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TOWARDS TRANSCRIPTOME-WIDE STUDIES OF mRNA TRANSLATION IN TISSUES FROM CANCER PATIENTS

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Towards transcriptome-wide studies of mRNA translation in tissues from cancer patients

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筚路蓝缕，以启山林。

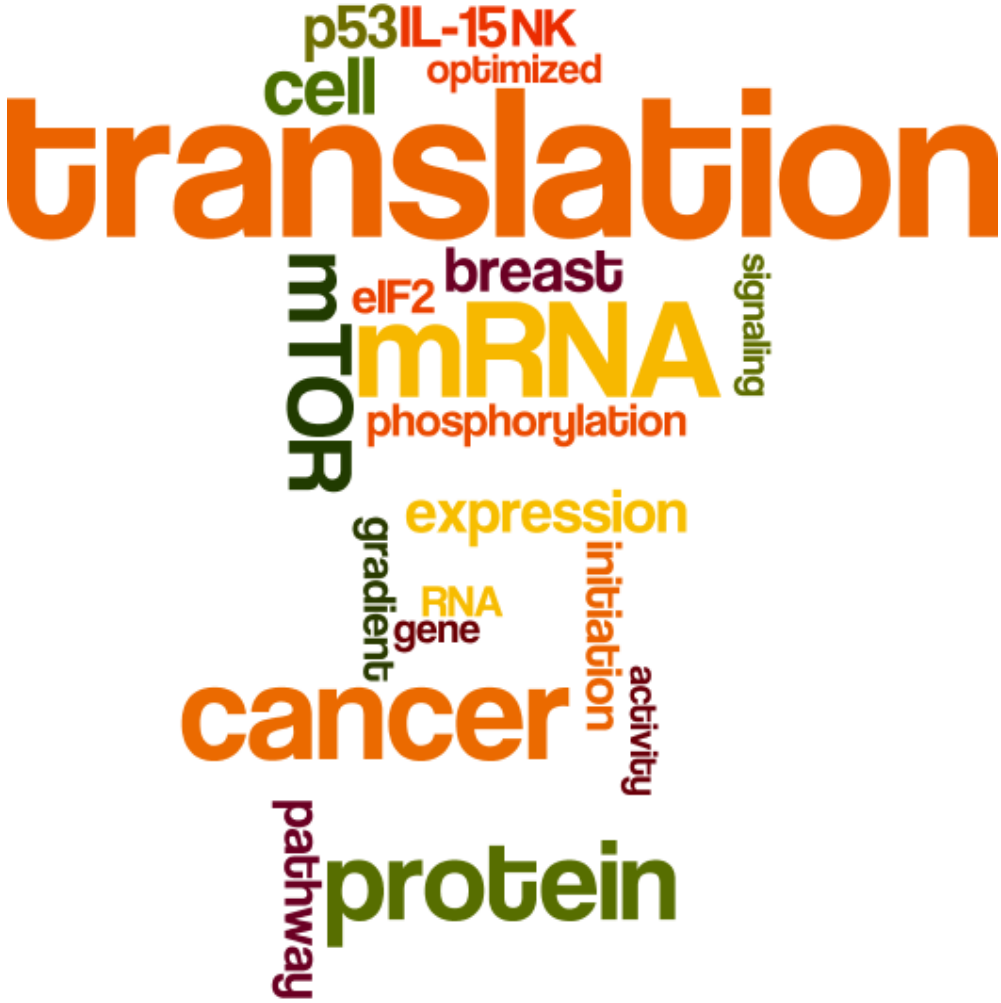
Driving a cart in rags to blaze a trail in a jungle.

《左传》

The Spring and Autumn Annals

To my beloved family

KEY WORDS



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ABSTRACT

Gene expression consists of multiple strictly regulated steps, including transcription, RNA modification, splicing, messenger RNA (mRNA) transport, mRNA degradation, mRNA translation and protein degradation. mRNA translation, the most energy consuming step, plays a critical role in gene expression via global and selective control of protein synthesis. Translation is a complex process that is commonly divided into initiation, elongation and termination. Among these, translation initiation is widely acknowledged as the rate-limiting step for mRNA translation. The mammalian/mechanistic target of rapamycin (mTOR) pathway, as one important regulator of translation initiation, delivers vital signaling by phosphorylating eIF4E binding proteins (4E-BPs) thereby facilitating eIF4F complex formation which participates in eukaryotic cap dependent translation. Increased mTOR activity and dysregulation of translation have been observed in many diseases, such as cancer as well as immune and metabolic disorders. Sequence and structure features of the mRNA, the translational apparatus and trans-acting proteins facilitate or restrict translation regulation of an mRNA. Moreover, these factors can potentially alter the translational efficiency of an mRNA thereby impacting protein levels without changes in mRNA levels. Accordingly, a well-established technique to study translatomes, polysome profiling, separates efficiently translated mRNA from total mRNA into multiple fractions based on the number of ribosomes bound on the mRNA. Extraction of these fractions is a time consuming and laborious process, which makes polysome profiling inconvenient for large experiments or samples with low RNA amounts. Until now, these shortcomings have prevented assessments of translatomes in patient tissue samples.

This thesis introduces an optimized non-linear sucrose gradient which consistently enriches the efficiently translated mRNA in merely one or two fractions, thus reducing sample handling 5-10 fold and saving time in the lab 10-20 fold. When combined with smart-seq2 RNA sequencing, translatomes can be obtained from samples with low amount of RNA and small bio-banked tissues. mRNA yields and translatomes acquired from the optimized gradients resemble those obtained from the standard linear gradients. Thus, this optimized polysome-profiling technique expands the usage of the methodology to small tissue samples and primary cells in large study designs.

Insulin sensitive mRNA translation has been observed in cancer cells derived from insulin insensitive organs, for instance breast. It is largely unknown that if this insulin sensitivity resembles that of cells from insulin sensitive organs or if cancer cells tailor a novel program. To this end, this thesis explored insulin’s effect on metabolomes and translatomes in human primary myotubes, human mammary epithelial cells immortalized with human telomerase (HMEC/hTERT) and the MCF7 breast cancer cells. The data indicates that MCF7 cells have developed pathological responses to insulin induction that differ from those observed in cells from insulin sensitive or insensitive organs. The exploration of mechanisms concealed behind this discrepancy would disclose a potential strategy for cancer treatment through annulment of cancer specific effects of insulin.

The role of mRNA translation during treatments with experimental anti-cancer drugs or those used in the clinic is largely unknown. We examined the effect on translation of one such experimental drug called “Reactivation of p53 and induction of tumor cell apoptosis” (RITA). The α subunit of eukaryotic initiation factor 2 (eIF2α) is a key regulator of translation initiation. We found eIF2α to be phosphorylated during RITA treatment and to be involved in RITA induced apoptosis and repression of mRNA translation. This activity of RITA is independent of TP53 and mTOR pathway. The inhibition of eIF2α phosphorylation counteracts the impact of RITA on apoptosis and clonogenicity.

Another aspect of this thesis explored regulation of translation in immune cells. Short post-infusion persistence restricts treatment of hematological malignancies via adoptive infusion of stimulated natural killer (NK) cells. Interleukin-15 (IL-15) was demonstrated to hold stronger ability than IL-2 to maintain antitumor functions of NK cells after cytokine deprivation. To explore the mechanism underlying these differences, a transcriptome wide study through polysome-profiling technique was applied. Further, the role of mTOR pathway in this superiority of IL-15 was also investigated. Coupled with clinical outcome of patients with B-cell lymphoma, IL-15 but not IL-2 is argued to be implemented in adoptive NK cell cancer therapy.

In conclusion, in order to facilitate studies of the translatome for samples with low amount of RNA and small bio-banked tissues, the optimized non-linear gradient was designed. Its performance in aforementioned samples for large experiment set and general applicability was verified to be satisfying. The study on cancer specific effects of insulin unraveled the prospect to selectively target insulin/IGF1 dependent effects on metabolomes and/or translatomes for cancer therapy. As two important pathways regulating translation initiation, the effect of mTOR in immune cell functions and eIF2α in RITA induced apoptosis were unveiled and explored.

LIST OF SCIENTIFIC PAPERS

I. **Liang S**[#], Bellato HM[#], Lorent J[#], Lupinacci FCS, Oertlin C, van Hoef V, Andrade VP, Roffé M, Masvidal L*, Hajj GNM*, Larsson O*.
Polysome-profiling in small tissue samples.
Nucleic Acids Res. 2018 Jan 9; 46(1):e3. doi: 10.1093/nar/gkx940.

II. **Liang S**, Hulea L, Saini A, Ristau J, van Hoef V, Masvidal L, Gustafsson T, Topisirovic I*, Larsson O*
Cancer specific effects of insulin on translatomes and metabolomes.
Manuscript

III. van Hoef V[#], Ristau J[#], **Liang S**, Peuget S, Topisirovic I, Selivanova G, Larsson O*
RITA-induced apoptosis requires eIF2α dependent modulation of mRNA translation.
Submitted

IV. Mao Y[#], van Hoef V[#], Zhang X, Wennerberg E, Lorent J, Witt K, Masvidal L, **Liang S**, Murray S, Larsson O*, Kiessling R*, Lundqvist A*.
IL-15 activates mTOR and primes stress-activated gene expression leading to prolonged antitumor capacity of NK cells.
Blood. 2016 Sep 15; 128(11):1475-89. doi: 10.1182/blood-2016-02-698027.

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CONTENTS

LIST OF ABBREVIATIONS

3'-UTR	Three prime untranslated region
4E-BPs	eIF4E binding proteins
4EHP	4E homologous protein
5’TOP	Five prime terminal oligopyrimidine tract
5'-UTR	Five prime untranslated region
Anota	Analysis of translational activity
AR	Androgen receptor
ARE	Adenylate-uridylate-rich elements
ATF4	Activating transcription factor 4
CREs	Cis-regulatory elements
CT	Computed tomography
DC	Dendritic cells
DCIS	Ductal carcinoma in situ
Dnd1	Dead end 1
EGF	Epidermal growth factor
eIF2α	α subunit of eukaryotic initiation factor 2
ER	Estrogen receptor
ERK	Extracellular signal–regulated kinases
FLT3L	FMS-related tyrosine kinase 3 ligand
FOXO1	Forkhead box protein O1
FRB	FKBP12-rapamycin binding
GAPs	GTPase-activating proteins
GEF	Guanine nucleotide exchange factor
GM-CSF	Granulocyte–macrophage colony-stimulating factor
GO	Gene Ontology
HER2	Receptor tyrosine-protein kinase erbB-2
hESCs	Human embryonic stem cells
HIF	Hypoxia-inducible factor
HIF1α	Hypoxia-inducible factor 1α
HPV	Human papillomavirus

IL-15	Interleukin-15
IRES	Internal ribosome entry sites
IRS-1	Insulin receptor substrate 1
ISR	Integrated stress response
ISRIB	Integrated stress response inhibitor
MAPK	Mitogen-activated protein kinase
Met-tRNAi	Initiator methionyl-tRNA
MLST8	Mammalian ortholog of the yeast LST8 protein 8
MNK	MAPK-interacting kinase
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
MSK	Mitogen and stress activated kinase
MSL2	Male-specific lethal2
MSL–DCC	MSL dosage compensation complex
mTOR	Mammalian / mechanistic target of rapamycin
NF-κB	Nuclear factor κB
NK	Nature killer cells
PABP	PolyA-binding protein
PARP	Poly ADP ribose polymerase
PDAC	Pancreatic ductal adenocarcinoma
PDCD4	Programmed cell death protein 4
PERK	Protein kinase RNA-like endoplasmic reticulum kinase
PGC1α	PPAR-γ coactivator 1α
PKCα	Protein kinase C-α
PIC	Pre-initiation complex
PIP_3	Phosphatidylinositide-3,4,5-P_3
PPARγ	Peroxisome proliferator-activated receptor-γ
PR	Progesterone receptor
PRTE	Pyrimidine-rich translational element
PUM1	Pumilio-1
RAC1	Ras-related C3 botulinum toxin substrate 1

RBPs	RNA-binding proteins
Rheb	Ras homolog enriched in brain
RIN	RNA Integrity Number
RITA	Reactivation of p53 and induction of tumor cell apoptosis
ROS	Reactive oxygen species
RPFs	Ribosome protected fragments
RPKM	Reads per kilobase per million mapped read
RSK	Ribosomal S6 kinase
S6K1	Ribosomal protein S6 kinase1
SREBPs	Sterol regulatory element-binding proteins
SXL	Sex-lethal
TC	Ternary complex
TLR	Toll-like receptor
TNBC	Triple negative breast cancer
TOP	Terminal oligopyrimidine
TOS	TOR-signaling
TRK	Tropomyosin receptor kinase
TSC	Tuberous sclerosis complex
TSG	Tumour suppressor gene
TSP1	Thrombospondin 1
UNR	Upstream of N-ras
uORFs	Upstream open reading frames
VEGF	Vascular endothelial growth factor
VIM	Vimentin
YY1	Ying-Yang 1

1 INTRODUCTION

1.1 REGULATION OF mRNA TRANSLATION

1.1.1 Regulation of gene expression

Mammalian gene expression is commonly divided into multiple regulatory steps including transcription (1), RNA modification (2), splicing (3), mRNA transport (4), mRNA degradation, mRNA translation (4) and protein degradation (5,6). Following transcription, the pre-mRNA is modified by e.g., 5' capping, 3' polyadenylation and RNA splicing to produce a mature mRNA. This is followed by export of the mRNA to the cytosol where it can be translated into proteins, stored or degraded. Each of these steps is elaborately regulated via different mechanisms to assure that the desired proteins are produced in proper amounts in response to extracellular signals (7), as is shown in Figure1. Moreover, correct regulation of gene expression is vital to maintain cell characteristics including differentiation state. As a key step in gene expression, regulation of mRNA translation represents a crucial line to yield biological functional macromolecules – proteins responding to cells' demand. Compared with regulation of transcription, regulation of translation can more rapidly affect protein levels as it does not require de novo synthesis (or degradation) of mRNA molecules to affect protein synthesis. Regulation of translation is involved in a wide range of biological processes such as cell growth, development (8), learning and memory (9,10) as well as cellular apoptosis (11). Its dysregulation has been observed in a variety of diseases, such as metabolic disorders and cancer, etc. (12). Benefitting from fast developing high throughput techniques and advances in data processing and analysis, the prominent discrepancy between transcriptome and translatome has been revealed, which indicates a complex regulatory mechanisms during mRNA translation. Therefore, the exploration of mechanisms underlying regulation of translation is necessary and significant to understand how it is controlled and holds a great therapeutic potential to treat the diseases with faulty translation.

