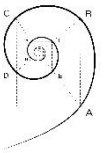
UNIVERSITÀ DEGLI STUDI
DI MILANO



DOTTORATO DI MEDICINA MOLECOLARE E TRASLAZIONALE

CICLO XXXIII

Anno Accademico 2019/2020

TESI DI DOTTORATO DI RICERCA

MED/04-Patologia Generale

MECHANISMS OF MIR-3189-3P-MEDIATED ANTITUMORAL ACTIVITY IN BREAST CANCER

Dottorando: **Cecilia VITTORI**

Matricola N°: R12041

TUTORE: Prof.ssa Daria **TRABATTONI**

CO-TUTORE: Prof.ssa Francesca **PERUZZI**

COORDINATORE DEL DOTTORATO: Prof. Michele **SAMAJA**

RIASSUNTO

Introduzione: *Il tumore al seno è uno dei tumori più diffusi in tutto il mondo. In US, il cancro al seno rappresenta il 30% tra tutti i nuovi casi di tumore diagnosticati nelle donne e rimane la seconda causa di morte, con 281550 nuovi casi nel 2021. A livello molecolare, esistono diversi sottotipi di cancro al seno: quello positivo per i recettori ormonali (HR+) è il più comune e il meno aggressivo mentre il triplo negativo è il più aggressivo e quello con la peggiore prognosi. Il tumore al seno triplo negativo (TNBC) è caratterizzato dall'assenza del recettore per gli estrogeni (ER), del recettore per il progesterone (PR) e del recettore HER2, e di conseguenza non può essere curato usando le terapie disponibili che agiscono contro tali recettori. Ad oggi, anche se alcuni pazienti con TNBC beneficiano dell'immunoterapia e degli inibitori dell'enzima Poly (ADP-ribose) Polymerase (PARP) non ci sono numerose terapie efficaci, e i soli trattamenti disponibili in prima-linea rimangono la chirurgia, la radioterapia e la chemioterapia.*

C-MYC, che è considerato un oncogene, regola numerosi processi biologici tra cui la proliferazione cellulare, la differenziazione, l'apoptosi e il metabolismo, ed è stato trovato up-regolato in diversi tumori incluso il cancro al seno. Per questo potrebbe essere considerato un possibile e promettente target nel trattamento contro il tumore al seno. Tuttavia, ad oggi non esistono terapie efficaci contro c-MYC.

I miRNA sono piccoli RNA endogeni di circa 21-25 nucleotidi in grado di regolare l'espressione genica. Il nostro laboratorio ha precedentemente caratterizzato il miR-3189-3p come una molecola con una forte attività antitumorale in due tumori molto aggressivi, glioblastoma e melanoma.

Visto quindi il ruolo dimostrato in questi tumori, abbiamo voluto studiare il suo effetto nel tumore al seno.

Metodi: *In questo studio è stata usata la linea cellulare MDA-MB-231 che è una linea di tumore al seno triplo negativo. Queste cellule sono state*

trasfettate con il miR-3189-3p o con un siRNA controllo. Il ruolo di questo miRNA è stato valutato con saggi di proliferazione cellulare, di migrazione ed invasione. La proteina 4E-Binding Protein 1 (4E-BP1) è stata validata come target diretto del miRNA tramite un saggio con luciferasi. La down-regolazione di c-MYC mediata dal miRNA è stata determinata tramite Real Time PCR e Western blots. Il ruolo di 4E-BP1 è stato valutato tramite over-espressione e down-regolazione del miRNA. L'effetto del miRNA sul metabolismo delle MDA-MB-231 è stato valutato tramite Seahorse XF96 Flux Analyzer.

Risultati: *in questo lavoro, abbiamo dimostrato che l'over-espressione del miR-3189-3p porta alla riduzione della proliferazione cellulare, della migrazione e dell'invasione. Sebbene MYC non sia un target predetto del miR-3189-3p, abbiamo scoperto che il nostro miRNA è in grado di down-regolare i livelli proteici di c-MYC sia in condizioni di crescita normali sia in condizioni di crescita sotto stress, attraverso la sua capacità di modulare l'espressione di 4E-BP1. Abbiamo trovato inoltre che il miR-3189-3p ha un effetto negativo sulla traduzione cap-dipendente e cap-indipendente. Successivamente abbiamo scoperto che le cellule trasfettate con il miRNA mostrano al loro stato basale un metabolismo differente, aumentando sia il metabolismo dell'ossigeno che la glicolisi.*

Conclusioni: *In conclusione, questi risultati dimostrano che il miR-3189-3p ha come target diverse proteine coinvolte nella regolazione della traduzione che hanno un effetto inibitorio sull'espressione di c-MYC, suggerendo l'utilizzo miR-3189-3p come nuovo possibile target terapeutico.*

SUMMARY

Background: *Brest cancer is one of the most diffused tumors worldwide. In US, breast cancer represents 30% of all newly diagnosed cancers in female and remain the second leading cause of death, with new 281,550 diagnosed cases in women. Invasive breast cancer can be divided into four subtypes, among which HR+ is the most common and the less aggressive. On the contrary, TNBC is the most aggressive subtype and the one with the worst prognosis. TNBC is characterized by the lack of estrogen receptor, progesterone receptor, and HER2. Consequently, TNBC cannot be treated with the available hormone therapies and receptor targeted treatments. Currently, even if some TNBCs do benefit from immunotherapy and PARP inhibitors, surgery and chemotherapy appear to remain the first-line treatments.*

The regulatory oncoprotein c-MYC regulate several pathways and is generally overexpressed in many tumors including TNBCs, making it a promising therapeutic target. Nevertheless, c-MYC is currently considered a non-drugable target.

MiRNAs are small endogenous ncRNAs, 18-25 bp long, that are able to regulate gene expression and our lab previously characterized miR-3189-3p as a miRNA with a strong anticancer activity against different tumor types, including glioblastoma and melanoma. Because of the role demonstrated in these tumors, we wanted to investigate the effect of miR-3189-3p in breast cancer.

Methods: *in this work, we used MDA-MB-231 cell line transiently transfected with miR-3189-3p or scramble control. The functional role of the miRNA was assessed by cell proliferation, cell migration and cell invasion assays. The miR-3189-3p gene target 4E-BP1 3'UTR was validated by luciferase assays. MiRNA-mediated c-MYC downregulation was assessed by Real time PCR and Western Blots. The role of 4E-BP1 in the miRNA-mediated effects was*

assessed through its overexpression or downregulation. The role of miR-3189-3p on the metabolism was assessed using the Seahorse XF96 Flux Analyzer.

Results: *We demonstrated that overexpression of miR-3189-3p reduces cell proliferation, migration and invasion. Additionally, although MYC 3'UTR is not a predicted target for miR-3189-3p, we discovered that our miRNA is able to downregulate c-MYC protein levels by targeting 4E-BP1 in normal growth conditions and in stress conditions. Indeed, miR-3189-3p has a negative effect on cap-dependent and cap-independent translation. Finally, we found that miR-transfected cells show a different metabolism at their basal state, increasing both oxygen metabolism and glycolysis.*

Conclusion: *Overall, results indicated that miR-3189-3p targets translational regulatory proteins that negatively affect expression of c-MYC, suggesting that miR-3189-3p can be a valuable therapeutic approach against a malignancy with few treatment options.*

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

HT: Hormone Therapy

ET: Estrogen Therapy

HR: Homologous Recombination

DSB: Double Strand Breaks

DBT: Digital Breast Tomosynthesis

MRI: Magnetic Resonance Imaging

ER: Estrogen Receptor

PR: Progesterone Receptor

HER2: Human Epidermal Growth Factor Receptor 2

DCIS: Ductal Carcinoma In Situ

TNBC: Triple Negative Breast Cancer

DC: Dendritic Cell

BCS: Breast-Conservative Surgery

CDK 1/4/6: Cyclin Dependent Kinase 1/4/6

APC: Antigen-Presenting Cells

CTLA-4: Cytotoxic T-Lymphocyte Antigen-4

PD-L1: Programmed Death-Ligand

PARP: Poly(ADP-Ribose) Polymerase

PFS: Progression-Free Survival

OS. Overall Survival

ncRNA: Non-coding RNA

lncRNA: Long non-coding RNA

siRNA: Small interfering RNA

miRNA: MicroRNA

UTR: Untranslated Region

DGCR8: DiGeorge Syndrome Critical Region Gene 8

XPO 5: Exportin 5

TRBP: Transactivation Response RNA Binding Protein

AGO: Argonaute Protein

RISC: RNA-Induced Silencing Complex

LDBR: Lariat Debranching Enzyme

Exp1: Exportin1

HIF1α: Hypoxia Inducible Factor 1 Subunit a

ZEB1/2: Zinc Finger E-box Binding Homeobox 2

MYOD1: Myoblast Determination Protein 1

EMT: Epithelial-Mesenchymal Transition

GSK3β: Glycogen Synthase Kinase 3b

DDX5/17: Helicase p68/p72

PIN1: Prolyl Isomerase 1

S6K: Ribosomal Protein S6 Kinase

HSC70: Heat Shock Cognate 70

HSP 70/90. Heat Shock Protein 90

SNP. Single Nucleotide Polymorphism

MECP2: 5-Methylcytosine-Binding Protein

5mC: 5-methylcytosine

m6A: 6-methyladenosine

PNPT1: Polyribonucleotide Nucleotidyltransferase1

MRE: miRNA-Responsive Element

tsmiR: tumor suppressor miRNA

oncomiR: Oncogenic miRNA

IGF2BP1: Insulin-like Growth Factor-2 mRNA-Binding Protein1

PTN: Pleiotrophin

RIP: RNA Immunoprecipitation

CLL: Chronic Lymphocytic Leukemia

SIRT1: Sirtuin1

SAHA: Suberoylanilide Hydroxamic Acid

EGCG: Epigallocathechin-3-Gallate

NPAT: Nuclear Protein Coactivator of Histone Transcription

DCLK1: Doublecortin-like Kinase 1

XIAP: X-linked Inhibitor of Apoptosis

CSC: Cancer Stem Cells

E2F1: E2F Transcription Factor 1

PTEN: Phosphatase And Tensin Homolog

PDCD4: Programmed Cell Death 4

GDF15: Growth Differentiation Factor 15

SF3B2: Splicing Factor 3B Subunit 2

Met-tRNA: Methionyl-tRNA

EIFs: Eukaryotic Translation Initiation Factors

4E-BP1: eIF4E-Binding Protein 1

VEGF: Vascular Endothelial Growth Factor

FGF2: Fibroblast Growth Factor 2

IRES: Internal Ribosome Entry Sites

ITAF: IRES-Transacting Factors

PTB: Polypyrimidine Tract Binding Protein

PAIP1: Polyadenylate-Binding Protein Interacting Protein 1

TCP80: Translational Control Protein 80

RHA: RNA Helicase A

METTL3/4: Methyltransferase Like 3 /4

WTAP: Wilms Tumor 1- Associated Protein

RBM15: RNA-Binding Motif 15

VIRMA: Vir-like m6A Methyltransferase Associated

FTO: Fat Mass And Obesity-Associated Protein

ALKBH5: Alpha-Ketoglutarate-Dependent Dioxygenase alkB Homolog 5

ASB2: Ankyrin Repeat and SOCS Box Containing 2

RARA: Retinoic Acid Receptor α

BNIP3: BCL2 Interacting Protein 3

YTHD: YTH Domain-containing Protein

SRSF10: Serine and Arginine Rich Splicing Factor 10

NSCLC: Non-Small Cell Lung Cancer

PDX: Patient-Derived Xenograft

OCR: Oxygen Consumption Rate

ECAR: Extracellular Acidification Rate

INTRODUCTION

1. BREAST CANCER

Worldwide, breast cancer is the most commonly diagnosed cancer and the second leading cause of death from cancer-related illnesses in women. While curable with currently available therapies in ~70–80% of patients with early-stage non-metastatic disease, advanced breast cancer with distant organ metastases is considered incurable [1].

In the US, breast cancer represents 30% of all newly diagnosed cancers in females [2]. The American Cancer Society estimates that breast carcinoma will remain the second leading cause of death in women in 2021, with 281,550 newly diagnosed cases of invasive breast cancer in women and 2.650 newly diagnosed cases in men (Figure 1) [2]. Rates of incidence vary depending on the ethnicity, with higher rates and higher numbers of metastatic breast cancer in African and African-American women compared to other ethnic groups [1].

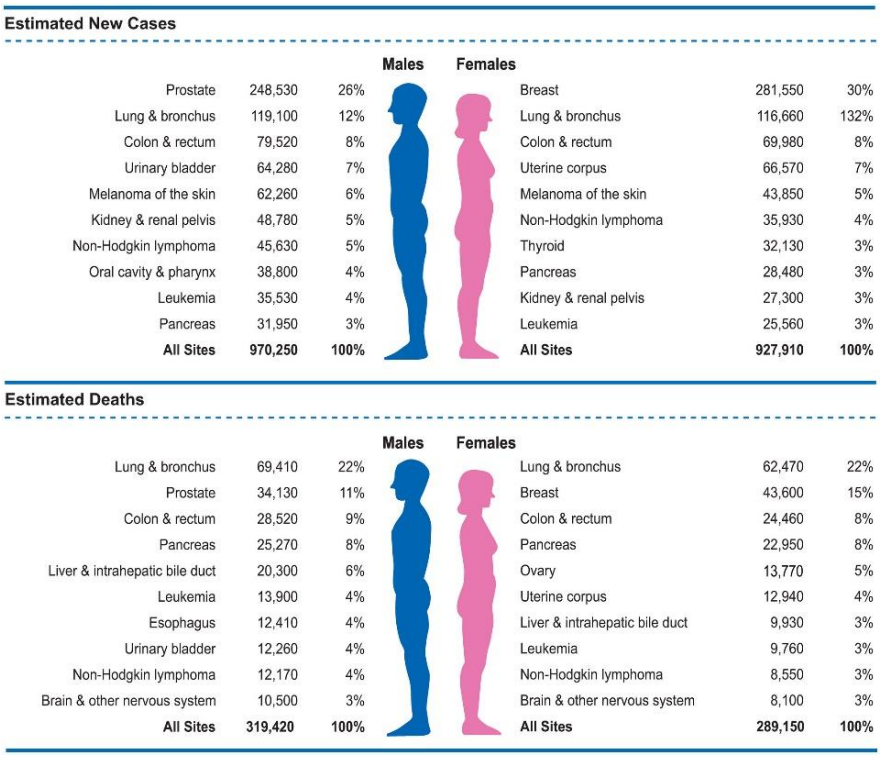


Figure 1. Estimated new cancer cases and death divided by sex in USA for 2021. The American Cancer Society estimates 281,550 new diagnosed cases of invasive breast cancer in women and 2.650 new diagnosed cases in men in 2021. The number estimations are based on projections and are rounded to the nearest 10. Adapted from Siegel RL et al. [2].

1.1 Risk factors

In general, for each type of cancer, there are multiple risk factors that can be divided into intrinsic or non-intrinsic, whether they are unmodifiable or modifiable, respectively. Intrinsic factors due to random error in DNA replication are the most common, representing up to 66% of cancer risk [3]. The relationship between intrinsic and extrinsic factors is not always clearly defined, since for some malignancies such as lung and skin cancer, non-intrinsic risk factors

can affect intrinsic risk factors. Examples of non-intrinsic risk factors that can affect intrinsic risk factors include exogenous (e.g. smoking, UV radiation, and viruses), or endogenous (e.g. aging, hormones, and genetic susceptibility) events. Interestingly, cancers with attributable lifestyle-related factors (breast, lung, and prostate cancers) occur more often in industrialized countries, whereas infection-related cancers are more common in less developed countries [4]. Overall, older age and being a female are the strongest intrinsic risk factors for breast cancer. Around 20% of breast cancers can be related to modifiable risk factors, such as physical inactivity and obesity. In fact, in an attempt to reduce those two types of risk factors, the American Cancer Society suggests 150 to 300 minutes of moderate-intensity or 75 to 150 minutes of vigorous-intensity activity each week. Consumption of alcohol daily increases the possibility of developing breast cancer by 7-10%, and women that consume 2-3 alcoholic drinks a day have about 20% more chances to develop breast cancer [1].

Other risk factors for breast cancer include early menarche, lack of breastfeeding, late-onset menopause, and hormone treatments. Some studies found that women using oral contraceptives have a higher possibility to develop breast cancer as well as women using combined hormone therapy (HT) and estrogen therapy (ET) after menopause [2, 5]. However, these studies were not conclusive and further investigation is needed to link hormone replacement therapy with increased risk of breast cancer.

Modifiable risk factors account for 20% of breast cancers, while 5-10% of worldwide breast cancers are genetic and associated with a family history. Several mutations can lead to inherited breast cancer (e.g.

mutations in ATM, CHEK2, PALB2, PTEN, STK11, and TP53 genes) [6], but the most common mutations are in the BRCA1 and BRCA2 genes, associated with 72% and 69% risk, respectively [7]. Even males can bring BRCA2 mutations that result in 6% increased risk of developing breast cancer [8]. BRCA1 and BRCA2 proteins participate in the DNA repair mechanism and their mutations lead to a defective homologous recombination (HR), a type of DNA repair that resolves double-strand breaks (DSB) [9]. The presence of mutated BRCA1 or BRCA2 genes in breast cancers is associated with less differentiated tumors and higher tumor grades [9].

1.2 Screening and diagnosis

In recent years, mortality has decreased due to increased prevention and early detection. The goals of screening are (I) to identify individuals with abnormalities or pre-cancerous lesions who haven't shown symptoms and (II) to identify individuals with cancers at the earliest possible stage, as well as refer them for diagnosis and treatment. The American Cancer Society recommends annual screening for women 40-54 years old and screening every 2 years when older than 55 years old [10].

Digital mammography (2D mammography), a low dose x-ray procedure, is the most common test used for screening breast cancer. Mammography, like many other types of screening, can give false negative or false positive results, especially in women with high-density breasts; around 12% of women are diagnosed with abnormalities but among those, only 5% are confirmed with breast

cancer [11]. Since its introduction in 2011, it is also possible to have 3D imaging, using the Digital Breast Tomosynthesis (DBT), alone or in combination with the normal mammography. For women that are at high risk, with a family history of breast cancer or BRCA1/BRCA2 mutations, Magnetic Resonance (MRI) is highly recommended, along with a genetic test for inherited mutations in the BRCA1/BRCA2 genes and the less common genes like the Phosphatase and Tensin Homolog (PTEN) or TP53.

Diagnosis is then made using three procedures: clinical examination, imaging, and needle biopsy [12].

The pathologic report will contain some essential information:

(I) Tumor size (T): the size is assessed by measuring the diameter of the tumor mass

(II) Lymph nodes status (N): determines if the nearby lymph nodes are affected or not

(III) Metastasis status (M): determines if metastasis is present in other parts of the body

(IV) Estrogen receptor status (ER): if 1% of the tested cells express estrogen receptor the tumor is ER+

(V) Progesterone receptor status (PR): if 1% of the tested cells express progesterone receptor the tumor is PR+

(VI) Human Epidermal Growth Factor Receptor 2 (HER2) status: based on the expression of HER2 the tumor can be positive, negative, or equivocal.

(VII) Histological Grade (G): the grade goes from 1 to 3 and is assessed following the Elston- and Ellis-modified Scarff–

Bloom–Richardson system [13]. The higher is the number, the faster cells are growing.

(VIII) Ki67 status: is an indicator of cell proliferation. The higher is the presence of Ki67, the faster the cells are proliferating.

1.3 Molecular subtypes

Breast cancers can be divided into two major categories, *in situ* breast cancers and invasive breast cancers. The first is also called ductal carcinoma *in situ* (DCIS) and accounts for 16% of total breast cancers [11]. Invasive cancers represent the majority of breast cancers and can be divided into four major molecular subtypes [14-16]:

1- Luminal A: the tumor is ER^+, PR^+ (>20%) and $HER2^-$(<10%). This is the most common subtype and represent ~50% of all breast cancers; it grows slowly and is less aggressive than the other subtypes. It also present low levels of Ki67 (<14%) and high expression of cytokeratins 7, 8, 18 and 19. Can be treated with specific therapies against hormone receptors (see next paragraph).

2- Luminal B: the tumor is ER^+, PR^+ (<20%) and/or $HER2^+$. This subtype is also highly positive for Ki67 (>20%) and tend to have a poorer outcome compared to the Luminal A.

3- HER2 enriched: this tumor is ER^-, PR^- and $HER2^+$(>10%) and it represent ~15% of all breast cancers. In the past, this subtype had the worst prognosis but currently it can be treated with targeted therapies against HER2 (see next paragraph).

4- Basal-like: the tumor is ER^-, PR^- and $HER2^-$. This group typically lacks the cytokeratins seen in the luminal subtype and it presents a high expression of cytokeratins 5, 6, 14 and 17. Since it is usually high grade with high proliferation index, this subtype has generally a poor outcome.

1.4 Triple negative breast cancer (TNBC)

Triple negative breast cancer (TNBC) is a tumor characterized by the absence of the three receptors ER, PR, and HER2. While majority of TNBCs can have characteristics of a basal-like subtype (80%), the remaining 20% belongs to the non-basal-like category [16, 17]. TNBCs can be divided in several subtypes, as shown in figure 2A. One of the early classifications (Lehmann classification) was based on gene expression profiling of 587 tumor samples and comprises: (I) the Basal-like 1 subtype (BL1), characterized by abnormal expression of cell cycle-regulating and DNA repair-related genes; (II) the Basal-like 2 subtype (BL2), characterized by abnormal activation of some signaling pathways such as EGFR, Wnt/β-catenin, and IGF-1R pathways; (III) the Mesenchymal subtype (M) defined by overexpression of cell migration-related signaling pathways, extracellular matrix–receptor interaction pathways, and differentiation pathways (Wnt pathway and TGF-β signaling); (IV) the Mesenchymal Stem-like subtype (MSL), characterized by low levels of cell proliferation-related genes and high levels of stemness-related genes; (V) the Immunomodulatory subtype (IM), marked by increased expression of immune cell-associated pathways such as Th1/Th2, NK

cell, B cell receptor signaling, dendritic cell (DC), and T cell receptor signaling pathways; and (VI) the Luminal androgen receptor subtype (LAR) typified by increased expression of genes involved in androgen receptor signaling [17, 18]. This classification has been recently revised to include only four subtypes (LAR, IM, BLIS and MES, FUSCC classification) (Figure 2A) [17, 19].

TNBC is the most aggressive type of breast cancer with the worst outcome. The survival of patients with TNBC has increased over time with the 5-years relative survival of about 90% and the 10-years relative survival of about 84% [2]. While those seem good numbers, in reality the survival strictly depends on the stage of the tumor at diagnosis, as the higher is the stage of the cancer, the lower is the survival. Specifically, the survival rate of TNBC patients at 2 years post-diagnosis drops drastically from 95% at stage I to 11.2% at stage IV (Figure 2B) [2, 20]. Finally, the basal-like phenotype of TNBC was found to be a factor that highly affects the survival; in fact, at 10 years post-diagnosis (Figure 2C), survival rates for TNBC patients with a basal-like phenotype is 10% lower than the ones with non basal-like TNBC [15].

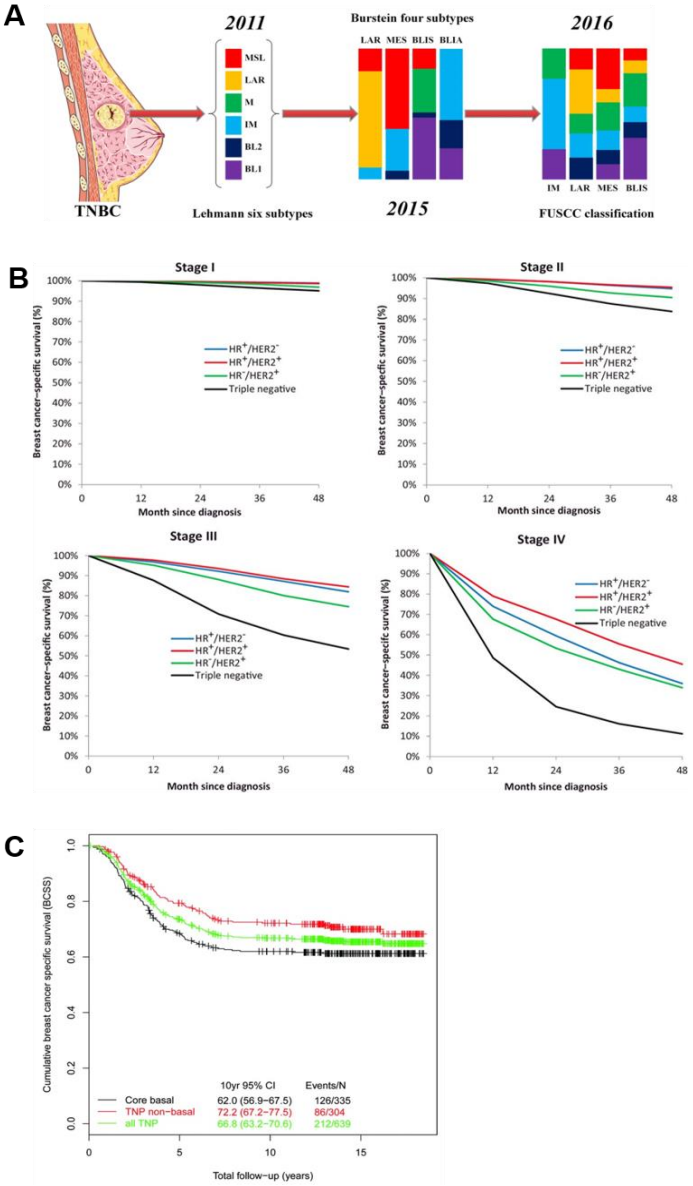


Figure 2. (A) TNBC classification. Following the Lehmann classification, TNBC was divided into six subtypes (BL1, BL2, M, MSL, IM, LAR). Recently this classification has been updated to include four subtypes (IM, LAR, MES, BLIS). Adapted from Yin L et al. [17]**, (B) Survival rates of the different subtypes of breast cancer.** If diagnosed at stage I, TNBC has a good survival rate (both short and long-term). With increasing stage, the survival drastically drops, especially for TNBC. Adapted from Howlader N. et al. [20], **(C) Differences in survival between basal-like TNBCs and non basal-like TNBCs**. The survival rate of basal-like TNBCs is lower than the non basal-like TNBCs (62% vs 72.2 %). Adapted from Seal MD. et al. [15].

1.5 Available treatments

Treatment decisions are made depending on the type of breast cancer, the molecular characteristic and the grade of cancer, the age of the patient, and the risks and benefits.

The first treatment option is surgery which can be total if removing the entire breast (mastectomy) or partial, also called breast-conservative surgery (BCS). However, BCS is often not an option for individuals with multiple tumors, inflammatory or advanced cancers. More commonly, women would prefer mastectomy for the fear of recurrence and the desire of symmetry, considering that breast reconstruction is generally offered during surgery. In the USA, the number of women that choose mastectomy is continuously increasing, both in young women (20-44 years) and women older than 45 years [21]. Following mastectomy, radiotherapy is widely used to remove the remaining cancer cells and to reduce the risk of recurrence. Again, the method through which radiotherapy is administered (external or internal radiation), is based on the characteristics of the tumor.

