the E, P and A-sites are indicated with yellow boxes (see section 1.2.4 for details. Polypeptide chains are depicted as a series of pink balls. Orange crosses represent transfer RNAs (tRNA) (see section 1.2.4 and 1.3.4 for more details on tRNAs).

1.2.3. Initiation In eukaryotes, 5' cap dependent initiation of mRNA translation consists of multiple stages. First, a ternary complex (TC) consisting of guanosine-tri-phosphate (GTP) bound eukaryotic initiation factor (eIF) 2 and methionine-initiator transfer RNA (met-tRNAi) is formed. This is followed by formation of the 43S pre-initiation complex (PIC). The PIC consists of a 40S ribosome subunit, the TC and, translation iniation factors eIF1, eIF1A, eIF3, eIF5 (Asano, Clayton, Shalev, & Hinnebusch, 2000). mRNAs then undergo "activation". Here, the 5' cap proximal structure is unwound by eIF4F together with eIF4B in an adenosine-tri-phosphate (ATP) dependent manner. eIF4F is the 5' cap binding complex consisting of: the 5' cap binding protein eIF4E, the RNA helicase eIF4A and, a scaffold protein eIF4G (Grifo et al., 1983). The Poly (A) binding protein (PABP) binds to the poly(A) tail of the 3' UTR and causes circularisation of the mRNA. The circularisation improves stability of the mRNA and aids in recruitment of translation initiation factors (Ivanov et al., 2016). The formed 43S PIC then binds to the unwound region of the mRNA and starts scanning in the 5' to 3' direction. Recognition of the translation initiation codon (AUG) induces formation of the 48S initiation complex. During this formation, displacement of eIF1 occurs which allows eIF5 to hydrolyse eIF2-bound GTP. The 60S ribosomal subunit then joins the 48S initiation complex which causes the release of eIF2-GDP and other initiation factors (eIF1, eIF3, eIF4B, eIF4F and eIF5). Subunit joining is mediated by eIF5B. After subunit joining the ribosome 80S ribosome is formed and the elongation process starts (*see figure 1.2.3*) (Asano et al., 2000; Hinnebusch, 2006; Jackson, Hellen, & Pestova, 2010). A mechanism for cap dependent translation initiation that is independent of eIF4E has been described. Here, eIF3d binds the 5' cap of mRNAs containing and RNA element that blocks eIF4E binding (A. S. Y. Lee, Kranzusch, & Cate, 2015,A. S. Y. Lee, Kranzusch, Doudna, & Cate (2016)). Next to 5'cap dependent initiation, mRNA translation can also be initiated in a 5' cap independent manner, e.g. via the internal ribosome entry site (IRES) (*see figure 1.2*). Mechanisms for 5' cap independent mRNA translation initiation are extensively reviewed elsewhere (Lacerda, Menezes, & Romão, 2017).

1.2.4. Elongation The 80S ribosome contains three sites important for decoding an mRNA: the aminoacyl (A), peptidyl (P) and exit (E) sites. During

elongation in eukaryotes, aminoacytelated tRNAs are delivered to the A-site in a TC with eukaryotic elongation factor (eEF) 1A and GTP. If the tRNA is cognate to the codon in the A-site of the ribosome, eEF1A is hydrolysed. This causes eEF1A to release and accommodates the aminoacetylated tRNA in the A-site. This is followed by a peptidyl transferase reaction. This reactions forms the peptide bond between the peptidyl tRNA in the P-site and amino group of the aminoacyl-tRNA in the A-site (aa-tRNA). After the peptide bond is formed a translocation step occurs through eEF2-GTP hydrolysis. The translocation step encompasses that the tRNA currently in the P-site moves into the E-site. Likewise the tRNA in the A-site moves to the P-site. Which leaves the A-site open for other tRNAs to join. The deacytelated tRNA in the E-site is then released from the ribosome (Dever & Green, 2012). This process is repeated until a stop codon (UAA, UGA or UAG) enters the A-site of the ribosome.
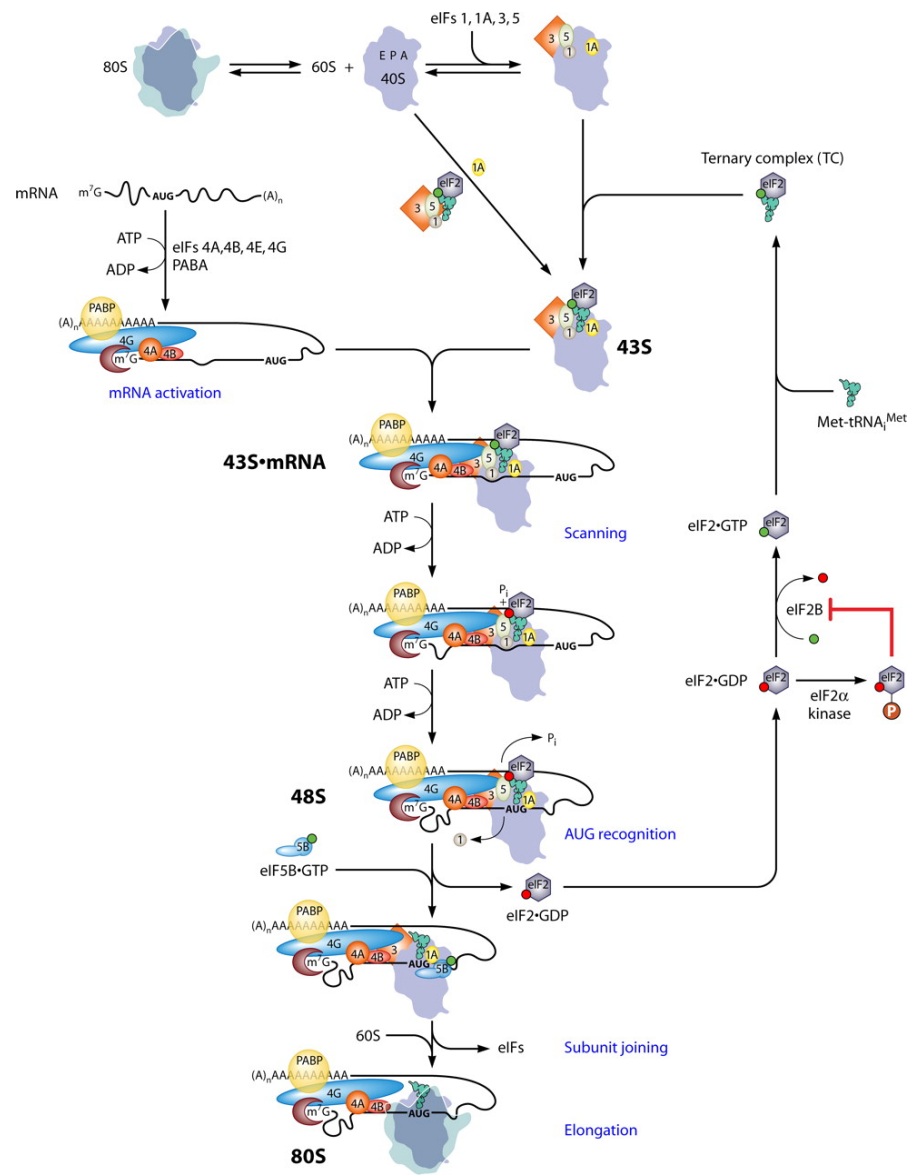
Figure 1.4: Pathway of eukaryotic translation initiation via ribosomal scanning.

1.2.5. Termination and recycling

mRNA translation termination is facilitated by two eukaryotic release factors (eRF), eRF1 and eRF3-GTP (E. Z. Alkalaeva, Pisarev, Frolova, Kisselev, & Pestova, 2006; I. Stansfield et al., 1995). The eRF1:eRF3-GTP complex binds to the A-site of the ribosome upon recognition of a stop codon. This causes an 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 (Hellen, 2018; Pisarev et al., 2010).

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.

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*) (Staehelin, Brinton, Wettstein, & Noll, 1963; Warner, Rich, & Hall, 1962). Therefore the translation efficiency of an mRNA depends on the number of ribosomes it is associated with. Experimental methods like polysome profiling and ribosome profiling are used to study translation efficiencies (*explained in detail in section 1.4*). While all steps of translation can affect the translation efficiency of an mRNA, it is thought to most commonly be regulated at the initiation step (Dever & Green, 2012; Jackson et al., 2010; J. D. Richter & Coller, 2015). This is supported by findings using polysome profiling in yeast that was cultured in nutrient rich medium where initiation was rate-limiting for most mRNAs (Arava et al., 2003). Furthermore, a recent ribosome profiling study assessed transcriptome-wide elongation rates. This revealed a similar rate of elongation for mRNAs of different classes. These classes were a stratification of mRNAs based on their UTRs or codon composition of the coding sequence (Ingolia, Lareau, & Weissman, 2011). The next section
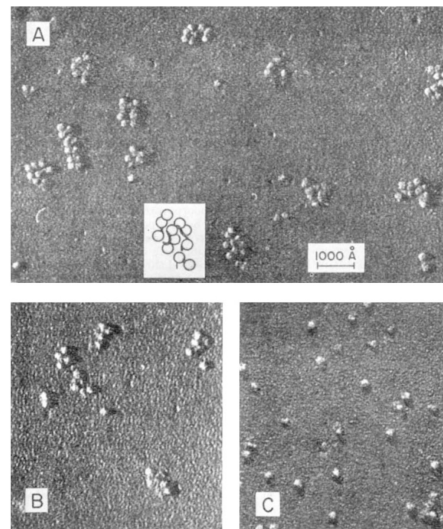
will go further into detail how translation initiation and elongation can be regulated.

1.3. Regulation of mRNA translation

mRNA translation is the most energy consuming process in the cell. In a study using concanavalin A stimulated rat thymocytes, it was estimated that translation accounts for ~20% of the cellular energy consumption (Buttgereit & Brand, 1995). The high energy consumption of mRNA translation and its central role in the gene expression pathway requires it to be tightly regulated.

Regulation of translation can be exerted at a global level, i.e. regulation of a large set of mRNA simultaneously. Global regulation of mRNA translation can be achieved by, e.g. perturbations of major signalling pathways impinging on mRNA translation. A strong feature of regulation of mRNA translation is that it can affect specific mRNA populations selectively. Selective translational regulation acts on characteristics of mRNAs, e.g. through 5' UTRs or RNA binding proteins (RBP) that bind to the 3' UTRs (**see figure 1.2**) (Leppek et al., 2018). Furthermore, regulation of initiation factors can also regulate translation selectively (Wolfe et al., 2014,Gandin et al. (2016b)). Below we will discuss multiple mechanisms that regulate mRNA translation globally or selectively.