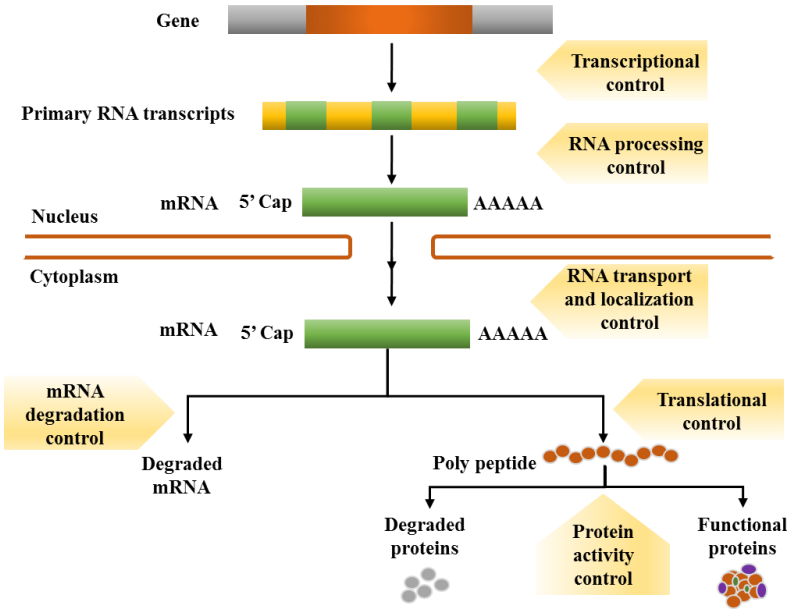

Figure 1. Levels of regulation of gene expression

1.1.2 An overview of regulation of translation

Protein synthesis consumes the majority of cells' energy (13). This requires tight and dynamic regulation of mRNA translation to ensure that energy demand is matched with energy production. Regulation of translation is defined as the modulation of translation efficiency (14). This can be exerted at multiple levels, including translation initiation, elongation and termination. Initiation of translation is acknowledged as a rate-limiting step in translation (15). This is consistent with the common sense that controlling the onset of one event is more energy efficient than modulating the speed. According to the scope of regulated transcripts, regulation of translation exhibits two regulatory modes – global control and selective control. "Global control" is defined as when translation of almost all mRNAs are switched on or off. This is in contrast to selective control, which only influences a subset of mRNAs. As mentioned above, sequence and structural features of mRNA, the state of the translational apparatus, and the availability and activity of trans-acting proteins affect translational efficiency (14). From a general perspective, the elaborate regulation of translation is realized through two main principles: one is modification of translation initiation factors depending on signaling networks involved in translation; the other is trans-acting factors that bind to RNA, such as RNA binding proteins and many micro RNAs (16). Below, these two principles will be introduced followed by two translation regulation modes of global control and selective control.

1.1.3 Translation initiation factors

Eukaryotic translation initiation is the process leading to assembly of an 80S ribosome on an mRNA (14). This process requires the small 40S and large 60S ribosomal subunits, most commonly a minimum of 12 eukaryotic initiation factors and ATP/GTP hydrolysis (14). eIF1, eIF1A and eIF3 facilitate the interaction of the ternary complex (TC) with a 40S subunit to form a 43S pre-initiation complex (PIC). Association of the 43S complex to the 5' end of mRNA requires the cooperation of eIF4F, eIF4B and polyA-binding protein (PABP) (17). eIF4F is a heterotrimer complex that contains eIF4A (an ATPase/RNA helicase of the DEAD-box family), eIF4G (a large modular protein as a scaffold which also binds with both eIF3 and PABP) and eIF4E which is a cap binding protein (18). eIF4E's binding to the m^7G-cap is widely acknowledged as the rate-limiting step of cap-dependent initiation (18,19). As a key constitutive part of the eIF4F complex which is required in translation initiation, eIF4E is involved in leading ribosomes to the cap structure of mRNAs (20). The binding of eIF4E to eIF4G acts in an ATP independent manner, while the activities of eIF4A and eIF4B requires ATP (21). eIF4F formation can be regulated by 4E-BPs, a family of translation repressors (22). The 4E-BPs compete with eIF4G to bind to eIF4E on an overlapping site. Thus, the 4E-BP binding to eIF4E causes dissociation of the eIF4F complex and thereby inhibits cap-dependent translation (23). After exposure to a series of extracellular stimuli, such as nutrients and mitogens, the 4E-BPs get hyper-phosphorylated on multiple sites such as threonines 37 and 46, the phosphorylation of Thr-37 and Thr-46 by mTOR primes the following phosphorylation of other Ser/Thr sites with the sensitivity to serum at the carboxy-terminal sites (24). Mothe-Satney et al. have demonstrated that the phosphorylation of Ser65

is subsequently relied on preceding phosphorylation of Thr 37, Thr46 and Thr70 (25,26). Another study revealed the order of 4E-BP1 phosphorylation in vivo: Thr37 and Thr46 are phosphorylated first, then Thr70, the last is Ser65 (27). Once phosphorylated, 4E-BP decreases its affinity to eIF4E, leading to the release of eIF4E and formation of the eIF4F complex. PABP interacts with eIF4F complex via eIF4G and binds the poly-A tail of eukaryotic mRNA and is thereby thought to promote circularization of the mRNA (28). The binding of PABP facilitates PIC attachment to mRNA. The PIC then scans towards the 3' end until it reaches the start codon (AUG) where translation is initiated (29). The process is shown in Figure 2.

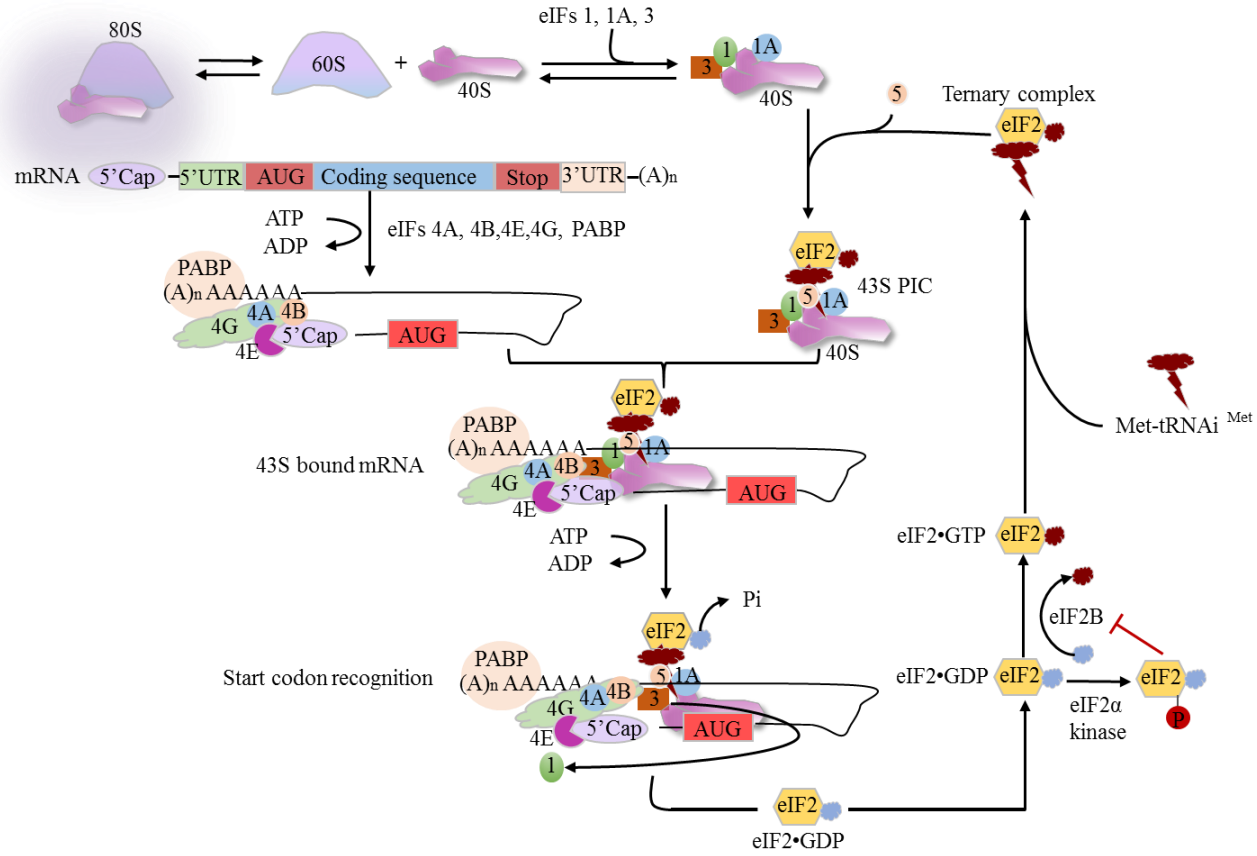

Figure 2. Sketch of eukaryotic translation initiation process

1.1.4 mTOR pathway

1.1.4.1 mTOR complex

mTOR is a serine/threonine protein kinase which belongs to phosphatidylinositol 3-kinase-related kinase protein family (30,31). A widely acknowledged function of mTOR pathway is to modulate the activity of cap-dependent translation initiation in eukaryotes. Activation of mTOR leads to phosphorylation of 4E-BP. This process is involved in many biological process such as cell growth, proliferation and survival, protein synthesis, autophagy, transcription, etc. (32). mTOR can bind to alternative multi-proteins leading to the formation of two distinct complexes: mTORC1 and mTORC2 which demonstrate different signaling activities (33), as shown in Figure 3. MTOR-GβL-Raptor (mTORC1) is composed of mTOR,

regulatory-associated protein of mTOR (Raptor) and mammalian ortholog of the yeast LST8 protein 8 (MLST8, also termed as GβL) (34). Rapamycin inhibits mTORC1 function by associating with the immunophilin FKBP12. The FKBP12-rapamycin complex blocks mTOR activity by binding directly to the FKBP12-rapamycin binding (FRB) domain of mTOR (35). mTORC1 can directly phosphorylate its downstream substrates which include eukaryotic initiation factor 4E-BP1 (36,37) and ribosomal protein S6 kinase1 (S6K1) (38,39) on distinct recognition motifs. For example, the TOR-signaling (TOS) motif at the C-terminal end of 4E-BP1 (40) binds to raptor, so also to mTOR. This motif on 4E-BP1 is responsible for phosphorylating the sites of Ser64/65 and Thr69/70 (Ser65 and Thr70 are on human 4E-BP1; Ser64 and Thr70 are in rodent 4E-BP1) in vivo (41). There has been a study showing that the RAIP motif (from the single letter code for its amino acid sequence) (42) plays a role in amino acids stimulated phosphorylation at the N-terminal sites of 4E-BP1 in a rapamycin-insensitive manner (41). Besides that, for all discovered S6 kinases, the TOS motif at their N terminus is proven to be vital for the phosphorylation and regulation of S6K1 activities (40). The TOS motif is of importance both for S6 kinases and 4E-BP1 to be regulated in the mTOR pathway since it functions as a site where mTOR and other upstream activators of S6K1 and 4E-BP1 can dock (40).

Instead of binding to Raptor, mTORC2 includes mTOR- GβL-Rictor (orthologous to yeast Avo3) which is insensitive to rapamycin (43,44). mTORC2 phosphorylates Ser-473 on the serine/threonine kinase Akt to influence metabolism and survival (45), PDK1 can phosphorylate a threonine T308 residue on Akt and this leads to full activation of Akt (46,47). By stimulating a series of factors such as protein kinase C α (PKCα), paxillin, Cdc42, F-actin stress fibers, Rac1 and RhoA, mTORC2 is reported to be a key regulator of the actin cytoskeleton (44).

Dysregulation of mTOR has been found in many diseases, including diabetes, obesity, Alzheimer's disease, Parkinson's disease and many cancers like breast cancer and ovarian cancer (48,49). As a key signal pathway affecting translation, the role of mTOR in diseases such as metabolic disorders, cancer and in immunology will be discussed next.

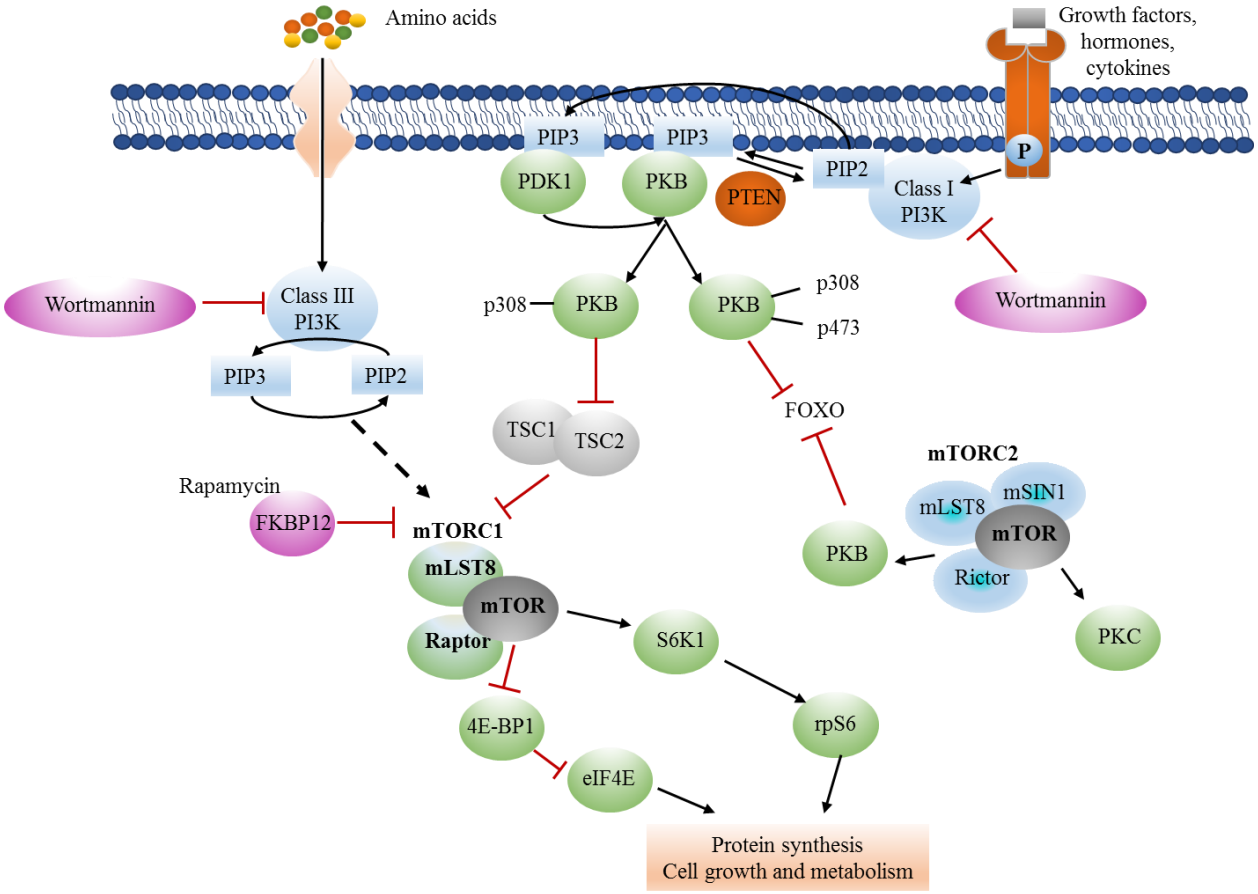

Figure 3. Overview of the PI3K/AKT/mTOR pathway

1.1.4.2 Insulin induction of mTOR signaling

Insulin is a hormone which maintains glucose homeostasis via modulating the utilization of glucose in peripheral tissues (50,51), where it stimulates anabolic process such as protein synthesis and ribosome biogenesis, which mostly relies on nutritional availability (32). Several studies have demonstrated that insulin's role in the anabolic process, most notably in ribosome biogenesis and protein synthesis that is mediated by the activation of mTOR/S6K1 signaling (52-54). The binding of growth factors such as insulin to its receptor makes specific tyrosine residues at the intermolecular receptor act as docking sites. One of these sites recruits PI3K (55) which facilitates the production of phosphatidylinositide-3,4,5-P_3 (PIP_3). Enhanced number of PIP3 recruits PKB (Also known as Akt) to the membrane where PKB becomes phosphorylated at Thr308 and Ser473 through the binding of its pleckstrin homology (PH) domain to PIP3 (56-58) (Also see in session 1.2.4.2). After activation, PKB phosphorylates tuberous sclerosis complex (TSC) protein 2, the phosphorylation of TSC2 inhibits its association with TSC1 (59) which interacts with TSC2, functioning as a tumor suppressor complex (60) (Figure 3). In mammals, the suppressive function of TSC1/TSC2 is due to the GTPase-stimulating activity of TSC2, which inactivates Ras homolog enriched in brain (Rheb) (61) into its GDP state. S6K1 activation induced by insulin was demonstrated to be inhibited by TSC1/TSC2 overexpression (62). Consistently, insulin has been proved to enhance the level of Rheb1-GTP (63) and elevates mTORC1's activity to signal to downstream factors like 4E-BP1 and S6K1 (64,65).