Another general type of treatment is chemotherapy, administered before and/or after surgery. TNBC and $HER2^+$ positive tumors seem to be more sensitive to chemotherapy, the efficacy of which depends on the type of the tumor [1].

Breast tumors that are positive for HR can be treated with hormonal therapies. If the tumor occurs in premenopausal periods, the standard drug regimen is 20 mg of Tamoxifen per day whereas for women in the postmenopausal period, aromatase inhibitors are preferred (letrozole, anastrozole, exemestane) [1, 22, 23].These treatments are considered standard care for at least 5 years after the diagnosis,

reducing the recurrence rate by approximately 50% [24]. In addition, in patients with luminal A (HR^+ and $HER2^-$) cancers, some clinical trials are aiming at evaluating the effect of cell cycle inhibitors, such as Cycline Dependent Kinase 4/6 (CDK4/6) inhibitors, in combination with endocrine therapy for at least 2-3 years, to block the progression of the cell cycle from G1 to the S phase [25-27].

For $HER2^+$ (luminal B and HER2 enriched) cancers, Trastuzumab and Pertuzumab are the first-line treatments, sometimes in combination with chemotherapy. Second-line treatment is represented by Trastuzumab plus a chemotherapy agent like Taxane (T-DM1) [1].

Patients with TNBC do not benefit from hormonal or trastuzumab-based therapies due to the absence of ER, PR, and HER2. While no targeted therapies are available for TNBC, an emerging field is represented by immunotherapy drugs. Considering that the tumor microenvironment in TNBC contains latent T and B lymphocytes and antigen-presenting cells (APCs), one of the most recent strategies has been developed to inhibit the cytotoxic T-lymphocyte antigen-4 (CTLA-4), which will lead to the activation of the immune response and slower progression of the tumor [28]. Nevertheless, this immune activation can be very aggressive and cause several side effects, since the antitumoral effect of CTLA-4 inhibitors seems to be due to direct activation of CD4+ and CD8+ effectors and not to the inhibition of regulatory T cells [29]. Indeed, in patients with advanced TNBC, a recent clinical trial showed the efficacy of the first FDA-approved immunotherapy drug Atezolizumab, which targets the Programmed Death-Ligand 1 (PD-L1), in combination with the chemotherapy drug nab-Paclitaxel (nanoparticle albumin-bound paclitaxel) [30]. Finally,

DNA-damaging drugs such as Cisplatin and Carboplatin and PARP inhibitors can be used in TNBC with BRCA1/BRCA2 mutations [31]. PARP inhibitors target the Poly (ADP-ribose) Polymerase (PARP), an enzyme that plays an important role in DNA repair [32]. If there is a mutation in the BRCA genes, cells rely on PARP for repair; consequently, the inhibition of PARP could kill cancer cells by preventing them from repairing DNA single-strand breaks. Olaparib and Talazoparib were the first two PARP inhibitors approved from the FDA as single-agents in patients with advanced $HER2^-$ breast cancer and with BRCA1/2 mutations [31, 32]. These drugs were evaluated in randomized phase III trials (OlympiAD for Olaparib and EMBRACA for Talazoparib), in which the progression-free survival (PFS) was significantly increased in patients treated with PARP inhibitors for both studies. However, no benefits were reported on the overall survival (OS). In summary, even if immunotherapy and PARP inhibitors are approved therapies against TNBC and can be beneficial for some individuals, surgery and chemotherapy, individually or in combination, appear to remain the first-line type of treatments.

2. NON-CODING RNAs

For many years RNA research focused on coding RNAs, considering non-coding RNAs (ncRNAs) as non-functional “junk RNA”. However, in the past 20 years ncRNAs have been thoroughly investigated and it is now clear their importance in regulating gene expression in several physiological processes and diseases. Indeed, the percentage of ncRNA content increases with the complexity of the organism, suggesting a critical function for these molecules in higher eukaryotes. In fact, the number of protein-coding genes in the human genome is only about 20000-25000, not much different from the 19,000 of *C. elegans* or from the 13,000 genes of Drosophila [33]. However, prokaryotic organisms have a limited number of ncRNAs (5-20%) [34] compared to mammals in which 80 % of the DNA is transcribed into RNA [35], the majority of which is ncRNAs (~98%) [36].

NcRNAs can be divided into 3 major classes: small (~18-30 nucleotides long), medium (~31-200 nucleotides long), and long ncRNAs (lncRNA, >200 nucleotides). Small ncRNAs are further divided into small interfering RNAs (siRNAs), microRNAs (miRNAs), and Piwi-interacting RNAs (piRNAs).

2.1 MiRNAs

MicroRNAs are small endogenous ncRNAs, 18-25 bp long, that inhibit gene expression through imperfect pairing with the 3' untranslated region (UTR) of target mRNAs. The inhibition of translation by miRNAs is reversible, indicating that miRNAs can function to stabilize mRNAs. *C. Elegans Lin-4* was the first miRNA identified and was found to negatively regulate the expression of LIN-14 protein in the first larval stage (L1) through binding complementary sequences in the 3'UTR of the LIN-14 mRNA [37]. Since that discovery, over 1900 hairpin precursors were identified and 2654 human mature miRNAs were annotated in miRBase, a database that provides unique identifiers for miRNAs [34, 38]. In respect to the nomenclature, the first three letters of a miRNA name identify the organism, so that "hsa" stands for human, "mmu" for mouse and "rno" for rat. Then the prefix "mir-" indicates the stem-loop sequence whereas the symbol "miR-" indicates the mature miRNA, both followed by a unique number that refers to the order of submission into the database (for example, if the last annotated miRNA is miR-500, the next miRNA that will be annotated will be named miR-501). Finally, the symbol MIR- denotes the gene of the miRNA [39]. Regarding the nomenclature of miRNA genes, when paralogous genes encode mature miRNA differing only by few nucleotides, the same unique identifier is used followed by a letter (for example, MIR-125a and MIR-125b). When multiple loci encode for the same mature miRNA, the same unique identifier is used followed by a number (for example, MIR-125b-1 and MIR-125b-2) (Figure 3) [39]. Additionally, miRNAs can be grouped in families on the basis of the full or partial conservation of the "seed sequence"

(nucleotides 2-8) of the functional mature miRNA. For example, miR 17-92 family is composed of three polycistronic clusters that encode for 15 different miRNAs [40]. Interestingly, miRNAs belonging to the same family can have similar functions, their genes are usually organized in clusters, and they can be co-regulated [41].

Finally, each miRNA can be designated as “5p” or “3p”, whether it is originated from the 5 prime (5’) strand or the 3 (3’) prime strand of the miRNA hairpin precursor, respectively.

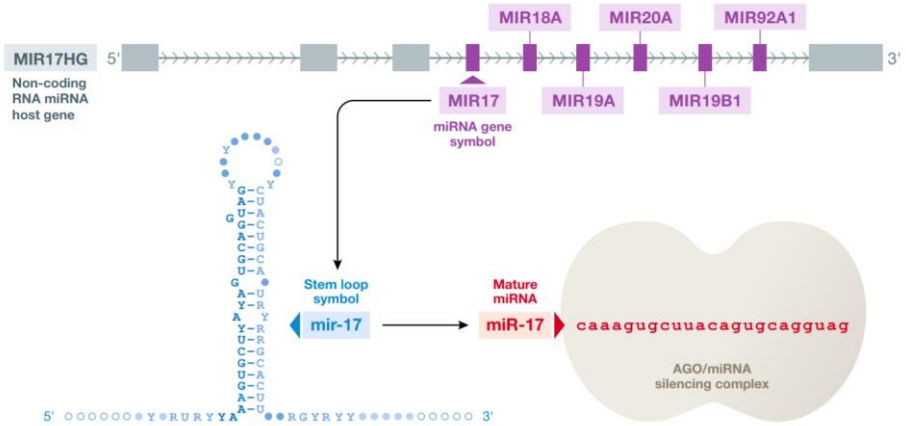


Figure 3. Nomenclature of miRNAs. Schematic representation of MIR17HG locus that contains the MIR17-92 family. The symbol MIR17 represents the gene; the symbol mir-17 represents the miRNA precursor stem-loop structure; the symbol miR-17 represents the active mature microRNA that is then incorporated into the RISC complex. Adapted from Seal RL. et al. [39].

2.1.1 Genomic organization of miRNAs

Depending on the gene from which they originate, miRNAs can be classified into intergenic or intragenic. MiRNAs belonging to the first class are located between genes (within the intergenic regions) and they are generally transcribed independently from the adjacent genes from their own RNA pol II- (RNA pol II) or RNA pol III- (RNA pol III) dependent promoters [42]. However, as indicated in Figures 4A and 4B, some studies have shown that a small percentage of intergenic miRNAs is co-transcribed with the closest genes by two mechanisms: (I) readthrough, where the transcription of one gene continues into intergenic regions overriding the termination site, and (II) divergent transcription, where two polymerases start transcription in both sense and antisense directions from the same promoter [42-45]. Since they are potentially co-transcribed with their neighboring genes, these two types of miRNAs can be classified also in the second category (intragenic). Most miRNAs are intragenic and some are localized in the antisense strand of a gene (antisense miRNAs, Figure 4C), in gene intron-exon junctions (junction miRNAs, Figure 4D), in exonic regions (exonic miRNAs, Figure 4E), or intronic regions (intronic miRNAs, Figure 4F) [42]. The last class is the most abundant, representing more than 85% of all intragenic miRNAs [46]. MiRNA genes can be also found in clusters and in this case different miRNAs are transcribed from a single polycistronic transcription unit [47].

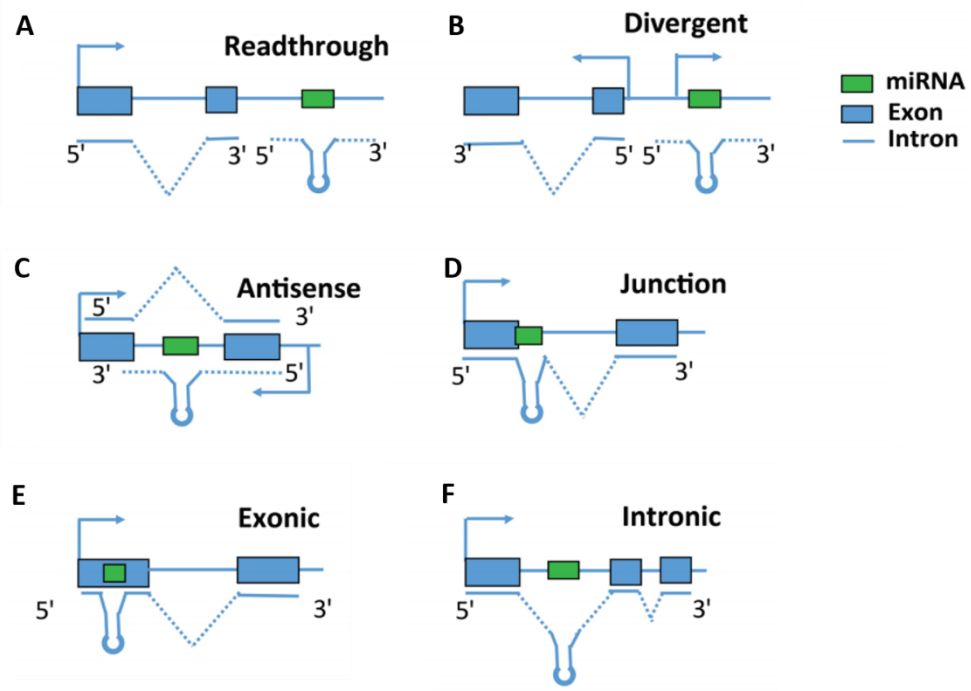


Figure 4. Intragenic miRNA classification. (A, B) These are two intergenic miRNAs that can be considered as intragenic since they are co-transcribed with their neighboring genes; **(C, D, E, F)** Intragenic miRNAs are divided into categories depending on the location of their genes: antisense miRNAs, exon-intron junction miRNAs, exonic miRNAs, and intronic miRNAs. Adapted from Liu B. et al.[42].

2.1.2 Canonical miRNAs biogenesis

The biogenesis of canonical miRNAs involves several steps, starting from transcription in the nucleus to maturation in the cytoplasm. At first, miRNA genes are transcribed by RNA pol II into a long primary transcript called pri-miRNA [48]. This transcript can be several thousand nucleotides long has a stem-loop structure and, similarly to mRNAs, it generally contains a 5' m^7 G cap and a 3' poly-A tail [49, 50]. Pri-miRNA transcripts can be monocistronic or polycistronic, whether they contain one or more miRNAs, respectively. Although RNA pol II is the most common type of polymerase for the transcription of miRNAs, a small part of them is transcribed by RNA pol III [51]. Regardless of the type of polymerase involved in the transcription, pri-miRNAs are then processed by Drosha- DiGeorge syndrome critical region gene 8 (Drosha-DGCR8) that cut the stem-loop in a shorter precursor miRNA (pre-miRNA) of about 70 nucleotides in length [52].

Pre-miRNAs are subsequently recognized by RanGTP-dependent nuclear transport reporter exportin 5 (XPO5) and transported into the cytoplasm through nuclear pores [53]. Once in the cytoplasm, pre-miRNAs are further processed by the RNase III enzyme (Dicer) and the transactivation response RNA binding protein (TRBP), to form ~ 22 nucleotides long miRNA duplexes [54, 55].

MiRNA duplexes are then associated with Argonaute proteins (AGO), forming the RNA-induced silencing complex (RISC) [55, 56]. One of the two strands remains into the RISC complex as the mature miRNA (originally called "guide strand"), while the other strand (or "passenger strand") was thought to be degraded. It was later demonstrated that

both strands can be functional, and the nomenclature has changed to -5p and -3p strands, with both strands being either guide or passenger [57].

Finally, within the RISC complex, the mature miRNA can bind the 3'UTR (and sometimes also the 5'UTR or the coding sequence) of the target messenger RNA (mRNA) [58] (Figure 5). The degree of complementarity between the miRNA and the mRNA generally determines if translational inhibition or mRNA degradation will occur.

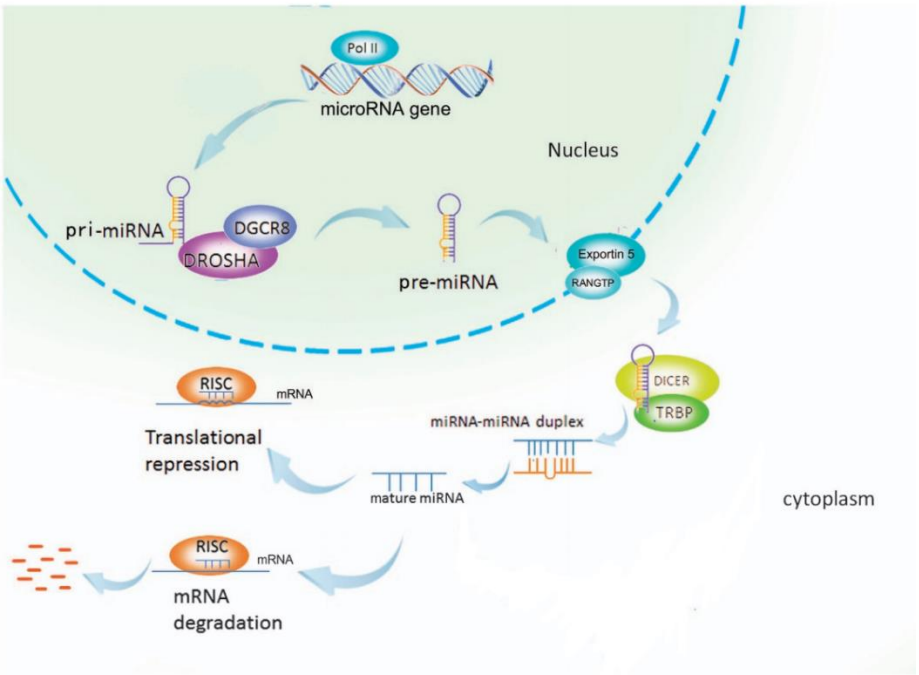


Figure 5. Canonical biogenesis of miRNAs. MicroRNAs are generally transcribed by the RNA pol II into a pri-miRNA and then processed by Drosha-DGCR8 to form the pre-miRNA. Exportin 5 will then bring the pre-miRNA into the cytosol through nuclear pores. At this point the pre-miRNA is further processed by Dicer, forming miRNA duplexes of ~ 22 nucleotides long. Finally, after association with AGO protein and incorporation into the RISC complex, the mature miRNA can bind the target mRNA. Adapted from Peng Y. et al.[59].

2.1.3 Non-canonical miRNAs biogenesis

2.1.3.1 Drosha-independent miRNAs processing

To date, several non-canonical miRNA biogenesis pathways have been discovered. One of these mechanisms is represented by the mirtron pathway, a Drosha-independent miRNA processing in which the miRNA originates within the intron. In this case, the pri-miRNA corresponds to the whole intronic sequence of the mRNA encoding genes where the miRNA is located [60]. Here, the processing step mediated by Drosha is bypassed and the small RNA precursor is generated through mRNA splicing [61]. After splicing and through the action of the lariat debranching enzyme (LDBR) [62], the intron assumes the typical pre-miRNA folding structure and can enter the "canonical pathway" of maturation, which includes its translocation to the cytoplasm by Exportin5, the processing by Dicer, and finally the binding to the mRNA target. Some mirtrons are tailed with extra sequences at the 5′ or 3′ end that need to be further trimmed by exonucleases, such as the multi-subunit complex RNA exosome, before their full maturation by Dicer [63].

Another example of Drosha-independent biogenesis is the 7-methylguanosine (m7G)-capped pre-miRNAs. These miRNA precursors are immediately exported into the cytoplasm by Exportin1 (Exp1) without being processed by Drosha [64].

2.1.3.2 Dicer-independent miRNAs processing

Although Dicer is a very important enzyme for the processing of miRNAs, some studies confirmed the existence of miRNAs processed by Drosha but not by Dicer [65]. For example, pre-miR-451 is processed initially by Drosha and, once in the cytoplasm, the processing is completed by AGO2, the only vertebrate Argonaute protein that has catalytic activity [65]. The pre-miR-451 stem-loop is too short to be recognized and processed by Dicer [66]. Instead, it is recognized by AGO2 that cleaves the precursor and generates a functional mature miRNA [67].

2.1.4 Multistep regulation of miRNA biogenesis

Biogenesis of miRNAs is a multistep and complex process subjected to a very strict control. In fact, dysregulation of some of the events that lead to the mature functional miRNA is often associated with human diseases, including diabetes, disorders of the nervous system, cardiovascular diseases, asthma, autoimmune diseases, and cancer.

2.1.4.1 MiRNA transcription regulation

Intragenic miRNAs are co-transcribed with adjacent genes in an RNA pol II-dependent manner, whereas transcription of intergenic miRNAs can be mediated by both RNA pol II or RNA pol III [49, 50]. As transcriptional mechanisms of miRNAs are elucidated, several miRNA databases, such as FANTOM4 EdgeExpress, MPromDb, ORegAnno, and EDGEdb catalog protein-DNA edges, started to incorporate

information about miRNA promoter sequences, transcription factors binding site predictions, and transcription factor protein-protein interactions [50]. In the past ten years, several transcription factors have been identified to positively or negatively regulate miRNA expression. This is particularly important in pathological conditions like cancer in which a dysfunctional expression of certain transcription factors can result in abnormal overexpression or downregulation of miRNAs, with consequent amplification or attenuation of specific pathways, respectively. For example, activation of the oncogenic protein p53 has been shown to increase miR-145 expression, a miRNA that is normally under-expressed in human tumors such as lung, colon, and breast cancers. Increased expression of miR-145 in turn reduces proliferation in breast cancer cells, promoting apoptosis [50]. Additionally, the hypoxia inducible factor 1 subunit alpha (HIF-1α) has been shown to increase the expression of miR-210, promoting cell survival under hypoxic conditions [68], a feature that allows tumor cells to survive in the tumor microenvironment where the quantity of oxygen is very low. Other factors like zinc finger E-box binding homeobox 1 (ZEB1), zinc finger E-box binding homeobox 2 (ZEB2), and myoblast determination protein 1 (MYOD1) can regulate miRNA expression [62]. For example, during myogenesis, MYOD1 and myogenin induce the transcription of miR-1 and miR-133 *via* direct binding to a region upstream of the locus encoding those miRNAs [47]. ZEB1 is instead involved in a feedback loop together with miR-200 family. In this mechanism, members of the miR-200 family (miR-200a, miR-200b and miR-429) prevent the expression of the transcriptional repressor ZEB1, which in turn inhibits the epithelial-mesenchymal transition

(EMT), while ZEB1 represses the expression of these miRNAs in mesenchymal cells [69].

2.1.4.2 Regulation of Drosha processing

The protein complex that processes miRNAs (microprocessor complex) is formed by Drosha, an RNase III-type endonuclease that cleaves the pri-miRNA, and DGCR8, a double-stranded RNA binding protein that recognizes pri-miRNAs and ensures the correct splicing by Drosha [70]. Interestingly, depletion of DGCR8 in humans is one of the causes of the genetic disorder called DiGeorge syndrome, characterized by heart problems, frequent infections, developmental delay, and learning disabilities [71]. Expression levels and activity of Drosha are both crucial in determining the abundance of miRNAs. Indeed, Drosha and DGCR8 regulate each other through different mechanisms: Drosha destabilizes DGCR8 mRNA whereas DGCR8 stabilizes Drosha through protein-protein interactions [72]. Additionally, nuclear localization and processing activity of the microprocessor complex are regulated by post-translational modifications. For instance, glycogen synthase kinase 3β (GSK3β) is essential for the nuclear localization of Drosha [73], while the serine/threonine kinase ERK increases the stability of DGCR8 [74]. Several RNA-binding proteins are interacting with Drosha, such as the helicase p68 (DDX5) and the helicase p72 (DDX17). In mice, these two proteins, which are both required for the processing of specific miRNAs, bind directly to the Drosha-DGCR8 complex forming a larger nuclear processing complex. Additionally, in humans, the two helicases can serve as scaffold proteins, for example acting as a

bridge between Drosha and p53 in response to DNA damage. This interaction will enhance the processing of miRNAs that exerts growth suppressive functions [75], such as miR-16-1, miR-143 and miR-145 [76].

2.1.4.3 Regulation of nuclear export and Dicer processing

In the nuclear export of miRNAs, the main player is XPO5. In some types of tumors, XPO5 has a truncated C-terminal domain and cannot export miRNAs into the cytoplasm, leading to a decreased number of mature miRNAs [77]. Additionally, in hepatocellular carcinoma, phosphorylation of XPO5 by ERK at T345/S416/S497 sites results in the interaction of this protein with the prolyl isomerase 1 (PIN1). This binding event changes the protein conformation, leading to the retention of XPO5 into the nucleus and inhibiting the nuclear export of pre-miRNAs [78].

The processing activity of Dicer has been thoroughly investigated and it is now clear that co-factors, such as TRBP, can be involved in its regulation. The gene encoding TRBP, TARBP2, is mutated in certain cancers, and it has been hypothesized that reduction of TRBP could destabilize Dicer, resulting in the decrease of miRNA levels [62]. Additionally, phosphorylation of Dicer by ERK increases the stability of the Dicer–TRBP complex, enhancing the stability of the miRNA-generating complex and further stimulating production of miRNAs [79]. An alternative mechanism links TRBP to the mTOR pathway and involves the phosphorylation of TRBP by the ribosomal protein S6

kinase (S6K), again strengthening the stabilization of the miRNA-processing complex [80].

2.1.4.4 Regulation of RISC and miRNA strand selection

After being processed by Dicer, miRNA duplexes are ready to be associated with members of the AGO family of enzymes and incorporated into the RISC complex.

During the process that loads the miRNA duplex into the RISC complex, AGO proteins undergo conformational changes: an “apo” state when they are not bound to the miRNA, a pre-RISC state where they bind to the miRNA duplex, and a third state where the AGO/miR pair is incorporated into the RISC complex. As previously mentioned, among the four known AGO proteins, only AGO 2 possesses the slicing activity that cleaves one strand of the miRNA duplex with the help of the endonuclease C3PO [62], while leaving the mature miRNA associated to AGO and incorporated into the functional RISC [79]. In humans, the other three AGO proteins, AGO1, AGO3 and AGO4, do not possess the slicing activity and the main mechanism through which they select the mature miRNA is by unwinding the duplex [81]. Additionally, the process of loading the miRNA into the RISC complex is an ATP-dependent process mediated by the heat shock cognate 70-heat shock protein 90 chaperone complex (HSC70-HSP90) and that results in a conformational change of AGO, which is now able to bind the miRNA duplex [82].

The strand selection depends on the stability of the two ends of the miRNA duplex. The strand of the miRNA duplex precursor with the weakest terminus at the 5’ end is generally selected as the guide

strand and incorporated into the RISC complex [62]. Interestingly, several studies have been demonstrated an alternative strand selection process called “arm switching” for a variety of miRNAs, such as miR-324, miR-362, miR-193a, and miR-140. MiRNAs generated through the arm switch can have completely different functions. For instance, miR-324-3p is upregulated in hepatocellular carcinoma where it promotes cell proliferation whereas miR-324-5p is downregulated in hepatocellular carcinoma and glioblastoma where it suppresses cell proliferation. The arm switching process can be caused by an alternative activity of Dicer: if pre-miR-324 is uridylated at the 3’ end, the cleavage site of Dicer is shifted of 3 nucleotides, generating a shorter duplex in which the 3’ strand is selected (miR-324-3p) instead of the 5’ strand (Figure 6) [83].

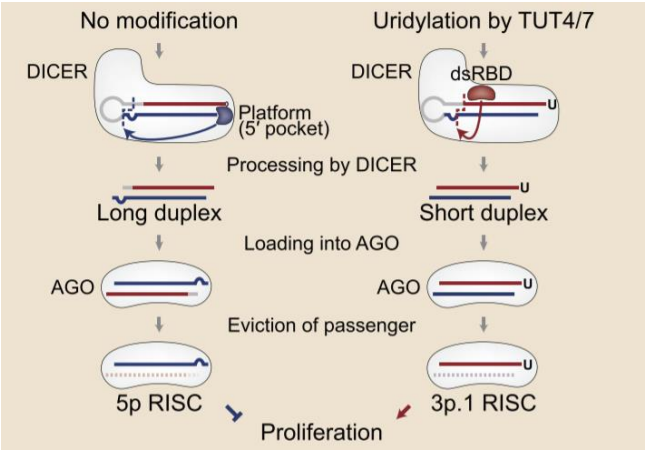


Figure 6. Arm switching of the miR-324. Normally, the selected strand for the mature miR-324 is the 5p. On the contrary, when the pri-miRNA is uridylated, the cleavage site of Dicer is shifted and this creates a shorter duplex, switching the selection of the strand for the mature miRNA from the -5p to the -3p. MiR-324-5p inhibits the proliferation and it has been found downregulated in hepatocellular carcinoma and glioblastoma. Instead, miR-324-3p increases cell proliferation and it is generally upregulated in both hepatocellular carcinoma and glioblastoma. Adapted from Haedong K. et al. [83].

