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UNTANGLING THE CONTRIBUTION OF UNTRANSLATED REGIONS TO mRNA TRANSLATION IN HEALTH AND DISEASE

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**Karolinska
Institutet**

Stockholm 2022

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Printed by Universitetsservice US-AB, 2022

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ISBN 978-91-8016-474-0

Cover illustration: David Ristau, 2022

UNTANGLING THE CONTRIBUTION OF
UNTRANSLATED REGIONS TO mRNA TRANSLATION
IN HEALTH AND DISEASE
THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Johannes Ristau

The thesis will be defended in public at the “Air and Fire” lecture hall at Science for Life Laboratory
in Solna,

11th February 2022, 9:00

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*Und jedem Anfang wohnt ein Zauber inne,
Der uns beschützt und der uns hilft, zu leben.*

- Hermann Hesse

To my family and friends.

In deep gratitude!

ABSTRACT

Protein homeostasis is essential for living organisms and a consequence of steps of the gene expression pathway such as transcription, mRNA translation as well as the degradation of mRNA and proteins. These major cellular events demand tight control, high maintenance, and require a large proportion of cellular resources. mRNA translation is regulated by a plethora of cellular pathways as well as features of the mRNA molecule itself and is implicated in malignancies such as cancer.

To decipher the contributions of mRNA elements in shaping the proteome, we developed a computational approach to identify mRNA features helping to understand their role in post-transcriptional gene regulation (**paper III**). While using mTOR-sensitive translation as a model, we characterized an mRNA subset, which despite its transcriptional regulation, does not lead to altered protein levels. This phenomenon, termed translational offsetting, associated with distinct features in the 3' untranslated region and mRNA stability.

The initiation step of mRNA translation is widely considered as rate-limiting and is greatly influenced by features in the 5' untranslated region. These characteristics can lead to impaired scanning and initiation and have been attributed to cellular cues such as the mTOR pathway and the integrated stress response. In **paper II**, we applied nano-cap analysis of gene expression (nanoCAGE) and identified 5'UTR variants containing upstream open reading frames. In combination with the development of a reporter-based high-throughput method we studied these variants in a 5'UTR-centric manner, which led to the discovery of an mRNA subset being stress-resistant due to precise transcription start site positioning.

In **paper IV**, we studied the coordination of gene expression upon depletion of the transcription factor ER α , known for its role in hormone-dependent cancers. Post-transcriptional regulation upon ER α depletion is characterized by extensive translational offsetting, which is largely assigned to features in the coding sequence of mRNAs. These mRNAs are enriched for codons requiring U34-modified tRNAs for their translation, while these modifications are regulated by ER α .

A large proportion of cancer types are characterized by aberrant tumor suppressor activity such as mutations or dysregulated protein levels of p53. Its reactivation by small molecules presents a promising strategy for cancer treatment. However, the exact mode of action of such compounds remains often elusive. RITA, a small molecule initially discovered for its induction of apoptosis upon p53 reactivation, induces cell death in a predominantly p53-independent manner. We studied RITA in the context of mRNA translation and found its activity is dependent on the phosphorylation of eIF2 α , a major regulator of mRNA translation (**paper I**).

LIST OF SCIENTIFIC PAPERS

- I. RITA requires eIF2 α -dependent modulation of mRNA translation for its anti-cancer activity

Johannes Ristau*, Vincent van Hoef*, Sylvain Peugot, Jianwei Zhu, Bo-Jhib Guan, Shuo Liang, Maria Hatzoglou, Ivan Topisirovic, Galina Selivanova, Ola Larsson
Cell Death & Disease 10, 845 (2019)

- II. Precise transcription start site selection in mRNAs with upstream open reading frames tunes stress-independent translation

Krzysztof J Szkop*, **Johannes Ristau***, Laia Masvidal, Baila Samreen, Laura Lee, Lynne-Marie Postovit, Ivan Topisirovic and Ola Larsson
Manuscript

- III. Anota2seqUtils uncovers widespread translational offsetting associating with 3'UTR features

Johannes Ristau*, Christian Oertlin*, Krzysztof J Szkop*, Valentina Gandin, Shannon McLaughlan, Marie Cargnello, Hayley Kim, Kristofferson Tandoc, Shan Chen, Georgios Mermelekas, Janne Lehtiö, Luc Furic, Michael Pollak, Ivan Topisirovic, Ola Larsson
Manuscript

- IV. Translational offsetting as a mode of estrogen receptor α -dependent regulation of gene expression

Julie Lorent*, Eric P Kusnadi*, Vincent van Hoef, Richard J Rebello, Matthew Leibovitch, **Johannes Ristau**, Shan Chen, Mitchell G Lawrence, Krzysztof J Szkop, Baila Samreen, Preetika Balanathan, Francesca Rapino, Pierre Close, Patricia Bukczynska, Karin Scharmann, Itsuhiro Takizawa, Gail P Risbridger, Luke A Selth, Sebastian A Leidel, Qishan Lin, Ivan Topisirovic, Ola Larsson, Luc Furic
EMBO J (2019) 38: e101323

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SCIENTIFIC PAPERS NOT INCLUDED IN THIS THESIS

Enhanced translation expands the endo-lysosome size and promotes antigen presentation during phagocyte activation

*Victoria E B Hipolito, Jacqueline A Diaz, Kristofferson V Tandoc, Christian Oertlin, **Johannes Ristau**, Neha Chaubhan, Amra Saric, Shannon McLaughlan, Ola Larsson, Ivan Topisirovic, Roberto J Botelho*

PLoS Biol. 2019 Dec 4; 17(12):e3000535

MNK2 governs the macrophage anti-inflammatory phenotype

Margarita Bartish, Dongmei Tong*, Yangxun Pan, Majken Wallerius, Hui Lin, **Johannes Ristau**, Sabrina de Souza Ferreira, Tatjana Wallmann, Vincent van Hoef, Laia Masvidal, Thomas Kerzel, Anne-Laure Johy, Christophe Goncalves, Samuel E J Preston, Talin Ebrahimian, Christina Seitz, Jonas Bergh, Kristian Pietras, Stephanie Lehoux, Luigi Naldini, John Andersson, Mario Leonardo Squadrito, Sonia V Del Rincón, Ola Larsson, Charlotte Rolny*

Proc Natl Acad Sci U S A. 2020 Nov 3; 117(44):27556-27565

Polysome-profiling for transcriptome-wide studies of mRNA translation

***Johannes Ristau**, Kathleen Watt, Christian Oertlin, Ola Larsson*

Methods in Molecular Biology, *under review*

Anota2seq for transcriptome-wide studies of mRNA translation

*Christian Oertlin, Kathleen Watt, **Johannes Ristau**, Ola Larsson*

Methods in Molecular Biology, *under review*

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LIST OF ABBREVIATIONS

3'	three prime
3'UTR	three prime untranslated region
43S PIC	43 Svedberg pre-initiation complex
4E-BP	4E binding protein
4EHP	eukaryotic translation initiation factor 4E family member 2
4E-T	4E transporter
5'	five prime
A	Adenine
Ago	argonaute RISC component
AKT	protein kinase B
anota	analysis of translational activity
Apaf-1	apoptotic protease activating factor 1
ARE	AU-rich element
ATF4	activating transcription factor 4
ATP	Adenosine triphosphate
BACE1	beta-secretase 1
BiP	heat shock 70 kDa protein 5
C	Cytosine
CAGE	cap analysis of gene expression
CCR4-NOT	carbon catabolite repression-negative on TATA-less
CDK2	cyclin-dependent kinase 2
CDKN1A	cyclin dependent kinase inhibitor 1A
CHOP	C/EBP homologous protein
CPEB	cytoplasmic polyadenylation element binding protein
DEK	DEK proto-oncogene
DENR	density-regulated protein

DNA	deoxyribonucleic acid
eEF	eukaryotic elongation factor
eIF	eukaryotic initiation factor
EPRS	glutamyl-prolyl-tRNA synthetase 1
ER	endoplasmic reticulum
eRF	eukaryotic release factor
G	Guanosine
GADD34	growth arrest and DNA damage-inducible protein GADD34
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GCN2	general control non-derepressible 2
GTP	Guanosine triphosphate
GW182	trinucleotide repeat containing adaptor 6A
HIF1 α	hypoxia-inducible factor 1 α
hnRNPK	heterogeneous nuclear ribonucleoprotein K
HRI	heme-regulated inhibitor
HuD	ELAV like RNA binding protein 4
HuR	ELAV like RNA binding protein 1
IRES	internal ribosome entry site
ISR	integrated stress response
ISRIB	integrated stress response inhibitor
KIF11	kinesin family member 11
lncRNA	long non-coding RNA
main ORF	main open reading frame
MAP	mitogen-activated protein
MAPK	mitogen-activated protein kinase

Mcl-1	induced myeloid leukemia cell differentiation protein Mcl-1
MCT-1	malignant T-cell amplified sequence 1
MDM2	mouse double minute 2 homolog
Met	methionine
miRISC	miRNA induced silencing complex
miRNA	micro ribonucleic acid
MNK	MAPK-interacting kinase
mRNA	messenger ribonucleic acid
mRNP	messenger ribonucleoprotein
MTORC1/2	mammalian/mechanistic target of rapamycin complex 1/2
MYC	MYC proto-oncogene
nanoCAGE	nano cap analysis of gene expression
nt	nucleotide
Oligo-dT	oligo-deoxy-thymidine
ORF	open reading frame
p38	Mitogen-Activated Protein Kinase 14
p53	tumor protein 53
PABP	polyA binding protein
PERK	protein kinase R (PKR)-like endoplasmic reticulum kinase
PI3K	phosphoinositide 3-kinase
polyA	poly-adenine
PRTE	pyrimidine-rich translational element
PTEN	phosphatase and tensin homolog
PTP	Protein tyrosine phosphatase
RAF	rapidly accelerated fibrosarcoma
RAS	rat sarcoma virus
Rheb	Ras homologue enriched in brain
RITA	reactivation of p53 and induction of tumor cell apoptosis

RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
S	Svedberg
S6K	ribosomal protein S6 kinase beta-1
SG	stress granule
shRNA	small hairpin ribonucleic acid
SILAC	Stable Isotope Labeling with Amino acids in Cell culture
siRNA	small interfering ribonucleic acid
SLC35A4	solute carrier family 35 member A4
SxL	protein sex-lethal
T	Thymine
TE	translational efficiency
TIA-1	TIA1 cytotoxic granule-associated RNA binding protein
TOP	terminal oligo-pyrimidine tract
TP53	tumor protein 53
tRNA	transfer ribonucleic acid
TSO	template switching oligo
TSS	transcription start site
TSC	tuberous sclerosis complex
TTP	tristetraprolin
U	Uracil
UMI	unique molecular identifier
uORF	upstream open reading frame
VEGF	vascular endothelial growth factor
XRN1	5'-3' exoribonuclease 1

PROLOGUE

Organisms are self-sustaining entities comprised of compartments, i.e., cells, as unicellular organisms such as prokaryotes (eubacteria, archaea) (Woese, 1987) and eukaryotes (Protozoa, unicellular fungi, unicellular algae; around 10% of all eukaryotes) (Boudouresque, 2015) OR a structure of innumerable cells called multi-cellular organisms termed eukaryotes (land plants, algae, fungi and animals) (Knoll, 2011). All these species can, to some extent, be identified and categorized by the blunt eye. However, these observable and unobservable differences are a consequence of invisible processes - **molecular biology**.

The work of two people established important concepts: Charles Darwin's theory about "the origin of Species" (Darwin, 1859), proposed that variations of species are heritable. Gregor Mendel, who laid out basic rules of genetics (Mendel, 1865), showed that genetic information is passed between generations within a species. Using salamander embryos, Walther Flemming identified threadlike structures in the nucleus (chromatin), which doubled when cells divided (mitosis) (Flemming, 1882). These threads, colored by basophilic dyes, were named chromosomes (from Gr. chroma, Eng. color, "colored bodies") (Waldeyer, 1888). The term "a gene" (from Gr. genos, Eng. birth, generation, category) was established by Wilhelm Johanssen, in an attempt to replace the ambiguous expression "Anlagen" (Eng. predisposition), which was commonly used during that time (Johanssen, 1909). The chemist Friedrich Miescher, working in the laboratory of Felix Hoppe-Seyler located in the castle of Tübingen, sought to identify the molecules building up cells. He collected old bandages from the nearby hospital to extract white blood cells from puss. He isolated a substance in 1869, which he named "das Nuklein" (from Lat. nucleus, Eng. kernel, core). Furthermore, he was able to demonstrate that this substance has an acid and a base component, which is composed of nitrogen, oxygen, phosphorous, and hydrogen (Miescher, 1871). Around 80 years later, James Watson and Francis Crick solved the structure of this substance: the DNA double helix. Presenting the basis for the understanding of the molecular mechanisms of **gene expression** (Watson and Crick, 1953).

Dear reader,

The above shall be a minuscule summary of important and often forgotten scientific findings today's scientists are basing their stories on. Thus, I see it as essential to mention these. Due to constraints in length and time, in this foolish compact form, omitting some researchers involved.

We will now dive – *ab initio* – into the context and content of my doctoral work. Welcome!

1 INTRODUCTION

1.1 THE DOGMA OF MOLECULAR BIOLOGY

Genetic information was initially thought to be encoded by proteins, since these were the only known macromolecules at the time which exposed high molecular complexity, in contrast to Deoxyribonucleic Acid (DNA). Work from Frederick Griffith showed that by infecting mice with strains of *Streptococcus pneumonia*, bacteria could transfer their, at that point unknown, genetic information (Griffith, 1928). Later, Avery and colleagues performed experiments, which showed that the extracted DNA from bacteria was the actual macromolecule containing genetic information (Avery et al., 1944). Experiments using radioactive labeled phosphorous and sulfur confirmed these results (phosphorus is found in DNA, sulfur is found in proteins) (HERSHEY and CHASE, 1952). Genetic information is stored in the nucleus of the cell in form of the DNA. It consists of 2 strands in which phosphate groups, as phospho-diester bonds between the C-5 and the C-3 of deoxyribose molecules, form a backbone. This structure results in the strand having a directionality, i.e. 5' to 3'. A double helix is formed by two independent DNA strands running against each other, while four different bases (adenine, thymine, cytosine and guanine) interact by pointing towards the strand's core, of which $A = T$ and $C \equiv G$ form hydrogen-bonds (number of dashes represent number of H-bonds), also known as Watson-Crick base-pairing (Watson and Crick, 1953). These bases make-up a “code”, the sequence which leads to an organism's uniqueness while containing the information for the organism's building blocks – proteins (Crick et al., 1961).

It was evident that DNA could not be involved in the synthesis of proteins, since it was absent at the sites in the cell where proteins were made. Moreover it became clear that levels of another large biomolecule, ribonucleic acid (RNA) increased when cells actively produced proteins (Brachet, 1942; Caspersson, 1941). The RNA molecule possesses similarities to the DNA molecule, however it contains ribose instead of deoxyribose, resulting in a hydroxyl group at the C-2 of the sugar. Furthermore, RNA uses the base uracil instead of thymine and does generally not form a double-strand (Allen, 1941). These findings were finally combined into the “central dogma of molecular biology” (Crick, 1970), which can be summarized as:

DNA can replicate itself (**replication**), under cell division (Lehman et al., 1958; Meselson and Stahl, 1958).

RNA is the transcript (**transcription**) of DNA and can be reverse-transcribed by viruses (Temin and Mizutani, 1970) and during telomere lengthening (Greider and Blackburn, 1989). Moreover, RNA can be replicated by RNA-dependent RNA polymerases in RNA viruses (Reich et al., 1961).

The gene product, a **protein**, is made from an RNA-template (**translation**).



Among the stages of the “Central Dogma”, mRNA translation will be the main subject discussed in this thesis. Hence, the processes preparatory to the synthesis of proteins will be discussed in less detail, in spite of their importance.

1.1.1 The genetic code

In 1902, Emil Fischer and Franz Hofmeister independently discovered that proteins are a chain of amino acids connected by peptide bonds (Fischer, 1902; Hofmeister, 1902). It was shown by induced mutagenesis in T4 bacteriophages that this information is encoded in triplets along the RNA (Crick et al., 1961) and gave rise to $(4 \times 4 \times 4)$ 64 possible triplets, also called codons. However, the number of identified amino acids in proteins was not 64, leading to the conclusion that the genetic code is degenerate, i.e. each amino acid is encoded by several codons (Crick et al., 1961; Jones and Nirenberg, 1966). The first characterized codon, UUU, encoding for phenylalanine (Matthaei and Nirenberg, 1961) paved the way to unravel the remaining codons (Nirenberg and Leder, 1964; Nirenberg et al., 1965) and led to the identification of the necessary adapters between the information encoded in the RNA and the amino acid, i.e. transfer RNAs (tRNAs) (Apgar et al., 1962; HOAGLAND et al., 1958; Holley et al., 1965). Of all 64 codons only 61 encode amino acids. The remaining 3 codons, stop-codons, lead to the termination of protein synthesis (Brenner et al., 1965, 1967). The start-codon, an AUG triplet, encoding for methionine, leads to peptide-bond formation (Clark et al., 1968; Levin et al., 1972). More recently other non-canonical start-codons have been identified (Ivanov et al., 2011). Proteins are composed of 20 amino acids (Ambrogelly et al., 2006), of which 9 are essential and must be supplied through the diet in mammals (Reeds, 2000). One class of proteins, selenoproteins, require an additional amino acid, selenocysteine, which has a specific catalytic activity necessary in redox-reactions (Johansson et al., 2005). This amino acid is encoded by a stop-codon, UGA, which in combination with a particular RNA motif in the 3' untranslated region (3' UTR), the SECIS Element (Berry et al., 1991), leads to incorporation of selenocysteine into the peptide-chain (Low and Berry, 1996).

1.1.2 RNA

RNAs exist in many different variants and abundances in the cell and can be broadly divided into “protein-coding” and “non-coding” classes. Non-coding RNAs such as small nuclear RNA (snRNA), tRNA and microRNA (miRNA), to a large extent, fulfill regulatory functions (Eddy, 2001). Long-non-coding RNAs (lncRNA) are another class of regulatory molecules that have been

discovered more recently. Interestingly, some lncRNAs have been found to be translated using methods to identify actively translated mRNAs (Aspden et al., 2014; Chen et al., 2020; Ingolia et al., 2011; Ji et al., 2015; Lee et al., 2012) and mass-spectrometry of proteins (proteomics) (Chen et al., 2020; Karunratanakul et al., 2019; Makarewich and Olson, 2017; Slavoff et al., 2013). However, currently, the functions of lncRNA-encoded proteins remain largely elusive. The most abundant RNA species in the cell is ribosomal RNA (rRNA), which comprises the machinery that synthesizes proteins, the ribosome. Another subclass is transfer RNAs (tRNAs), which act as adaptors between the genetic code (in the form of an mRNA) and the amino-acids used for protein synthesis (HOAGLAND et al., 1958). However the RNA subclass which will be most discussed in this thesis are messenger RNAs (mRNAs) which, as the transcript of protein-coding genetic information (protein-coding genes), are the blueprints for proteins.

1.1.3 Ribosomal RNA, the Ribosome

The macromolecule responsible for the synthesis of proteins is the ribosome. Ribosomes are composed to 40% of ribosomal proteins and to 60% ribosomal RNA (Frank, 2000). Since rRNAs are the most abundant RNA species in the cell (80%) (Warner, 1999), ribosomes are very abundant, with to 10^5 - 10^6 ribosomes per cell (Raveh et al., 2016). The discovery of the ribosome was closely connected to the advancements of electron microscopy. George Palade discovered small structures, closely associated with the endoplasmic reticulum (PALADE, 1955). These were initially described as ribonucleic particles (LITTLEFIELD et al., 1955) and later defined as ribosomes (ALLFREY et al., 1953; Roberts, 1958). Further structural studies using electron microscopy revealed that several ribosomes perform protein synthesis on a single mRNA. The uncovering of these structures, first named ergosomes and later polysomes, contributed greatly to basic understanding of mRNA translation (Slayter et al., 1963; Staehelin et al., 1963; Wettstein et al., 1963). The first structure of the bacterial ribosome was determined much more recently using x-ray crystallography (Ban et al., 2000; Harms et al., 2001; Wimberly et al., 2000). Additional insights into ribosomal structures including tRNA and the mRNA (Yusupov et al., 2001) and subsequent increased resolution (Schuwirth et al., 2005) widened the mechanistic understanding of protein synthesis. Moreover, these advancements revealed that peptide bond-formation was catalyzed by RNA without the direct involvement of proteins (Nissen et al., 2000) classifying the ribosome as a ribozyme, a RNA molecule with the ability to catalyze chemical reactions (Kruger et al., 1982). More recently, the first structure of the eukaryotic ribosome was obtained in *Saccharomyces cerevisiae* using x-ray crystallography (Ben-Shem et al., 2010, 2011) and cryo electron microscopy (cryo-EM) (Anger et al., 2013; Khatter et al., 2015). These structural insights have not only contributed to the understanding of translation mechanisms but greatly enabled the development of antibiotics targeting bacterial protein synthesis (Steitz, 2005; Yonath, 2005).

Ribosomes consist of two subunits, named after their sedimentation coefficient in Svedberg units [S]. In eukaryotes, these comprise the small subunit (40S) and the large subunit (60S), which together form the 80S monosome (Anger et al., 2013). Moreover, there are 80 ribosomal proteins and around 5,500 nucleotides of ribosomal RNA required for the assembly of one eukaryotic ribosome (Armache et al., 2010). These ribosomal proteins, are characterized by high abundance and protein stability (Nikolov et al., 1987) and encoded by particularly abundant and stable mRNAs containing a specific motif in their 5' untranslated regions, the terminal oligo pyrimidine tract (TOP), whose protein abundance is mainly controlled by mRNA translation (Meyuhas, 2000). Ribosomal RNA is characterized by high RNA-stability (Kaempfer, 1969; Meselson et al., 1964) and the maintenance of its RNA levels requires 60% of the cellular transcriptional output, in addition to the 50% of RNA-polymerase II activity for ribosomal proteins (Warner, 1999). All these features renders the ribosome synthesis as highly energy consuming and leads to an impressive ribosomal half-life of 12 days (Nikolov et al., 1987).

1.1.4 Messenger RNA

Messenger RNA is, in contrast to rRNA, one of the least abundant RNA molecules in the cell and was initially thought to be an unstable intermediate in *E.coli* bacteria upon phage infection (Brenner et al., 1961; Gros et al., 1961). Moreover, it was shown that apart from extracted ribosomes from bacteria, RNA from the soluble fraction was also necessary for protein synthesis (Matthaei and Nirenberg, 1961). These findings together revealed that apart from ribosomes another RNA species existed carrying the information derived from DNA to the site where proteins are made - mRNA. mRNA molecules (**Figure 1**) are typically 1000 – 3000 nt in length and can be divided into three distinct regions: the 5' untranslated region (5'UTR), the open reading frame (ORF) and the 3' untranslated region (3'UTR). Additionally, mRNAs are characterized by a 5'cap structure (m⁷GpppN) (SHATKIN, 1976) and a long stretch of adenosine bases at the 3'end referred to as the polyA-tail (Darnell et al., 1971; Lee et al., 1971). Both structures stabilize the mRNA and prevent degradation by exonucleases (Schoenberg and Maquat, 2012), but also play an important role during the initiation step of mRNA translation.

The 5'cap is added to the first nucleotide of the nascent mRNA strand during transcription from its DNA template, while the polyA-tail is added (polyadenylation) after transcription termination in the nucleus prior to mRNA transport to the cytoplasm (Edmonds et al., 1971; SHATKIN, 1976; Wickens, 1990). However, in germ-line cells polyadenylation can occur in the cytoplasm (Belloc et al., 2008). The open reading frame (ORF) is defined by a start codon (AUG) and a stop codon. The 3'UTR, situated between the stop codon and the polyA-tail harbors binding sites for RNA

binding proteins (RBPs), long non-coding RNAs (lncRNAs) and microRNAs (miRNA) (Filipowicz et al., 2008). Additional features in untranslated regions will be discussed throughout this thesis.

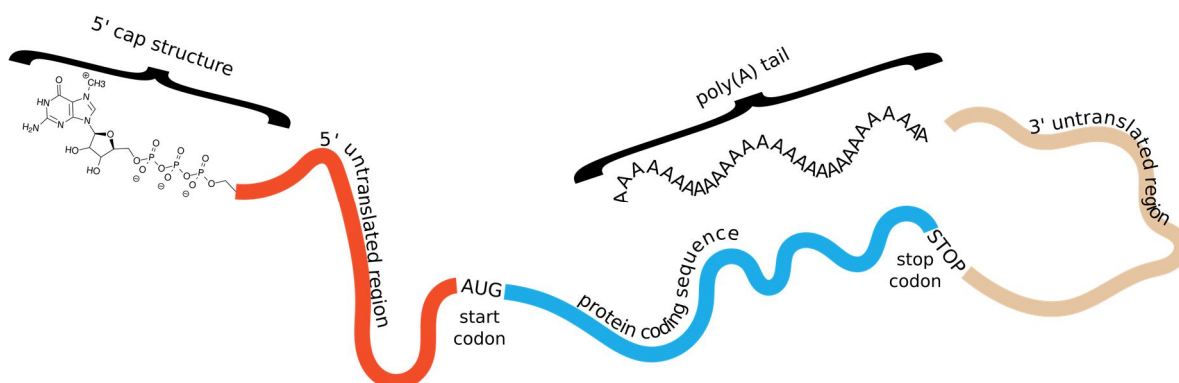


Figure 1 - The mRNA molecule consists of three distinct regions, which will be discussed in detail in this thesis. The 5' cap structure is added during transcription (1.1.5) and is important for the initiation of mRNA translation (1.2.1). Features in the 5' untranslated region (5'UTR) are widely implicated in translational control (1.3.2) and are the focus of **paper II** and **III**. The 3' untranslated region (3'UTR) plays, among other functions, a role in mRNA stability (1.3.3 and **paper III**). The coding region contains the information for the protein sequence and its codon composition plays a role in the regulation of mRNA translation (1.3.4) and **papers III and IV**.

1.1.5 Transcription

The process of copying genetic information from DNA to RNA in the nucleus is called transcription (**Figure 2**). Specific RNA-polymerases catalyze the formation of RNA molecules by forming phospho-diester bonds using RNA bases as triphosphates (Roeder and Rutter, 1969; Stevens, 1960). The activity of these polymerases is regulated by a plethora of transcription factors (Lambert et al., 2018). These act in concert with enzymes modifying histones post-translationally, proteins on which DNA is space-efficiently stored, leading to in- or decreased accessibility of the chromatin (Venkatesh and Workman, 2015). Chromatin, an assembly of nucleosomes, is a macromolecular structure containing DNA, proteins (Kornberg, 1974) and RNA (Holmes et al., 1972). Positioning of nucleosomes leading to open chromatin makes sections of the DNA accessible for regulators (Lorch et al., 1987), such as transcription factors (Segall et al., 1980). This is commonly associated with the initiation of transcription (Lambert et al., 2018). The sites, at which transcription starts (transcription start-sites, TSS) are of importance when studying 5'UTRs (1.6.4 and **paper II**). Different RNA species are transcribed with different RNA polymerases, of which RNA polymerase II (RNA-pol II) is responsible for the transcription of mRNAs and miRNAs, RNA polymerase III for tRNAs and the 5S rRNA. RNA-polymerase I produces the remaining rRNAs (Barba-Aliaga et al., 2021). In particular, RNA polymerase II is intrinsically regulated by its C-terminal domain (CTD). This domain is the target of many different kinases, and its phosphorylation pattern steers RNA polymerase II activity towards different activation states (Hsin and Manley, 2012). For instance, phosphorylation at Serine 5 of the CTD recruits the

capping enzyme, leading to 5'capping of the nascent mRNA strand (Cho et al., 1997; Ho and Shuman, 1999). This event is of importance when transcription start sites are analyzed using cap analysis of gene expression (CAGE) or nanoCAGE techniques. This will be discussed in more detail later in this thesis (**1.6.4**). The newly synthesized and capped mRNA molecule (pre-mRNA) is then spliced by the spliceosome (**Figure 2**), consisting of a multitude of snRNAs and proteins (Fica and Nagai, 2017). This event serves to further increase the complexity of the genome with its 3 billion bases since multiple protein isoforms can be generated from the same mRNA molecule (mRNA isoforms) (Chow et al., 1977). This is achieved by the removal of introns from the pre-mRNA and the joining of exon sequences generating the final mRNA molecule (Shi, 2017). In addition, genomic complexity is further increased by alternative splicing (Nellore et al., 2016; Pan et al., 2008) that can occur as a result of, for instance, overexpression of the splicing factor SRSF1 in breast cancer (Anczuków et al., 2012).