1.3.1. mTOR mTOR is a conserved Ser/Thr kinase and is found in two structurally and functionally distinct complexes, mTORC1 and mTORC2 (Pearce et al., 2007; Saxton & Sabatini, 2017). In a growth promoting environment mTOR regulates cell metabolism to increase protein synthesis, lipids and nucleotides, while suppressing catabolic pathways, e.g. autophagy. mTORC2 promotes survival via signalling through protein kinase A (AKT), anabolic metabolism, and cytoskeleton regulation (Sarbassov, Guertin, Ali, & Sabatini, 2005,Zoncu, Efeyan, & Sabatini (2011)).

mTOR activity is modulated via hormone and growth factor signalling, e.g. insulin and insulin-like growth factor (IGF1). This signalling is predominantly mediated through the phosphoinositide 3-kinase (PI3K) / AKT pathway. PI3K activation generates phosphatidylinositol-3,4,5-trisphosphate (PIP3). Phosphatase and tensin homologue (PTEN) can counteract this by hydrolsis

of PIP3 to phosphatidylinositol 4,5-bisphosphate (PIP2). PIP3 recruits phosphoinositide-dependent kinase 1 (PDK1) and AKT to the plasma membrane. At the plasma membrane AKT is activated through phosphorylation of PDK1. AKT in turn increases activity towards its substrate tuberous sclerosis complex (TSC). TSC consists of a scaffold protein, TSC1, and a GTPase, TSC2. TSC negativeley regulates mTOR activity. This negative regulation occurs through hydrolsis of Ras homologue enriched in brain (Rheb) that leads to its inactivation. Rheb binds to mTOR to promote its activity (*see Figure 1.6*). Furthermore, signalling through the RAS/ERK pathway can also activate mTOR (reviewed here (Reuben J. Shaw & Cantley, 2006)).

The PI3K pathway is involved in oncogenic signalling and under investigation as therapeutic target 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 catalytic p110α subunit of PI3K (PI3KCA) is frequently mutated or amplified (J. W. Lee et al., 2005; D. A. Levine et al., 2005; Samuels et al., 2004). The E545K mutation leads to a reduced inhibitory effect of p85 on PI3KCA. 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). Hyperactivity of PI3K/AKT signalling has been reported in multiple cancers and has been linked to anti-cancer therapy resistance (Pópulo, Lopes, & Soares, 2012; Salaroglio, Mungo, Gazzano, Kopecka, & Riganti, 2019; Tan & Yu, 2013). Furthermore, both PTEN and the TSC act as tumour suppressors and are frequently mutated in cancer (Mak & Yeung, 2004; M. S. Song, Salmena, & Pandolfi, 2012). mTOR is as a downstream target of these factors and integrates their signals. Therefore, mTOR has become a focus of anti-cancer therapy by either targeting mTORC1 or using dual inhibitors for PI3K and mTOR (Bhat et al., 2015).

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

mTOR also fulfills a central role in metabolic signalling. Here, mTOR integrates signals arising through amino acid availability, glucose metabolism and cellular oxygen levels and forms an appropriate response. For example, increased amino acid availability induces relocalisation of mTOR into proximity of Rag GTPases leading to its activation through Rheb (Sancak et al., 2008). Furthermore, Glucose deprivation leads to increased adenosine-mono-phosphate kinase (AMPK) signalling via phosphorylation of serine/threonine kinase 11 (LKB1) (Kimball, 2006; M. J. Sanders, Grondin, Hegarty, Snowden, & Carling, 2007; R. J. Shaw, 2009). In turn, AMPK phosphorylates TSC2 leading to its activation (Kimball, 2006). LKB1, by activating TSC2, acts as a tumor suppressor. LKB1 mutations have been found in cancer and studies evaluate its role in anti-cancer therapy (R.-X. Zhao & Xu, 2014). Hypoxia also inhibits mTOR via regulated in development and DNA damage response 1 (REDD1) which stabilises the TSC (Brugarolas et al., 2004). While hypoxia (i.e. deprivation of oxygen) inhibits

protein synthesis in normal cells, in breast cancer, protein synthesis was not inhibited during hypoxia which is attributed to uncontrolled mTOR signalling (Connolly, Braunstein, Formenti, & Schneider, 2006).

In cancer, cellular metabolism, proliferation as well as growth are often dysregulated (Hanahan & Weinberg, 2011). Given the central of mTOR governing proliferation, growth and metabolism it is vital to comprehensively understand mTOR signalling in this disease (P. P. Roux & Topisirovic, 2018).

1.3.1.1 Global regulation of translation via mTOR

Well studied downstream targets of mTOR for the regulation of mRNA translation are 4E-binding proteins (4E-BP) and ribosomal protein S6 kinases (S6Ks). mTOR phosphorylates 4E-BP leading to the release of eIF4E that then can engange in eIF4F complex formation (A.-C. Gingras et al., 1999). Therefore, inhibition of mTOR leads to a down regulation of 5' cap dependent mRNA translation.

S6Ks have been shown to regulate phosphorylation of multiple components of the translation machinery, e.g. ribosomal protein S6 (rpS6), programmed cell death protein 4 (PDCD4), eEF2 and eIF4B. S6K phosphorylates rpS6 which has been implicated in the regulation of cellular growth and protein synthesis (Ruvinsky et al., 2005). Furthermore, S6K/rpS6 signalling was suggested to be involed in ribosome biogenesis. Another S6K target is eEF2 which, as mentioned above, facilitates transocation of peptidyl-tRNAs and therefore affects ribosome elongation rates (X. Wang et al., 2001). Furthermore, phosphorylation of PDCD4 by S6K triggers its degradation. PDCD4 blocks eIF4G-eIF4A interactions repressing eIF4A activity and cap dependent mRNA translation (Dorrello et al., 2006; A. Göke et al., 2002). Lastly, phosphorylation of eIF4B by S6K stimulates the unwinding activity of eIF4A (Rogers, Richter, Lima, & Merrick, 2001).

Collectively, through acting on its downstream targets, mTOR regulates translation globally via cap dependent mechanisms, translaton elongation and ribosome biogenesis.

1.3.1.2 Selective or "mTOR sensitive" regulation of translation

Selective or "mTOR sensitive" regulation of translation acts on transcripts with a terminal oligo pyrimidine motif (TOP mRNAs). This TOP motif consists of a C followed by a stretch of 4-15 pyrimidines directly after the 5' cap. TOP mRNAs show near complete dissociation from ribosomes under conditions when mTOR is inhibited and are enriched for genes encoding for components of the translation machinery (Meyuhas, 2000; Thoreen et al., 2012; Yamashita et al., 2008). Recent works indicate the importance of La ribonucleoprotein domain family member 1 (LARP1) in regulation of TOP mRNAs with contradictory findings (B. D. Fonseca et al., 2015; Hopkins et al., 2016; Jia et al., 2021; Maraia, Mattijssen, Cruz-Gallardo, & Conte, 2017). A panel of researches were asked to evaluate these findings which led to the establishment of a model for translational regulation via LARP1 (Berman et al., 2021). Herein, LARP1 binds to the 5' mRNA cap of TOP mRNAs via its DM15 domain. Binding of DM15 represses translation of TOP mRNAs by obstructing eIF4E binding. This binding occurs in instances where mTOR activity is abolished. In environments where mTOR is active, phosphorylation of the DM15 occurs by mTOR. This causes DM15 to release the 5' cap. However, the la domain of LARP1 which is bound to the 3' UTR remains. The still bound la domain stabileses the mRNA to facilitate translation (Berman et al., 2021). Other instances of selective translation by mTOR are explained in the next section.

1.3.2. Selective regulation through members of the eIF4F complex

As mentioned above, the eIF4F complex consists of eIF4E, eIF4A and eIF4G and is required for cap dependent mRNA translation (A. G. Hinnebusch, 2014). The availability of the eIF4F complex is normally limited due to that eIF4E is bound by 4E-BPs. Therefore, under normal conditions mRNAs must compete for translation. Such competition is affected by characteristics of 5' UTRs that introduce variation to how well mRNAs can be translated. Benedetti et. al. derived and expanded on a model for mRNA competition origally proposed by Lodish (De Benedetti & Graff, 2004 ; Lodish, 1974). Herein, "strong" mRNAs are widely expressed and represent the majority of cellular mRNAs and are characterised by optimally long and unstructured 5' UTRs, e.g. β-actin. An optimal 5' UTR length of ~80 nucleotides was proposed by Kozak (Kozak,

1987). On the other hand, weak mRNAs show long and structured 5' UTRs. "Strong" mRNAs encode for potent growth and survival factors, e.g. c-Myc, ornithine decarboxylase (ODC1) and vascular endothelial growth factor (VEGF). Translation of "strong" mRNAs would remain effective in conditions where eIF4F complex availability would be limited. However, "weak" mRNAs show sensitivity to eIF4F availability which is dependent on eIF4E expression (Graff, Konicek, Carter, & Marcusson, 2008). Elevated eIF4E expression is common in cancer and drives malignancy due to selective induction of translation of tumor promoting mRNAs (De Benedetti & Graff, 2004).