2.1.4.5 MiRNAs intrinsic regulation

In addition to extrinsic regulatory mechanisms, intrinsic modifications such as alterations in the RNA sequence can cause a defective maturation and/or turnover of miRNAs. For instance, certain single nucleotide polymorphisms (SNPs) can affect miRNA processing and target specificity. As showed in Figure 7, pri-miRNA hairpin precursors contain two particular motifs at the basal junction: UG motif and CNNC motif [62]. Accordingly, the substitution of a single nucleotide (C to T) in the first C of the CNNC motif of pri-miR-16-1 can change the efficiency of processing by Drosha, resulting in lower amounts of mature miR-16 [84].

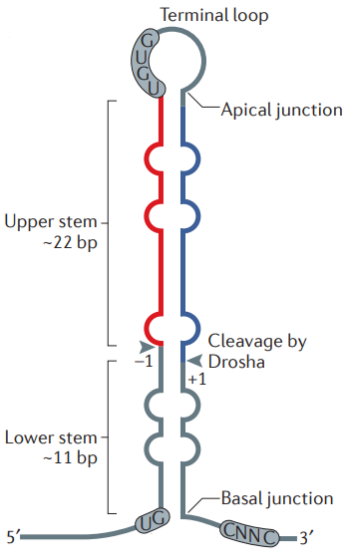


Figure 7. MiRNA hairpin before Drosha cleavage. Drosha cuts pri-miRNAs at approximately ~11bp away from the basal junction and ~22bp from the apical junction. At the basal junction are present 2 motifs: CNNC and UG. A single C to T mutation in the CNNC sequence compromises the efficiency of cleavage by Drosha, resulting in lower amounts of the mature miRNA. Adapted from Ha M. et al. [62].

Another mechanism of miRNA regulation is methylation of DNA at their genes and/or RNA methylation of their precursors or mature sequence. For instance, the methyltransferase BCDIN3D O-methylates the 5´ end of pre-mir-145 and pre-mir-23b, interfering with Dicer processing [85]. A recent study showed that the activity of the microprocessor complex is influenced by DNA methylation. In fact, the 5-methylcytosine-binding protein (MECP2) binds to methylated MIRNA loci, reducing the elongation by RNA pol II and enhancing the activity of Drosha in processing pri-miRNAs. This leads to increased levels of mature miRNAs [86]. There are multiple mechanisms of miRNA regulation through methylation, including methylation of cytosine residues to form 5-methylcytosine (5mC) and methylation of adenine residues to form the 6-methyladenosine (m6A). Interestingly, methylation of specific miRNAs can affect their biogenesis, leading to a change in their biological function. In glioblastoma, miR-181-5p has been shown to inhibit cell proliferation and invasion whereas when the miRNA is 5mC methylated, it loses its repressive functions [87].

Another important mechanism of post-transcriptional regulation that can affect the abundance and turnover of some miRNAs is their stability. In the mammal retina, although the nucleases involved are unknown, several miRNAs, such as miR-183, miR-96, miR-192, miR-204, and miR-211, are subjected to a rapid turnover [62]. In melanoma cells instead, the nuclease Polyribonucleotide Nucleotidyltransferase 1 (PNPT1) is able to specifically degrade miR-221, miR-222, and miR-106 [62]. This rapid turnover seems to be important for miRNA homeostasis, altering miRNA levels and consequently their activity [88].

Finally, some viral RNAs have been shown to destabilize their host miRNAs, both in mouse and human. In humans, certain strains of Cytomegalovirus express a lncRNA, UL144-145, that binds host miR-17 and miR-20a, causing their decay [89]. In fact, one of the functions of lncRNAs is to act as “sponges” for miRNAs, thus sequestering them from their target mRNAs. LncRNAs can bind miRNAs through the miRNA-responsive elements (MREs) present in their sequences. In gastric cancer, for example, the lncRNA TRPM2-AS acts as a sponge for the tumor suppressor miRNA (tsmiR) miR-612. In this way, miR-612 cannot target the insulin-like growth factor-2 mRNA-binding protein 1 (IGF2BP1), finally resulting in impaired degradation of c-MYC mRNA [90]. LncRNAs have been found upregulated in several tumors, including gastric and breast cancers [91]. For instance, MACC1-AS1 lncRNA promotes cell proliferation and breast cancer progression by “sponging” multiple miRNAs, including miR-145-3p and miR-384, to modulate the expression of pleiotrophin (PTN) and c-MYC, respectively [92].

2.1.5 Mechanism of action of miRNAs

MiRNAs are able to regulate several different biological processes such as cell cycle, cell growth and differentiation, cell proliferation, apoptosis, energetic metabolism, and stress response [93]. Since miRNAs require only 70% of homology with their target sequence, a single miRNA can target multiple mRNAs, regulating multiple pathways; likewise, one mRNA can be targeted by several miRNAs [48]. Currently, there are two known mechanisms used by miRNAs to regulate protein synthesis: (I) mRNA destabilization and (II) translational repression [58].

In general, miRNAs bind the 3'UTR of target mRNAs through a sequence of 2-8 nucleotides called the "seed sequence". When the complementarity of the binding between mRNA and miRNA is perfect, the mRNA is degraded; more commonly however, partial complementary of the binding results in the inhibition of translation, which can occur at the level of initiation, post-initiation and elongation [58] (Figure 8). Since the demonstration that miR-369 is able to repress or activate the translation depending on the growth conditions, it is believed that in serum starvation or in the absence of growth factors, some miRNAs can shift from inhibition to activation of translation, increasing the protein level of their target mRNA [94].

MiRNAs can bind to the 5'UTR of mRNA targets (e. g. miR-122 and miR-103a-3p), or can bind to both the 3' and the 5'UTRs [95, 96]. Some miRNAs such as miR-10a can activate translation through their binding to the 5'UTR of the target mRNA. In this model, the binding of miR-10a to the 5'UTR of ribosomal mRNAs, enhances the translation

of ribosomal proteins (RPs) with a consequent increase in ribosome biogenesis [97].

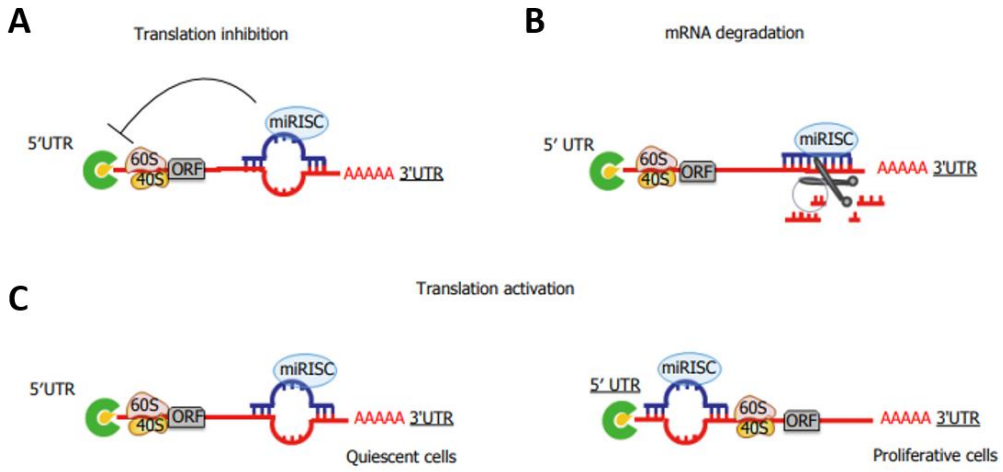


Figure 8. Mechanisms of action of microRNAs. (A) Partial binding of a miRNA (in blue) to the 3'UTR of a target gene through its seed sequence (nucleotides 2-8 at the 5'end of the miRNA sequence) results in the inhibition of translation; **(B)** When the binding between the miRNA and the mRNA is perfectly complementary, miRNAs induce mRNA degradation; **(C)** Under specific conditions, miRNAs can also function as activators of translation. MiRNAs can bind to non-canonical binding sites, such as the 5'UTR, and activate the translation of their mRNA target when cells are quiescent or in a proliferative state.
ORF: Open reading frames. Adapted from Oliveto S. et al. [58].

2.1.6 MiRNA targets identification

The biological role of each miRNA can be investigated through the identification of its gene targets, which are estimated to be over 200 [98]. Bioinformatic approaches that predict miRNA gene targets are a good and rapid starting point toward the identification of a putative miRNA target. Originally based only on the seed sequence, the ranking of miRNA gene targets is now determined by including other parameters, such as the free energy of the binding and the secondary structure of 3'UTRs [99]. In addition to miRNA gene target prediction, online databases contain information related to the miRNA sequences, such as their genomic localization and their expression levels in different cell lines or tissues.

While gene target analysis is important, it is crucial to validate experimentally the effect of a miRNA on its predicted targets. One of the most popular approaches to validate miRNA/3'UTR interaction is to use a reporter plasmid in which the 3'UTR of the predicted gene target is cloned in frame with a reporter gene, such as renilla or luciferase. Another approach is RNA immunoprecipitation (RIP), which can be used to identify the binding between the RISC and the mRNA target.

Finally, an omics-based approach can be used to identify a target through differential proteomic and/or transcriptomic analyses after inhibition or over-expression of a given miRNA [99].

2.1.7 Role of miRNAs in tumors

MicroRNAs can regulate different cellular processes, in both physiological and pathological conditions, and dysregulation of a single miRNA or a subset of miRNAs can play key roles in the development of diseases. The first evidence of microRNA-cancer relationship was found through the discovery that two miRNAs, miR-15a and miR-16-1, were deleted in 69% of patients with chronic lymphocytic leukemia (CLL) [58].

There are multiple mechanisms through which the expression of a miRNA can be dysregulated in a tumor. Since many miRNA genes are located in cancer-associated genomic regions [59], the increased or decreased expression of miRNAs in tumors is often attributed to deletion or amplification of miRNA genes. In addition to the previously cited miR-15-a and miR-16-1, other examples are represented by miR-143 and miR-145, whose gene deletion was found in lung cancer, and by the miR-17-92 cluster, whose gene amplification was found in B-cell lymphoma and lung cancer [59].

In addition to amplification or deletion, abnormal expression of miRNAs in tumors can be also caused by dysregulation of key transcription factors, such as p53, c-MYC, nuclear receptors, and RAS. The tumor suppressor p53, often mutated in cancer, increases the expression of miR-34a through direct binding to its promoter. Upregulation of this miRNA in turn triggers apoptosis through several mechanisms, such as the suppression of Sirtuin1 (SIRT1) pathway [100]. Another oncoprotein, c-MYC, is frequently up-regulated in tumor cells in which it regulates a variety of miRNAs. For example, c-MYC activates the transcription of the miR-17-92 cluster, an oncogenic

cluster, and represses the transcription of mir-15a, miR-26, miR-29, mir-30, and let-7 families, which are considered tumor suppressor miRNAs [101]. Additionally, in hepatocellular carcinoma c-MYC has been found to inhibit the transcription of miR-148a-5p/ miR-363-3p genes through direct binding to their promoter, inducing G1 to S phase progression and enhancing tumorigenesis [102].

Similar to all other genes, miRNA gene transcription is under epigenetic control, such as methylation and histone modification, and tumors may be induced by epigenetic changes in miRNAs [60]. The importance of epigenetic modifications in miRNA transcription is documented for example by a study showing that a therapy targeting an histone deacetylase inhibitor (suberoylanilide hydroxamic acid or SAHA) in combination with a DNA methyltransferase (epigallocatechin-3-gallate or EGCG), resulted in the downregulation of two oncogenic miRNAs, miR-221 and miR-222 in TNBC cells [103]. Given the growing number of miRNAs found to associate with tumorigenesis, cancer-associated miRNAs have been divided into two types: oncogenic miRNAs (oncomiRs) and tumor suppressor miRNAs (tsmiRs). As shown in Figure 9, aberrant miRNA expression can result in increased levels of oncomiRs that inhibit tumor suppressor genes, or it can result in decreased tsmiRs that cause the upregulation of oncogenes. Consequently, all these events lead to tumorigenesis, stimulate uncontrolled cell proliferation, and induce anti-apoptotic responses, invasion, and metastasis [48]. The identification of such powerful miRNAs (oncomiRs and tsmiRs) has important implications for therapeutic approaches against a variety of diseases, including cancer. Drugs that inhibit or downregulate oncomiR expression can

trigger the reactivation of tumor suppressor genes, resulting in tumor regression or eradication. On the other hand, the upregulation of tsmiRs expression can block cell proliferation, invasion, and metastasis, leading to tumor regression or eradication [42].

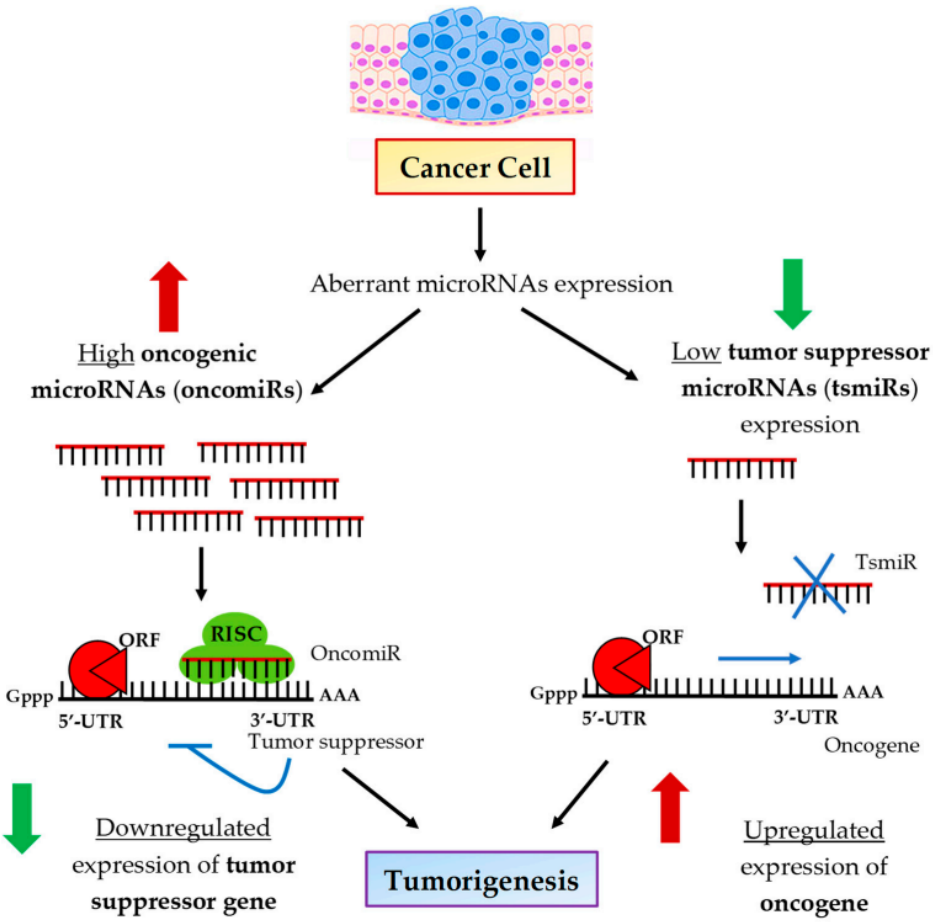


Figure 9. Regulatory mechanisms of oncomiRs and tsmiRs in cancer. Increased expression of oncomiRs leads to the inhibition of tumor suppressor genes and decreased expression of tsmiRs leads to the upregulation of oncogenes; in turn, these events will lead to tumorigenesis and will stimulate cell proliferation, anti-apoptotic response, invasion and metastasis. Adapted from Loh HY. et al. [48].

2.1.7.1 Role of miRNAs in breast cancer

Dysregulation of cell proliferation is an important hallmark of cancer cells [104]. Several studies demonstrated that miRNA can regulate cell proliferation and cell cycle events in breast cancer through their interaction with cyclin proteins, protein kinases, growth factor promoters, and tumor suppressor genes [48]. Cyclin E1, an important cell cycle regulator of the G1–S transition, was shown to be a target of several tsmiRs such as miR-497, miR-16, and miR-30c-2-3p. Overexpression of these miRNAs was sufficient to inhibit cell proliferation and cell cycle progression of breast cancer cells [48]. Indeed, miR-483-3p has been found to target directly Cyclin E1, preventing DNA synthesis initiation by the nuclear protein coactivator of histone transcription (NPAT), further inhibiting the entrance of breast cancer cells into the S phase of cell cycle [105]. Since miR-424-5p is commonly downregulated in breast cancer, its overexpression was shown to target the cyclin-dependent kinase 1 (CDK1), arresting the cells in the G2-M phase of the cell cycle [106]. Interestingly, in TNBC cell lines, the same miRNA is able to downregulate the proliferation and the metastatic behavior by binding to its downstream target doublecortin-like kinase 1 (DCLK1) [107].

MiRNAs can influence another important hallmark of cancer, the metastatic and invasive potential of tumor cells, by regulating the epithelial-mesenchymal transition (EMT) and other genes responsible for cell invasion and motility. EMT is the biological process that allows cancer cells to acquire migratory capabilities and, as such, it has a central role in the dissemination of metastasis. In this context, one of the most important miRNA families is the family of tumor suppressor

miRNAs, miR-200. In breast cancer stem cells and in TNBC, low expression of miR-200 family members is associated with EMT, tumor progression and a more aggressive phenotype. MiR-200a, miR-200b, and miR-200c target the transcriptional repressors of E-cadherin ZEB1 and ZEB2 increasing the levels of E-cadherin and consequently suppressing EMT [108]. In breast cancer, miR-200b blocks EMT and metastasis formation, inhibiting Kindlin2, a positive regulator of metastasis [109]. Furthermore, another component of the miR-200 family, miR-200c, maintains the epithelial phenotype suppressing genes involved in cell motility and promotes apoptosis targeting the X-linked inhibitor of apoptosis (XIAP) [110]. Nevertheless, some studies report that other members of the miR-200 family are oncomiRs, since they are increased in some tumors, such as colorectal cancer where they contribute to increased cell proliferation [111].

Chemotherapy is one of the main treatments for breast cancer, especially for TNBC. However, several tumors develop drug resistance in which miRNAs can play an important role, influencing pathways involved in drug transporters, apoptosis, activation of EMT, and in an increased number of cancer stem cells (CSC) (Figure 10). For example, in doxorubicin-resistant breast cancers, miR-505, miR-128 and miR-145 are downregulated and miR-663, miR-181a, and miR-106b-miR-25 clusters are upregulated [112]. Regarding cisplatin, another drug used in the treatment of breast cancer that inhibits DNA replication, several experiments indicated that miR-345 and miR-7 are associated with the cisplatin-resistant phenotype whereas miR-302b can sensitize cells to cisplatin by regulating E2F transcription factor 1 (E2F1) and the cellular DNA damage response [113, 114]. As breast

cancer cells acquire drug resistance, tsmiRs can be interesting molecular targets in the therapy of breast cancer [48]. Indeed, some studies revealed how reintroducing some of the downregulated miRNAs into the tumor cells can revert the resistance phenotype. One such example is given by miR-129-5p that, that if re-introduced, it sensitizes the cells to Trastuzumab by decreasing the expression of the ribosomal protein S6 (RPS6) [115]. In Paclitaxel-resistant TNBC, overexpression of miR-5195-3p was shown to re-sensitize the tumor to chemotherapeutic drug by depleting the Eukaryotic translation initiation factor 4 alpha 2 (EIF4A2) transcript. Similarly, the miRNA-449 family has been shown to partially reverse Doxorubicin-resistance by targeting cell cycle genes [116-118]. Beside intervening by overexpressing miRNAs, an anti-miRNA technology has been also investigated in the treatment of TNBC. The technology consisted of targeted delivery to the cancer stem cells of RNA nanoparticles containing anti-miR-21. Promising results showed inhibition of expression of miR-21 and consequent up-regulation of its targets, the tumor suppressors PTEN and programmed cell death 4 (PDCD4). In addition, the anti-miR-21 delivery with this technology inhibited tumor growth in TNBC xenograft models [117, 119].

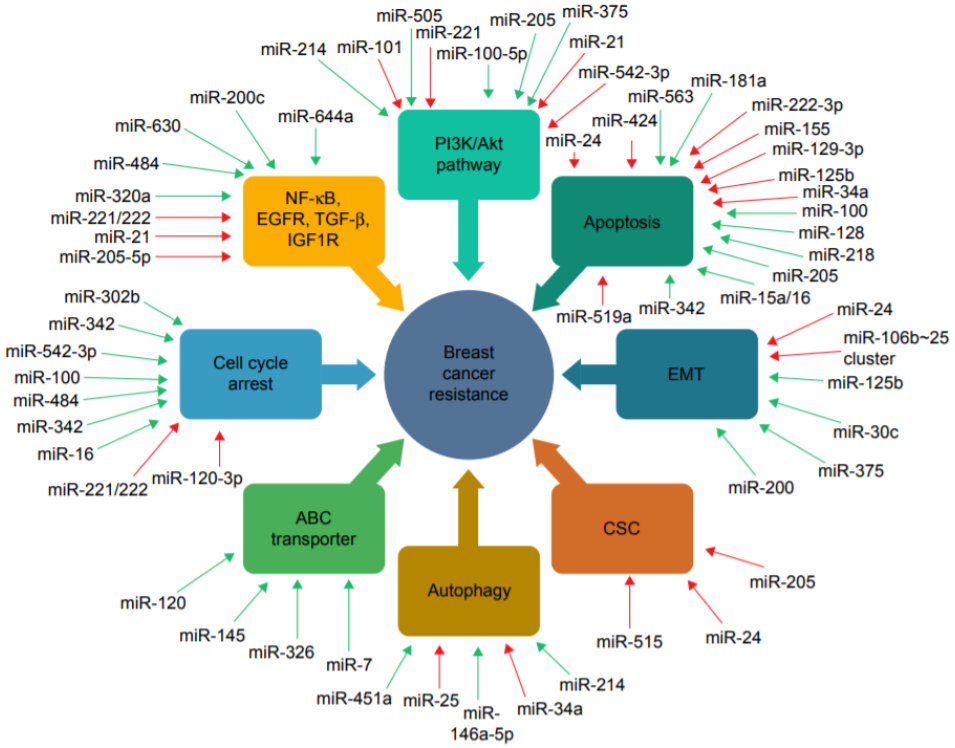


Figure 10. MiRNA-mediated mechanisms involved in breast cancer drug resistance. MiRNAs can influence drug resistance and they can affect many different pathways. The red arrows indicate oncomiRs and the green arrows indicate tsmiR. Adapted from Hu W. et al. [112].

2.1.8 The role of miR-3189-3p

MiR-3189 is a miRNA located in the single intron of the growth differentiation factor 15 (GDF15) gene (Figure 11) [120]. The precursor sequence contains two mature miRNAs: miR-3189-3p and miR-3189-5p, respectively 21 and 25 nucleotides long [120].

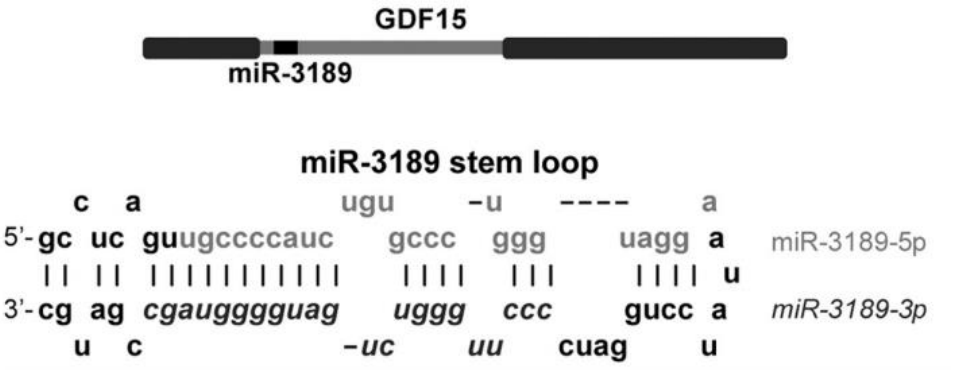


Figure 11. Location of the miR-3189 sequence. MiR-3189 sequence is located into the single intron of the GDF15 gene. The pre-miRNA contains two mature miRNAs: miR-3189-3p (21 nucleotides long) and miR-3189-5p (25 nucleotides long). Adapted from Jeansonne D. et al.[120].

Dr. Peruzzi's laboratory has previously shown the anti-tumoral effects of miR-3189-3p in glioblastoma [120] and melanoma (unpublished data). Overexpression of miR-3189-3p in the glioblastoma cell lines LN229 and U87MG resulted in a 50% reduction of cell proliferation whereas the overexpression of miR-3189-5p did not have the same effect [120]. Additionally, cells expressing miR-3189-3p showed less migratory capacity when compared to control cells (~58%). Impairment of growth and migration by the miR-3189-3p was mediated by the downregulation of two targets of the miRNA, the splicing factor 3b subunit 2 (SF3B2) and the guanine nucleotide exchange factor

p63RhoGEF, respectively. Importantly, miR-3189-3p was under-expressed in astrocytoma and glioblastoma samples when compared to normal brain tissue, whereas the above cited targets showed an opposite trend. In the same study, we have also demonstrated the antitumoral activity of miR-3189-3p *in vivo*. In fact, after intracranial injection, mice injected with miR-3189-3p transfected U87MG cells showed reduced tumor growth or no tumor [120]. Later, miR-3189-3p was found to inhibit cell proliferation in colorectal and gastric cancer [121, 122], confirming the activity of this miRNA in a variety of tumors. Nevertheless, additional studies are needed to clarify if miR-3189-3p can work as a therapeutic agent.

The absence of a well-defined targeted therapy available for TNBC patients and the pressing need to devise novel therapies, prompted us to investigate the role of miR-3189-3p in TNBC, which is the topic of this thesis.

3. TRANSLATION

The translation of mRNAs into proteins is a complex, multistep process that requires the coordination of many components. It can be grossly divided into four phases: initiation, elongation, termination, and ribosome recycling [123]. In the first step, the ribosome 80S is assembled at the start codon, generally AUG, with a methionyl-tRNA (met-tRNA) in the peptidyl site (P site) of the ribosome. During elongation, the ribosome moves further on the mRNA, synthetizing one amino acid at the time through the action of a tRNA bound to the proper amino acid. Apart from the starting methionyl-tRNA that is localized in the P site, all other new tRNAs enter the ribosome in the aminoacyl site (A site), then they move to the P site where the poly-peptide chain is formed, and finally they exit from the ribosome through the exit site (E site). In the termination step, the ribosome will reach a stop codon (UAA, UAG or UGA), and the protein will be released from the tRNA. Finally, the 2 subunits of the ribosome are recycled to start a new process (Figure 12) [123].