1.1.4.3 mTOR signaling in metabolism

mTOR activity is highly dependent on nutrients and mitogens such as insulin (66), which is consistent with that mTOR plays important roles in mammalian metabolism and physiology, especially in tissues such as brain, muscle, liver and white and brown adipose tissues. Studies have shown that insulin receptor substrate 1 (IRS-1) deficiency can cause insulin resistance in brown adipocytes by affecting insulin oriented lipid synthesis. Moreover, IRS-2 deficient hepatocytes fail to respond to insulin due to being incapable to activate PI3K-Akt-mTOR pathway (67). The insulin receptor is indispensable for regulation of the mitogenesis of foetal pancreatic β-cell mediated by complete activation of mTOR/p70S6K through PI3 kinase and MEK-1 pathway. However, glucose induced β-cell mitogenesis, independent of PI3 kinase, is induced by MEK-1, which converges on mTOR/p70S6K pathway to regulate foetal β-cell proliferation (67).

As a key mediator of most metabolic processes, mTORC1 is involved in enhancing glycolytic flux by stimulating the expression of hypoxia inducible factor 1α (HIF1α) which is an enhancer of many glycolytic genes (68-71); by facilitating the association of transcription factor Ying-Yang 1 (YY1) and peroxisome proliferator-activated receptor-γ (PPARγ) coactivator 1α (PGC1α). mTORC1 has also been reported to enrich mitochondrial DNA content and participate in oxidative metabolism by stimulating the expression of corresponding genes (72). Further, lipid biosynthesis is mediated by mTORC1 through its effects on transcription factors of sterol regulatory element-binding protein 1/2 (SREBP1/2), which regulates the translation of a lot of genes e.g., ACC, FASN and SCD1, participating in the synthesis of fatty acid and cholesterol (73). Several studies have revealed that for certain cell types, mTORC1 control SREBP function through S6K1 (69,74,75). Glycolysis and mitochondrial respiration is also mediated by mTORC1 through 4E-BP-dependent translational regulation to supply the cellular energy (76). There has been a study demonstrating that mTORC1 modulates the activity and biogenesis of mitochondria by prioritizing selective translation of nucleus-encoded mitochondria-related mRNAs by suppressing 4E-BPs. This selective translation of nucleus-encoded mitochondria-related mRNA provides an eligible capacity for ATP production, as an energy source for mitochondrial activities (77). Similarly, mTORC2 stimulates AKT to increase glycolytic metabolism, and dephosphorylates class IIa histone deacetylases (78,79), which causes inactivation of the forkhead box protein O1 (FOXO1) and FOXO3 by acetylation, this further activates MYC transcription (76).

1.1.4.4 mTOR pathway in immunology

The intercellular homeostasis is central for cell development and proliferation, the innate immune system functions as a guardian in response to different kinds of perturbations, thus to maintain the cells' homeostasis (80). There have been several studies demonstrating that mTOR is implicated in mediating the functions of innate immune cells by participating in a wide network of cellular and metabolic activities affecting immune effector responses.

When innate immune cells become activated, they require a reprogramming to remodel their metabolism and energy consumption to undergo a series of activities such as changing their morphology, migration and secreting cytokines, chemokines and lipid mediators (76). Most of these functions act via the activation of the mTOR pathway. mTOR in activated innate immune cells is stimulated by growth factors, cytokines and Toll-like receptor (TLR) ligands. There have been several studies showing that in dendritic cells (DC) and neutrophils, mTORC1 is activated by FMS-related tyrosine kinase 3 ligand (FLT3L) and the growth factors granulocyte–macrophage colony-stimulating factor (GM-CSF) (81-83); both mTORC1 and mTORC2 are stimulated by TLR ligands in human and mouse macrophages, monocytes and DCs (81,84-90); mTORC1 and mTORC2 are activated by IL-4 in mouse macrophages (91,92), and IL-15 can induce mTOR activity in both human and mouse natural killer (NK) cells (93). As already discussed, mTORC1 signaling is central in regulating translation and protein synthesis by modulating the activities of its downstream effectors eIF4E and S6K. Therefore, mTOR is of importance in induction of protein synthesis in activated innate immune cells.

In addition, many studies have illustrated that mTORC1 is involved in myeloid cell development (94). TLR-stimulated primary myeloid immune cells exhibit an immuno-stimulatory effect by suppressing mTORC1 or mTORC2.

NK cells are a type of innate immune cells with cytotoxic lymphocyte capability, they play an important role in immune surveillance of cancer and infections (95). In mouse models, the proliferation and cytotoxicity of NK cell mediated by IFNγ and granzyme B requires the participation of mTORC1 (93,96,97). In NK cells under the inhibition of mTOR by rapamycin, the cells stop expressing IFNγ and granzyme B and halt their proliferation (93,97). This suppressive influence of mTOR knock down on NK cells' proliferation and cytotoxicity has also been observed in rapamycin-treated transplant human recipients (98). Once mTOR is reactivated in NK cells, the cells carry out a multistep of mTOR dependent metabolic reprogramming, which leads to increased glucose uptake and more aerobic glycolysis (99). The NK cells' cytotoxicity relies directly on the rate of glycolysis (99). Therefore, NK cells' growth and functionality relies heavily on glucose availability and utilization regulated by mTORC1 (76).

1.1.4.5 mTOR in cancer

As mentioned above, the disorder and malfunction of mTOR pathway has been observed in carcinogenesis of many tumors (100). As a well-known tumor suppressor, TP53 deficiency has been found to stimulate mTORC1 activation, while activated TP53 suppresses mTOR activity and controls its downstream effects, such as autophagy induced tumor suppression. TP53 performs its influence on mTOR by requiring AMP kinase activation and TSC1/TSC2 complex participation, which both act in energy-deprived cells (101). Furthermore, a growing number of studies have exhibited that majority of factors upstream of mTOR signaling are mutated in a series of cancers, such as Akt (101,102), TSC1/2 (103), neurofibromatosis type 1 (Nf1) (104), serine threonine kinase 11 (Lkb1) (105) and Pten (106). The dysregulation of

translation initiation downstream of mTORC1, more specifically, 4E-BP1 and eIF4E disorder has been considered as a key in cancer formation. These two factors have been demonstrated to participate in transmitting the oncogenic effects of Akt on mTOR pathway in protein synthesis and tumor development (107). Cells lacking 4E-BPs and thus with overwhelmed cap-dependent translation lose their control over cell proliferation, which is originally realized by selectively suppressing the translation of mRNAs that encode for proteins with stimulatory functions in proliferation and cell cycle progression (108). S6K1 as another important target downstream of mTORC1 appears to contribute the maintaining of fast cell growth of cancer by being involved in ribosome synthesis, thus it offers a sufficient supply of translation machinery (109). As mentioned above, mTORC1 is also involved in lipid biosynthesis through transferring growth factor signaling to SREBP1/2 (69,73), the lipid biogenesis is highly required in cancer cell proliferation since fatty acids must be created to reach the rapid membrane synthesis requirement (110). Even though mTORC2 is not as widely studied as mTORC1, several studies have illustrated that mTORC2 is highly involved in cancer development. Rictor as a component of mTORC2, has been found to be overexpressed in glioma and to endow cells with proliferative and invasive capability (111,112). Hence, the activation of oncogenes conveys a series of signaling to mTOR complexes which increases a variety of activities in protein synthesis, cell proliferation, survival, anti-apoptosis required for cancer initiation and development.

1.1.5 eIF2α pathway

The eIF2α pathway, as another important signal pathway in addition to mTOR, also participates in eukaryotic cap-dependent initiation. eIF2 is a G-protein switch which delivers the initiator methionyl-tRNA (Met-tRNAi) to ribosome. The process starts with the binding of the TC which involves the met-tRNAi and GTP bound eIF2 to the small (40S) ribosomal subunit, this forms the 43S PIC (113). Comparing to eIF2-GDP, eIF2-GTP has stronger affinity to Met-tRNAi (114). There has been a study showing that eIF2γ can bind directly to GTP and Met-tRNAi, eIF2α and eIF2β subunits are involved in increasing this affinity to Met-tRNAi almost one hundred times. However, the exact mechanism behind this is still largely unknown (115,116). Under the help of a series of aforementioned initiation factors such as PABP, eIFs, 4B, 4H and 4F, 43S PIC binds to the mRNA at the location of the 5'-7-methylguanosine cap and then scan the five prime untranslated region (5'-UTR) until it reaches the start codon AUG. Base-pairing between the start codon AUG and anticodon of Met-tRNAi is considered as the beginning of start codon recognition (117-119). Then eIF2 will release phosphate which turns itself from eIF2-GTP to eIF2-GDP, following this process with presence of other initiation factors, the large 60S subunit binds to the complex to form the 80S, which represents the end of translation initiation and a start of elongation (120).

1.1.6 Trans-acting factors — RBPs in regulation of translation

Regulation of translation is under elaborate control by a complex of molecular mechanisms. Among which, RNA-binding proteins (RBPs), trans-acting factors and small RNAs revamp mRNA translatability by binding to certain regions of the mRNA (121). Cis-regulatory

elements (CREs) are one of these regions of non-coding sequence that RBPs bind to. These regions are tightly involved in regulation of translation and more often found in 5'-UTR and/or three prime untranslated regions (3'-UTR) (16). The 3'-UTR contains binding sites for RBPs and miRNAs which usually have inhibitory effects on translation or cause degradation of the transcripts (121,122). At the step of translation initiation, eIF4G, as a scaffold protein, binds with initiation factors and PABP which binds to polyA tail of 3'-UTR to form a closed loop. The formation of this loop is often affected by RBPs. A study in drosophila, for example, has demonstrated that bicoid binds to the 3'-UTR of mRNA, this leads to the recruitment of 4E homologous protein (4EHP, in mammals known as eIF4E2) to the 5' cap, which decreases the translation due to 4EHP's low affinity with eIF4G (121). Moreover, there have been studies showing that RBPs also cooperate with other factors to regulate translation, such as miRNAs (121). For example, the regional secondary structure of the 3'-UTR of p27 mRNA is altered by binding of pumilio-1 (PUM1), which facilitates miR-221 and miR-222 to target the sites to perform their inhibitory functions (123). In contrast, the protein Dead end 1 (Dnd1) competitively binds to the overlapping sites to inhibit miRNA-mediated silencing (124).

One example of an RBP is upstream of N-ras (UNR), as a conserved RBP in drosophila, executes its functions of controlling mRNA translation and maintaining stability through the similar mechanism like other RBPs via binding to specific sequences in UTRs. Along with the non-coding RNA roX, male-specific lethal2 (msl2) mRNA bound by UNR is acknowledged to be involved in regulating drosophila dosage compensation which is a process whereby genes on the male X chromosome is hyper-transcribed to compensate and equalize the expression level of genes on X chromosome between female (XX) and male (XY) (125). The suppressed expression of msl2 is indicated to repress dosage compensation in females. This suppression of msl2 is mediated by another RBP — sex-lethal (SXL), which in the nucleus, inhibits mRNA splicing by binding to oligo uridine segments of the 5'UTR of msl2, while in cytoplasm, SXL exerts its inhibitory function via adhering to 3'UTR (126). Several studies have revealed that UNR engages itself in different mechanisms by combining distinct regulatory sequence to control mRNA translation in a gender specific style (127,128). A study has illustrated that UNR inhibits MSL2 mRNA translation via disrupting the packaging of MSL dosage compensation complex (MSL–DCC) in females (129). However for males, UNR stimulates and facilitates the MSL–DCC to target the X-chromosome in an MSL independent manner (128,130). Moreover, UNR is acknowledged of its function in facilitating the translation mediated by internal ribosome entry sites (IRES) and retaining the stability of mRNA in mammals (131,132). Wurth, et al. have indicated that the expression of UNR is increased in melanoma tumors which stimulates tumor's invasion and metastasis (133). Besides, UNR coordinately regulates novel pro-metastatic RNA regulons. As a RBP, UNR not only plays a role in maintaining RNA steady-state levels, but also participates in regulating some of its targets such as VIM (Vimentin) and RAC1 (Ras-related C3 botulinum toxin substrate 1) mRNAs on the translation elongation level (133). There has also been a study demonstrating that the low expression of UNR by immunohistochemistry using a tissue

microarray was significantly associated with poor prognosis after surgery in patient with pancreatic ductal adenocarcinoma (PDAC) (P = 0.010) (134).

As another important RBP, HuR selectively binds to and stabilizes mRNAs containing adenylate-uridylate-rich elements (ARE). This stabilizing capability is realized by binding to AREs and preventing their degradation. It transports mRNAs containing ARE in the 3'UTR from the nucleus to the cytosol. There has also been a study showing that HuR antagonizes miRNA function by self-oligomerization along the 3'-UTR and cause the detachment of miRNA (135). HuR plays a vital role in stabilizing the mRNA of central molecules or cytokines involved in carcinogenesis (136) and subsequent progression such as cell proliferation, angiogenesis, invasion, metastasis and immune evasion (137). Due to HuR's preventive effect on mRNAs from degradation, it indirectly enhances protein production and is involved in the control of differentiation process. HuR was found to enhance the expression of many growth-promoting, proliferative and proto-oncogenic factors like epithelial growth factor (EGF), c-myc and c-fos, GM-CSF, cyclin A, B1 and D1, pro-angiogenic factors such as HIF-1a and VEGF, and anti-angiogenic factors like thrombospondin 1 (TSP1). Thus, as an RBP, HuR is involved in regulation of translation and influences the expression of numerous traits vital to the development and progression of cancer.