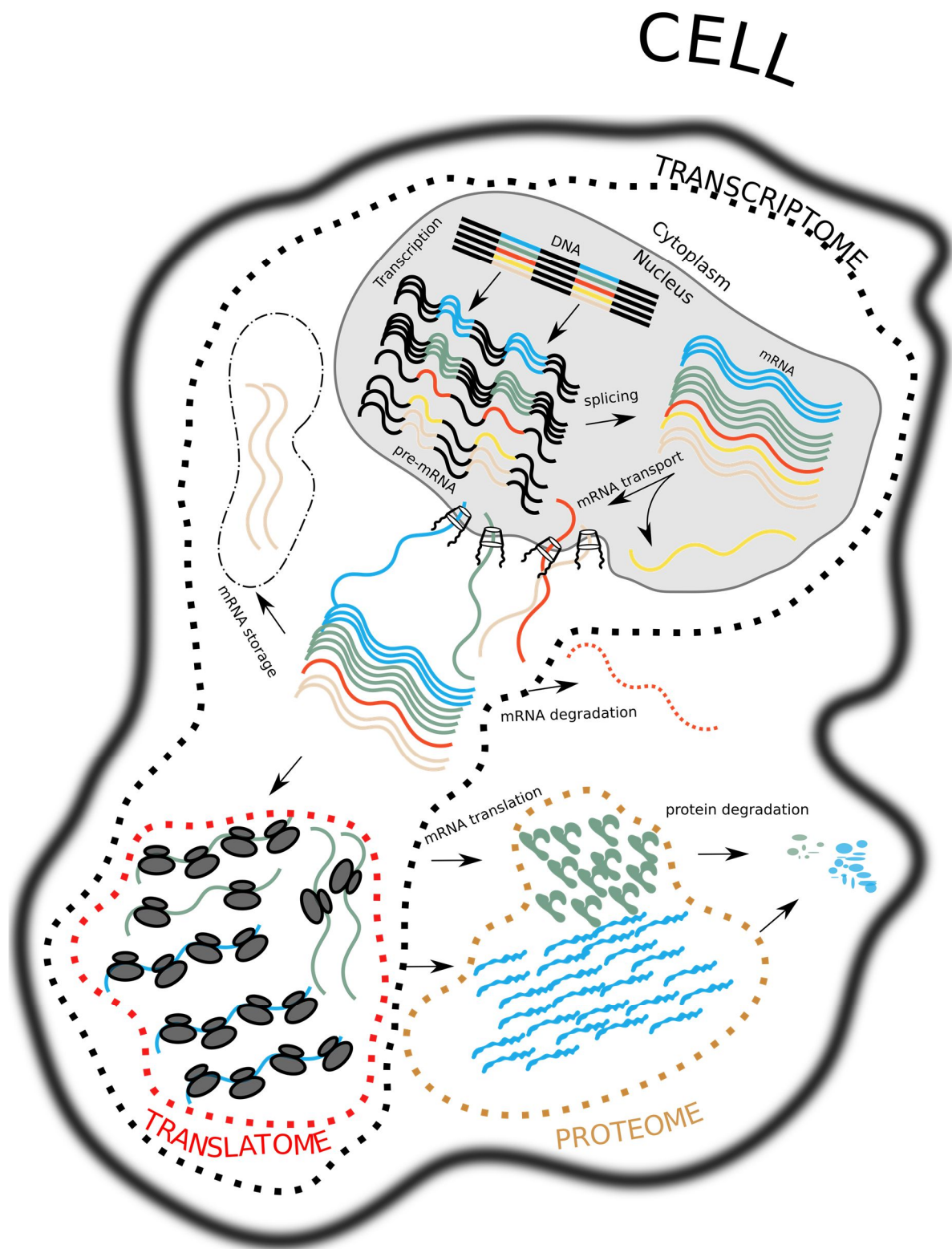


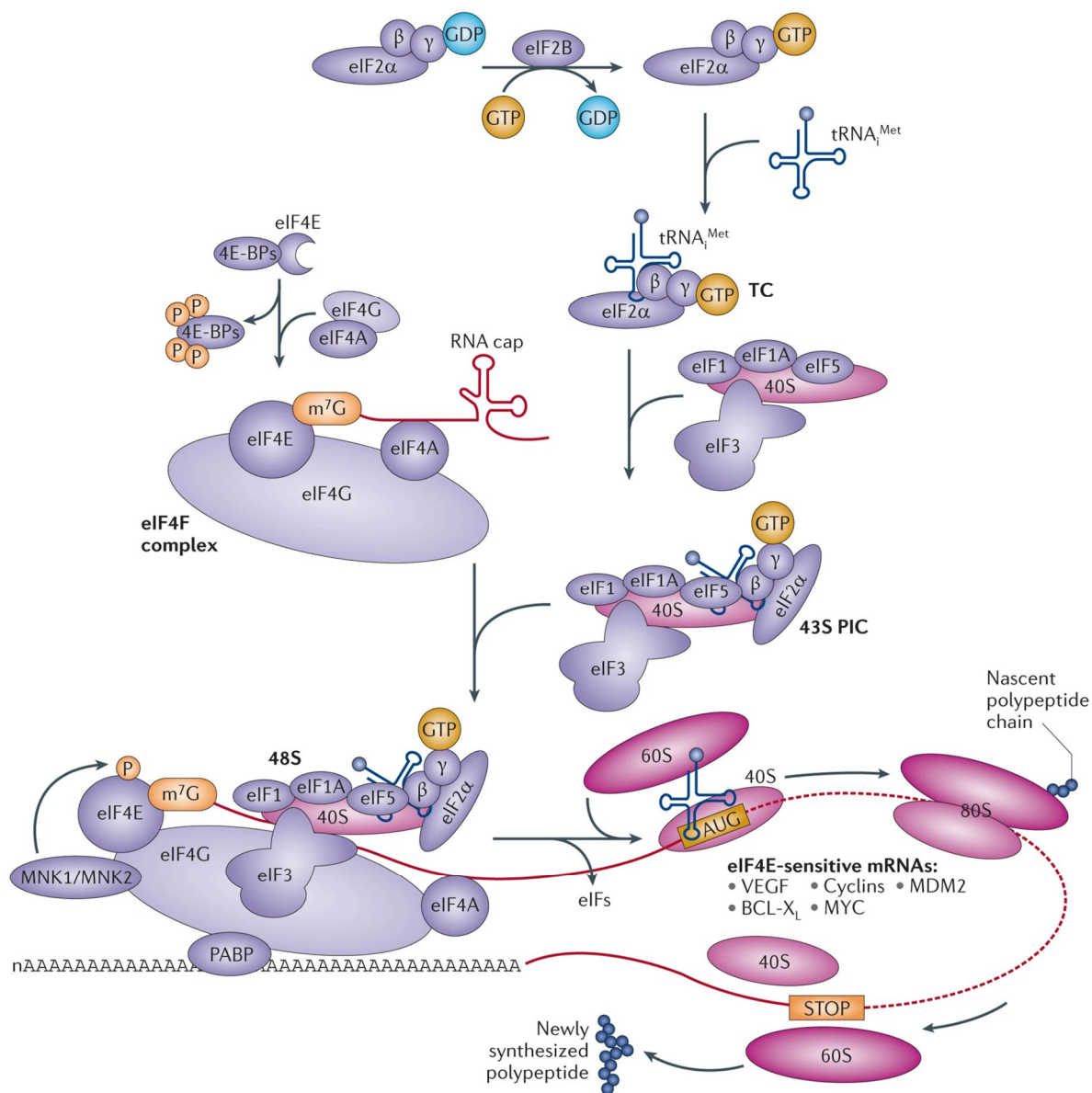
Figure 2 – A scheme of the gene expression pathway: Pre-mRNAs are transcribed from the genome (DNA) followed by splicing (1.1.5). mRNAs are transported or retained in the nucleus (1.1.6). In the cytoplasm, mRNAs can be stored, degraded or used for mRNA translation (1.2). Protein synthesis is performed by ribosomes, which associate with mRNAs and, due to translational control (1.3), give rise to different amounts of the respective protein. Protein levels (*the proteome*) are mediated by the rate of mRNA translation and protein degradation (1.5). The *transcriptome* is defined by all RNAs in the cell, while the *translatome* contains all mRNAs associated with ribosomes.

1.1.6 mRNA transport from the nucleus

Once capped and spliced, mRNAs are exported into the nucleus by export-factors like TREX and TREX-2 in a transcription-coupled manner via nuclear pore complexes residing in the nuclear membrane (Köhler and Hurt, 2007) (**Figure 2**). This process is tightly coupled to cellular mechanisms of mRNA-quality control (Hieronymus et al., 2004; Hilleren et al., 2001) and has been shown to be co-regulated with mRNA-transcription and mRNA-splicing (Köhler and Hurt, 2007). Interestingly, mRNAs related to cell cycle progression and survival, such as ODC1, Cyclin D1 and c-myc require binding of the cap-binding protein eIF4E including a 3'UTR-motif and do not underlie the transport mechanism for bulk mRNA, linking mRNA transport and regulation of mRNA translation (Culjkovic et al., 2006; Rousseau et al., 1996).

1.2 mRNA TRANSLATION

mRNA translation, a post-transcriptional mechanism of gene expression regulation, is the process through which proteins are synthesized by the ribosome, using the mRNA as a template and is uni-directional, i.e., in 5' to 3' manner. Early experiments showed that proteins are synthesized on polysomes, i.e., several ribosomes associated with an mRNA (WARNER et al., 1963). Thus one mRNA gives rise for several molecules of the respective protein. The relative rate at which this occurs can be measured as translational efficiency of an mRNA, which describes the proportion of each mRNA in the cell associated with ribosomes and is therefore a proxy for the resulting protein levels (Larsson et al., 2013). The proteome is shaped by the synthesis and degradation of proteins and, given the fact, that maintaining cellular protein levels (protein homeostasis or proteostasis) of an organism is essential for its survival (Balch et al., 2008), mRNA translation is an omnipresent process. Moreover, the rate of protein production per time ranges widely between 10 to 10,000 proteins per mRNA in comparison to the production of 0.1 to 100 mRNAs per hour by transcription (Hausser et al., 2019; Liu et al., 2016; Schwanhäusser et al., 2011) illustrating the importance of mRNA translation in mediating rapid changes to the proteome. As such, mRNA translation and the necessary energy supply to sustain it are subject to complex mechanism of regulation. Indeed, mRNA translation is considered to be the most energy demanding process in the cell (Buttgereit and Brand, 1995), up to 28% of the energy stored in adenosine triphosphate (ATP) is consumed for the production of proteins (Rolfe and Brown, 1997) and the formation of one peptide bond requires 4 high-energy bonds (triphosphates), corresponding to approximately 25 kcal/mol (Mathews et al., 2000). mRNA translation can be divided into three major steps: initiation, elongation and termination including ribosome recycling. Each step is highly regulated of which the initiation step is considered to be rate limiting (Mathews et al., 2000; Sonenberg and Hinnebusch, 2009).



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Figure 3 – Initiation of mRNA translation (1.2.1): The binding of eIF4E to the m⁷G cap structure is rate limiting for cap-dependent mRNA translation. This is followed by formation of the eIF4F complex comprised of the scaffold eIF4G and the DEAD-box helicase eIF4A. Its assembly is regulated downstream of the mTOR pathway (1.3.1.1) by the regulatory 4E binding proteins (4E-BPs). The 43S pre-initiation complex is formed between the 40S ribosomal subunit, eIF1, eIF1A, eIF5 and eIF3 and the ternary complex of eIF2α, -β, -γ carrying the initiator tRNA (tRNA_i^{Met}). The 48S pre-initiation complex scans the 5'UTR until a start-codon is recognized, followed by assembly of the 80S ribosome and translation elongation (1.2.2). Upon stop codon recognition, translation is terminated (1.2.2) and the 40S and 60S subunits are recycled for a new round of mRNA translation. Specific oncogenic mRNAs, such as Vascular endothelial growth factor (VEGF), Cyclins, Mouse double minute 2 (MDM2), B-cell lymphoma-extra large (BCL-X_L), and MYC proto-oncogene are sensitive to eIF4E protein levels, leading to their aberrant protein synthesis when eIF4E is elevated (1.4). The phosphorylation of eIF4E by MAP kinase-interacting serine/threonine-protein kinase 1/2 (MNK1/2) regulates translation of a subset of mRNAs and is implicated in cancer (1.3.1.3).

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1.2.1 Translation initiation

There are two main mechanisms through which initiation of mRNA-translation is thought to occur. The first and most prevalent being highly dependent on the 5'cap-structure (Pelletier et al., 2015). A secondary mechanism of initiation requires a specific RNA structure, an internal ribosome entry site (IRES) (Pelletier and Sonenberg, 1988). Cap-dependent translation initiation requires a plethora of initiation factors (eIFs) (**Figure 3**). The eukaryotic initiation factor 4E (eIF4E) binding the 5'cap (Sonenberg et al., 1978), is the rate-limiting step (Duncan et al., 1987) and allows recruitment of eukaryotic initiation factors 4G and 4A (eIF4G and eIF4A), which together comprise the eIF4F complex (Sonenberg and Hinnebusch, 2009). Recent studies revealed additional mechanisms of initiation by DAP5 and eIF3d for a subset of mRNAs. The latter is another cap-binding protein requiring a distal stem-loop for eIF4F-independent initiation (Lee et al., 2016). Furthermore, DAP5 was identified as stimulating IRES-driven translation (Liberman et al., 2015) and, in combination with eIF3d, modulating translation in a cap-dependent manner without requiring the eIF4F complex (de la Parra et al., 2018) controlling T-cell phenotypes (Volta et al., 2021). Following cap-recognition the pre-initiation-complex (43S PIC), harboring the initiation tRNA (met-tRNA_i), together with translation factors eIF1, eIF1A, eIF2 (including several subunits such as eIF2 α), eIF2B, eIF3 and eIF5 and the 40S ribosomal subunit, scans in 5' to 3' directionality until a start codon (AUG) is recognized. This leads to subsequent GTP-hydrolysis of eIF2 bound GTP by eIF2B and the release of the pre-initiation complex. Next the 60S ribosomal subunit is recruited including eIF6, inhibiting the association with free 40S in the cytoplasm, and eIF5B, which in turn is released after GTP-hydrolysis and initiation ends by forming the 80S initiation complex (Jackson et al., 2010; Sonenberg and Hinnebusch, 2009). Translation takes place in a closed loop formation, thought to be important for its efficiency and selectivity for intact mRNAs (Gingras et al., 1999b). The interaction of the poly A binding protein (PABP) with eIF4G leads to closed loop formation and enhances translation (Gallie, 1991; Svitkin and Sonenberg, 2006). Indeed, mRNAs lacking a polyA-tail, e.g. of replication dependent histones, harbor 3'UTR stem-loops, bound by RNA-binding proteins to facilitate closed-loop formation (Cakmakci et al., 2008). These well characterized steps (**Figure 3**) are not only mediated by availability and functionality of initiation factors, but likewise by features and structures in the 5'UTR which will be discussed later in this thesis.

1.2.2 Translation elongation and termination

When the 80S ribosome is formed at initiation codons (start-codons), the initiator-tRNA (Met-tRNA_i^{Met}) is recruited to the P-site of the ribosome, while the tRNA_i anticodon pairs with the AUG of the mRNA. Next, eukaryotic elongation factor (eEF) 1A in a ternary complex with a tRNA complementary to the next codon of the mRNA enters the A-site and the interaction between

codon and tRNA triggers eEF1A catalyzed GTP-hydrolysis (Dever et al., 2018). Peptide bond formation by nucleophilic attack of the free amino group of the amino acid loaded on the aminoacyl-tRNA (A-site) and the ester bond between the peptidyl-tRNA (P-site) occurs by transferring the nascent poly-peptide chain onto the tRNA in the A-site (Moore and Steitz, 2003; Trobro and Åqvist, 2005). This reaction is catalyzed by interaction with rRNA of the large ribosomal subunit (Ben-Shem et al., 2011; Trobro and Åqvist, 2005), including stabilization of the peptidyl-tRNA by eIF5 (Gutierrez et al., 2013) and repositioning of both tRNAs by subunit rotation of both ribosomal subunits (Moazed and Noller, 1989). eEF2, a GTP-dependent translocase, leads to translocation of tRNAs in P- and A-site to E- and respectively P-site, allowing the next aminoacyl-tRNA to accommodate the A-site (Spahn et al., 2004).

Translation is terminated by the interplay of eukaryotic release factors (eRFs) 1 and 3 (Hellen, 2018). eRF1 is a protein with structural similarity of a tRNA. Its amino-terminal domain recognizes a stop codon (Bulygin et al., 2010; Chavatte et al., 2002) and its middle domain promotes hydrolysis of the peptide-chain from the tRNA in the P-site (Song et al., 2000). The ribosome is recycled by ABCDE1, which requires eEF1 (Pisarev et al., 2010), and splits the 40S and 60S subunit in an ATP-dependent manner (Pisarev et al., 2010; Shoemaker and Green, 2011). Recycling of the 40S subunit mainly relies on eIF3 and its interplay with eIF1, eIF1A (Pisarev et al., 2007). Together with binding of eIF6 to the 60S subunit prevents premature joining of the ribosomal subunits (Ceci et al., 2003; Strunk et al., 2011) allowing for a new cycle of mRNA translation beginning with translation initiation.

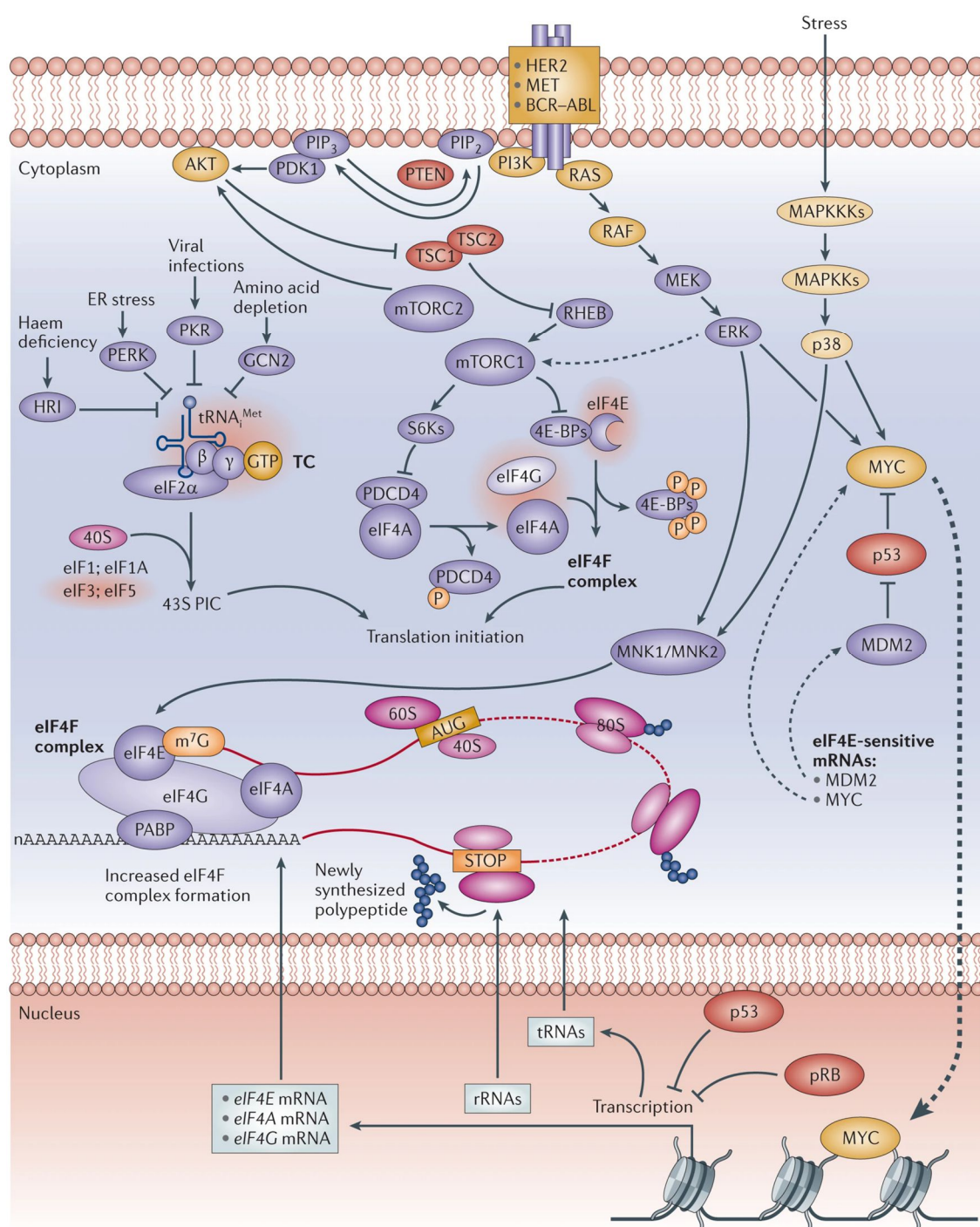
1.3 TRANSLATIONAL CONTROL

As illustrated above, translation is an advanced process requiring a large proportion of cellular energy and a multitude of translation factors. Understanding how mRNA translation is controlled began with the observation that cells alter their protein production upon external stimuli. The discovery of the lac-operon established that external signals are essential for cellular survival (JACOB and MONOD, 1961). Later experiments showed, that protein production in sea urchin eggs, when fertilized, was uncoupled from transcription of new RNA (Hultin, 1961). Reticulocytes, stimulated with heme and iron ions, presented a dramatic increase in alpha- and beta-globin protein (Bruns and London, 1965). This observation was of particular importance, since reticulocytes do not contain nuclei; hence protein production is singularly reliant on translational control (Kruhi and Borsook, 1956). Translational control is not exclusively attributed to global activation or reduction of protein synthesis, but can also occur in a selective manner. This is achieved by regulation via cellular cues impinging on mRNA translation, including intrinsic features of the mRNA (cis-factors) or the interaction of the mRNA with proteins or RNAs (trans-factors). These aspects will be discussed in the following sections.

1.3.1 Pathways regulating translation

1.3.1.1 *The mTOR pathway*

There are two major cellular cues that regulate mRNA translation. These are pathways downstream of the phosphatidylinositol-3 kinase (PI3K) and mitogen activated protein kinase (MAPK) (Roux and Topisirovic, 2018). The mammalian target of rapamycin (mTOR) is an evolutionary conserved Ser/Thr-kinase, and acts as a major regulator for cell proliferation and cell growth. There exist three distinct complexes of mTOR; mTORC1, 2 and 3. Of these complexes, the mechanisms for mTORC1 and 2 are most studied. (Saxton and Sabatini, 2017) (**Figure 4**). mTORC3 is the most recently described of the complexes and is comprised of the transcription factor ETV7 in complex with mTOR. It exhibits similar activity to mTORC1 and 2, while lacking a number of their subunits (Harwood et al., 2018). mTORC1 mainly regulates mRNA translation, whereas mTORC2 is involved in mediating the stability of protein kinase C (PKC), phosphorylates nascent AKT polypeptides and plays a role in actin and cytoskeleton reorganization (Oh and Jacinto, 2011). Hormones and growth factors activate mTORC1 by the PI3K pathway upon stimulation of receptor tyrosine kinases (RTK), like the insulin receptor (Fruman et al., 2017; Proud and Denton, 1997). Downstream signaling in this cascade signals occurs via AKT (protein kinase B) and tuberous sclerosis complex 1 and 2 (TSC1/TSC2) to RAS-homologue enriched in brain (Rheb), which, acting as a GTPase, activates mTORC1 in its GTP-bound state (Dibble and Cantley, 2015). The activation of mTORC1 is associated with an increase in mRNA translation mainly by phosphorylating the p70-S6 kinase and eIF4E binding proteins (4E-BPs) (**Figure 4**). S6Ks play an important role in regulating cellular and organismal size downstream of mTORC1 (Fingar et al., 2002). These kinases phosphorylate rpS6, involved in ribosome biogenesis (Chauvin et al., 2014) and eukaryotic elongation factor 2 kinase, which is a negative regulator of mRNA translation by inhibiting eEF2 (Kenney et al., 2014). Insulin stimulation leads to inhibition of eEF2K activity resulting in increased protein synthesis due to higher elongation rates (Redpath et al., 1996; Wang et al., 2001). The other axis of mTORC1's translational regulation occurs 4E-BPs, which, when hypo-phosphorylated under low mTORC1 activity, bind to eIF4E and thereby reduces its affinity for the 5'cap, abolishing binding of eIF4G and therefore inhibiting translation initiation resulting in decreased protein synthesis (Gingras et al., 1999a; Pause et al., 1994).



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Figure 4 – Cellular pathways regulating mRNA translation: Mitogenic signals are transferred into the cell by receptor tyrosine kinases, such as the Bcr-Abl tyrosine-kinase, Receptor tyrosine-protein kinase erbB-2 (HER2) and tyrosine-protein kinase Met (MET), which activate the mTOR pathway via PI3K and the RAS-RAF cascade upstream of MNK1/2 (1.3.1.3). mTOR activity regulates eIF4F complex formation via 4E-BPs and eIF4A activity via S6K leading to increased translation initiation (1.3.1.1). Ternary complex (TC) formation is negatively regulated by the kinases: Heme-regulated inhibitor kinase (HRI), protein kinase R (PKR)-like endoplasmic reticulum kinase (PERK), protein kinase R (PKR) and general control non-depressible 2 (GCN2) (1.3.1.2). Proteins encoded by eIF4E-sensitive mRNAs (i.e. MYC, MDM2) regulate transcription via p53 (MDM2) (1.7) or as transcription factor (MYC) leading to transcription of mRNAs encoding translation initiation factors of the eIF4F complex, ribosomal RNA (rRNA) and transfer RNA (tRNA).

