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MICRORNAS AND THEIR PROCESSING FACTORS IN CAENORHABDITIS ELEGANS AND HUMAN CANCERS

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MicroRNAs and their processing factors in Caenorhabditis elegans and human cancers

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To my friends and family, this would not be possible without you!

It is difficult to sum up the contributions all of you made into realizing this thesis. I am grateful for your encouragement and support for allowing me to follow science.

To borrow the words of Sir. Isaac Newton:

“If I have seen further, it is by standing upon the shoulders of giants”.

Thanks for letting my see further than I could imagine. Thanks for letting me stand on your shoulders.

ABSTRACT

MicroRNAs (miRNAs) are small noncoding RNAs approximately 20-22 nucleotides (nt) long. These small RNAs (sRNAs) was initially discovered in *Caenorhabditis elegans* (*C. elegans*), but is conserved in over 50 animal species. According to miRbase, a database of miRNA sequences from various species, humans have over 2500 different miRNAs and estimated to regulate over 60% of the protein coding genes in humans. Due to their role in gene regulation, they are important for controlling key processes in the cell, and changes in miRNA expression in cell can have catastrophic consequences.

In this thesis we explore the role of miRNA and their biogenesis factor. We begin by studying the first miRNA to be identified in humans, *let.7*. We use the *C. elegans* model system with different genetic strains to describe an unknown role for this miRNA. The unravelling of this new role highlights the importance of miRNA as important regulator in cells not only for protein coding transcripts but also transcripts that do not code for proteins. The findings of this study opens up new and exciting research possibilities for miRNAs.

Because of miRNAs importance in controlling key cellular processes in cells. Changes in their expression patterns can lead to severe effect. In study II we explore one such event, development of human tumors. We characterize the miRNA profile of different types of ovarian tumors to identify biomarkers that can be used in clinics and to understand the pathology of the tumors to find an effective cure. Using only a few miRNAs we can characterize different types of ovarian tumors based on their miRNA profile. We also identify a miRNA target that could potentially be explored for therapy.

The miRNA biogenesis factors are important to ensure proper production of miRNAs. Their role in the miRNA biogenesis is well characterized, but very little is known if they possess other biological function besides miRNA production. In study III we describe a new role for TARBP2, which is responsible for miRNA maturation. Hundreds of thousands RNAs are transcribed from a single cell, and each RNA molecule have different sequences or structures that are important for their function/regulation. We used computational biology to predict all the potential TARBP2-interaction sites on messenger RNAs, and found several in the 5' untranslated region (5'UTR). The 5'UTR are just upstream of the initiation site of protein synthesis, and usually contain important regulatory sequences. We explore how the TARBP2 interaction with the 5'UTRs can affect gene expression. In particularly we found a gene that is important for regulation of a process in the cell called autophagy.

In this thesis we investigate the role of miRNAs and their processing factors in *C. elegans* and humans. We describe a new role for miRNA as regulator of noncoding transcript, and we explore the possibility of using them as biomarkers in ovarian tumors. Finally, we describe a new role for the miRNA biogenesis factor TARBP2 as a regulator of autophagy.

LIST OF SCIENTIFIC PAPERS

The thesis is based on the following papers, which will be referred throughout the text by their Roman numerals.

I. Zisoulis DG, Kai ZS, **Chang RK**, Pasquinelli AE.
Autoregulation of microRNA biogenesis by let-7 and Argonaute.
Nature, 486:541-4 (2012)

II. **Chang RK**, Li X, Mu N, Hrydziusko O, Garcia-Majano B, Larsson C, Lui WO.
MicroRNA expression profiles in non-epithelial ovarian tumors.
Int J Oncol., 52:55-66 (2018)

III. **Chang RK**, Gao J, Li X, Garcia-Majano B, Wiman K, Lui WO.
TARBP2 regulates autophagy through post-transcriptional regulation of ATG2A expression.
Manuscript

CONTENTS

LIST OF ABBREVIATIONS

ATG	Autophagy-related gene
bp	Base pairs
C. elegans	*Caenorhabditis elegans*
cDNA	Complementary deoxynucleic acid
CLIP	Crosslinking RNA immunoprecipitation
CPU	Central processing unit
dNTP	Deoxynucleoside triphosphate
ddNTP	Dideoxyneocleoside triphosphate
ds-cDNA	Double-stranded complementary DNA
dsRNA	Double-stranded ribonucleic acid
GB	Gigabytes
GFP	Green fluorescence protein
HITS-CLIP	High-throughput sequencing of RNA isolated by crosslinking immunoprecipitation
Hz	Hertz
LTR	Long terminal repeat
miRNA	microRNA
mOGCT	Malignant ovarian germ cell tumor
mosSCI	Mos1-mediated single copy insertion
mRNA	Messenger RNA
nt	Nucleotides
OGCT	Ovarian germ cell tumor
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
Pre-let-7	Precursor let7 transcript
Pri-let-7	Primary let-7 transcript
RBP	RNA binding protein
RFP	Red fluorescence protein
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid

RNAi	RNA interference
RNA-IP	RNA immunoprecipitation
RT-PCR	Reverse transcription-polymerase chain reaction
SCST	Sex-cord stromal tumor
TAR	Trans-activation responsive
UTR	Untranslated region
wt	Wild-type

1 *CAENORHABDITIS ELEGANS* AS A BIOLOGICAL MODEL SYSTEM

In 1963 Sydney Brenner expressed his concern for the future of molecular biology in a letter to Max Perutz, the chairman of the medical research council's laboratory of molecular biology. He was concerned that the classical problems of molecular biology could not be solved using conventional methods, and if molecular biology would progress we needed to look beyond the prokaryotic genetic. He proposed that a small eukaryotic model organism, the roundworm with notable development and nervous system would take molecular biology into the next phase of molecular biology[1].

In fact, the thought of using nematodes as model system was already born in 1940s. Two research groups, Nigon and Doherty, worked close together to refine cultivating conditions for nematodes by analyzing the animal's reproduction and nutrition. Despite their success, they were unable to receive funding for their projects. At the time, Sydney Brenner was a well-known bacterial geneticist who understood the limitations of bacteria and was inspired by the work of Richard Goldschmidt's work on neuronal cell invariance and nervous system connectivity of small intestinal roundworm. Sydney Brenner experimented with many species of nematodes, and managed to refine the cultivating methods for *C. elegans*[1]. Sydney Brenner's fame helped him secure funding where his predecessors failed, but thanks to his work we have procured critical insight into many key cellular and biological processes including cell death[2-5], temporal cell fate determination[6], RAS signaling[7; 8], nonsense-mediated decay[9] and RNAi mechanism[10].

C. elegans are small, inexpensive, easy to store, short life span, and has the ability of self-fertilization, which make it an ideal genetic model system to cultivate and study in labs. The transparent body of *C. elegans* together makes it ideal to track developmental processes (cell differentiation and proliferation). Having the entire *C. elegans* genome sequenced makes it extremely useful to understand human genetics, due to its extensive homology with our genome [11-13]. The ease of performing forward and reverse genetics in *C. elegans* have made it an invaluable model system to understand basic human cellular and biological processes. The use of tagged fluorescent proteins allows one to follow developmental processes, screen for mutants affecting cell development and function, isolate cells, and characterize protein interactions in vivo[14-16].

The ease of cultivating and maintaining genetic strains of *C. elegans* makes it a popular model organism among geneticist. The animals are cultivated on agar plates containing a lawn of *Escherichia coli* (*E. coli*) which they feed on. Usually the animals are cultivated in 20ºC, but

by altering the temperature, one can control the rate of development of the animals. Shorter exposures to higher temperature can aid in the production of male progenies. To study biochemical processes, it can be advantageous to synchronize the animals to ensure they are in the same developmental stage. This is achieved by dissolving the cuticle of gravid adult animals with bleach. After removing the bleach, eggs are harvested and incubated until they hatch into L1 larvae. Putting the animals on agar plates with bacteria allow them to develop through all the life cycles of the animals[1].

1.1 LIFE CYCLE OF C. ELEGANS

Embryogenesis starts with cell division from a single cell to over 600 undifferentiated cells[17]. After the larvae crawls out of the eggshells the animals have a functioning feeding apparatus, gut, nervous system and muscles. The animals begin to eat and develop through four larval stages (L1-L4). Lethargus is a sleep-like stage, which marks the end and the beginning of each larval stage. During lethargus the animals remain in a quiescent state[18]. After L4 stage, adult animals will produce progenies either asexually or sexually, and the adults can continue living for several weeks before dying of senescence. Scarcity of food can push L2 larvae into a larval stage called dauer[19]. During dauer, animals form cuticles throughout their body, which protect them from environmental stress. However, the cuticle also prevents the animals from eating and causes developmental arrest. While in dauer, the animals can survive for months without food, but once they are transferred to agar plates with food the cuticle starts to molt, and they progress into L4 stage (Figure 1).

The ability to self-fertilize makes *C. elegans* a highly attractive model organism to study genetics. Genetic changes introduced in adult animals (P0) can be easily maintained and propagated in first (F1) and second (F2) generations progenies without mating[1]. The animals can also be frozen, which make the recovery and maintenance of different genetic strain simpler. The Caenorhabditis Genetics Center (CGC) was formed in 1978 with the aim of curating and distribute different genetic strains of *C. elegans*[20]. Today, any *C. elegans* lab can request thousands of different genetic strains, submitted by others, in their studies.