1.1.7 Regulation of global translation

Fertilized invertebrate eggs and mammalian iron-starved reticulocytes were the cases studied earliest on global translational regulation in which all mRNAs are regulated in unison (14). This united regulatory pattern of protein synthesis that turning on in fertilized eggs and turning off in iron-starved reticulocytes happens in absence of transcription (120). The global translation is usually realized via changes of protein synthesis machinery components (14). For example, a wide variety of stresses conveying the signals to phosphorylate eIF2α through activating nuclear factor κB (NF-κB) which regulates gene transcription (138,139). Such stress conditions include pro-inflammatory cytokines exposure, UV irradiation, microorganism infection, and damaged protein folding in the endoplasmic reticulum (ER) (138). As a key to regulate protein synthesis, guanine nucleotide exchange factor (GEF) eIF2B changes eIF2-GDP to its transnationally active eIF2-GTP form (140,141). Once eIF2α is phosphorylated at serine 51, as a constitutional functional group in the regulatory subcomplex of eIF2B, this complex converts eIF2 to a competitive inhibitor of GEF (142). As a result, eIF2-GTP levels are reduced and translation initiation is globally suppressed to reserve energy, and cells adapt themselves to a new gene expression program to avoid cell damage caused by aforementioned stresses (143). The other signaling pathway controlling global translation is mTOR pathway. It is mTORC1 that regulates global protein synthesis via phosphorylating specific effector proteins 4E-BP1, this results in eIF4E released from 4E-BP1 and then facilitates translation initiation by forming eIF4F complex. These consecutive events are generally considered as the dominating mechanism by which mTOR mediates global translation (18). It is worth mentioning that mTOR also regulates selective translation by preferentially accelerating the translation of some selected groups of mRNAs, among

them, one subset with relatively long and structured 5' UTRs are termed as "eIF4E sensitive" mRNAs (144), which will be discussed in the next section.

1.1.8 Regulation of selective translation

In contrast to global control, selective control regulates the translation of a subset of mRNAs in a cell, or even merely a single mRNA species under extreme condition. Such regulation can occur in tune with factors specific to individual mRNAs or classes of mRNAs (such as RBPs discussed above), or via modulating the activity of certain translation machinery components, such as eIF4E (14). The mRNA subsets translated preferentially via modulation of eIF4E activity are termed as "eIF4E-sensitive" mRNAs which commonly have long and structured 5'UTRs (144-146). This feature makes eIF4E sensitive mRNAs rely more on the unwinding activity of eIF4A (A DEAD (Asp-Glu-Ala-Asp)-box RNA helicase) in eIF4F complex (147). The mRNAs in this subset encode proteins such as vascular endothelial growth factor (VEGF) (148), cyclins (149), c-Myc (150) and ornithine decarboxylase (151), which participate in cell survival and proliferation (108). A recent study identified a new subset of mRNAs produced from nuclear encoding proteins involved in mitochondrial functions (such as ATP5O, ATP5G1) as being sensitive to eIF4E, but this new subset of mRNAs lack a long 5'UTR (152). These short 5'UTR mRNAs have plenty of translation initiator of short 5'UTR (TISU) elements (153). Another example of selective regulation of translation is integrated stress response (ISR) dependent eIF2α phosphorylation, which also induces the translation of select transcripts. For example, activating transcription factor 4 (ATF4), whose activation stimulates the transcription of genes subject to ISR (143). For more advanced eukaryotes, such as mammalian cells, mitogen-activated protein kinase (MAPK)-interacting kinases (MNK) 1 and 2 bind to the C-terminal region of eIF4G, and phosphorylate eIF4E under stress and mitogen stimulation condition (154-156). eIF4E phosphorylation selectively stimulates the translation of mRNAs involved in survival (157) and tumor invasion (158). There has been a study showing that mTORC1 tends to initiate translation of mRNAs with a 5′ terminal oligopyrimidine tract (5′ TOP) or with a pyrimidine-rich translational element (PRTE, whose position is recently reported to be not strictly within the 5' UTRs) (159). These structures are manifested to encode the components of translational apparatus (160,161) and proteins involved in translation and metabolism (162,163). Another study shows that in oxygen deprived cells, TOP mRNAs show a TSC-Rheb-mTOR dependent manner, but independent of 4E-BPs (164). This was proven by experiment setup in 4E-BP loss- and gain-of-function studies that the phosphorylation status of 4E-BP did not contribute to a cause for the translation inhibition of TOP mRNAs under growth factor deficiency or hypoxia conditions (164).

1.1.9 Regulation of translation in cancer

The dysregulation of translation can lead to a variety of abnormalities, such as imbalance in proliferation, aberrant angiogenesis, prolonged survival, disorder in immune response and cancer energetics. The dysregulation of translation has been found in many types of cancers (165-168). Cancer is frequently found with a series of amplified and/or dysregulated

translation initiation factors (146). For example, eIF4E overexpression causes poor prognosis in breast (169), head and neck (170), stomach (171), bladder (171), liver (172) and prostate cancers (172); overexpression of 4E-BP1 is oppositely correlated with tumor grade (173); eIF4G overexpression correlates with lung (174), breast (175) and cervical cancers (176); increased expression of eIF4A can be found in lung (174) and cervical cancers (176); loss of PDCD4 is associated with poor outcome in breast (174), lung (177), colon (178) and ovarian cancers (179). Moreover, enhanced expression of eIF2α is associated with aggressive lymphoma subtypes (179); overexpression of eIF3a is associated with breast, esophageal, stomach, lung and cervical cancers (179). All these dysregulated translation initiation factors in cancers are listed in Table 1. Breast cancer shows elevated eIF4E phosphorylation (180), 4E-BP1 phosphorylation (173,181), overexpression of eIF4G (175), decreased level of PDCD4 (182), increased level of eIF3a (183), b (184), h (185), i (186) subunits, decreased expression of eIF3e (187) and f subunits (188). Moreover, some of the most common cancer-related mutations, such as mutations of MYC, RAS and PIK3CA have been found to affect the translation machinery (146). Indeed, many oncogenic signals affect translation machinery components, and most cancer cells show an increased activity of the translation machinery. Thus, this suggests that tumors are addicted to selective changes in protein synthesis (146). Therefore, the treatment targeting these translational programs may be a promising strategy to treat cancer (189).

Table 1. Dysregulation of translation initiation factors in cancers

Factors	Dysregulation	Consequences in cancers
eIF4E	Overexpression	Poor prognosis in breast, head and neck, liver, prostate, bladder and stomach cancers
4E-BP1	Overexpression	Oppositely correlated with tumor grade
eIF4G	Overexpression	Correlates with lung, breast and cervical cancers
eIF4A	Up-expression	Overexpressed in lung and cervical cancers
PDCD4	Loss	Associated with poor outcome in breast, lung, colon, and ovarian cancers
eIF2α	Enhanced expression	Associated with aggressive lymphoma subtypes
eIF3a	Overexpression	Associated with breast, cervical, esophageal, lung and stomach cancers

References are listed in the paragraph of section 1.1.9.

The dysregulation of translation has been proven to be involved in many human disorders and diseases. Cancer, as a spectrum of severe and sharply increasing syndrome in recent decades, will be further discussed in this thesis from here on.

1.2 CANCER

1.2.1 Cancer hallmarks and therapeutic challenge

Cancer is a large variety of diseases arising from uncontrolled cell growth with the characteristic to invade and/or metastasize to adjacent and distance sites from the original lesion. Cancerization as the replacement of the normal cell population by a cancer-primed cell population (190), commonly starts as a long period ahead of a clinically detectable mass and certain obvious symptoms. Cancerization is a complex and multistep process including genetic mutations and epigenetic alterations. These genetic and molecular changes differentiate cancer from normal tissues by rendering them a series of distinctive and complementary capabilities to fuel the growth and metastatic dissemination of a tumor. Those capabilities, in other words, hallmarks can be categorized and summarized as below: imbalance between proliferative and inhibitory signaling, avoidance of apoptosis, immortalized replication, sustained angiogenesis, tissue invasion and metastasis, escaping from immune surveillance, tumor-oriented inflammation, energy metabolism reprogramming, genome instability and mutation (191), and replication stress (Figure 4) (192).

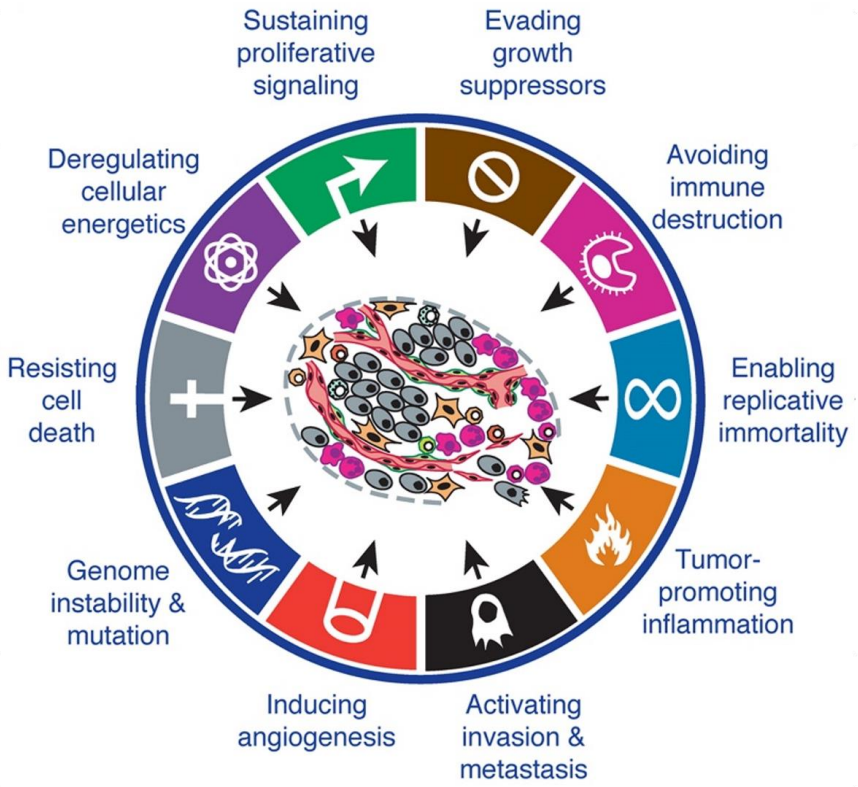

Figure 4. Hallmarks of cancer.

Adapted from Hallmarks of Cancer: The Next Generation. Hanahan, D. and Weinberg, R.A. (191)

The therapeutic strategy targeting the mechanism behind each hallmark has naturally been introduced and studied widely for ages. For example, VEGF signaling inhibitors can be used to offset the induction of angiogenesis; replicative immortality can be suppressed by telomerase inhibitors; selective anti-inflammatory drugs can extinguish tumor induced

inflammation, etc. It is worth mentioning that most of the hallmark-targeting drugs were designed to act specifically against one particular cancer capability, in order to reduce off-target effects and to avoid nonspecific toxicity. However, this presumed virtue has failed to produce a long lasting clinical response, for most cases being followed by an inevitable relapse (191).

According to Hanahan and Weinberg, one explanation for this failure is that many signaling pathways converge to contribute to one particular hallmark capability. Therefore, only a simplex therapeutic drug targeting one specific pathway is not sufficient to occlude a hallmark capability. On the other hand, the cancer cells can adapt themselves to the stress imposed by the applied therapeutic agent, adjust and survive to be more relied on the other hallmark capabilities to overcome the potencies of the targeting drugs. As a consequence, only a combination of therapeutics targeting multiple hallmark capabilities can generate a more effective and non-relapsed clinical results (191).

1.2.2 Genetic and epigenetic alterations in cancer

A mutation is a stable alteration of the nucleotide sequence in the genome. It can happen by chance during DNA replication and be triggered by exposure to various mutagens, like carcinogens or radiation. Due to the variety and complex of mutations, there are several ways to classify mutation types from different aspects. Based on the scale of nucleotides affected, they are categorized as small-scale mutations or large-scale mutations. For small-scale mutations, only one gene or a few nucleotides are changed. This includes deletion, insertion and substitution of one or several nucleotides in DNA. Depending on which kind of erroneous codon being produced, the point mutations which happen in the protein coding region can be classified into silent mutations which yield the same or a highly similar amino acid, missense mutations that encode for a disparate amino acid, and nonsense mutations which result in a stop codon or a shorter protein. The large-scale mutations are the structural and/or numerical alterations of chromosomes or chromosome loci. This includes for example the deletions of large chromosomal regions, duplications causing double or multiple copies of certain region within a chromosome, inversions changing the orientation of a chromosomal segment, substitutions as a region from a chromosome becomes a new region in a non-homologous chromosome, and translocations as interchange of a segment from non-homologous chromosomes.

Mutations exist widely in both normal and cancer cells. The biological activity of normal cells is under strict control through a series of molecular networks from their birth to death, thus the normal cells are able to repair or eliminate the genetic errors. If cells are overwhelmed by the errors, they will start programmed cell death which leads to apoptosis. However, cancer cells lost this rigorous control, instead of apoptosis, they keep accumulating genetic errors and entail them to their progeny. However, not all mutations retain the ability to transform normal cells into cancer cells. Normally only 25% of them are the “driver mutations” which can lead to a cancer initiation and progression (193). Eventually, the accumulation of these genetic disorder leads to an alteration of their genome, they gain the

capability to invade and metastasize to distance tissues, and a series of malignant characteristics aforementioned.

Unlike the genetic alteration, epigenetics is defined as a heritable phenotype due to alterations in chromosome without changes in the DNA sequence (194). Epigenetic alterations can either cause an activation or a silencing of certain gene, which guarantee the cells to express the genes that are essential to their utility in a differentiation process. The most common epigenetic changes involve DNA methylation, histone modification and micro-RNA gene silencing (195). An epigenetic malfunction plays an even more vital role than genetic mutations in transforming normal cells to cancer cells (196). Compared to normal cells, the hyper-methylation of CpG islands (epigenetic promoter) in cancer, appears over ten times more frequent to cause a transcription silencing than by genetic sequence alterations (193). Due to the variety of epigenetic disorders and their crucial role in cancer development, controlling and correcting epigenetic malfunction discloses a promising therapeutic strategy for cancer prevention and treatment.

1.2.3 Oncogenes and tumor suppressor genes

In 1941, Theodor Boveri firstly indicated the concept of oncogene in his book "The origin of malignant tumors". He described the oncogene as substances amplified during tumor development. Src (proto-oncogene tyrosine-protein kinase Src) was the first discovered oncogene in 1970, which was found in a chicken retrovirus (197). An oncogene is derived from a proto-oncogene with certain accumulated mutations and an increased level of carcinogenesis, which endows normal cells the ability to turn themselves into cancer cells. A proto-oncogene, as a normal gene exists in the genome, encodes for proteins largely involved in cell proliferation and differentiation. When the proto-oncogenes undergo a series of structure modifications, such as mutations within the regulatory region, gene duplication or chromosomal translocation, they become oncogenes (198). The most common proto-oncogenes are for example MYC, RAS, WNT, extracellular signal–regulated kinases (ERK), and tropomyosin receptor kinase (TRK) (199). When the normal cells are accumulating mutations in proto-oncogenes, with the overcome of apoptosis and restriction of tumor suppressor genes, these abnormalities will coordinately turn the normal cells into cancer cells (200). The classification of oncogenes has not reached to a united agreement yet, based on factors they affect, they are categorized as growth factors (c-Sis), receptor tyrosine kinases (EGFR, PDGFR, VEGFR) (201), cytoplasmic tyrosine kinases (Src-family, Syk-ZAP-70 family), cytoplasmic serine/threonine kinases and their regulatory subunits (Raf kinase), regulatory GTPases (Ras protein) (202), transcription factors (myc gene) (203).