Studies investigated the effects of eIF4A inhibition on mRNA translation and the underlying characteristics of eIF4A dependent mRNAs. Therein, mRNAs that dependent on eIF4A showed characteristics of "weak" mRNAs, i.e. these mRNAs had long and structured 5' UTRs (Rubio et al., 2014; Waldron, Raza, & Le Quesne, 2018; Wolfe et al., 2014). Stuctural elements in the 5' UTRs include classical hairpins formed through Watson-Crick basepairing (*see Figure 1.2*) (Leppek et al., 2018). Other structures using hoogsteen basepairing have also been proposed to regulate eIF4A dependent mRNA translation (Wolfe et al., 2014). These structures are called G-quadruplexes and are stable structures formed by stacking two or more G-tetrads (Kwok & Merrick, 2017). However, whether G-quadruplexes form in cells is still debated (Biffi, Di Antonio, Tannahill, & Balasubramanian, 2014; J. U. Guo & Bartel, 2016; Laguerre et al., 2015; Weldon, Eperon, & Dominguez, 2016). Some predict formation of G-quadruplex structures based on occurrences of GGC_4 motif repeats in the 5' UTR of mRNAs (Singh et al., 2021; Wolfe et al., 2014). Indeed, multiple studies report enrichments of GGC_4 motifs in mRNAs whose translation is dependent on eIF4A (Modelska et al., 2015; Rubio et al., 2014; Singh et al., 2021; Waldron et al., 2018). However, Waldron et al. show that GCC_4 motifs fail to form G-quadruplexes in their reporter mRNA system. The authors concluded that eIF4A dependence of mRNAs with GGC_4 motifs enriched in their 5' UTR was likely mediated by classical hairpin-like structures (Waldron et al., 2018). In addition, in Rubio et. al. one third of eIF4A dependent mRNAs exhibited multiple 5' UTR variants, while for eIF4A independent mRNAs this was $< 1\%$ (Rubio et al., 2014). Translation of mRNAs with different 5' UTR variants has recently been implicated to drive cancer cell plasticity towards more "stem cell-like" phenotypes during hypoxia (Jewer et al.,

14

2020). Notable here is the use of eIF4A inhibitors with different modes of action as a means to test eIF4A dependency of mRNAs. Hippuristanol inhibits RNA interaction of selective binding to eIF4A (Bordeleau et al., 2006; Lindqvist et al., 2008). In contrast rocaglates and its derivatives, e.g. silvestrol and CR-1-31-B, clamp eIF4A on polypurine sequences on the mRNA (Iwasaki, Floor, & Ingolia, 2016).

Recently, a more nuanced picture for translation of mRNAs sensitive to inhibition of components of the eIF4F complex has been shown (Gandin et al., 2016b). Therein, a comparison of treatments with a mTOR and eIF4A activity inhibitors was evaluated. This revealed that mTOR sensitve mRNAs encompass two functionally distinct sets of mRNAs. These sets were characterised by different 5' UTR features. Here, mRNAs characterised by long and structured 5' UTRs and endoced for pro surival proteins and were dependent on both eIF4E and eIF4A (hereafter referred to as mTOR-eIF4A sensitive). The other subset showed less sensitivity to eIF4A but more so to eIF4A (here after referred to as mTOR-eIF4E senstive mRNAs). mTOR-eIF4E sensitive mRNAs encoded for proteins in metabolic functions. The authors concluded that inhibiton of mTOR-eIF4A dependent programs would lead to cytotoxic effects. Whereas, inhibition of mTOR-eIF4E mRNAs would lead to metabolic dormancy and a cytostatic effect (Gandin et al., 2016b).

1.3.3. The integrated stress response The integrated stress response (ISR) is a pathway activated through kinases responding to various stress signals. These kinases include Protein kinase R-like endoplasmic reticulum kinase (PERK) activated by misfolded peptides in the endoplasmatic reticulum (ER). Heme regulated eIF2alpha kinase (HRI) activated during oxidative stress. Protein kinase R (PKR) which is activated in response to certain viral infections by binding to double-stranded RNA (dsRNA). As well as general control nonderepressible 2 (GCN2) which is activated when cells are deprived of amino acids (Dmitry E Andreev et al., 2015; Guan et al., 2017; Kapur, Monaghan, & Ackerman, 2017; Lemaire, Lary, & Cole, 2005; Taniuchi, Miyake, Tsugawa, Oyadomari, & Oyadomari, 2016). (*see also figure 1.4*).

1.3.3.1 Global and selective regulation of translation via the ISR

Global and selective regulation of translation via the ISR is, similar to mTOR signalling, achieved at a global and selective level. During the ISR the α subunit of eIF2 is phosphorylated. Phosphorylated eIF2α directly engages the guanine nucleotide exchange factor eIF2B and prevents conversion of inactive eIF2-GDP to active eIF2-GTP needed for met-tRNAi incorporation in the TC. This reduces TC availability and causes a global downregulation of mRNA translation (Sonenberg & Hinnebusch, 2009) 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 the coding sequence ORF (cdsORF) (*see Figure 1.2*). uORFs can be out of frame with the cdsORF and, when translated, lower the expression of the cdsORF (Kozak, 1984). Activating transcription factor 4 (ATF4), that regulates expression of stress response genes, contains multiple uORFs of which one partially overlaps with the cdsORF. Under normal conditions ATF4 translation is initiated at uORF1 and reinitiation at uORF2 occurs. The overlap of uORF2 with the cdsORF causes ribosomes to synthesise protein from uORF2 thereby inhibiting the translation of the coding sequence. Limitation of TC availability during ISR causes longer ribosome scanning times leading to that ribosomes scan past uORF2 and initiate at the cdsORF (i.e. delayed reinitiation) (Pakos-Zebrucka et al., 2016).

Ribosome profiling studies indicate that 50% of mammalian mRNAs harbour uORFs (*see section 1.4 for detials on ribosome profiling*). mRNAs containing uORFs include oncogenes and transcripts important in differentiation and cell cycle (Calvo, Pagliarini, & Mootha, 2009; Ingolia et al., 2011). Apart from delayed reinitiation, uORF translation can also be due to "leaky scanning". The surrounding sequence of the uORF is important for initiation of translation. An AUG in the classical Kozak context (i.e. RNN**AUG**G) is most efficient for translation initiation due to better recognition by the met-tRNAi (Calvo et al., 2009; Kozak, 1986). Unfavored flanking sequences of the AUG can cause the ribosome to scan past the AUG, this process is called "leaky scanning". An example of this is DNA-inducible gene 34 (GADD34) which increases its translation upon ER stress, i.e. a condition where eIF2α is phosphorylated. In humans, GADD34 contains two uORFs separated by 30 nucleotides (Y.-Y. Lee,

Cevallos, & Jan, 2009). In contrast to the uORFs in ATF4, the uORFs in GADD34 are to close together to promote reinitiation. Under basal conditions uORF2, which has a poor kozak context, represses translation of the CDS. However, under stress causing eIF2α phosphorylation, ribosomes scan past uORF2 to translate the CDS (Young, Willy, Wu, Sachs, & Wek, 2015). Furthermore, both uORF1 seems to initiate translation to repress the cdsORF under basal conditions. Lastly, structural elements in proximity of the uORF can also influence its translation (H. Ruan, Hill, Fatemie-Nainie, & Morris, 1994).

1.3.4. 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 the ribosome. In eukaryotes, 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 1.3.4**). 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
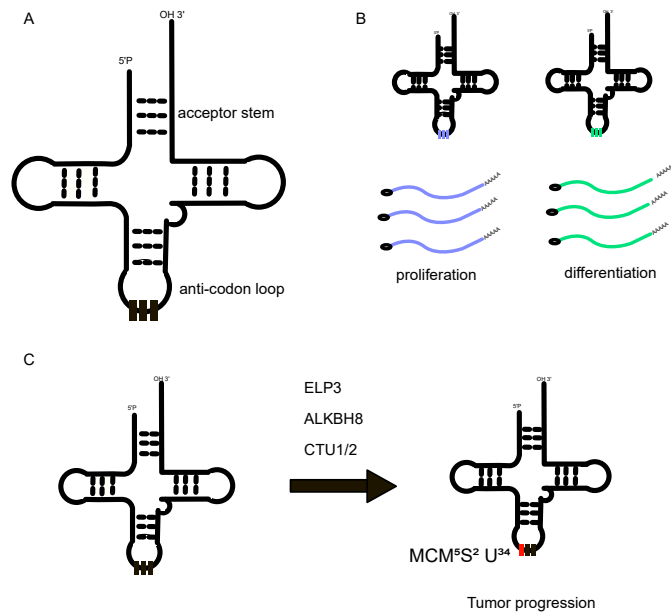

Figure 1.7: Schematic representations of (A) the tRNA cloverleaf structure with indicated anti-codon and amino acid acceptor sites; (B) the proliferation and differentiation mRNAs dependent on distinct tRNA subsets; (C) the U34 wobble position and the catalytic enzymes involved in this modification that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

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 and thus protein synthesis.

This supply (i.e. tRNA availability) and demand (i.e. codon composition) relationship has been found to vary across different cellular states, e.g. proliferation and differentiation. Gingold et. al. observed two differen tRNA subsets. One

subset is induced under proliferation and is otherwise repressed. And the other subset is induced under differentiation and repressed otherwise. Both tRNA subsets match the codon demand of the transcriptome under their respective cellular state (Gingold et al., 2014). This model has been disputed and it was proposed 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 using small RNAseq (i.e. for identification of tRNAs) and protein samples across 17 tissues reported a tRNA signature stratified by proliferation marker Ki67 staining. The identified tRNA signature has 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 (Gingold et al., 2014; Hernandez-Alias et al., 2020; Z. Zhang et al., 2018). For instance, increased expression of $tRNA_{CCG}^{Arg}$ and $tRNA_{UUC}^{Glu}$ has been observed in breast cancer cell lines and proposed to drive metastasis (Goodarzi et al., 2016).

Others report of a role of tRNAs in cancer attributed to tRNA modifications, specifically at the U34 anti- codon (or wobble) position which is highly conserved (El Yacoubi, Bailly, & de Crécy-Lagard, 2012; Lorent et al., 2019; Rapino, Delaunay, Zhou, Chariot, & Close, 2017). The ability to wobble was proposed by Francis Crick and refers to 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. The URM includes the cytosolic thiouridylase homolog 1 and 2 (CTU1/CTU2) (Kalhor & Clarke, 2003; Karlsborn et al., 2014). These enzymes ultimately modify the U34 position into 5-methoxycarbonyl-methyl-2-thiouridine (mcm^5s^2U) which ensures cognate codon recognition. This modification is thought to occur in tRNAs with a U in the wobble position, e.g. $tRNA^{UUU}$, $tRNA^{UUC}$, $tRNA^{UUG}$, $tRNA^{UCC}$, and $tRNA^{UCU}$.

Loss of the ability to modify U34 has been shown to reduce translation elongation

18

rates with varying effects on protein expression (Deng et al., 2015; Nedialkova & Leidel, 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), others reported a subtle dowregulation of proteins encoded by mRNAs requiring U34-modified tRNAs (Deng et al., 2015). U34 modification dependent tRNAs have been shown to play a role in cancer. For example, ELP3 is important in tumor initiation in intestinal epithelia and promotes breast cancer invasion as well as progression to metastasis (Delaunay et al., 2016; Ladang et al., 2015).

1.3.5. RNA binding proteins and trans-acting factors The UTRs of an mRNA contain sequence elements to which small RNA and RNA binding proteins (RBPs) bind and exert translational regulation (*see figure 1.2*).