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1.3.1.2 The integrated stress response

A response to cellular stresses regulating global protein synthesis is the phosphorylation of the alpha subunit of eIF2 (eIF2 α) leading to a global decrease of mRNA translation (Prostko et al., 1993) (**Figure 4**). Four different kinases mediate this phosphorylation (protein kinase R (PKR)-like endoplasmic reticulum kinase [PERK], general non-derepressible 2 [GCN2], double-stranded RNA-activated protein kinase [PKR], heme-regulated inhibitor kinase [HRI]) each activated by different cellular stresses such as endoplasmic reticulum stress, low amino acid availability, heme levels or as part of antiviral responses (Sonenberg and Hinnebusch, 2009; Wek, 2018). These stresses and subsequent translational decrease can fine tune protein synthesis and reveal distinct translational programs to protect from, for example, proteotoxicity, which under extreme circumstances can lead to apoptosis (Harding et al., 1999; Walter and Ron, 2011). The translation of specific mRNAs induced by phospho-eIF2 α span from specific transcription factors (ATF4, CHOP), protein processing and degradation (BiP, BACE1), Cell cycle control (CDKN1A), feedback control for eIF2 α phosphorylation (GADD34), nutrient transport (SLC35A4) and tRNA charging (EPRS) (Baird et al., 2014; Koritzinsky et al., 2006; Wek, 2018). Recently it has been shown using the ER-stress inducer thapsigargin, that one such translational program acts as a first response, and chronic stresses leads to transcriptional reprogramming (Guan et al., 2017).

1.3.1.3 MNKs and eIF4E phosphorylation

The activity of the cap-binding protein eIF4E can also be regulated by MAP kinase signal-integrating serine/threonine-protein kinases (MNKs) which phosphorylate eIF4E by docking onto eIF4G (Roux and Topisirovic, 2018). MNKs, exist in two isoforms, and are activated directly by ERK and p38, in which ERK is downstream of the RAS pathway and p38 downstream of the MAP kinase pathway (Proud, 2015). Furthermore MNK2 plays a role in resistance to rapamycin in cancer cells due to sustained mTORC1-activity (Brown and Gromeier, 2017). MNKs are the only presently known kinases that phosphorylate eIF4E at Serine 209 (Flynn and Proud, 1995; Joshi et al., 1995). However, to date, the precise role of eIF4E-phosphorylation in context of global mRNA translation remains unclear.

1.3.2 Translational control by 5' untranslated regions

1.3.2.1 Scanning and initiation

As previously mentioned, the 5'UTR is comprised of the cap-structure followed by a stretch of RNA-nucleotides (5'UTR), until the start-codon (AUG) of the main open-reading-frame (coding sequence). 5'UTRs differ in length between different species. The median length of 5'UTRs in budding yeast is 53nt, however in human the median length is 218nt but can reach up to approximately 1200nt (Leppke et al., 2018). These large variations in length and the combinatorial

possibilities in nucleotide composition, result in many possibilities for regulation. Additionally, 5'UTR complexity can be modulated by RNA splicing, since around 35% of 5'UTR sequences contain introns (Pesole et al., 2001), which have been found to be longer compared to those in coding regions (Hong et al., 2006). The actual 5'UTR characteristics are determined by usage of transcription start sites (TSS), who can be differentially selected in response to altered cellular conditions (Livingstone et al., 2015). Efforts have been made to map those TSSs in a transcriptome-wide manner (Forrest et al., 2014; Suzuki and Sugano; Suzuki et al., 2015). To date, the available information in data-bases on TSSs in cell-lines and tissues is limited and 5'UTRs have been shown to be inaccurately annotated to a large extend (Gandin et al., 2016).

Translation initiation is commonly considered the rate limiting step in protein synthesis, which involves scanning of the 5'UTR in 5' to 3' directionality, putting the first AUG in a favor for initiation. How scanning contributes to translation initiation was initially addressed by Marilyn Kozak who described scanning based on the fact that most eukaryotic mRNAs are mono-cistronic, i.e., encode for one protein, and lack upstream initiation sites. The insertion of upstream (proximal) AUG codons in the pre-proinsulin mRNA, led to the idea that the 43S pre-initiation complex (43S PIC) scans the 5'UTR until start codon recognition (Kozak, 1983, 1986a). Introducing point-mutations surrounding the AUG codon especially at position -3 relative to the AUG led to a dramatic effect on translation initiation such that an AUG can be completely bypassed giving rise to the term “leaky scanning” (Kozak, 1986b). These findings ultimately defined the Kozak sequence for vertebrates,

5'-GCCGCC(A/G)CCAUGG-3' (Kozak, 1987a)

as the most favorable sequence context for translation initiation. By modifying the canonical AUG to single-nucleotide mutants it was shown that initiation is reduced. Non-canonical start-codons initiate at a frequency of 1-10% compared to canonical AUGs depending on gene and study. The codons AAG and AGG were shown as non-functional (Clements et al., 1988; Peabody, 1989). In some cases, non-canonical start codons can be decoded by the leucyl-tRNA requiring eIF2A instead of eIF2, which is necessary for loading of antigenic precursors on major histocompatibility complexes (Starck et al., 2012). Start codon recognition in its strong and weak Kozak contexts is controlled by different eukaryotic initiation factors in which eIF1 (Pestova et al., 1998) and eIF5 play two opposite roles. eIF1 promotes scanning and inhibits recognition of non-AUG codons and eIF5, a GTPase-activating protein for eIF2 helps to dissociate eIF1 from the arrested scanning 43S PIC also supporting start-codon recognition (Llácer et al., 2018; Zeman et al., 2019). The translation of these two initiation factors is tightly auto regulated. The eIF1 mRNA harbors an AUG in weak context leading to increased eIF1 translation when eIF1 protein levels are low. The

eIF5 mRNA in contrast contains an inhibitory upstream open reading frame (uORF) in weak context, which is translated under low eIF1 conditions and bypassed when eIF1 is high (or eIF5 levels are low) leading to increased eIF5 levels (Ivanov et al., 2010; Loughran et al., 2012). Since start-codon recognition is essential to accomplish translation initiation, it is not surprising that mRNAs with very short 5'UTRs (≤ 20 nt) show lower translational efficiency resulting from increased leaky scanning, which can result in N-terminal truncated proteins (Kozak, 1991b, 1991a; Pestova and Kolupaeva, 2002). However some mammalian mRNAs with very short 5'UTRs contain an element that promotes cap-dependent but scanning independent initiation. The translation initiation of short UTRs (TISU) element, often found in mitochondrial related mRNAs (Sinvani et al., 2015), promotes translation of 5'UTRs as short as 5nts and relies on specific sequences up and downstream of the AUG, in which downstream nucleotides compensate for lacking 5'UTR sequence (Elfakess and Dikstein, 2008; Elfakess et al., 2011). Paradoxically, the TISU element relies on eIF1 (Sinvani et al., 2015), which normally prevents initiation at AUGs close to the cap (Pestova and Kolupaeva, 2002). A recent study described translation of a group of mRNAs with very short 5'UTRs (< 50 nts) containing a TISU element as highly mTOR sensitive. These transcripts encode for proteins with mitochondrial functions and elements of the respiratory chain (Gandin et al., 2016) and their regulation relies on eIF4E, however not on the integrity of the TISU element. Nevertheless, many mitochondrial proteins contain such an element, suggesting that their regulation is connected to energy metabolism (Sinvani et al., 2015).

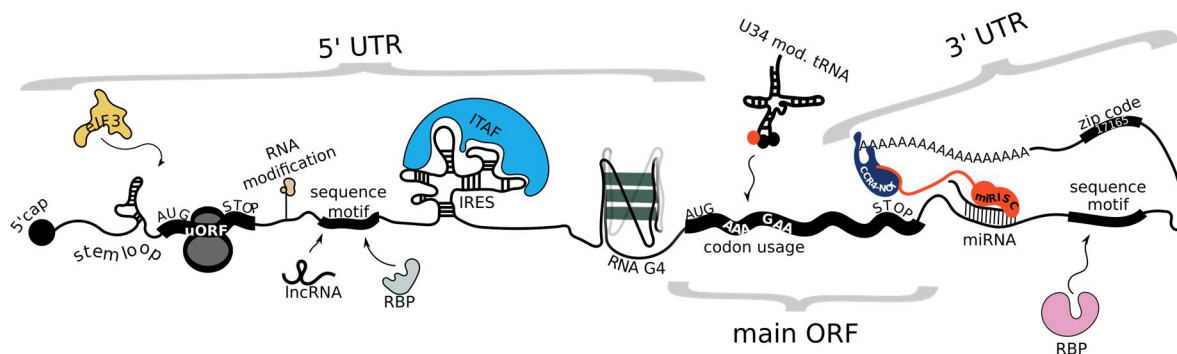


Figure 5 – Intrinsic features of the mRNA molecule: Sequence elements in the 5'UTR regulate primarily translation initiation (1.3.2.2-5, paper III). Upstream open reading-frames (uORFs) regulate initiation at main open reading frames (main ORF) (1.3.2.4, paper II). The decoding of specific codons by modified tRNAs plays a role in translational regulation (1.3.4, paper III, IV). 3'UTR features alter translation and mRNA stability (paper III) mediated by miRNAs and RBPs (1.3.3.1-2). Zip-codes, as binding sites for RBPs, lead to localization of mRNAs (1.3.3.3).

1.3.2.2 Structures and sequence motifs in the 5'UTR

Due to their sequence-complexity, 5'UTRs are prone to form secondary structures that can influence translation initiation. Longer 5'UTRs are more likely to form structures, show different requirements for translation initiation factors compared to shorter sequences. Higher levels of eIF4E increase translation of mRNAs with higher structural content in the 5'UTR due to higher

demand of eIF4F complex formation, involving RNA helicases like the ATP-dependent RNA helicase eIF4A (Svitkin et al., 2001) (**Figure 3**). Shorter, less structured 5'UTRs show lower dependency on eIF4A, while at the same time being to some extent more mTOR-dependent (Gandin et al., 2016). Secondary structures, related to GC-content, proximal to the 5'cap impede eIF4F complex formation (Manzella and Blackshear, 1990; Pelletier and Sonenberg, 1985). These structures are more dependent on RNA helicases DDX3 (a member of the DEAD-box family), which can resolve this stem loop (Soto-Rifo et al., 2012) and DHX29 (Abaeva et al., 2011). However, particularly long 5'UTRs in yeast are highly dependent on Ded1 (homolog of DDX3), which acts in a mRNA but also eIF4F dependent manner (Gupta et al., 2018). eIF4A however, contributes more to global translation initiation as shown in yeast (Sen et al., 2015; Yourik et al., 2017). Secondary structures such as G-quadruplexes (RNA G4), a secondary structure formed by G-rich sequences (Burge et al., 2006) (**Figure 5**), and commonly occurring in oncogenic mRNAs, are highly dependent on eIF4A activity, when co-varying with other structures such as hair-pins (Waldron et al., 2018). This led to the development of specific inhibitors for eIF4A as possible cancer treatment (Rubio et al., 2014; Wolfe et al., 2014). Interactions of 5'UTRs with lncRNA have been described but appear as a rare phenomenon (**Figure 5**). The murine Uchl1 mRNA is targeted by a lncRNA from the same locus leading to increased ribosome recruitment. This intriguing mechanism is controlled by mTOR activity leading to increased cytoplasmic Uchl1 levels, which allows a bypass of cap-dependent translation utilizing an alternative initiation mechanism (Carrieri et al., 2012). An alternative mechanism involving a well-studied initiation factor to by-pass cap-dependent initiation, is a stem-loop in the 5'UTR recruiting eIF3 (**Figure 5**), known for its important role in the 43S PIC. Cross-linking experiments found eIF3 bound to around 3% of all mRNAs. Mechanistically, eIF3 binds a hairpin in the 5'UTR of c-Jun, a regulator of proliferation, which when disrupted reduces eIF3-dependent translation (Lee et al., 2015). This is challenged by a previously mapped internal ribosomal entry site (IRES) in proximity, potentially being required for initiation by eIF3 (Blau et al., 2012). Structural elements in 5'UTRs can also be a sensor for nutrient levels in organisms. One example of this mode of regulation is the iron-responsive element (IRE) in the human ferritin 5'UTR, which forms a binding site for RNA binding proteins such as the Iron-responsive element-binding protein (IRP), when iron levels are low. Increased iron levels release IRP binding and induce translation of the iron storage protein ferritin (Hentze et al., 1987). Some viral mRNAs have the ability to by-pass the scanning mechanisms by recruiting the PIC to specific structural sites in the 5'UTR called internal ribosomal entry site (IRES) (Jackson et al., 2010; Pelletier and Sonenberg, 1988). While extensive biochemical characterization has been carried out on specific mRNAs containing these features, a few genome-wide studies are available (Baird et al., 2007), albeit approximately 10% of randomly selected mRNAs contain these structures (Weingarten-Gabbay et al., 2016). Cap-independent initiation by IRES play an important

role in adaptation to nutrient depletion in yeast (Gilbert et al., 2007) and when cells reprogram translation under apoptotic conditions, which leads to reduced global translation and a switch from cap-dependent to cap-independent initiation. This is mediated by cleavage of the translation factors eIF4G, eIF4B and 4E-BPs by caspases and altered phosphorylation of 4E-BPs and eIF2 α (Spriggs et al., 2005). In parallel rates of mRNA degradation increase (Bushell et al., 2004). To maintain apoptotic signaling, selective translation of pro-apoptotic factors is necessary. These specific translational programs are initiated by IRES-mediated translation initiation and IRES trans-acting factors (ITAFs) (**Figure 5**). These RNA binding proteins facilitate the assembly of initiation complexes independent of the canonical scanning mechanism mediated by eIF4F (Spriggs et al., 2005). Examples are hnRNPK stimulating c-MYC's IRES-mediated translation (Evans et al., 2003) and PTP binding the IRES of Apaf-1 (Mitchell et al., 2001) and BiP (Kim et al., 2000).

1.3.2.3 RNA modifications in the 5'UTR

RNAs can be modified by covalently bound chemical groups, generating N⁶-methyladenosine (m⁶A) or N¹-methyladenosine (m¹A) (Dominissini et al., 2012). These reversible modifications are generally deposited by methyltransferases ("writers"), read by RBPs ("readers") and removed by demethylases ("erasers") (Harcourt et al., 2017; Zhao et al., 2016) (**Figure 5**). m⁶A can stimulate translation, in a cap-independent but scanning-dependent manner utilizing eIF3 (Meyer et al., 2015) suggesting a similar role to the actual 5'cap for m⁶A. Cellular stresses such as heat shock have been shown to induce an altered m⁶A pattern across 5'UTRs (Meyer et al., 2015) leading to stress induced translation mediated by eIF3 (Meyer et al., 2015). Translation of m⁶A modified mRNAs can also be impaired due to impeded decoding of the CDS by tRNAs at m⁶A sites (Chio et al., 2016), in contrast to m¹A modifications in 5'UTRs, shown to be associated with increased initiation (Dominissini et al., 2016). However this has been challenged by methodological refinement, yielding in an opposite conclusion, such that m¹A modifications are low abundant and regulate translation in a negative manner (Safra et al., 2017).

1.3.2.4 Upstream open reading frames

The development of next-generation sequencing methods and subsequent sequencing of whole genomes and transcriptomes of different organisms, enabled the mapping of translation start-sites on a transcriptome-wide level. However most sites were initially annotated by AUG-codons at the beginning of the longest open reading frame (Saeys et al., 2007) (**Figure 5**). Studies using the ribosome-profiling technique revealed ribosomes present in short open reading frames in 5'UTRs. These sequences, termed upstream open reading frame (uORF), predicted to be found in around 50% of transcripts (Resch et al., 2009) and evolutionary conserved (Brar et al., 2012; Iacono et al., 2005), were therefore found to be translatable due to ribosomes present in the initiation and

elongation state (Chen et al., 2020; Ingolia et al., 2009; Lee et al., 2012). To this end, however still challenging, efforts have been made to optimize the use of translation inhibitors and computational analysis of data derived from ribosome profiling experiments in terms of quality control of datasets and identification of actively translated ribosomes (Calviello et al., 2015; O'Connor et al., 2016). The small peptides encoded by uORFs are not well functionally characterized (Akimoto et al., 2013; Chen et al., 2020; Parola and Kobilka, 1994), due to ongoing challenges in detection on a proteome-wide level (Oyama et al., 2004; Vanderperre et al., 2013), arguably raising the question around their presence and function in general. Furthermore it should be noted that the detection of uORFs can underlie biases introduced by inhibitors of initiating and elongating ribosomes especially under stress conditions in which mRNA translation is limited (Gerashchenko and Gladyshev, 2014).

uORFs are generally considered to regulate translation by reducing initiation of main ORFs (Calkhoven et al., 1994; Kozak, 1984; Werner et al., 1987) (**Figure 5, paper II**), especially when several uORFs are present (Brown et al., 1999; Hinnebusch, 1984; Mueller and Hinnebusch, 1986; Thireos et al., 1984). This translational repression leads to alterations in protein levels between 30-80%, as assessed by comparing datasets from mouse in different developmental stages (Calvo et al., 2009). However ribosome profiling performed in mouse embryonic stem cells, human and zebrafish only show a modest reduction of 15-30% in translational efficiency (Chew et al., 2016). Nevertheless, the ability to repress is more pronounced, when the AUG codon of the uORF lies in a strong Kozak context (Lee et al., 2012). Increasing distance between 5'cap and uORF-AUG codon results also in stronger reduction of main-ORF translation supporting the notion of more efficient initiation at uORFs while having a more robust scanning of the 43PIC (Calvo et al., 2009; Kozak, 1991a). Interestingly, there is no substantial difference between 5'UTRs harboring an isolated uORF versus uORFs overlapping with the main ORF (Calvo et al., 2009; Johnstone et al., 2016). With decreasing distance of the uORF in respect to the main ORF a reduced reinitiation potential could be observed using the pre-proinsulin mRNA (Kozak, 1984, 1987b). This was confirmed in mouse, human and zebrafish, which showed a depletion of uORF start-codons towards the main ORF (Chew et al., 2016). The start codons of uORFs, as defined by mapping translation initiation sites, are composed of 25% AUG, 30% CUG, with the majority being AUG variants (UUG, GUG, AGG, AACG, AAG, AUC, AUA, AUU) of around 41% (Lee et al., 2012). Importantly, despite the different variations of start-codons, robust inhibition of translation by uORFs has mainly been shown for AUG start-codons (Arribere and Gilbert, 2013; Calvo et al., 2009; Ingolia et al., 2012; Johnstone et al., 2016). Nevertheless, yeast cells show a dramatic reshaping of transcription start sites and translation during meiosis involving initiation at non-canonical AUG codons, with a preference of UUG and CUG (Brar et al., 2012). Moreover,

terminating ribosomes at uORF stop codons can induce non-sense mediated decay, emphasizing the inhibitory potential of uORFs and connecting their regulatory potential to mRNA stability (Gaba et al., 2005). In contrast to the high genome-wide occurrence in different species, there is only a small number of genes which contain a characterized uORF. Among these, two main mechanisms of translational control have been identified. First, the PIC initiates at the uORF AUG leading to increased translation of the uORF and subsequent lower translation of the main ORF. This is supported by the observed correlation of stronger Kozak contexts of more inhibitory uORFs (Calvo et al., 2009; Lee et al., 2012). The second mechanism involves a peptide originating from the uORF stalling the 80S ribosome therefore acting as a roadblock to the scanning 43PIC, which did not initiate at the uORF AUG due to leaky-scanning (Wei et al., 2012; Werner et al., 1987). Additionally, an inhibitory peptide functions as an antizyme for the expression of different ornithine-decarboxylase homologs mediating polyamine-induced repression of the main-ORF, in which interestingly the start codon of the regulatory uORF is an AUU codon (Ivanov et al., 2008).

As previously mentioned, phosphorylation by four different kinases on Serine 51 at the alpha subunit of eIF2 is one of the key regulatory mechanism of translation initiation resulting in global reduction of mRNA translation (**Figure 3**). This leads, however, to induction of a specific translational program to prime cells for stress recovery. eIF2 α -phosphorylation is mediated by different kinases in response to various cellular stresses (Wek et al., 2006) and results in lower GTPase-activity of eIF2B leading to decreased availability of functional ternary complexes. A proportion of PIC proceeds to scan without available ternary complex resulting in increased leaky scanning on start codons. This bypassing of uAUG codons subsequently leads to initiation at the main ORF under stress (Hinnebusch, 2014). Most transcripts encoding for such genes with low susceptibility to stresses contain these uORFs leading to their low translational efficiency of their main ORF in stress-free conditions, in contrast to transcripts without uORFs underlying the stress-induced reduction of global protein synthesis (Andreev et al., 2015; Lawless et al., 2009; Vattem and Wek, 2004). Among the examples for this phenomenon is GADD34, the regulatory subunit of the phosphatase PP1, exhibiting auto regulatory activity, which antagonizes eIF2 α phosphorylation (Choy et al., 2015). Another example is ATF4, a transcription factor leading to induction of CHOP and GCN4, leading to cell death if stress conditions persist (Han et al., 2013). Two major uORFs in the ATF4 mRNA modulate translation by initiating at a short uORF upstream of a second uORF, which overlaps with the main ORF. The remaining ternary complex at the stop codon of this uORF, initiates after 60S dissociation at the overlapping uORF preventing translation of ATF4. When ternary complex is limited, initiation at the overlapping uORF is decreased leading to ATF4 expression (Vattem and Wek, 2004). One of the first characterized examples is GCN4 in yeast, which is activated by GCN2 upon amino acid starvation. Here four

uORFs regulate translation of the main ORF, demonstrating the same phenomena as in ATF4 when the remaining 40S subunits reinitiate with ternary complexes at the three following uORFs. However this reinitiation is again limited when phospho-eIF2 α levels are high, 43S PICs resume leaky scanning leading to recognition of the GCN4 start-codon (Hinnebusch, 1997). One common feature of these short uORFs is their strong Kozak context leading to robust inhibition and subsequently initiation at main ORFs when uORF recognition is moderately impaired. This allows fine-tuning of the proteome by reducing global translation and induction of key-transcription factors (Hinnebusch, 2005). There has been evidence that eIF3 plays a role in reinitiation after short uORFs (Park et al., 2001; Roy et al., 2010), by interacting with structures in the 5'UTR in the case of GCN4 and ATF4 mRNAs (Hronová et al., 2017). However, additional factors like DENR and MCT-1 are necessary for reinitiation after longer uORFs (Schleich et al., 2014). The role of eIF2 α phosphorylation is also implicated in different neurological conditions and liver development. Loss of Gcn2 in mice and a subsequent decrease in phospho-eIF2 α to reduce translation of ATF4 mRNA is leading to increased memory (Costa-Mattioli et al., 2005). Furthermore, the mutation of the uAUG in the CCAAT/enhancer-binding protein beta (C/EBP β) impairs liver development in mice, herein by dysregulating the translation of different N-terminal isoforms of C/EBP β (Wethmar et al., 2010). In **paper II**, we identified 5'UTR variants harboring TSSs in distinct distances upstream of uORFs rendering those resistant to the integrated stress response.

1.3.2.5 TOP mRNAs

A class of mRNAs harboring a C-residue at the cap-site followed by a stretch of 4-15 pyrimidines, called “terminal oligo pyrimidine tract” - TOP (Avni et al., 1994; Meyuhas and Dreazen, 2009; Perry, 2005), exhibit distinct modes of regulation. The TOP-motif is conserved among all vertebrates and the ribosomal proteins in *Drosophila melanogaster*, however not in yeast and *Caenorhabditis elegans* (Meyuhas and Kahan, 2015). Its function as a cis-regulatory element is strictly relying on the integrity and composition of the TOP-motif. Removal of the C at the cap-site and/or the replacement of the following base to an A abolishes TOP-functionality (Avni et al., 1994). This class of mRNAs is highest expressed (Gandin et al., 2016) and dominated by 80 ribosomal proteins with a smaller proportion of 5 eukaryotic elongation factors (eEFs) and 2 eukaryotic initiation factors (eIFs) (Meyuhas and Kahan, 2015). However there are likely more mRNAs harboring a TOP-motif. Translation regulation of TOP-mRNAs can be described by an “all-or-nothing”-mechanism, in which mRNAs switch between extremely efficient translated under normal growth conditions to largely repressed under cellular stress (Gandin et al., 2016; Hornstein et al., 2001). Additionally around 30% of TOP-mRNAs remain translationally repressed in optimal growth conditions (Patursky-Polischuk et al., 2009). This might be explained by the fact

that ribosome production and maintenance of the protein-synthesis machinery is extreme energy consuming (Granneman and Tollervey, 2007). Cells can therefore tune the production of protein-synthesis components in a dramatic and efficient manner. Indeed, unfavorable growth conditions, such as serum starvation or contact inhibition, will cease TOP-mRNA translation (Stolovich et al., 2002). An unsuccessful attempt to relieve the translational repression of ribosomal proteins by over-expression of eIF4E in quiescent cells underlines the unique mechanism of translational regulation of this class of transcripts (Shama et al., 1995). The signal transduction regulating TOP-mRNA translation relies strongly on the integrity of the PI3K-AKT pathway (Stolovich et al., 2002), furthermore requiring TSC1/2 and their downstream target, the GTPase Rheb (Bilanges et al., 2007; Miloslavski et al., 2014). These findings suggest that TOP-mRNA translation is highly dependent on activity of mTORC1, moreover as depletion of amino acids leads to mTORC1 inactivation, reduced activity of S6K and rpS6 phosphorylation. Subsequently it was shown that alterations of S6K and P-rpS6 do not impact TOP-mRNA translation (Tang et al., 2001). Studies using Ribosome profiling show that 4E-BPs play a major role in regulating TOP-mRNAs, as mouse embryonic fibroblasts lacking 4E-BPs exhibited less repression on TOP-mRNA translation upon inhibition with mTOR inhibitors such as Torin1 and INK128 (Hsieh et al., 2012; Thoreen et al., 2012). This stands in stark contrast to TOP-mRNAs being shown un-sensitive to eIF4E (Shama et al., 1995). Stimuli like oxygen, nutrients and growth factors alter TOP mRNA-translation in an mTOR dependent but 4E-BP-independent manner (Miloslavski et al., 2014), suggesting another mechanism of mTOR not involving 4E-BPs. TIA-1 and TIAR, factors associated with stress granules, have been found to regulate translation of TOP-mRNAs depending on amino acid levels mediated by GCN2 and activation of mTOR (Damgaard and Lykke-Andersen, 2011). In recent years, La-related proteins (LARP) have been heavily studied and shown to regulate TOP mRNA translation (Tcherkezian et al., 2014). The RNA binding protein LARP1, phosphorylated by mTORC1 upon association with raptor (Fonseca et al., 2015), has been found to interact with the TOP motif in 5'UTRs of TOP-mRNAs via its DM15 domain regulating translation (Fonseca et al., 2015; Lahr et al., 2015) and mRNA stability of TOP-mRNAs (Aoki et al., 2013; Blagden et al., 2009). More recently, it was proposed that LARP1 also interacts with the 5'cap of TOP-mRNAs proposing a competitive effect with eIF4E impeding the assembly of the eIF4F complex (Lahr et al., 2017; Philippe et al., 2018). Moreover, cap binding of LARP has been shown to be mediated by mTORC1 (Hong et al., 2017; Jia et al., 2021). However it has so far been debated if LARP1 acts as a repressor or activator of TOP-mRNA translation. While it has been shown to repress translation downstream of mTORC1 (Fonseca et al., 2015; Lahr et al., 2017) an activating role has been described suggesting a context dependent effect. In this study LARP1 binds the 5'cap and PABP and associates with polysomes in a mTOR-dependent manner (Tcherkezian et al., 2014). Moreover, LARP1 plays a role in TOP-mRNA stability (Gentilella et al., 2017). To this end,

it remains unclear to what extent LARP1 is the key-modulator of TOP-mRNAs and if there are other factors at play.