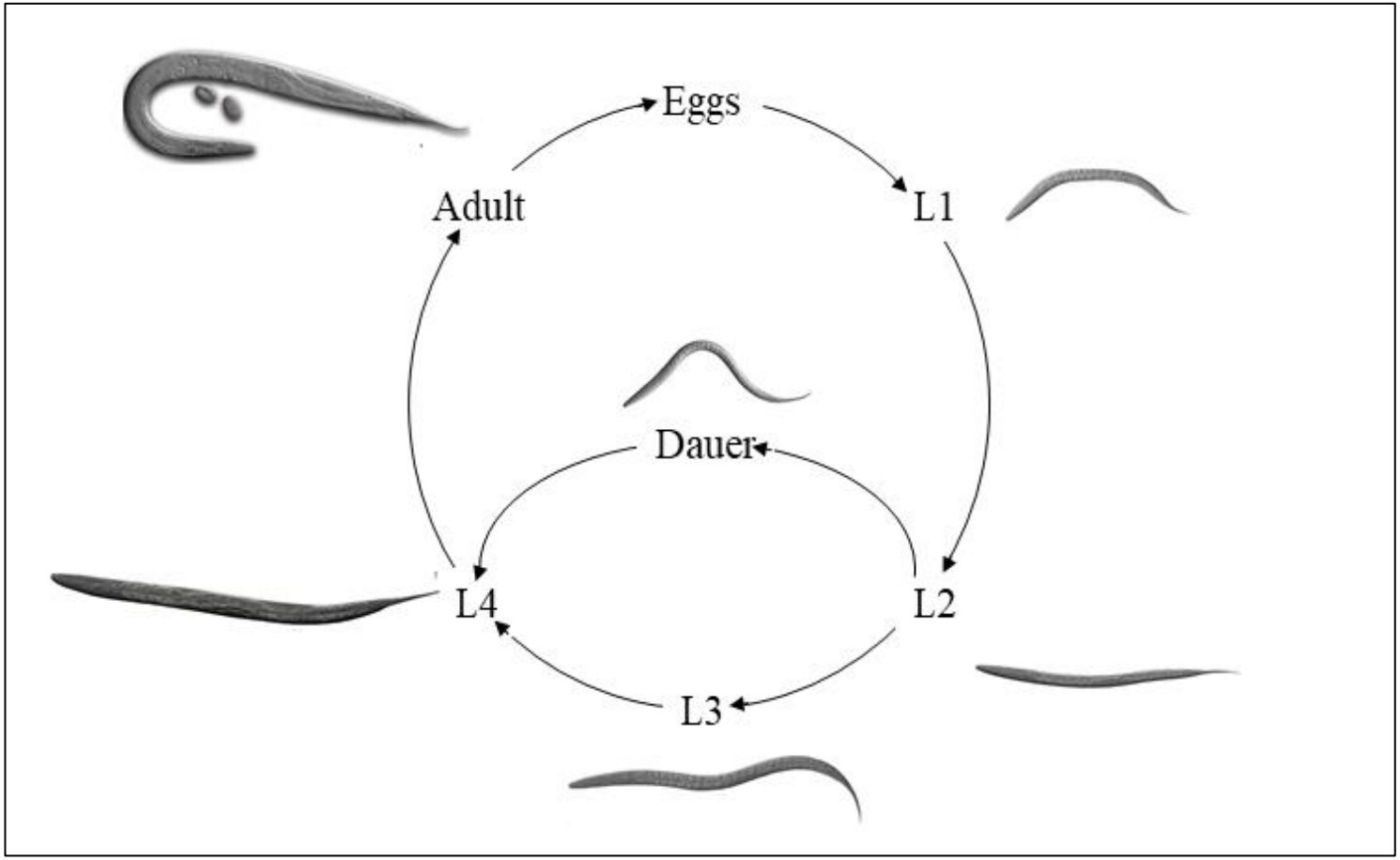

Figure 1. Life cycle of *C. elegans*

After embryogenesis, *C. elegans* begins to eat and develop through four larval stages L1-L4. Starvation forces *C. elegans* to go into dauer, and once food is re-introduced the dauer animals develop into L4 animals. L4 animals develop into adult animals and can reproduce asexually or sexually.

2 MICRORNA

MicroRNAs (miRNAs) are endogenous small RNAs ~21 nucleotides (nt) that regulate gene expression post transcriptionally by binding to 3' untranslated (UTR), coding sequences or 5'UTR of target messenger RNAs (mRNAs), and can lead to translational inhibition or degradation of mRNA[21]. Before the discovery of miRNAs, the scientific efforts have been mainly focused on protein coding transcripts. After it was discovered that miRNA can act as gene regulators that control various processes such as cell fate specification[22], apoptosis[23] and metabolism[24]. It became evident that the noncoding regions of the genome contained genetic material that are important for cell's functions.

2.1.1 *lin-4*, the first miRNA discovered in *C. elegans*

In *C. elegans lin-14* expression levels are important for stage-specific cell fate in the L1 through L3 stage. Animals without *lin-14* retain L1 specific cells in L3 stage. LIN-14 protein levels oscillate in L1 through L2 while the mRNA remain constant. Suggesting that post transcriptional regulation of *lin-14* was important for temporal fate of stage-specific cells[6]. Genetic studies of *lin-4* revealed it is a regulator of *lin-14*[25]. Furthermore, animals with mutations in the 3'UTR of *lin-14* showed no decrease in LIN-14 levels when *lin-4* was expressed, suggesting that the mechanism of *lin-4*-mediated downregulation of *lin-14* mRNA is a post transcriptional event and *lin-14* 3'UTR is necessary for this mechanism[26; 27].

Surprisingly, when Lee and colleagues cloned the *lin-4* gene they discovered that the gene does not encode for any proteins[26]. Instead it contains two 22 nt long untranslated RNAs, and the more abundant form is expressed during early L1 stage[28]. Coincidentally this is the time when LIN-14 protein decreases, implicating that *lin-4* RNA might be directly involved in reducing the level of LIN-14 protein. The discovery that the *lin-4* noncoding RNA can interact with the *lin-14* 3'UTR through complementary Watson-Crick base-pairing was the first evidence that small noncoding RNAs can regulate expression of genes[26; 27].

2.1.2 *let-7*, the first miRNA discovered in humans

In 1999, *lethal-7* (*let-7*), a small noncoding RNA was discovered in *C. elegans* that could control the expression of several heterochronic genes. *let-7* is expressed during L3 stage but peaks at L4 stage, and genetic strains of *let-7* mutants revealed that *let-7* was important for L4-to-adult transition. Without the expression of *let-7* in L4 the animals die due to bursting of the vulva[29]. The *let-7* became the second known miRNA after *lin-4*, but more importantly *let-7* was the first miRNA to be identified in humans. Bioinformatical analysis further revealed that *let-7* was conserved across several species, suggesting that miRNAs have a gene regulatory

role that expands beyond *C. elegans*[30]. Although *let-7* is well conserved from *C. elegans* to humans, several differences can be found between various animal species. For example, nematodes and fruit flies have one copy of *let-7* miRNA, while higher animals have several copies of *let-7* miRNAs throughout their genome. The copies of *let-7*, found in higher animals, usually have overlapping function and share sequence similarity, and are categorized as *let-7* family members[31-34]. Studies have shown that *let-7* family members can promote differentiation during development and function as tumor suppressors in various cancers[29; 35-38].

2.2 MIRNA BIOGENESIS PATHWAY

MiRNA are endogenous small RNAs that are produced from non-coding transcripts with hairpin structure. miRNAs are first transcribed as a long RNA transcript called primary-miRNA (pri-miRNA). The secondary structures of pri-miRNAs are recognized and bound by DGCR8 (Pasha in flies and worms) and Drosha. Drosha is an endoribonuclease that will release the precursor-miRNA (pre-miRNA) from the pri-miRNA. Exportin 5 exports pre-miRNAs from the nucleus to the cytoplasm. In the cytoplasm the pre-miRNA is processed into mature miRNA duplex by the endonuclease Dicer together with TARBP2 and PACT. The mature miRNA is loaded into Argonaute protein by strand selection, and the miRNA guides the Argonaute protein to its targets by forming an imperfect binding to the target mRNA that lead to translation repression either by degradation of the mRNA or blocking of translational initiation/elongation[39] (Figure 2).

2.2.1 The non-canonical miRNA biogenesis pathway

While the majority of miRNAs are generated through the canonical miRNA biogenesis pathway, a small subset of miRNAs is processed independent of Drosha or Dicer.

Mirtrons are encoded in intronic regions, and is generated through an mRNA splicing mechanism independent of Drosha. The spliced-out intron is converted to a pre-miRNA-like structure that can be cleaved by Dicer[40-42].

miR-451 is an unique miRNA, it requires Drosha but not Dicer for its biogenesis[43-45]. Drosha processed *pre-miR-451* is too short for Dicer cleavage. Instead, *pre-miR-451* is loaded directly onto Argonaute and the 3' end is trimmed to generate the mature *miR-451*[46].

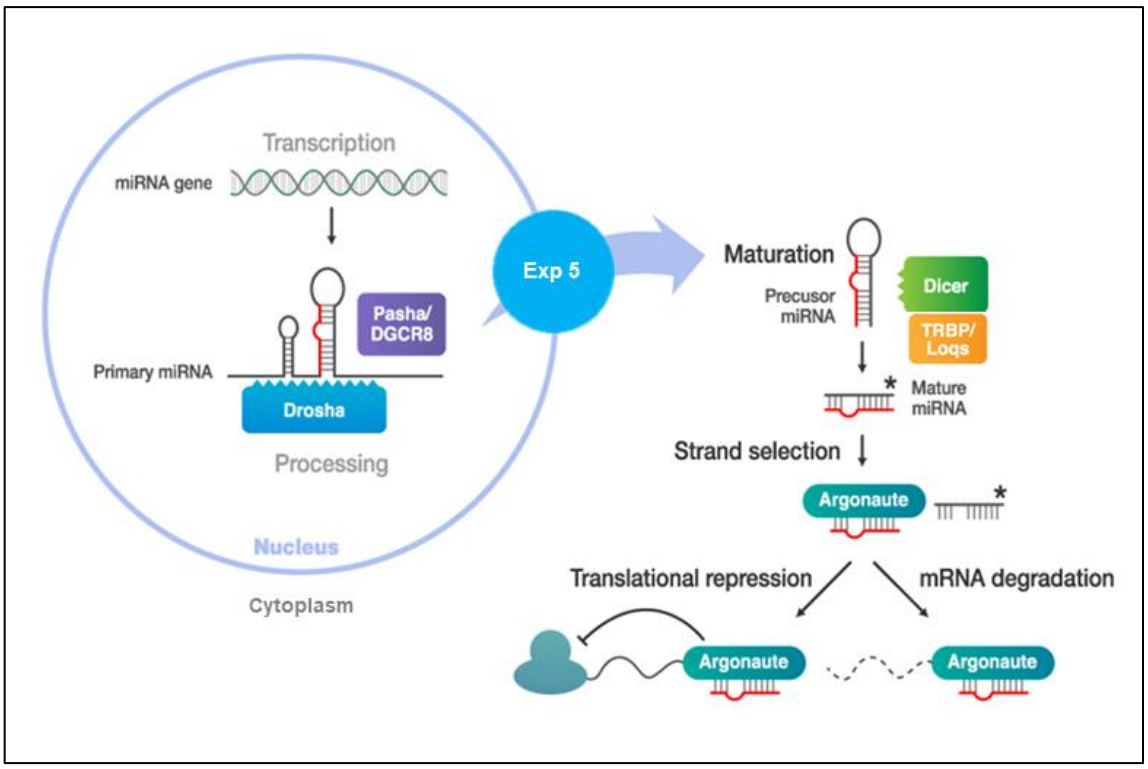

Figure 2. Canonical miRNA biogenesis pathway

Primary miRNAs are transcribed, and processed into precursor miRNAs by Drosha and DGCR8, Pasha in flies and worms. Precursor miRNAs are transported to the cytoplasm by Exportin 5. Dicer and TARBP2, Loquacious in flies, cleaves precursor miRNA to produce a mature miRNA duplex. The miRNA is loaded into Argonaute, and the miRNA guides Argonaute to its target transcript where it either initiates translational repression or mRNA degradation.