1.3.5.1   microRNAs

microRNAs (miRNA) are a class of small non-coding RNA. The precise role and mechanisms in regulation of translation by miRNAs is still under active investigation (Oliveto, Mancino, Manfrini, & Biffo, 2017). However, they can directly bind to other mRNAs and silence them accomplished through translational repression or destabilisation [Jonas2015]. Regulation of gene expression by miRNAs has been observed in cancer. Here, miRNAs have been implicated to promote tumorgenesis or act as tumor suppressors (Muniyappa et al., 2009; Nagpal et al., 2015; Sampson et al., 2007; Tian et al., 2010).

1.3.5.2   RNA binding proteins

RBPs are a class of proteins involved in many regulatory steps of gene expression and account for ~7.5% of the protein coding genes. RBPs bind to elements in the 3' UTRs, e.g. the poly (A) tail. One RBP binding the poly(A) tail is PABP. As explained in *section 1.2.3* PABP is involved in mRNA translation initiation and stabilisation of the mRNA (Afonina, Myasnikov, Shirokov, Klaholz, & Spirin, 2014; Amrani, Ghosh, Mangus, & Jacobson, 2008) (**see also figure 1.4**).

Another site in the 3' UTR is the U-rich cytoplasmic polyadenylation (CPE) site to which cytoplasmic polyadenylation element binding proteins (CPEBs)

can bind (*see Figure 1.2*). Studies in *Xenopus* oocytes indicate that CPEB associates with a non-canonical poly(A) polymerase (Gld2) and a poly(A) deadynelase (PARN). PARN has a higher activity than Gld-2 and thus binding of CPEB to an mRNA leads to shortening of the Poly(A) tail (Barnard, Ryan, Manley, & Richter, 2004). However, hormonal stimulation leading to CPEB phosphorylation removes PARN from the complex. Removal of PARN promotes poly(A) tail elongation through Gld-2 (J. H. Kim & Richter, 2006). Furthermore, in *Xenopus* oocytes, CPEB associates with an eIF4E binding protein, i.e. maskin. Maskin bound to eIF4E prevents eIF4F complex formation which represses mRNA translation (Ivshina, Lasko, & Richter, 2014; Stebbins-Boaz, Cao, de Moor, Mendez, & Richter, 1999). Therefore, CPEBs can regulate translation by altering 3' UTR lengths and are involed in transaltional repression by blocking eIF4A association with the 5' cap in *Xenopus* oocytes. While the role of CPEB mediated regulation has been described mostly in *Xenopus* oocytes, dysregulation of CPEBs has been observed in glioblastoma, colorectal and pancreatic cancer (Y.-T. Chang et al., 2014; Ortiz-Zapater et al., 2011; Villanueva et al., 2017).

Another important RBP implicated in regulation of translation is Human antigen R (HuR). HuR preferentially binds to AU-rich sequences in the 3' UTR and acts as a stabilising agent and is involved in RNA-processing (Baou, Norton, & Murphy, 2011; X. C. Fan & Steitz, 1998; T. D. Levine, Gao, King, Andrews, & Keene, 1993; S. S.-Y. Peng, Chen, Xu, & Shyu, 1998). In colorectal carcinoma cells HuR has been shown to enhance protein synthesis of p53 after exposure to short-wavelength UV light (UVC) by binding the 3' UTR (Mazan-Mamczarz et al., 2003). The enhanced effect on protein synthesis was not mediated by stability as HuR failed to stabilise p53 upon UVC exposure. In human bone osteosarcoma epithelial cells, overexpression of HuR led to a dose dependent increase in eIF4E protein levels. The increase in protein levels was atrributed to eIF4E transcript stabilisation through binding of HuR to AU-rich elements (AREs) in the 3' UTR of eIF4E (Topisirovic et al., 2009). Other known drivers of tumor progression targetted by HuR include hypoxia inducible factor 1 (HIF-1), VEGF, c-Myc (Denkert et al., 2004; López de Silanes et al., 2003; López de Silanes, Lal, & Gorospe, 2005). Furthermore, studies in breast, colon and lung cancer observed correlation between HuR and malignancy (Denkert et al., 2004; López de Silanes et al., 2003, 2005).

1.4. Expertimental methods to measure mRNA translation

Two methods are predominantly used for measuring mRNA translation to estimate changes in translation efficiencies across conditions, namely polysome profiling and ribosome profiling (**see figure 1.8**). These methods capture the number of ribosomes an mRNA is associated with. The number of bound ribosomes is an adequate estimator changes in translation efficiencies when initation is rate limiting. Indeed, as explained above this is a common scenario.

1.4.1. Polysome profiling

Polysome profiling allows for separation of polysomes from monosomes, ribosomal subunits and messenger
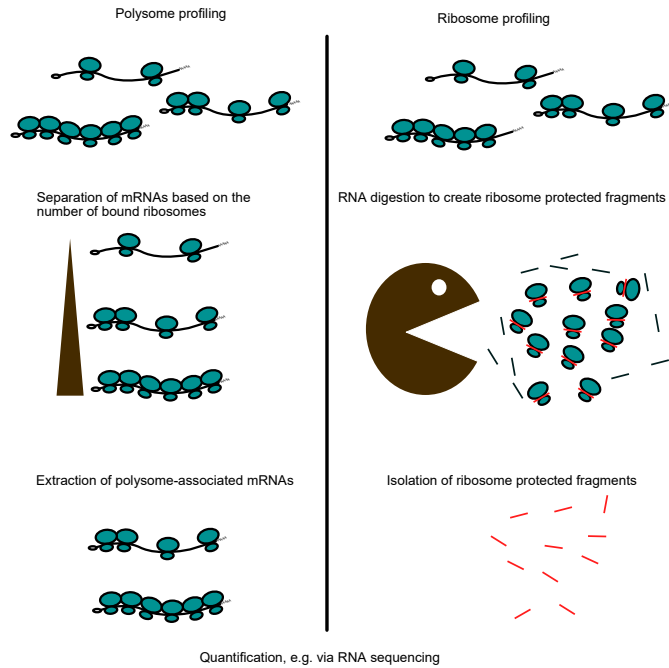

Figure 1.8: Polysome profiling and ribosome profiling workflows. In polysome profiling a fraction from whole cytoplasmic RNA is loaded onto a sucrose gradient on which they get separated by sedimentation into the sucrose gradient using ultra centrifugation. Fractions corresponding to efficiently translated 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 breaks the RNA. Ribosomes will protect fragments thereby creating ribosome protected fragments. These fragments are then isolated and can be sequenced.

ribonucleoprotein particles (mRNPs). During the assay, ribosomes are immobilized on the mRNAs using translation elongation inhibitors, e.g. cycloheximide (CHX). Cytoplasmic RNA extracts sediment into a linear sucrose gradient (5-50%) using ultra centrifugation. The resulting gradient is fractionated and mRNAs with different numbers of bound ribosomes can be extracted and analyzed for changes in translational efficiency (Gandin et al., 2014). Typically fractions belonging to mRNAs with more than 3 bound ribosomes are pooled. A 3-ribosome cut off has been chosen as it is thought to capture most biologically relevant changes in translation efficiency (Gandin et al., 2016b).

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 2 or more bound ribosomes. mRNAs are typically normally distributed along the fractions, i.e. in a pool of the same mRNA many will be associated with different numbers of ribosomes (Gandin et al., 2016b). Changes in mRNA abundance may lead to an overall increase in the amount
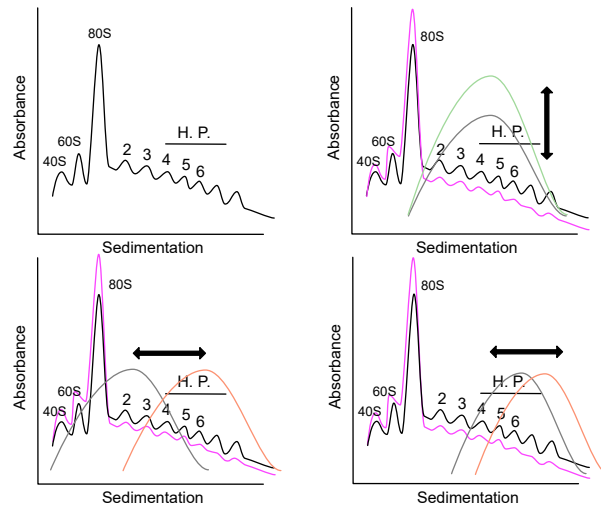

Figure 1.9: Polysome proﬁles - (top left) Schematic representation of a polysome profile using linear sucrose gradient fractionation. Indicated in the polysome profiles are the 40S, 60S ribosomal subunits as well as the 80S monosome. H.P. indicates heavy polysome fractions.Between conditions (i.e. black 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.

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 along the polysome fractions. For example, if the mRNA shift from the light (inefficiently translated) towards the heavy (efficiently translated) polysome fractions or vice versa in the absence of changes in total mRNA levels (**Fig 1.9 bottom left**). Shifts within the heavy polysome fractions (i.e. with a mean distribution around 4 bound ribosomes to 7 bound ribosomes) can also occur (**Fig 1.9 bottom right**). 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 efficiently translated mRNAs are pooled and quantified using either DNA-microarrays or RNA sequencing.

Pooling of mRNAs as well as collection of multiple fractions makes polysome profiling inconvenient when dealing experiments with low amounts of input RNA or with large samples sizes. Therefore, an optimized sucrose gradient was developed where mRNAs bound to >3 ribosomes 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 that impact to pool of mRNAs that can associate with ribosomes e.g. transcription and mRNA stability. Therefore, to identify true changes in translation efficiency it is important to collect cytoplasmic mRNA and polysome-associated mRNA from the same sample in parallel to correct for such mechanisms during downstream analysis.

1.4.2. Ribosome profiling For R17 bacteriophage ribosome protected RNA fragments (RPFs) have been obtained in the 1960s using ribonucleosases to trim away mRNA sequences not protected by ribosomes (J. A. Steitz, 1969). More recently ribosome profiling has been developed. Ribosome profiling enables sequencing of RPFs on a transcriptome-wide scale (Ingolia, 2016; Ingolia, Ghaemmaghami, Newman, & Weissman, 2009). Similar to polysome profiling. in the ribosome profiling assay ribosomes are immobilised on mRNAs using translation elongations inhibitors (e.g. CHX).