1.3.3 Translational control by 3' untranslated regions

The mRNA sequence downstream of the stop-codon of a main-ORF is defined as the 3'-untranslated region (3'UTR). These sequences are longer, in comparison to 5'UTRs, ranging between several hundred to several thousand of nucleotides (Siepel et al., 2005; Xie et al., 2005). Interestingly, 3'UTR length has been increasing during the evolution of mRNAs, suggesting they play important functional roles (Chen et al., 2012; Jan et al., 2010). In contrast to 5'UTRs, 3'UTRs contain proportionally fewer introns (Hong et al., 2006). This can be explained by the induction of non-sense mediated decay by introns downstream of stop codons and the presence of splicing signals in 3'UTRs leading to negative selection (Scofield et al., 2007). Similar to 5'UTRs, these sequences are responsible for a plethora of regulatory functions, i.e. translational control, mRNA degradation and mRNA localization.

1.3.3.1 *microRNAs*

microRNAs (miRNAs), are short RNA molecules of approximately 20nt length (Hutvagner et al., 2001), which are involved in a myriad of cellular processes and diseases (Calin et al., 2004; Chang and Mendell, 2007; Esquela-Kerscher and Slack, 2006; Krützfeldt and Stoffel, 2006) and present promising treatment strategies (Krützfeldt et al., 2005; Setten et al., 2019; Soutschek et al., 2004). These small RNA molecules bind to sequence motifs in the 3'UTR (**Figure 5**) and regulate gene expression post-transcriptionally by RNA-mediated interference, resulting in altered mRNA- (Farh et al., 2005; Friedman et al., 2009; Lim et al., 2005; Sood et al., 2006) and protein-levels (Baek et al., 2008; Guo et al., 2010; Selbach et al., 2008). However, miRNA binding sites have also been found in coding regions of mRNAs (Chi et al., 2009; Hafner et al., 2010; Hausser et al., 2013; Schnall-Levin et al., 2010). RNA interference (RNAi) was initially observed in petunia flowers, where transgenic increased expression of chalcone synthetase to increase pigmentation, resulted in an inverse effect (Napoli et al., 1990). Later it was observed that only transfection of double-stranded RNA yielded in gene-silencing effects (Fire et al., 1998). The first miRNA was characterized in *Caenorhabditis elegans*, by the identification of the non-coding gene *lin-4*, whose expression reduced LIN-14 protein levels (Lee et al., 1993). The sequence complementarity of *lin-4* to the 3'UTR of the LIN-14 mRNA suggested a RNAi mechanism (Wightman et al., 1993). This was identified as evolutionary conserved, since the *let-7* miRNA (Reinhart et al., 2000) was identified among many organisms (Pasquinelli et al., 2000) including mammals (Elbashir et al., 2001). These findings paved the way for several tools for genetic-engineering and treatment against diseases, i.e. shRNA and siRNA (Davidson and McCray, 2011). miRNAs are transcribed from a

single transcription unit or from introns of their target mRNA (Ramalingam et al., 2014) and exported to the cytoplasm (Yi et al., 2003), where they are processed by endonucleolytic cleavage (Schwarz et al., 2003; Zeng et al., 2005), allowing target binding and assembling of the miRNA induced silencing complex (miRISC) (Iwasaki et al., 2010; Khvorova et al., 2003; Kwak and Tomari, 2012). The miRISC then recruits the CCR4-NOT complex, which contains exonucleases to remove the polyA-tail of the mRNA (deadenylation) (Fabian et al., 2010) (**Figure 5**) leading to subsequent removal of the 5'cap (decapping) by DCP2 and ultimate degradation of the transcript by XRN1 in 5'-3' directionality (Huntzinger and Izaurralde, 2011). mRNAs targeted by miRNAs are, besides being degraded, also translationally repressed. Among all effects induced by miRNAs 6-29% account for translational repression (Eichhorn et al., 2014) and it is widely accepted that miRNAs repress cap-dependent translation (Eulalio et al., 2008; Mathonnet et al., 2007; Ricci et al., 2013). Despite the idea that mRNA degradation is a consequence of translational inhibition by miRNAs (Bazzini et al., 2012; Béthune et al., 2012), mRNAs can be degraded independently of translation (Wakiyama et al., 2007), of which the CCR4-NOT complex has been proposed to be translationally repressive in the absence of deadenylation (Eulalio et al., 2008; Zekri et al., 2013). More recent studies have presented a wide role for CCR4-NOT beyond mRNA-translation and -stability, including mRNA localization and codon-usage (Gillen et al., 2021). Translational repression has also been linked to DEAD-box helicases like eIF4A1/2 and DDX6 (Fukao et al., 2014; Nicklas et al., 2015), where structured 5'UTRs remain unresolved due to interference by miRISC or the interaction of NOT1, a member of the CCR4-NOT complex, with eIF4A2 (Meijer et al., 2013; Wilczynska et al., 2019). Conversely, studies in *Drosophila melanogaster* have shown a release instead of a recruitment of eIF4A and eIF4E (Fukaya et al., 2014). In contrast, mRNAs with unstructured 5'UTRs are unsusceptible to miRNA induced translational repression (miRNA silencing) (Meijer et al., 2013). Indeed, mRNAs containing IRES, which do not require scanning, are rendered as resistant to miRNA silencing, supporting the model of interference with the scanning-mechanism of the 43 PIC (Fukao et al., 2014; Fukaya et al., 2014; Meijer et al., 2013). This is in contrast to IRES requiring eIF4A, being repressed by miRNAs (Fukao et al., 2014; Meijer et al., 2013). DDX6 has also been proposed to play a role in silencing since abolishing the interaction between DDX6 and NOT1 reduced the silencing ability (Rouya et al., 2014) and repressed translation independent of mRNA degradation (Presnyak and Coller, 2013). Potentially, DDX6 represses translation via binding to 4E-T, a transport protein of eIF4E, which in turn competes for the interaction with eIF4G disrupting the eIF4E-eIF4G binding. This model is challenged by only partial loss of silencing when 4E-T is depleted (Kamenska et al., 2013). Moreover, DDX6 has been shown to not associate with miRNA targeted transcripts in a transcriptome-wide study (Wilczynska et al., 2019), as opposed to loss of DDX6 in embryonic stem cells leading to up-regulation of miRNA targeted transcripts (Freimer et al., 2018). Taken

together, the detailed mechanisms of miRISC induced translational repression have yet to be delineated.

1.3.3.2 RNA-binding proteins in 3'UTRs

The functional elements encoded by 3'UTRs (cis-factors), besides miRNA binding sites, are sequence motifs as hubs for RBPs (**Figure 5**) (Baltz et al., 2012; Dominguez et al., 2018; Ray et al., 2013). The sequences required for binding are often short (3-8 nucleotides) and repeated (Hennig and Sattler, 2015). A class of sequence motifs that has been studied to a large extent are AU-rich elements (ARE). These elements were identified when studying the transformation ability of the viral fos-gene (v-fos), whereby the viral isoform is able to transform cells, the cellular gene (c-fos) is not. Both genes differed only in the presence of AREs, which led to its degradation, rendering c-fos less stable compared to v-fos. This leads to cellular transformation upon v-fos expression in mammalian cells (Meijlink et al., 1985; Shaw and Kamen, 1986). ARE's are generally found in 3'UTRs of transcripts encoding for short-lived factors such as oncogenes, growth factors and cytokines (Caput et al., 1986) and play a role in mRNA stability or translational regulation (Kruys et al., 1989; Lindstein et al., 1989; Meijlink et al., 1985; Shaw and Kamen, 1986). These functions are mediated by binding of trans-factors (RBPs), such as HuR and TTP. HuR has been found to stabilize ARE-containing mRNAs (Fan and Steitz, 1998), by inhibiting the recruitment of the exosome complex to the mRNA (Chen et al., 2001), whereas TTP destabilizes mRNAs by recruiting the exosome (Carballo et al., 1998; Hau et al., 2007). Interestingly, TTP has also been shown to be a translational repressor by recruiting 4EHP, a cap-binding protein competing with eIF4E (Morita et al., 2012). HuD, also binding to AREs, induces cap-dependent mRNA translation, thought to bind the polyA-tail and eIF4A in the closed loop formation (Fukao et al., 2009). Other factors binding to 3'UTRs leading to altered mRNA translation have been identified in *Drosophila melanogaster* embryos. Here, Bicoid binds to a 3'UTR motif of the caudal mRNA and to 4EHP to repress translation (Cho et al., 2005). Cup, another eIF4E-binding protein, represses translation of the oskar mRNA via the 3'UTR-binding protein Bruno (Igreja and Izaurralde, 2011; Kim-Ha et al., 1995; Nakamura et al., 2004). These repressive mechanisms are important for *Drosophila* development, which requires protein gradients with precise location along the oocyte for polarity prior to fertilization (Johnston and Nüsslein-Volhard, 1992). Studies in *Xenopus* oocytes revealed that CPEB, which binds to the CPE motif in 3'UTRs regulating their translation, associates with eIF4E through Maskin leading to translational repression similar to the mechanism of Cup in *Drosophila*. This repression is released upon signals inducing maturation (Stebbins-Boaz et al., 1999). In order to compensate the expression of X-chromosomal linked genes in organisms containing different numbers of X-chromosomes between females and males (X-chromosome dosage compensation) in *Drosophila melanogaster*, 5' and 3'UTRs are utilized. This is achieved by the

RBP Sxl binding to uridine-rich sequences in both UTRs of the *msl2* mRNA and inhibiting translation independent of 5'cap and polyA-tail (Gebauer et al., 1999, 2003), while impairing scanning by the 43S PIC (Hennig et al., 2014). Another mechanism resulting in down regulation of translation initiation by inhibited association of the 43S PIC is mediated by the GAIT'-complex. The 3'UTR of the ceruloplasmin mRNA is bound by a multimeric complex, induced by interferon- γ , consisting of the ribosomal protein L13a including GAPDH, NS-associated protein 1 and Glu-Pro-tRNA-synthetase. This complex blocks interaction of eIF3 with eIF4G and impairs binding of the 43S PIC leading to decreased translation initiation despite a formation of the closed loop between PABP and eIF4G (Kapasi et al., 2007).

In summary, these mechanisms show that, despite the presence of cis-factors in 3'UTRs (sequence-motifs), the resulting effect is mediated by trans-factors (RBPs). The diversity of cis-elements in mRNAs is further increased by alternative cleavage and polyadenylation of 3'UTRs (Mayr and Bartel, 2009; Sandberg et al., 2008; Tian et al., 2005), often in a tissue-specific manner, leading to several 3'UTR variants of the same mRNA (Lianoglou et al., 2013).

1.3.3.3 mRNA localization and transport

The localization and transport of mRNAs is attributed to features in their 3'UTRs (Jansen, 2001), which is mediated by the binding of RBPs into complexes of mRNA and protein – messenger ribonucleoproteins, mRNPs. These complexes are then transported to cellular compartments by associating with motor proteins. A well-described example for mRNA-transport in the developing *Drosophila* oocyte is the Oskar mRNA. This mRNA is required to be translationally repressed by Bruno (1.3.3.2) (Kim-Ha et al., 1995) and localized by microtubules through interactions with tropomyosin, Staufen and components of the exon junction complex (EJC) (Micklem et al., 2000). In mammalian cells, mRNAs are transported via Zipcode-binding protein 1, binding to zipcode sequences in the 3'UTR (Figure 5), as illustrated in the transport of beta-actin mRNA (Ross et al., 1997). This movement is facilitated by KIF11, a motor-protein associated with tubulin (Song et al., 2015).

Spatial organization of mRNAs also occurs by localization to membrane-less granules, such as stress granules (SG) or processing-bodies (P-bodies). These, are characterized by aggregating translationally silent mRNAs (Ivanov et al., 2019). P-bodies are linked to mRNA-silencing and decay, since the exonuclease XRN1 and silencing factors such as GW182, CCR4-NOT and Ago co-localize with these granules and mRNAs lack polyA-tails (Bashkirov et al., 1997; Eulalio et al., 2007; Sheth and Parker, 2006). SGs are associated with translational inhibition upon cellular stresses by phosphorylation of eIF2 α (Kedersha et al., 2005; Stoecklin et al., 2013) and are mainly characterized by translation initiation factors (Kedersha et al., 2002, 2005). mRNAs in SGs have

polyA-tails and can therefore resume being translated (Kedersha et al., 2000). Both intracellular aggregations of mRNAs and proteins are considered responses to cellular stress (Stoecklin et al., 2013) and therefore related to spatial organization of mRNA translation to mediate localized protein and mRNA levels. However, a deep mechanistic understanding of these processes is still lacking.

1.3.4 Translational control by tRNAs

The influence of the coding sequence on mRNA translation is currently not well understood, nevertheless it has been shown that elongation of synonymous codons (codons decoding the same amino acid) is not equally efficient (Rudorf and Lipowsky, 2015), elongation fidelity can be impaired by neighboring codons (Gamble et al., 2016) and ribosomal pausing alters mRNA stability (Buschauer et al., 2020; Gillen et al., 2021; Radhakrishnan et al., 2016). Efforts have been made to investigate the relationship between codon composition and the availability of the respective decoding tRNA. This revealed that, given that each amino acid can be decoded by several codons, expression level of tRNAs correlates with the frequency of codons in mRNAs in bacteria and unicellular eukaryotes (Ikemura, 1985; Percudani et al., 1997). The advent of high-throughput methods enabled the quantification of tRNAs on a transcriptome-wide level, resulting in the identification of tissue-specific transcription of tRNAs reflecting codon-adaptation of highly expressed genes (Dittmar et al., 2006; Plotkin et al., 2004). The influence of global tRNA levels on phenotypes has been studied in several cell models, describing distinct tRNA expression related to proliferation, metastatic potential and differentiation (Aharon-Hefetz et al., 2020) with concomitant codon-composition (codon-usage) in subsets of mRNAs (Gingold et al., 2014; Goodarzi et al., 2016; Zhang et al., 2018). However, the impact of tRNAs on translational fidelity is still under thorough investigation. Indeed global protein synthesis is linked to mTOR-dependent stimulation of tRNA synthesis by RNA pol III (Michels et al., 2010) and translational decrease mediated by phosphorylation of eIF2 α by GCN2 due to increasing levels of uncharged tRNAs (Dever et al., 1992). However, these mechanism alter translation globally. Therefore, the mechanisms through which translation is regulated specifically by tRNAs are of great interest. Studies in a melanoma model revealed tumorigenesis and resistance to targeted therapy related to altered codon usage of HIF1 α - dependent on posttranscriptional modifications of tRNAs (Rapino et al., 2018). These modifications (wobbling-modifications) (**Figure 5 and 25**) at the U34 position of tRNAs, are catalyzed by a cascade of 3 different enzymes complexes, generating a 5-methoxycarbonyl-methyl-2-thiouridine (mcm⁵s²U) at position 34 in the tRNA, which leads to increased decoding efficiency of AAA and AAG (Lysine, tRNA^{UUU}); CAA and CAG (Glutamine, tRNA^{CUU}); GAA and GAG (Glutamic Acid, tRNA^{UUC}), rendering mRNAs enriched in these codon sensitive to U34 modifications (Rapino et al., 2017). These modifications have been shown not to

be necessary for maintenance of global protein synthesis, but rather play a role in different pathophysiological phenotypes such as the unfolded protein response (UPR) during brain development (Laguesse et al., 2017), breast cancer progression (Delaunay et al., 2016) and tumorigenesis in the intestine (Ladang et al., 2015). Wobbling leads to decoding of the aforementioned codons ending with G, and interestingly the cognate tRNAs for these codons (tRNA^{CUU} (Lysine), tRNA^{CUG} (Glutamine), tRNA^{CUC} (Glutamic Acid)) are expressed as well. This raises the question to which extent U34 modifications are required for sufficient translational elongation. Indeed, loss of U34 modifications in yeast led to increased ribosomal densities at AAA, CAA and GAA codons (Nedialkova and Leidel, 2015; Zinshteyn and Gilbert, 2013), suggesting ribosomal-pausing at codons ending with A. These effects did not reduce global protein levels, rendering translation initiation as rate-limiting. However, impaired elongation has been shown to affect protein folding (Nedialkova and Leidel, 2015) and has been proposed to be related to a hydrophilic pentapeptide encoded by the mentioned A-ending codons leading to protein aggregation and decreased expression (Rapino et al., 2021). Distinct translational programs can also be related to levels of U34 modifying enzymes and mRNAs requiring U34 modifications (**paper III, IV**).

1.4 mRNA TRANSLATION IN CANCER

As mentioned previously, protein synthesis is one of the most energy-demanding processes in the cell (Buttgereit and Brand, 1995; Rolfe and Brown, 1997) and must be tightly controlled (Hershey et al., 2012; Sonenberg and Hinnebusch, 2007). Aberrant proliferation, survival, angiogenesis, energy metabolism and alterations in immune response are considered as hallmarks of cancer and can occur as a consequence of dysregulated mRNA translation (Hanahan and Weinberg, 2011; Tahmasebi et al., 2018). Initially, differences in transcription rates of genes, leading to changes in the transcriptome, were thought to be mainly contributors to altered protein levels and subsequently shaping development of cancer and the outcome of cancer patients (van 't Veer et al., 2002; Hawkins and Ren, 2006). However many of the pathways altered in cancer converge on mRNA translation. Among those oncogenes are MYC, RAS, PTEN, AKT and tumor suppressors like TP53 (**Figure 4**) (Bhat et al., 2015). Additionally alterations in ribosomal proteins or ribosome biogenesis, referred to as ribosomopathies are connected to higher cancer prevalence (Ganapathi and Shimamura, 2008). Hyperactivation of the upstream kinases of MNKs, the oncogenes RAS and RAF, occurs in many cancer types resulting in increased levels of eIF4E phosphorylation (Siddiqui and Sonenberg, 2015). Moreover it was shown that eIF4E phosphorylation levels are elevated in human prostate cancer (Furic et al., 2010) and drive translation of SNAIL and MMP3, leading to increased metastasis (Robichaud et al., 2014). These findings complemented earlier studies, showing that overexpression of eIF4E in NIH-3T3 cells led to malignant transformation

(Lazaris-Karatzas et al., 1990). Additionally, its overexpression in prostate cancer is contributing to poor patient outcome (Graff et al., 2009) and synergistic effects between eIF4E and c-MYC have been found to lead to lymphomagenesis (Ruggero et al., 2004). A more recent study showed, that eIF4E-phosphorylation is attributed to polarization of tumor-associated macrophages, depending on Mnk2 (Bartish et al., 2020). Here increased phosphorylation levels of eIF4E are associated with an anti-inflammatory macrophage phenotype, proposed to counter cancer-progression. Interestingly, this macrophage phenotype is driven by differential mRNA-translation (Bartish et al., 2020). Taken together, these findings underpin the role of eIF4E in cancer development and progression. Besides eIF4E, a plethora of translation factors are altered in expression and activity in cancers. Among these, the scaffold protein eIF4G (increased expression); the helicase eIF4A (increased expression), regulatory proteins 4E-BP (increased and decreased expression) and others (Silvera et al., 2010). Many mRNAs encoding proto-oncogenes harbor long and highly structured 5'UTRs or upstream open reading frames (uORFs), which in turn require higher levels and activity of cap-dependent translation and initiation factors such as eIF4E, eIF4G and eIF4A (eIF4F complex) or alterations of ternary complex and start codon recognition by eIF2 α , eIF1 and eIF5 (Hinnebusch et al., 2016; Schuster and Hsieh, 2019; Silvera et al., 2010; Sonenberg and Hinnebusch, 2009). In greater detail, oncogenic mRNAs such as c-MYC, CDK2, Mcl-1, survivin and VEGF show a high dependency on eIF4E for their translation (Hsieh and Ruggero, 2010), rendering some cancers highly dependent on protein synthesis. Tumor cells present dramatically different genetic landscapes resulting in big heterogeneity, making the establishment of targeted therapies challenging. Therefore, targeting pathways of the translational machinery, which unite many oncogenic signals, is a promising strategy. Additionally, due to frequent alterations of translation factors and higher demand for protein synthesis, tumor cells are prevalent targets for these therapy approaches (Ruggero, 2013). Considerable big efforts have been made to develop therapeutic agents targeting different key steps in translation. Among those, are inhibitors for the kinase complexes mTORC1 and mTORC2 such as: asTORin (INK128, Torin1, PP242), inhibitors of the initiation factors eIF4E by cap-analogues or eIF4A by silvestrol (Chu et al., 2016), Hippuristanol (Bordeleau et al., 2006a) or Pateamin A (Bordeleau et al., 2006b). Additionally, inhibition of upstream kinases such as MNK by for example cercosporamide has been shown to have promising effects on metastasis mediated by phosphorylation of eIF4E (Konicek et al., 2011). The implications of ternary complex formation in cancer are less well understood than the formation of the eIF4F complex. However a non-phosphorylatable mutant of eIF2 α leads to transformation of NIH-3T3 cells (Donzé et al., 1995), and increased levels of phosphorylation of eIF2 α promote apoptosis (Donzé et al., 2004). This suggests increased phosphorylation of eIF2 α could be a strategy for anti-cancer treatment (Han et al., 2013). Compounds like BTdCPU (Chen et al., 2011) induce phosphorylation of eIF2 α by the HRI kinase

and salubrinal (Boyce et al., 2005) inhibits its dephosphorylation, showing promising potential in sensitizing cancer cells to undergo apoptosis, thus suggesting utility as combination-therapy with other anticancer agents (Jeon et al., 2016). Intriguingly a recent study suggested the phosphorylation state of eIF2 α is a prognostic marker for prostate cancer, which when elevated, leads to tumor progression in a model carrying PTEN and MYC mutations bolstering the burden of high protein synthesis rates in tumors. These effects could be reversed by ISRIB a small molecule that reactivates eIF2 α function despite its phosphorylation state by binding to eIF2B (Zyryanova et al., 2018), leading to selective cell death of prostate cancer cells (Nguyen et al., 2018).

1.5 CONTRIBUTION OF TRANSCRIPTION AND TRANSLATION IN SHAPING THE PROTEOME

So far, mechanisms regulating mRNA translation have been discussed. However, the general contribution of transcription and translation to the composition of the proteome will be the focus of this section.

Cellular phenotypes are to a large extent shaped by their proteome, i.e. the composition of all proteins in the cell. The composition of the proteome is a result of several steps: transcription and mRNA transport give rise to an mRNA pool that underlies mRNA degradation or is used for protein synthesis. Protein levels are determined by mRNA translation and protein degradation (Liu et al., 2016; McManus et al., 2015). It has raised substantial interest to which extend steps in the gene expression pathway contribute to shaping the proteome. Several studies have shown that mRNA levels and protein levels correlate imperfectly (Cheng et al., 2016; Schwanhäusser et al., 2011; Vogel and Marcotte, 2012; Wang et al., 2019), suggesting mRNA translation playing an important role in determining protein levels. This was challenged by other studies promoting the concept of protein-levels being mainly explained by mRNA levels in a context-dependent manner (Battle et al., 2015; Jovanovic et al., 2015; Li and Biggin, 2015; Li et al., 2014). Nevertheless, mRNA translation has been shown to be responsible for massive changes of the proteome in perturbed systems, such as intracellular stresses (Andreev et al., 2017; Baird et al., 2014; Guan et al., 2017) and rapid adaptations to extracellular stimuli (Gerashchenko and Gladyshev, 2014; Hulea et al., 2018; Jewer et al., 2020). To assess the contribution of transcription and translation systematically, a range of methods can be applied. In order to capture changes of the proteome, mass-spectrometry (Aebersold and Mann, 2016) in combination with labeling methods for newly synthesized proteins, such as SILAC can be used, (Mann, 2006; Schwanhäusser et al., 2011). This allows to calculate protein degradation rates and can be paralleled by nucleic-acid quantification in combination with labeling of newly synthesized RNAs using 4-thio Uridine (4sU) (Rabani et al., 2011). Notwithstanding these methods providing kinetic information for both RNA and protein, methods to capture nucleic-acids on a transcriptome-wide level have different characteristics

compared to mass-spec based proteomics, due to the ability to amplify nucleic-acids and the dynamic-range of measurement (Wang et al., 2009; Wilhelm and Landry, 2009). Moreover, the differences of mRNA and protein half-lives (proteins 46h, mRNA 9h) (Schwanhäusser et al., 2011; Vogel and Marcotte, 2012) and methodologies can lead to obscured results and cannot distinguish between translation and protein degradation. To interrogate the contribution of mRNA translation, the research community has developed experimental and computational methods. These will be discussed in the following sections.

1.6 METHODS TO STUDY mRNA TRANSLATION

The complete sequencing of the human genome (The Human Genome Project) (Craig Venter et al., 2001; Lander et al., 2001) and the advancements in identifying and measuring genes and their transcripts on a genome-wide level has enabled to answer many complex biological questions (Goodwin et al., 2016). Two now widely used methods revolutionized the way to study gene expression: A hybridization based method using known DNA-probes, i.e. DNA-microarrays (Brown and Botstein, 1999; Fodor et al., 1991; Niemitz, 2007; Schena et al., 1995), and a newer method called RNA-sequencing, largely using a “sequencing-by-synthesis” approach (Bainbridge et al., 2006; Mortazavi et al., 2008; Stark et al., 2019; Wang et al., 2009). To date, these methods have become available to almost any researcher due to the wide-range of available platforms (Stark et al., 2019) and their increasing cost-efficiency [(National Human Genome Research Institute (NHGRI), 2021), <https://www.genome.gov/sequencingcosts/> (14.12.2021)]. The application of these techniques in combination with methods to study mRNA translation enables researchers to understand not only the effects of cellular pathways or translation factors impinging on protein-synthesis, but also allows to integrate the contribution of certain cis-elements of the mRNA (**paper II,III and IV**).

1.6.1 Polysome profiling

As previously described, one mRNA molecule gives rise to several molecules of proteins while being associated with several ribosomes, e.g. polysomes (Staehelin et al., 1963; Wettstein et al., 1963). This in turn, allows to relate the efficiency of mRNA translation to the number of associated ribosomes on an mRNA (Larsson et al., 2013). Cycloheximide, a fungicide produced by *Streptomyces griseus*, leads to inhibition of translation elongation by binding to the E-site of the 60S subunit of the ribosome resulting in stalled ribosomes on the mRNA (KERRIDGE, 1958; Schneider-Poetsch et al., 2010; Sisler and Siegel, 1967). While cycloheximide is commonly used to stall ribosomes on mRNAs, artifacts related to its usage in yeast have been reported (Gerashchenko and Gladyshev, 2014; O'Connor et al., 2016). However, a comparison between yeast and human cells indicated that these biases are specific to the usage of the organism (Sharma et al., 2021). The separation of

heavier mRNAs due to increased numbers of associated ribosomes (polysomal mRNA) from the total (cytosolic) mRNA pool allows to analyze the proportion of efficiently translated mRNAs under a certain condition. The polysome profiling technique entails this separation by loading the lysate derived from cells or tissue on a sucrose gradient, which after sedimentation of the heavier polysomal mRNAs by ultracentrifugation, can be analyzed by gradient profiling while measuring the absorbance at 254nm (Gandin et al., 2014). This allows to collect fractions containing mRNAs associated with distinct numbers of ribosomes followed by analysis using northern blotting, RT-qPCR, micro array or next-generation sequencing based methods. Generally fractions of mRNAs with more than 3 ribosomes are considered as efficiently translated mRNAs, which can be pooled together. The mean number of associated ribosomes can be considered as normally-distributed along the sucrose gradient (Gandin et al., 2016) (**Figure 6**), thus changed mean ribosome association resulting in shifting along the gradient can be captured by polysome profiling. This is also true in cases where mRNAs shift in an extreme manner, e.g. for TOP mRNAs, who are largely excluded from polysomal fractions when their translation is inhibited (Amaldi and Pierandrei-Amaldi, 1990; Tang et al., 2001). However when pooling heavy fractions, measurements of mRNAs shifting within heavy polysomal fractions can be obscured and fractions should be analyzed independently. Such transcripts have been identified but remain to be further characterized (Hulea et al., 2018). Polysome profiling in combination with cycloheximide treatment can also be used to quantify global changes of mRNA translation, in cases when regulation occurs at the initiation step. Here the area under the polysomal fractions can be quantified after normalization. It is however recommended to verify these findings by other methods to quantify global protein synthesis such as the incorporation of S35-labeled methionine/cysteine (**paper I**) (Leblond et al., 1957), puromycin (Schmidt et al., 2009) and “clickable” amino acid or puromycin derivatives (Dieterich et al., 2007; Nagelreiter et al., 2018). Besides cell lines, polysome profiling can be used with tissue samples, e.g. from biobanks (Liang et al., 2018; Sandri et al., 2019; Shin et al., 2021). To increase the feasibility especially when sample amounts are low an optimized gradient protocol has been developed (Liang et al., 2018) (**paper II**). This strategy concentrates polysomal mRNA to only two fractions, eliminating low mRNA yield due to dilution across many fractions when material is limited and making RNA extraction less time consuming (Liang et al., 2018).