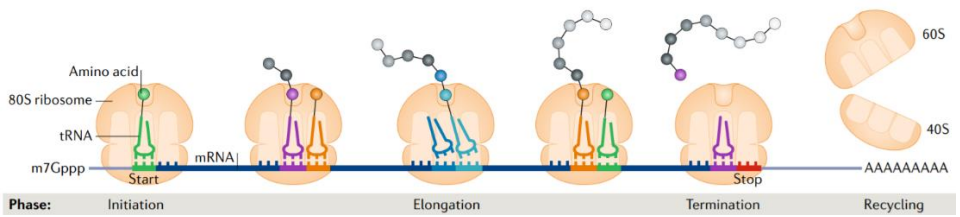


Figure 12. Overview of eukaryotic translation. The process of translation can be divided into four phases: initiation, elongation, termination and ribosome recycling. In the initiation phase, the ribosome 80S is assembled on the start codon with the met-tRNA in the site P of the ribosome. During elongation, the ribosome moves further on the mRNA and a new tRNA bound to an amino acid enters the site A of the ribosome and the polypeptide chain starts to form. In the termination phase the ribosome encounter the stop codon and the protein is released from the ribosome. After the termination, the subunits of the ribosome are recycled for a new translation process. Adapted from Schuller A.P. et al. [123].

3.1 Canonical translation

There are several types of translation, but they can be classified into two major sub-groups: canonical and non-canonical. The canonical translation is also called cap-dependent translation and is mediated by the presence of a 7-methylguanosine cap structure (m7 G cap) at the 5' end and a poly(A)-tail at the 3' end of mRNA. The majority of mRNAs undergo cap-dependent translation. The non-canonical type of translation is cap-independent and relies on other RNA structures or modifications, as described in the chapter 3.2.

3.1.1 Canonical translation initiation

At the end of a translation process, the eukaryotic translation initiation factors (eIFs) eIF3, eIF1, and eIF1A are associated with the 40S subunit of the ribosome, as illustrated in Figure 13 [124]. The binding of eIF1 and eIF1A induces a conformational change in the 40S subunit, opening the mRNA-binding channel [125]. Then the ternary complex, composed of GTP, eIF2, and a met-tRNA, is subsequently attached to the 40S subunit to form the 43S preinitiation complex [124]. The binding of the 43S complex to the mRNA requires the cooperative action of the eIF4F complex to unwind the 5'cap region of the mRNA and prepare it for the ribosomal attachment. In this phase, the multidomain "scaffold" protein EIF4G binds the cap-binding protein eIF4E, the DEAD-box RNA helicase eIF4A, the poly(A)-binding protein (PABP) and eIF3 [124]. Additionally, eIF4B and eIF4H are recruited to the 43S complex to enhance the helicase activity of eIF4A [124].

After the attachment to the mRNA, the 43S preinitiation complex binds the mRNA forming the 48S preinitiation complex, which then scans the mRNA sequence downstream of the cap in search of the start codon. The factors eIF1 and eIF1A are required for the scanning activity and the movement of the 43S complex on the mRNA [125, 126]. EIF3 may additionally contribute to the scanning activity of the complex by interacting with mRNA upstream of the A site of the ribosome and by forming an extension of the mRNA-binding channel, increasing translation efficiency [127].

The recognition of the correct start codon is fundamental and eIF1 has been shown to play a key role in this event, allowing 43S complexes to discriminate against non-AUG triplets and dissociating aberrant ribosomal complexes [128]. Once the start codon is recognized, eIF5 binds eIF2 and induces the hydrolysis of GTP [124], which results in a reduced affinity of eIF2 for met-tRNA leading to the partial dissociation of eIF2–GDP from 40S subunits [128, 129].

In the final step, the 60S subunit is recruited to the 40S, eIF5B induces the dissociation of eIF1, eIF1A, eIF3, and the elongation can proceed [130, 131].

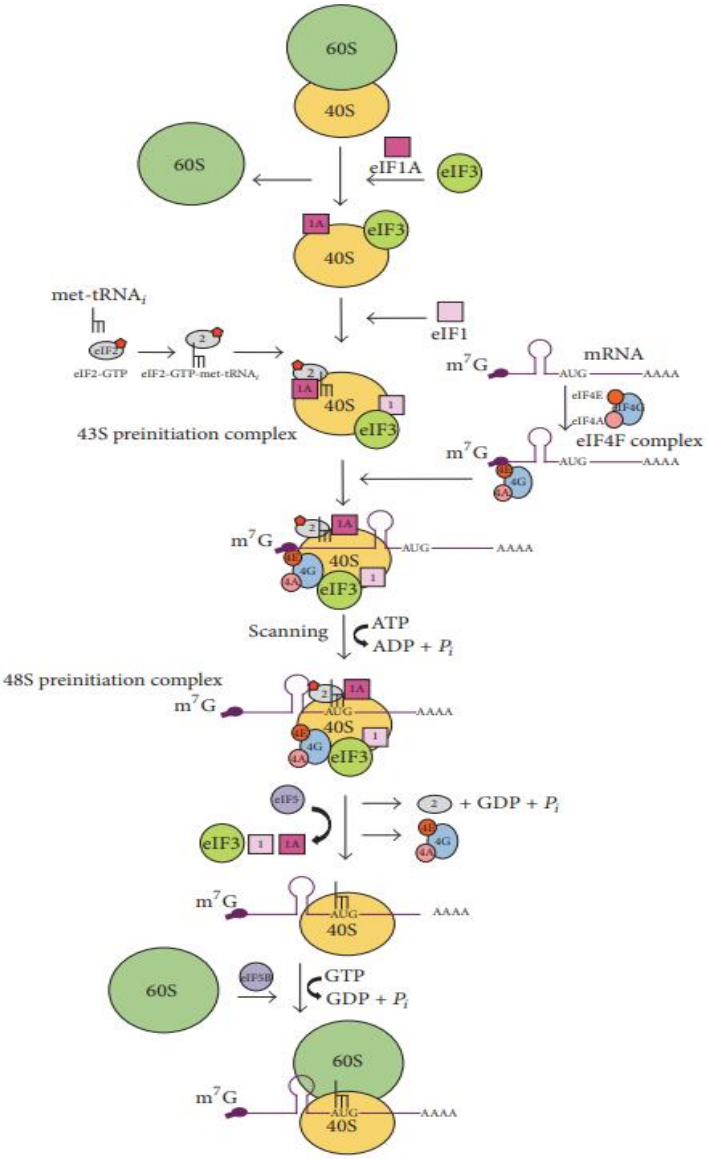


Figure 13. Cap-dependent translation initiation and its regulation. During the ribosome recycling phase, eIF1A, eIF1 and eIF3 are associated with the 40S subunit of the ribosome. Then the ternary complex, composed of eIF2, a methionine charged transfer RNA (met-tRNA), and GTP, binds the 40S subunit to form the 43S preinitiation complex. At this point the eIF4 complex, composed of the cap-binding protein eIF4E, the DEAD-box RNA helicase eIF4A and the multidomain scaffold protein eIF4G bind the 43S preinitiation complex forming the 48S preinitiation complex. Once the start codon is recognized, the hydrolysis of GTP from the ternary complex allows the detachment of all the eIFs and the recruitment of the 60S subunit to the ribosome. Adapted from Sharma DK. et al. [132].

3.1.2 Regulation of the canonical translation initiation

Translation is a very important biological process and it is often dysregulated in a variety of diseases such as metabolic disorders, neurological disorders, immunodeficiency, and cancer [133].

The mechanisms that regulate translation are grossly divided into mechanisms influencing the initiation factors and mechanisms influencing the mRNA itself. One of the most investigated mechanisms is the one that regulates the availability of active eIF2. After GTP hydrolyzation, eIF2B normally mediates guanine nucleotide exchange on eIF2, preparing this factor for the new translation cycle. Serine phosphorylation of eIF2α subunit blocks the activity of eIF2B and inhibits the recycling capabilities of an active eIF2-GTP form, ultimately reducing the general level of translation (Figure 14) [124, 134]. EIF2 is a central molecule in cellular stress responses such as UV radiation, amino acid starvation and endoplasmic reticulum (ER) stress. Phosphorylation of eIF2 results in a reduction in global protein synthesis with a consequent increase in the expression of NF-κB-dependent genes [134]. Similarly to eIF2, phosphorylation of eIF4E plays critical roles in translation [124] and will be explained in detail in the chapter 3.1.3.

Another RNA-binding protein that participates in the translation initiation complex is PABP, which binds the poly(A)-tail while interacting with eIF4G, therefore creating a “circular” mRNA that facilitates translation [135, 136].

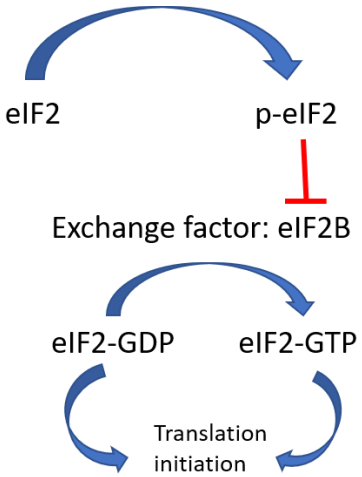


Figure 14. Cap-dependent translation initiation and its regulation. EIF2 is part of the ternary complex together with GTP and met-tRNA. This complex participates in the translation initiation by binding the 40S subunit of the ribosome and consequently forming the 43S pre initiation complex. During the translation, the GTP bound to eIF2 is hydrolyzed to GDP. EIF2B then mediates the guanine nucleotide exchange on eIF2, preparing this factor for the new translation cycle with new GTP. Phosphorylation of eIF2 blocks the activity of eIF2B, which in turn inhibits the attachment of new GTP and leads to a decreased global translation. Adapted from Wek RC. et al. [134].

3.1.3 EIF4E and its regulation through 4E-BP1

EIF4E is required for the translation of almost all eukaryotic mRNAs. It is a component of the eIF4F complex that binds eIF4G and the m7 G cap on the 5' end of the mRNA. The activity of eIF4E is limited by eIF4E-binding protein 1 (4E-BP1), which prevents its interaction with eIF4G [132]. The mechanism involves 4E-BP1 phosphorylation. The non-phosphorylated form of 4E-BP1 binds eIF4E and inhibits the formation of the 48S preinitiation complex, further inhibiting the cap-dependent translation (Figure 15) [137, 138]. For this repressive function on translation, 4E-BP1 is considered a tumor suppressor [138,

139]. Nevertheless, in many tumors, such as breast cancer [140, 141], bladder carcinoma [142], colon cancer [143], esophageal cancer [144], pediatric glioma [145], lung cancer [146], ovarian cancer [147], and prostate cancer [148], 4E-BP1 is hyperphosphorylated and therefore unable to bind eIF4E. Furthermore, in all these types of tumors, the levels of phospho-4E-BP1 (p-4E-BP1) positively correlate with poor prognosis, poor differentiation and tumor progression.

This dual role of 4E-BP1 in tumors can be explained, at least in part, by the fact that tumor cells need to adapt to different growth conditions in the tumor microenvironment. In this context, 4E-BP1 could favor cell survival allowing their adaptation to stress conditions through the translation of specific stress response genes [138]. Indeed, several studies have found that the 4E-BP1/eIF4E axis can preferentially regulate some transcripts. mRNAs with short and less complicated 5'UTR, such as housekeeping genes mRNA, are unaffected by eIF4E changes, while mRNAs with longer and more complex 5'UTR, generally found in growth factors, angiogenesis factors and proto-oncogenes, are not efficiently translated when the activity of eIF4E is limited [149]. Consequently, as showed in Figure 15A, when 4E-BP1 is phosphorylated (inactive) and eIF4E activity is increased, vascular endothelial growth factor (VEGF), fibroblast growth factor 2 (FGF2), MYC proto-oncogene (c-MYC), and cyclin D1 transcripts are increased. In contrast, when 4E-BP1 is not phosphorylated (active) there will be a selective inhibition of translation of the above-mentioned transcripts (Figure 15B) [138, 149, 150]. Furthermore, under hypoxic conditions, 4E-BP1 can increase the translation of HIF-1α and VEGF

(Figure 15B) through a cap-independent translation, mainly an IRES-dependent translation [151], as detailed in the next chapter.

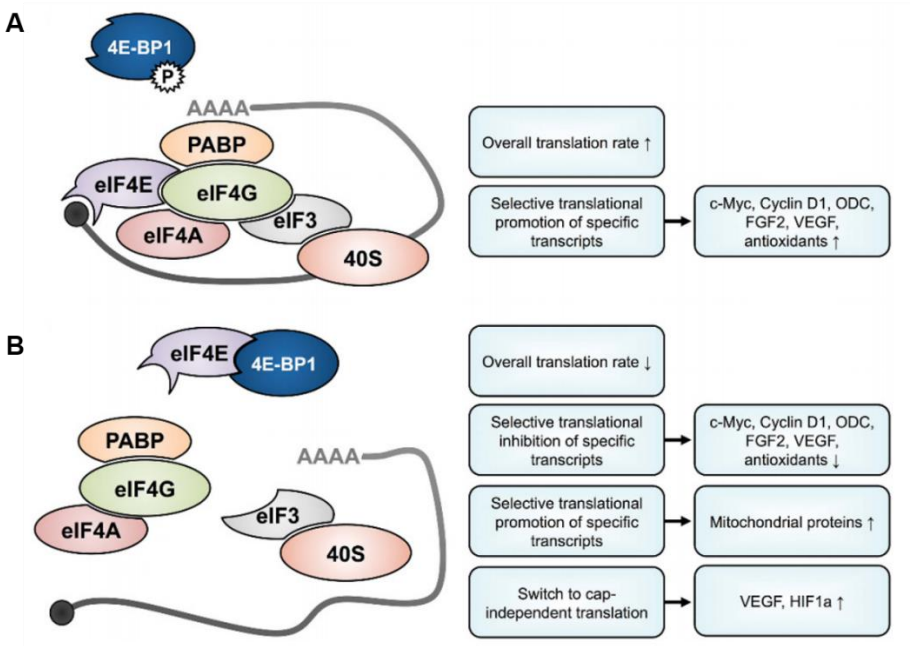


Figure 15. Schematic illustration of 4E-BP1 regulating eIF4F assembly and its impact on global and selective translation rates. (A) Phosphorylated and inactive 4E-BP1 does not bind eIF4E, which is now able to promote overall translation, including translation of specific mRNAs with long and more complicated 5'UTR, such as pro-oncogenic transcripts and growth factor transcripts; **(B)** Non-phosphorylated and active 4E-BP1 binds eIF4E, inhibiting eIF4F complex formation and decreasing the overall translation rate, including the translation of pro-oncogenes and growth factors. However, under stress conditions, active 4E-BP1 can also induce cap-independent translation, promoting the translation of HIF1α and VEGF. Adapted from Musa J et al. [138].

3.2 Non-canonical translation

Under stress conditions, such as hypoxia, starvation and endoplasmic reticulum stress, the canonical translation is inhibited. However, cells still require some essential RNAs, which are then translated through a non-canonical or cap-independent mechanism [152]. There are several types of non-canonical translation: leaky scanning, ribosomal shunting, translation re-initiation, IRES-dependent translation and m6A-dependent translation.

3.2.1 IRES-dependent translation

In 1998, Johannes G. and Sarnow P. showed that the 40S subunit of the ribosome can bind the specific internal structures of mRNA without the need to bind the 5- cap [153]. These internal structures are called internal ribosome entry sites (IRES) and they were first discovered in viruses of the *picornaviridae* family (hepatitis A virus, human rhinovirus, poliovirus, and foot-and-mouth disease virus), whom mRNAs lack the cap and the poly(A)-tail [154, 155]. Analogous sequences were later found in cellular mRNAs [156] and it is now estimated that about 10% of cellular mRNA is translated in an IRES-dependent manner [157, 158]. In fact, IRES sequences have been found in several cellular mRNAs, such as c-MYC, FGF, VEGF and p53 [159-162], and are summarized in Table 1. A more detailed list of cellular and viral IRESs can be found at http://iresite.org/IRESite_web.php.

Cellular pathway	Name	References
Amino acid starvation	Cationic amino acid transporter 1 (CAT-1)	Fernandez et al. 2001
	Sodium-coupled amino acid transporter (SNAT2)	Gaccioli et al. 2006
Nutrient signaling hypoxia	Human insulin receptor (HIR)	Spriggs et al. 2009
	Hypoxia inducible factor 1α (HIF-1α)	Schepens et al. 2005
	Vascular endothelial growth factor (VEGF)	Morris et al. 2010
	Fibroblast growth factor 2 (FGF2)	Bonnal et al. 2003
Apoptosis survival	Apoptotic protease activating factor 1 (Apaf-1)	Sella et al. 1999
	B-cell lymphoma 2 (Bcl-2)	Marash et al. 2008
	BAG family molecular chaperone regulator 1 (Bag1)	Pickering et al. 2004
	Cellular inhibitor of apoptosis 1 (cIAP1)	Graber et al. 2010
	X-linked inhibitor of apoptosis (XIAP)	Riley et al. 2010
Oncogene	c-myc	Le Quesne et al. 2001
	c-Jun	Blau et al. 2012
Mitosis	p58 PITSLRE	Cornelis et al. 2000
	Cyclin-dependent kinase 1 (CDK1)	Marash et al. 2008
	p120 catenin (p120)	Silvera et al. 2009
DNA damage response	p53	Yang et al. 2006
	Serine hydroxymethyltransferase 1 (SHMT1)	Fox et al. 2009
	Replication protein A2 (RPA2)	Yin et al. 2013
Differentiation	Homeobox transcription factor (Hox)	Xue et al. 2015
	Platelet-derived growth factor 2 (PDGF2)	Sella et al. 1999
	Runt-related transcription factor 1 (Runx1)	Pozner et al. 1999
	Lymphoid enhancer-binding factor 1 (LEF-1)	Jimenez et al. 2005
Cold shock	Cold inducible RNA-binding protein (CIRP)	Al Fageeh and Smales 2009

Table 1. Mammalian cellular IRES. List of some of the most important IRES sequences discovered in mammals. Adapted from Kwan T. et al. [163].

While the viral IRES can be divided into four sub-classes, the cellular IRES can be classified as type I or type II, based on the mechanism of ribosome recruitment. Type I IRES, also called “land and scan IRES“ [152], interacts with the ribosome through IRES-transacting factors (ITAFs) to initiate translation (Figure 16A) whereas type II IRES recruits the ribosome through direct interaction with the 18S ribosomal RNA (rRNA) of the 40S subunit (Figure 16B) [164].

ITAFs were initially identified as nuclear proteins and proteins that can shuttle between the nucleus and the cytoplasm, and that can participate not only in the translation process but also in the transcription. One example of ITAF shuttling between nucleus and

cytoplasm is the polypyrimidine tract binding protein (PTB) that normally promotes the translation through viral and cellular IRES but can also participate in RNA splicing, stability and transport [165].

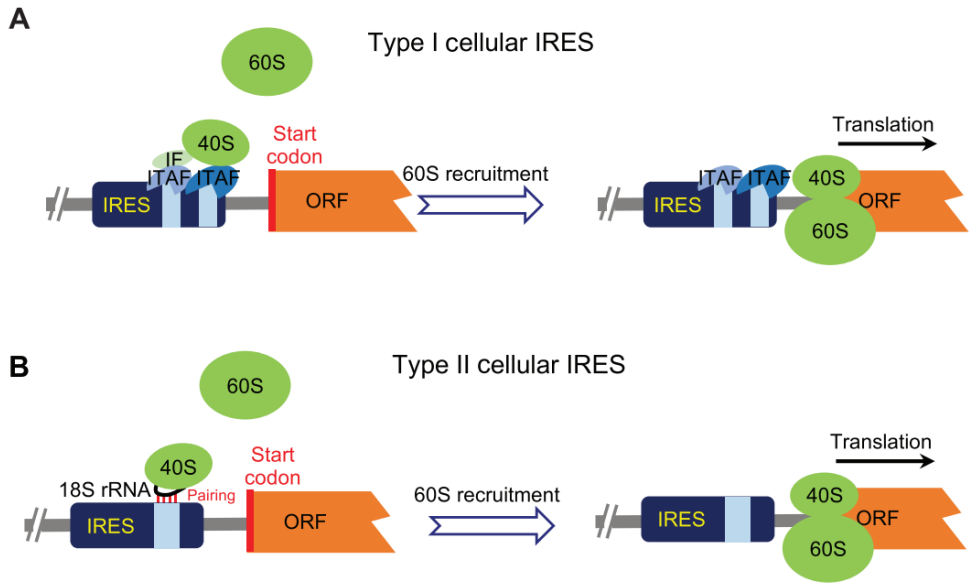


Figure 16. Types of cellular IRES. (A) Type I cellular IRES recruits the 40S subunit of the ribosome through IRES-transacting factors (ITAF). Then the 60S subunit joins the complex and the translation starts; **(B)** Type II cellular IRES can directly interact with the ribosome. More specifically, it recruits the 40S subunit through an element that pairs with the 18S rRNA. Then the 60S subunit joins the complex and translation starts. Adapted from Yang Y. et al. [164].

The mechanism through which ITAFs can facilitate ribosomal recruitment is not still completely understood. Some studies suggest that ITAFs function as chaperone proteins, remodeling RNA structures and allowing the attachment of the ribosome. Other studies refer to an alternative mechanism by which ITAFs could function as adaptors, interacting with the ribosome or eIFs [156, 166]. Accordingly, as shown

in Figure 17B, the IRES sequence of *XIAP* mRNA recruits the ribosome close to the AUG codon with the cooperation of ITAFs, eIF3 and PABP. The Interaction between eIF3 and PABP through the polyadenylate-binding protein-interacting protein 1 (PAIP1) enhances ribosome recruitment allowing circularization of the mRNA and binding to the 40S subunit [167]. EIF3 is also required for the activity of c-MYC IRES. More specifically, IRES-mediated translation of c-MYC requires the interaction of eIF4G with eIF4A and eIF3, although this protein appears to be recruited directly to the mRNA and not via eIF4G [168]. However, this mechanism has yet to be fully clarified.

Interestingly, one ITAF can regulate simultaneously more than one IRES and one given IRES can be regulated by several ITAFs in a positive or negative manner. For example, *p53* mRNA has two known IRES sequences and, in normal condition, when the cap-dependent translation is preferred, two ITAFs, nucleolin and PDCD4, act as negative regulators of the IRES-dependent translation. In contrast, in stress conditions, translational control protein 80 (TCP80) and RNA helicase A (RHA) bind the IRES sequence, enhancing the IRES-dependent translation [159].

In stress conditions, the cap-dependent translation is inhibited and the cells use alternative mechanisms of translation to survive. Inside the tumor, cells are subjected to several stress conditions, including nutrient deprivation and hypoxia, and consequently, oncogenes and tumor suppressor genes are tightly regulated by IRES translation. IRES translation of certain mRNAs, such as c-MYC, p53, XIAP and c-JUN, is required for tumor cell growth, tumor angiogenesis and

evasion from apoptosis [169]. For example, c-JUN levels have been found elevated in glioblastoma without an increase in its mRNA, indicating an increase in the translation process [170].

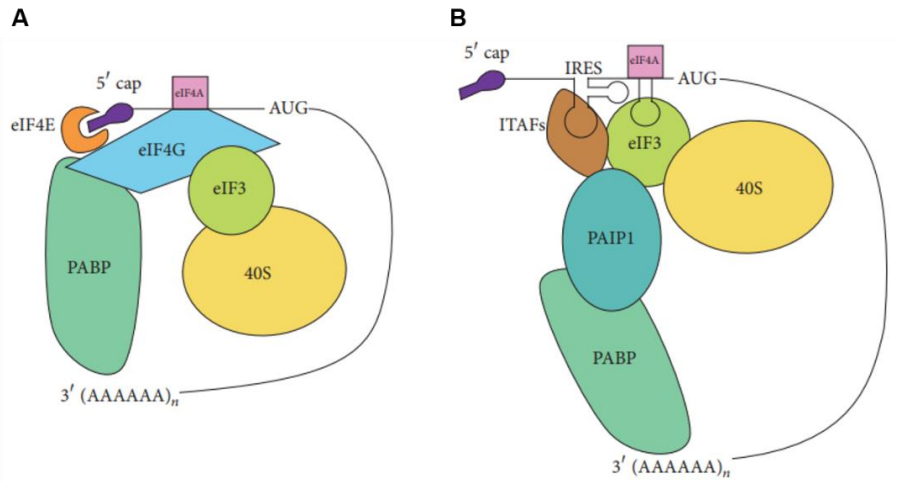


Figure 17. Comparison between the cap-dependent translation and the IRES translation in recruiting initiation factors. (A) In the cap-dependent translation, the initiation complex is recruited on the 5' end of mRNAs with the help of several eIFs, such as the eIF4F-complex (eIF4E, eIF4G, and eIF4A), eIF1A and eIF1, and eIF3; **(B)** In the IRES-mediated translation, the initiation complex is formed on the IRES sequence with the help of ITAFs and eIFs. In this example, XIAP IRES interacts with eIF3 and recruits the ribosome via eIF3-PAIP1-PABP bridge. Adapted from Sharma DK. et al. [132].

3.2.2 m6A-dependent translation

N6-methyladenosine (m6A) modification is one of the most abundant RNA modifications, representing 80% of all RNA base modifications [152] and it has been implicated in several cellular and physiological events including cortical neurogenesis, embryogenesis, spermatogenesis and tumorigenesis [171]. At first, m6A was discovered mainly in the 3'UTR of mRNAs, suggesting a role for RNA

stabilization [172]; however, in recent years, m6A has been found also in the 5'UTR, indicating a possible role in the translation initiation [173, 174]. Indeed, some studies showed that mRNAs containing the m6A modification can be translated even without the presence of the eIF4F complex, confirming a new role of the m6A in the translation initiation [173, 175, 176]. Similarly to the IRES-mediated translation, the m6A-dependent translation is used by the cells that are under stress when the canonical translation is decreased or inhibited. Inside a tumor, for example, cells are subjected to multiple stresses, such as hypoxia, starvation and oxidative stress, and they rely on cap-independent translation to synthesize proteins that allow their survival and growth in the tumor microenvironment.

Although the m6A RNA modification can occur in both the nucleus and in the cytoplasm, it takes place mainly in the nucleus on the nascent mRNA [177]. In the m6A-dependent translation, the recruitment of the 43S pre initiation complex requires only eIF1, eIF1A, eIF3 and the 40S subunit of the ribosome, with eIF3 being the factor that binds the m6A modification in the mRNA to start the translation [173] (Figure 18). For example, in response to heat-shock stress, there is an increase in the methylation of the heat-shock protein 70 (HSP70) 5'UTR [178]; this event triggers the recruitment of the 40S subunit and the binding of eIF3 to m6A residues, with consequent translation initiation [179]. Indeed, the fact that the presence of m6A modifications recruits the ribosome directly to specific sites, could represent a potential mechanism for the translation of truncated protein [178].