On the contrary, a tumor suppressor gene (TSG), also termed as antioncogene, is a type of genes encoding for proteins that are involved in cell cycle and apoptosis, thus can protect cells from turning into cancer. Its existence was firstly discovered by Knudson in 1969 (204). Carcinogenesis initiates when gain-of-function mutations happen in proto-oncogenes and loss-of-function mutations occurs in tumour suppressor genes (205). Most antioncogenes follow the principle of "two-hit hypothesis", which means an effect can only appear when

both alleles coding for a specific protein are influenced (204). However, not all the TSGs obey the "two-hit hypothesis", such as *TP53*, which is the most widely studied antioncogene (206). The p53 protein encoded by one mutated allele can counteract the effect of normal protein encoded by non-mutated allele, this indicates that the mutation of only a single allele of *TP53* enhances the possibility to cancer development (207). The inactivation and loss of *TP53* has been found in a variety of cancers, including leukaemia, lymphomas (208), sarcomas (209), brain tumors (210), breast (211), colon (212) and lung carcinomas (213), etc. Nevertheless, the first TSG was *Rb* instead of *TP53* which was discovered in retinoblastoma (214). With more studies of *Rb*, it has been found to be involved in a series of cancers, such as bladder, breast and lung carcinomas. Other TSGs include for example *BRCA1/2* (215), *NF1/2* (216), *PTEN* (217), *VHL* (218) and *WT1* (219).

1.2.3.1 TP53 gene overview

TP53 gene which resides on the short arm of chromosome 17 (17p13.1) in humans, encodes tumor suppressor p53. The most important function of this protein is to prevent normal cells from transformation to cancer cells (220). Due to the ability to maintain genome stability, *TP53* has been honored as "the guardian of the genome" (221). The given name of p53 was because it appeared as a 53-kilodalton (kDa) protein on SDS-PAGE when it was first discovered. But the real mass of p53 protein is 43.7 kDa. This deviation is because of high content of proline in p53 which drags its migration on SDS-PAGE. p53 functions as a central pivot in a series of networks. Its inactivation and malfunction has been observed in more than 50% human tumors, such as prostate, breast, colon and lung cancers, etc. (222,223).

1.2.3.2 Functions of p53

p53 shows a variety of antitumor functions, including inducing apoptosis (224), maintaining genomic stability (225) and inhibition of angiogenesis (226). These biological functions have been widely studied. There have been studies demonstrating that p53 usually associates with its negative regulator mdm2 as a complex, this association maintains p53 in an inactive status in normal cells (227). Once when cells suffers from a variety of stresses, such as UV radiation, ionizing radiation, chemicals, oxidative stress, osmotic shock, and DNA damage, p53 will dissociate from mdm2 and become activated (227). The activated p53 on one hand acts as a promoter to enhance the activity of DNA repairing proteins to mend the damaged DNA, if the damage cannot be repaired, p53 induces initiation of apoptosis (228); on the other hand, p53 binds to DNA and activates the expression of genes for instance microRNA miR-34a (229), p21 encoded by WAF1/CIP1 and numerous of other genes, among these genes, p21 inactivates the G1-S/CDK complex which plays an important role in G1/S transition, thus to stop cell proliferation and maintain the cell at the G1/S regulation point (228), providing more time for DNA repairing proteins to mend the damaged DNA. However, wild type p53 is very unstable with many folded and unstructured regions, resulting in a status of continuous synthesis and degradation. Once p53 is mutated, e.g., on R175H and R249S (230), it will lose its anti-proliferation ability by decreasing its affinity to DNA. As a result, less p21 will be produced to slow down the speed of cell proliferation

(231). In addition, p53 is also indispensable in differentiation of human embryonic stem cells (hESCs) and the maintenance of stemness in adult stem cell niches (232). There has been a study demonstrating that hESCs keep p53 in a low active status (233), the increased activity of p53 will cause a fast differentiation of hESCs (234). Knocking out p53 delays the differentiation of hESCs, and the rescue of p53 stimulates a spontaneous differentiation of hESCs. This explicates that p53 plays an indispensable role in hESCs' differentiation.

1.2.3.3 Regulation of p53

As p53 locates at the key position of several biological networks, its regulation also requires the participation of a variety of factors. Upon activation of p53, the N-terminal domain of p53 will be phosphorylated, this domain has a lot of phosphorylation sites which function as the primary targets for protein kinases conveying stress signals (235). There are mainly two groups of proteins kinases which interact with the transcriptional activation domain of p53. One group is the MAPK family, and the other is ATR, ATM, and CHK1 (236). As previously mentioned, mdm2, as a negative regulator of p53, binds to and covers the p53 transactivation domain, then prohibits p53 to induce target genes' transcription (237). Moreover, mdm2 is also an E3 ubiquitin ligase which captures p53 and recruits small ubiquitin proteins to the p53, leading the poly-ubiquitinated p53 to be degraded by the proteasome system (238). The ubiquitin/proteasomes system is a highly regulated machinery which undergoes intracellular protein degradation and turnover by proteolysis.

In addition, p53 also induces the transcription of mdm2. This forms a negative feedback loop for these two factors, once mdm2 is produced, it will lead to more p53 degradation (239). The excessive mdm2 leads to less functional p53, this could increase the possibility of carcinogenesis. There has been a study showing that many tumors are found to be with an increased level of mdm2, such as sarcomas (240). On the contrary, an organism without mdm2 is not able to survive. Mice without mdm2 during embryonic development has been found to end up in death caused by overwhelmed apoptosis. The lethality can be rescued by backcrossing to p53 null mice (241,242). Besides mdm2, p53 is also regulated at transcriptional and post-translational level; modifications at the post-translational level involves for example, phosphorylation, acetylation, ubiquitination and methylation, etc.(243).

p53 can both function as a transcriptional activator and a transcriptional repressor by directly binding to DNA through its DNA binding domain (244). Transcriptional machinery will be summoned by p53 to the promoter-enhancer region of its target genes. These activated target genes will then be transcribed into corresponding microRNAs and translated to their corresponding proteins, thus to transduce and execute p53 functions. By binding to DNA, p53 can in some situation act as a transcriptional repressor, inhibiting the transcription of certain genes, such as bcl-2 gene which is with the function of anti-apoptosis (245).

1.2.3.4 p53 and diseases

Considering the important functions of p53, its suppression and deletion has been found in many types of diseases. If the mutation of p53 occurs in germ line cells, it can cause a severe

hereditary disease named as Li–Fraumeni syndrome (Firstly found by two U.S physicians, Frederick Pei Li and Joseph F. Fraumeni, Jr.) (246). As a rare autosomal dominant disorder, it causes sarcoma, breast, leukemia and adrenal gland (SBLA) syndrome (246). In addition, p53 mutation and malfunction has been found in over half of all human tumors. One of the most common infectious viruses — human papillomavirus (HPV) suppresses the p53 protein function by secreting proteins E6 and E7, E7 also inactivates pRB and CKIs (247), causing the escape of cell apoptosis and unrestricted cell proliferation. The infection of the low risk HPV subtypes leads to warts, and the high risk HPV subtypes infection, such as types 16 and 18, causes a cervical dysplasia. The accumulative influence of high risk HPV infection for a long time will finally initiate a cervical carcinoma in situ and followed up by a metastatic cancer (248).

1.2.4 Signaling pathways in cancer

Cancer is a spectrum of diseases involving disorders of many signaling pathways. Such pathways include for example regulating cell growth and proliferation responding to cellular environment. Ras, PI3K and mTOR signaling plays important roles in this matter; nuclear factor-κB transcription factors are involved in tumor development and progression; tumor vascularization and metastasis is orchestrated by hypoxia-inducible factor (HIF), functioning as a transcriptional factor in nutrient stress signaling. Further, a lot of signaling pathways participate in tumors' distant metastasis. However, no thesis of this length can pretend to completeness covering all molecular disorders of signaling pathway involved in cancer initiation and progression. So, the next two sections will be briefly focused on the Ras-ERK and PI3K-Akt signaling pathways that are largely implicated in many steps associated with cancer. Also, both of them impinge on mRNA translation and therefore are of interest here,

1.2.4.1 Ras-ERK pathway

The activation of Ras-ERK pathway is either through mutations in Ras or Raf gene, which leads to activation of these proteins consecutively, or via inactivating GTPase-activating proteins (GAPs), such as NF1 (249), DAB21P (250) and RASAL2 (251). These proteins promote the hydrolysis of GTP bound to Ras, thus they cause the inactivation of Ras (252). As an important transcription factor, Myc which is downstream of Ras-ERK and many other pathways, can be phosphorylated by ERK, this phosphorylation prevents Myc from degradation caused by ubiquitylation (253). The abnormal expression of Myc has been found in many types of cancers, such as Burkitt lymphoma (254), cervical, colon, breast, lung and stomach carcinomas. Myc plays a multi-functional role as it is involved in cell cycle progression, cellular differentiation and apoptosis (255). The role of Myc in inducing cell proliferation is realized by stimulating a variety of genes that function as promoters in cell proliferation. These includes G1/S cyclins, CDKs, and the transcription factors in E2F-family which drives the cell cycle progression (256). Besides, Myc also stimulates the expression of genes functioning in increasing translation and anabolic metabolism, and Myc suppresses the genes that induce cell differentiation and blocks the activity of cell cycle inhibitors (252).

In addition to Myc, ERK can also phosphorylate a series of kinases, such as mitogen and stress activated kinase (MSK), multiple kinases in the ribosomal S6 kinase (RSK), MAPK and MNK. Once phosphorylated, these kinases will further enhance the activities of transcription factors which participate in cell cycle progression. Under the induction of mitotic stimuli, MSKs mediate the phosphorylation of histone H3 at S10 (257). There has been a study showing that in mice with the deletion of MNKs phosphorylation site, the cells are not able to transform themselves into tumors (258). This revealed the important role of MNKs in tumor initiation. It has been demonstrated that by phosphorylation of translation initiation factor eIF4E, MNKs are involved in translation initiation (257). Moreover, mTOR pathway can also become activated by RSK activation stimulated by ERK. The activated RSK can phosphorylate TSC2 that binds with TSC1 as a complex to block the mTOR activation. Upon phosphorylation of the complex, the inhibition on mTOR will be relieved. Besides, RSK also phosphorylates eIF4B, which further binds with translation initiation factor eIF3, thus to increase the translation initiation. An overview of this pathway can be seen in Figure 5.

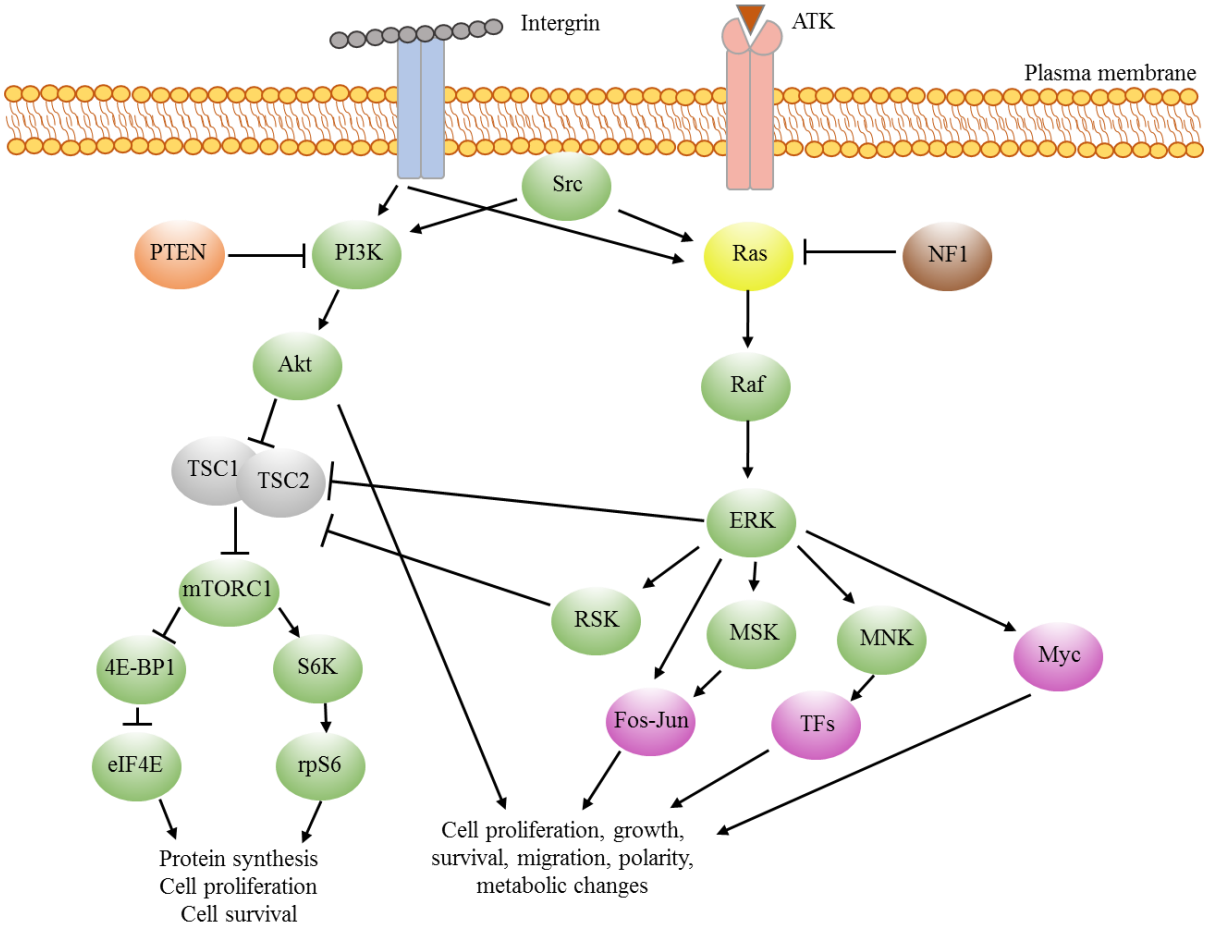

Figure 5. The Ras-ERK and PI3K pathways.

1.2.4.2 PI3K-Akt signaling pathway

The PI3K/AKT pathway functions as an indispensable signaling in controlling cell cycle and cell proliferation, and its aberrant activation has been largely implicated in many cancers with

reduced apoptosis and hyper-proliferation. PI3K signaling is initiated by the growth factor and cytokines that bind to the tyrosine kinase receptor, and this leads to the receptor dimerization. The factors that have been proven to stimulate this pathway include EGF (259), insulin (260), IGF-1 (261) and CaM (262). However, genetic mutations hold the capability to activate PI3K-Akt pathway even without growth factors. A lot of mutated genes in cancer commonly influence PI3K-Akt pathway by encoding targets or components of this pathway (252). A series of proteins involved in this pathway can be affected either through amplification or activation of gene mutations, such as PIK3CA which is the type I PI3K isoform, adaptor protein PIK3R1 and Akt; or via deletion or inactivating mutations which happens in the phosphatases with hydrolyzing function on phosphatidylinositol 3,4,5-trisphosphate (p1p3) which functions as a PTEN and an INPP4B tumor suppressor (252). Once activated, lipid kinase PI3K is recruited to the internal docking site and becomes activated. The activated PI3K then stimulates membrane lipids PIP2 to transform into the active PIP3 form, this leads to activation of the key signaling kinase AKT. AKT activation initiates several downstream processes such as activating CREB (261), inhibiting p27 (260), blocking FOXO activity by localizing FOXO in the cytoplasm (260), activating PtdIns-3ps (262) and promoting cell growth through protein synthesis by activating mTOR (260), which further affect transcription of p70 or 4EBP1(260). On the contrary, there are also a series of factors antagonizing this pathway such as PTEN (263), GSK3B (261) and HB9 (259).