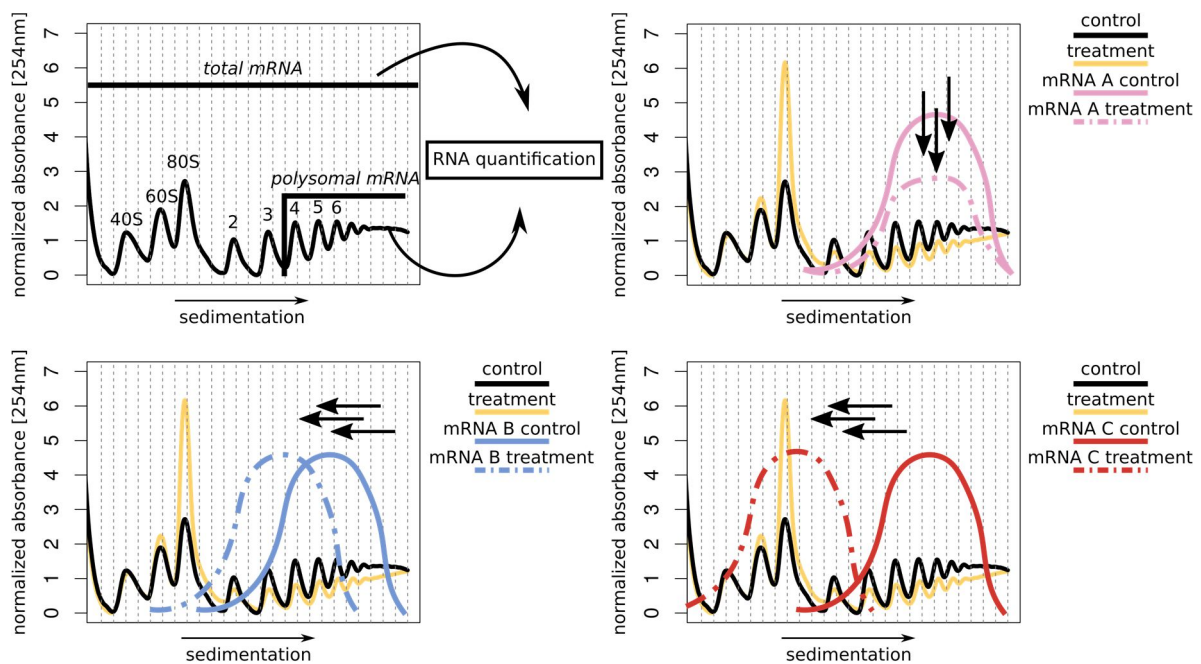


Figure 6 – Polysome profiling (1.6.1): Tracings of MCF7 cells subjected to polysome profiling under a control (DMSO) and a treatment condition (torin1) for 4h. Upper left: mRNAs are separated using ultracentrifugation on a linear sucrose gradient (5-50%). mRNAs associated with increasing number of ribosomes are heavier and sediment faster into the gradient. This is visualized by measuring the absorbance at 254nm. Fractions collected during gradient profiling are indicated by vertical dashed lines. When the translatoome is assessed by RNA-sequencing or micro-array analysis, total (cytosolic) mRNA and mRNAs associated with more >3 ribosomes (polysomal mRNA) are extracted and analyzed in parallel. Peaks indicate the small (40S), big (60S) ribosomal subunits as well as a monosome (80S), and mRNAs with 2, 3, 4, etc. associated ribosomes. Upper right: ribosome association is considered to be normal distributed along the sucrose gradient. mRNA A is regulated by mRNA abundance (1.6.3.1), leading to decreased mRNA levels under the treatment condition and no shift of ribosome association towards lighter fractions. Lower left: mRNA B is regulated by translation (1.6.3.2) and the distribution of ribosome association shifts towards lighter fractions of the sucrose gradient. Lower right: mRNA C shifts extremely under the treatment, leading to near exclusion from polysomal fractions. This has been observed for TOP mRNAs under inhibition of mTOR (1.3.2.5).

1.6.2 Ribosome profiling

Hence, mRNAs are associated with ribosomes during translation, parts of the mRNA molecule are exposed for endnucleolytic cleavage, while the regions protected by ribosomes are not accessible. This is exploited by ribosome profiling when upon cycloheximide treatment, mRNA is extracted and digested using RNases (Ingolia et al., 2009, 2012) (**Figure 7**). This approach was initially used to remove excessive mRNA not involved in translation initiation when identifying ribosomal binding sites in R17 phages (Steitz, 1969) or the distribution of ribosomes along the mRNA (Wolin and Walter, 1988). Later, this approach was combined with next-generation sequencing techniques (Ingolia et al., 2009). Key steps in this work-flow are the following: Similar as in polysome profiling, Ribosomes are stalled using cycloheximide. Next, the extracted mRNAs are subjected to endonucleolytic digestion with RNases, while unprotected regions of mRNAs are cleaved, leading to mRNA fragments protected by ribosomes (**Figure 7**). Monosomes are then purified by a sucrose gradient to yield ribosome protected fragments (RPFs, approximately 28 nts

in length). Since, total mRNA samples are necessary for analysis of translation, polyA-selected mRNA is randomly fragmented using alkaline hydrolysis in parallel followed by depletion of ribosomal RNA. Subsequently, fragments are separated using gel-electrophoresis and bands of similar sized fragments of RPFs and fragmented mRNA are excised. These are then used for the generation of sequencing libraries. Despite being commonly used to study translomes and other aspects of mRNA translation, ribosome profiling is confronted with several caveats and requires important considerations: Cells are often pre-treated with cycloheximide, which can lead to artifacts by enriching ribosomes close to initiation sites leading to arbitrary stalling upstream of initiation sites (Gerashchenko and Gladyshev, 2014; Hussmann et al., 2015; Jackson and Standart, 2015). Similarly, harringtonine, which inhibits the transition from initiation to elongation, enriches ribosomes at initiation sites (Jackson and Standart, 2015). Moreover, cycloheximide treatment allows ribosomal translocation to occur for one cycle prior to stalling (Schneider-Poetsch et al., 2010) obscuring codon-dependent analysis (Nedialkova and Leidel, 2015). Generally, it is advised to omit pre-treatment with elongation inhibitors since quick cooling of cells followed by harvesting in the presence of elongation inhibitors is sufficient in stalling ribosomes on mRNAs ((Gonzalez et al., 2014; Guo et al., 2010) and **paper I, II, III**). The choice of nuclease can be critical, since a comparative study of different nucleases revealed biased ribosomal coverage along mRNAs and in some cases complete digestion of ribosomes leading to increased contamination with ribosomal RNA (Gerashchenko and Gladyshev, 2017). This underlines, that strategies to remove ribosomal RNA prior to library preparation is essential. The conformational change of ribosomes during elongation can lead to fragments of 21nt (Lareau et al., 2014), hence the stringent size selection at 28 nts can influence the capturing of the underlying translome (Andreev et al., 2017). Larger fragments have been observed when ribosomes stack together due to ribosome stalling (disomes, 40-65 nt) and different conformational changes (Jackson and Standart, 2015). Moreover, a study comparing several datasets generated by ribosome profiling in different organisms revealed substantial biases related to library construction due to sequence composition affecting the identified ribosome positioning (Artieri and Fraser, 2014a). While the initial protocols for ribosome profiling describe the fragmentation of total mRNA using alkaline hydrolysis of polyA-selected mRNAs followed by depletion of ribosomal RNA (Ingolia et al., 2012) (**Figure 7**), this can introduce additional biases. In **paper III**, sequencing of total mRNA is performed using smartseq2 (Picelli et al., 2013), which circumvents those biases due to sequencing of full-length transcripts.

In order to assess regulation of gene expression at the level of translation using ribosome-profiling data, datasets have to be subjected to computational quality-control to eliminate intrinsic biases. RUST (Ribo-seq Unit Step Transformation), a method to reduce data-variance, has been shown

to detect biases in ribosome profiling datasets and allows for more robust analysis (O'Connor et al., 2016). Translating ribosomes are predicted to be found on in-frame codons, such that the periodicity of ribosome densities on mRNAs is used as another measure of quality for these datasets (Lauria et al., 2018).

Albeit commonly used to generate data to study translationalomes, polysome and ribosome profiling both have its advantages and disadvantages. When performing polysome-profiling, intact mRNAs associated with ribosomes are extracted, however the information about positioning at codon-resolution is lost. This information is preserved when conducting ribosome profiling, where positioning of the ribosome on the mRNA is maintained. Therefore ribosome profiling can be used to study translation elongation (Anthony Schuller et al., 2017; Lareau et al., 2014; Riba et al., 2019), the influence of codon-composition (Frye and Bornelöv, 2021; Gamble et al., 2016; Nedialkova and Leidel, 2015; Yu et al., 2015; Zinshteyn and Gilbert, 2013), identify upstream open-reading frames (Andreev et al., 2015; Brar et al., 2012; Chen et al., 2020; Chew et al., 2016; Kulkarni et al., 2019), frame-shifting (Rato et al., 2011; Yordanova et al., 2018) and termination (Anthony Schuller et al., 2017; Lobanov et al., 2017; Selmi et al., 2020; Wangen and Green, 2020). In contrast, polysome-profiling allows studying of isoform-specific translation, since intact mRNAs can be subjected to deep RNA-sequencing (Floor and Doudna, 2016) while ribosome-profiling only provides information of “snippets” of mRNAs. A recent study presented an approach to derive isoform specific translational data from ribosome profiling data-sets, however this relies on previously annotated isoforms (Reixachs-Solé et al., 2020). In line with the increased efforts to study single cells stemming from cell populations or tissues, ribosome profiling has been made applicable to study ribosome positioning in single-cells (VanInsberghe et al., 2021). However, this does not enable studying translational efficiencies, since total mRNA expression cannot be assessed in parallel. Another method, which reveals ribosome positioning, 5Pseq (Pelechano et al., 2015), maps ribosomes at the 5'end of co-translationally degraded mRNAs without the need for translation inhibitors (Pelechano et al., 2016). This been used to study ribosomal pausing in yeast upon oxidative stress (Pelechano et al., 2015) or eIF5A depletion (Pelechano and Alepuz, 2017).

In cases where translational regulation is studied transcriptome-wide, ribosome-profiling is widely applied, albeit several problems: Polysome profiling captures both large and moderate shifts of mRNAs across a sucrose gradient (described above, **Figure 6**). Using ribosome profiling, translational efficiencies are determined by ribosome-bound mRNA fragments of more- and less-efficiently translated mRNAs, whereas polysome profiling uses the whole mRNA molecule and its distribution. In contrast, ribosome profiling is biased towards heavy shifting mRNAs, such as TOP mRNAs under modulation of mTOR activity (Gandin et al., 2016). In turn translational efficiencies are influenced by largely shifting mRNAs when using ribosome profiling, leading to misleading

biological conclusions (Gandin et al., 2016; Masvidal et al., 2017). In conclusion, the choice of methodology has to be made carefully and in line with the strengths and weaknesses of the available techniques.

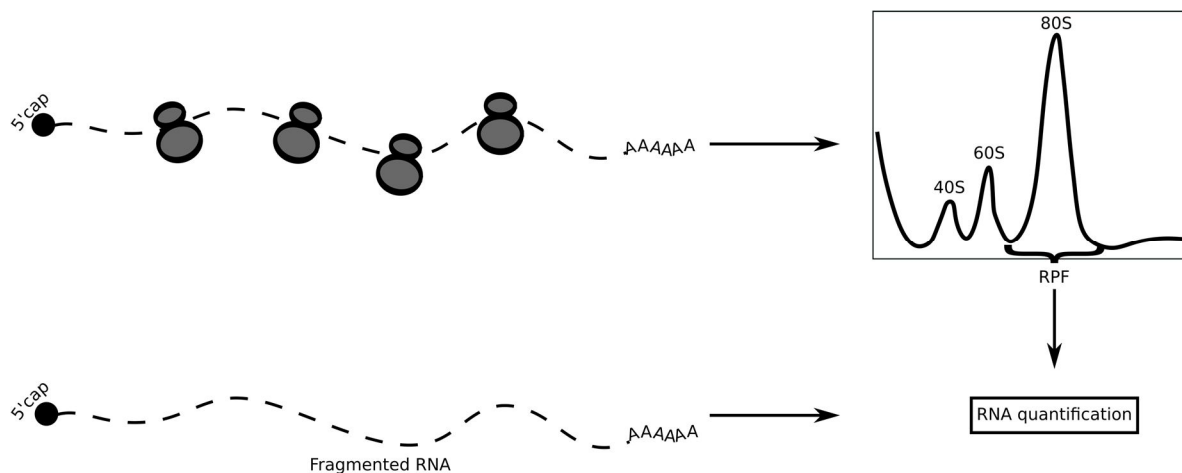


Figure 7 – Ribosome profiling (1.6.2): (Upper) mRNAs are digested, which generates ribosome protected fragments (RPFs). Ultracentrifugation of a sucrose gradient is used to purify ribosome protected fragments from the 80S monosome peak. (Lower) In parallel, total mRNA is polyA-selected and randomly fragmented using alkaline hydrolysis. The mRNA are used to prepare RNA-sequence libraries for RNA-quantification.

1.6.3 Analysis of modes of translation using anota2seq

The rapid development of high-throughput methods to study gene expression require appropriate methods to analyze the generated data. Albeit a plethora of methods being available, this section will only discuss anota2seq. Applicable to all methods for analysis of translomes, it is required to obtain measurements for both total mRNA populations, which contains data for all transcribed mRNAs and mRNAs associated with ribosomes using polysome or ribosome profiling. Subsequently, analysis is performed between two conditions or more. Moreover, it should be noted that total mRNA samples contain both total and translated mRNAs and polysomal mRNA represents the proportion with ribosome association at a certain cut-off (**Figure 6**). The algorithm behind anota2seq, analysis of translational activity (anota), was developed, since analysis of datasets assessing gene expression using simple log-ratios between translated (polysomal) and total RNA led to correlations identified as spurious, i.e., a relationship between two variables seemingly based on causality but uncorrelated in reality (Pearson, 1897). Consequently, the obtained conclusions using these methods were shown to be strongly influenced by total mRNA levels (Larsson et al., 2010). To overcome these biases, anota uses a by-transcript linear regression approach between expression of total and translated mRNAs in combination with analysis of partial variance (APV) (Larsson et al., 2010). In other words a linear model per transcript is fitted for each group of replicates per condition, such that the slopes for each condition are equal. The difference of the intercept on the y-axis (translated mRNA) corresponds to the difference in translational efficiency

(Δ TE, **Figure 10**). This model describes the changes of translated mRNA levels occurring independently of changes on total mRNA levels per condition:

$$\text{translated mRNA} \sim \text{total mRNA} + \text{condition}$$

Initially, anota was developed for analysis of micro-array derived data and was later modified for analysis of data generated by RNA-sequencing methods upon polysome or ribosome profiling (Oertlin et al., 2019). Anota2seq has the ability to decipher coordination of gene expression by defining three regulatory modes: mRNA abundance, translation and translational buffering.

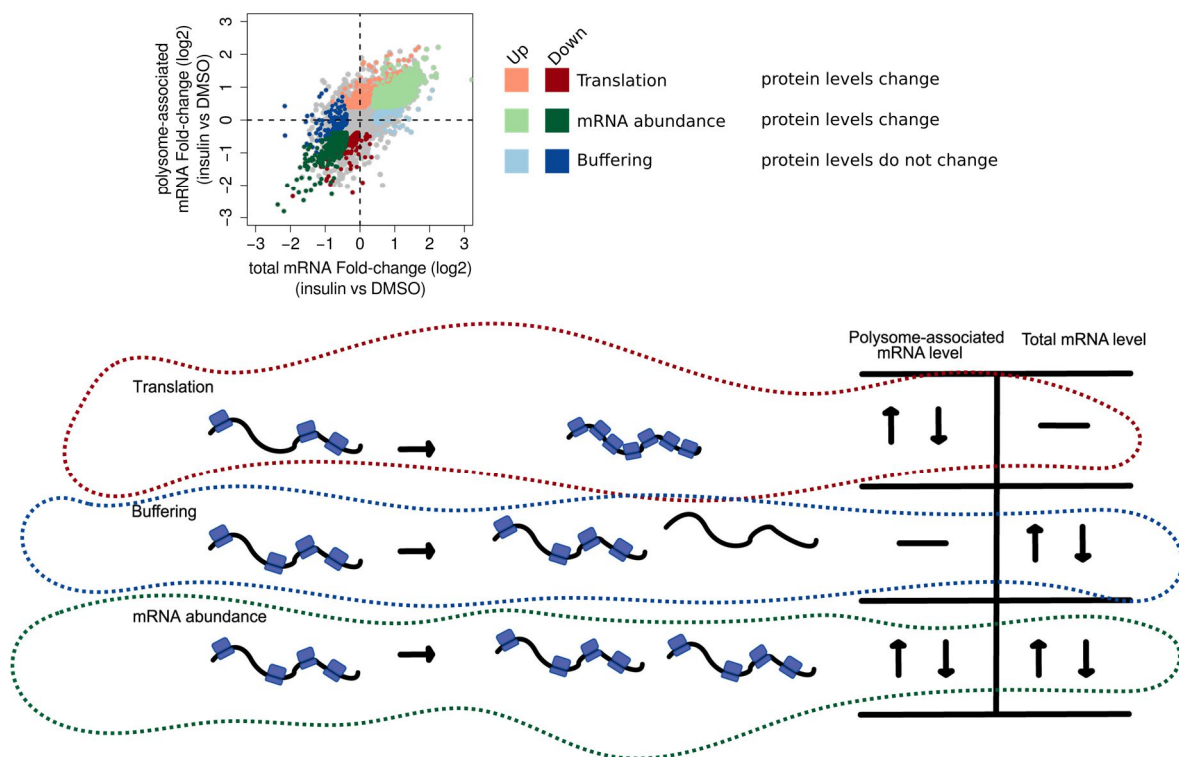


Figure 8 – Overview of regulatory modes of translation identified by anota2seq: (Upper) Fold-change plot of polysome-associated mRNA (or RPF; i.e., translated mRNA) vs. total mRNA. MCF7 cells have been treated with insulin for 4h followed by polysome profiling, RNA-sequencing and anota2seq analysis (insulin vs DMSO). Each dot represents one mRNA whereas grey mRNAs have been identified as non-regulated by anota2seq. Red dots represent mRNAs which, are regulated by translation (1.6.3.2), e.g. a change of polysomal mRNA levels independent of total mRNA levels leading to altered protein levels. mRNAs regulated by abundance (1.6.3.1) are colored in green and largely lay on the diagonal indicating congruent changes on both: total and polysome-associated mRNA levels followed by changes on protein levels. mRNAs whose total mRNA level change while their polysome-associated mRNA levels remain constant are colored in blue and are identified as translationally buffered (1.6.3.3). Here, protein levels remain unchanged. (Lower) Depiction of mRNAs and their change in ribosome association in the respective regulatory mode. Arrows indicate the observed changes on total and polysome-associated mRNA levels.

Modified from Oertlin C. et al. "Generally applicable transcriptome-wide analysis of translation using anota2seq." *Nucleic Acids Res.* 2019 Jul 9;47(12):e70. doi: <https://doi.org/10.1093/nar/gkz223>

1.6.3.1 Regulation by mRNA abundance

Changes in mRNA abundance are characterized by the in- or decrease of total mRNA levels, due to either increased transcription or degradation of a certain mRNA. These changes are paralleled by the association of ribosomes, such that the observed protein output follows the same directionality as the altered total mRNA level. When assessing this using the anota-model, the changes of polysome-associated mRNA levels are not independent of the changes of total mRNA levels, e.g. the difference in translational efficiency (ΔTE , intercept on the y-axis) is close to 0.

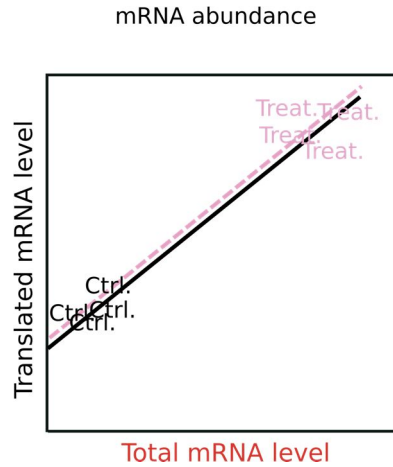


Figure 9 - Anota model of regulation by mRNA abundance: Translated mRNA levels vs. total mRNA levels are plotted for replicate per condition. A linear model with identical slope is fitted for each sample group. The intercept with the y-axis reflects the change in translational efficiency.

1.6.3.2 Regulation by translation

When total mRNA levels change to a smaller extent than levels of translated mRNA, transcripts are regulated by translation. Here, the distribution of mRNAs is shifted between heavy and light fractions of the polysome profile, such as the regulation of TOP-mRNAs (Gandin et al., 2016), inhibition of eIF4A (Chan et al., 2019) or selective translation during the integrated stress response (Guan et al., 2017; Kaspar et al., 2021). Using the anota-model, fitting of linear models results in a difference of the intercepts on the y-axis, indicating the difference in translational efficiency, (ΔTE). $\Delta TE \neq 0$.

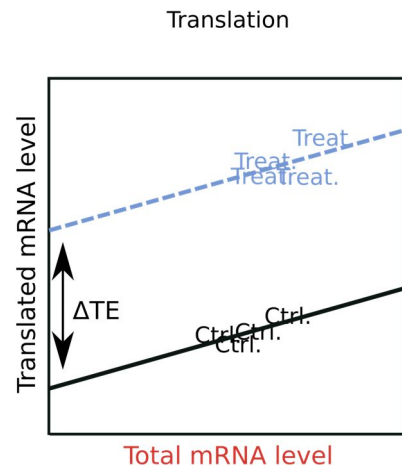


Figure 10 – Anota model of regulation by translation: Translated mRNA levels are plotted against total mRNA levels. The fitting of linear models with identical slopes reveals a difference in translational efficiency illustrated by different intercepts on the y-axis (ΔTE).

1.6.3.3 Regulation by (translational) buffering

Changes by mRNA abundance and translation are defined by changes of translated mRNA levels, either with concomitant (abundance) or independent (translation) changes of total mRNA. In contrast, translational buffering is defined by changes of total mRNA levels, while the polysome association remains unchanged. This can occur in two directions, since during buffering a decrease in total mRNA levels leads to an increase in polysome association, while increasing total mRNA levels are paralleled by decreased polysome association. In other words, the change in net-polysome association between the two conditions is near zero. When assessing this regulatory mode using the anota-model, total mRNA levels and translated mRNA levels are switched, and intercepts with the y-axis are assessed. This reveals if changes reflected on total mRNA are independent of changes in translated mRNA:

$$\text{total mRNA} \sim \text{translated mRNA} + \text{condition}$$

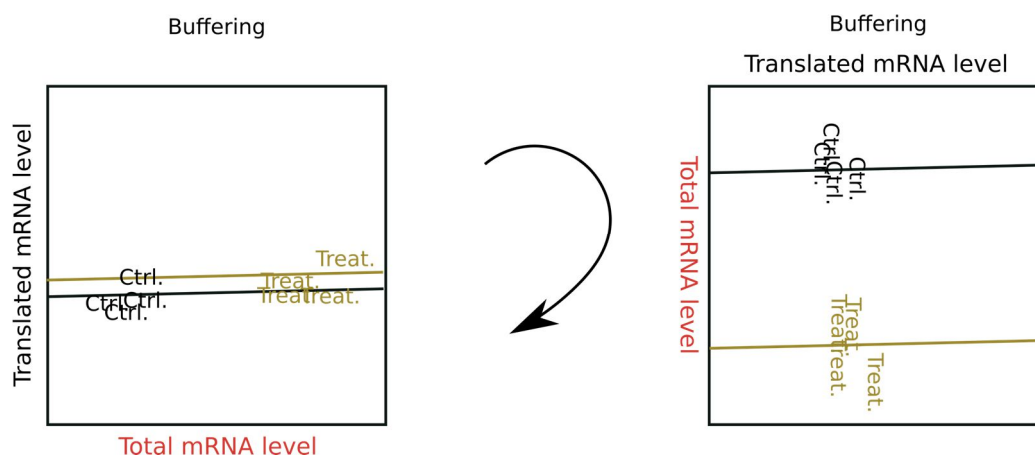


Figure 11 - Anota model for regulation by translational buffering: Translated mRNA levels are plotted against total mRNA levels. (Left) Fitting of similar slopes reveals no change of translated mRNA levels. To assess translational buffering, the axis are flipped (right) and the intercept with the y-axis reveals a change of total mRNA levels, which is independent of altered levels of polysome-associated mRNA leading to the identification of translational buffering.

Translational buffering is an intriguing mechanisms, since it entails that changes of total mRNA levels do not lead to altered protein levels. While analysis using anota2seq designates those mRNAs as buffered, this mode can be further defined in respect to biological contexts. Stimulations or perturbations can lead to changes on total mRNA levels, while translation is “offset”, leading to no changes of polysome association. This counter-balancing of changes of specific mRNAs stabilizing the corresponding protein level has been shown in response to growth factor stimulation in HeLa cells (Tebaldi et al., 2012) or upon TP53-loss in HCT116 cells (Liang et al., 2018). Neuronal stem cells upon loss of Fmr1, leading to the Fragile X syndrome, showed offsetting of synaptic proteins despite increased mRNA levels in combination with mitochondrial

mRNAs, whose mRNA level decreased (Liu et al., 2018). **Paper III** and **IV** will further decipher translational offsetting and its underlying mechanism. Transcriptional fluctuations between species or organisms belonging to the same family can be seen as compensation through buffering, such as differences in ploidy in *Drosophila* cells (Zhang and Presgraves, 2017) and between yeast strains (Artieri and Fraser, 2014b; McManus et al., 2014) lead to similar protein levels by translational-regulation. Similarly, transcriptional-variation in human tissue (Perl et al., 2017) and between individuals (Cenik et al., 2015) has been suggested to be mediated by translational-buffering. Finally, a study comparing a range of different species (human, macaque, mouse, opossum and platypus) revealed extensive translational regulation between species leading to less divergence of the translome and the proteome as compared to the species-dependent transcriptome (Wang et al., 2020). Examples in which components of cellular pathways or protein complexes are stoichiometrically buffered upon transcriptional fluctuations have been described in *Bacillus subtilis* and *Escherichia coli*, whose protein-levels have been shown to be diverging to a lesser extent than mRNA levels (Lalanne et al., 2018). This mechanism of buffering by equilibration has also been shown for pathways in eukaryotes, while functionally unrelated but genomically neighboring and co-expressed genes were buffered on the protein-level (Kustatscher et al., 2017). Finally, the identification of mechanisms leading to buffering require adequate analysis-tools (Oertlin et al., 2019) and can be observed using measures of mRNA and protein-levels, however not capturing the contribution of mRNA translation or protein-stability (Taggart et al., 2020).