2.3 MECHANISMS OF MIRNA REGULATION

MiRNAs are described as regulators of gene expression. miRNAs recruit RISC complex to target mRNAs through Watson-Crick base-pairing between miRNAs 5'-proximal "seed" (nucleotide 2-8) sequence to matching sequences in the mRNA[39]. Argonaute and glycine-tryptophan repeat-containing protein of 182 kDa (GW182) are central components in the miRISC complex. The biological outcome of the miRISC-mRNA interaction leads to several consequences. The most common are either mRNA degradation or translational repression[47-53].

2.3.1 mRNA degradation

mRNAs usually contain a 7-methylguanylate cap (m^7G) at their 5' end and a stretch of adenosine monophosphates (poly(A)) at their 3' end. Both the m^7G and poly(A) are required for stabilization of mRNA and cap-dependent translation of mRNAs, and removal of either of the two initiates degradation of mRNAs[54-56]. Functional studies show that GW182 recruit

CCR4-NOT and PAN2-PAN3 deadenylase complexes, which facilitates mRNA decay via deadenylation of poly(A) followed by subsequent decapping of m^7G[57; 58]. Without m^7G and poly(A) the mRNA is vulnerable to degradation by exonucleases[59].

2.3.2 Translational repression

Poly(A) binding proteins (PABP) interact with eukaryotic initiation factor 4G (eIF4G) to initiate translation[60; 61]. GW182 blocks the PABP-eIF4G interaction thus preventing translational initiation[62; 63]. Another study demonstrated that mammalian Ago2 can associate with m^7G, and outcompete eIF4E to associate with m^7G of mRNAs, which also leads to translational initiation block[64]. Fruit flies have five different Argonaute proteins: *Ago1*, *Ago2*, *Ago3*, *piwi* and *aub*[65]. AGO1 and AGO2 display different mechanism of miRNA-mediated mRNA regulation[66]. AGO1 elicit deadenylation and lead to degradation of target mRNA whereas AGO2 induces translational repression through interaction with eIF4E[66; 67]. Humans have eight different Argonaute proteins[68], but it does not seem humans employ the same strategy for miRNA-mediated mRNA regulation as flies. It is still unclear how miRNAs initiate mRNA degradation or translational repression in humans.

3 TARBP2, A RNA BINDING PROTEIN INVOLVED IN MIRNA BIOGENESIS

TARBP2 (Trans-Activation-Responsive RNA binding protein 2) was first discovered as a cellular protein that activates expression of the HIV-1 virus through binding to the TAR RNA loop structure of the LTR (long terminal repeat) together with the viral tat-protein[69]. The *TARBP2* gene is located on human chromosome 12 and produces two different isoforms. TARBP2 isoform 1 has additional 21 amino acids at the amino-terminus that is known to interact with Dicer for miRNA processing (Figure 3A). The function of TARBP2 isoform 2 is still unclear. TARBP2 has two double-stranded RNA (dsRNA) binding domains and a domain called C4 that is responsible for protein-protein interactions[70].

TARBP2 has been found to associate with PKR (Protein Kinase RNA-activated) through an RNA-independent interaction and inhibits its activities[71]. PKR is a dsRNA-dependent kinase that phosphorylates eIF2α causing a blockage of global mRNA translation in response to cellular stress or infection. Using a proteomic approach, Chi et al. identified numerous TARBP2-interacting proteins that are known to be involved in protein synthesis, RNA processing, DNA transcription and cell growth[72].

Besides its protein-protein interactions, TARBP2 is also known to bind to other RNA stem-loop structures in addition to pre-miRNAs. One of the examples is the HIV-1 TAR RNA, as described above. TAR RNA element is a stable stem-bulge-loop structure that is present at the 5' end of all HIV-1 mRNAs[73]. This structure was originally discovered as the target for the Tat protein (the trans-activator of HIV)[74] (Figure 3B). TAR-containing transcripts can be translationally inhibited through PKR, however the translation repression can be alleviated by interaction with TARBP2[75], La autoantigen[76], Staufen 1[77] or DDX3[78]. TAR RNA is not restricted to translational regulation; it can also interact with nuclear transcription factors[79], and regulates transcription of viral genes[80-82].

Interestingly, TAR RNA-like structures can also be present in cellular genes. In mouse, protamine RNA binding protein (Prbp), the mouse homolog of human TARBP2, can bind to the TAR RNA-like structure located in the 3'UTR of protamine 1 (Prm-1) and control its translation during spermatogenesis[83; 84] (Figure 3C). Goodarzi *et al.* also showed that TARBP2 binds to structural RNA stability elements (sRSE) of *APP* (amyloid precursor protein) and *ZNF395*, and destabilizes the transcripts[85] (Figure 3D).

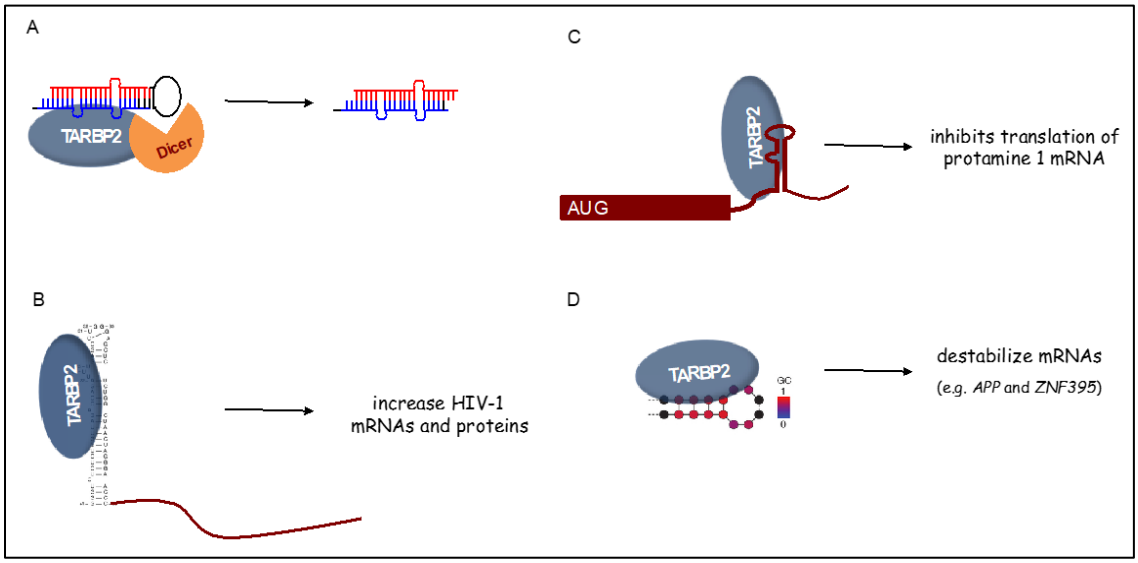

Figure 3. TARBP2 binds to specific RNA structures

A, TARBP2 binds to pre-miRNAs and regulates pre-miRNA processing by Dicer. **B**, TAR RNA element is found in all HIV-1 mRNAs that can interact with TARBP2 for translational/transcriptional regulation. **C**, The mouse homolog of human TARBP2, Prbp, can bind to specific stem-loop structure in the 3'UTR of protamine 1 mRNA and inhibits its translation. **D**, TARBP2 destabilizes the sRSE-containing transcripts *APP* and *ZNF395* that promotes metastasis.

4 AUTOPHAGY

Degradation of cytoplasmic components within lysosomes is orchestrated by a cellular process called autophagy. It is a nonselective degradation that differs from the ubiquitin-proteasome degradation pathway. Autophagy play many roles such as starvation-adaption, intracellular protein and organelle clearance, development, anti-aging, elimination of microorganisms, cell death, tumor suppression and antigen presentation[86-90]. The most notable function of autophagy is maintaining the homeostasis of cytosolic components in the cells, "basal autophagy", or response to produce amino acids during starvation, "induced autophagy".

4.1 MTORC1 A REGULATOR OF AUTOPHAGY

When cells senses low levels of amino acids, mammalian target of rapamycin complex 1 (mTORC1) signaling is inhibited. mTORC1 is a major regulator of autophagy, which in presence of amino acids and growth factors inhibits the initiation of autophagy[91]. Inhibition of mTORC1 triggers autophagy activation of downstream proteins needed to execute the autophagic program. Once amino acids are released from autophagic breakdown of proteins, reactivation of mTORC1 leads to inactivation of autophagy.

4.2 AUTOPHAGOSOME BIOGENESIS

The autophagosome biogenesis consists of initiation of autophagy signaling sequestration of cytoplasmic components by the phagophore or isolation membrane, and the formation of the autophagosome followed by fusion with the lysosome. Once the autophagosome-lysosome forms degradation process can begin[92]. A number of proteins are involved in autophagosome biogenesis, and the process of autophagy is tightly controlled. Genetic studies of autophagy machinery in yeast have identified 37 factors known as ATG (autophagy-related genes), less than half of these are thought to be involved in autophagosome biogenesis[93; 94]. Autophagosome biogenesis can be broken up into three stages: initiation, nucleation and expansion (Figure 4).