RPFs are obtained by RNAse treatment that degrades the links of RNA between ribosomes leaving single ribosomes with a ~28 nucleotide long RNA fragment within each ribosome. However, as ribosomal RNAs (rRNA) are also degraded during this process they represent a big fraction herein. The RPFs are then isolated using ultra centrifugation through a sucrose cushion. During this step other RNA fragments such as rRNAs, non-coding RNAs (ncRNAs) or large ribonucleoprotein complexes can co-migrate and contaminate the sample. Typically RPFs with a size ranging from 25-30 nucleotides are selected for quantification. However, among these sizes could be RNA protected by RBPs that have no ribosome associated to it. Furthermore, ribosomes undergoing conformational changes have been shown to protect fragments corresponding to a length of 21nt when translation elongation is blocked by e.g. CHX (L. F. Lareau, Hite, Hogan, & Brown, 2014). Therefore, size selection can distort the estimation of translation efficiency from ribosome profiling data (Dmitry E. Andreev et al.,

2017). From the resulting RPFs libraries can be constructed and quantified using RNA sequencing. During library construction additional biases due to enzyme sequence preferences can be introduced that can lead wrong estimations of codon positions within the ribosome (Artieri & Fraser, 2014a).

Recently, Ribo-seq Unit Step Transformation (RUST) has been developed (O'Connor, Andreev, & Baranov, 2016). This algorithm can reveal mRNA sequence features affecting RPF density globally. The authors applied RUST to 30 publicly available data sets and identified substanial sequence heterogeneity affecting RPF densities. Thus, sequence bias is prominent in ribosome profiling data (O'Connor et al., 2016).

Initially, fragmented total mRNA using alkaline hydrolysis of the same size were retrieved in paralell to RPFs. This was achieved by extraction of total mRNA from cell lysate followed by purification via recovery of polyadenylated messages or removal of ribosomal RNA (Brar & Weissman, 2015; Ingolia et al., 2009). The random fragmentation of total mRNA has been shown to underlie experimental bias. Therefore, now sequencing of unfragmented total mRNA in parallel is preferred.

1.4.3. Comparing ribosome and polysome profiling Albeit both methods generate count data after quantification with RNA sequencing, there are some key aspects that differ between the techniques. Polysome profiling separates efficiently translated mRNAs from non- efficiently translated mRNAs along a sucrose gradient thereby creating an mRNA based perspective for analysing 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 can have implications for identifaction of transcript variants. For example, if a ribosome would not protect a fragment spanning a variant deducing site the information would be lost during ribosome profiling (S. N. Floor & Doudna, 2016). Whereas in polysome profiling the quantification is based on the whole mRNA. This gives polysome profiling the advantage in cases where transcript variants with different 5' UTR lengths are of interest.

Shifts in ribosome association can be dramatic (e.g. near complete dissociation

of ribosomes from an mRNA) or subtle (e.g. shifts from 2 to 4 ribosomes). Such different shifts be due to different properties of the mRNAs (Gandin et al., 2016b). When dramatic and subtle changes in ribosome association are present in parallel, ribosome profiling is biased towards identification of dramatic shifts and masks the subtle ones (Gandin et al., 2016b). Gandin et. al. showed under mTOR inhibition ribosome profiling studies would predominantly identify TOP mRNAs (i.e. heavy shifters). In contrast, polysome profiling identified both TOP and non-TOP mRNAs when mTOR is inhibited (Gandin et al., 2016b). Therefore, in scenarios where global mRNA translation is affected, application of ribosome profiling could lead to imprecise biological conclusions. The masking of subtle changes has been attrituted to the indirect estimation of translation efficiencies from counting RPFs for ribosome profiling, which is highly dependent on the abundance of mRNAs. In polysome profiling this effect is much less pronounced as changes in translation efficiency are directly estimated from the mRNAs associated with heavy polysomes (Masvidal, Hulea, Furic, Topisirovic, & Larsson, 2017). Therefore, polysome profiling is more suitable in studies that aim to analyse global changes in translation efficiences. (Gandin et al., 2016b; Masvidal et al., 2017).

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 is located at the mRNA. Polysome profiling cannot reveal ribosome locations along the mRNA. The single nucleotide resolution of ribosome profiling is necessary in contexts studying events such as ribosomal frame shifts or uORF translation (Dmitry E Andreev et al., 2015; Rato, Amirova, Bates, Stansfield, & Wallace, 2011). One limitation of ribosome profiling to identify features such as uORFs, is the use of elongation inhibitors in the protocol, e.g. CHX. After CHX treatment elongation is not immediatly inhibited but continues for several cycles (Hussmann, Patchett, Johnson, Sawyer, & Press, 2015). Studies investigating stress in yeast showed that increases in ribosome occupancy were due to CHX treatment rather than stress (Gerashchenko & Gladyshev, 2014). Therefore, CHX treatment can introduce biases in ribosome profiling obscuring indentification of sequence features that are potentially important for translational regulation (Gerashchenko & Gladyshev, 2014; Hussmann et al., 2015).

Both methods have their strengths and weaknesses. Thus, when designing an

experiment each method should be considered and chosen depending on the underlying biological question.

1.5. Modes for regulation of gene expression through mRNA translation

From transcriptome-wide assessments of translation using ribosome or polysome profiling expression levels are obtained for both cytoplasmic and polysome-associated mRNAs (or RPFs). For 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
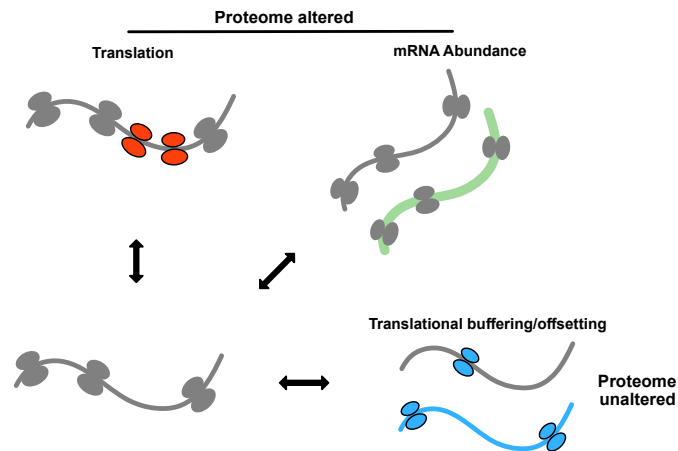

Figure 1.10: Modes for regulation of gene expression - Schematic representation of modes by which mRNA translation regulates gene expression in a fold-change scatter plot. Indicated in red are changes in translation (i.e. changes in translated mRNA but not total mRNA), in green changes in mRNA abundance (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.

interrogation at two steps of the gene expression pathway as well as their interaction. The interplay of total mRNA with translated mRNA can give valuable insights about the underlying mechanisms that govern gene expression in the studied system.

When comparing perturbed systems to their corresponding control state three "modes" in which translated mRNA and total mRNA distinctly interact can be observed (*see figure 1.5*). We refer to these modes as "mRNA abundance", "translation" (i.e. changes in translation efficiencies leading to altered protein levels) and, "translational buffering" (i.e. changes in translation efficiencies ensuring protein homeostasis).

1.5.1. mRNA Abundance A change in mRNA abundance is observed when the translated mRNA level changes to a similar magnitude as the total mRNA level (*see figure 1.5*). For these mRNAs the translation efficiency is unaltered, as the change in total mRNA levels explains the change in translated mRNA levels. This is in line with the model of "weak" and "strong" mRNAs where the abundance of mRNAs alters the ability to compete for translation initiation factors. The underlying biological implications for this mode are often related to transcription or mRNA stability. While genes of the mRNA abundance mode do not change their translation efficiency, the change in overall translation is expected to reshape the proteome.

1.5.2. Translation A change in translation occurs when translated mRNA levels either increase or decrease, while corresponding total mRNA levels remain constant or change to a lesser extent (*see figure 1.5*). The change in ribosome association indepedent of total mRNA levels is therefore a change in their translation efficiency. A prominent example of this mode can be observed for TOP mRNAs. Under conditions when mTOR is inhibited, TOP mRNAs show a near complete dissassociation from ribosomes (Gandin et al., 2016a). Furthermore, during the ISR, translation of ATF4 is altered due to eIF2α phosphorylation. Similar to changes in mRNA abundance, mRNAs under the translation mode are expected to reshape the proteome (*see figure 1.5*).

1.5.3. Translational buffering The third mode of regulation of gene expression is termed translational buffering. Under this mode, a change in total mRNA levels is observed, whereas polysome-associated mRNA levels remain constant between conditions. Translational buffering also reflects a change in translation efficiency as the proportion of mRNAs associated with ribosomes is altered. Notable, the change in translation efficiency upon translational buffering (i.e. ribosome associated is unaltered) is distinct to that from changes in translation where ribosome association is explicitly modulated. In contrast to changes in translation and mRNA abundance, translational buffering has been shown to maintain protein homeostasis rather than reshape the proteome (*See figure 1.5*) (Lorent et al., 2019; McManus, May, Spealman, & Shteyman, 2014).

Currently the literature supports multiple forms of translational buffering.

Translational buffering can compensate for differences in mRNA levels due to e.g. differences in gene dosages, so that protein levels remain similar (Dassi et al., 2015; Lalanne et al., 2018). Furthermore, rather than compensating it can "offset" modulations of total mRNA levels at the level of translation to temporarily alter the mRNA:protein ratio (Lorent et al., 2019).

Translational buffering in its compensation form has been observed at steady state levels. A study comparing co-evolution of transcription and translation across seven different organs and mammals showed overall greater divergence of transcription as compared to translation (Z.-Y. Wang et al., 2020). Similar compensation at the level of translation has been observed between individuals, tissues and prokaryotes (Artieri & Fraser, 2014b; C. Cenik et al., 2015; Dassi et al., 2015; Lalanne et al., 2018; Perl et al., 2017).

Compensation via translational buffering can also enforce equilibration of pathway or protein complex stoichometry (Lalanne et al., 2018; Li, Burkhardt, Gross, & Weissman, 2014). An example of this was observed in evolutionary distant bacteria, i.e. B. subtilis and E. coli. In B. subtilis translation related factors rpsP and rplS are located in different operons, whereas in E. coli they lie within an operon together with rimM and trmD. While B. subtilis can fine tune transcription at the different operons, in E. coli these will be transcribed together. However, rimM and trmD are only needed in low protein abundance, whereas rpsP and rplS are required in high abundance. E. coli compensates the transcriptional input at the translational level and thus equilibriates for requirements in pathway stoichometry (Lalanne et al., 2018).