1.6.4 nanoCAGE

As described in **1.3.2**, 5'UTRs and its features are involved in a wide-range of translational control mechanisms. The in- or exclusion of such features is mediated by the usage of transcription-start sites and subsequent capping (**1.1.5**). In order to understand the impact of 5'UTRs, methods to identify and measure these sequences are required. Among those are a 5'-specific version of serial analysis of gene expression (5'-SAGE) (Hashimoto et al., 2004), a cap-specific and PCR-free single molecule sequencing (HeliscopeCAGE) (Kanamori-Katayama et al., 2011), and Cap analysis of Gene Expression (CAGE) (Shiraki et al., 2003; Takahashi et al., 2012). CAGE was developed to identify promoters on a genome-wide level (Shiraki et al., 2003) and, due to the lack of current RNA-sequencing methods, it relied on Sanger-sequencing (Shiraki et al., 2003). A central feature of these methods is the exact mapping of the 5'cap of the mRNA, which in turn allows to map the position at which transcription starts with nucleotide precision – the transcription start-site (TSS). During CAGE, this is achieved by cap-trapping, which involves biotinylation of the 5'cap-structure followed by a pull-down with streptavidin coated beads (Carninci and Hayashizaki, 1999; Carninci et al., 1996). This, in combination with polyA-selection by oligo-dT priming, leads to complete cDNAs, which are subjected to sequencing (Shiraki et al., 2003). Moreover, the performance of

reverse transcription was improved by adding Trehalose and Sorbitol to the reaction. This led to higher thermostability of the reverse transcriptase and allowed higher reaction temperatures to resolve structures in the mRNA (Carninci et al., 1998, 2002). In order to capture transcripts lacking polyA-tails and ensure that the 5'cap was reached, random priming during reverse-transcription was introduced (Kodzius et al., 2006). For Sanger-sequencing, CAGE-tags were concatenated by molecular cloning and bacterial clones were sequenced (Kodzius et al., 2006). Later, this method was adapted for pyro-sequencing for improved throughput (Valen et al., 2009). In line with the advancement of sequencing-technologies, CAGE was subsequently adapted for next-generation sequencing on the Illumina platform (Takahashi et al., 2012). CAGE, using the cap-trapper and restriction-digestion, requires high amounts of RNA-input (5 µg (Takahashi et al., 2012), 25 µg (Shiraki et al., 2003)), which restricted this application to large amounts of material. Therefore, nanoCAGE, using only nanograms of RNA, was developed (Plessy et al., 2010). This method relies on template-switching to capture the 5'cap of transcripts. Upon reaching the 5'cap, reverse transcription introduces a stretch of cytosines at the 3'end of the nascent cDNA strand (Hirzmann et al., 1993). Next, a template-switching oligo (TSO) containing three guanine ribo-nucleotides (ribo-G) at its 3' end hybridizes with the C-stretch. This leads to an extension of the cDNA with the TSO (Matz et al., 1999; Zhu et al., 2001) and to template-switching between the RNA template and the TSO while eliminating the requirements of adaptor ligations and allowing the introduction of random sequences (unique-molecular identifiers (UMIs) and barcodes) (Kivioja et al., 2012). However, this strategy can lead to artifacts, which occur from hybridization between the TSO and the cDNA strand due to complementarity of UMI and/or barcode sequences with motifs in the nascent strand (Tang et al., 2013). These shorter artifacts, generated by “strand invasion”, depend highly on the last six nucleotides of the TSO and can be identified during downstream computational analysis of the sequencing data (Tang et al., 2013). Experimentally, strand invasion can be avoided by adding a generic spacer between the UMI upstream of the ribo-G at the 3'end of the TSO (Tang et al., 2013). The generated cDNA is further synthesized into a double-strand by semi-suppressive PCR and adapters are ligated followed by sequencing (Plessy et al., 2010). The semi-suppressive PCR uses partial-complementary primers. This excludes short fragments and fragments with the same adapters (Plessy et al., 2010). In this thesis applied, and latest iteration of nanoCAGE uses a transposase-based method to introduce sequencing adapters. Here, tagmentation leads to sequencing libraries compatible with the Illumina platform ((Poulain et al., 2017) and **paper II, IV**). A key-feature in today's nanoCAGE is the usage of barcodes and finger prints (UMIs). Barcodes allow for multiplexing samples for sequencing, whereas finger prints are used to filter PCR artifacts (Poulain et al., 2017). Sequencing is performed using a specific sequencing primer, which binds directly upstream of the barcode-sequence allowing precise mapping of TSSs (Poulain et al., 2017; Salimullah et al., 2011).

The application of CAGE by the Fantom consortium led to the first genome-wide identifications of promoters and transcription start sites (Carninci et al., 2006; Forrest et al., 2014; Kawai et al., 2001) and showed that transcripts contain several transcription start-sites, i.e., a range of mRNA isoforms (**Figure 13**, (Forrest et al., 2014)). This data is characterized by tags aligning to the reference genome, which generates clusters containing peaks of TSS and allows to identify new mRNA isoforms as well as new mRNAs (Carninci et al., 2006; Forrest et al., 2014). Moreover, the number of tags per transcript can be inferred as a measure of expression of transcripts (Kawaji et al., 2014). Due to its cap-specificity other transcripts containing 5'caps have been identified by CAGE, such as lncRNAs (Hon et al., 2017) and enhancer RNA (eRNAs) (Andersson et al., 2014; Kim et al., 2010). Methods to analyze this data have been developed, such as “TSRchitect” (Raborn and Brendel, 2019), “CAGEr” (Haberle et al., 2015) and CAGEfightR (Thodberg et al., 2019).

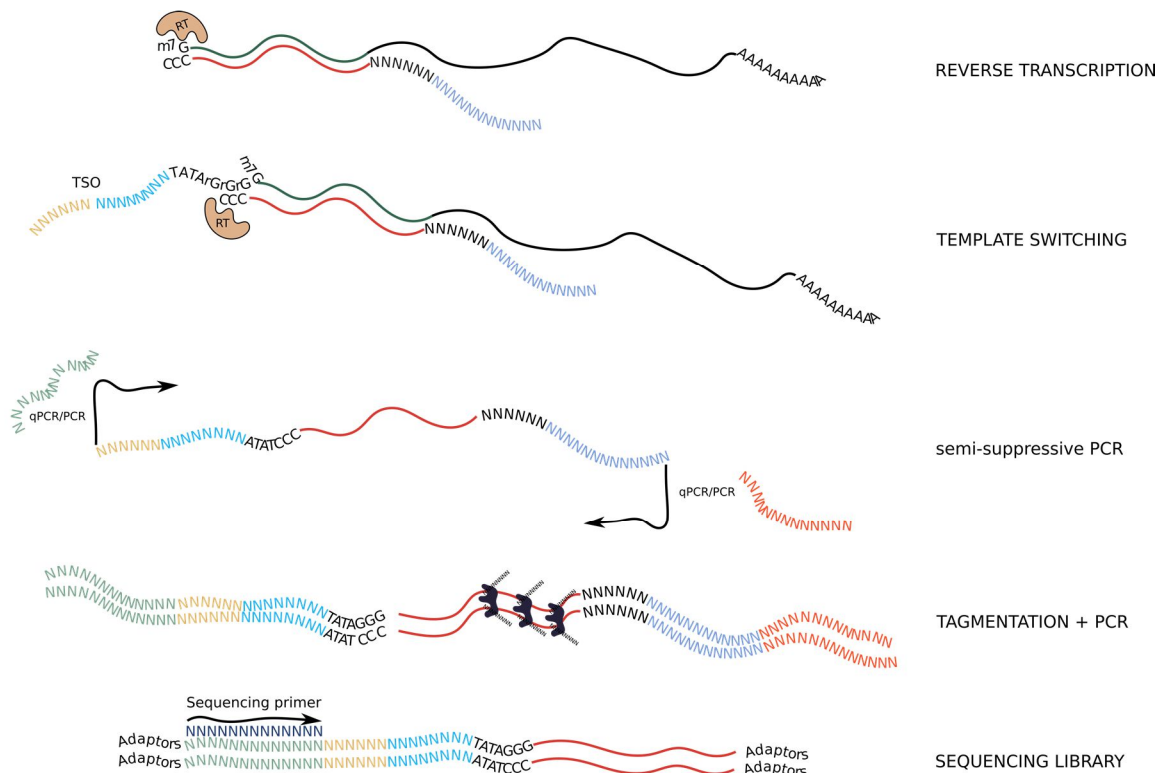


Figure 12 - Preparation of nanoCAGE sequencing libraries: *Reverse transcription* is performed using a random hexamer primer (black N), including a tail for semi-suppressive PCR (blue N), to amplify mRNAs including their 5'UTR (dark green line). When the cap-structure is reached, *template switching* occurs. The template switching oligo (TSO) containing three ribo-Gs at the 3'end binds to the C-overhang and the reaction extends the cDNA strand (red line) incorporating the TSO. For early multiplexing of samples, the TSO contains a barcode sequence (yellow N) and unique molecular identifiers (UMIs, finger prints) to remove PCR artifacts in the downstream analysis (light blue N). A diagnostic *qPCR* is performed to evaluate the efficiency of the reverse-transcription, followed by *semi-suppressive* PCR (green and red N) to generate a DNA double strand. Next, *tagmentation* leads to the introduction of adapters for RNA-sequencing. Sequencing is performed using a primer (dark blue N) directly upstream of the 5' barcode leading to mapping of the 5'cap/ TSS with nucleotide precision.

It seems a rather obvious choice to apply these methods in the context of mRNA translation, since 5'UTR features play an important role and are determined by TSS (1.3.2). Transcription start-sites mapped by CAGE exist as wide or narrow, mediated by either CpG island or TATA boxes

(Carninci et al., 2006). CpG islands containing promoters are rich in Cytosine and Guanine and give rise to broad TTS (Deaton and Bird, 2011), whereas TATA rich core promoters lead to sharp transcription-initiation (Ponjavic et al., 2006) and sharp CAGE clusters. However, this distinction has been challenged and promoters containing both elements have been identified (Ponjavic et al., 2006). In regards to mRNA translation, precise TTS-selection is necessary. One example are TOP mRNAs, which have been shown to be transcribed from precise positions (Eliseeva et al., 2013; Gandin et al., 2016; Parry et al., 2010; Perry, 2005; Yamashita et al., 2008) due to their requirement of distinct sequences at the very 5' end of the transcript for their regulation (1.3.2.5). Similarly to uORFs, whose presence in the 5'UTR can dependent on TSS-selection (paper II). 5'UTR isoforms have been demonstrated in breast-cancer, where the stemness factors NANOG, SNAIL and NODAL are transcribed with alternative 5'UTRs (Jewer et al., 2020). Each expresses a 5'UTR variant which is preferentially translated in a eIF2 α -dependent manner under hypoxia leading to a stem-like phenotype in breast-cancer (Jewer et al., 2020). This shows that apart from the identification of 5'UTR variants their quantification is important. Generally, transcriptome-wide studies rely on adequate annotations. In the case for 5'UTRs the longest identified variants are usually annotated in databases such as RefSeq or UTRdb (Gandin et al., 2016). However the identification of TSSs in MCF7 cells using nanoCAGE revealed that around 30% of annotated TSSs give rise to shorter 5'UTRs (Gandin et al., 2016), illustrating the importance of the systematical identification of 5'UTRs. The contribution of 5'UTRs to translation initiation on a genome-wide level has been studied to some extent by using CAGE (Li et al., 2019; Wang et al., 2016) and other methods in combination with polysome profiling such as TrIP-seq (Floor and Doudna, 2016) or CapSeq

(Tamarkin-Ben-Harush et al., 2017). In order to systematically assess the contributions of sequence contexts in a high-throughput manner, reporter-based methods have been used. These use constructs largely containing libraries of random 5'UTRs upstream of reporter start-codons, whose protein expression is either measured by fluorescence (Dvir et al., 2013), growth rate (Cuperus et al., 2017) or polysome

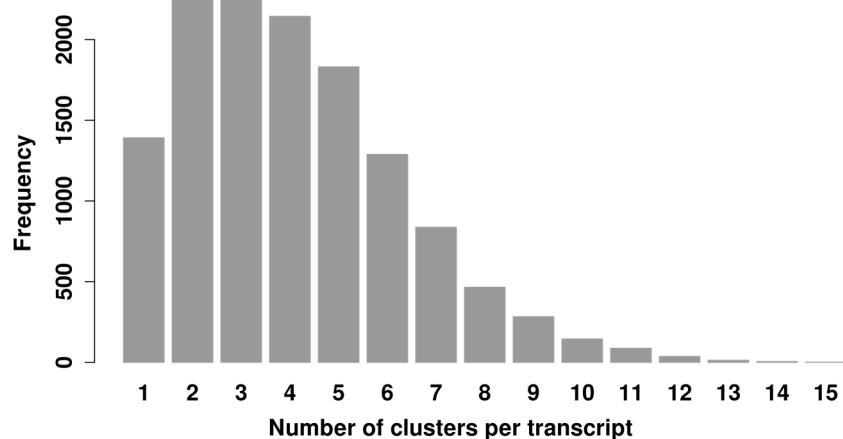


Figure 13 - Histogram of TSS clusters per transcript in T47d breast cancer cells generated by transcriptome-wide nanoCAGE. A total number of 53597 clusters were identified, while 89.39% transcripts contained more than one TSS. In average 4.09 TSS per transcript were found. This Figure was kindly provided by Krzysztof Szekop.

profiling (Sample et al., 2019). These methods in combination with network analysis helped to understand the contribution of 5'UTR sequence composition to mRNA translation, however rely only to a small extend on actual expressed 5'UTR variants and do not address commonly studied 5'UTR features regulating translation initiation (**1.3.2 and Figure 5**). In **paper II** we apply nanoCAGE to identify 5'UTRs derived from narrow TSS selection and use a newly developed approach called targeted-nanoCAGE (tgNC). This method uses a mammalian expression vector and cloning of previously identified 5'UTR variants up to 182nt upstream of a luciferase open-reading frame. Luciferase-specific nanoCAGE sequencing in combination with polysome profiling allows to study the impact of 5'UTR features independent of other mRNA features such as coding region or 3'UTRs. In summary, 5'UTR specific sequencing methods in combination with polysome profiling can be a powerful tool to study the influence of 5'UTRs on mRNA translation.

1.7 P53

P53 (TP53 gene) is one of the most studied genes and proteins in cancer-research. Its discovery as an oncogene (Lane and Crawford, 1979; Linzer and Levine, 1979), followed by its characterization as a tumor-suppressor (Finlay et al., 1989), has led to a huge body of research and enabled a better understanding of cancer on a molecular level. Mice lacking TP53 are characterized by spontaneous tumor formation after 6 months (Donehower et al., 1992), in contrast to a mouse model constitutively expressing a N-terminal truncated P53 isoform showing growth defects and early onset of ageing (Maier et al., 2004). P53 is a transcription factor (Farmer et al., 1992) binding to a 10 base-pair DNA-motif in the genome (El-Deiry et al., 1992; Kern et al., 1991), regulating a plethora of cellular pathways (Vogelstein et al., 2000) and as the “guardian of the genome” counteracts genome-instability (Lane, 1992) a hallmark of cancer (Hanahan and Weinberg, 2000,

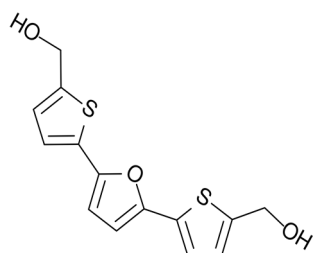


Figure 14 - Reactivation of P53 and Induction of tumor cell apoptosis (RITA). Molecular structure of the small molecule used in **paper I**.

2011). This is achieved by activation of p53 upon extracellular and intracellular stresses such as hyper proliferative signals, oxidative stress, DNA damage, hypoxia and nutrient depletion (Bieganski et al., 2014). As a result, p53 induces DNA-repair (Tang et al., 1999), cell cycle arrest (Martinez et al., 1991), and apoptosis transcriptionally (Symonds et al., 1994; Yin et al., 1997). This illustrates p53's ability to suppress tumorigenesis and leads to the selection of cells with non-functional p53 during cancer progression (Lutzker and Levine, 1996). TP53 is mutated in over 40% of cancers (Hollstein et al.,

1991), mainly at its DNA-binding domain leading to non-functional p53 due to impaired DNA binding, protein folding and altered protein-protein interactions (Cho et al., 1994; Kasthuber and Lowe, 2017; Muller and Vousden, 2013; Pavletich et al., 1993). Moreover, truncated p53

isoforms have been shown to be implicated in cancer due to alternative splicing, alternative promotor usage or alternative translation initiation (Aoubala et al., 2011; Bourdon, 2007; Candeias et al., 2016; Khoury and Bourdon, 2011). p53 is destabilized by binding to MDM2 resulting in proteasomal degradation and decreased protein levels (Kubbutat et al., 1997). Upon DNA damage or replication stress, p53 levels are increased by either phosphorylation leading to reduced MDM2 binding (Shieh et al., 1997) or MDM2 deactivation by ARF (Pomerantz et al., 1998). However under malignancy, these mechanisms are dysregulated by overexpression of MDM2 resulting in decreased p53 levels despite its activation (Fakharzadeh et al., 1991; Finlay, 1993). p53 acts as tetramer, and expression of p53-wt and p53-mut in TP53-wt and TP53-mut heterozygous tumors results in hetero dimerization and abolishes p53-wt activity by a dominant-negative mechanism (Willis et al., 2004). These mechanisms result in decreased p53-wt activity in cancer and gave rise to the development of strategies reinstate p53-wt. Conceptually, p53-mut is to gain p53-wt activity and p53-wt levels are to be elevated (Binayke et al., 2019; Wiman, 2010; Zawacka-Pankau and Selivanova, 2015). High-throughput screening of small molecule libraries have succeeded in finding compounds, which lead to p53 mediated cell death in cancer cells. One such compound is RITA (Reactivation of p53 and induction of tumor cell apoptosis), initially identified as binding directly to p53-wt leading to impaired binding of MDM2 and MDMX and restoring p53 protein levels (Enge et al., 2009; Issaeva et al., 2004). RITA was initially thought to act in a p53-dependent manner, however additional studies revealed substantial p53-independent effects ((Surget et al., 2014; Wanzel et al., 2015; Weilbacher et al., 2014) and **paper I**). RITA has also been shown to inhibit p53 degradation by inhibiting E6 ligase mediated ubiquitylation in cervical carcinoma (Zhao et al., 2010a) and reactivating p53-mut in a range of cancer cells (Zhao et al., 2010b). Thioredoxin reductase 1 (Trx1) was found to be non-covalently bound by RITA leading to p53-dependent cell-death in cancer cells (Hedström et al., 2009). This was challenged by a more recent study using thermal protein profiling (TPP), to identify RITA's target in a proteome-wide manner. This revealed that RITA treatment is mainly associated with impaired transcription and mRNA processing (Peuget et al., 2020). Taken together, the mechanisms by which RITA potently leads to cell death in cancer cells still remain to be fully understood. **Paper I** studies RITA's impact on mRNA translation in combination to its antineoplastic effects. Other compounds such as nutlins, which bind directly to MDM2 and increase p53-wt levels (Vassilev et al., 2004) are used in clinical trials (NCT00623870, NCT00559533) and have been shown to act in a largely TP53-dependent manner (Tovar et al., 2006; Vassilev et al., 2004). Compounds such as Prima-1/Prima-1^{MET} (p53 reactivation and induction of massive apoptosis) reactivate p53-mut by leading to an active conformation of p53 and promote classical p53 downstream effects upon DNA binding such as apoptosis (Bykov et al., 2002). Mechanistically, PRIMA-1 binds covalently to cysteine residues in p53 upon conversion to a methylene quinuclidinone, a Michael acceptor (Lambert et al., 2009).

This leads not only to reactivation of p53-mut but also to increased ROS levels due to thiol-binding of Prima-1 to Glutathione (Ceder et al., 2021; Tessoulin et al., 2014) and Trx1 (Peng et al., 2013). PRIMA-1 is to date the only compound reactivating p53-mut with positive results in clinical trials (Nahi et al., 2006; Rao et al., 2013).

Empirical in the narrower sense is the knowledge that stops at effects without being able to arrive at the causes. For practical purposes it often suffices, as for example in therapeutics.

The nonsense of the natural philosophers of Schelling's school on the one hand, and the results of empiricism on the other, have provoked in many such a dread of system and theory that they expect progress in physics entirely by hand without the aid of the head and would, therefore, like best of all simply to experiment without giving any thought to the matter. They imagine that their physical or chemical apparatus should do their thinking for them and itself should express the truth in the language of mere experiments. For this purpose, experiments are now multiplied ad infinitum and again in these conditions, so that operations are carried on solely with extremely complicated and in the end utterly absurd, experiments, namely with such as can never furnish a simple and straightforward result. Nevertheless, they are to act as thumb-screws applied to nature in order to force her even to speak.

The genuine research worker, on the other hand, who thinks for himself, arranges for his experiments to be as simple as possible so that he may plainly hear nature's clear statement and judge accordingly. For nature appears always only as witness.

*From "On philosophy and natural science", § 76 in Parerga and Paralipomena II, (Oxford University Press, 1974)
Arthur Schopenhauer (1788 – 1860)
Translated by E. F. J. Payne*

2 AIMS OF THIS THESIS

The here presented thesis work aims to characterize regulatory modes of mRNA translation and to develop new methods to study the contribution of mRNA elements to protein synthesis.

In **paper I** we study the mechanism of RITA, a small molecule reactivating p53, and its effect on mRNA translation.

5'UTR specific mRNA sequencing is applied in **paper II** to identify mRNAs with narrow transcription start sites (TSS). We use a newly developed approach, 5'-centric targeted TSS sequencing in combination with polysome profiling to contextualize these findings.

Paper III presents a computational approach to study mRNA features in concert. We use this method to characterize translation and translational offsetting.

In **paper IV** we investigate the role of the well-studied transcription factor ER α in the regulation of gene expression at the level of mRNA-translation in cancer.

3 RESULTS AND DISCUSSION

3.1.1 Study I - RITA requires eIF2 α -dependent modulation of mRNA translation for its anti-cancer activity

P53 is a key tumor suppressor, and its loss of function is a common phenotype across many different cancer types (1.7). Therefore, the reactivation of p53 by small molecules has been shown to be a promising strategy for cancer treatment (1.7.1). Despite many small molecules being initially discovered by their ability to reactivate p53, they have been shown to exert their antineoplastic activities independent of TP53-status (Wanzel et al., 2015) and target a multitude of cellular pathways such as MEK (Lu et al., 2016), the balance of glutathione and reactive oxygen species (Tessoulin et al., 2014), JNK/SAPK and p38 (Weilbacher et al., 2014). RITA, a small molecule initially identified as inhibiting the MDM2-mediated proteasomal degradation of p53, (Issaeva et al., 2004) has been shown to act independently of TP53 status (Wanzel et al., 2015; Weilbacher et al., 2014). Moreover a study using NMR-spectroscopy revealed that binding between p53 and MDM2 was not abolished by RITA *in vitro* (Krajewski et al., 2005). It was therefore hypothesized that RITA and other small molecules reactivate p53 as a secondary effect or in cooperation with other mechanisms.

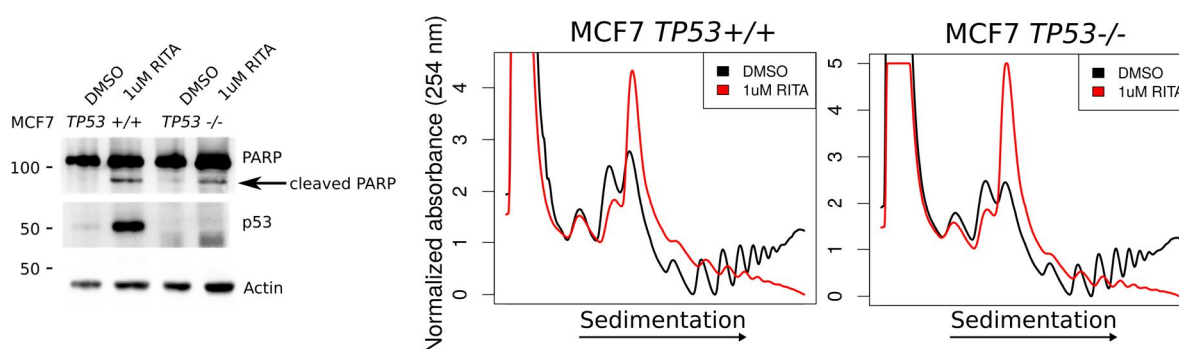


Figure 15 - (left) Western blot analysis of MCF7 TP53 +/+ and -/- cells illustrating pro-apoptotic effects of RITA by PARP-cleavage, which are independent of TP53 status. (right) Polysome profiling of MCF7 cells reveals TP53 independent reduction of global protein synthesis upon RITA treatment, as judged by the reduced area under the curve of polysomal mRNA and the increased 80S monosome peak.

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This was previously shown for compounds such as Nutlin-3 inducing the DNA damage response (Valentine et al., 2011), Prima-1 activating the unfolded protein response (Teoh et al., 2016) and Prima-1^{Met} leading to increased reactive oxygen species (ROS) paralleled by p53-reactivation (Tessoulin et al., 2014). We treated MCF7 cells harboring p53-wt with RITA, leading to induction

of apoptosis and monitored its effect on mRNA translation using polysome profiling, which led to a global reduction of translation. This effect was independent of TP53 status as S35 labeling of newly synthesized proteins and polysome profiling in TP53 $-/-$ and $+/+$ MCF7 cells showed a comparable results paralleled by PARP cleavage as measure of apoptosis (**Figure 15**). Since RITA leads to elevated ROS (Shi et al., 2014) we hypothesized that RITA-induced translational inhibition was ROS-dependent. While monitoring global protein synthesis under RITA treatment in combination with an anti-oxidant N-Acetyl Cysteine (NAC), which decreased ROS-levels, translational repression and induction of apoptosis could not be released. Next, we investigated classical pathways regulating mRNA translation, such as the mTOR pathway. The activity of downstream targets of mTOR, S6K and 4E-BP1, were not altered upon treatment with RITA. Polysome profiling using MCF7 cells lacking 4E-BP1 exhibited the same translational repression as the WT cells. This concluded that RITA inhibits translation independent of the mTOR pathway.