4.2.1 Initiation

Three main complexes are required for the initiation and formation of an autophagosome. The first complex is the unc-51-like kinase 1 (ULK) complex, which consist of ULK1, ATG13, FAK family kinase-interacting protein of 200 kDa (FIP200) and ATG101. Induction of autophagy releases the ULK complex from mTORC1, the ULK complex translocates to autophagy initiation sites, and regulates recruitment of a second kinase complex, vacuolar protein sorting 34 (VPS34) complex[95-97].

4.2.2 Nucleation

The VPS34 complex consist of VPS34, Beclin 1, VPS15 and ATG14-like (ATG14L), and leads to production of phospholipid phosphatidylinositol 3-phospate (PI3P) where autophagosome formation is initiated. The PI3P acts as a signaling molecule for the recruitment of WD repeat domain phosphoinositide-interacting protein 2 (WIPI2B) and double FYVE containing protein 1 (DFCP1). Together with other proteins this lead to formation and expansion of the phagophore[98-102].

4.2.3 Expansion

The third complex, consisting of the ATG16L1–ATG5–ATG12 conjugation machinery, is essential for the lipidation of microtubule-associated proteins 1A/1B light chain 3B-II (LC3-II). The lipidated LC3-II associates with newly forming autophagosome membrane. LC3-II remains on mature autophagosomes until its fusion with lysosomes[103; 104].

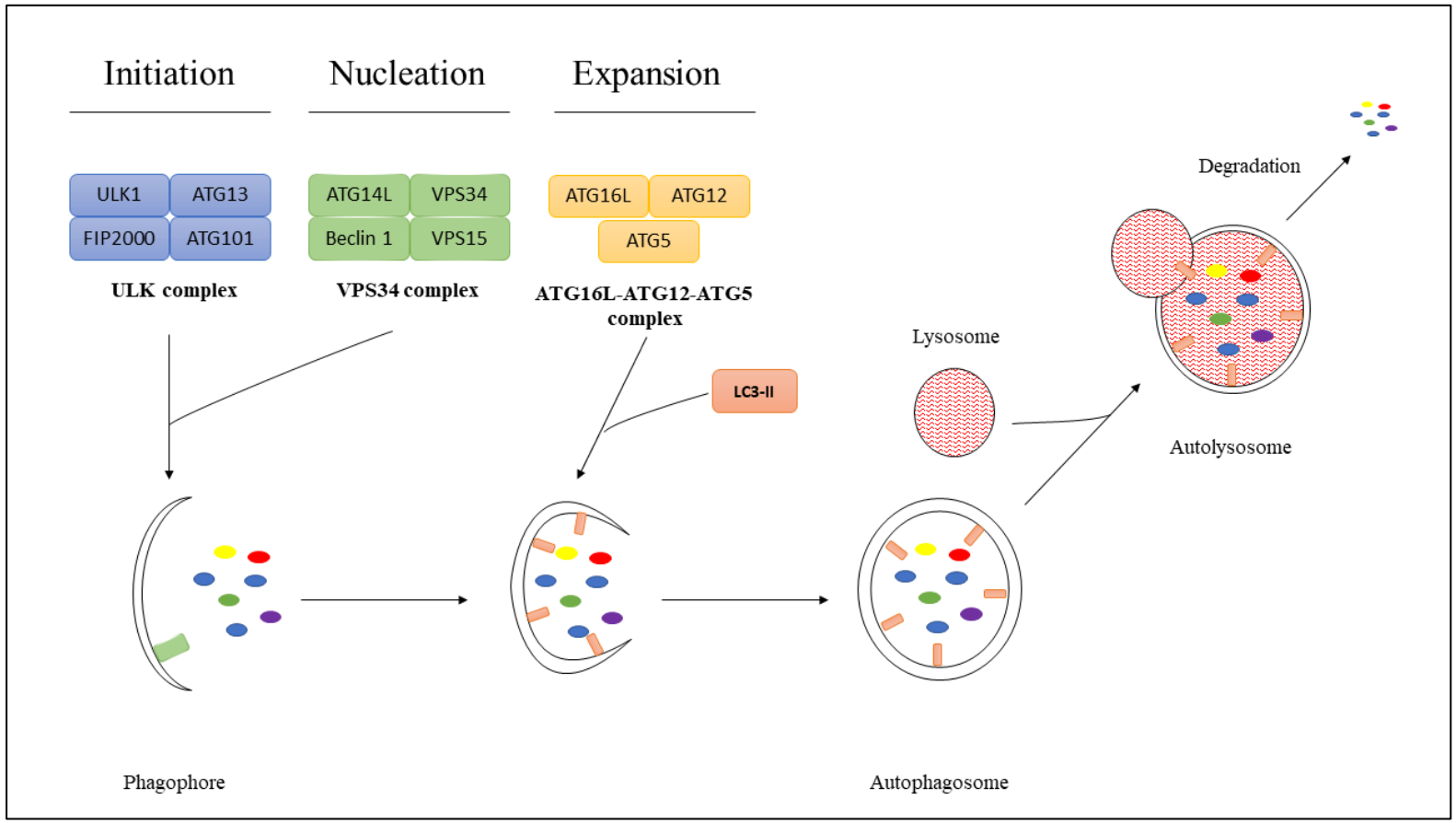

Figure 4. Autophagosome biogenesis

Initiation and formation of autophagosome starts with ULK complex. The ULK complex recruits a second complex, the VPS34 complex. This leads to production of PI3P, which leads to expansion of the phagophore. Lipidated LC3-II associates with the autophagosome membrane. After fusion with lysosome, the autolysosome starts degradaing cargo inside the membrane and amino acids are released and can be reused in protein synthesis.

5 AIMS OF THE STUDY

5.1 STUDY I:

To understand the underlying mechanism of *let-7* and ALG-1 binding to primary *let-7*.

5.2 STUDY II:

To characterize miRNA profile in non-epithelial ovarian tumors.

5.3 STUDY III:

To understand the underlying mechanism of TARBP2 binding to ATG2A.

6 MATERIALS AND METHODS

6.1 NEMATODE CULTURE AND STRAINS

Our study of ALG-1 and *let-7* required different strains of *C. elegans*. The advantage for nematode aficionados is the availability of different genetic worm strains that are stored and curated by Caenorhabditis Genetics Center (CGC)[20].

Wild-type (N2 Bristol), *let-7* mutant (n2853), *alg-1* null (gk214) and *let-7* null (mn112) were obtained through CGC. Other strains used in study I were generated by mos1-mediated Single Copy Insertion (mosSCI) system and crossing.

6.1.1 MosSCI

The mosSCI system allow us to introduce a DNA template through homologous recombination to a site where DNA break occurred. This insertion is directed to a specific site in the worm genome[105] (Figure 5).

Worm strains PQ-320, 402 and 404 were generated by mosSCI. PQ-320 express two copies of wt *pri-let-7,* from chromosome X and II. PQ-402 and PQ-404 express wt *pri-let-7* and *pri-let-7* transcript without the ALG-1 binding site (Δ*alg-1*) from chromosome X and II respectively.

6.1.2 Crossing

PQ-425 and PQ-426 were created by backcrossing PQ-320 and PQ-404 with mn112 worms. mn112 worms are *let-7* null, and adult worms without *let-7* will die due to bursting of vulva. The viable worms are screened and crossed with n2853 worms. This creates a transgenic worm that express wt *pri-let-7* (from PQ-320) or *pri-let-7* without the ALG-1 binding site (from PQ-404) from a single copy transgene integrated in chromosome II and the endogenous *let-7* (n2853) transcript from the X-chromosome.

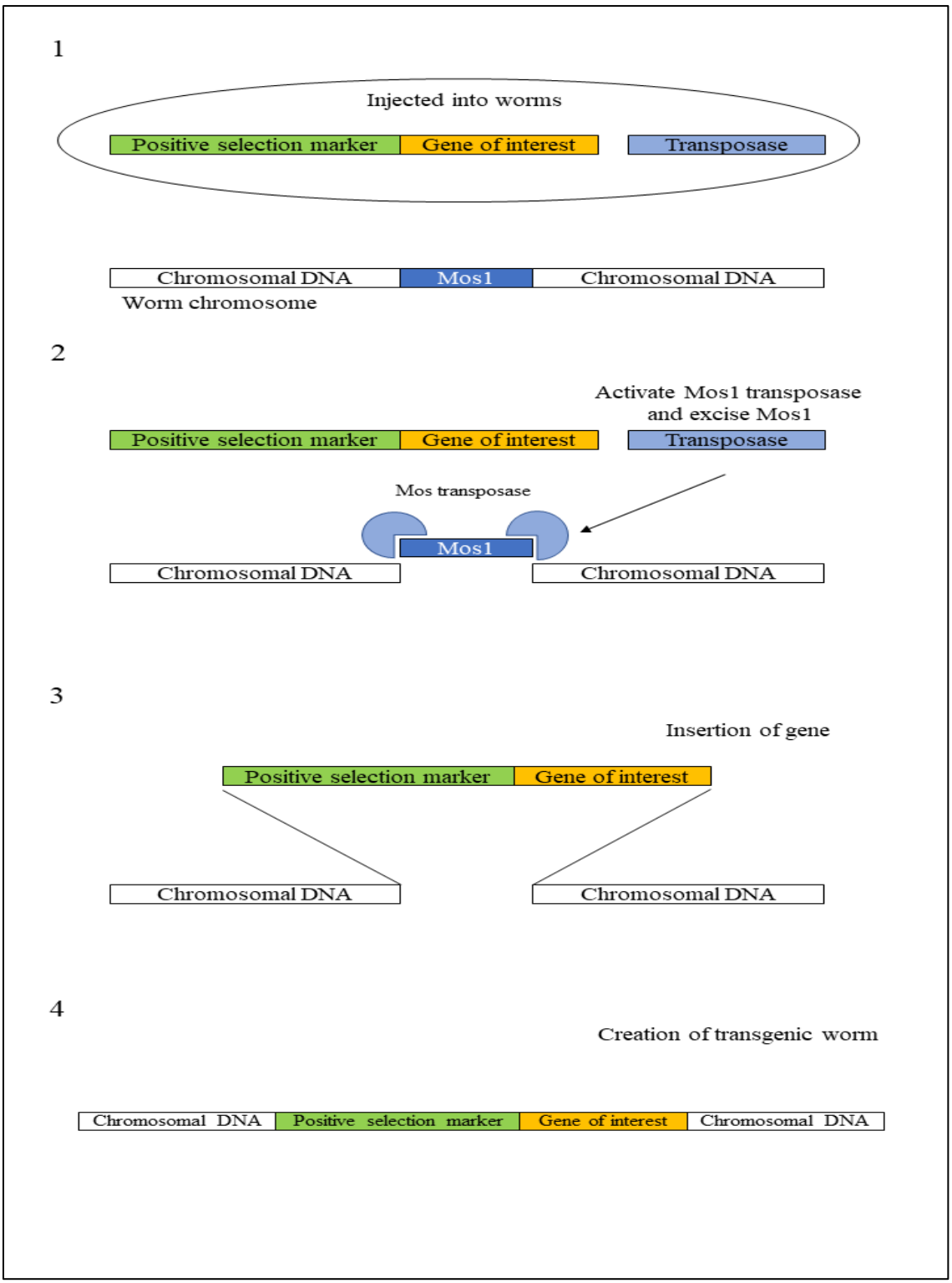

Figure 5. MosSCI

Plasmids containing gene of interest and positive selection marker is injected into worms together with plasmid expressing Mos1 transposase. Once Mos1 transposase is expressed, a double stranded break is created at a specific site and gene can be inserted into the chromosome of the worm.