1.2.5 Breast cancer

1.2.5.1 Breast cancer snapshot

Breast cancer is a spectrum of diseases with heterogeneous clinical and morphological entities, which are distinctive in clinical history and prognosis. The optimized polysome-profiling mentioned in the first constituent paper aimed at dealing with hundreds of bio-banked breast cancer tissues, thus to explore the characterization of breast cancer by differential translation; the second and the third constituent papers also enrolled breast cancer cell line MCF7. Therefore, breast cancer will be introduced next in brief.

Majority of breast cancer originate from the lining of the lobules and milk ducts, which are named as lobular carcinomas and ductal carcinomas, respectively (264). Risk factors for breast cancer include family history, benign breast disease, inherited mutations in the BRCA1 or BRCA2 genes (265), obesity, hormone replacement therapy during menopause, overdose of radiation and aging (264,266). Several tests are used to diagnose breast cancer including physical exam, mammogram, ultrasound exam, computed tomography (CT) or magnetic resonance imaging (MRI) and biopsy. In addition, the level of hormone receptors such as estrogen, progesterone and human epidermal growth factor type 2 receptors can also provide information regarding aggressiveness, prognosis and therapeutic regimen (264). Histologically, based on the degree of tumor cell differentiation, breast cancer is classified into well, moderately and poorly differentiated (marked as Grade 1,2,3 correspondingly). Staging is based on the extent and distribution of the tumor in the body and therefore affects treatment strategies. The most acknowledged staging method is TNM (T: tumor size; N:

lymph node involvement; M: tumor metastasis). Stages of breast cancer are classified into Stage 0 (Carcinoma in situ) to IV (Metastasis to other part of the body). The standard methods to treat breast cancer mainly include surgery, chemo-radiotherapy, hormone therapy and targeted therapies (267).

1.2.5.2 Epidemiology

Breast cancer is a highly frequent cancer among women in developed countries (268). With increased life expectancy, urbanization and adoption of a western life style, the incidence of breast cancer is swiftly rising in traditional low-incidence Asian countries such as Japan (269), Singapore (270) and China (271). The Surveillance, Epidemiology, and End Results Program of the National Cancer Institute (SEER) showed 246,660 estimated new cases in 2016, which accounted for 14.6% of all new cancer cases. Moreover there were 40,450 estimated deaths in 2016 which was equivalent to 6.8% of all cancer deaths (272).

1.2.5.3 Classification

As mentioned above, the main breast cancer category considers tumor's histopathology and receptor status. Histopathological classification is the category that pathologists use most to describe tumors. According to the 2012 World Health Organization (WHO) classification of breast tumors (273), breast lesions mainly include invasive breast carcinomas, mesenchymal tumors, male breast tumors, malignant lymphoma, metastatic tumors, precursor lesions, benign epithelial lesions, myoepithelial lesions, fibro-epithelial tumors, benign and malignant tumors of the nipple. Among which, the invasive ductal carcinoma almost accounts for 55% of breast cancer, while invasive lobular carcinoma is rare (5%). Another major type is ductal carcinoma in situ (DCIS, 13%) (274), which is characterized by the trait that the surrounding tissue is not invaded by cancer cells.

Receptor status classification is also commonly used as a reference for treatment. For example, ER and progesterone receptor (PR) are expressed in some breast cancer cells and are considered as prognostic markers (275). Based on the expression of ER and PR, breast cancer is diagnosed as ER positive or negative, and PR positive or negative types. Patients with ER expression usually receive the treatment of anti-hormone drugs such as Tamoxifen and Toremifene (Fareston®) to block the estrogen receptors. In addition, receptor tyrosine-protein kinase erbB-2 (HER2) or HER2/neu, a member of the human epidermal growth factor receptor family, is also an indispensable indicator for patients' prognosis (275). About 20% of breast cancer overexpress HER2 protein commonly via amplification of the gene which plays a vital role in initiation and progression of certain aggressive breast cancer types (276). This excessive expression of HER2 can be antagonized with drugs that target HER2, such as trastuzumab (Herceptin®) and lapatinib (Tykerb®). Triple negative breast cancer (TNBC: ER-, PR- and HER2-) is a type with poor prognosis, which is more common in younger women with early metastasis. Hormone therapy is invalid for this type since the cancer cells lack hormone receptor expression.

Molecular subclasses consider tumor histology, receptors status, grade, stage and molecular signatures to classify the tumors as subclasses below. The luminal subclass is characterized as ER positive, which can be further divided into A, B and ER-/AR+ (androgen receptor) types. The name "luminal" originates from the discovery that the gene expression of this pattern is most similar to normal luminal cells from the inner side of breast ducts and glands. Luminal A type has a better prognosis than B type. The ER-/AR+ type is also termed as "molecular apocrine" and the androgen receptor has been reported to be expressed in 12-50% in all ER- cases (277). The HER2 subtype has been discussed above. Majority of TNBC belongs to basal type with the worst prognosis. In contrast to TNBC, normal like tumors grow slowly and show a good prognosis. Their gene expression pattern is mostly similar to normal breast epithelial cells.

A more detailed classification for breast cancer facilitates the discovery of individualized treatments. Commonly, gene expression patterns obtained from total RNA have been used to identify tumor subgroups in multiple cancers including breast, ovarian, liver, lymphoma and soft tissue sarcomas (278-280). These subgroups are considered to reflect cell origin of the cancer, a distinct tumor microenvironment and/or other cell biology aspects. Observers' perspective restricts the definition of relevant tumor subtype. If data from total RNA levels are adopted as input for identification of subtypes, then only subtypes revealed at this level will be observed. Hence, alternative means of studying tumors may identify additional clinically relevant entities. Since the hyper-activation of eIF4E in breast cancer and that eIF4E affects translation of distinct subsets of genes, it is assumable that mRNA translation may provide an alternative perspective on breast cancer subtypes. Considering the vast number of RNA binding proteins (>700), it is also possible that non-eIF4E related translation is dysregulated in breast cancer. So, only a transcriptome wide approach can conclusively answer whether differential translation is manifested in breast cancer in vivo or not.

1.2.5.4 Therapeutic strategy & Challenge

The intrinsic diversity of breast cancer requires a multidisciplinary therapeutic strategy. Surgery, chemotherapy, radiation therapy, hormone therapy, targeted therapy, or selective combination of several of them is currently used to treat breast cancer in clinic. This is done in the adjuvant setting, i.e. after surgery, and the neo-adjuvant setting, i.e. before surgery to shrink the tumor's size and to facilitate excision of the tumor. These treatments lead to side effects especially following radiation and chemotherapy due to their low specificity to cancer cells.

1.3 TECHNIQUES TO STUDY TRANSLATION EFFICIENCY

1.3.1 Polysome profiling

A well-established technique to study genome wide patterns of mRNA translation is polysome-profilng. Polysome-profiling involves immobilization of ribosomes on mRNA by employing translation elongation inhibitors (e.g. cycloheximide) followed by isolation of efficiently translated mRNA. A cytosolic lysate is first loaded on 5%-50% linear sucrose

gradient. Next, during ultracentrifugation, mRNAs sediment according to their association with ribosomes which allows for separation of efficiently translated mRNAs (associated with >3 complete ribosomes) from inefficiently translated mRNAs (associated with ⩽ 3 complete ribosomes) (281). Alteration in the distributions of mRNAs over the gradient among different conditions can be determined from the mRNA extracted from each fraction. For example, the comparison between efficiently translated mRNA from HCT116 p53+/+ in starvation and complete medium demonstrated the mRNAs shift across the polysome-profile, as shown in Figure 6. For genome wide experiments, mRNA from fractions corresponding to >3 associated ribosomes are commonly pooled (281). This >3 threshold is used because most mRNAs with translational efficiency alteration show a change across this threshold. The reason behind this is that the distribution of ribosome association applies to normal distribution for both on-off regulation and continuous shifts (152). Moreover, 80% newly synthesized polypeptides will be captured by this threshold (282,283). During polysome-profiling, the efficiently translated mRNAs are commonly obtained by pooling about 10 fractions corresponding to 5 ml solution collected in 10 Eppendorf tubes. The isolation of these mRNAs is done from one fraction to another separately, and the pooling of these fractions is during re-suspension of purified RNA pellet. For small samples such as those from bio-banked tissues, such extensive dilution may cause sample loss. For larger experimental setup, pooling this number of fractions is not only labor intensive and time consuming, but also may lead to mistakes of mislabeling samples or erroneous pooling of fractions. Therefore, an optimized non-linear sucrose gradient was designed to enrich the efficiently translated mRNAs in only 1 or 2 fractions to reduce sample handling 5-10 fold and saving time 10-20 fold, which was a successful solution to the aforementioned shortcomings.

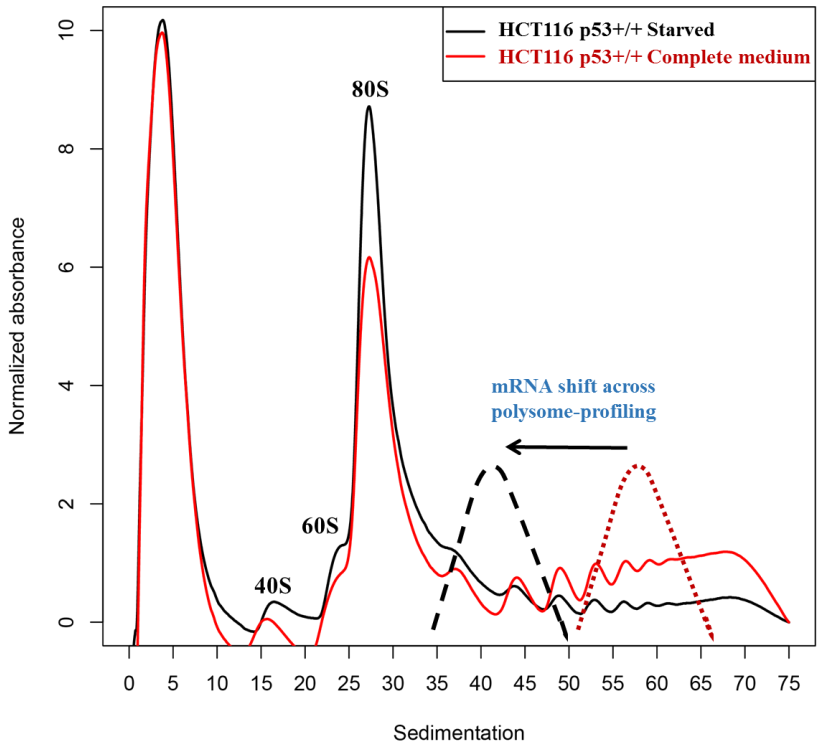

Figure 6. mRNA shifts across the polysome-profilng under starvation via changes in translation efficiency. The polysome-profilings of HCT116 p53+/+ under serum starvation and under

complete medium are shown in black and red respectively. The black and red dash lines display the mRNA shift across polysome-profilling.

1.3.2 Ribosome profiling

Ribosome profiling is an emerging tool using deep sequencing of ribosome-protected mRNA fragments to determine ribosome positioning in a genome wide pattern (284). An 80S ribosome protects around 30 nucleotides from RNase digestion (285,286). Accordingly, such ribosome protected fragments (RPFs) can be isolated, sequenced and mapped backwards to the original mRNA to pinpoint the location of the ribosome. The mapping of RPFs can help to identify translation products by locating translation starting and ending sites, explore translation mechanism, such as to find translated upstream open reading frames (uORFs) and to identify subsets of ribosomes participating in translation by spotting their physical location in cells and monitoring their interacting molecules (284). Although primarily designed to study ribosome positioning, translation efficiency can also be assessed by comparing RPFs to mRNA levels across conditions. Thus, ribosome profiling annotates the coding regions, facilitates the discovery of gene expression regulation underlying diverse biological processes, and reveals the key mechanism underlying the protein synthesis, also helps to identify unknown proteins (284).

1.3.3 Comparison between polysome and ribosome profiling

Because ribosome profiling provides precise position information of ribosome footprints, it can be applied to facilitate identification of ribosomal frame shifting, translation initiation at non-AUG codons, stop codon readthrough, ribosome pausing and uORF translation (284,287-291). However, there are also several disadvantages of ribosome profiling that should be considered. Firstly, the translation pausing can cause a signal blurring and an experimentally introduced accumulation of ribosomes at a specific location if inhibition is slow (284). Secondly, RNA structures or large ribonucleoprotein complexes also lead to increased RPFs, which thereby contaminates ribosome footprints and yields false readouts of translation (284). Further, short size of ribosome footprints leads to difficulties in determining the right alignment position for reads from highly similar or repetitive regions (284). Finally, ribosome profiling requires larger mRNA amounts as compared to mRNA-seq (284). Applying ribosome profiling to study differential translation has resulted in conflicting results as compared to polysome profiling. Hsieh et al. and Thoreen et al. used ribosome-profiling to study mTOR-sensitive translation and suggested that mTORC1/EIF4EBP/EIF4E pathway exclusively regulates translation of 5'TOP and 5'TOP like mRNAs (163,292). There has been a study showing that the translation of TOP mRNAs is independent of 4E-BPs (164). This was in stark contrast to a study employing polysome-profiling which suggested that mTOR pathway also regulates the translation of non-TOP mRNAs (152). This discrepancy was later explained as that ribosome-profiling is more biased towards mRNAs showing large shifts in translation efficiency (such as TOP-mRNAs) and less sensitive for detection of mRNAs that show intermediate shifts co-occurring with those showing large shifts (152). In contrast, polysome profiling shows less bias and can therefore identify differential translation

of mRNAs showing large and small shifts in translation efficiency even when these occur simultaneously (152).

1.3.4 Computational methods to analyze translational efficiency

DNA microarrays and RNA sequencing are used to quantify polysome-associated RNA to study regulation of translation from a transcriptome-wide level. To analyze the differential expression from quantification of total RNA (293), the large sets of high throughput data require specialized computational methods. It is noteworthy that cells' overall transcription level also influences the analysis of the following translation step. Thus in order to study translation per se, besides the efficiently translated mRNAs, the cytosolic mRNAs should also be measured to counteract the potential effect of cytosolic mRNA levels to differential levels of efficiently translated mRNA (294). A lot of previous studies correct the effect of cytosolic mRNA level by calculating the log ratio of efficiently translated mRNA levels by cytosolic mRNA levels acquired in parallel (295). However, this log ratio method generates a great number of biological false positive and negative results (295). Analysis of translational activity (Anota) was developed to address these drawbacks (294,295). Anota was initially designed to analyze DNA-microarray data but recently, our lab developed anota2seq which allows the analysis of data both from ribosome- or polysome-profiling quantified by RNA sequencing or DNA-microarray (296). In addition, anota2seq also allows the interrogation of a previously unexplored regulation mechanism of gene expression — translational buffering which polysome-associated mRNA level maintains the same (as well as protein levels in the absence of differential protein degradation) despite alterations of total mRNA level (296). Anota2seq was implemented as an R package which was used to analyze the RNA-seq data generated from the constituent research in this thesis.

2 AIMS OF THE THESIS

The overall aim of this thesis:

Dysregulated translation is a key factor in tumor biology that contributes to patients' prognosis and guide individualized cancer treatment. This thesis aims to explore the possibility that cancer is characterized by differential translation. This would provide some clues guiding future therapies targeting faulty translation in cancer.

The specific aims of the included papers:

Paper I: To design an optimized polysome profiling method to enrich efficiently translated mRNA in less fractions, validate the yield, reproducibility and general applicability in cell lines and small clinical tissues as compared to the standard linear gradient.