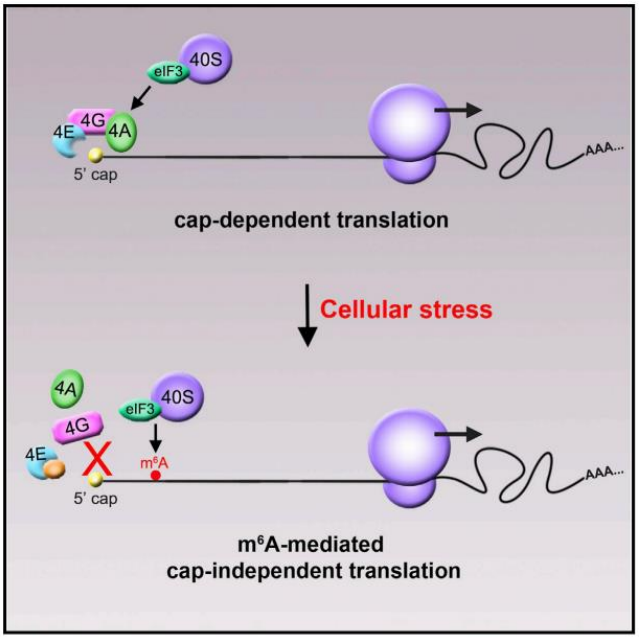


Figure 18. m6A-mediated cap-independent translation. In normal conditions, the cap-dependent translation is favored. In contrast, under cellular stress the canonical translation is inhibited and m6A modifications can direct a cap-independent translation. In this case, the translation initiation requires only some eIFs, such as eIF3, and the 40S subunit of the ribosome. Adapted from Meyer KD. et al. [173].

The quantity of m6A modifications deposited on the mRNAs is very dynamic and, similarly to other types of methylation, it is regulated by "writers", "erasers" and "readers" [179] (Figure 19). The writers are methylases that deposit the m6A methylation on the mRNA. The heterodimer composed of methyltransferase like 3 (METTL3) and methyltransferase like 4 (METTL4) plays a central role in the process [180-182]. The heterodimer METTL3/4 associates with other proteins such as the Wilms tumor 1-associated protein (WTAP), implicated in the recruitment of the m6A methyltransferase complex to mRNA targets [183], the RNA-binding motif 15 (RBM15) that help this

complex to move towards the appropriate m6A sites [184], and the Vir-like m6A methyltransferase-associated (VIRMA), the molecular function of which remains unknown [185]. One study suggested that VIRMA can influence the m6A modification in the 3'UTR and nearby stop codons and associate with the polyadenylation cleavage factor CFIm to participate in alternative polyadenylation [186].

Erasers are demethylases that remove the m6A modifications. Currently, two erasers have been identified: the fat mass and obesity-associated protein (FTO) and the α-ketoglutarate-dependent dioxygenase alkB homolog 5 (ALKBH5). Although FTO was originally discovered in obesity, several studies suggest that it preferentially participates in the demethylation of residues close to the 5'cap affecting mRNA stability [187]. Interestingly, FTO has been shown to be overexpressed in different cancers. In leukemia, it has an oncogenic role in reducing methylation levels of the ankyrin repeat and SOCS box containing 2 (ASB2) and the retinoic acid receptor alpha (RARA). In doing so, FTO inhibits the action of ASB2 and RARA, enhancing cell transformation and leukemogenesis [188]. Additionally, FTO has been found upregulated in breast cancer and its downregulation has been shown to inhibit breast cancer tumor growth in mice. Here, again, FTO acts as an oncogene, downregulating the expression of BCL2 interacting protein 3 (BNIP3), a pro-apoptotic gene [189].

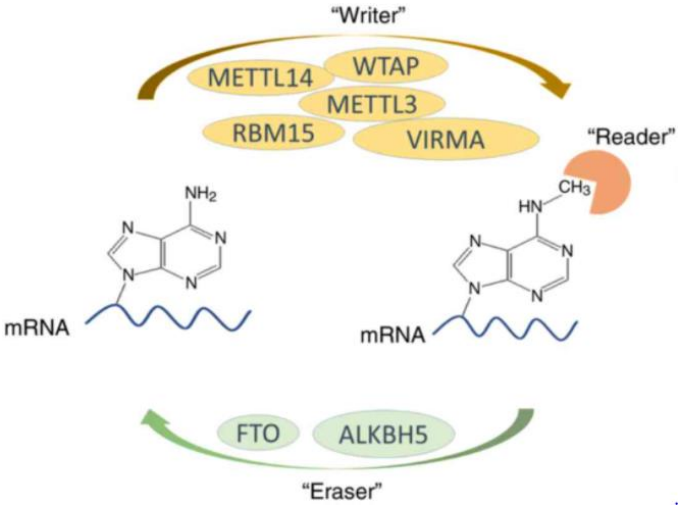


Figure 19. Roles of the N6 -methyladenosine writer, eraser, and reader proteins. RNA m6A modifications are regulated by several proteins. The writers deposit the methylation, the erasers remove the methylation and the readers are the effectors, as they recognize and mediate several biological processes. Adapted from Zhu W. et al. [179].

The third group of factors that regulate methylation through direct recognition and binding of the m6A modification is called "readers". This group includes YTH domain-containing proteins (YTHDF1, YTHDF2, YTHDF3, YTHDC1, and YTHDC2) [171]; among these, YTHDC1 is localized in the nucleus whereas the other YTH factors are localized in the cytoplasm. Since the m6A modification is more likely to be found in introns and exons that undergo alternative splicing, this type of methylation can play an important role in regulating RNA splicing (Figure 20a). In fact, mRNAs with multiple isoforms originated *via* alternative splicing contain more m6A than mRNAs with only one spliced isoform [178]. Additionally, FTO and YTHDC1 influence RNA splicing. FTO can bind pre-mRNAs in the nucleus triggering the inclusion of alternatively spliced exons [190] and YTHDC1 can

promote the binding of the serine and arginine rich splicing factor 3 (SRSF3) to mRNAs and antagonize the binding of the serine and arginine rich splicing factor 10 (SRSF10), leading to exon inclusion events [191].

As shown in Figure 20, m6A can regulate other nuclear events such as secondary structure switching (20b), mRNA export (20c), miRNAs maturation (20d), mRNA stability (20e) and XIST-dependent X chromosome inactivation (20f). Instead, in the cytoplasm, it can regulate translation (20g and 20h) and mRNA decay (20i) [192].

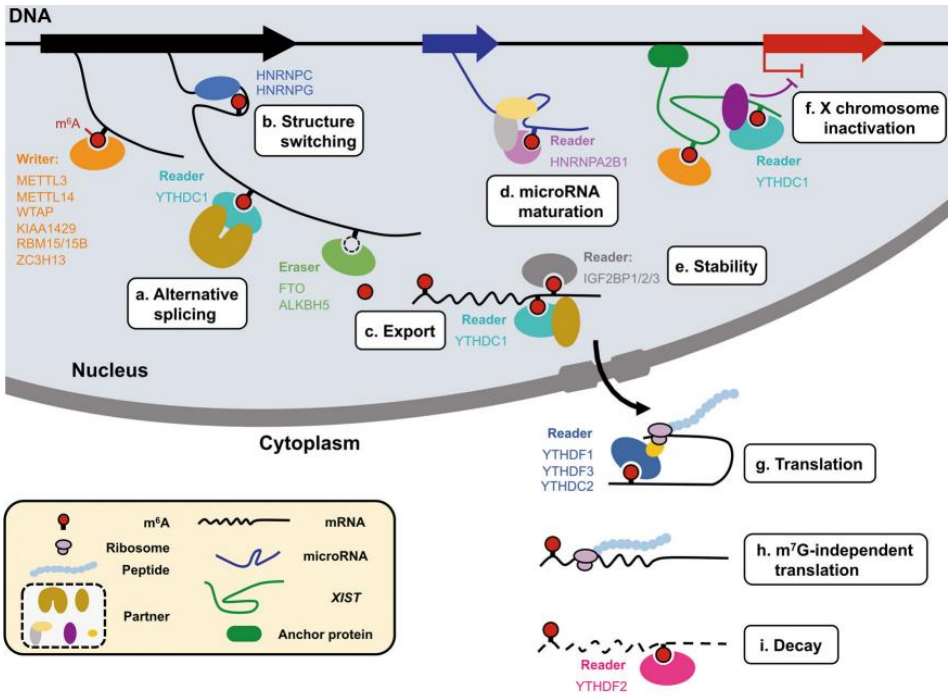


Figure 20. Diverse molecular functions of m6A. In eukaryotic cells, RNA m6A levels are regulated directly or indirectly by writers, erasers, and readers. Different readers contribute to the diversity of cellular processes involving m6A. In the nucleus m6A can modulates **(a)** mRNA alternative splicing, **(b)** secondary structure switching, **(c)** mRNA export, **(d)** miRNAs maturation, **(e)** mRNA stability and **(f)** XIST-dependent X chromosome inactivation. The cytoplasmic m6A enhances **(g,h)** mRNA translation and **(i)** accelerates mRNA decay. Adapted from Yang Y. et al. [192].

Since m6A modifications can regulate mRNA metabolism, it is thought that they play an important role in human tumorigenesis. Dysregulation of m6A modifications is involved in initiation and progression of several tumors, such as lung carcinoma, hepatocellular carcinoma and acute myeloid leukemia [193].

As illustrated in figure 21, several studies have been focused on molecular mechanisms of m6A-mediated mRNA regulation in cancer, causing mainly increased translation or mRNA decay [194].

Among the several mechanisms, in non-small cell lung cancer (NSCLC), METTL3 is highly expressed and acts as a reader of the m6A modification, enhancing the translation of specific oncogenic mRNA and increasing cell proliferation and invasion [195, 196]. In acute myeloid leukemia and hepatocellular carcinoma, the m6A reader YTHDF2 is overexpressed and it plays a critical role in tumor progression destabilizing some mRNAs, such as the suppressor of cytokine signaling 2 (SOCS2) in hepatocarcinomas [197, 198].

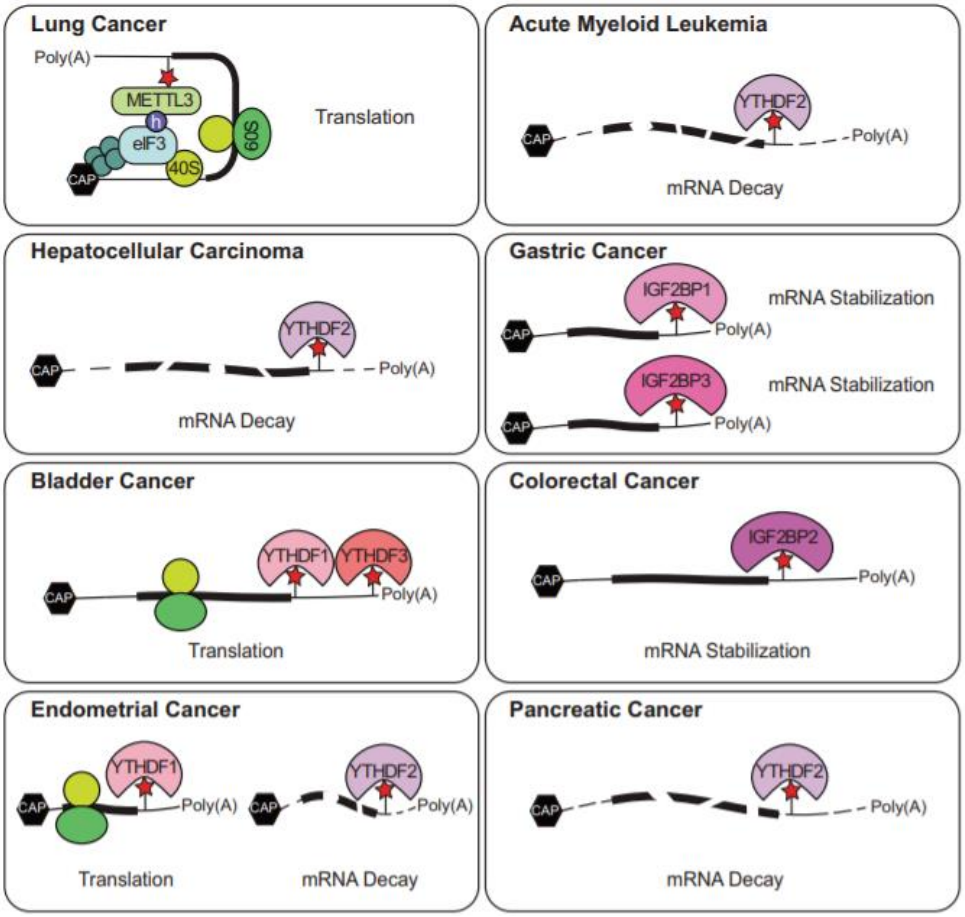


Figure 21. Molecular mechanisms of m6A-mediated mRNA regulation in tumorigenesis. Several studies have found that m6A-mediated mRNA regulation can cause increased translation or mRNA decay. Multiple m6A reader proteins are involved in these mechanisms, including YTHDF2, YTHDF2 and YDHF3. The red stars represent an m6A modification of the mRNA. Adapted from Han SH. et al. [194].

AIMS

Breast cancer is one of the most commonly diagnosed tumors worldwide. TNBC is the most aggressive subtype of invasive breast cancer with very few treatment options. Even if in some cases the patients benefit from immunotherapy and PARP inhibitors, surgery and chemotherapy remain the best first-line treatment options. As Dr. Peruzzi's laboratory has previously shown a strong anticancer activity of miR-3189-3p in glioblastoma and melanoma, in this study we evaluated the effects of this miRNA in triple negative breast cancer.

The aims of this thesis are the following:

- evaluate the anti-cancer effects of miR-3189-3p by the analysis of proliferation, migration, and invasion
- investigate the mechanisms of miR-3189-3p-mediated inhibition of translation through the analysis of c-MYC expression
- evaluate the effect of miR-3189-3p on cell metabolism by measuring mitochondrial respiration and glycolysis

MATERIALS AND METHODS

Cell culture, transfection, and reagents

MDA-MB-231 cells were obtained from the America Type Culture Collection (HTB-26; Manassas, VA) and cultured in Dulbecco's modified eagle medium (DMEM) with low glucose (1g/L) and 10% Fetal Bovine Serum (FBS). For transfection experiments, cells were seeded at a density of 4 to $6x10^5$ cells/60 mm dish, and transfected using Lipofectamine 2000 (Life Technologies, Grand Island, NY) per manufacturer's instructions. The miR-3189-3p mirVana miRNA mimic was purchased from Life Technologies (Carlsbad, CA) and used at a final concentration of 50 nM. The scramble control siRNA was purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and used at the final concentration of 50 nM. eIF4E-BP1 and eIF4E siRNAs were purchased from Santa Cruz Biotechnology and were used at the final concentration of 50 nM. The proteasome inhibitors MG-132 and ALLN were from Calbiochem (MilliporeSigma, Burlington, Massachusetts) and were used at the concentration of 1μM and 5μM respectively. The proteasome inhibitor Lactacystin was from Santa Cruz Biotechnology and was used at the concentration of 8μM. Chariot protein delivery reagent was from Active Motif (Carlsbad, CA). Immobilized γ-aminophenyl-m^7GTP was from Jena Bioscience (Jena, Germany). The vectors containing HPV E7 protein and the empty vector used as control were a gift of Dr. Lin Zhen (Tulane University Health Sciences Center, Louisiana Cancer Research Center, Department of Pathology). For this experiment, E7 vector or empty vector were transfected together with the miR-3189-3p mimic with the transfection conditions described above.

Patient-derived cells (PDX), from a Paclitaxel-resistant metastatic TNBC patient, were a gift of Dr. Suresh K. Alahari (LSU Health Sciences Center, Department of Biochemistry & Molecular Biology).

Cell Proliferation Assay

MDA-MB-231 cells were plated at a density of 2.5x104 cells/well in a 12-well plate and transfected with siRNA scramble control or miR-3189-3p mimic. 72 h after transfection, cells were incubated with medium containing 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) reagent (Promega) following the manufacture instructions. Cells were then incubated at 37 °C for 30–60 min, and absorbance was measured at a wavelength of 490 nm using the Sinergy™ Neo2 Multi-mode Microplate Reader (BioTek, Winooski, VT).

Scratch Assay

MDA-MB-231 cells were plated in a 35 mm glass bottom dish (MatTek Corporation, Ashland, MA) at a density of $1.8x10^5$ cells/dish and left to adhere overnight. Cells were then transfected with miR-3189-3p for 72h and the scratch assay was performed by scraping the cell monolayer with a pipette tip. Migration into the cell-free area was monitored for up to 24 h using live cell time-lapse imaging through a VivaView FL incubator fluorescent microscope (Olympus, Center Valley, PA).

Migration Assay

The migration assay was performed using 6.5 mm transwell with 8.0 μm pore polycarbonate membrane inserts from Corning (Corning, NY). Cells were plated at the density of $6x10^5$/60 mm dish and transfected the following day with miR-3189-3p or scramble control using Lipofectamine 2000. After 72h, cells were detached from the plate, washed 2 times with PBS, counted and then resuspended in serum-free medium. Cells were then added into the insert at the final concentration of $3x10^4$ cells per chamber in 200 μl of medium. 600 μl of complete culture medium with 10% FBS as chemoattractant were added to the lower chamber, being careful not to form bubbles between the insert and the well, and cells were incubated at 37 °C for 24h. The inserts were gently washed with cold PBS 3 times and fixed with 100% methanol. Non migrated cells were removed from the top of the inserts using cotton swabs. After fixation, the cells migrated on the bottom side of the insert were washed again with PBS and stained with 0.4% cristalviolet. The number of migrated cells was counted from three random fields per insert at the magnification of 20X and averaged from at least three biological replicates.

Invasion Assay

The invasion assay was performed using Corning BioCoat Matrigel Invasion Chambers with 8.0 μm PET Membrane from Corning (Corning, NY). The cells were transfected with miR-3189-3p or the scramble control as described for the migration assay. After 72 hours, cells were detached from the plate, washed, counted and

resuspended in serum-free medium. Cells were then added to the insert at the final concentration of $8x10^4$ cells per chamber in a total of 500 µl. 750 µl of complete culture medium containing 10% FBS as chemoattractant were added to the lower chamber, being careful not to form bubbles between the insert and the well, and cells were incubated at 37 °C for 40h. The inserts were gently washed with cold PBS 3 times and fixed with 100% methanol. Non migrated cells were removed from the top of the matrigel using cotton swabs. After fixation, the invaded cells on the bottom side of the insert were washed again with PBS and stained with 0.4% cristalviolet. Cells attached to the bottom well were also fixed and stained. The number of invaded cells was counted from three random fields per insert at the magnification of 20X and averaged from at least three biological replicates.

Quantitative RT-PCR

Total RNA was isolated using the mirVana miRNA extraction kit (Ambion, Austin, TX, USA) and 500 ng of total RNA were reverse transcribed to cDNA using the High Capacity cDNA Reverse Transcription kit for mRNAs (Applied Biosystems, Waltham, MA). For the cDNA reaction, samples were incubated for 10 min at 25 °C, 120 min at 37 °C and 5 min at 85 °C. Quantitative real-time PCR was performed using a Roche LightCycler 480 Real-Time PCR System (Indianapolis, IN, USA). 50 ng of cDNA were assessed for the expression levels of c-MYC with the specific TaqMan Gene Expression Assay (containing primers and probes) and TaqMan 2X Universal PCR Master Mix (Applied Biosystems). PCR conditions

were: 10 min at 95 °C and then 15 sec at 95 °C and 1 min at 60 °C for 50 cycles. Each sample was assessed in duplicate and GAPDH was used as reference gene. The relative quantification of gene expression was calculated using the comparative Ct ($2^{-\Delta\Delta Ct}$) method as we previously described [120, 199-201].

Western Blot analysis

Cells were washed twice in cold PBS (Gibco, Life Technologies) and collected by gently scraping in the presence of RIPA lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM EGTA, pH 7.4, 0.25% sodium deoxycholate) supplemented with 1 mM PMSF, 1 mM sodium orthovanadate (NA_3VO_4), and phosphatase and protease inhibitor cocktails (Sigma). Whole-cell lysates were kept on ice for 20 min and then centrifuged for 30 min at 14,000 rpm at 4 °C in a microcentrifuge to remove genomic DNA and membranes. Next, lysates (30 to 70 µg) were separated on a 4–15% SDS-PAGE gel (Mini-PROTEAN TGX Precast Gel, Bio-Rad, Hercules, CA) and transferred to a 0.2 µm nitrocellulose (Trans-Blot Turbo Mini 0.2 µm Nitrocellulose Transfer Packs, Bio-Rad, Hercules, CA) using the Trans-Blot TURBO apparatus (Bio-Rad, Hercules, CA). C-MYC, 4E-BP1, and eIF4E antibodies were purchased from Cell Signaling Technology (Beverly, MA). GAPDH and 14-3-3ζ antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA) and were used as loading control.

Cloning and mutagenesis of the 4E-BP1 3'UTR

The genomic sequence corresponding to the 3'UTR of 4E-BP1 was PCR-amplified from MDA-MB-231 cells and cloned downstream of the Renilla luciferase reporter gene in the psiCHECK-2 vector (Promega, Madison, WI). This vector also contains the firefly luciferase for normalization. The primers used for cloning were: forward, 5'-CCGCTCGAGATGGACATTTAAAGCACCAGCCATC, and reverse, 5'-ATAAGAATGCGGCCGCCTTGGCCCTAGGGCGAAGG. The underlined nucleotides represent the restriction sites of XhoI and NotI, respectively.

Mutation of the miR-3189-3p putative binding site in the 4E-BP1 3'UTR sequence was generated using the QuikChange Lightning site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA) using the psiCHECK2/3'UTR plasmid as a template. The oligonucleotides used for the mutagenesis of 4E-BP1 3'UTR were: 5'-AGGAGCTGCCACCCCTTCCGGAGTGACCCCTGCC, and 5'-GGCAGGGGTCACT-CCGGAAGGGGTGGCAGCTCCT. The underlined nucleotides represent the mutated bases in the miR-3189-3p binding sites.

Dual Luciferase Assay

MDA-MB-231 cells were plated at a density of $8x10^4$ cells/well in a 12-well plate and transfected with psiCHECK-2 vector expressing the eIF4E-BP1 3'UTR (160 ng/well) alone or together with the miR-3189-3p mimic (50 nM), or the mutated 3'UTR together with the miRNA mimic using Lipofectamine 2000. After 24 h, cells were harvested, and

lysates were assayed for luciferase activity with the Dual-Luciferase reporter assay system (Promega) using a Synergy 2 microplate reader (BioTek Instruments, Inc., Winooski, VT). For each sample, relative units of Renilla luciferase activity were normalized to the firefly luciferase internal control. Data represent the average of at least three independent experiments, each in duplicate.

Delivery of recombinant proteins in cell culture

Chariot Protein Delivery Reagent (Active Motif, Carlsbad, CA) was used to deliver 4E-BP1 protein into the cells. For protein delivery experiments, MDA-MB-231 cells were seeded at a density of $4x10^5$ cells/60 mm dish or $1.75x10^5$ cells/35 mm dish. After one wash with PBS, cells were incubated with 4E-BP1 protein/Chariot complex in serum free medium according to manufacturer's instructions. After 1h, medium with or without serum was added to the cell cultures. Cells were harvested after 2h as described above for Western blotting.

Cloning of c-MYC IRES into pYIC plasmid

The pYIC vector was obtained from Addgene. The plasmid contains a bicistronic fluorescent reporter gene in which EYFP expression is driven by a cap-dependent mechanism while ECFP translation is under the control of a 587 bp EMCV-IRES sequence. The 395 bases 5'UTR of human c-MYC (c-MYC mRNA sequence accession number: NM_002467.6) was amplified by PCR and cloned into the pYIC vector by the Klenow approach, after removal of the EMCV-IRES sequence through digestion with EcoRI and BstXI restriction enzymes. The

primers used for the cloning were: forward 5'-GCCTGACTGACTAAGTAATTCCAGCGAGAGGCAGA-3' and reverse 5'-TTGCTCACCATGGTTGTCGCGGGAGGCTGCT. The 5′-end of each primer is complementary to the vector used for cloning (underlined bases). The region of interest was then confirmed by sequencing.

Cap Pulldown Assay

Cap pulldown assays were performed using γ-aminophenyl-m^7GTP beads (Jena Bioscience). Briefly, cells were washed twice in cold PBS and lysed in RIPA buffer supplemented with protease and phosphatase inhibitors as described for Western blot analysis. Whole-cell lysates were centrifuged for 10 min at 14,000 rpm at 4°C in a microcentrifuge. To pulldown cap-binding proteins, 30 µL of γ-aminophenyl-m^7GTP beads were first resuspended in 500 µL of wash buffer (0.5% BSA in PBS). Following centrifugation at 1000 rpm for 1 min, the wash buffer was removed and beads were incubated with 350 µL of protein lysates diluted with RIPA buffer. After 1 h of incubation on a rotator at 4°C, supernatants were collected and beads/proteins complexes were washed three times with RIPA buffer. After the last wash, beads/proteins complexes were resuspended in 50 µL of gel loading buffer and incubated at 95°C for 5 min. Beads were centrifuged at 12,000 rpm for 5 min and supernatants containing cap-binding proteins were loaded on a 4–15% SDS-PAGE gel for Western blot analysis.

Metabolism Assay

Metabolism of MDA-MB-231 cells was evaluated using the Seahorse XF96 Flux Analyzer (Seahorse Bioscience). Cells were plated at the concentration of $6x10^5$ per 60 mm dish and transfected with miR-3189-3p or scramble control using Lipofectamine 2000. Untransfected cells were used as an additional control. After 48h, cells were detached, counted, seeded into 96-well XF96 cell culture microplate (Seahorse Bioscience) in normal growth media at the final concentration of $3x10^4$ cells/ well, and incubated at 37 °C (with 5%CO_2) for 4-5 hour to allow attachment. Then the medium was replaced with the Seahorse medium and the plate was incubated for at least 1 hour at 37 °C without CO_2 before proceeding with the Seahorse assay. Mitochondrial function was analyzed by performing three baseline oxygen consumption rate (OCR) measurements, followed by three measurements each of the consecutive injections with oligomycin (ATP synthase inhibitor; 2 µM final concentration), carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP; mitochondrial oxidative phosphorylation uncoupler; 2 µM final concentration), and rotenone (mitochondrial complex I inhibitor; 0.5 µM final concentration) together with Antimycin A (mitochondrial complex III inhibitor; 0.5 µM final concentration). The last injection included Hoecst dye for cell counting and normalization. Each measurement cycle consisted of 3 min mix and 3 min measure. Extracellular acidification rate (ECAR) was measured at the same time as OCR. Data were normalized to total number of cells present in the well by extrapolation using a standard curve previously made by plating a different number of cells. The plate was read at 350 nm excitation and 461 nm emission in the Sinergy

Neo2 Multi-mode Microplate Reader (BioTek). Results are presented as the average of two independent experiments in which each sample was assessed in quadruplicates.