As mentioned above, a different form of translational buffering can be observed in perturbed systems that offset changes in total mRNA levels at the level of translation temporarily. For example, translational offsetting was observed in prostate cancer cells where a transcriptional program was induced under estrogen receptor α (ERα) depletion that showed no increase in polysome-associated mRNA. mRNAs whose transcription was translationally offset required the tRNA u34 modification. ERα has been shown to regulate the expression of the catalytic enzymes required for the u34 modification (Lorent et al., 2019). Thus, depletion of ERα led to that tRNAs could not be properly modified at the U34 position. Therefore the translation efficiency of mRNAs requiring the modification was

reduced despite increased total mRNA levels. The authors confirmed that protein levels of translationally offset mRNAs were unaltered between conditions (Lorent et al., 2019).

1.6. Algorithms for analysis of changes in translation efficiencies

As discussed above, mRNA translation can reshape the proteome via multiple modes for regulation of gene expression. Furthermore, these modes can have different underlying biological mechanisms. It is therefore warrented to distinguish them in analysis of translation efficienies. In this section discusses methods to analyse polysome-profiling and ribosome profiling data to estimate changes in translation efficiencies across 2 or more conditions.

Initially analysis of transcriptome-wide translation studies used an approach called the translation efficiency score (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 gives an overview of the relationship between a change in TE-score and each gene expression mode (*see also figure 1.10*). Changes in mRNA abundance will lead to a ΔTE close to 0 in log space (i.e. no change in translation efficiency). This is due to that total mRNA and translated mRNA change with a similar magnitude. However, in the case of both translation and translational buffering, the nominator and denominator in the TE-score equation change leading to a ΔTE (TE < 0 or TE > 0). This leads to 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 (**see**
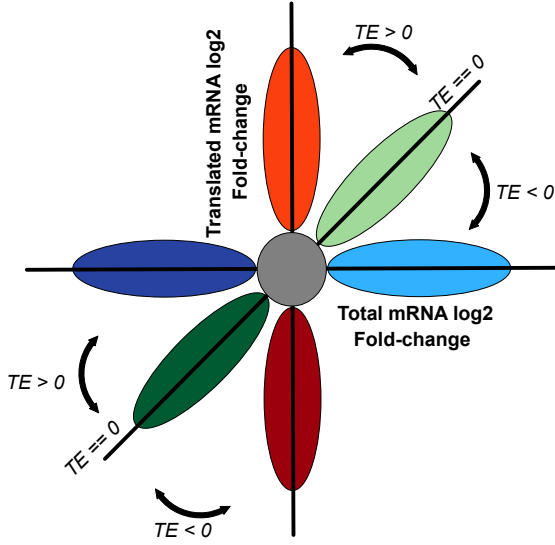
also section **1.5**).

Figure 1.11: TE-scores for modes for regulation of gene expression - Schematic representation of modes for regulation of gene expression in a fold-change scatter plot. The x-axis indicates the fold-change for total mRNA levels. The y-axis indicates the fold-change for translated mRNA levels. 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 relationship for the TE-score and the modes for regulation of gene expression is indicated.

The TE-score approach was questioned when proposing the Analysis of Translation Activity (anota) algorithm which was developed for DNA-microarray data (Larsson et al., 2010). 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) samples classes sharing a common slope, (iii) homoscedasticity of residuals and (iv) normal distribution of per gene residuals. In the second step anota performs analysis of changes in translational activity using the following model:

$$y_{gi} = \beta_g^{RNA}\ X_i^{RNA} + \beta_g^{cond}\ X_i^{cond} + \varepsilon_{gi}$$

here y_{gi} is the polysome associated mRNA expression, β_g^{RNA} describes the relationship to total RNA for the *gth* gene of the *ith* sample column of model matrix X; β_g^{cond} represent the difference in intercept between treatment classes and ε_{gi} denotes the residual error.

Within anota a common slope for the treatment classes that describes the relationship between translated mRNA and total mRNA is calculated. The difference between the slope intercepts is then interpreted as the change in translation efficiency. A simplified view of this model is provided in (*Figure 1.12 top left*). Here expression for translated mRNA and total mRNA are modeled over two sample classes that each has 4 replicates. Changes in translation efficiencies can also be observed when translated mRNA expression is modulated to a larger extent than the total mRNA levels (*Figure 1.12 top right*). Identification of genes in this category can be a challenge, especially in highly variable data sets, as they resemble mRNA
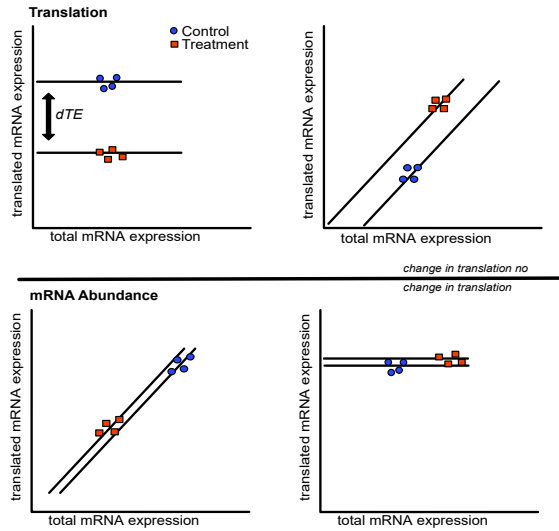

Figure 1.12: anota gene models - Schematic representation of the anota analysis models. Translated mRNA expression is set out against total mRNA expression for each biological replicate and treatment condition. Top left shows the model of a gene that is differentially translated (i.e. change in translated but not total mRNA). The difference in the slope intercepts are used to estimate changes in translation efficiencies between conditions. 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).

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*). Here, a change in total mRNA but not translated mRNA levels is observed. For this gene, the difference in slope intercepts between sample classes is small and will not be identified as differentially translated as would be the case in the TE-score approach. Anota was developed at a time where translational buffering was not considered in data sets. Naturally, the method lacks a setting to analyse translational buffering. This was addressed in anota's successor, anota2seq, and will be discussed in *Study 1*.

Advances in experimental methods warrant for appropriate statistical approaches to analyse data resulting from them. DNA-microarray was the dominant platform to assess transcriptome-wide changes before the advent of RNA sequencing. DNA-microarrays measure intensity after hybridisation events which is an indicator of expression. In contrast, in RNA sequencing reads of

constructed libraries 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 generalised linear models (GLMs) (Love, Huber, & Anders, 2014; Robinson, McCarthy, & Smyth, 2010). 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 column of the model matrix X and ε_{gi} denotes the residual error. When analysing changes in translation effiencies additional parameters 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 applying this exact model. A notable difference is that Ribodiff allows dispersion estimation for translated mRNA and total mRNA separately. Differences in of within-RNA variances between RNA types can be expected due to varying experimental protocols (Liang et al., 2018; Zhong et al., 2017). 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-variables 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 (see TE-score equation above). Therefore these methods suffer from similar issues as the TE-score which will be discussed in *Study 1*.

2. Aims of this thesis

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

In **Study I**, we adapted the ANalysis Of Translation Activity data (anota) algorithm so that it could be applied to next-generation sequencing data. Furthermore, we implemented the analysis of translational buffering a recently described mode for regulation 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 spurious correlations leading to elevated false positive identification that can result in false biological conclusions (Larsson et al., 2010). 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, Sonenberg, & Nadon, 2011). The Analysis of Translation Activity (anota) algorithm facilitates analysis of translational efficiencies that are corrected for changes in total mRNA levels (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 is due to the mean variance relationship, a characteristic of RNA sequencing data. 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 mRNA levels 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).
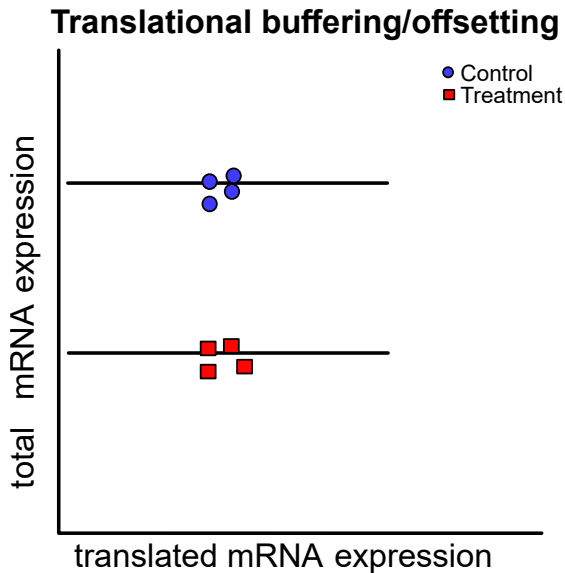

Translational buffering/offsetting

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 efficiencies. 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**).

Application of anota2seq has successfully 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 characteristics.

The aim of this study was 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 this we used simulated data. While it is arguable to what extent conclusion drawn from simulated data can be extended towards empirical 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 empirical data (Guan et al., 2017). Furthermore, during testing of our simulation we used an additional data set to estimate parameters from to generate data sets. Using these simulated data to compare the performance of the above mentioned algorithms showed almost identical results.

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 sampled reads from the same NB distribution for both the control and treatment conditions in both the translated and total mRNA. This category represents 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 sampled 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 samled 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 sampled 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 identification). 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. DESeq2 was marginally affected by this also. 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 simulated 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 shortcoming 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 (Chan et al., 2019; Chaparro et al., 2020; Hipolito et al., 2019; Lorent et al., 2019). Furthermore, more recently anota2seq has been used to compare mRNA levels between cytoplasmic mRNA and mRNA stored in P-bodies showcasing that anota2seq is generally applicable beyond analysis of translation efficiencies (Bearss et al., 2021).

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

Pancreatic cancer is considered a lethal malignancy and has limited treatment options. While other cancers (e.g. ovary, breast and stomach) showed a decline in mortality rates, no major overall reduction in mortality was observed for pancreatic cancer in the period of 1970-2021 (Carioli et al., 2021).

Pancreatic ductal adenocarcinoma (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 of the pancreas (Luchini, Capelli, & Scarpa, 2016). So far treatment options are mostly limited to surgical removal, which often is impossible due to the anatomical location of the pancreas head. The survival rate for this disease is less than 10% (Rawla, Sunkara, & Gaduputi, 2019). A Dutch nationwide study indicated that in cases were surgical removal was possible survival only increased from 9.1% to 16.5% (Latenstein et al., 2020).