Paper II: To identify changes in mRNA translation downstream of insulin that are dependent or independent of mTOR signaling in cells from tissues regarded as insulin sensitive or insulin insensitive and transformed or non-transformed cells. To ultimately identify effects from insulin that are specific to cancer cells and that are important components to their phenotypes.

Paper III: To elucidate the mechanisms of RITA's anti-cancer activity, specifically to investigate whether this anti-tumor activity is dependent on TP53 and its implication on translation through eIF2α phosphorylation.

Paper IV: To compare the ability of IL-2 and IL-15 to maintain human NK-cell functions following cytokine withdrawal to model post-infusion performance. Use the polysome profiling to explore the mechanism from a translational perspective and to investigate the role of mTOR and STAT-5 pathways in antitumor functions of IL-15 induced NK cell.

3 RESULTS AND DISCUSSION

3.1 PAPER I

Polysome-profiling in small tissue samples

The standard linear gradients of polysome-profiling yield many fractions per sample to isolate efficiently translated mRNA, this causes a major limitation by leading to a broad dilution of the efficiently translated mRNA, which could lead to a sample loss and technical inconstancy, These disadvantages further cause an underestimation of quantification and reproducibility of the translatome when performing studies in primary cells or small tissue samples. For large study design with over hundreds of samples, this causes laborious work on thousands of fractions to pool to get the efficiently translated mRNA.

3.1.1 The optimized non-linear sucrose gradient reproducibly and consistently isolates the efficiently translated mRNA in high quality.

An optimized non-linear sucrose gradient was invented based on that the number of bound ribosomes on the mRNA is associated with their translational efficiency. Moreover, ultracentrifugation poses a linear relationship between the log2 number of associated ribosomes and sedimentation distance. This facilitates the calculation of the sucrose concentration that differentiates mRNAs associated with less than 3 ribosomes from those bound with more than 3 ribosomes (efficiently translated mRNA in this scenario). The calculation result was 34% (Figure 7A). We set a layer of 55% sucrose at the bottom of 34% layer to prevent polysome from further sedimentation and a layer of 5% sucrose above the 34% sucrose to facilitate sample entry into the gradient. Next, we tried to optimize the volumes for each sucrose layers. The objectives are that the optimized gradient should have high reproduction and elution time ought to be reduced. We used the BioComp gradient maker that is a cylinder to indicate the desired level on tube. The sketch of the optimized gradient is shown as in Figure 7B. The pilot experiment of the optimized non-linear gradient separated the ribosomal subunits of 40S, 60S and the monosome 80S, following these peaks, a high peak appeared between the 34% and 55% sucrose interface.

The exploration of the fractions around the high peak at the interface of 34% and 55% sucrose indicates that the fraction right under the peak and the one after are strongly enriched in mRNA with >3 ribosomes while the fraction before the peak, is enriched in mRNA associated with <3 ribosomes even though it contains a bit of efficiently translated mRNA. We reproducibly observed this pattern in another two independent experiments. This optimization collects the efficiently translated RNA in only two fractions. By pooling these two fractions and dividing them equally into two parts (one sample and one backup), only one tube with efficiently translated mRNA needs to be worked on.

We assessed the mRNA extracted from human colon cancer cell lines HCT-116 which differ in their p53 status (HCT-116p53+/+ and HCT-116p53-/-) after 16h serum-starvation. Serum starvation influenced global translation for both cell lines observed as a similar reduction in

polysome-associated RNA companied with an enhancement in free ribosome subunits and 80S. Cytosolic lysates from 6 plates (15 cm) of each cell type was equally divided, each equivalent was loaded on the linear gradient and on the optimized non-linear gradient. We did four independent experiments. We then extracted mRNA from the linear gradient and the optimized non-linear gradient. These two types of gradients produce similar amounts of efficiently translated mRNA. And these two gradients both endow persistent isolation of essentially intact RNA evaluated by Agilent Bioanalyzer which grades each sample with a RNA Integrity Number (RIN).

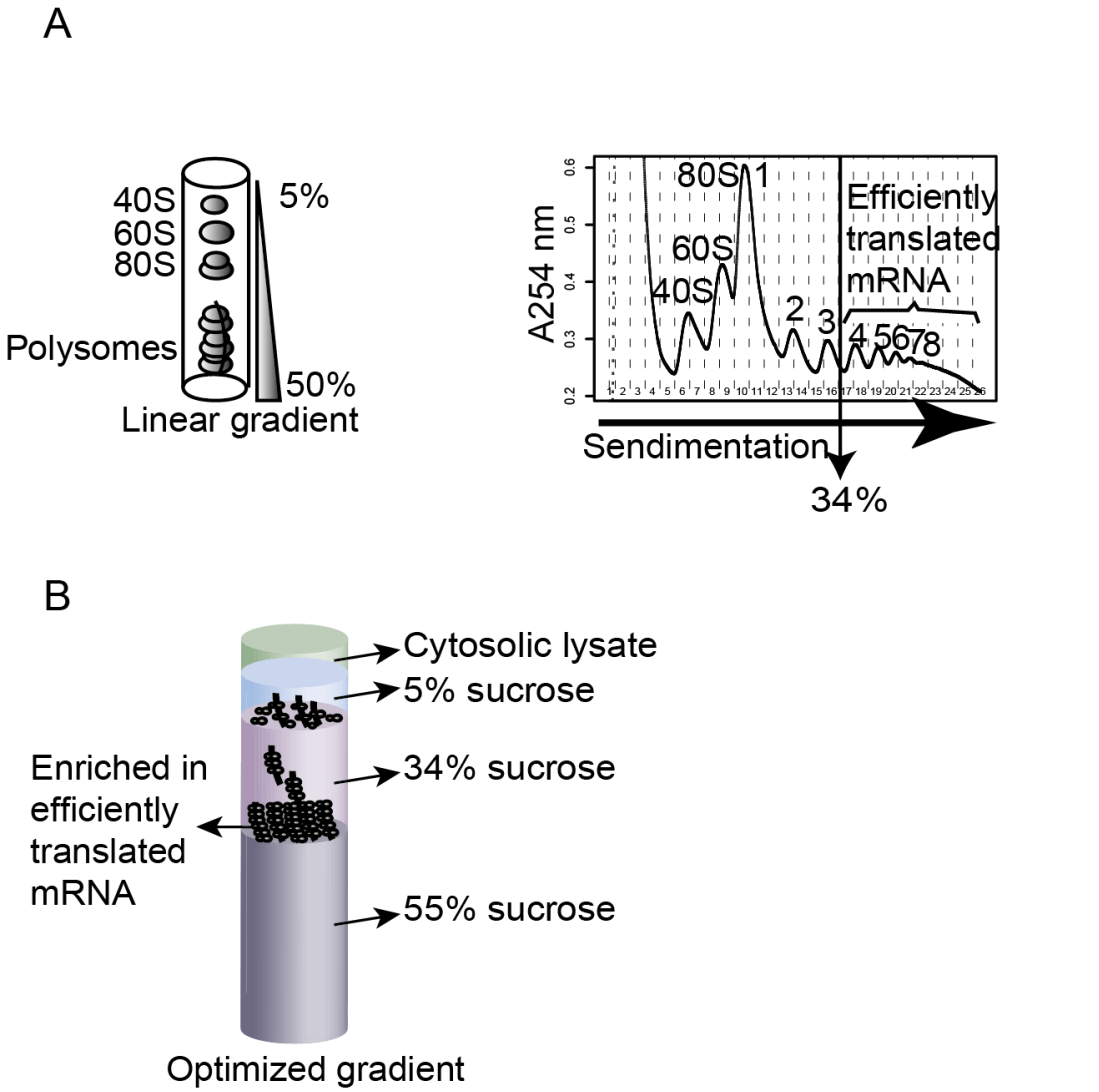

Figure 7. The sketch of the linear and the optimized non-linear gradients. A. The setup of 5% to 50% linear sucrose gradient and polysome profiling from the linear gradient. Ultracentrifugation separates ribosome subunits of 40S and 60S, monosome 80S and polysomes from cytosolic lysate loaded on the linear gradient. (UV signal was captured at the absorbance of 254 nm across the sucrose gradient). Efficiently translated mRNA (associated with more than 3 ribosomes) corresponding to 34% sucrose concentration is isolated from polysome-fractions. B. The optimized non-linear sucrose gradient is composed of 5%, 34% and 55% sucrose layers. This design is to enrich mRNA>3 ribosomes at the interface between 34% and 55% sucrose solution.

3.1.2 Similar translatomes can be obtained from the optimized non-linear and the standard linear sucrose gradient.

Smart-seq2 (297) with an input of 10 ng RNA was performed to construct cDNA libraries for cytosolic RNA and efficiently translated RNA isolated from the optimized non-linear and standard linear gradients, also for cytosolic RNA. These RNAs were from HCT-116 cells with and without p53 (serum starved for 16h). The principal component analysis of sequencing data showed that the first component capturing the main source of variance (52.1%) discriminated RNA source between cytosolic mRNA from polysome-associated mRNA. The following principal components differentiate samples regarding replicate (16.7% of the variance) and p53 status (6.1% of the variance) respectively. This is consistent with that results obtained from the two gradients are comparable. We compared gene expression of polysome-associated mRNA from the optimized and the linear gradient. When FDR threshold was set at 0.1, more differential expression was associated with the optimized gradient approach compared to the linear gradient. However, mRNAs identified as differentially expressed by the optimized non-linear-gradient covers almost all those identified by the linear gradient. Between the two methods, the obtained fold-changes (HCT-116 $p53^{+/+}$ vs. $p53^{-/-}$ cells) showed good correlation with spearman coefficient 0.74. And lower FDRs were obtained by performing the optimized non-linear gradient.

3.1.3 Gene expression is affected by P53 status through various mechanisms including translational buffering.

The optimized non-linear sucrose gradient captures more changes in polysome associated mRNA, which can be seen from the number of mRNAs showing low FDRs for p53-status dependent expression, compared to changes in cytosolic mRNA levels. Correspondingly, more mRNAs (682 mRNAs) exhibits regulation in translational efficiency influencing protein levels as compared to alteration in mRNA abundance (438 mRNAs). Interestingly, many alterations in cytosolic mRNA (373 mRNAs) levels were buffered at the level of mRNA translation. Gene Ontology (GO) enrichment analysis demonstrated that mRNAs with mRNA abundance pattern in $p53^{+/+}$ cells were more involved in functions corresponding to development, migration and extracellular matrix. Neural related functions were more targeted by the genes whose cytosolic mRNA levels were buffered at the level of translation. Therefore, it demonstrated selectivity that certain gene expression mechanism targets certain cellular functions.

3.1.4 The performance of optimized non-linear gradients coupled with smartSeq2 on breast cancer tissues from bio-bank.

The optimized non-linear gradient was applied on a cohort of 161 breast cancer tissues to isolate their efficiently translated mRNA. The Pearson correlation between RINs of the efficiently translated and cytosolic mRNA pools is 0.66. RINs for efficiently translated mRNA were higher than RINs in cytosolic input samples, which cleared the suspicion of isolation technique that caused a low RIN for the pool of efficiently translated mRNA. We

performed RNA sequencing for a set of 5 breast cancer tissues from the cohort. The breast cancer translatomes exhibit a high coverage as proven by that an RPKM (Reads per kilobase per million mapped read) >0.2 that was obtained by mRNAs from >12 000 genes, and an RPKM >1 for >10 000 genes. Hence, the combination of the optimized non-linear gradient with RNAseq2 can be used to comprehensively explore the translatomes from bio-banked small tissue samples.

3.2 PAPER II

Cancer specific effects of insulin on translatomes and metabolomes

Insulin sensitive mRNA translation has been observed in cancer cells which originates from insulin insensitive organs, such as breast. However, whether cancer cells obtain a response resembling those observed in cells from insulin-sensitive organs or whether cancer cells tailor pathological responses is largely unknown. In addition, how insulin orchestrates effects on metabolic program with changes in mRNA translation in cells from insulin sensitive and insensitive organs is neither characterized. Hence, this study is to investigate the effects of insulin and IGF-1 on selective translation and metabolism in cells from insulin sensitive and insensitive organs, coupled with cancer cells originating from an insulin insensitive organ.

3.2.1 Insulin mediated modulation of mTOR-pathway activity in insulin sensitive and insensitive cells

After 12hs starvation, MCF7 and HMEC/hTERT cells displayed nearly complete lack of phosphorylated 4E-BP1 and S6K1. Insulin/IGF1 stimulated such phosphorylation (Fig.8). While starved myotubes expressed the phosphorylated 4E-BP1 and S6K1 which was enhanced by insulin/IGF1 (Fig.8). In all cell types, torin1 nearly completely abrogated the phosphorylation of 4E-BP1 and S6K1 stimulated by insulin/IGF1, for myotubes this level of phosphorylation was substantially lower comparing with the starved condition. Therefore, insulin/IGF1 regulates activity of the mTOR pathway in cells of insulin sensitive or insensitive.

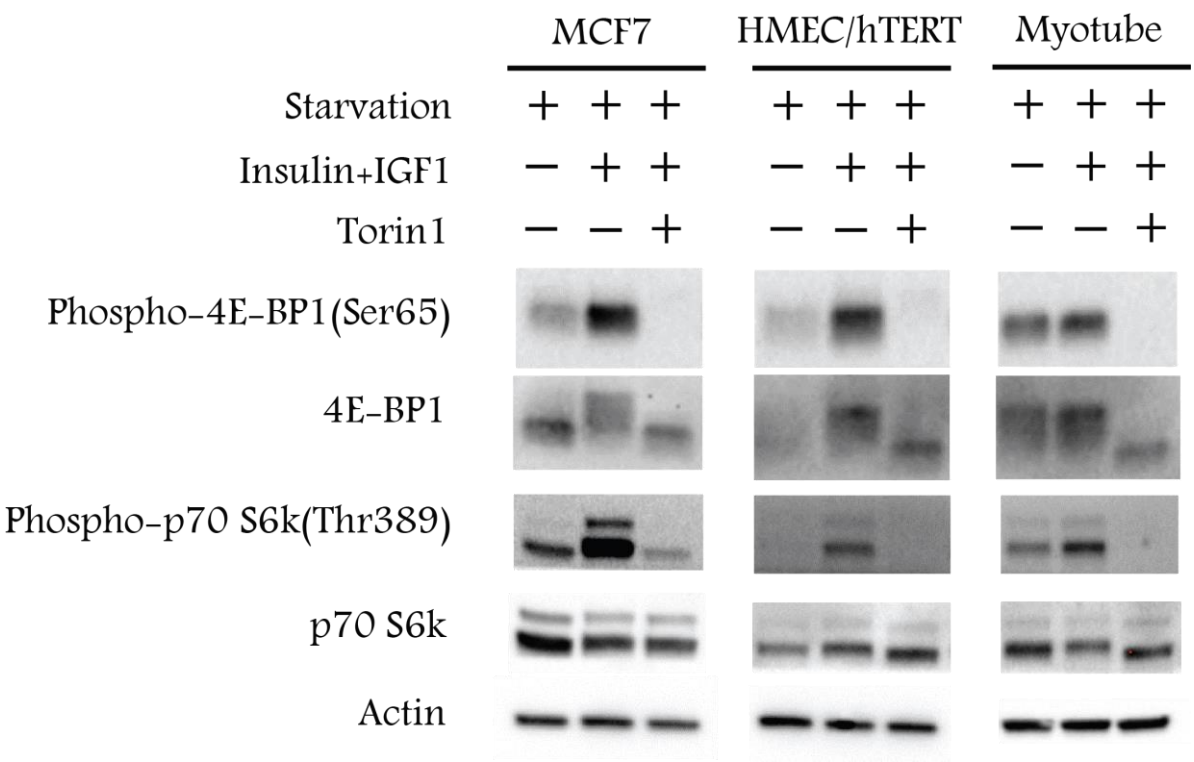

Figure 8. Activity of the mTOR pathway is modulated in cells from both insulin sensitive and insensitive organs following insulin/IGF1 stimulation. Western blotting using extracts from MCF7, HMEC/hTERT and myotubes starved for 12hs followed by stimulation with vehicle or insulin+IGF1 in the presence or absence of torin1. Beta-actin was used as a loading control for MCF7 and HMEC/hTERT cells while alpha-actin was used for myotubes.