Statistical analysis

Data are presented as mean +/- S.D. All data were graphed using GraphPad Prism and Excel software packages. Comparisons between two experimental groups were performed using the unpaired two-tailed Student's t test. One way ANOVA was used to compare three or more groups. P-values ≤ 0.05 were considered statistically significant.

RESULTS

MiR-3189-3p impairs TNBC growth and migration

Dr. Peruzzi's laboratory has previously shown that miR-3189-3p has anticancer activity in glioblastoma, where it reduces cell proliferation of 50% and cell migration of 58% [120]. In the present study we investigated the effects of miR-3189-3p in TNBC.

Similarly to the results obtained with glioblastoma, after 48 hours, MDA-MB-231 cells transfected with miR-3189-3p mimic changed their morphology from a spindle/triangle-like to a more round shape when compared to untransfected cells (Figure 22A).

To determine the effects of this miRNA on cell proliferation, we transfected cells for 72 hours prior to cell count, and we found that miR-3189-3p inhibits proliferation of 60% when compared to cells transfected with an RNA scramble control (Figure 22B). To further strengthen our hypothesis, we repeated the same experiment with mammospheres, a 3D sphere model of cancer. Although less than in a monolayer, also in this model, proliferation of mammospheres was lower in cells transfected with miR-3189-3p (25%) compared to control cells (Figure 22C).

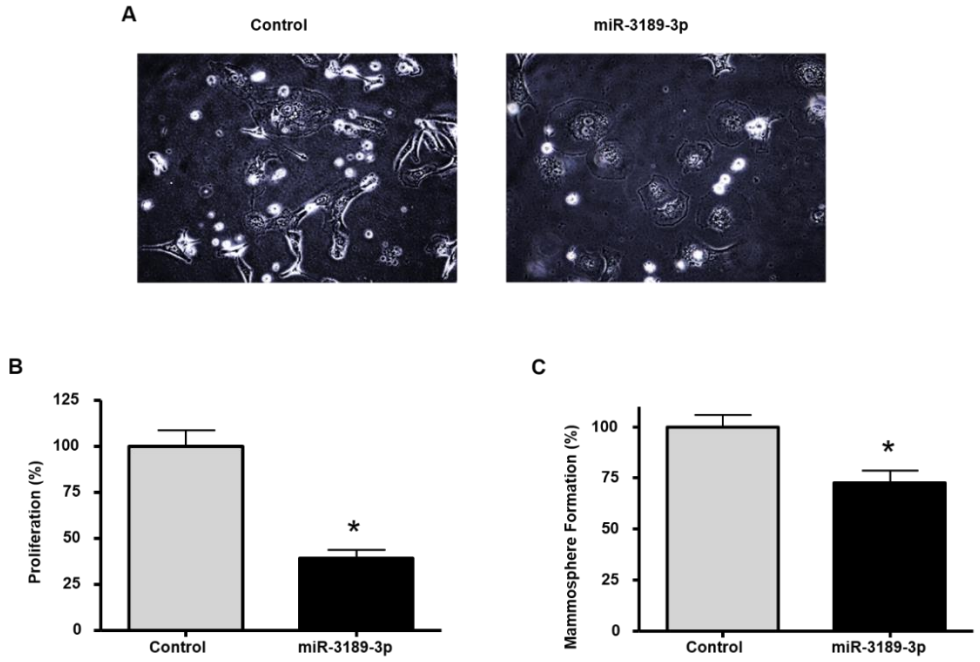


Figure 22. miR-3189-3p reduces cell proliferation on MDA-MB-231 cells. (A) Phase contrast imaging showing a change in the morphology of MDA-MB-231 cells after transfection with miR-3189-3p. Images were acquired 48 hours post-transfection. Original magnification 20X; **(B)** Cell proliferation assay for MDA-MB-231 cells, performed 72 hours post-transfection with the miR-3189-3p or the scramble control. Results are expressed as percent growth of cells transfected with miR-3189-3p/ control-treated cells; **(C)** Cell proliferation assay for mammospheres, performed 72 hours post-transfection with the miR-3189-3p or the scramble control. Results are expressed as percent of the number of mammospheres obtained from cells transfected with miR-3189-3p/mammospheres of control cells. Asterisks indicate statistical significance.

Next, we evaluated the effect of miR-3189-3p on cell migration. To this end, we transfected MDA-MB-231 cells with miR-3189-3p or the scramble control and we tested their migration on a monolayer (scratch assay) and through a transwell. For the scratch assay, while control-transfected cells populated the entire scratched area of the monolayer in 12 hours, cells transfected with the miR-3189-3p showed a 75% decrease in the ability to close the scratch in the same amount

of time (Figure 23A). In the transmigration assay, miR-3189-3p reduced the ability of MDA-MB-231 cells to migrate across the 8 μm pores of the filter of about 80% when compared to the same cells transfected with the scramble control (Figure 23B). Additionally, as previously observed, microscopy analysis of the miR-3189-3p-transfected cells showed altered morphology, with a less elongated and more rounded shape.

Overall, these results confirmed the previous data obtained in glioblastoma [120] and in melanoma (unpublished data).

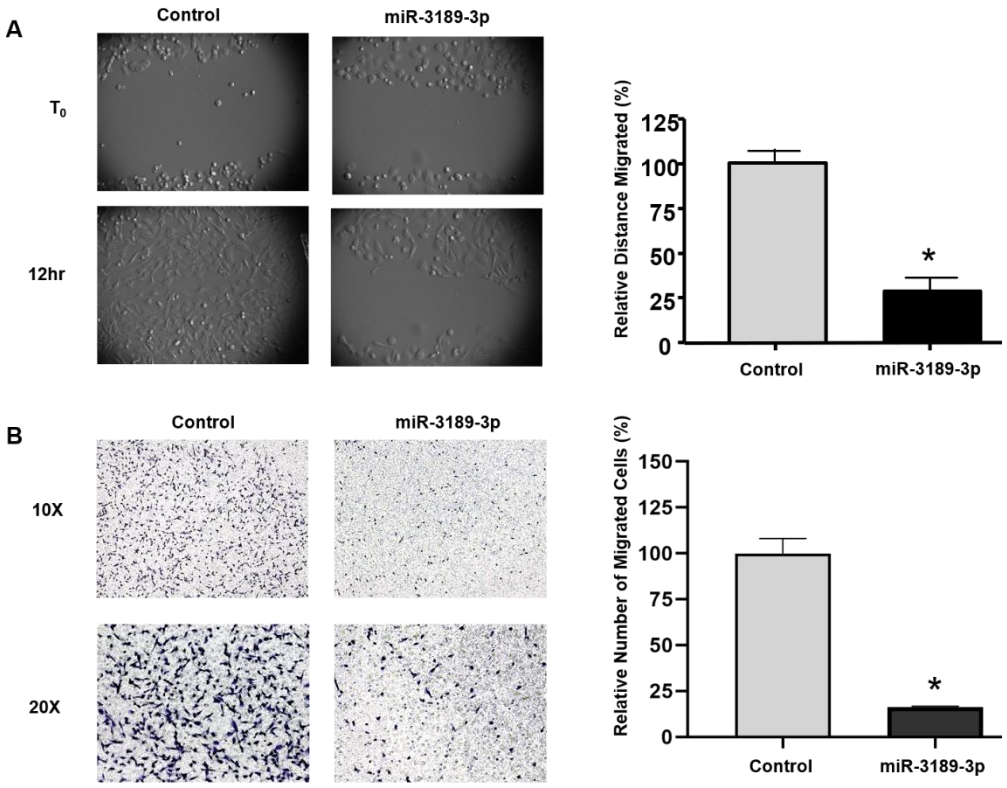


Figure 23. miR-3189-3p reduces migration of MDA-MB-231 cells. (A) Representative images of a scratch assay 72 hours post-transfection with miR-3189-3p or scramble control. Migration into the cell-free area was monitored by time-lapse imaging in a VivaView incubator. Original magnification 10X; **(B)** Representative images of transwell migration assay 72 hours post-transfection with the miR-3189-3p or scramble control. Migrated cells were stained with crystalviolet and pictures were acquired with an optic microscope. Original magnification 10X and 20X. Asterisks indicate statistical significance.

MiR-3189-3p reduces TNBC cells invasion

To further investigate the effect of miR-3189-3p on cell invasion, we transfected MDA-MB-231 cells with the miRNA or the scramble control and we analyzed the ability of the transfected cells to invade Matrigel-coated transwells, as the Matrigel mimics the extracellular

environment containing laminin, collagen, heparan sulfate proteoglycans, and several growth factors. 72h post-transfection cells were reseeded on the top of the inserts and left undisturbed for 40 hours. After this time, non-invaded cells were removed from the top of the filter and both, the inserts and the bottom wells were stained with crystalviolet. Results showed that the number of invaded cells in the miR-trasfected samples was decreased of 75% when compared to the control transfected cells (Figure 24). Overall, this experiment demonstrates the ability of miR-3189-3p to inhibit migration and invasion of MDA-MB-231 cells, suggesting a potential use of this miRNA against breast cancer.

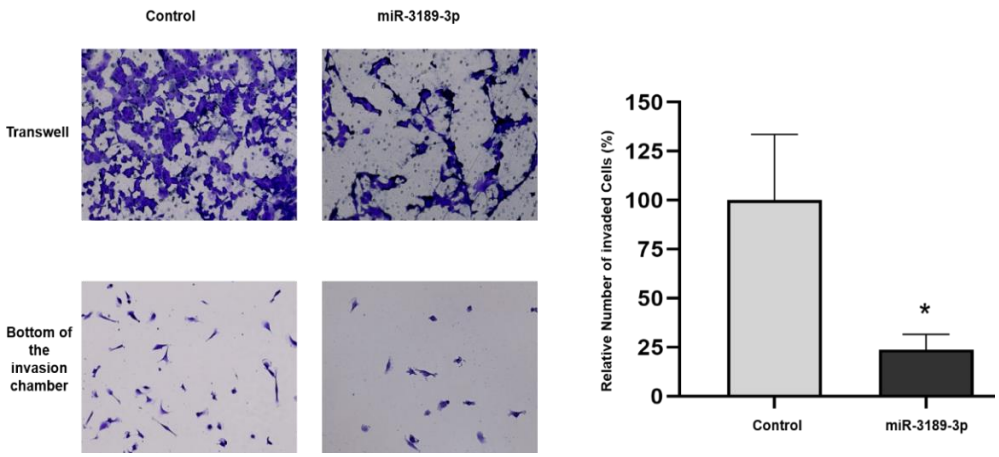


Figure 24. miR-3189-3p reduces invasion of MDA-MB-231 cells. Representative images of transwell invasion assay 72 hours post-transfection with the miR-3189-3p or scramble control. Both the inserts and the bottom wells were stained and pictures were acquired with an optic microscope. Original magnification 20X. Asterisk indicates statistical significance.

MiR-3189-3p mediates c-MYC downregulation

C-MYC is a regulatory gene involved in cell growth, cell death, cell survival, cell differentiation, metabolism, microRNA regulation, cell cycle progression and ribosome biogenesis. Furthermore, c-MYC has been found upregulated in several tumors and it is considered a marker of poor prognosis [202-205]. Since no therapies currently exist against c-MYC, it could represent an attractive therapeutic target [203]. Using data extracted from the Cancer Genome Atlas (TCGA), METABRIC project, we found that, among all breast cancer subtypes, TNBC had the higher expression of c-MYC mRNA (Figure 25A). Considering this high expression and the strong anticancer effect of miR-3189-3p, we decided to investigate if c-MYC was a downstream target of our miRNA, even if it is not a predicted gene target. MDA-MB-231 cells transfected with miR-3189-3p and scramble control were collected after 72 hours for protein and RNA analysis. Interestingly, while c-MYC mRNA expression did not change after transfection with the miRNA (Figure 25B), c-MYC protein was downregulated. Quantitatively, densitometric analyses revealed an average of 75% +/- 5% reduction of c-MYC protein levels in miR-transfected cells when compared to control transfected cells (Figure 25C). Since levels of the protein but not the mRNA of c-MYC seem to be regulated, we hypothesized a post-transcriptional regulation of c-MYC by the miR-3189-3p. We repeated the same experiment using patient-derived xenograft (PDX, a gift from Dr. Suresh K. Alahari), which are models of cancer where the tissues or cells from a patient's tumor are implanted in mice, allowed to grow *in vivo* and then harvested and used for *in vitro* experiments. We collected PDX cells after 72 hours of

transfection with miR-3189-3p or control and results showed a slight decrease of c-MYC protein expression in miR-transfected cells, although not as strong as in MDA-MB-231 (Figure 25D). Although preliminary, these results indicate the ability of miR-3189-3p to impair c-MYC expression in patient-derived cells.

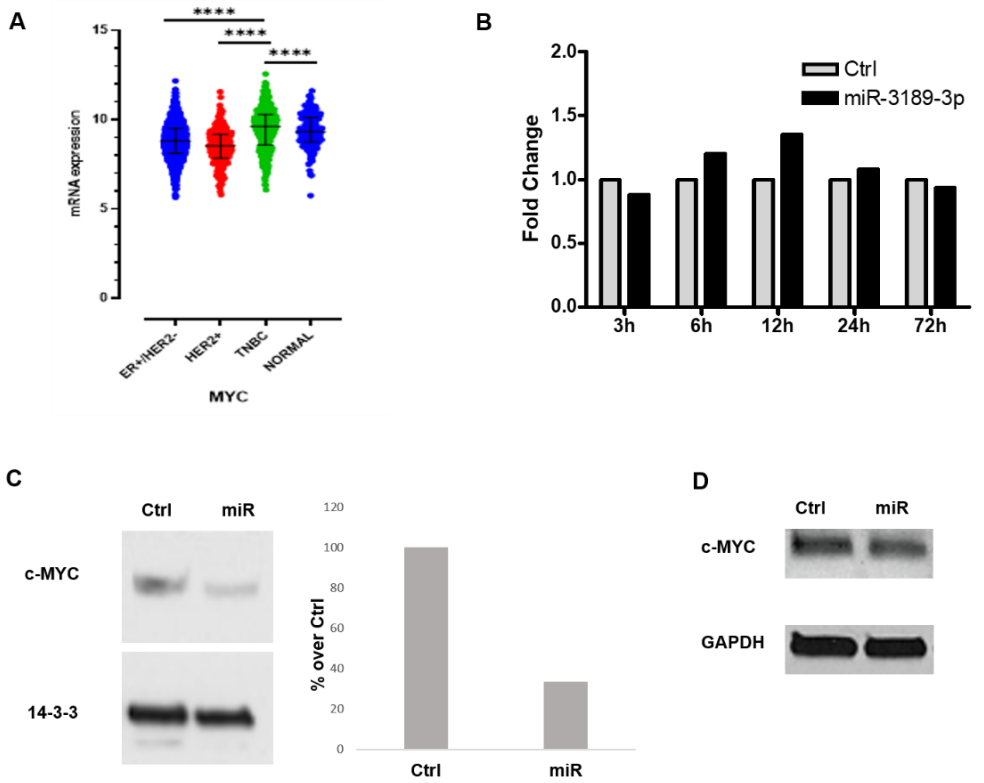


Figure 25. MiR-3189-3p mediated downregulation of c-MYC. (A) Expression of c-MYC mRNA in normal breast and different subtypes of breast cancer. Data were obtained through the TCGA database, METABRIC project. Asterisks indicate statistical significance; **(B)** Real-time PCR data showing the expression of c-MYC mRNA at different time points after transfection with miR-3189-3p or scramble control. Results are expressed as fold change of the mRNA in miR-treated cells compared to the control treated cells; **(C)** Western blot for c-MYC protein from MDA-MB-231 cells transfected with miR-3189-3p or scramble control for 72 hours. 14-3-3 was used as loading control. Densitometric analysis of the bands relative to c-MYC was done using Image J; **(D)** Western blot to detect c-MYC protein in PDX cells transfected with miR-3189-3p or scramble control for 72 hours. GAPDH was used as loading control.

To better explore the mechanisms of c-MYC downregulation in miR-3189-3p transfected cells, we investigated the proteasome pathway through the use of inhibitors. MDA-MB 231 cells were transfected with miR-3189-3p or control RNA for 48 hours and incubated with the proteasome inhibitors MG-132 (1 µM), Lactacystin (8 µM), or ALLN (5 µM) for an additional 24 hours before protein extraction. Western blot results showed that only MG132 was efficiently inhibiting the proteasome pathway, preventing c-MYC downregulation in control transfected cells. However, in miR-transfected cells, none of the inhibitors was effective in stabilizing c-MYC protein (Figure 26), indicating that miR-3189-3p mediated c-MYC downregulation is independent from the proteasome pathway.

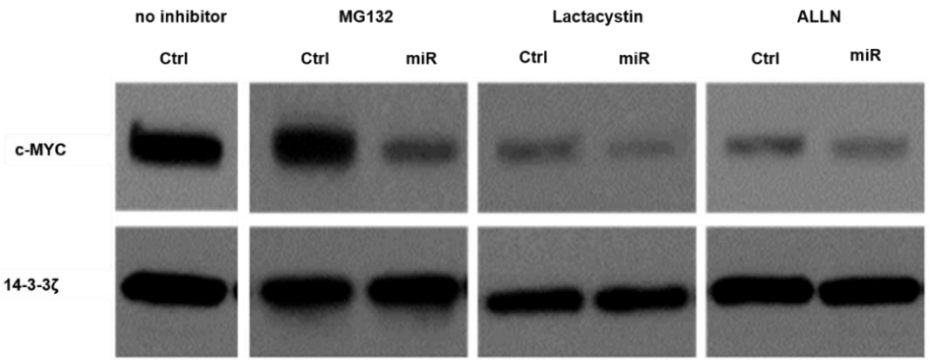


Figure 26. MiR-3189-3p mediated downregulation of c-MYC is independent from the proteasome pathway. Western blots performed on MDA-MB-231 cells that were transfected with control miRNA (Ctrl) or miR-3189-3p (miR) in the absence or presence of the proteasome inhibitors MG132, Lactacystin or ALL. 14-3-3 was used as loading control.

MiR-3189-3p directly targets 4E-BP1

Since miR-3189-3p downregulates c-MYC protein but not its mRNA, we focused our attention on miRNA targets that can mediate the translation of c-MYC mRNA.

According to the database TargetScan (www.targetscan.org), two eukaryotic initiation factors, eIF4E and 4E-BP1, are predicted gene targets of miR-3189-3p, although eIF4E ranks low in the cumulative weighted context scores of the prediction [206]. Since eIF4E and 4E-BP1 are involved in translation, including c-MYC translation [138], we selected these proteins for further experiments. Expression of miR-3189-3p in MDA-MB-231 cells resulted in reduced levels of 4E-BP1 protein compared to control-transfected cells (Figure 27A). Expression levels of total eIF4E did not change in the same experimental conditions (Figure 29), indicating that this gene is not a validated target of the miRNA, at least in these cells. The 3'UTR of 4E-BP1 contains one putative binding site for the miR-3189-3p that was evaluated in a miRNA-target validation experiment using a luciferase-based reporter assay. The direct binding of the miRNA to the 3'UTR of 4E-BP1 resulted in a reduction of approximately 30% ($p=0.000099$) of luminescence in cells overexpressing miR-3189-3p (Figure 27B). Conversely, a 4E-BP1 3'UTR mutated in the miRNA binding sequence showed an approximate 13% reversal of this reduction in luminescence signal. Overall, these results demonstrate that the direct binding of miR-3189-3p to the 3'UTR of 4E-BP1 is specific.

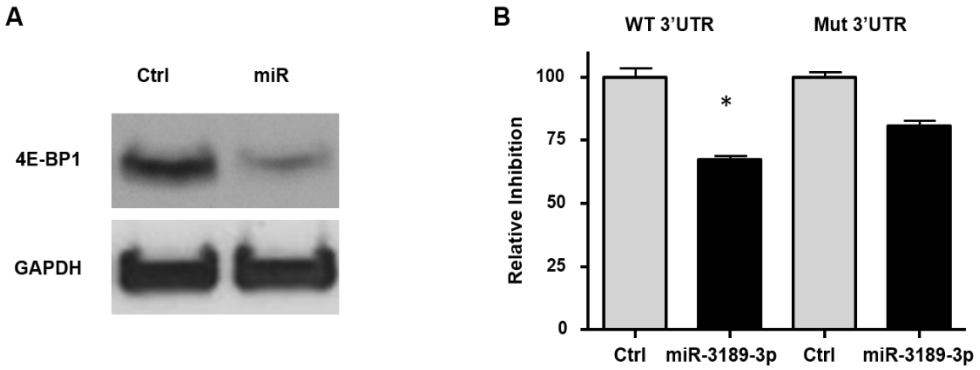


Figure 27. MiR-3189-3p directly targets 4E-BP1. (A) Western blot to detect 4E-BP1 protein in MDA-MB-231 cells transfected with miR-3189-3p (miR) or scramble control (Ctrl). GAPDH was used as loading control; **(B)** Luciferase assay of MDA-MB-231 cells co-transfected with miR-3189-3p and either 4E-BP1 3'UTR (WT 3'UTR) or the mutated 4E-BP1 3'UTR (Mut 3'UTR). The scramble sequence of the miRNA was used as control. The asterisk indicates statistical significance.

Role of 4E-BP1 in miR-3189-3p-mediated c-MYC downregulation

4E-BP1 and c-MYC are often overexpressed in tumors, including breast cancer [138, 202, 203, 207]. Although 4E-BP1 is considered a tumor suppressor and an inhibitor of translation, its levels can influence the upregulation of c-MYC. We then investigated whether c-MYC levels are dependent on 4E-BP1 expression using a siRNA-mediated approach. At first, we transfected the cells with a siRNA against 4E-BP1 or control. As shown in Figure 28A, 4E-BP1 was efficiently silenced and the silencing of 4E-BP1 resulted in a decrease of c-MYC protein levels. Interestingly, levels of eIF4E remained unchanged in both 4E-BP1 and control silenced cells, suggesting that the downregulation of c-MYC is not due to the loss of eIF4E (Figure 28A). Then we tested the dependence of c-MYC from eIF4E levels,

using a siRNA against eIF4E. As shown in Figure 28B, eIF4E was efficiently silenced and the silencing of eIF4E resulted in a dramatic reduction of c-MYC protein levels. Of note, the silencing of eIF4E resulted in a parallel downregulation of 4E-BP1 (Figure 28B).

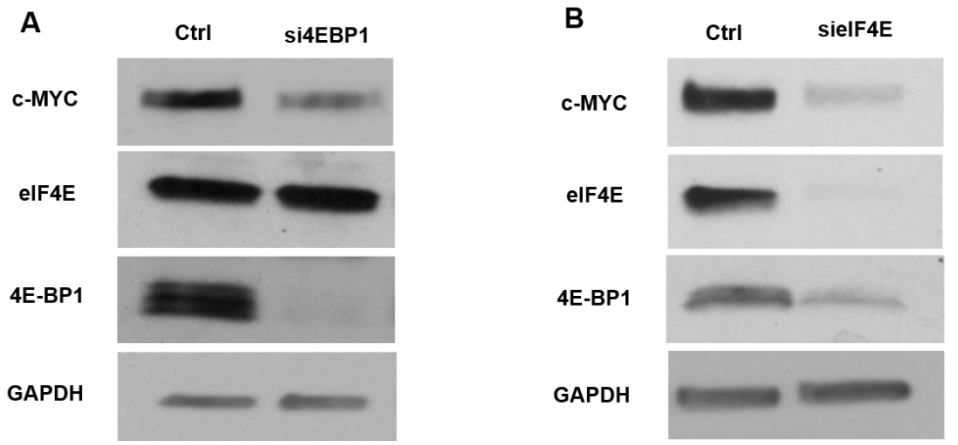


Figure 28. MiR-3189-3p targets c-MYC translation in a 5'cap-dependent manner. Western blots showing levels of the indicated proteins after silencing 4E-BP1 **(A)** or eIF4E **(B)**. Cells transfected with the scramble RNA were used as control (Ctrl). GAPDH was used as loading control.

Since our data suggest that 4E-BP1 is critical for c-MYC expression, we decided to deliver 4E-BP1 protein to cells in the presence or absence of miR-3189-3p, reasoning that overexpression of 4E-BP1 might reverse the downregulation of c-MYC. We transfected cells with miR-3189-3p or control and we collected protein lysates 3 hours after the addition of 4E-BP1 recombinant protein (r4E-BP1) to the cells using the Chariot system (Figure 29A). Again, c-MYC and 4E-BP1 were downregulated in miR-transfected cells whereas the levels of eIF4E remained unchanged. However, even if some r4E-BP1

successfully entered the cells, it did not rescue expression of c-MYC (Figure 29A, compare lanes 1 and 3). Next, we decided to repeat the same experiment using a plasmid for the overexpression of 4E-BP1, previously generated in our laboratory, instead of the recombinant protein (Figure 29B). Differently from the results obtained with the recombinant protein, overexpression of 4E-BP1 partially rescued the miRNA-mediated downregulation of c-MYC (Figure 29B, lane 3). This discrepancy in the results obtained with the recombinant protein and the plasmid, is likely due to the presence of serum inhibiting the entry of r4E-BP1 into the cells, as explained in the next section.

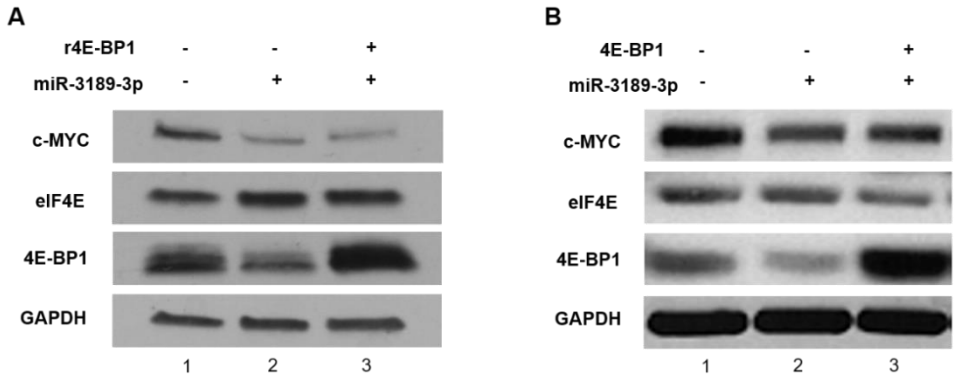


Figure 29. Role of 4E-BP1 in the downregulation of c-MYC. Western blots showing c-MYC, eIF4E and 4E-BP1 proteins in MDA-MB-231 cells transfected with miR-3189-3p or scramble control. **(A)** When indicated, r4E-BP1 was added 3 hrs prior to cell lysis; **(B)** Where indicated, cells were co-transfected with 4E-BP1 expression vector. GAPDH was used as loading control.