With the increasing understanding of tumor heterogeneity anti-cancer therapy improved (Biankin & Hudson, 2011). For example, in breast cancer stratification

by histological, molecular and gene expression features identified 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 cancer treatment strategies benefit from a rather well established understanding of the molecular subtypes, in pancreatic cancer transcriptomic based subtyping is still ongoing (P. Bailey et al., 2016; Collisson, Bailey, Chang, & Biankin, 2019; Collisson et al., 2011; Moffitt et al., 2015; Puleo et al., 2018). Therefore, insufficient understanding of molecular mechanisms that underpin PDAC hinder development of more efficient therapeutic approaches.

While intricacies of molecular subtypes are still being investigated, research has shown that oncogenic mutations in KRAS as well as inactivation of tumor suppressor TP53 are commonly shared among PDACs (S. Jones et al., 2008). Furthermore, PDACs have been shown to be dependent on increased protein synthesis mediated induced via KRAS mutations (Chio et al., 2016). This indicates an important role of mRNA translation in PDAC.

The aim of *study 2* was to investigate the therapeutic effects of targeting eIF4A in a murine three dimensional PDAC organoid cell culture with mutations in the $Kras^{LSL-G12D}$, $Trp53^{LSL-R172H}$ and Pdx1-cre alleles that has been shown to recapitulate PDAC tumor progression (Boj et al., 2015). Pancreatic and duodenal homeobox 1 (PDX1), is an important factor for pancreatic differentiation. PDX1 knock out mice failed to develop a pancreas (Hale et al., 2005). The inhibition of eIF4A was carried out using a synthetic rocaglate, CR-31. Rocaglates have been shown to inhibit eIF4A helicase function and displayed anti-tumor activity (Cencic et al., 2009).

We first wanted to establish the therapeutic validity of targeting eIF4A in PDAC. *In vitro* experiments comparing treated PDAC organoids (KP) to their normal (N) counter parts revealed heightened sensitivity of KP organoids to CR-31 treatment relative to N organoids. OP-puromycin incorporation showed reduced protein synthesis in KP organoids, whereas N organoids were affected to a lesser extent. Furthermore, similar effects were found *in vivo* for PDAC tumours. Here CR-31 reduced protein synthesis (assessed by SUnSET assay), tumor growth (assessed by ultra sound imaging) and increased survival of mice. The effect on protein

synthesis was not due to inhibition of oncogenic signalling pathways which was evaluated via western blot assessing the phosphorylation of e.g. AKT, mTOR and 4E-BP1. From these findings we concluded that there is therapeutic validity in targeting eIF4A in PDAC.

Using polysome profiling, we then sought to decipher the mechanisms explaining the increased sensitivity to CR-31 in KP organoids. First we investigated the differences in gene expression between untreated KP organoids and N organoids. Analysis of changes in translation efficiencies using anota2seq revealed massive modulation at both total mRNA levels and translation indicative of underlying differences in e.g. genomic stability and enhanced oncogenic signalling impinging on protein synthesis reported in PDAC (Boj et al., 2015). Consistent with the *in vitro* OP-puromycin incorporation and *in vivo* SUNsET experiments, CR-31 strongly impacted global protein synthesis in KP organoids, while only exerting a modest effect in N organoids.

We then compared the translatomes of untreated KP organoids to that of N organoids. This revealed differences at both total mRNA levels as well as translation. Treatment of KP organoids with CR-31 reversed these changes. Thus the translational program of KP organoids is reversed when treated with CR-31. mRNAs affected by CR-31 in KP organoids showed modulated total mRNA levels in N organoids, that were offset at the level of translation. Translational offsetting maintain protein homeostasis (Lorent et al., 2019). The ability for N organoids to modulate mRNAs levels 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 mRNAs whose translation was affected 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) (Gandin et al., 2016b; Rubio et al., 2014; Wolfe et al., 2014). Furthermore, a mechanisms by which rocaglates would clamp eIF4A to mRNAs with [A,G] repeats in their 5 UTR was described (Iwasaki et al., 2016). However, mRNAs sensitive to CR-31 treatment herein had short 5' UTRs that were more structured when corrected for their length without enrichment for 4G-quadruplexes or [A,G] repeats. Therefore, CR-31 sensitive mRNAs in

KP organoids show 5' UTR characteristics different from those reported in the literature (*see section 1.3.2*). However, based on our polysome profiling and 5' UTR analysis we concluded that eIF4A supports an oncogenic translation program in PDAC cells for mRNAs with shorter but structured 5' UTRs.

Translation of mRNAs harbouring shorter 5' UTRs has been shown to be sensitive to eIF4E expression and encode for metabolic functions (Gandin et al., 2016b). 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 and consequently increased ability of eIF4E to engage in the eIF4F complex (Y. Martineau et al., 2014). 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). Indeed, CR-31 treatment in KP organoids reversed the translational profile for eIF4E sensitive mRNAs.

We further inspected the regulated gene sets in treated and untreated KP organoids compared to N organoids. Here we could see an enrichment in metabolic pathways, e.g. Oxidative phosphorylation. This pathway was upregulated at the polysome associated mRNA levels in untreated KP compared to N, whereas in KP organoids CR-31 treatment reversed the translational profile of this pathway. Furthermore, CR-31 treatment in KP organoids lead to reduced oxygen consumption rates, whereas N organoids where affected to a lesser extent. While measuring oxygen consumption rates do not rule out that non-mitochondrial sources are affected we attributed the observed decrease in oxygen consumption to defective oxydative phosphorylation.

A way to counter loss of energy production through oxidative phosphorylation is to increase activity of other metabolic pathways, i.e. glycolysis. However, in CR-31 treated KP porganoids we could not detect an upregulation of glycolysis measured by $U - C^{13}$ glucose labeling and extra cellular acidification rates nor did CR-31 treatment affect expression of glycolytic enzymes (e.g. HK1,HK2, LDHA, SLCA1, SLCA3). Furthermore, glucose deprivation did not further sensitise to CR-31 treatment. However, the polysome profiling 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 PDAC cell lines treated with CR-31. Glutamine can be converted into α-ketoglutarate and funneled into the citric acid cycle and therefore can serve to increase energy production (D. Xiao et al., 2016). Indeed, using gas chromatography mass spectrometry (GC/MS) to quantify metabolites after culturing PDAC cells in C_5^{13}- glutamine, we identified a shift towards reductive carboxylation of α-ketoglutarate obtained from C_5^{13}- glutamine to produce citrate. Notably, the reductive 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 PDAC cells to CR-31 treatment indicating that glutamine utlisation is important therein. Therefore, our study suggests an eIF4A dependent translational program in PDAC that can act as a theurapeutic target in PDAC. 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 PDAC.

Nevertheless, the same study indicated differences on the underlying regulated mRNA subsets (Singh et al., 2021). They report, in line with the literature, that eIF4A dependent mRNAs show long and structured 5' UTRs containing GGC_4 sequence motifs they propose to form G-quadruplexes (Singh et al., 2021). This raises some questions about the differences between experimental setups and their potential influence on biological outcomes and interpretation thereof. 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). Furthermore, by measuring IC50 concentrations for CR-31 in a panel of pancreatic cancer cell lines, Singh et. al. show a ~6-fold difference in susceptability to CR-31 between the cell lines of which PANC1 cells were most sensitive to CR-31. Dosage dependent viability experiments of patient derived PDAC cells in our study revealed that at 10nM CR-31 treatment cell viability was reduced by ~30%, whereas treatment with 25nM reduced viability by > 50%. Furthermore, in patient derived PDAC at 25nm CR-31 a combination treatment of CR-31 and CB839 did not alter cell viability compared to CR-31 treatment alone. However, at 12.50nM CR-31 treatment, combined treated with CR-31 and CB839 further reduced viability. Therefore, combining the findings of these two studies indicate that CR-31 treatment in PDAC indeed has a therapeutic effect. However the underlying mechanisms that are observed in the transcriptome wide analysis of translation efficiencies could be dependent on the experimental method to assess mRNA translation, the model system and drug concentrations.

3.3. Study 3 - Insulin signalling gene expression landscapes distinguish non-transformed vs. BCa cells

Breast cancer is an umbrella term for a heterogeneous disease with numerous molecular subtypes with different clinical behaviour. Currently, breast cancer is classified into five major groups; luminal A, luminal B, HER-2 enriched, triple negative/basal-like and normal-like (Dai et al., 2015). The classification is based on histology and molecular features. Histopathological features are determined by the degree of tumor differentiation (tubule formation), nuclear pleomorphism and proliferation (mitotic count). These charactersitics are then scored into a histological grade (I-III) (Eliyatkin, Yalçin, Zengel, Aktaş, & Vardar, 2015). As mentioned earlier, receptor status of the progesterone (PR), estrogen (ER) and HER-2 are evaluated and have implications for neo adjuvant and adjuvant treatment strategies. Advances in technologies lead to classification of breast cancer subtypes using gene expression profiles,e.g. the PAM50 classification, that allow for unbiased classification of breast cancer (Parker et al., 2009). A study that correlated the gene expression profiles of the PAM50 classification found a significanlty higher mRNA:protein correlation for PAM50 genes, indicating a prognostic value (Johansson et al., 2019). Nevertheless, the authors observed intermixed luminal B and HER2 clustering based on protein expression

(Johansson et al., 2019). This is in line with the literature where HER2 subtypes receive conflicting mRNA based classifications (Prat et al., 2014). Thus, the understanding of breast cancer subtypes has increased tremendously leading to improved and targeted treatment options, however there is need for further research to understand the full breast cancer spectrum on a molecular level.

Another important factor to consider in breast cancer treatment are life style and other health related issues that could impact cancer progression or response to treatment, e.g. obesity. Studies in the 1970 observed unfavorable prognosis for breast cancer in obese women (Abe, Kumagai, Kimura, Hirosaki, & Nakamura, 1976). Obesity can pose an increased risk to develop metabolic disorders such as metabolic syndrome or type 2 diabetes that can lead to hyperinsulinemia, i.e. elevated physiological insulin levels (Saltiel, 2001).