3.2.2 Selective mTOR dependent modulation of metabolomes in cells from insulin sensitive and insensitive organs upon insulin/IGF1 stimulation

Regarding the metabolomes of myotubes, HMEC/hTERT and MCF7, cells from insulin sensitive organs, such as myotubes differ in their insulin/IGF1 and mTOR dependent changes as compared to cells from insulin non-sensitive organs, like HMEC/hTERT cells. Intriguingly, insulin/IGF1 and mTOR sensitive metabolomes of cancer cells, for example, MCF7 cells appear to be different as compared to cells from both insulin sensitive and insensitive organs.

3.2.3 Pervasive modulation of translatomes in cells from both insulin sensitive and insensitive organs

Myotubes displayed abundant changes in both mRNA translation and mRNA abundance after insulin/IGF1 treatment. Notably, with mTOR inhibitor torin1, translational buffering turned out to be predominant pattern which indicates that the mTOR pathway modulates translation of the transcriptional program downstream of insulin/IGF1. In contrast, HMEC/hTERT cells showed a prominent buffering pattern independently of whether insulin/IGF1 stimulation was performed with or without torin1. At last, insulin/IGF1 modulated translational efficiencies and to a lesser extent of mRNA abundance and buffering

in MCF7 cells. A similar pattern was also discovered with torin1. Hence, cells from insulin sensitive and insensitive organs modulate gene expression using different modes. Also, mTOR seems to play an important role in deciding which of the mRNAs whose abundance changes upon insulin/IGF1 stimulation will be translated.

3.3 PAPER III

RITA-induced apoptosis requires eIF2α dependent modulation of mRNA translation

TP53 as a famous tumor suppressor gene is commonly mutated in majority of cancers. Thus the reactivation of TP53 by small molecules such as RITA is a promising therapeutic strategy. However, how RITA suppresses cell growth and induces apoptosis is still largely unknown. This paper explored the mechanism underlying these effects of RITA.

3.3.1 RITA induces apoptosis and represses mRNA translation by stimulating eIF2α phosphorylation, independently of TP53 status, oxidative stress and mTOR pathway.

1μM RITA treatment showed a time-dependent increase in phosphorylation of eIF2α in MCF7 cells. eIF2α phosphorylation was also induced by 8hs treatment of 1μM RITA in colon cancer cell lines GP5d and HCT116 both with wild-type TP53. Integrated stress response inhibitor (ISRIB) rescues eIF2B GEF activity independently of the phosphorylation-state of eIF2α. ISRIB reestablished translation following RITA treatment which was confirmed by polysome profiling and quantified by Met-S35 incorporation. So RITA-suppressed translation is dependent on the phosphorylation of eIF2α.

8h treatment with 1μM RITA in MCF7 cells leads to a strong TP53 accumulation and cleavage of the apoptosis marker PARP. RITA reduced the amount of efficient translation mRNA two fold with a concomitant increase in 80S monosomes. The assessment of dependence between RITA's effect and TP53 showed that RITA has a similar effect on PARP cleavage in MCF7 TP53-/- as compared to MCF7 TP53+/+cells, and the same for the proportion of ribosomes engaged in efficient translation. Incorporation of S35-labeled methionine in nascent proteins was also measured; this confirmed a similar reduction of protein synthesis in TP53+/+ and TP53-/- MCF7 cells upon RITA treatment. Thus, RITA induces apoptosis and suppresses global mRNA translation independently of TP53.

Translation remained suppressed even when RITA-induced reactive oxygen species (ROS) accumulation is completely reversed by anti-oxidant NAC. Thus, RITA-associated accumulation of ROS does not explain its effects on mRNA translation. Cells lacking 4E-BPs showed a similar reduction in the proportion of ribosomes engaged in efficient translation as compared to their control cells; no change in phosphorylation of mTOR targets 4E-BP1 or S6K was observed following RITA treatment. Thus, RITA modulates translation independently of 4E-BPs and the mTOR pathway.

3.3.2 PERK activity is required for RITA-mediated suppression of mRNA translation.

Protein kinase RNA-like endoplasmic reticulum kinase (PERK) inhibition by inhibitor GSK2606414 reduced RITA-induced eIF2α phosphorylation in a dose-dependent manner, which rescued RITA-suppressed translation and decreased apoptosis. ER-stress inducer thapsigargin but not RITA stimulated a strong phosphorylation of PERK. Thus, RITA does not induce ER stress nor activate PERK via phosphorylation at the commonly assessed site threonine 980 and it seems that RITA does not appear to induce PERK activity through the ER-stress mechanism.

3.3.3 Modulation of eIF2α phosphorylation largely accounts for RITA's anticancer effects.

Salubrinal, as an inhibitor of eIF2α phosphatases was used to augment eIF2α phosphorylation. 32μM salubrinal acted as an enhancer to eIF2α phosphorylation in MCF7 in addition to a range of RITA concentrations. Comparing with only RITA treatment, 32μM salubrinal with 1μM RITA treatment greatly strengthened induction of apoptosis and inhibited colony formation, and 1μM GSK2606414 combined with 1μM RITA leads to an increased colony formation as compared to only RITA being used. Therefore, RITA's inductive ability on eIF2α phosphorylation is necessary to efficiently induce apoptosis and inhibit colony formation.

3.4 PAPER IV

IL-15 activates mTOR and primes stress-activated gene expression leading to prolonged antitumor capacity of NK cells

Activated NK cells by interleukins have been recently used to treat hematological malignancies. Nevertheless, the therapeutic effect of activated NK cells is largely reduced by the limited post-infusion persistence. In this study, the ability of interleukin-2 and interleukin-15 to maintain the anti-tumor capability of NK cells was compared by a genome wide analysis, the implication of mTOR and STAT-5 signaling was investigated as well. The results revealed that mTOR is of importance in regulating metabolic signaling in immune cells, and comparing with IL-2, IL-15 showed priority in adoptive NK cell treatment for cancer.

3.4.1 Survival and cytolytic activity is primed by IL-15 in human NK cells.

A comparable enhancement in primary human NK-cell cytolytic activity and proliferation was induced by activated IL-15 or IL-2 at a preset concentration ($P<0.05$). While when the cytokine concentration is less than 9.15 ng/mL, IL-15 was more capable to retain NK-cell proliferation as compared to IL-2. Under cytokine deprivation, NK cells treated by IL-15 preserved a greater level of cytotoxicity ($P<0.05$) and underwent a less apoptosis ($P<0.05$) than IL-2-treated NK cells. Retreat IL-15–treated NK cells with IL-15 leaded to higher levels of CD251/CD1371–activated NK than IL-2–treated NK cells re-exposed to IL-2. This indicates that IL-15 and IL-2 hold the different ability to maintain cytokine signaling and/or stimulate the expression of cytokines and/or their corresponding receptors.

3.4.2 After cytokine withdrawal, IL-15 and IL-2 differentially regulate steady-state levels of mRNA, translational efficiencies in NK cells; IL-15 controls the expression of genes involved in mitochondrial function and cell cycle through priming mechanism.

Before and after cytokine withdrawal, cytosolic and polysome-associated IL-2Rα mRNA levels were elevated in NK cells treated with IL-15 as compared to the ones treated with IL-2, while CD56 expression was kept mostly unaffected.

1212 mRNAs exhibited significantly distinct polysome association (FDR<0.15 and fold change>1.5) with slight heterogeneity among donors in NK cells treated by IL-15 versus IL-2 after deprivation of cytokine (573 downregulated genes and 639 upregulated genes), and among them, 29% of the genes (350 genes) were translated differentially. GO analysis showed selected upregulation of genes involved in cell cycle and mitochondrial functions, and it was IL-15 that upregulated majority of mitochondria-related genes.

Four different groups were identified by the clustering of differentially expressed genes spotted under both before and after cytokine deprivation: we found 466 cytokine-primed up-regulated genes (IL-15 vs IL-2), 286 cytokine-primed down-regulated genes, 173 cytokine-induced up-regulated genes and 287 cytokine-induced down-regulated genes. Hence, intriguingly, IL-15 mainly modulates gene expression in NK cells through cytokine priming mode with 62% of all genes. Also, cytokine-primed genes were more inclined to be modulated by differential translation as compared to cytokine-induced genes (with 1.7 fold, P=0.0002 by fisher exact test).

The up-regulated genes by cytokine-induced pattern were mainly involved in cell cycle functions; Cytokine-induced down-regulated genes primarily participate in cell development, motility and cell signaling. Accordingly, genes up-regulated by cytokine-primed pattern were more involved in the functions in metabolic processes, respiration and translation; genes down-regulated by cytokine-primed pattern mainly took part in cell signaling, transcription and developmental progress. Therefore, through cytokine-induced and cytokine-primed modes, IL-15 modulates gene expression programs by addressing different cellular functions, which is consistent with refined NK-cell activity.

3.2.3 mTOR, instead of STAT-5, primarily modulates metabolic and cytotoxic functions in NK cells activated by IL-15 after cytokine deprivation; and IL-15-stimulated NK cells are resistant to cytokine deprivation.

IL-15 induced elevated phosphorylation of S6K which is a substrate of mTOR. Even after 24 hours of cytokine deprivation, comparing with IL-2, IL-15 stimulated S6K phosphorylation was still maintained at a minor level.

IL-15-treated NK cells exhibited a higher capability in basal and maximal cellular respiration as compared to the ones treated with IL-2. Torin-1 treatment deleted a series of biological effects of NK cells induced by IL-15. For example, Torin-1 diminished the S6K phosphorylation associated with IL-15 before and after cytokine deprivation, it also

weakened IL-15 induced respiratory activity ($P<0.05$). Hence, amplified mTOR activity is of independency for refined cytotoxic and metabolic activities of IL-15-treated NK cells after cytokine deprivation. In addition, STAT-5 plus mTOR inhibition decreased the cytolytic functions of NK cells activated by IL-15 at all effector-to-target ratios ($P<0.05$). Prominently, concurrent suppression of mTOR and STAT-5 did not affect NK cells' cytolytic function after cytokine deprivation as compared to using torin-1 only, revealing that prolonged NK-cell activation primed by IL-15 is STAT-5 independent but mTOR dependent. Even though STAT-5 influences some phenotypes of IL-15–activated NK-cell, after cytokine deprivation, metabolic and cytotoxic functions are primarily dependent on mTOR instead of STAT-5 signaling.

A clinically approved protocol was used to assess the functionality of NK cells induced by IL-15. NK cells expanded with IL-15 leaded to a higher expression of CD25 than IL-2. After cytokine deprivation, CD25 expression and certain other activating receptors such as, NKp30, NKp44, CD69, NKG2D and CXCR3 maintained increased on NK cells expanded by IL-15 as compared to IL-2–expanded NK cells.

3.2.4 The expression of IL-15 predicates a better clinical prognosis in B-cell lymphoma patients.

We reanalyzed a pre-published mRNA dataset obtained from tissue samples of B-cell lymphoma patients. We plotted the residuals of a blank Cox model against IL-15 expression to investigate the influence of IL-15 expression on prognosis. The results indicated a poor prognosis for patients with low expression of IL-15.

4 CONCLUSION

Paper I: Polysome-profiling in small tissue samples

The optimized non-linear sucrose gradient collects and enriches the efficiently translated mRNA (>3 ribosomes associated) in merely one or two fractions. The sample handling was largely reduced by 5-10 fold and time was saved for RNA extraction by 10-20 fold. By combining with Smart-seq2, which is developed for single-cells cDNA library construction, this optimized polysome profiling provides the possibility to produce data on translatomes from bio-banked or clinical small tissue samples and from low amount of cells. Notably, this method yields very similar data on translatomes as compared to the standard linear gradient method. Therefore, polysome profiling can be performed on RNA-amount-limited small tissues samples or primary cells.

Paper II: Cancer specific effects of insulin on translatomes and metabolomes

The metabolomes of myotubes and HMEC/hTERT cells were modulated by insulin/IGF1 in an mTOR dependent manner, but their metabolic pathways affected were different. Intriguingly, MCF7 cells tailored their metabolic response to insulin/IGF1 stimulation. Upon insulin/IGF1 stimulation, HMEC/hTERT, myotubes and MCF7 cells displayed distinct changes in mRNA abundance, translation and translational buffering. So, MCF7 has programmed pathological responses to insulin stimulation, which differs from those discovered in insulin sensitive or insensitive cells.

Paper III: RITA-induced apoptosis requires eIF2α dependent modulation of mRNA translation

RITA induces apoptosis and represses mRNA translation by inducing eIF2α phosphorylation independently of TP53 status and the mTOR pathway. Suppression of eIF2α phosphorylation by inhibition of the upstream kinase PERK rescues mRNA translation with a concomitant reversal of RITA's effects on apoptosis and clonogenicity. Correspondingly, RITA's anti-cancer activity can be enhanced by inhibiting dephosphorylation of eIF2α. Hence, modulation of mRNA translation via phosphorylation of eIF2α is required for RITA's anti-cancer properties.

Paper IV: IL-15 activates mTOR and primes stress-activated gene-expression leading to prolonged antitumor capacity of NK cells

The studies in this paper prompt the understanding of the establishment and maintenance of cytokine-activated NK cells; the paper also reveals the significance of mTOR-mediated metabolic and cytotoxic effects of immune cells by examining the cytokine-mediated gene expression programs and downstream cellular functions of NK cells. The paper argues for the application of IL-15 for adoptive NK-cell therapy, and the research on NK cells also enlightens scientists to perform similar studies on other immune cells.

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With passion and curiosity about science, I took off from China and landed in Sweden in September 2013 to pursue my Ph.D at Karolinska Institutet. The first taste of Stockholm in early autumn was sweet and beautiful; everything seemed new and exciting for me, especially my research life in my new lab and new working atmosphere at KI. My group and department are international and I met people from different countries with their own culture. Each one has his or her own background and working style, which also code for their specific merit that I can learn and benefit from. During this four and half years long journey, there was excitement and depression, joy and sadness, progress and retrogress, success and failure, gain and loss, all these feelings display as a contradiction like the saying in philosophy that contradiction exists wherever and whenever, and this period of study will definitely be a valuable experience in my life.

From a newborn Ph.D student with M.D background, many techniques and specific knowledge in mRNA translation was new to me, but the new things always trigger my great interest. Polysome fractionation by ultracentrifugation was my favorite at the beginning; western blot came the second, Smart-seq2, cell culture, Bioanalyzer and Qubit for RNA quality and quantity measurement, etc. They were not friendly towards me at the beginning, but now after thousands of times trying and groping, they became my everyday accompanying friends.

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