6.2 RNAI TREATMENT IN *C. ELEGANS*

The RNAi mechanism was first described by injecting dsRNA into C. elegans[106] (Fire et al. 1991). Later it was discovered that soaking nematodes in dsRNA solution or feeding worms with bacteria producing dsRNA also lead to efficient degradation of mRNA[10; 107]. Injection with dsRNA is performed on young adult hermaphrodite, and the progeny is scored for mutant phenotype. Injection-based RNAi is more labor intensive than soaking or feeding, and not suitable if you want to treat many animals. Soaking with dsRNA is favorable for high-throughput, screening phenotypes for many animals. Feeding nematodes with dsRNA is also suitable for high-throughput, and is the least labor intensive and inexpensive method, but produces slightly more variable results compared to soaking or injection. Both soaking and feeding with dsRNA can be performed on worms at all larval stages.

In study I worms was fed with *E. coli* producing dsRNA for *xpo-1*.

6.3 RNA DETECTION METHODS

For RNA biologists being able to detect and track the changes of expression of different RNAs during development and different conditions is crucial to understand their function. The early RNA methodologies that were developed in 1977 by James Alwine, David Kemp, and George Stark involved the use of electrophoresis to separate RNA species by size followed by detection with a hybridization probe complementary or partially complementary to the target RNA[108]. Because of the similarity the method had with a DNA detection method called Southern blotting, they named the RNA detection method Northern blotting. Only a few years after the development of Northern blot another popular RNA detection method was introduced. Thanks to the discovery of the enzyme reverse transcriptase (RT) from RNA viruses[109; 110], scientist around the world were able to convert single stranded RNA into complementary single stranded DNA, which is more stable than RNA. This opened the possibilities to use RNA in molecular cloning, PCR, micro-array and RNA sequencing. The simplicity and efficacy of RT-PCR has made it into a popular tool to study RNAs.

6.3.1 Northern Blotting

In study I we used polyacrylamide gel electrophoresis (PAGE) and agarose northern blotting to detect smaller and larger RNA species. The major difference between these two gels lies in their composition and ability to allow molecules to wander. PAGE gel comprises of large molecules, which makes it narrower for the RNA molecule to travel, thus making it ideal to separate small-sized RNAs and difficult to separate large-sized RNAs compared to agarose gel. Northern blotting has the advantages that it is simple and relatively high specificity. Sequences

with even partial homology can be used as hybridization probes. mRNA transcript size can be detected. With one probe one can detect different sizes of splice variant and processed RNAs. Disadvantages are risk of RNA degradation during electrophoresis, the sensitivity of northern blot is relatively low in comparison with that of RT-PCR and difficult with detection using multiple probes.

6.3.2 RT-PCR

In study I, II and III we used RT-PCR with different variations. RNA template is converted to a complementary DNA (cDNA) with reverse transcriptase. The cDNA can be further amplified with PCR. RT-PCR is more sensitive than northern blot as it requires fewer RNA molecules for detection and quantification. The drawbacks with using RT-PCR is difficulties distinguishing sizes difference and modification of different RNA species.

6.3.2.1 SYBR green

SYBR green was used in study I and is the most economical method for quantitative PCR. It contains a fluorescent dye that binds to double stranded DNA molecules. The fluorescence is measured at the end of each PCR cycle to determine how much DNA has been amplified.

6.3.2.2 Taqman assay

Taqman assay was used in study III and are predesigned oligos and a gene-specific probe with fluorophore and a quencher attached. The close proximity of the fluorophore and the quencher prevents fluorescence. During amplification the fluorophore is cleaved resulting in emittance of light that is registered.

6.3.2.3 miRNA taqman assay

miRNAs are 20-22 nt long, which makes amplification impossible. To quantify miRNAs with RT-PCR miRNA sequences are extended by a hairpin probe with an overhang that can hybridize to the miRNA of interest. RT will create a chimeric cDNA with the mature miRNA sequence and taqman probe sequence. PCR amplification is performed with oligos spanning the miRNA and a sequence on the taqman probe.

Although both Northern blotting and RT-PCR are still widely used RNA methodologies around the world, their limitation lies in the ability to study multiple RNA species. To truly understand how the RNA profile of a cell looks like or what happens within a cell during development or changes in condition of multiple RNA species, a high-throughput method is required. An example of such method used in this thesis is the sequence-based approach.

6.3.3 Sequence-based approach

Sequencing technologies have played a major part in our understanding of human genome and transcriptome. Initial efforts on sequencing nucleotides were performed on RNA species, such as microbial ribosomal or transfer RNA, or the genomes of single stranded RNA bacteriophages. The reason for sequencing these molecules was because they could be produced in mass quantities, they were single-stranded, and significantly shorter to eukaryotic DNA. Early attempts of sequencing could only distinguish composition and not the order[111]. RNase enzymes can cut RNA chains at specific sites[112]. These enzymes were used to produce fully or partially degraded RNA pieces that could be sequenced[113]. In 1965 scientist managed to sequence the entire nucleotide sequence of alanine tRNA from Saccharomyces cerevisiae[114].

The discovery and development of recombinant DNA technologies and polymerase chain reaction (PCR) were the major contributors of advancing sequencing technologies. The Sanger's chain termination technique utilizes dideoxynucleotides (ddNTPs) that are chemical analogues of deoxy ribonucleotides (dNTPs)[115]. Dideoxynucleotides (ddNTP) lack the 3' hydroxyl group that is required for the extension of DNA chains, and therefore cannot form a bond with the 5' phosphate of the next dNTP, making the extension of DNA polymerase impossible[116]. The initial method involved use of non-radioactive NTP and radioactive ddNTP which enabled visualization of the various sizes of DNA fragments. The technique was further developed, and radioactive ddNTPs were later replaced with fluorometric based detection and capillary based electrophoresis[117-121]. This lead to development of the first generation of automated sequencing machines to be used in sequencing genomes of various organism[122].

The early sequence-based approach used Sanger sequencing of cDNA or expressed sequence tag (EST) libraries[123]. However, this was relatively low throughput, expensive and not quantitative. To overcome this, tag-based sequencing approaches were being explored. Tag-based sequencing approach like serial analysis of gene expression[124] (SAGE) that relies on poly(A) tail, cap analysis of gene expression[125] (CAGE) that relies on m^7G, or RNA-Paired End Tag[126] (RNA-PET) and Transcript isoform sequencing[127] (TIF-seq) that allows sequencing from both 5' and 3' end of RNAs could offer sequencing that were cost-effective and high-throughput. An advantage of both paired-end tag approach is the detection of fusion transcripts. However, they still have technical limitations obtaining information from long full-length transcripts.

In 1990 the scientific community wanted to sequence the entire human genome, which consist of over 3 billion bases. The aim was to identify genetic variants that could increase the risk for common diseases like cancer and diabetes. The aim was to understand our biology but also use

this information in healthcare to sequence patient's DNA to investigate if they were prone to certain diseases[128; 129]. However, sequencing the patient's entire genome was not feasible due to sequencing cost. The National Institutes of Health (NIH) wanted a cost-effective sequencing method that would only look for genetic variants that carried diseases. Later, NIH found the idea of breaking up the genome into pieces (shotgun) followed by sequencing most attractive[130; 131]. Shotgun sequencing is a fast and efficient method to capture the transcriptomic profile. RNAs are converted to cDNAs. The larger cDNAs are fragmentized into smaller fragments and cloned into a cDNA library followed by sequencing. The reads are pieced together like a giant jigsaw puzzle bioinformatically. An advantage with shotgun sequencing is that it is does not rely on 5' cap or poly(A) tail. It can also distinguish expression levels of each exons within a transcript, and detect alternative splicing events. However, the drawbacks with shotgun sequencing compared to tag-based approach is that it is not strand specific, meaning that the read you obtain from shotgun sequencing does not discriminate the 5' end or the 3' end.

The next breakthrough in sequencing technologies came during the discovery of luminescent method for measuring pyrophosphate synthesis[132]. The light produced production was proportional to the pyrophosphate levels. By measuring pyrophosphate production during the DNA polymerization reaction as each nucleotide is pumped through while the DNA template is attached to a solid surface it would reveal which nucleotide is being incorporated and how many[133]. The pyrosequencing technique could be performed using natural nucleotides instead of heavily modified dNTPs, and observed in realtime[134]. However, it has difficulties detecting stretches of DNA sequence with more than 4 identical nucleotides. The pyrosequencing technique was later licensed to 454 Life Sciences.

6.3.3.1 454 sequencing

Massive parallelization of sequencing reactions, also called Next-Generation Sequencing (NGS) or second-generation sequencing was led by the technology of 454 sequencing machines. Preparation for the 454 sequencing required attachment of DNA library to an adaptor sequence annealed to a bead, one molecule per bead, which is then amplified with an adaptor-specific primer. Each bead is placed in a single well on a reaction plate. For each sequencing round enzymes and specific nucleotide are pumped and washed onto the reaction plate. The release of pyrophosphate is monitored and registered[135].