MiR-3189-3p targets c-MYC translation under cellular stress

The above data show that miR-3189-3p-mediated downregulation of c-MYC involves downregulation of 4E-BP1, which plays a fundamental role in 5'cap-dependent translation. Since several studies demonstrated that under stress conditions, 4E-BP1 can also promote cap-independent translation [138, 151, 208], we tested whether miR-3189-3p could block the expression of c-MYC under conditions of cellular stress. Since c-MYC is up-regulated under stress due to an increase in the cap-independent translation [138, 159, 169], we first confirmed the upregulation of c-MYC under conditions of serum starvation. MDA-MB-231 cells grown in complete medium were switched to a medium without serum and the upregulation of c-MYC was observed at 6 and 24 hours (Figure 30A). Next, we wanted to determine if administration of r4E-BP1 to cells transfected with miR-3289-3p could prevent miRNA-mediated downregulation of c-MYC. Similar to the experiments performed in the presence of serum we observed a significant decrease in the expression of c-MYC and 4E-BP1 in cells treated with miR-3189-3p when compared to cells transfected with the control (Figure 30B, compare lanes 1 and 2). Interestingly, the addition of r4E-BP1 prevented this inhibition (lane 3). Altogether, since the translation of c-MYC in the absence of serum is likely IRES-dependent, our data suggest that the miRNA-mediated downregulation of c-MYC occurs in a cap-dependent as well as cap-independent manner through downregulation of 4E-BP1. Data shown in Figure 30B also confirmed our hypothesis that the presence of serum could inhibit an efficient entry of r4E-BP1 into the cells (see also Figure 29A).

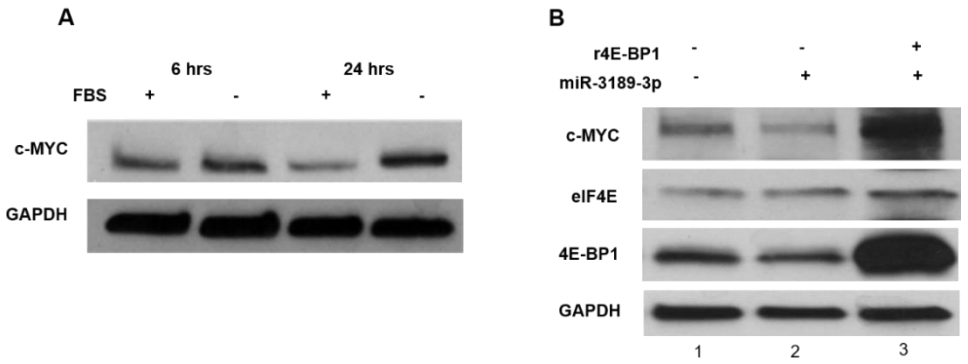


Figure 30. MiR-3189-3p targets c-MYC translation under cellular stress. (A) Western blots showing c-MYC protein in cells cultured in serum-free medium for 6 hours and 24 hours. GAPDH was used as loading control; **(B)** Expression of c-MYC, eIF4E and 4E-BP1 proteins in cells transfected with miR-3189-3p or scramble control in the absence or presence of r4E-BP1. GAPDH was used as loading control.

MiR-3189-3p decreases the binding of eIF4E to the 5'CAP

Since miR-3189-3p appears to inhibit cap-dependent translation of c-MYC without strongly affecting the levels of eIF4E, we wanted to determine whether the miRNA could reduce the association of eIF4E with the mRNA 5'cap. We then performed a cap pull down assay using γ-aminophenyl-m7GTP beads and protein lysates obtained from MDA-MB-231 cells transfected with the miRNA or scramble control in the presence or absence of r4E-BP1. EIF4E was efficiently immunoprecipitated in all conditions, since no protein is detected in the supernatant of the pull down (Figure 31, lanes 4, 5, 6). After pull down, approximately 10% reduction in eIF4E binding to the cap is observed when the cells were transfected with miR-3189-3p (Figure 31, lane 2), suggesting a decrease in cap-dependent translation, which was prevented by the addition of r4E-BP1 (Figure 31, lane 3).

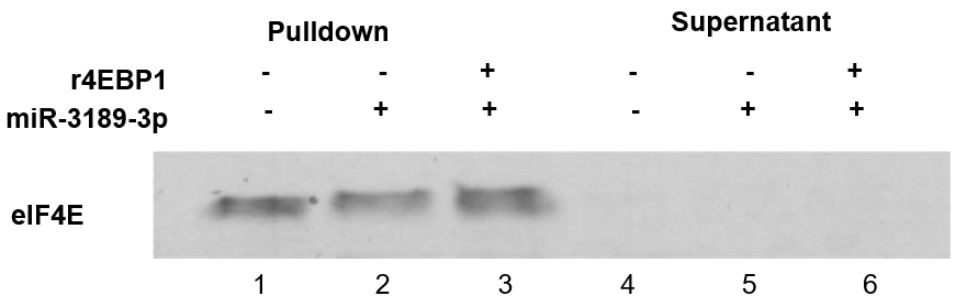


Figure 31. MiR-3189-3p reduces the binding of eIF4E to the cap of the mRNA. Western blot to detect eIF4E after pull-down of γ-aminophenyl-m7GTP beads from lysates of cells transfected with miR-3189-3p or scramble control or miRNA + r4E-BP1.

Negative effect of miR-3189-3p on cap-independent translation

After establishing that c-MYC expression was impaired by miR-3189-3p in MDA-MB-231 cells both in normal growth conditions and under stress conditions, we investigated whether this miRNA might affect also the cap-independent translation. As IRES sequences have been found in several mRNA, including c-MYC, FGF, VEGF and p53 [159, 163], we tested if IRES-dependent translation of c-MYC was affected by miR-3189-3p. We utilized a bicistronic vector (pYIC) encoding a single transcript containing a yellow fluorescent protein (EYFP, myc-tagged) and a blue fluorescent protein (ECFP, HA-tagged). In normal growth conditions, a cap-dependent translation will result in the expression of yellow fluorescent proteins. However, a viral IRES (EMCV-IRES) is also contained in the sequence upstream of ECFP, so that in conditions that impair cap-dependent translation and favor IRES-dependent translation, the expression of ECFP should increase. Cells were co-transfected with pYIC and miR-3189-3p or scramble control. Results showed a dramatic downregulation of EMCV-IRES-driven ECFP protein in cells transfected with the miRNA (Figure 32A). Next, considering that c-MYC mRNA contains an IRES in its 5'UTR, we replaced the EMCV-IRES with the 395 bp c-MYC 5'UTR in the pYIC vector. Figure 32B shows a strong inhibitory effect of miR-3189-3p on the c-MYC-IRES sequence. Next, we wanted to determine if 4E-BP1 would increase IRES-dependent translation. Since EMCV-IRES has been found to be stronger than c-MYC IRES [209], we utilized the pYIC vector to perform this experiment. We co-transfected cells with

the miRNA or control and 4E-BP1 expression plasmid or control plasmid (pcDNA3). As expected, miR-3189-3p downregulated the expression of EMCV-IRES driven protein and the addition of 4E-BP1 at least partially rescued this downregulation (Figure 32C).

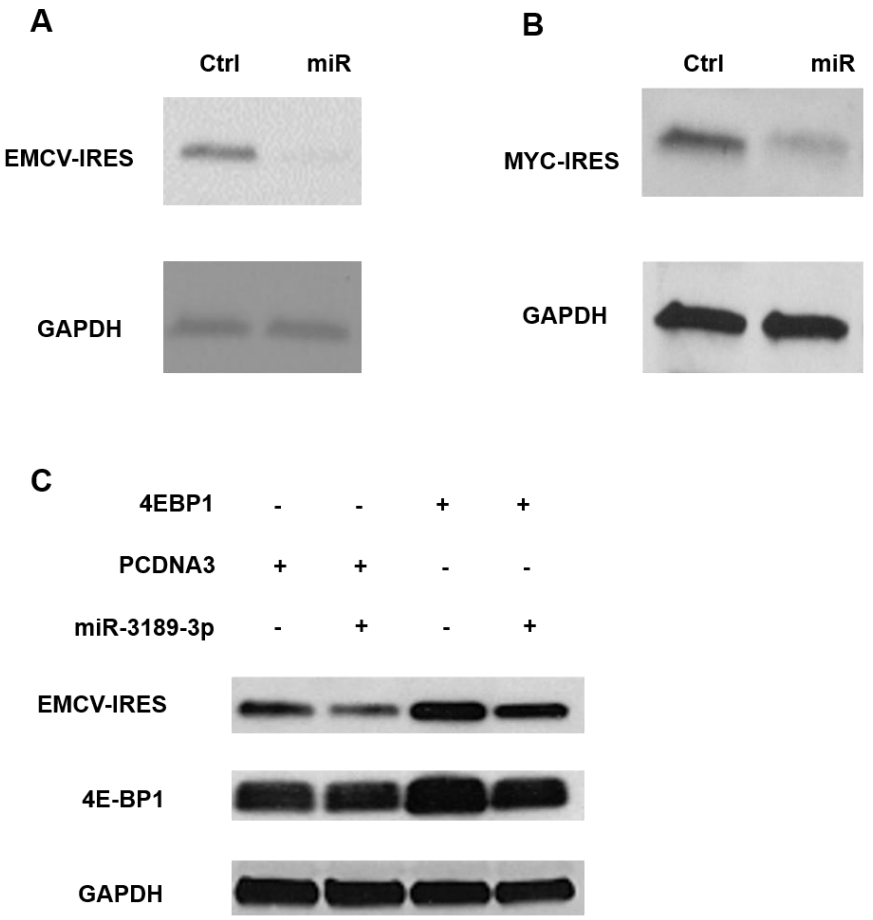


Figure 32. MiR-3189-3p impairs the IRES-dependent translation. Western blots to detect IRES-dependent translation of ECFP after co-transfection of MDA-MB-231 cells with pYIC/EMCV-IRES **(A)** or pYIC/c-MYC-IRES **(B)** and miR-3189-3p or scramble control; **(C)** In the same experimental conditions, overexpression of 4E-BP1 partially prevented miR-mediated EMCV-IRES downregulation. In all blots, GAPDH was used as loading control.

Finally, as increasing m6A levels in c-MYC mRNA transcript by METTL3 can bring to increased c-MYC expression [210, 211], we determined whether miR-3189-3p could impair m6A-dependent translation, another type of cap-independent translation. We used a plasmid encoding a circular RNA with m6A driving the expression of the HPV protein E7 (a kind gift of Dr. Zhang Lin). We co-transfected MDA-MB-231 cells with miR-3189-3p or scramble control together with the E7 reporter construct or the empty vectors (EV) and we collected cell lysates after 24 and 48 hours. Results showed downregulation of E7 protein levels in the miR-transfected cells compared to control transfected cells, both at 24 and even more at 48 hours, indicating an impairment in m6A-mediated translation by miR-3189-3p (Figure 33). Taken together, these results suggested that miR-3189-3p can interfere with both cap-dependent and cap-independent translation of c-MYC.

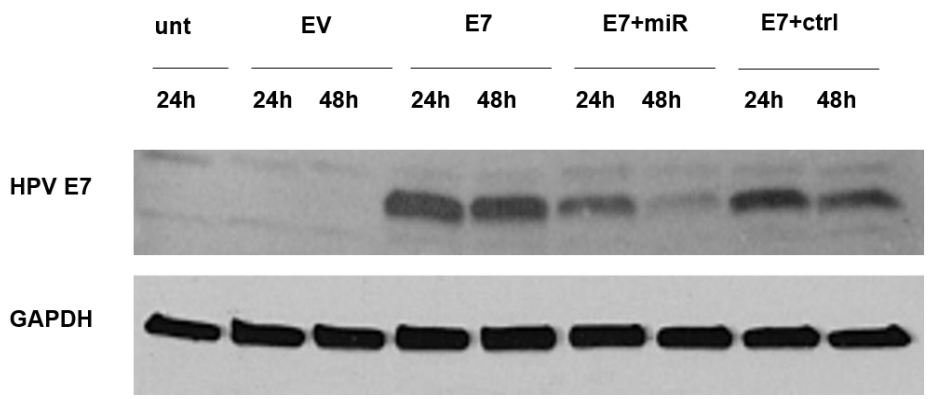


Figure 33. MiR-3189-3p impairs m6A-dependent translation. Western blot of MDA-MB-231 cells co-transfected with miR-3189-3p or scramble control together with a plasmid encoding a circular RNA containing E7 under m6A translation (E7) or the empty vector (EV) for 24 and 48 hours. GAPDH was used as loading control.

MiR-3189-3p impairs MDA-MB-231 cells metabolism

Cellular metabolic changes are strictly related to growth abnormalities in cancer. Malignant transformation is often accompanied by glycolysis and one of the main drivers of glycolysis is c-MYC [204]. Considering that miR-3189-3p downregulates c-MYC expression, we investigated the effect of the miRNA on the cellular metabolism of MDA-MB-231 cells. Since our miRNA decreases proliferation and since it is critical to perform the metabolic experiment with the same number of cells in each well, we transfected MDA-MB-231 cells with the miRNA or the scramble control for 48 hours and seeded them in the Seahorse plate the day of the experiment. Oxygen consumption rates (OCR) and extracellular acidification rates (ECAR) flux were measured simultaneously (Figure 34) and they are respectively indicative of mitochondrial respiration and glycolysis. Basal OCR and basal ECAR are displayed as the average of the first 3 measurements before the first injection with oligomycin and results showed that cells transfected with the miR-3189-3p have statistically significant increased levels of both basal OCR and basal ECAR (Figure 35A and Figure 35B). The spare respiratory capacity represents the ability of cells to respond to an energetic demand and it was calculated as the difference between the maximal respiration and the basal respiration. As shown in Figure 35C, miR-transfected cells displayed a higher spare respiratory capacity when compared to control-transfected cells or untransfected cells. Finally, energy maps showed that miR-3189-3p changed the metabolism of MDA-MB-231 cells, making them both more energetic and more glycolytic at both basal (Figure 36A) and metabolically stressed states (Figure 36, B, C, D). Taken together, these findings

revealed that cells transfected with miR-3189-3p are more metabolically active, increasing the usage of mitochondrial respiration and glycolysis. In addition, as demonstrated by the increased response to the uncoupling reagent FCCP (second injections), this metabolic analysis revealed an increased mitochondrial fitness of miR-transfected cells.

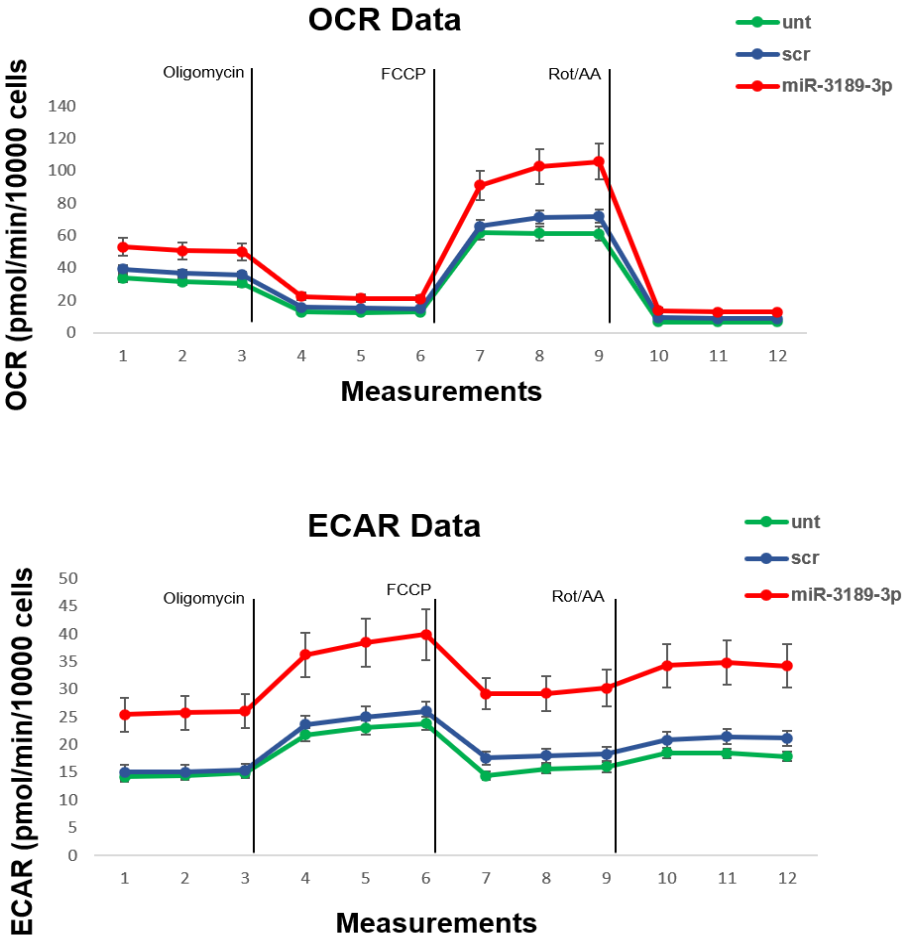


Figure 34. OCR and ECAR measurements. MDA-MB-231 cells were transfected with miR-3189-3p or scramble control and were subjected to the Seahorse XF Cell Mito Stress Test. OCR and ECAR were measured simultaneously. Data are represented as the mean ± standard error of technical replicates.

Injections were Oligomycin (2 µM, line a), FCCP (2 µM, line b), and Rotenone/Antimycin A (0.5 µM, line c).

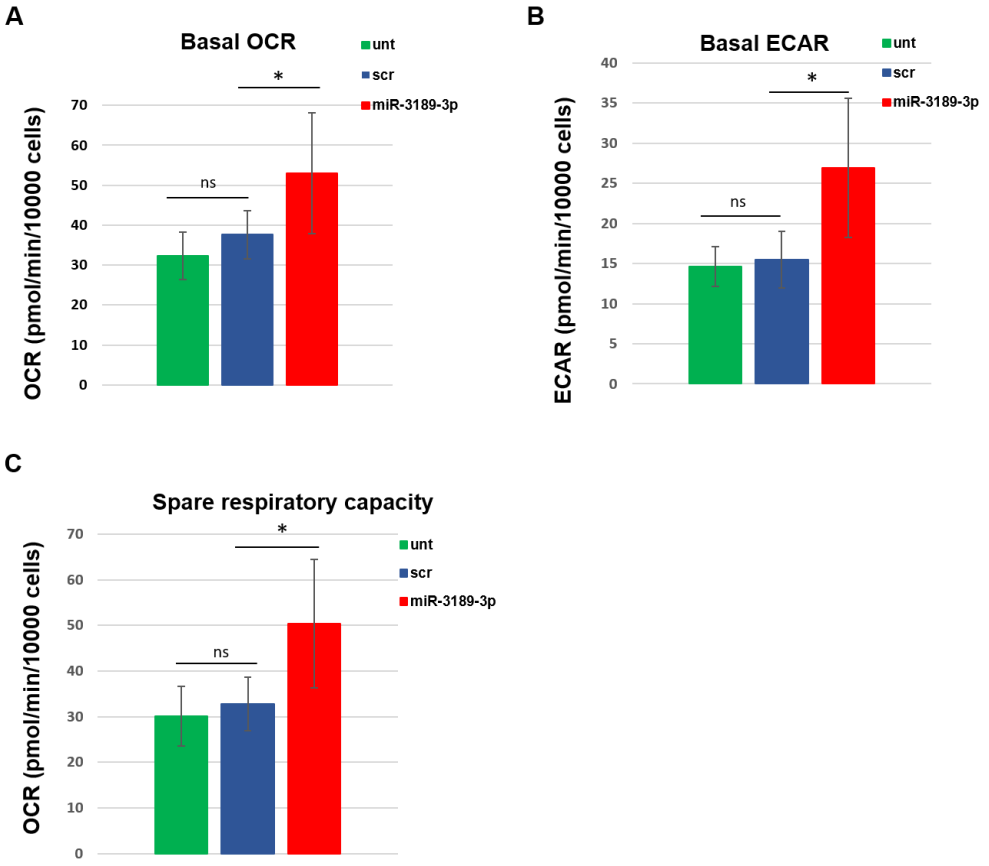


Figure 35. Basal OCR, basal ECAR and spare respiratory capacity measurement. MDA-MB231 cells were transfected with miR-3189-3p or scramble control and were subjected to the Seahorse XF Cell Mito Stress Test. **(A)** Basal OCR was calculated as the average of the three first measurement of the OCR flux before the injection of Oligomycin; **(B)** Basal ECAR was calculated as the average of the three first measurement of the ECAR flux before injection of Oligomycin; **(C)** Spare respiratory capacity was calculated as the difference between maximal respiration and basal respiration

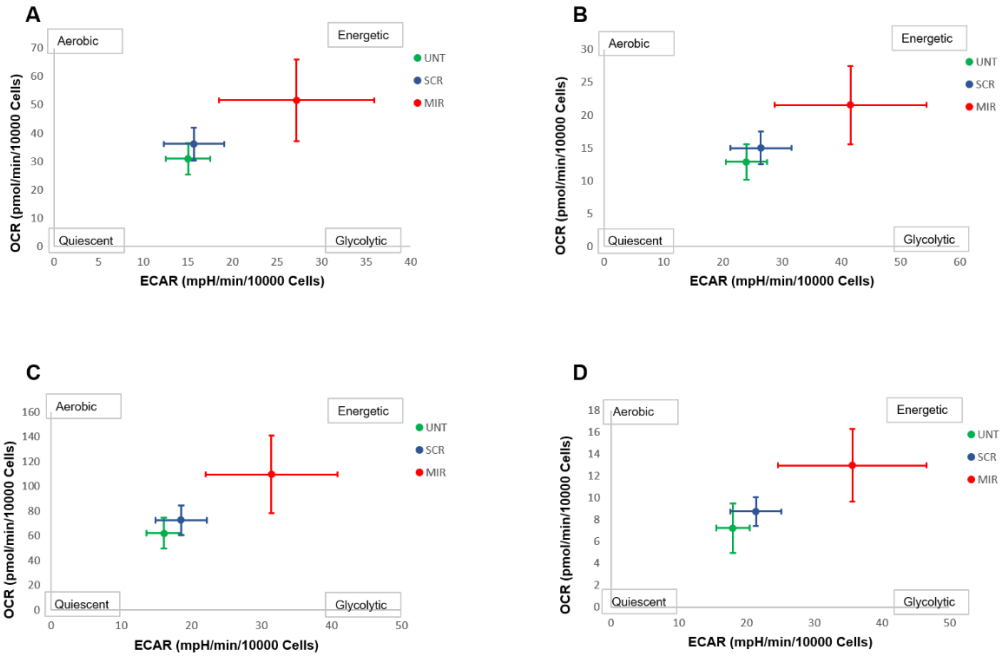


Figure 36. Energy maps from OCR and ECAR measurements. MDA-MB-231 cells were transfected with miR-3189-3p or scramble control and were subjected to the Seahorse XF Cell Mito Stress Test. Data are represented as ECAR/OCR **(A)** basal measurements, **(B)** measurement after Oligomycin injection, **(C)** measurements after FCCP injection, **(D)** measurements after Rot/AA injection.

DISCUSSION

Dr. Peruzzi and others have demonstrated a strong anti-cancer activity of miR-3189-3p [120-122, 212]. Specifically, Dr. Peruzzi's laboratory was the first to demonstrate that miR-3189-3p has anti-tumoral activity in glioblastoma, both *in vitro* and *in vivo*. The mechanism involved direct downregulation of the splicing factor SF3B2 and the rho guanine nucleotide exchange factor p63RhoGEF [120]. Considering these results and considering that TNBC is a very aggressive tumor with few available treatments, we decided to investigate the effects of miR-3189-3p in TNBC. Similarly to the data obtained in glioblastoma [120], we confirmed the inhibitory role of miR-3189-3p in breast cancer cell proliferation (~60%) and migration (~75-80%) (Figure 22 and Figure 23). Additionally, we demonstrated that miR-3189-3p impaired cell invasion *in vitro*, strengthening the anti-cancer function of this miRNA (Figure 24). To better understand the mechanism of its anti-proliferative effect, we decided to investigate whether c-MYC could be regulated by the miR-3189-3p. C-MYC is a transcription factor that regulates many biological processes, including cell proliferation, cell differentiation, metabolism and apoptosis. In normal cells, c-MYC is strictly regulated, whereas in cancer cells is generally activated through direct alterations of the MYC gene, including amplification or chromosomal translocation [213]. C-MYC has been found dysregulated in many tumors, including breast cancer [202-204]; however, it is considered "undruggable" and many therapeutic alternatives have been explored, including transcriptional, translational and post-translational targeting [202, 214]. Interestingly, although c-MYC is not a direct target of the miR-3189-3p, we found that the miRNA downregulated c-MYC, not at the mRNA level as shown in

Figure 25B but at the protein level (Figure 25C). Additionally, this miRNA was able to downregulate c-MYC in breast cancer PDX cells, which are cancer cells originated from a patient (Figure 25D). This is important, since PDX models are considered more relevant models compared to tumor cell lines because they are thought to preserve gene expression patterns and tissue histology, and to maintain chromosomal architecture [215]. However additional experiments are needed to better address the mechanisms of c-MYC downregulation in this model.

Next, considering that c-MYC has a very short life of 15-20 minutes [216, 217], we asked whether miR-3189-3p could downregulate c-MYC through the proteasome pathway. Treating MDA-MB-231 cells with different proteasome inhibitors, however, did not rescue miRNA-mediated c-MYC downregulation (Figure 26). As these results suggested a translational regulation of c-MYC by the miRNA, we focused our attention on direct target of the miR-3189-3p that might affect the translation. MiR-3189-3p has numerous putative targets that play a role in protein translation, including several eukaryotic initiation factors and 4E-BP1. 4E-BP1 is considered an inhibitor of cap-dependent translation. When it is non-phosphorylated, it inhibits the cap-dependent translation by binding eIF4E, which is a cap-binding protein fundamental for the initiation of translation [137, 138]. For its inhibitory function, 4E-BP1 is considered a tumor-suppressor and it is reasonable to think that 4E-BP1 expression should be lower in carcinomas than in normal tissue. On the contrary, like c-MYC, 4E-BP1 has been found upregulated in many tumors, suggesting its cooperation with c-MYC in the tumorigenesis [138, 139, 207]. Our

results showed the downregulation of 4E-BP1 by miR-3189-3p (Figure 27A). A direct inhibitory effect of this miRNA on the 3'UTR of 4E-BP1 was further demonstrated by a luciferase assay (Figure 27B); however, differently from what we expected, mutation of the miRNA binding site did not fully prevent gene downregulation and the modest reversal effect may suggest a general, binding-independent effect of the miRNA on translation.