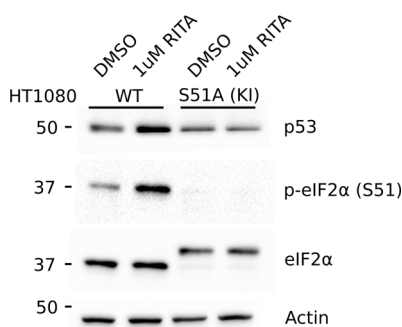


Figure 16 - Western blot analysis of HT1080 cells under RITA treatment. The induction of p53 depends on the phosphorylation of eIF2 α . HT1080 KI cells contain a non-phosphorylatable mutant at S51.

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Another major cue in regulating protein synthesis is the formation of the ternary complex. We assessed this by monitoring phosphorylation of eIF2 α and observed a time-dependent increase in eIF2 α phosphorylation in MCF7 cells.

This was also true for the colon cancer cells HCT116 and GP5d both harboring p53-wt. Treatment with the integrated stress response inhibitor (ISRIB), which restores eIF2B

activity followed by increased protein synthesis, in combination with RITA alleviated the translational repression underlining RITA's effect via ternary complex formation. Since the phosphorylation of eIF2 α is mediated by different kinases (**1.3.1.2**) such as PERK, we treated MCF7 WT cells with RITA in combination with the PERK inhibitor GSK2606414. This led to decreased phosphorylation of eIF2 α and reduced translational repression. Intriguingly, using MCF7 WT and GP5d cells,

induction of apoptosis and p53 protein levels were also reduced upon PERK inhibition. Additionally, long term cell growth under RITA treatment in MCF7 TP53 $-/-$, $+/+$ and WT cells was diminished upon RITA treatment, whereas RITA in combination with GSK2606414 did not lead to a noticeable effect. This was not mediated by ROS since concomitant PERK inhibition led to rather higher ROS-levels compared to the control. Since these experiments revealed an interplay between pro-apoptotic effects of RITA and phosphorylation status of eIF2 α , we wondered if modulation of eIF2 α -phosphorylation results in altered pro-apoptotic effects by RITA. Salubrinal, a phosphatase inhibitor of eIF2 α , led to increased eIF2 α phosphorylation in combination with

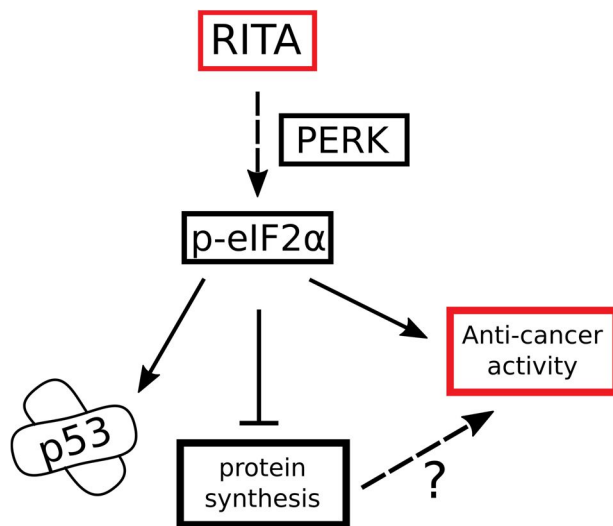


Figure 17 - The model describing the RITA-induced activation of p53, its anti-cancer activity and reduction of protein synthesis downstream of eIF2 α , which is mediated by PERK activity.

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RITA. When assessing early and late apoptosis by staining with Annexin V and Propidium iodide, eIF2 α -phosphorylation associated with the pro-apoptotic effects of RITA. This relationship was further evaluated using colony formation as a measure of clonogenicity in MCF7 cells. Here RITA treatment led to reduced colony formation, which followed the modulation of eIF2 α phosphorylation, i.e. lower colony formation eIF2 α -phosphorylation is high. These results underlined the TP53 independent effect of RITA, since colony formation was regulated independently of TP53 status. Lastly, we applied RITA on HT1080 fibrosarcoma cells (**Figure 16**), harboring TP53-WT and

overexpress a non-phosphorylatable eIF2 α -mutant (S51A). This led to the expected reduced p53 levels in the absence of eIF2 α phosphorylation. Moreover, these cells exhibited a reduced vulnerability to long-term RITA treatment under a wide range of concentrations in the absence of p53 reactivation. This study illustrates how the reactivation of p53 and antineoplastic effects by RITA are downstream of eIF2 α (**Figure 17**). Increased PERK activity, in general a pro-survival response in context of the integrated stress response, exhibits pro-apoptotic effects upon RITA treatment. This underlines the importance of major cellular cues in the context of p53 reactivation and can be of great help to improve drug efficacy and stratify treatment. However, the exact mechanism how p53 levels are increased upon RITA treatment downstream of eIF2 α remain to be resolved. Potentially, MDM2, a negative regulator of p53, is translationally or transcriptionally down-regulated leading to increased p53 protein levels. A more recent study showed RNA pol-II degradation upon RITA treatment (Peuget et al., 2020), which could lead to decreased MDM2 mRNA levels, whereby a mechanism downstream of eIF2 α suggests a regulation by mRNA translation.

3.1.2 Study II – Precise transcription start site selection in mRNAs with upstream open reading frames tunes stress-independent translation

The features of 5'UTRs are involved in a wide range of translational regulation (**1.3.2**) and their characteristics are dependent on the usage of transcription start sites. While transcription start sites

are generally dependent on the genomic region of core-promoters, CpG islands and TATA boxes give rise to wide or respectively more narrow TSS (Carninci et al., 2006; Ponjavic et al., 2006). A

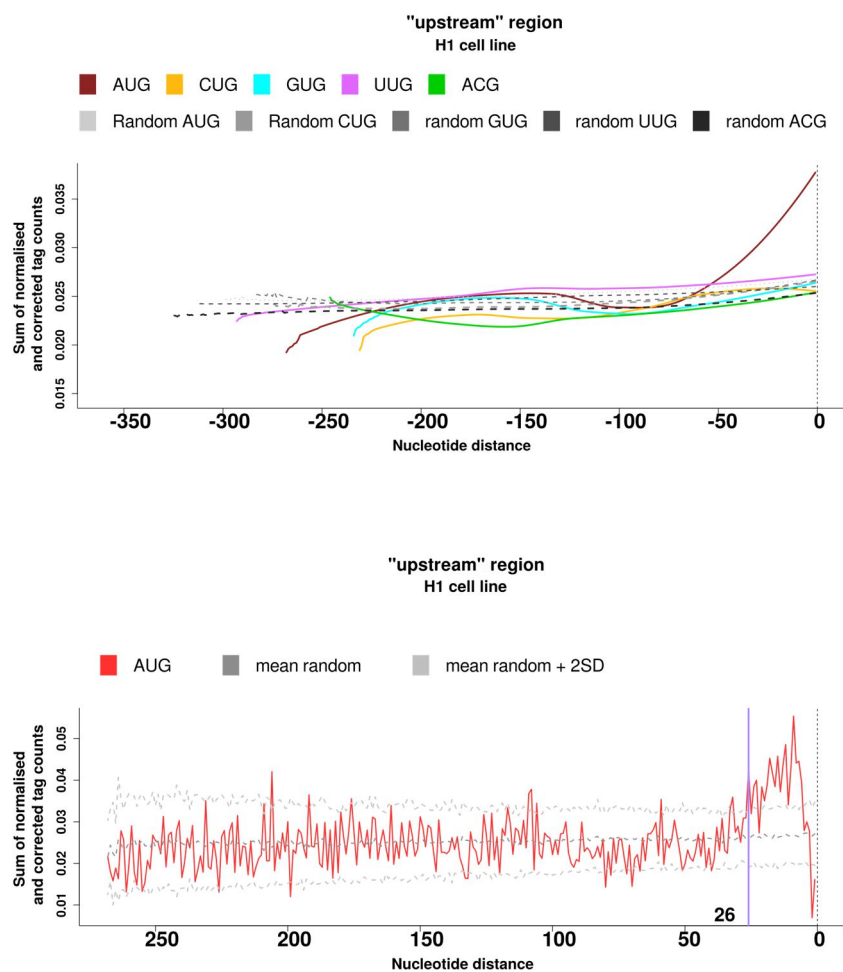


Figure 18 – nanoCAGE identified 5'UTR variants with narrow TSS upstream of uORFs: (Upper) Density plots of identified TSS in H1 cells upstream of uORFs characterized by the indicated start codons. We observed an enrichment of TSS in close proximity to the canonical AUG start codon (brown line). (Lower) Unsmoothed distribution of TSS upstream of AUG codons in H1 cells. The enrichment exceeded the background + 2SDs (grey dotted lines) at 26nts upstream of the start codon. This was reproducible across all studied cell lines.

containing a TOP motif. Instead, those transcripts contained a start codon in their 5'UTR, suggesting the presence of an upstream open-reading frame. Analysis of the surrounding sequences, the Kozak context, showed these AUG codons are surrounded of a strong context and therefore suggested those as translatable. These findings were true for a range of cells lines (stem cells, cancer cells and breast epithelia cells) underlining the generality of these findings. Next, we analyzed the regions upstream, downstream and within the identified uORFs. Firstly, the presence of canonical start-codons, i.e. AUG, was enriched upstream of the uORF in all cell lines, in comparison to randomly positioning of the respective codon along the 5'UTR as background (Figure 18). In contrast to non-canonical start codons, such as CUG, GUG, UUG and ACG. This

well characterized subset of mRNAs requiring precise TSS selection are TOP mRNAs (1.3.2.5). These are generated from precise TSSs (Gandin et al., 2016; Parry et al., 2010; Perry, 2005; Yamashita et al., 2008), which generates a canonical motif downstream of the 5'cap and is of importance for its translational regulation (Avni et al., 1994; Levy et al., 1991). We applied nanoCAGE to a range of cell lines and mapped transcription start sites in a

transcriptome wide manner to identify capped transcripts with narrow TSS. This led, as expected, to the identification of TOP mRNAs. Intriguingly, we found more mRNAs who exhibited sharp TSS not

enrichment was followed by a depletion within the uORF sequence and even in the region downstream of the uORF stop codon. These findings, reproducible across all cell lines, were further evaluated by plotting TSS enrichment in an un-smoothed fashion. Strikingly the “change-point” at which enrichment increased over the background occurred reproducibly

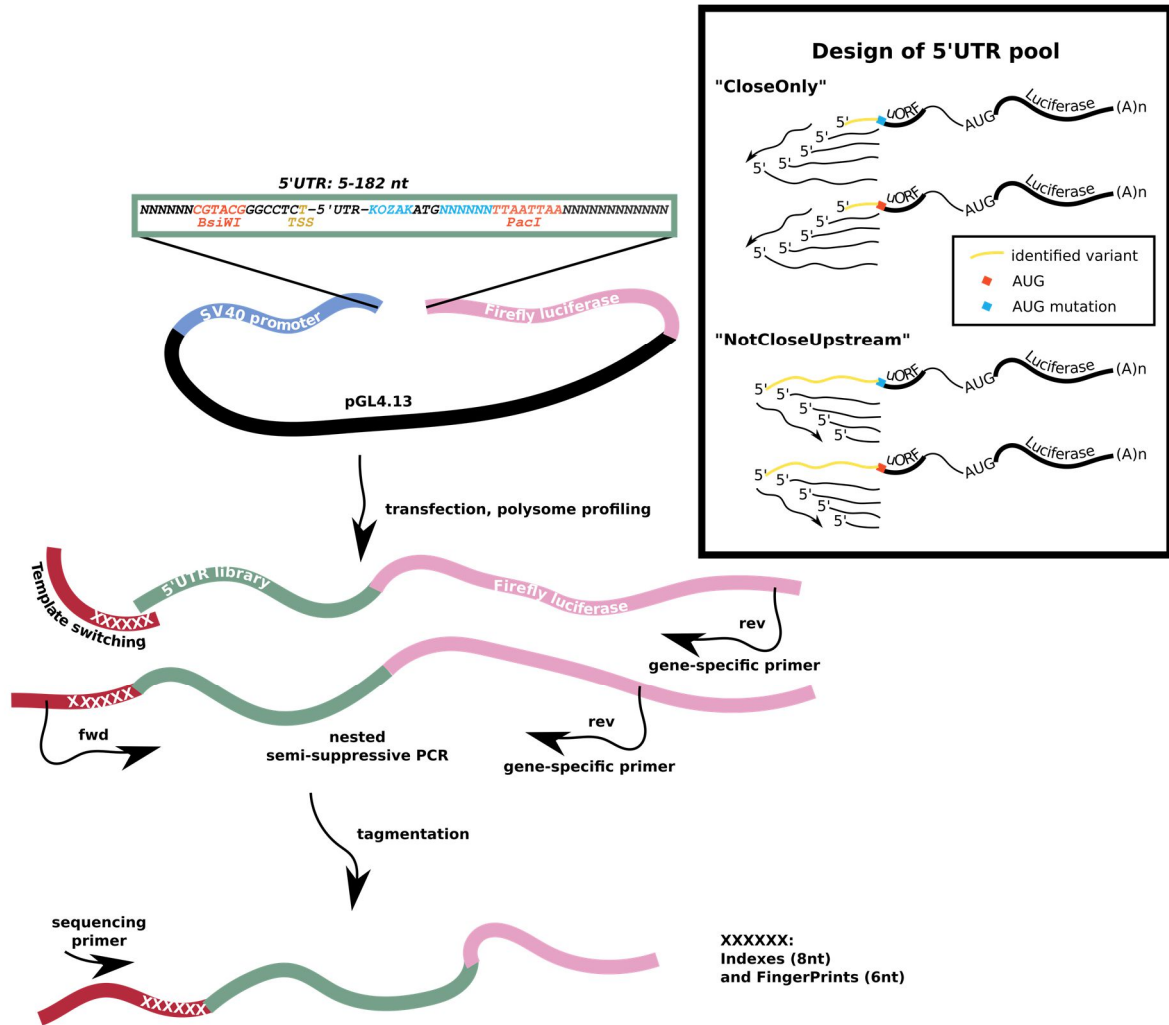


Figure 19 – targeted-nanoCAGE: A mammalian expression vector (based on pGL4.13) was modified such that a 5'UTR library could be cloned downstream of the SV40 promoter and upstream of a firefly luciferase open reading frame. The positioning of the PacI restriction site allowed the inclusion of the native Kozak context for each 5'UTR variant. After transfection into MCF7 cells and polysome profiling, targeted-nanoCAGE (tgNC) was applied to generate luciferase-specific sequencing libraries. tgNC uses reverse transcription followed by a nested PCR with gene-specific primers. At the 5'end template switching was performed as described in (1.6.4). Black box: The strategy to study identified 5'UTR subsets with “CloseOnly” and “NotCloseUpstream” TSS upstream of uORFs. The identified variant was stepwise extended (“CloseOnly”) or shortened (“NotCloseUpstream”) to assess the length dependency and the uORF start codon was mutated in order to study the dependency of the uORF.

between 25-27 nt upstream of the uORF start codon (Figure 18). While 5'UTR lengths are important in how mRNA translation is initiated (1.3.2), we hypothesized that this conserved distance plays a role in how these transcripts are translationally regulated. To assess this, we generated three subsets according to the nanoCAGE-derived distance between TSS and uORF: A

subset containing only transcripts with TSS close to the uORF (“closeOnly”) and a subset containing TSS far upstream of the uORF (“NotCloseUpstream”). The third subsets consists of transcripts with two different 5’UTR isoforms, close and upstream of the uORF (“CloseUpstream”). Moreover, these subsets showed distinct biological functions when conducting a Gene Ontology analysis. In order to study these subsets in context of mRNA translation, a new method was required.

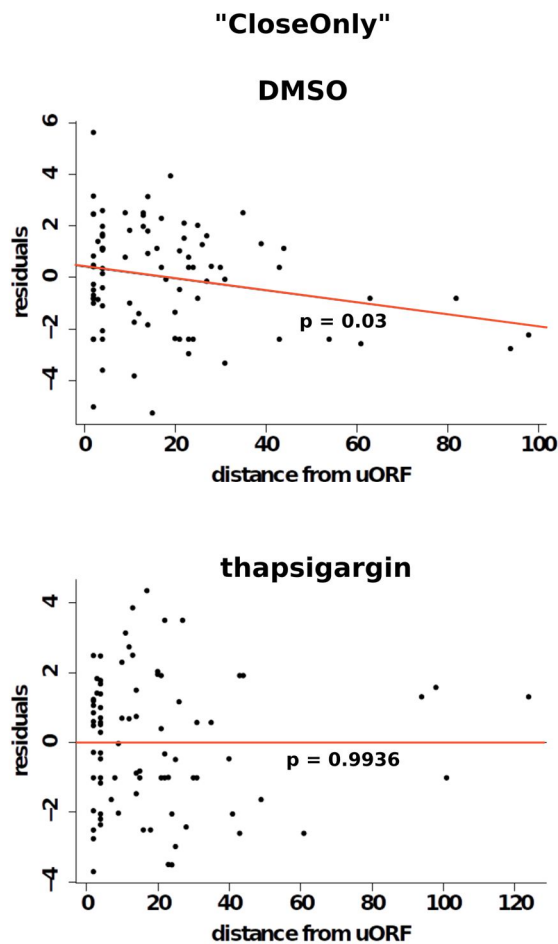


Figure 20– targeted-nanoCAGE revealed the stress independent translation of “CloseOnly” variants: Translational efficiency (residuals) was estimated using total and polysome-associated mRNA levels upon sequencing of targeted-nanoCAGE libraries under control (DMSO) and induction of the integrated stress response (1.3.1.2) by thapsigargin. Each dot represents a variant of specific length. Linear models assess the contribution of distance upstream of the uORF to the translational efficiency across all variants. Here, the positioning of the TSS close to the uORF (“CloseOnly”) ensures efficient translation under non-stressed and stressed conditions.

We developed a system, which allows to interrogate these 5’UTR variants independently of the mRNA features downstream of the 5’UTR (targeted-nanoCAGE) (Figure 19). To achieve this a mammalian expression vector was modified such that a collection of 5’UTR of different lengths was cloned directly downstream of its TSS. Moreover, the cloning strategy allowed to include the endogenous Kozak context for each transcript. The collection of variants was designed such that the initially identified variant was stepwise extended (“CloseOnly”) or stepwise shortened (“NotCloseUpstream”). Additionally, we included similar variants lacking a functional uORF start codon (AUG mutation to AGU) to assess the contribution of the uORF. After transfection of MCF7 the integrated stress response was induced by thapsigargin to assess stress-dependent translation (1.3.1.2, 1.3.2.4). Torin1 was used to inhibit mTOR and assess the mTOR-dependency of the 5’UTR variants. In order to understand the impact on protein output of the identified 5’UTR variants, we used polysome profiling to measure the

translational efficiency of the main-ORF (Firefly luciferase) by extracting total and polysome associated mRNAs. The nanoCAGE protocol used to generate 5’UTR-specific sequencing

libraries on a transcriptome wide level was modified to specifically sequence mRNAs containing the luciferase main ORF. This was achieved using two gene-specific primers and a nested semi-suppressive PCR. Linear models were used to analyze translational efficiencies as a function of distance between TSS and uORF, by class (“CloseOnly” or “NotCloseUpstream”) and treatments (thapsigargin, torin1). Short 5’UTRs (“CloseOnly”) revealed a significant relationship between length and translational efficiency (**Figure 20**), whereas the extension of 5’UTRs led to lower main-ORF translation. This effect was uORF dependent, since variants containing a non-translatable uORF (AUG mutation) showed no significant effect of altered 5’UTR length. These effects were independent of the mTOR-pathway as treatment with torin1 led to similar results. As discussed earlier in this thesis (**1.3.2.4**), uORFs play an important role in translational regulation under cellular stresses. Upon stress-induced phosphorylation of eIF2 α , ternary complex formation is decreased and uORF start codons are by-passed due to leaky-scanning. This ensures the translation of specific main-ORFs under stress conditions. In this study, treatment with thapsigargin for 1h led to similar translational efficiencies of short 5’UTRs in comparison to longer variants (**figure 20**). This indicates that the uORF-dependent translation of transcripts belonging to the “CloseOnly” subset are not susceptible to cellular stresses by positioning their TSS close to the uORF. In stark contrast to the “NotCloseOnly” subset, which exhibited no length-dependent and uORF-dependent change in translational efficiency under non-stressed conditions. While treatment with torin1 did not alter translational efficiency, treatment with thapsigargin led to a gain in translational efficiency when shortening the distance between TSS and uORF. Additionally, this relationship was dependent on initiation at uORF start-codons, since the AUG-mutation showed no significant length-dependency and indicates the requirement of longer 5’UTRs upstream of a uORF for appropriate translational regulation under stress. This suggests, besides uORF-dependent regulation, other elements between TSS and uORF are required for stress-induced translational repression.

This study identified a subset of uORF-containing mRNAs with short TSS-to-uORF distance due to precise TSS selection. Here, the short distance and the uORF are required for efficient translation under non-stressed conditions, while under stress main ORF translation is not impaired due to close positioning. uORF-containing mRNAs with longer upstream sequences are translated length-dependent under stress, while requiring a uORF and other unknown mRNA elements presenting a dramatic difference to mRNAs with close TSS and are devoid of length-dependent regulation under non-stressed conditions. These findings suggest two distinct strategies to design stress-resistant and stress-sensitive mRNA architecture certainly regulated by more detailed mechanisms. To this end, we assessed length dependencies, however the effect of the sequences upstream or downstream of the uORFs is to be investigated as well. This could be assessed by

exchanging 5'UTR regions, such as upstream regions, uORFs and their downstream sequences, between the identified subsets ("CloseOnly" and "NotCloseUpstream") in a systematic manner.

3.1.3 Study III – Anota2seqUtils uncovers widespread translational offsetting associating with 3'UTR features

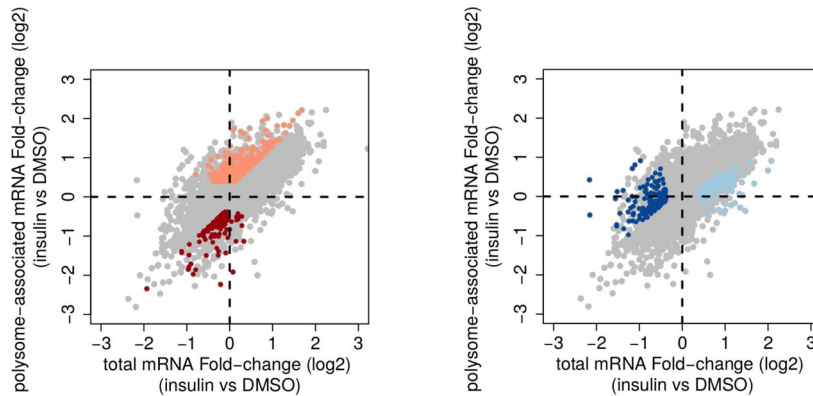


Figure 21– *Anota2Seq analysis (1.6.3) reveals changes by translation and translational offsetting in MCF7 cells after treatment with insulin for 4h upon 16h serum-starvation.*

mRNA translation is regulated by cellular pathways (Roux and Topisirovic, 2018) as well as by intrinsic features of mRNAs. These features encompass features in the 5'UTR such as TOP

motifs (Meyuhas and Kahan, 2015), GC content (Pelletier and Sonenberg, 1985) and

uORFs (Lu et al., 2004) as well as binding sites for miRNAs (Jonas and Izaurralde, 2015) and RBPs (Szostak and Gebauer, 2013) in the 3'UTR. Albeit these mRNA features being widely studied independently, methods to study these in concert are lacking. We developed anota2seqUtils, a computational approach, to analyze the contributions and interdependencies of mRNA features in translome data. This is achieved by applying linear models on regulated mRNA subsets and assesses the contribution of a certain element in a hierarchical manner. Herein, subsets derived from analysis by anota2seq (Oertlin et al., 2019) are used, i.e. translation and offsetting (a variant of translational buffering, 1.6.3.3, Figure 21). In order to test this computational approach we generated a dataset characterized by mTOR sensitive translation. We employed MCF7 cells and stimulated mTOR with insulin after starvation. mTOR inhibition was achieved by the active-site inhibitor torin1 (Thoreen et al., 2009). Next, we performed polysome profiling followed by RNA sequencing and analysis of the translome using anota2seq. We identified mRNAs regulated by translation, mRNA abundance, but intriguingly also translational offsetting. In order to assess mTOR dependency we compared the mRNAs of the identified subsets with their regulation under mTOR inhibition. mRNAs regulated by translation and abundance were largely mTOR dependent, moreover a sizeable number of mRNAs remained offset when mTOR was inhibited. Next, we sought to characterize the differences between mRNAs regulated by translation and offsetting, since offsetting remains an overlooked mode of regulation. Indeed, data generated by ribosome profiling in mouse embryonic fibroblasts (MEFs), polysome profiling upon torin1 treatment in neuroblastoma Be2 cells, and published data of MCF7 cells treated with the mTOR inhibitor

PP242 and analyzed by micro array (Larsson et al., 2012), showed ample translational offsetting. Since total mRNA levels in- or decrease and polysome association remains unchanged when mRNAs are offset, we validated this by using Hi-RIEF mass-spectrometry. This revealed that albeit total mRNA levels change, the resulting protein levels remain constant when translation is offset. Recently the insulin receptor has been shown to directly regulate transcription upon translocation to the nucleus (Hancock et al., 2019). We tested this by assessing the regulation of previously identified insulin-receptor sensitive mRNAs in our dataset. This showed that mRNAs regulated by the insulin receptor are largely regulated by mRNA abundance, indicating that translational offsetting upon insulin stimulation is independent of the transcriptional activity of the insulin receptor. Next, we used anota2seqUtils on mRNAs regulated by translation and offsetting. Since the 5'UTR plays a major role in mTOR dependent translation and different features, such as TOP-motifs, PRTE, 5'UTR GC content, 5'UTR length, and uORFs have been described as modulating translation (Avni et al., 1994; Hinnebusch et al., 2016; Hsieh et al., 2012; Pelletier and

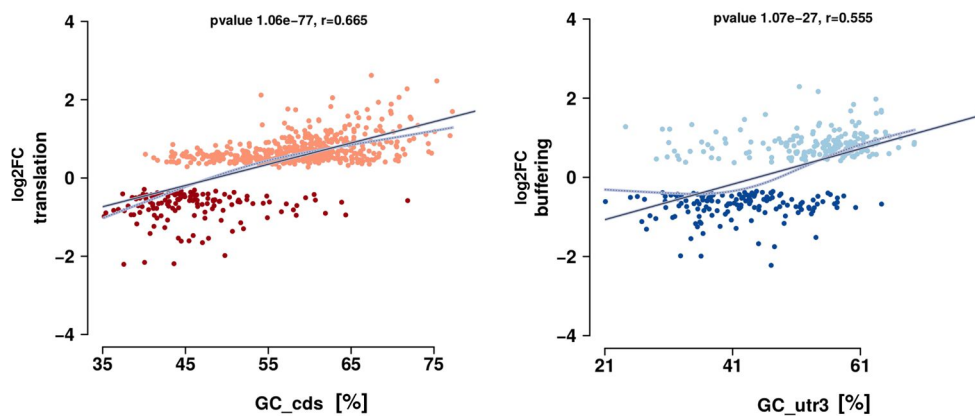


Figure 22 – Anota2seqUtils output of mRNAs regulated by translation, illustrating the relationship of GC content of the coding sequence with regulation by translation (light red: translation up, red: translation down) upon insulin stimulation (left). (Right) Similar analysis illustrating the relationship between GC content of the 3'UTR of mRNAs whose total mRNA level change upon insulin treatment but polysome-association remains constant, i.e. translational offsetting (light blue: increasing total mRNA levels, blue: decreasing total mRNA levels).