6.3.3.2 Illumina/Solexa sequencing

Not long after 454 sequencing technology another sequencing method was developed, Solexa sequencing. The Solexa technology immobilizes clusters of sequencing templates on a solid surface covered with complementary adapter sequences. A process called bridge amplification can generate clusters of amplified DNA clones within a surface. These clusters are sequences with nucleotides with fluorophores attached to the 3' hydroxyl position. The fluorophores must be cleaved away before incorporation of a new nucleotide[136; 137]. The Solexa technology was acquired by Illumina, and the technology is referred to as Illumina sequencing.

6.3.3.3 SOLiD sequencing

A sequencing approach based on DNA ligase to detect and incorporate bases in a very specific manner was developed by George Church in 2005. The method is called small oligonucleotide ligation and detection system (SOLiD) and differ from the sequencing by synthesis employed by 454 sequncing and Illumina. Instead DNA fragments are attached and amplified on beads. Specific probes complementary to the adaptor sequences are hybridized to the amplified fragments. These probes allow ligation of fluorescently labeled probes which consist of eight bases, which contain ligation site and cleavage site. If the first two bases are complementary to the template than ligation will occur, and the rest will be cleaved of. The fluorophore is removed and another set of fluorescently labeled probes are added. The ligation and removal of fluorophore is then registered[138].

In study I and II Illumina 1G Genome Analyzer was used for sequencing of *c. elegans* CLIP-sequences and small RNAs of ovarian tumor samples. In study III Illumina HiSeq 2000 was used. The 1G Genome Analyzer from Illumina can generate 35-bp reads and produce at least 1 GB of sequence per run in 2-3 days. The Illumina HiSeq 2000 can generate up to 25 GB of 100-bp long paired-end reads per day.

6.4 BIOINFORMATICAL ANALYSIS

In 2001 the cost of sequencing the human genome was 100 million USD. In less than 20 years the cost of sequencing has dropped to remarkable 1000 USD[139]. Advancement in sequencing technologies and recombinant DNA technologies, and its' application in healthcare are the major reasons for this dramatic change. However, without the ability to properly interpret the vast output of biological data then all the advancement in sequencing would be in vain. The advancement in computer technology and mass production of biological data paved way for a

new research field in biology, using computers to understand and interpret biological data, bioinformatic.

In 1969 when NASA guided a space shuttle carrying two astronauts from earth to the moon and safely back to earth, they had access to computing power of 64kbyte memory and CPU 0.043MHz[140]. If we compare that to a smart phone in 2017, which have 256Gbyte memory and CPU 2.39 GHz[141] it is not surprising that our computers can process more data and perform more difficult task which we could not 50 years ago. These advancements have contributed significantly to our understanding of human biology and genetic.

Initially when human genome was sequenced with shotgun method. Billions of short reads had to be assembled to a complete genome. By finding reads that would overlap partially scientist tried to puzzle all the pieces together. This was a daunting task, and many compared it to putting together a million pieces puzzle with only partial information. In this case researchers used DNA sequences that partially overlapped to put together an entire genome.

The major challenge in assembling reads from shotgun sequencing lied in the fact that human genome contains many repetitive regions, inability to distinguish orientation of each read, and insufficient coverage of the entire genome. During assembly step numerous problems were encountered. (1) Reads containing small adapter sequence, that has not been removed by the sequencing machine's software. (2) Chimeric reads, that arise from separate pieces of DNA fused together. (3) Contaminant from other species that does not belong to the genome[142].

Since sequencing short reads was the cheapest strategy to acquire information about the human genome. Determining the accuracy of our mapping needed to be statistically calculated. Thanks to the effort of producing reference genomes we can now map reads to locations in the genome. However, we are still faced with several problems.

The first problem is the practical matter. Even the most powerful computers today would need months to map millions of reads to the human genome, which consist of over 3 billion bases. The size of the reference genome imposes a problem in term of computing power. The algorithm needs to be memory efficient when mapping millions of short reads to the reference genome. The second problem is if reads comes from a repetitive region from the genome. It would be difficult to determine the exact location of the read with high confidence. The third problem is mapping of chimeric reads or alternative spliced reads with different junctions[143].

The new sequence alignment algorithms rely on building indexes (fragmentizing) of reference genome. By indexing the reference genome, it improves the computational time significantly.

The short sequence alignment tools employ different strategies to match reads to reference genome. One of the more common strategy is the Burrows-Wheeler alignment (bwa). In study II and III we mapped sequencing reads to the human genome using Bowtie and TopHat2. Both algorithm utilizes bwa[144; 145].

6.4.1 Burrows-Wheeler alignment

Burrows-Wheeler alignment algorithm compresses and collapses the reference genome. This means that sequences are fragmentized permutated and sequences that share similarity are collapsed. When aligning reads, the algorithm maps short reads to all the sequences in the reference genome that are similar. It then sorts out the sequences with the highest possibility of match. If several locations in the genome matches then the algorithm assigns reads to the location with best alignment, but this is not always correct. [146].

6.4.2 Tophat2, discovering splice junctions

Tophat2 is used to identify splice junctions in RNA-seq without relying on known splice sites from reference genome.

6.5 TRANSFECTION OF DNA OR RNA INTO MAMMALIAN CELLS

6.5.1 Lipid-based transfection

The positively charged head group of the synthetic lipid reagent forms a complex together with the negatively charged nucleic acid. These complexes can be delivered to the cells through endocytosis and then released into the cytoplasm. DNA will be translocated to the nucleus for transcription, while RNA and antisense oligonucleotides remain in the cytoplasm[147]. In study III, a lipid-based transfection was used to deliver plasmid DNA in HeLa, PC3, HEK293T, NoDice 2-20 and NoDice 4-25 cells.

6.5.2 Polyamine-based transfection

Polyamines have been explored for gene delivery. Compared to lipid-based transfection they can deliver nucleotides with minimal cytotoxicity[147]. In study III polyamine-based transfection was used to deliver siRNA to HeLa and PC3 cells.

6.6 PROTEIN-RNA INTERACTION

RNA binding proteins (RBP) that binds to double- or single stranded RNAs have a crucial role in various cellular processes, such as gene regulation, transport and cell division[148; 149]. They are especially important in post-transcriptional control of RNAs, such as alternative splicing, transport of RNA, RNA editing and translation[148; 149]. Approximately 1240 genes are annotated

to encode RBPs in mammals and 880 in nematodes based on the presence of domains capable of interacting with RNA[150; 151]. Comprehensive studies of mRNA-bound interactome in human cell lines identified several proteins that were capable of binding to mRNA without the presence of known RNA binding domains[152]. Suggesting that there are more RNA binding proteins in the human and nematode genome than first predicted. To study protein-RNA interaction biochemically, several methods can be used like RNA electrophoretic mobility shift assay[153] (EMSA), RNA pull-down assay[154], oligonucleotide-targeted RNase H protection assay[155; 156] (RPA) and fluorescent in situ hybridization co-localization[157]. These methods can identify the proteins that interact with a specific RNA species. However, to identify the RNAs that are bound to a specific protein it is necessary to employ another strategy.

A method was developed in the Darnell laboratory where they sequenced RNAs bound to Nova proteins from mice brains. Nova proteins regulate alternative splicing in neurons. Using this method, they were able to identify Nova RNA targets. Using crosslinking and immunoprecipitation the authors identified over 300 Nova binding sites. Because RNA targets were crosslinked to the proteins it was named CLIP[158]. The development of sequencing technology enabled the use of high-throughput sequencing together with CLIP (HITS-CLIP). miRNAs guide Argonaute to RNA targets that are partially complementary to the miRNA, and repress translation or degrade mRNA. HITS-CLIP of Argonaute was quickly adopted to identify miRNA targets in humans, mice, c. elegans and other organisms[159-161].

Years after other method appeared with variation to the original CLIP method; photoactivatable ribonucleoside-enhanced CLIP[162] (PAR-CLIP), individual-nucleotide resolution CLIP[163; 164] (iCLIP), crosslinking, ligation and sequencing of hybrids CLASH[165] and RNA hybrid and individual-nucleotide resolution CLIP[166] (hiCLIP) to study protein-RNA interaction.

ALG-1 HITS-CLIP was used in study I to identify the ALG-1 binding site in let-7 primary in c. elegans (Figure 6). TARBP2 HITS-CLIP was used in study III to identify the binding site of TARBP2 in ATG2A and TARBP2 in human cancer cell lines. Variation of the CLIP method (RNA-CLIP/RNA-IP) with or without crosslinking was also used in study I and III where RNase digestion, and sequencing primer ligation was omitted. Instead full-length RNA targets were isolated and converted to cDNAs, which were detected with either semi-quantitative RT-PCR or quantitative RT-PCR.