The role of insulin in the body is to regulate glucose and lipid metabolism as well as protein synthesis. Protein synthesis and metabolism are often dysregulated in cancer (Hanahan & Weinberg, 2011; Saltiel, 2001). Insulin can bind to its receptors insulin receptor (IR) A and IR-B that activate the PI3K/AKT/mTOR and RAS/ERK signalling pathways. A role of insulin in cancer progression has initially been observed in long term tissues cultures where it increased metabolism as well as growth (Osborne, Bolan, Monaco, & Lippman, 1976). IGFs (i.e. IGF1 and IGF2) carry out similar roles as insulin and bind to insulin-like growth factors receptor 1 and 2. Insulin and IGFs can bind to either IRs or IGFRs. Furthermore, IRs and IGFRs have been shown to form homo- and heterodimers, e.g. IR-A/IGF1R dimers. Studies indicate that insulin and IGF singalling is nearly identical (Boucher, Tseng, & Kahn, 2010; Pollak, 2008). Additionally, IGF1 plays a role in cancer progression and its levels are elevated upon hyperinsulinemia (Bailyes et al., 1997; Christopoulos, Msaouel, & Koutsilieris, 2015; Gallagher & LeRoith, 2010; A. Molinaro et al., 2019).

The importance of both IGF1 and insulin signalling in cancer has been well established now and led to development of therapeutic strategies by e.g. targeting both IGF1R and INSR or the PI3K signalling pathway (Kuijjer et al., 2013; Mayer et al., 2017). Yet, to this day the full mechsanisms of insulin/IGF action in cancer remain poorly understood. This study aims to bridge this gap in knowledge by elucidating the effects of insulin signalling on gene expression using a multi-omics

(including transcriptomics, translatomics and proteomics) approach to capture multiple steps of the gene expression pathway simultaneously. Furthermore, we asses insulin signalling in cancer cells as well as non-transformed epithelial cells.

We first investigated the effects of insulin on gene expression in a luminal A breast cancer cell line, i.e. MCF7 cells. MCF7 cells harbour the PI3KC mutatation and are senstive to insulin stimulation. Polysome-profiling revealed a strong modulation of total mRNA levels and translational response upon insulin stimulation. Among the translationally activated mRNAs were mRNAs with short 5' UTRs that harboured TOP motifs which is in accordance that insulin signalling leads to activation of mTORC1 and phosphorylation of its downstream targets. When visualising the mRNAs in the data where MCF7 cells were stimulated with insulin in the presence of torin1, an mTOR active site inhibitor, we observed that the changes in mRNA translation were almost fully reversed. This led to the conclusion that the effects of insulin on gene expression are to a great extent dependent on mTOR activity.

What was surprising to us was the observation that a subset of mRNAs exhibited translational buffering upon insulin stimulation while mTOR is inhibited. For these mRNAs, the total mRNA levels were increased, whereas the polysome-association was unaltered between conditions effectively. Thus their changes in total mRNA levels where offset at the level of translation. Using HiRIEF LC/MS we suggest that translationally offset mRNAs maintain constant protein expression across conditions (R. M. M. Branca et al., 2014; Lorent et al., 2019). The ability of translational offsetting to maintain protein homeostasis has been shown by others before (Lorent et al., 2019). These findings indicate that mTOR can act as a gatekeeper for transcriptional programs to fine tune translation in response to extra cellular or intra cellular signals.

MCF7 cells are of epithelial origin and therefore not "classical" insulin sensitive such as adipose tissue or muscle cells. Their strong response to insulin prompted us to investigate whether this could be due to cellular plasticity in cancer. To assess this we chose to compare the effects found in MCF7 to a non-malignant epithelial cell type, i.e. HMEC cells. We found that insulin alone was not sufficient to stimulate the PI3K/AKT/mTOR pathway in HMEC to a similar extent as in MCF7. However, a combination treatment of insulin and IGF1 in HMEC cells

46

elicited a similar reponse as insulin treatment in MCF7 alone. We therefore opted to compare the combined insulin + IGF1 treatment in HMEC to that of MCF7 assuming their signalling cascades are nearly identical as proposed in the literature (Boucher et al., 2010; Pollak, 2008).

Polysome profiling of the insulin + IGF1 stimulated HMEC cells revealed a strong translational response which was similar to MCF7 cells. Translationally activated mRNAs in HMEC showed 5' UTR features similar to those in MCF7 cells. Consequently, their translational activation was dependent on mTOR signalling evident from their translational suppression under conditions when mTOR was inhibited during insulin + IGF stimulation. Furthermore, comparing the mRNA signatures of HMEC and MCF7 in the opposite cell line we suggested that changes at the level of translation were almost fully in accord across cell types.

In contrast to MCF7 cells, HMEC did not elicit a strong strong modulation of total mRNA levels as shown by the small number of changes in mRNA abundance. When comparing a recently described transcription signature induced after IR translocation to the nucleus, we could see that in both HMEC and MCF7 these mRNA show changes in total mRNA levels but of differing magnitude (Hancock et al., 2019). A possible explanation for the different response in total mRNA levels could be due to differences in, e.g. chromosome instability between the cell lines that expose different parts of the DNA to trans acting factors. While not assessed herein, a transcription factor analysis paired with chromatin immunoprecipitation (ChIP) sequencing could provide insight into this (P. J. Park, 2009; Solomon, Larsen, & Varshavsky, 1988). Assuming a consequence of having a weaker modulation of total mRNA leves, HMEC cells did not elicit translational offsetting upon insulin + IGF1 stimulation when mTOR was inhibited. Thus the effects of insulin and IGF1 signalling on mRNA translation are foremost mTOR dependent, however total mRNA responses differ between malignant and non-malignant epithelial cells.

The translational offsetting in MCF7 drove us to investigate this phenomenon more. To assess differences dependent on mRNA characteristics we defined two subsets. The "reversed" and the "uncoupled" (that is translationally buffered when mTOR is inhibited) subsets that only differed in their total mRNA response

when mTOR was inhibited during insulin stimulation. To rule out that the observed effects on total mRNA are technical artifacts we validated total mRNA levels for two genes from each subset. The differences in regulation of gene expression were not dependent on codon usage which has been described before in a different context (Lorent et al., 2019). However, overall uncoupled mRNAs had shorter 3' UTRs with a higher GC content and were depleted for HuR binding sites.

The depletion of HuR binding sites in the 3' UTRs of the uncoupled subset prompted us to investigate mRNA stability differences. Using a time series experiment under actinomycin D treatment to block transcription quantified using nanoString, we found significant longer mRNA half lifes for the uncoupled subset as compared to the reversed subset. From these data we hypothesised that there are different underlying mechanisms that regulate gene expression under conditions where mRNA translation is dampened between these subsets. Under this hypothesis the reversed subset is likely regulated through mRNA stability, whereas the more stable uncoupled subset requires to be regulated by translation as their total mRNA level remains high for longer periods of time.

The involvement of HuR in this cannot be fully supported with our current data as the analysis only supports a correlation between the occurrence of HuR binding sites and the 3' UTRs of the reversed subset. The effect on stability could be due to other trans acting factors, e.g. miRNAs and other RBPs (Valinezhad Orang, Safaralizadeh, & Kazemzadeh-Bavili, 2014). A way to increase confidence is to investigate HuRs involvement experimentally, e.g. we could use a single guide (sgRNA) to silence HuR and measure total mRNA levels of the reversed and uncoupled mRNAs previously validated by qPCR. If HuR is involved, we would expect that the reversed mRNAs retain higher total mRNA levels under the condition where mTOR is inhibited during insulin stimulation in MCF7 cells as compared to control. Furthermore, while the differences in total mRNA levels between the insulin and the insulin and torin1 treated conditions in the RNAseq data imply a treatment effect on the mRNA stability we did not observe this in the time chase experiment quantified by nanoString. This raises the question whether the transcription block induced by actinomycin D could influence the regulation of mRNA stability between treatments. We could address this by including

actinomycin D in the sgHuR experiment and see if effects thereof differ or setup an experiment independent of sgHuR. Presence of an effect of actinomycin D on mRNA stability could indicate a cross talk between transcription and regulation of mRNA stability.

Since the translational offsetting identified herein was only observed in insulin treated cancer cells, we wondered whether this is only specific to this system. Cellular plasticity in cancer allows cancer cells to obtain stem cell like features (Jewer et al., 2020; Quail, Taylor, & Postovit, 2012; Wahl & Spike, 2017). Therefore, we reasoned that a system were we study gene expression of stem cells could give some insight whether cancer cells obtained stem cell features that normal epithelial cells do not have. Furthermore, we wanted to investigate whether other means of mTOR inactivation, e.g. hypoxia, would lead to similar effects on gene expression. To assess these aspects we cultured H9 stem cells in medium with insulin present in normoxia and hypoxia. This experimental setup differs to that of MCF7 and HMEC cells as these were serum starved (i.e. no insulin in medium) prior to induction with insulin.

Studying this system in H9 we could observe changes for all three modes for regulation of gene expression. Most notably, we observed a large fraction of translationally buffered mRNAs with similar 3' UTR characteristics to that of the uncoupled subset in MCF7 cells. Using publicly available data on mRNA stability we saw that the translationally buffered mRNA were overall more stable as compared to their background. Furthermore, visualising the reversed and uncoupled subset identified in MCF7 in the H9 data we observe differences in their regulation of total mRNA levels, indicating that these subset underlie different modes of regulation even across these two models. Furthermore, these data argue for that translational buffering observed in insulin treated MCF7 cells during mTOR inhibition is not limited to that system but can also occur under more physiological conditions.

Here we present an unprecedented and comprehensive investigation of the effects on insulin on gene expression in cancer cells and non- transformed epithelial cells across multiple steps of the gene expression pathway. Our results indicate that cancer cells have acquired an increased sensitivity to insulin signalling as compared to non-transformed epithelial cells that is largely dependent on mTOR

in both cell types. Furthermore, we observed that cancer cells have the ability to translationally buffer mRNAs which is a feature they share with stem cells.

4. Conclusions

Cancer is a vastly heterogeneous disease that is characterised by uncontrollable growth and proliferation as well as dysregulated metabolism that can evade therapy through acquiring resistance.   mRNA translation is a common denominator of these processes and it is therefore paramount to understand the precise mechanisms by which mRNA translation is regulated to better formulate therapeutic strategies against cancer.

This thesis provides an advance in methodology to analyse transcriptome-wide changes translation efficiencies that can be applied to study cancer models. Using this methodology in the context of pancreatic cancer we could propose a possible new therapeutic strategy for this lethal disease where treatment options are limited. Lastly, we explore the effects of insulin on gene expression in an unprecedented study that highlighted an adapted insulin responsiveness of cancer cells. Furthermore, we illuminate the ability of cancer cells to translationally buffer genes which is a feature they share with stem cells and could therefore be an acquired mechanism.

Taken together this thesis provides insight on gene expression in two different cancer models that could potentially lead to clinical applications. While these studies are a step forward, more research is needed to grasp the full extent of the mechanisms involved in cancer.

Acknowledgments

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