Sonenberg, 1985), we tested the contribution of those features. This showed that 5'GC content explained the biggest contribution in insulin dependent translation followed by TOP motifs and 5'UTR length, all associating with increased translational efficiency.

In the last step of anota2seqUtils the independent contribution of each mRNA feature is analyzed, which revealed that 5'GC content, the presence of a TOP motif, and 5'UTR length contribute independently. Similar assessment for mRNAs regulated by offsetting under insulin stimulation revealed that 5'GC content and 5'UTR length explained the biggest variance and contribute independently, while they associate with increased total mRNA levels, which entails reduced translational efficiency, i.e. opposite to insulin dependent translation. Since 5'UTR features

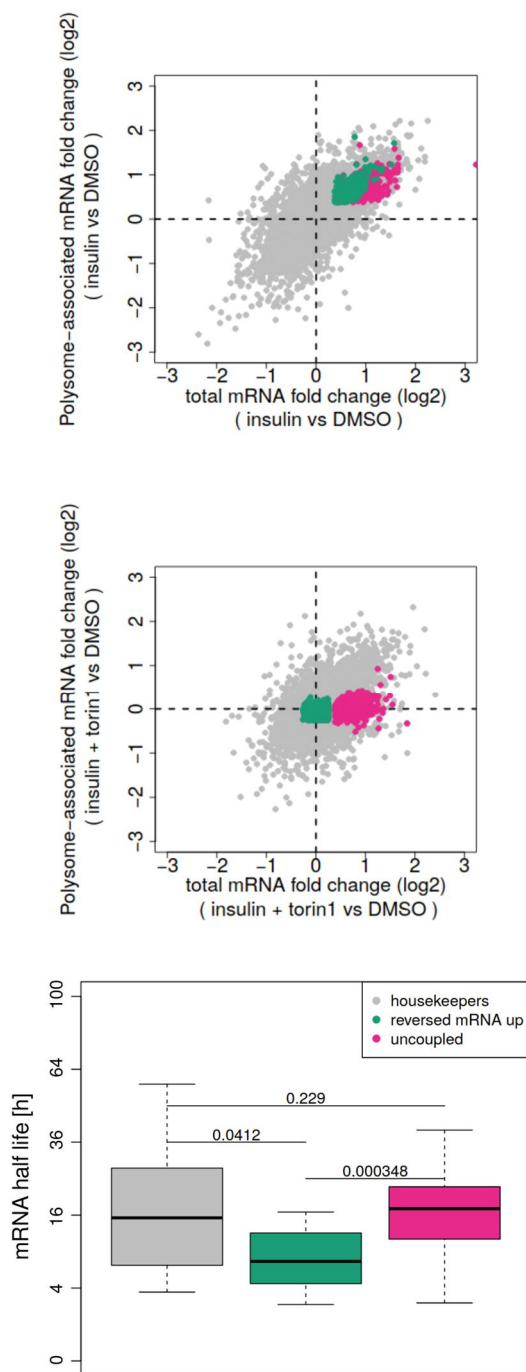


Figure 23 – (Upper) *Anota2seq* fold change plot (1.6.3) of MCF7 cells stimulated with insulin. The indicated subsets are (pink) mRNAs, which are translationally offset in comparison to mRNAs regulated by mRNA abundance (green). (Middle) similar subsets under stimulation with insulin and mTOR inhibition by torin1. The pink subset remains translationally offset and its total mRNA levels are “uncoupled” from mTOR activity, while reversed mRNAs are regulated in an mTOR-dependent manner. (Lower) NanoSTRING analysis after Actinomycin D treatment assessing mRNA half-lives. Translationally offset mRNAs, whose total mRNA levels are uncoupled from mTOR are more stable in comparison to mRNAs by mTOR inhibition.

explained around 20% of translation and offsetting respectively, we went further and combined features of 5' and 3'UTRs. Here, 3'GC content explained the biggest variance followed by 5'GC content, TOP motifs and 3'UTR length for mRNAs regulated by translation (40% of the total variance).

Similar analysis for translationally offset mRNAs showed significant and independent contributions for 3' and 5'GC content only, accounting for around 40% of the total variance as well. This indicated that translation and offsetting are regulated by features in the 5' and 3'UTR, which contribute independently. Next, we performed the analysis using a large panel of mRNA features encompassing untranslated regions as well as the coding sequence. Surprisingly, insulin-sensitive translation was mainly explained by GC content of the coding sequence and codons (**Figure 22**), which are enriched in translationally down regulated mRNAs. Previously identified features, 5' and 3'UTR GC content, were also identified and exhibited a strong covariance with features in the coding region, indicating potential structure formation between these sequences. Similar

analysis for offset mRNAs resulted in substantial differences, since the 3'GC content explained the largest variance. This lays in contrast to translational offsetting mediated by codon usage upon ER α depletion (**paper IV**). Since 3'GC content was found to play a major role in insulin induced translational offsetting upon *anota2seqUtils* analysis and 3'UTR features are related to mRNA degradation (1.3.3.1-2), we hypothesized that offset mRNAs

might be characterized by altered mRNA stability. Indeed, by using previously published datasets, we could show that offset mRNAs with increased GC content have higher mRNA half-lives in comparison to non-regulated mRNAs. This was further tested using nanoSTRING analysis upon Actinomycin D chase in MCF7 cells. Herein, we measured mRNA half-lives of two subsets: a subset, whose regulation on total mRNA level appeared to be mTOR independent (uncoupled) despite mTOR-dependent polysome association in contrast to a subset regulated completely by mTOR (reversed) (**Figure 23**). Albeit, mRNA half-lives were not regulated by mTOR activity, these two subsets showed substantial differences in mRNA stability, since mTOR-uncoupled mRNAs were found more stable in comparison to mTOR-reversed mRNAs and the control. This is in line with the previously identified lower proportion of A and U (AREs) in 3'UTRs of offset mRNAs, which are target for RBPs altering mRNA stability (**1.3.3.2**). Lastly, we analyzed published data of patient derived glioblastoma stem cells (GSC). This dataset consists of RNA-seq data generated by polysome profiling of GSCs of different glioblastoma subtypes, whose translome was analyzed upon differentiation. Anota2seq analysis revealed substantial offsetting with stark differences in 3'UTR GC content between the offset subsets (Offsetting mRNA up and down). Analysis of mRNA stability revealed increased half-life for offset mRNAs with higher 3'GC content versus the background, while mRNAs characterized by decreased total mRNA levels and 3'GC content being translationally offset revealed significant lower mRNA stability as well. In **paper IV** we identified tRNAs, which are modified at their U34 position for efficient decoding of codons of offset mRNAs when ER α is depleted (**paper IV**). While codon usage did not explain offsetting in mRNAs having increased half-lives, we tested if codon usage could play a role in translational offsetting in glioblastoma for offset mRNAs having lower 3'GC content. Indeed, in this glioblastoma model the latter showed an enrichment for U34-modified codons in comparison to mRNAs regulated by decreased abundance, suggesting an alternative mechanism for offsetting.

In aggregate, this study presents a novel approach to analyze the interplay of mRNA features in mRNA translation. Notwithstanding the need of experimental validation of the identified mRNA features, anota2seqUtils provides information about the complexity of regulation of mRNA translation and eliminates erroneous conclusions due to covariation between mRNA elements. We use this approach to decipher the mechanisms of translational offsetting, which is fundamentally different to translation, since protein levels do not change. We find that 3'UTR GC content and altered mRNA stability associates with offset mRNAs. Intriguingly, **Paper IV** describes offsetting, upon depletion of a transcription factor, dependent on features in the coding region, which associates with mTOR-sensitive translation in this study. This suggests that offsetting, despite being a general mode of translational regulation, associates with wide-spread mechanisms illustrating the importance of precise identification of mRNA elements in respective biological

settings. While this study confirms the role of previous identified mRNA elements such as 5'UTR features in mTOR-sensitive translation, it also identified features in the 3'UTR and codons challenging the classical view of 5'UTR-mediated translational regulation and indicates an interplay between these mRNA elements. Therefore, the application of anota2seqUtils in other biological contexts can shed light on the underlying mRNA elements contributing to regulation of mRNA translation and improves our understanding of post-transcriptional gene-expression.

3.1.4 Study IV – Translational offsetting as a mode of estrogen receptor α -dependent regulation of gene expression

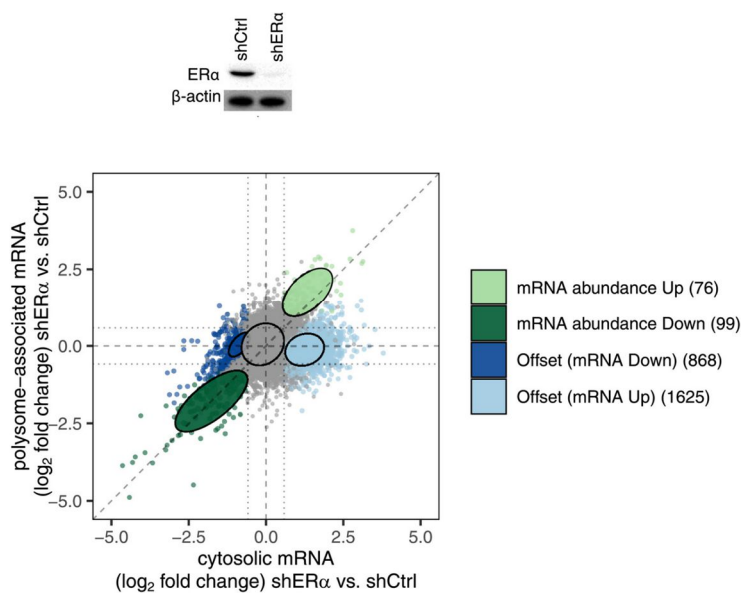


Figure 24– *Anota2seq analysis (1.6.3) upon shERα knock-down in BM67 cells followed by polysome profiling and RNA sequencing shows wide-spread translational offsetting upon loss of ERα.*

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Estrogen receptor α (ER α) is a transcription factor, which is associated with increased proliferation in prostate cancer (Takizawa et al., 2015). Generally, prostate cancer is treated by hormonal depletion to inhibit the androgen receptor (AR) (Mohler et al., 2021). However, there is increasing evidence, that prostate cancers are also driven by estrogen-mediated ER α signaling (Bosland et al., 1995; Ricke et al., 2006; Takizawa et al., 2015). Apart from its transcriptional activity upon DNA-

binding, ER α has been shown to be involved in signaling of the PI3K/AKT/mTOR-axis in prostate (Takizawa et al., 2015) and other tissues such as breast (Levin, 2009), which suggests a role in mRNA translation. In this study, we used the BM67 prostate cancer cell line lacking ER α upon shRNA mediated knock-down. Translatome data was obtained by polysome profiling followed by DNA micro-array analysis, which was further validated by RNA-sequencing (**Figure 24**). Anota2seq analysis revealed wide-spread changes of gene expression, of which most mRNAs were regulated by translational offsetting. Since translational offsetting leads to changes on total mRNA levels despite changes on the resulting protein levels (Oertlin et al., 2019), we validated selected targets by qPCR, NanoString using total and polysomal mRNA. Next, the corresponding changes on protein levels were validated by western blotting including CHX chase to assess

potential effects on protein degradation of offset mRNAs upon ER α depletion. In summary, regulations resulting from micro-array and RNA-sequencing analysis could be validated on mRNA and protein levels and alterations of ER α levels did not lead to changes of protein stability for

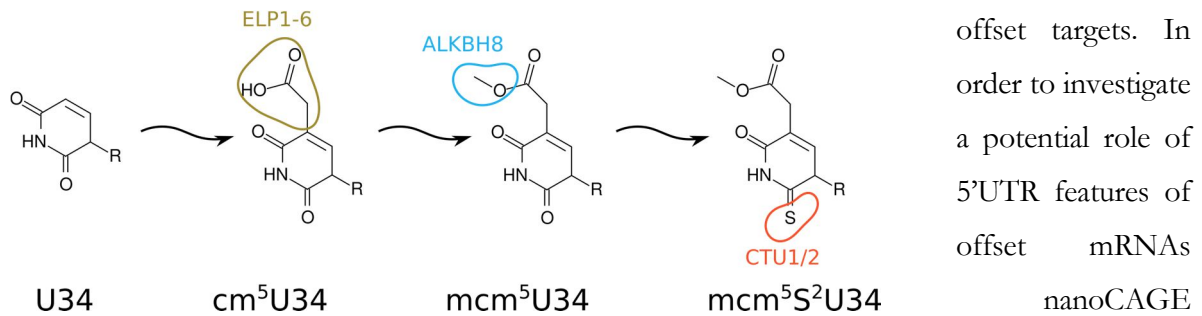


Figure 25 – tRNA modification pathway leading to U34 modifications by the elongator complex proteins 1-6 (ELP1-6), Alkylated DNA repair protein alkB homolog 8 (ALKBH8) and Cytoplasmic tRNA 2-thiolation protein 1-2 (CTU1-2). R corresponds to the RNA backbone of the tRNA.

offset targets. In order to investigate a potential role of 5'UTR features of offset mRNAs nanoCAGE sequencing was performed. This revealed 5'UTR differences (decreased 5'UTR length and increased

fold energy) for mRNAs whose total mRNA levels decreased and translation was offset in comparison to mRNAs regulated by decreased abundance. In contrast, mRNAs with increased total mRNA levels and offset translation only showed differences when comparing 5'UTR folding energies. Abundance of uORFs in strong Kozak context was not different between mRNAs offset or regulated by abundance upon ER α depletion. Since ER α regulates the expression of a wide range of miRNAs, small RNA sequencing was conducted in order to assess miRNA expression upon loss of ER α . While total expression of miRNAs was not altered, a subset of miRNAs was differentially expressed. Next, we assessed if translationally offset mRNAs were enriched or depleted of target sites for miRNAs with ER α dependent expression, in order to explain miRNA-dependent changes of total mRNA levels. This revealed that down-regulated but offset mRNAs were not enriched in miRNA target sites of upregulated miRNAs and up-regulated but offset mRNAs lacked down-regulated miRNA target sites. Since features of the 5'UTR and miRNA target sites in the 3'UTR could not explain the observed translational offsetting, we analyzed the coding sequence. The codon usage, i.e. the requirement of distinct tRNAs, was assessed. Strikingly, mRNAs with increased mRNA levels but offset showed a clear enrichment for distinct tRNA subsets in comparison to mRNAs regulated by increased abundance. When assessing global tRNA levels upon ER α depletion, no change was observed. This led to the assumption that tRNAs require post-transcriptional modifications for their function, as described previously (El Yacoubi et al., 2012). DEK, whose mRNA levels increase but translation is offset, requires the 5-methoxycarbonyl-2-thiouridine modification at U34 (**Figure 25**), also known as the wobble position (Delaunay et al., 2016). Moreover, the codons requiring these modifications were enriched in upregulated but translationally offset mRNAs (**Figure 26**). We therefore hypothesized if the expression of the enzymes, necessary for tRNA modification at U34 (Rapino et al., 2017) was

altered in a ER α -dependent manner. Indeed, the factor catalyzing the first step in the modification cascade (ELP3) showed decreased protein levels upon loss of ER α . Upon knockout of ELP3 in BM67 cells, DEK mRNA levels were increased and protein levels remained unchanged, which illustrated a role of tRNA modifying enzymes in translational offsetting. Next, ELP3 KO BM67 cells were treated with fulvestrant and estradiol, leading to respective degradation and stimulation

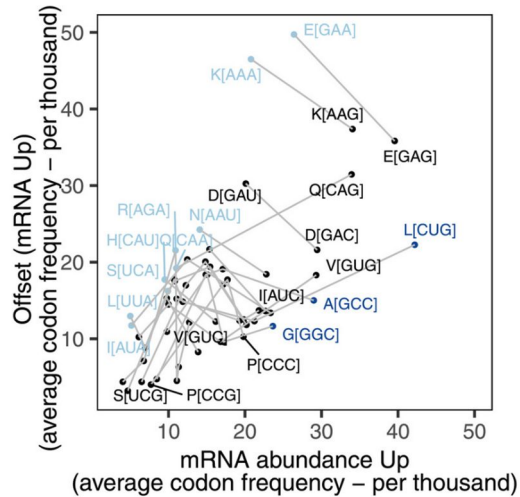


Figure 26 – Average codon frequency of mRNAs translationally offset in comparison to mRNAs whose levels increase with concomitant ribosome association (abundance up). Codons requiring U34 modifications (1.3.4) are enriched in offset mRNAs.

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of ER α . This revealed that proliferation of BM67 cells lacking ELP3 was not altered by either treatment, while control cells showed an increase upon estradiol and a repression upon fulvestrant treatment suggesting ELP3-mediated proliferation downstream of ER α . Finally, in order to distinguish long-term effects mediated by ER α , we used MCF7 as a model for estrogen-mediated stimulation of ER α . We could show by using a previously published data set that stimulation with estradiol led to modulation of mRNA levels and protein levels of ELP3, ALKBH8, CTU2. Moreover, CHIP-Seq data measuring DNA binding of ER α revealed increased association of ER α on the ELP3 locus upon stimulation with estradiol in MCF7 cells.

Finally, we measured the aforementioned U34 modifications by mass-spectrometry under induced inhibition of ER α (72h treatment with fulvestrant). This revealed altered levels of U34 modifications upon inhibition with fulvestrant in comparison to the control.

Taken together, this study presents distinct post-transcriptional regulation of gene expression upon depletion of ER α dominated by translational offsetting. In this model, translational offsetting upon increased mRNA levels is mediated by the usage of codons requiring U34 modifications, whose catalysis depends on ER α activity. Since these offset mRNAs underlie an increase of total mRNA levels, their association with polysomes remains constant. This raises the question about the relationship between impaired decoding and similar polysome-association. Potentially, a result of impaired elongation can lead to decreased initiation due to ribosomes stalling. This can lead to maintained ribosome association opposing increased mRNA levels, which has to be further studied. While offset mRNAs whose levels decrease upon ER α depletion are characterized by a depletion of target sites for upregulated miRNA and optimal codons, it indicates two different

mechanisms leading to offsetting. Moreover, these findings provide new ideas on treatment strategies for cancer with aberrant ER α activity.

3.2 CONCLUSIONS

mRNA translation is regulated by a plethora of cellular pathways and plays a major role during the adaptation of gene expression pathways upon extra- and intracellular signals (Piccirillo et al., 2014; Roux and Topisirovic, 2018; Sonenberg and Hinnebusch, 2009). Its wide-spread implication in diseases such as cancer (Tahmasebi et al., 2018) has led to a range of therapy approaches (Bhat et al., 2015). Despite the role of cellular pathways, features of the mRNA molecule contribute strongly to translational regulation (Hinnebusch et al., 2016; Jonas and Izaurralde, 2015; Szostak and Gebauer, 2013). This thesis presents methodological advancements, which help to understand the contributions of mRNA elements to translational regulation of the gene expression pathway. Anota2seqUtils enables to disentangle the contributions of mRNA features to modes of translational regulation, such as offsetting. NanoCAGE leads to the identification of regulatory elements in 5'UTRs and targeted-nanoCAGE allows 5'UTR-centric analysis of mRNA features and their impact on translation. This approach is essential to validate nanoCAGE-derived 5'UTR isoforms. Moreover, the work in this thesis provides new mechanistic insights into regulation of translation by precise positioning of 5'UTR elements such as uORFs and small molecule-induced p53 reactivation through modulation of the translation factor eIF2 α . The depletion of the transcription factor ER α leads to altered mRNA levels, which are translationally offset. Here, offsetting is a consequence of features in the coding-region of mRNAs requiring specific tRNA modifications for efficient translation, while insulin-induced offsetting is characterized by features in the 3'UTR and altered mRNA stability. Since mRNA features have been studied to a large extend independently, today's era of next-generation sequencing in combination with new computational approaches such as anota2seqUtils helps to generate a deeper understanding of gene-expression with emphasis on post-transcriptional mechanisms in health and disease.

ACKNOWLEDGEMENTS

This thesis marks the end of a journey, which took me through moments of joy and frustration and taught me invaluable lessons about science, life and most of all myself. This book illustrates the work of 6 years (from my side) and many more years of work of others. But it is in essence a collection of research, which has been performed in collaboration with many incredibly talented people, a huge team effort. I want to thank everyone who has been involved in my research work, all teachers at Karolinska Institutet and SciLifeLab for the great courses and everyone creating this great research environment. I feel very privileged that I had the chance to get trained by outstanding colleagues and peers in the spirit of such high-level research institutions. I want to thank Martin Bushell, who promptly agreed on being the opponent for my defense and the members of my examination board: Catharina Larsson, Cristian Bellodi and Carsten Daub for evaluating this work.

I am very grateful, **Ola**, that you happened to be my main supervisor. Working in your group under your guidance has given me seemingly endless possibilities to develop and to “do what I want”. You taught me so many valuable things that I left almost every meeting with new knowledge and you never got tired explaining things over and over again. You supported me at any end and gave me important constructive advice. When I met you the first time, you impressed me with your excitement for research and, after coming to Sweden, for your dedication and determination. You always gave me new perspectives, encouragement and ideas when I was stuck, doubted my abilities and thought that I will never finish this PhD. You genuinely cared about my happiness, which I appreciate a lot. You have a great heart! Thank you!

I also want to thank my co-supervisor **Galina Selivanova** for her input on my work on paper I and for the collaboration on that project. **Johan Hanson** for being at my half-time seminar despite the fact that, unfortunately, our collaboration never happened.

Ivan, thanks for your input, the help, your knowledge in science and beyond, the insights about scientific publishing and the challenges, which taught me important lessons.

A big “thank you” to all collaborators of the studies presented in this thesis. This team effort would not have been possible without your help. Especially to **Sylvain Peugeot** and **Jiawei Zhu** for important experiments on the RITA project. **Yujie Zhang**, **Vicent Pelechano** and the **NGI** facility for help with sequencing.

Thanks to all the people at SciLife for a great atmosphere! The site support for fixing things, **Mats and his gang** for taking care of the goods, being handy and always helping out. Also to the people at the **IT!** **Frida** and **Axel**, they are still doing the BAM seminars!!! It would be great to meet again!

All the former and present colleagues: Thanks to all the people at alfa1: **Jun, Christian, Rozbeh, Fabio, Matthias, Georgios, Haris and the rest** for the great atmosphere and help! **Vincent**, thanks for all the work on the RITA project, for introducing me to the world of polysome profiling and R programming. Also for the far too few after work runs and your constant positive attitude. **Laia**, for the help in the lab, teaching me how to make libraries, for your work on nanoCAGE, taking care for the ordering, which I inherited and started to understand how much “fun” it is. **Shuo**, it was a pleasure to work with you Sir! I really enjoyed the conversations with you, your Chinese music tips and I am still puzzled by your karaoke skills. **Baila**, thanks for your positivity and kindness. **Margarita**, your determination while working on this macrophage project has impressed me a lot. I really like your scientific thinking and think you will have a great future!! It was great fun to have you around in the beginning and then again! **Dongmei**, thanks for always being a helping hand and for the Chinese food, especially the steam balls. I hope you have a new cat and a dog by now. **Yangxun**, it was great to have you here! **Hui**, working with you has been very entertaining and I hope you have found something fun to work on in China now! **Charlotte**, thanks for your feedback on my research, I hope the Mnk-eIF4E axis will provide you with a bright future. **Inci**, I wish you all the best for your PhD, all the work will fall into place eventually! And thanks for the great memes you’re sending me from time to time! **Kiana**, I’m very happy you joined the group! And I hope the “large-scale experiments” will work out. You are “PhD-material” but don’t forget to train! **Shan**, thanks for your help in optimizing nanoCAGE your humor and your endless help doing miniseq runs. **Sabrina**, thanks for the brunch, the beer, Brazilian liquor and for your great humor. All the best for you! Dear **Kathleen**, it is unfortunate that you joined the group so late (on my time scale). I will miss the talks about science and just other important unscientific things (shit)! Thanks for your help, your smart and important input and going through the introduction of this thesis (the sentence consisting of 33,33% RNA is still there). Even though you have a backpack full of projects, I know you will focus on the right thing and eventually you will have your own lab! **Christian**, man this insulin project is long-lasting..... But Ola’s (and my?!) almost endless optimism says that this will eventually be published..... Thanks for your work on the insulin analysis and all the projects filled with madness, reading sections of this thesis, your help with R, reviewing papers and your ideas. All the best in space, or at least in the related research. **Julie**, there is so much I have to thank you for. I think I would not have survived this without you, you as desk neighbor and helping me with science, R!! and dealing with life as a PhD student. The discussions with you were incredible important for my understanding of data/science/”people in science” and I really hope we will always have a lunch date from time to time in the future. **Krzysztof**! The work in this thesis contains so much of your work. Thanks for all the effort in putting nanoCAGE and anota2seqUtils on the table and making it actually work from a data point of view! It seems like it has taken forever (and I think it’s actually true... ☺), but

it paid off!!! Your healthy optimism and especially patience is impressive and I am sure it will rain papers for you, soon! I hope we will see each other in the near future!!!

To all the people from outside of science, whose support and friendship has made this thesis possible:

Thanks **Patrick** for all these rides around Stockholm. You have no idea how much those were needed for forgetting about science and to increase my FTP. **Hanni** and **Armin**, thanks for your thoughtfulness, I'm happy you are a part of the family now. To **Matilda** and **John**, thanks for the friendship, your big heart and warmth. To all the **Tübinger**. I hope we will always be in touch and meet! **Linn**, **Ola** and **Erik**, the weekends and dinners with you are so much fun and give so much energy! **Jon** and **Erik**, thanks for all these dinners with wine, drinks, occasional dance, discussions and your curiosity and caring! **Elin**, **Martin** and **Sofia** (Fantastic5) thanks for your friendship, Tylösand, Njutånger, Halmstad, Uppsala, every time we meet I'm a happy person. **Kristina!!** You are a sunshine and I hope we will forever be friends, go out and eat and drink! Thank you for you being you!!!! To my Swedish family: **Gonzalo**, **Clara**, **Hanna**, **Åsa**, **Bobo**, **Lena**, **Kirre**, **Harald**, **Tore**, **Tuva**. Thank you for embracing me into your family! I never imagined moving to Sweden would gift me with this family extension. **Lena** and **Kirre**, thank you for all the support and the dinner invitations always filled with great discussions! **Gonzalo**, thanks for being my Swedish pappa and being so caring. **Åsa** and **Bobo** for always welcoming me på Ljusterö and your warmth. To all my friends in Germany: **Jugendwerkler**, you know who you are. More specifically: **Stella**, **Miri**, **Jochen**, **Philip**, **Lukket**, **Felix**, **Kon** and the Ragaz gang. These trips mean so much. **Johannes L**, thanks for your open ear, your help in any situation, your understanding about the difficulties in science and all the trips. It's about time for the next one! **Tomma**, for your year-long friendship for listening and supporting. **Till**, my friend! We meet so seldom but if we do it's like back in the days! **Steffen**, thanks for being there, your support, you listening and for all the travels on and off the bike. One day we will live in the same city. To my family: my brother **David**, who I am so grateful for and proud to have. I'm impressed about your patience and determination. It will all fall into place in the end. Trust me! To my parents **Ulrike** and **Manfred**: it's hard to put into words how much you have done for me! Thank you for the endless love and support. This thesis shall be foremost dedicated to you! Finally, I want to thank you, **Clara**! For your understanding, your compassion, your support, your warmth, your heart, your love. You're my best friend and the love of my life. I am excited about all the adventures ahead!

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