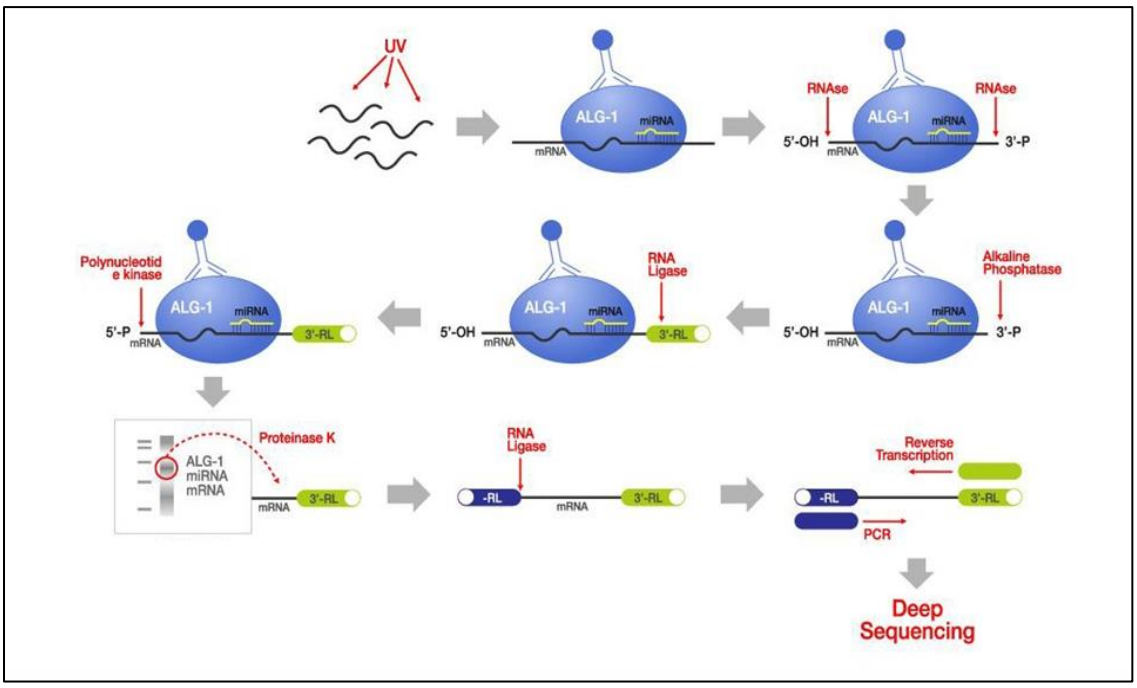

Figure 6. Flow chart of ALG-1 HITS-CLIP

Stabilization of ALG-1/RNA interaction with ultraviolet light in vivo followed by lysis and immunoprecipitation of ALG-1/RNA complex. RNAs bound by ALG-1 are protected from degradation by RNase digestion. After RNase digestion a 5'-hydroxyl group and a 3'-phospate group are formed. The 3' ends of the RNA can be ligated with sequencing primers after removal of phosphate group by alkaline phosphatase. A radiolabeled P-γ-ATP is added to the 5' end with polynucleotide kinase and run on native SDS-PAGE to isolate the band corresponding to ALG-1/RNA complexes. ALG-1 protein is digested by proteinase K to release the crosslinked RNAs followed by RNA extraction. After ligation of 5' sequencing primer to RNA conversion to cDNA and cloned into cDNA library.

6.7 MONITORING AUTOPHAGY IN MAMMALIAN CELLS

To monitor the activity of autophagy three methods are mainly used. Electron microscopy, immunoblotting of LC3 and tandem fluorescent tagged LC3 reporter. In this study we used two of the methods to monitor the activity of autophagy.

6.7.1 Monitoring LC3 turnover by immunoblotting

LC3 is the only known protein that can be found in all autophagic membranes, including phagophore, autophagosome and autolysosome. The amount of LC3-II is a good indicator of the number of autophagosomes, which is a good index of autophagy induction. The full-length LC3 is cleaved by ATG4 to form cytoplasmic LC3-I. LC3-I is then conjugated to PE to form LC3-II, which involves ATG7 and ATG3. LC3-II binds to both the inner and outer membrane of autophagosome. After fusion with the lysosome LC3-II in the inner membrane is degraded while the outer LC3-II is deconjugated by ATG4 and returns to the cytosol. By measuring the

level of LC3-II with immunoblotting we can monitor the progression of autophagy either by LC3-I to LC3-II conversion or lysosomal degradation[167].

Since autophagy is a highly dynamic process. The detection of LC3-II levels at a specific time-point is insufficient for an overall estimation of autophagic flux. Autophagic flux referring to the complete process of autophagy, which includes sequestration of target proteins within the autophagosome, the fusion of autophagosome with lysosome, and finally breakdown of the target proteins. An increase of LC3-II could reflect either increase of autophagosome due to autophagy induction or a blockage in the downstream step of autophagy, either fusion or breakdown of target proteins. Thus, we detect LC3-II using immunoblots together with drugs that alter the lysosomal pH and blocks the autophagosome-lysosome fusion, bafilomycin A1. An alternate to monitor autophagic flux is detecting the levels of p62, also known as SQSTM1. It anchors ubiquitinated proteins to the autophagic machinery and promotes clearance of proteins through the lysosome. Degradation of p62 occurs mainly through autophagy, which makes it a good marker for autophagic activity. Accumulation of p62 indicate suppression of autophagy while decrease of p62 indicate autophagic activation. Like LC3-II, measuring p62 levels with immunoblotting require treatment with lysosomal inhibitor to evaluate the true effect of autophagy[167].

6.7.2 Monitor autophagic flux with tandem fluorescent LC3 reporter

Using confocal microscopy, we can measure the LC3 puncta, which corresponds to autophagosomes when cells are transfected together with a LC3 reporter. However, the increase of LC3 puncta does not invariably correspond to increased levels of autophagosomes, because autophagy is a highly dynamic process. The accumulation of autophagosome at a specific time point can reflect either increase of autophagosome formation or suppression of autophagic flux. By using a tandem monomeric RFP-GFP-tagged LC3[168] we can measure the levels of both autophagosome formation and autophagic flux. The GFP fluorescent signal is quenched by the low pH within the lysosome, whereas mRFP maintain their fluorescence in the lysosome. Yellow puncta (colocalization of green and red signals) on the confocal microscope indicate the tandem reporter is located in phagophore or autophagosome whereas red puncta (only red signals) indicate the tandem reporter is located in lysosome (autolysosome).

6.8 STATISTICAL METHODS

Several statistical tests were applied in this thesis, which is described below:

6.8.1 Student t-test

Student t-test is used to determine if two groups or observed phenomena are significantly different from each other, by comparing the means between the groups or phenomena. It assumes that our data are normally distributed. *P*-value is calculated to determine if the outcome occurred by chance. Low *P*-value means that the observed outcome was not due to chance, and null hypothesis can be rejected. Student t-test was used in study I and III to calculate if the difference observed between two groups were significant.

6.8.2 Mann-Whitney U test

Unlike student t-test, Mann-Whitney U test is a nonparametric test of the null hypothesis, and applied when data do not follow normal distribution. Mann-Whitney U test was used in study II to calculate if the difference observed between two groups were significant.

6.8.3 Kruskal-Wallis test

Kruskal-Wallis test is used to assess for significant difference among three or more groups, which was used in Study II.

7 RESULTS AND DISCUSSION

7.1 STUDY I

In this study, we explored a novel role of miRNAs in *C. elegans*. The role of miRNA and Argonaute in regulation of protein coding transcripts and function in the cytoplasm have been well characterized; however, not much is known about their role in the nucleus and their interaction with noncoding transcripts. This is the first study to describe a new role for nuclear miRNA and Argonaute, in which they bind noncoding RNAs and interfere with their function.

HITS-CLIP of ALG-1 in *C. elegans* revealed an ALG-1 binding site at the 3' end of *pri-let-7*. To our surprise this site contained a *let-7* complementary site (LCS), which suggested that interaction of *pri-let-7* with ALG-1 could be mediated by its own mature *let-7* miRNA. Further inspection revealed that the Argonaute binding site at *pri-let-7* was conserved in other worm species and in a subset of *pri-let-7*s in humans, suggesting that the site might have an important function in other species as well.

To understand the mechanism of ALG-1 and *let-7* interaction with *pri-let-7*, we used various worm strains that either lacked ALG-1 (*alg-1*), ALG-1 binding site on *pri-let-7* (Δalg-1) or *let-7* (n2853). This enabled us to investigate how removing one of these components would affect *pri-let-7*.

Without ALG-1, Δalg-1 and *let-7*, animals displayed an impairment in processing of *pri-let-7*, which could be seen with increase of either *pri-let-7* and *pre-let-7* or *pri-let-7* alone. This suggests that ALG-1, ALG-1 binding site and *let-7* are needed to ensure efficient processing of *pri-let-7*.

Normally, the *let-7* (n2853) animals display low levels of *let-7*[29]. This is caused by a point mutation in the seed sequence of let-7, which makes the miRNA less stable but also affect the processing of its own *pri-let-7*. Introduction of wild-type *let 7* resulted in an increase of mutated *let-7* levels. Further strengthening our evidence that *let-7* is needed to ensure proper processing of *pri-let-7*.

Lastly, we demonstrate that the ALG-1 interaction to the *pri-let-7* occurs in the nucleus. Processing of primary miRNAs occur in the nucleus. Purified nuclear extract contained both ALG-1 and miRNAs, and ALG-1 can retain its binding capacity to *pri-let-7* in the nucleus. XPO-1 is a nuclear transport receptor and has been shown to decrease the levels of *let-7*[169]. Silencing of XPO-1 resulted in a decrease of nuclear ALG-1 and reduced association of ALG-1 and *pri-let-7*, but did not affect the overall level of ALG-1. These results demonstrate that

ALG-1 nuclear export is mediated by XPO-1, and that ALG-1 binding to *pri-let-7* occurs in the nucleus (Figure 7).

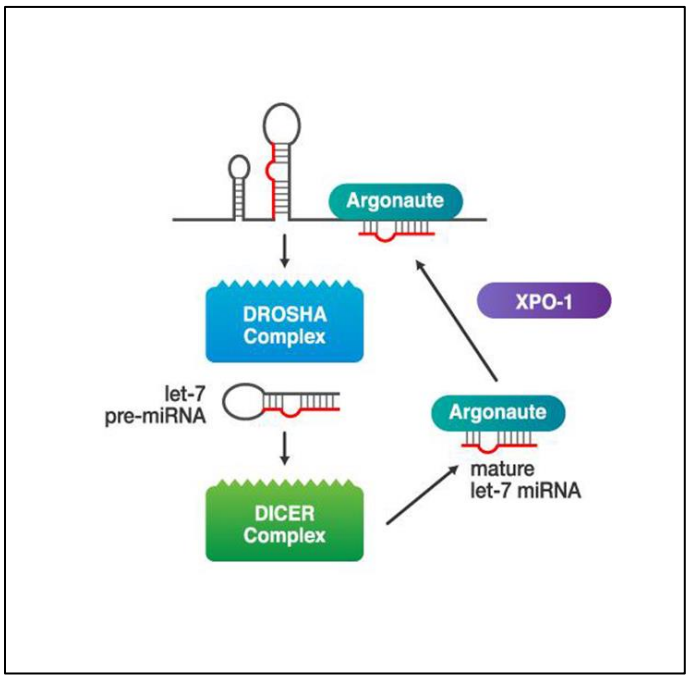

Figure 7. Auto-regulation of *let-7* processing by mature *let-7* and Argonaute

The mature *let-7* miRNA is loaded onto Argonaute and guides them to the *let-7* complementary site (LCS) close to the 3' end of the primary *let-7* transcript, which promotes processing of mature *let-7*. Exportin-1 protein (XPO-1), a nuclear transport factor, shuttles Argonaute with mature *let-7* to the nucleus thereby facilitating the interaction between Argonaute and primary *let-7*.

The key findings of this study:

1. miRNAs and Argonaute can target noncoding transcripts
2. A new role for nuclear miRNAs and Argonaute
3. *let-7* can promote its own expression by binding to *pri-let-7*

Since the discovery of miRNAs in 1993[26], their ability to bind to protein-coding transcripts and impose regulation have been well characterized[26; 27; 29; 36; 38; 170]. It was not known whether miRNAs could target other transcripts besides mRNA, which is surprising considering that a majority of eukaryotic transcriptome is noncoding RNAs[171]. Our study re-evaluates the role of miRNAs and emphasizes that miRNA targets should not only be focused on protein-coding transcripts but noncoding transcripts as well. Since the publication of this study numerous lab have demonstrated how miRNAs can bind and target noncoding transcripts.

let-7 can target long noncoding RNAs in various tissues and promote their degradation[172-175]. Others have shown that noncoding transcripts contain miRNA binding site and can sequester and prevent miRNAs from binding to mRNAs, i.e. sponging effect[176-181]. For example, *miR-1* can bind to the 3'UTR of the lncRNA urothelial cancer associated 1 (*UCA-1*) and effectively inhibit its expression[182]. Interestingly, another study showed that *UCA-1* could sequester *miR-16*, and prevent it from binding to mRNAs[183]. Together, these findings suggest that there is a crosstalk between various miRNAs, and they can regulate expression and function of each other.

To fully understand the complex network of miRNA interaction, Li and colleagues analyzed hundreds of CLIP-seq experiments, and combined their results into a database of miRNA interaction network. Although many miRNA targets were protein-coding transcripts, thousands of interactions between miRNAs and noncoding RNAs were also found[184].

7.2 STUDY II

This study aimed to characterize the miRNA profiles of various non-epithelial ovarian tumors. miRNA profile is altered in various tumors[185-189] and can predict outcome and classification of histological subtypes[190]. Given that non-epithelial ovarian tumors are challenging for clinicians and pathologists due to their histologically, genetically and clinically heterogeneous. Advantages of using miRNAs as diagnostic tool in cancer are several. miRNAs are stable in formalin-fixed paraffin-embedded (FFPE) cancer tissues[191], and in serum, plasma and blood which could be used as a sensitive non-invasive biomarkers[192; 193].

Our cohort of non-epithelial ovarian tumors consists of frozen tumor tissues with different histology. To characterize the miRNA profiles, we isolated small RNAs and cloned them into small cDNA libraries. We performed RNA-seq and aligned the sequences to miRbase, which is a database that contain sequences of thousands of mature and precursor miRNAs. Initially we performed an unsupervised clustering of the tumor samples, and found a distinct miRNA profile for malignant ovarian germ cell tumors (mOGCT) compared to benign OGCT (bOGCT) and sex-cord stromal tumors (SCST). This suggests that SCST and bOGCT are genetically more similar than mOGCT.

To strengthen our findings, we validated 12 miRNAs by RT-qPCR in an extended cohort of samples. These miRNAs were selected because they were known to be dysregulated in various tumors. The results from the RT-qPCR confirmed our findings from RNA-seq for many of the miRNAs. *miR-199a-5p*, which was downregulated in mOGCT, is known to target Beclin 1 (BECN1), a key component in autophagy[194; 195]. BECN1 protein level was evaluated in different tumor groups, and BECN1 was shown to be significantly higher in the mOGCT in comparison to bOGCT. Autophagy plays a role in maintenance of female germ cells[196; 197], suggesting that dysregulation of autophagy could contribute to tumorigenesis in female germ cells.

The key findings of study II are:

1. Potential use of miRNAs as biomarkers in non-epithelial ovarian tumors
2. Identification of miRNAs that could be important for tumorigenesis
3. Autophagy may have an important role in the pathogenesis of non-epithelial ovarian tumors

The promise of using miRNAs as biomarkers in the clinics led oncologist to identify and validate miRNAs profile in various tumors[185-189]. miRNAs can also be used to diagnose different tumors as well as monitoring the progression of specific tumors[190]. Additionally, miRNA expression profile can help oncologist to determine the best treatment for the patient. Certain tumors are more difficult to classify and grade, which makes it difficult to determine a proper treatment. There are multiple types of ovarian cancer, which are classified by the cell from which the tumor originates from[198]. Most non-epithelial ovarian tumors are cured but a few persist and do not respond to traditional treatment. Understanding the genetic profile of different ovarian tumors can help us to understand the pathology of the disease as well as developing better treatments.

Autophagy is important to maintain cellular homeostasis. Dysregulation of autophagy can lead to various pathologies[199; 200]. Especially in female germ cells autophagy seems to be important for the survival of germ cells[196; 197]. In aggressive female germ cell tumors, we found lower

expression of *miR-199-5p* that is a direct target of BECN1, a key component in autophagy. This suggests that tumor aggressiveness might be caused by dysregulation in autophagy, and could be explored either as a biomarker or a new treatment option. Recently, autophagy inhibitors have been used in combinations with other drugs to treat cancer[201; 202]. Perhaps autophagy inhibitors could be explored as a novel therapy for germ cell tumors.

7.3 STUDY III

This study uncovers a novel role of TARBP2, as a regulator of ATG2A and autophagy. TARBP2 is a double-stranded RNA binding protein (dsRBP), and is involved in cleavage of precursor miRNA to mature miRNA[203; 204]. It was initially discovered to stimulate expression of HIV-1 by binding to TAR in the viral long terminal repeats[69]. Approximately over 1200 RNA binding proteins are annotated in the human genome[150]. They are especially important in posttranscriptional control of RNAs, such as alternative splicing, transport of RNA, RNA editing and translation[148; 149]. 5'UTR is a region of mRNA that is upstream of the translational start site. This region is often GC rich and contain secondary RNA structures that impact regulation of translation[205; 206]. However, it remains elusive how 5'UTR can impact regulation of translation, and which are the factors involved. In this study we attempted to address this question and revealed that TARBP2-bound transcripts are associated with several important biological processes, including gene regulation and autophagy. Autophagy is a cellular process that sequester and deliver cytoplasmic cargo targeted for degradation through envelopment of double membrane vesicle to the lysosome[207]. It is responsible for maintaining cellular homeostasis. Dysregulation of autophagy can lead to diseases, it is therefore important for the cell to tightly regulate this process[90; 199; 200].

A study by Goodarzi et al. identified several TARBP2-bound transcripts and they demonstrate that TARBP2 can promote metastasis in cancer cell lines by binding to ZNF395 and APP, which lead to destabilization of these transcripts[85]. We re-analyzed their data and found several genes with TARBP2 binding site at their 5'UTR. We noticed several of these genes are involved in autophagy. Among them, ATG2A is important for autophagic degradation[207]. Immunoprecipitation of TARBP2 protein showed a strong association to *ATG2A* mRNA, and surprisingly also to *TARBP2* mRNA. This demonstrates that TARBP2 binds *ATG2A* and *TARBP2* mRNAs.

To determine whether *ATG2A* mRNA interaction with TARBP2 have an impact on its expression, we modulated the levels of TARBP2 either by silencing or overexpression and visualized the effect with immunoblotting. TARBP2 overexpression resulted in decrease of ATG2A, and silencing of TARBP2 had reverse effect, increase of ATG2A. Taken together our results show that TARBP2 influence ATG2A expression.

Given the role of ATG2A in autophagosome formation, we assessed whether TARBP2 regulation of ATG2A influenced autophagy. LC3-II levels correspond with the number of autophagosomes, which is commonly used as autophagy marker[167]. Indeed, immunoblotting revealed that TARBP2 overexpression resulted in decrease of LC3-II whereas silencing of TARBP2 resulted in increase of LC3-II, which suggest that TARBP2 can regulate autophagy. To evaluate if autophagy regulation mediated by TARBP2 was due to inhibition of autophagosome formation or autophagic flux (rate of autophagic degradation) we blocked the autophagosome-lysosome formation by drugs and analyzed the LC3-II levels by immunoblotting. Blocking the autophagosome-lysosome formation also led to decrease in LC3-II levels upon TARBP2 overexpression and reverse effect with silencing of TARBP2 suggesting that TARBP2 can regulate autophagic flux. The tandem mRFP-GFP-LC3 reporter can be used to detect the number of autophagosomes and autolysosomes in the cell. Ectopic expression of TARBP2 revealed a slight increase of autophagosomes and a decrease in autolysosomes. Together, these findings suggest that TARBP2 has a role in regulation of autophagic flux.

Since TARBP2 is involved in miRNA biogenesis, we wanted to determine if the regulation of ATG2A by TARBP2 was dependent of miRNAs. Dicer is an endoribonuclease and responsible of cleaving precursor miRNA into mature miRNA duplex[203; 204]. Dicer knockout (KO) cells are unable to produce high levels of mature miRNAs[208]. Modulating TARBP2 levels in Dicer KO cells showed similar results as previously observed. TARBP2 overexpression resulted in decrease of ATG2A and LC3-II levels, and reverse effect with silencing of TARBP2. This suggests that ATG2A and autophagy regulation mediated by TARBP2 is miRNA-independent.

The key findings of study III:

1. TARBP2 can bind ATG2A and suppress its expression

2. TARBP2 can also bind TARBP2 mRNA
3. TARBP2 is a regulator of autophagy

Our genome encodes over thousands of RBPs[150]. They are described as important regulators of RNA processing[148; 149]. However, their role in the cell and their function remains elusive for majority of these proteins. In this study we describe a novel role for one such protein, TARBP2.

TARBP2 is important in many cellular processes[70]. Perhaps it is mostly known for its role in miRNA biogenesis and interaction with protein kinase R (PKR)[209]. We found several TARBP2 binding sites throughout the entire human transcriptome. Many of these binding sites are found at introns and CDS, but perhaps the most interesting sites are in the 5'UTRs and 3'UTRs. They are known to contain many regulatory regions and can control expression of genes[205; 206]. Here, we revealed a new mRNA target of TARBP2 and a novel function in autophagy regulation.

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