Next, we were interested in understanding the role of 4E-BP1 in the miR-3189-3p-mediated downregulation of c-MYC protein in MDA-MB-231 cells. Silencing 4E-BP1 revealed a strong downregulation of c-MYC, suggesting a dependence of c-MYC on 4E-BP1 levels (Figure 28A). Since 4E-BP1 inhibits the activity of eIF4E, silencing 4E-BP1 should result in enhanced activity of eIF4E and increased global translation, including c-MYC translation. However, while c-MYC was downregulated the levels of eIF4E remained unchanged, suggesting that the downregulation of c-MYC is independent from the expression of eIF4E. Alternatively, our data may indicate a dependence of eIF4E from 4E-BP1 or, in other words, eIF4E requires a minimal expression of 4E-BP1 to be functional [218, 219]. A direct correlation between eIF4E and 4E-BP1 could indeed exist, since in cells where eIF4E is silenced, active and not-phosphorylated 4E-BP1 is ubiquitinylated and degraded by the proteasome, allowing the residual eIF4E to start the translation [220]. We did not evaluate general translation after eIF4E downregulation, nor the proteasomal degradation of 4E-BP1, but we observed a simultaneous downregulation of 4E-BP1 and c-MYC (Figure 28B). This may indicate that in our experimental setting the residual eIF4E, if any, is not promoting c-MYC translation.

If 4E-BP1 levels are strictly related to c-MYC levels, adding back 4E-BP1 should restore the normal expression of c-MYC. As shown in Figure 29A, we delivered the recombinant protein directly into the cells and, unexpectedly, this did not rescue the expression of c-MYC. On the contrary, adding back 4E-BP1 *via* a plasmid rescued the normal c-MYC expression (Figure 29B). This discrepancy in the results might be due to the Chariot system used to deliver the recombinant protein, since the presence of serum can interfere with the entry of r4E-BP1 into the cells. Indeed, when the experiment was repeated in serum free conditions, the recombinant protein efficiently entered the cells and promoted c-MYC expression in the presence of miR-3189-3p (Figure 30B).

An additional mechanism may involve again the interaction between 4E-BP1 and eIF4E. As demonstrated by a 5'cap pull down assay, miR-3189-3p reduces the binding of eIF4E to the cap of about 10% (Figure 31). It is reasonable to think that if miR-3189-3p downregulates the expression of 4E-BP1, there is less 4E-BP1 available to interact with eIF4E, which is now unbound and ready to initiate cap-dependent translation. However, since our data suggest that c-MYC is downregulated while total eIF4E levels remain consistent, we speculated that miR-3189-3p could affect the phosphorylation, and thus activity, of eIF4E. EIF4E has been found to be overexpressed and hyperphosphorylated in several cancers, and it is considered a marker of poor prognosis [221-223]. Unphosphorylated and active 4E-BP1 can bind eIF4E and block its phosphorylation, which has been shown to have more affinity for the 5'cap [224-226]. In this case, we would expect more cap-dependent translation to prevent the miRNA-

mediated downregulation of c-MYC. As our data show the contrary, it is reasonable to think that miR-3189-3p, in addition to downregulating 4E-BP1, may reduce the phosphorylation of eIF4E, making it less efficient and resulting in less expression of c-MYC.

C-MYC regulates many cellular processes and it is often upregulated in tumors, where it promotes cell survival and proliferation in conditions of stress, such as hypoxia or nutrient deprivation [138, 159, 169, 213]. We then asked if miR-3189-3p could downregulate c-MYC also in these conditions. First, we confirmed the upregulation of c-MYC when cells were cultured in the absence of serum (Figure 30A). We next determined the effect of miR-3189-3p on c-MYC expression in those cell growth conditions and found that our miRNA downregulated the expression of both c-MYC and 4E-BP1 also in serum-starved cells (Figure 30B). Additionally, adding back r4E-BP1 rescued the expression of c-MYC, suggesting an important role of 4E-BP1 in the translation of c-MYC under stress conditions (Figure 30B, lane 3). Those data are important, since they strengthen the possibility of using miR-3189-3p as a therapeutic approach in conditions where most chemotherapeutic drugs fail.

Furthermore, considering that under stress conditions the cap-dependent translation is reduced while the cap-independent translation is favored [152], we tried to understand the role of miR-3189-3p in two different types of cap-independent translation, the IRES-dependent translation and the m6A-dependent translation. We utilized a bicistronic vector in which ECMV-IRES drives the expression of a fluorescent protein (ECFP). Bicistronic vectors are vectors containing two ORFs separated by an IRES within a single

transcription unit. After the transcription of a single mRNA, the first protein is translated through the cap-dependent while the second protein is translated by the IRES-dependent translation. Although useful, this type of vectors have the potential of giving false results: (I) the DNA sequence of the IRES may be considered a cryptic promoter and used to generate a new mRNA, (II) alternative splicing can produce alternative forms of the proteins, (III) readthrough or re-initiation mechanism can continue or restart the translation after finishing the first ORF [164]. Our results showed that miR-3189-3p efficiently downregulated the expression of ECFP (IRES-dependent) compared to the miRNA negative control (Figure 32A). To better understand the effect on the IRES-dependent translation of c-MYC, we cloned the c-MYC 5'UTR (containing the IRES) in the same vector and again found that miR-3189-3p downregulates the expression of the IRES-driven ECFP (Figure 32B). Interestingly, the addition of 4E-BP1, resulted in increased expression of ECFP (Figure 32C), suggesting that 4E-BP1 may support c-MYC expression under stress through the IRES-dependent translation.

Next, as increased levels of m6A in c-MYC RNA can increase c-MYC expression [210, 211], we reasoned that our miRNA could impair also m6A-dependent translation. To this end, we used a plasmid encoding a circular RNA in which m6A drives the expression of the HPV protein E7. The protein encoded by the circular RNA can be expressed only if the plasmid is transcribed and back splicing of mRNA occurs; when mRNA is circularized, the protein is translated through a cap-independent translation because circular RNAs do not possess a 5'cap [227]. We co-transfected MDA-MB-231 cells with the E7 vector

or empty vector (EV) and the miRNA or scramble control and our results clearly showed a reduction of the E7 protein in the miR-transfected cells (Figure 33). This suggests that miR-3189-3p impairs the m6A-dependent translation.

Finally, as c-MYC can regulate cell metabolism, we checked the effect of miR-3189-3p on the metabolism of MDA-MB-231 cells. As specified in the results paragraph, tumorigenesis is always accompanied by a change in metabolism, mostly by glycolysis transformation. Several studies demonstrated that triple negative breast cancer cells have a heterogeneous metabolism. For example, cells like MDA-MB-468 use more oxygen metabolism than glycolytic metabolism while MDA-MB-231 and Hs578T rely more on glycolysis than on mitochondrial respiration [228, 229]. To determine the effect of miR-3189-3p on cellular metabolism, we used the Seahorse XF96 Flux Analyzer and found that miR-3189-3p increases significantly oxygen metabolism and glycolysis at both basal and stressed state (Figure 34, Figure 35, Figure 36), uncoupling high energy production to cell proliferation. Interestingly, the unique metabolic profile of cancer cells allows them to react to drugs in different ways. For example, cells that rely more on mitochondrial respiration like MDA-MB-468, are more sensitive to Metformin than MDA-MB-231 cells [228]. Considering that miR-3189-3p makes cells more metabolically active, a change in cell metabolic phenotype could sensitize cells to other chemotherapeutic agents, allowing a better drug approach.

Our results about the anti-tumoral effect of miR-3189-3p in breast cancer are promising, but additional studies are needed to clarify whether this miRNA can be used as a therapeutic agent. RNA

technology (siRNA, miRNA) emerged in the past as a promising gene therapy approach. However, since naked RNAs are quickly degraded and eliminated from the blood, consequently diminishing the bioavailability, they need to be protected [119, 230]. To this end, several type of nanoparticles, such as gold and silver nanoparticles, liposomes and aptamers, are under evaluation as potential RNA carriers. While miRNA therapies for breast cancer remain in preclinical stages, several clinical studies have been conducted for miRNA therapy in other types of cancer. For example, a siRNA targeting KRAS is currently in Phase III clinical trial for the treatment of pancreatic cancer in combination with chemotherapy. The technology consists of a miniature biodegradable polymeric matrix inserted into the tumor through a biopsy. The polymeric matrix protects the siRNA from degradation while slowly releasing the siRNA over a period of several months [117, 231, 232].

In summary, results from this study suggest that miR-3189-3p can be a valuable therapeutic target in a malignancy with few targeted therapeutic options. Data presented here will be included in a manuscript in preparation (Duane Jeansonne, Cecilia Vittori, Adam Lassak, Celeste Faia, Zhang Lin, Krzysztof Reiss, and Francesca Peruzzi).

CONCLUSION

Dr. Peruzzi's previous studies have demonstrated an anti-tumoral activity of miR-3189-3p in glioblastoma. Here, we further explored its role in breast cancer cells. We confirmed that miR-3189-3p impairs cell proliferation, migration, and invasion. Additionally, we found that miR-3189-3p exerts its anti-cancer function through downregulation of c-MYC at the protein level, by targeting the inhibitor of cap-dependent translation 4E-BP1. We experimentally validated this target, demonstrating that miR-3189-3p binds directly to the 3'UTR of 4E-BP1 using a reporter assay. We further demonstrated that through targeting 4E-BP1, miR-3189-3p can downregulate c-MYC by impairing the 5'cap-dependent translation in either normal growth conditions or in stress conditions. Indeed, we demonstrated that miR-3189-3p can also impair the cap-independent translation downregulating both IRES-dependent and m6A-dependent translations.

Finally, we found that metabolic reprogramming induced by miR-3189-3p in MDA-MB-231 cells promotes oxygen metabolism and glycolysis at the basal state, uncoupling high energy production to cell proliferation.

Overall, results indicated that miR-3189-3p targets translational regulatory proteins that negatively affect expression of c-MYC. Considering that c-MYC is a potent survival factor in cancer cells, our findings may provide new therapeutic approaches that target the tumor microenvironment, often characterized by hypoxia and nutrient deprivation. Additionally, the metabolic changes mediated by the miRNA could sensitize TNBC cells to other chemotherapeutic drugs. Altogether, these findings suggest that miR-3189-3p can be a valuable therapeutic agent against a malignancy with few treatment options.

BYBLIOGRAPHY

1. Harbeck, N., et al., *Breast cancer.* Nat Rev Dis Primers, 2019. **5**(1): p. 66.
2. Siegel, R.L., et al., *Cancer Statistics, 2021.* CA: A Cancer Journal for Clinicians, 2021. **71**(1): p. 7-33.
3. Tomasetti, C., L. Li, and B. Vogelstein, *Stem cell divisions, somatic mutations, cancer etiology, and cancer prevention.* Science, 2017. **355**(6331): p. 1330-1334.
4. Wu, S., et al., *Evaluating intrinsic and non-intrinsic cancer risk factors.* Nature communications, 2018. **9**(1): p. 1-12.
5. Siegel, R.L., K.D. Miller, and A. Jemal, *Cancer statistics, 2020.* CA: A Cancer Journal for Clinicians, 2020. **70**(1): p. 7-30.
6. Taylor, A., et al., *Consensus for genes to be included on cancer panel tests offered by UK genetics services: guidelines of the UK Cancer Genetics Group.* Journal of medical genetics, 2018. **55**(6): p. 372-377.
7. Kuchenbaecker, K.B., et al., *Risks of breast, ovarian, and contralateral breast cancer for BRCA1 and BRCA2 mutation carriers.* Jama, 2017. **317**(23): p. 2402-2416.
8. Balmana, J., et al., *BRCA in breast cancer: ESMO Clinical Practice Guidelines.* Annals of Oncology, 2011. **22**: p. vi31-vi34.
9. Liu, L., et al., *BRCAness as a prognostic indicator in patients with early breast cancer.* Scientific Reports, 2020. **10**(1): p. 1-8.
10. Oeffinger, K.C., et al., *Breast cancer screening for women at average risk: 2015 guideline update from the American Cancer Society.* Jama, 2015. **314**(15): p. 1599-1614.
11. Siegel, R.L., K.D. Miller, and A. Jemal, *Cancer statistics, 2019.* CA: a cancer journal for clinicians, 2019. **69**(1): p. 7-34.
12. Irwig, L., P. Macaskill, and N. Houssami, *Evidence relevant to the investigation of breast symptoms: the triple test.* The Breast, 2002. **11**(3): p. 215-220.
13. Cw, E., *Pathological Prognostic Factors in Breast Cancer. I. The Value of Histological Grade in Breast Cancer: Experience From a Large Study With Long-Term Follow-Up. CW Elston & IO Ellis. Histopathology 1991; 19; 403-410.* Histopathology, 2002. **41**(3A): p. 151-153.
14. do Nascimento, R.G. and K.M. Otoni, *Histological and molecular classification of breast cancer: what do we know?* 2020.
15. Seal, M.D. and S.K. Chia, *What is the difference between triple-negative and basal breast cancers?* The Cancer Journal, 2010. **16**(1): p. 12-16.
16. Tsang, J. and G.M. Tse, *Molecular classification of breast cancer.* Advances in anatomic pathology, 2020. **27**(1): p. 27-35.
17. Yin, L., et al., *Triple-negative breast cancer molecular subtyping and treatment progress.* Breast Cancer Research, 2020. **22**(1): p. 1-13.

18. Lehmann, B.D., et al., *Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies.* The Journal of clinical investigation, 2011. **121**(7): p. 2750-2767.
19. Zhao, S., et al., *Molecular subtypes and precision treatment of triple-negative breast cancer.* Annals of translational medicine, 2020. **8**(7).
20. Howlader, N., et al., *Differences in breast cancer survival by molecular subtypes in the United States.* Cancer Epidemiology and Prevention Biomarkers, 2018. **27**(6): p. 619-626.
21. Nash, R., et al., *State variation in the receipt of a contralateral prophylactic mastectomy among women who received a diagnosis of invasive unilateral early-stage breast cancer in the United States, 2004-2012.* JAMA surgery, 2017. **152**(7): p. 648-657.
22. Harbeck, N. and M. Gnant, *Breast cancer.* Lancet, 2017. **389**(10074): p. 1134-1150.
23. Moo, T.-A., et al., *Overview of breast cancer therapy.* PET clinics, 2018. **13**(3): p. 339-354.
24. Waks, A.G. and E.P. Winer, *Breast cancer treatment: a review.* Jama, 2019. **321**(3): p. 288-300.
25. Finn, R.S., et al., *Palbociclib and letrozole in advanced breast cancer.* New England journal of medicine, 2016. **375**(20): p. 1925-1936.
26. Goetz, M.P., et al., *MONARCH 3: abemaciclib as initial therapy for advanced breast cancer.* Journal of Clinical Oncology, 2017. **35**(32): p. 3638-3646.
27. Hortobagyi, G.N., et al., *Ribociclib as first-line therapy for HR-positive, advanced breast cancer.* New England journal of medicine, 2016. **375**(18): p. 1738-1748.
28. Medina, M.A., et al., *Triple-negative breast cancer: a review of conventional and advanced therapeutic strategies.* International journal of environmental research and public health, 2020. **17**(6): p. 2078.
29. Maker, A.V., P. Attia, and S.A. Rosenberg, *Analysis of the cellular mechanism of antitumor responses and autoimmunity in patients treated with CTLA-4 blockade.* The Journal of Immunology, 2005. **175**(11): p. 7746-7754.
30. Schmid, P., et al., *Atezolizumab and nab-paclitaxel in advanced triple-negative breast cancer.* New England Journal of Medicine, 2018. **379**(22): p. 2108-2121.
31. Kawaji, H., et al., *Comprehensive molecular profiling broadens treatment options for breast cancer patients.* Cancer Medicine, 2020.
32. Gonçalves, A., A. Bertucci, and F. Bertucci, *PARP inhibitors in the treatment of early breast cancer: the step beyond?* Cancers, 2020. **12**(6): p. 1378.
33. Consortium, I.H.G.S., *Finishing the euchromatic sequence of the human genome.* Nature, 2004. **431**(7011): p. 931.

34. Mattick, J.S. and I.V. Makunin, *Non-coding RNA.* Human molecular genetics, 2006. **15**(suppl_1): p. R17-R29.
35. Li, J. and C. Liu, *Coding or Noncoding, the Converging Concepts of RNAs.* Frontiers in Genetics, 2019. **10**(496).
36. Slack, F.J. and A.M. Chinnaiyan, *The role of non-coding RNAs in oncology.* Cell, 2019. **179**(5): p. 1033-1055.
37. Lee, R.C., R.L. Feinbaum, and V. Ambros, *The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14.* cell, 1993. **75**(5): p. 843-854.
38. Kozomara, A., M. Birgaoanu, and S. Griffiths-Jones, *miRBase: from microRNA sequences to function.* Nucleic Acids Research, 2018. **47**(D1): p. D155-D162.
39. Seal, R.L., et al., *A guide to naming human non-coding RNA genes.* The EMBO journal, 2020. **39**(6): p. e103777.
40. Concepcion, C.P., C. Bonetti, and A. Ventura, *The miR-17-92 family of microRNA clusters in development and disease.* Cancer journal (Sudbury, Mass.), 2012. **18**(3): p. 262.
41. Kamanu, T.K., et al., *Exploration of miRNA families for hypotheses generation.* Scientific reports, 2013. **3**(1): p. 1-8.
42. Liu, B., et al., *Interplay between miRNAs and host genes and their role in cancer.* Briefings in functional genomics, 2019. **18**(4): p. 255-266.
43. Georgakilas, G., et al., *microTSS: accurate microRNA transcription start site identification reveals a significant number of divergent pri-miRNAs.* Nature communications, 2014. **5**(1): p. 1-11.
44. Seila, A.C., et al., *Divergent transcription from active promoters.* science, 2008. **322**(5909): p. 1849-1851.
45. Seila, A.C., et al., *Divergent transcription: a new feature of active promoters.* Cell cycle, 2009. **8**(16): p. 2557-2564.
46. Galatenko, V.V., et al., *Comprehensive network of miRNA-induced intergenic interactions and a biological role of its core in cancer.* Scientific reports, 2018. **8**(1): p. 1-12.
47. Kim, V.N., J. Han, and M.C. Siomi, *Biogenesis of small RNAs in animals.* Nature reviews Molecular cell biology, 2009. **10**(2): p. 126-139.
48. Loh, H.-Y., et al., *The regulatory role of microRNAs in breast cancer.* International journal of molecular sciences, 2019. **20**(19): p. 4940.
49. Lee, Y., et al., *MicroRNA genes are transcribed by RNA polymerase II.* The EMBO journal, 2004. **23**(20): p. 4051-4060.
50. Schanen, B.C. and X. Li, *Transcriptional regulation of mammalian miRNA genes.* Genomics, 2011. **97**(1): p. 1-6.

51. Borchert, G.M., W. Lanier, and B.L. Davidson, *RNA polymerase III transcribes human microRNAs.* Nature structural & molecular biology, 2006. **13**(12): p. 1097-1101.
52. Han, J., et al., *The Drosha-DGCR8 complex in primary microRNA processing.* Genes & development, 2004. **18**(24): p. 3016-3027.
53. Yi, R., et al., *Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs.* Genes & development, 2003. **17**(24): p. 3011-3016.
54. Chendrimada, T.P., et al., *TRBP recruits the Dicer complex to Ago2 for microRNA processing and gene silencing.* Nature, 2005. **436**(7051): p. 740-744.
55. Pong, S.K. and M. Gullerova, *Noncanonical functions of micro RNA pathway enzymes–Drosha, DGCR 8, Dicer and Ago proteins.* FEBS letters, 2018. **592**(17): p. 2973-2986.
56. Yoda, M., et al., *ATP-dependent human RISC assembly pathways.* Nature structural & molecular biology, 2010. **17**(1): p. 17.
57. Meijer, H.A., E.M. Smith, and M. Bushell, *Regulation of miRNA strand selection: follow the leader?* Biochemical Society Transactions, 2014. **42**(4): p. 1135-1140.
58. Oliveto, S., et al., *Role of microRNAs in translation regulation and cancer.* World journal of biological chemistry, 2017. **8**(1): p. 45.
59. Peng, Y. and C.M. Croce, *The role of MicroRNAs in human cancer.* Signal transduction and targeted therapy, 2016. **1**(1): p. 1-9.
60. Annese, T., et al., *microRNAs Biogenesis, Functions and Role in Tumor Angiogenesis.* Frontiers in Oncology, 2020. **10**(2610).
61. Ruby, J.G., C.H. Jan, and D.P. Bartel, *Intronic microRNA precursors that bypass Drosha processing.* Nature, 2007. **448**(7149): p. 83-86.
62. Ha, M. and V.N. Kim, *Regulation of microRNA biogenesis.* Nature reviews Molecular cell biology, 2014. **15**(8): p. 509-524.
63. Flynt, A.S., et al., *MicroRNA biogenesis via splicing and exosome-mediated trimming in Drosophila.* Molecular cell, 2010. **38**(6): p. 900-907.
64. O'Brien, J., et al., *Overview of MicroRNA Biogenesis, Mechanisms of Actions, and Circulation.* Frontiers in Endocrinology, 2018. **9**(402).
65. Desvignes, T., et al., *miRNA nomenclature: a view incorporating genetic origins, biosynthetic pathways, and sequence variants.* Trends in Genetics, 2015. **31**(11): p. 613-626.
66. Yang, J.-S., et al., *Conserved vertebrate mir-451 provides a platform for Dicer-independent, Ago2-mediated microRNA biogenesis.* Proceedings of the National Academy of Sciences, 2010. **107**(34): p. 15163-15168.
67. Cheloufi, S., et al., *A dicer-independent miRNA biogenesis pathway that requires Ago catalysis.* Nature, 2010. **465**(7298): p. 584-589.

68. Huang, X., et al., *Hypoxia-inducible mir-210 regulates normoxic gene expression involved in tumor initiation.* Molecular cell, 2009. **35**(6): p. 856-867.
69. Bracken, C.P., et al., *A double-negative feedback loop between ZEB1-SIP1 and the microRNA-200 family regulates epithelial-mesenchymal transition.* Cancer research, 2008. **68**(19): p. 7846-7854.
70. Roth, B.M., D. Ishimaru, and M. Hennig, *The core microprocessor component DiGeorge syndrome critical region 8 (DGCR8) is a nonspecific RNA-binding protein.* Journal of Biological Chemistry, 2013. **288**(37): p. 26785-26799.
71. Shiohama, A., et al., *Molecular cloning and expression analysis of a novel gene DGCR8 located in the DiGeorge syndrome chromosomal region.* Biochemical and biophysical research communications, 2003. **304**(1): p. 184-190.
72. Han, J., et al., *Posttranscriptional crossregulation between Drosha and DGCR8.* Cell, 2009. **136**(1): p. 75-84.
73. Tang, X., et al., *Glycogen synthase kinase 3 beta (GSK3β) phosphorylates the RNAase III enzyme Drosha at S300 and S302.* PloS one, 2011. **6**(6): p. e20391.
74. Herbert, K.M., et al., *Phosphorylation of DGCR8 increases its intracellular stability and induces a progrowth miRNA profile.* Cell reports, 2013. **5**(4): p. 1070-1081.
75. Shen, J. and M.-C. Hung, *Signaling-mediated regulation of MicroRNA processing.* Cancer research, 2015. **75**(5): p. 783-791.
76. Suzuki, H.I., et al., *Modulation of microRNA processing by p53.* Nature, 2009. **460**(7254): p. 529-533.
77. Melo, S.A., et al., *A genetic defect in exportin-5 traps precursor microRNAs in the nucleus of cancer cells.* Cancer cell, 2010. **18**(4): p. 303-315.
78. Sun, H.-L., et al., *ERK activation globally downregulates miRNAs through phosphorylating exportin-5.* Cancer cell, 2016. **30**(5): p. 723-736.
79. Treiber, T., N. Treiber, and G. Meister, *Regulation of microRNA biogenesis and its crosstalk with other cellular pathways.* Nature Reviews Molecular Cell Biology, 2019. **20**(1): p. 5-20.
80. Warner, M.J., et al., *S6K2-mediated regulation of TRBP as a determinant of miRNA expression in human primary lymphatic endothelial cells.* Nucleic acids research, 2016. **44**(20): p. 9942-9955.
81. Sheu-Gruttadauria, J. and I.J. MacRae, *Structural foundations of RNA silencing by Argonaute.* Journal of molecular biology, 2017. **429**(17): p. 2619-2639.

82. Iwasaki, S., et al., *Hsc70/Hsp90 chaperone machinery mediates ATP-dependent RISC loading of small RNA duplexes.* Molecular cell, 2010. **39**(2): p. 292-299.
83. Kim, H., et al., *A Mechanism for microRNA arm switching regulated by uridylation.* Molecular Cell, 2020. **78**(6): p. 1224-1236. e5.
84. Auyeung, V.C., et al., *Beyond secondary structure: primary-sequence determinants license pri-miRNA hairpins for processing.* Cell, 2013. **152**(4): p. 844-858.
85. Xhemalce, B., S.C. Robson, and T. Kouzarides, *Human RNA methyltransferase BCDIN3D regulates microRNA processing.* Cell, 2012. **151**(2): p. 278-288.
86. Glaich, O., et al., *DNA methylation directs microRNA biogenesis in mammalian cells.* Nature communications, 2019. **10**(1): p. 1-11.
87. Cheray, M., et al., *Cytosine methylation of mature microRNAs inhibits their functions and is associated with poor prognosis in glioblastoma multiforme.* Molecular cancer, 2020. **19**(1): p. 1-16.
88. Rüegger, S. and H. Großhans, *MicroRNA turnover: when, how, and why.* Trends in biochemical sciences, 2012. **37**(10): p. 436-446.
89. Lee, S., et al., *Selective degradation of host MicroRNAs by an intergenic HCMV noncoding RNA accelerates virus production.* Cell host & microbe, 2013. **13**(6): p. 678-690.
90. Xiao, J., et al., *Long noncoding RNA TRPM2-AS acts as a microRNA sponge of miR-612 to promote gastric cancer progression and radioresistance.* Oncogenesis, 2020. **9**(3): p. 1-15.
91. Yousefi, H., et al., *Long noncoding RNAs and exosomal lncRNAs: classification, and mechanisms in breast cancer metastasis and drug resistance.* Oncogene, 2020. **39**(5): p. 953-974.
92. Zhang, X., et al., *LncRNA MACC1-AS1 sponges multiple miRNAs and RNA-binding protein PTBP1.* Oncogenesis, 2019. **8**(12): p. 1-13.
93. Mallanna, S.K. and A. Rizzino, *Emerging roles of microRNAs in the control of embryonic stem cells and the generation of induced pluripotent stem cells.* Developmental biology, 2010. **344**(1): p. 16-25.
94. Vasudevan, S., Y. Tong, and J.A. Steitz, *Switching from repression to activation: microRNAs can up-regulate translation.* Science, 2007. **318**(5858): p. 1931-1934.
95. Roberts, A.P., A.P. Lewis, and C.L. Jopling, *miR-122 activates hepatitis C virus translation by a specialized mechanism requiring particular RNA components.* Nucleic acids research, 2011. **39**(17): p. 7716-7729.
96. Zhou, H. and I. Rigoutsos, *MiR-103a-3p targets the 5′ UTR of GPRC5A in pancreatic cells.* Rna, 2014. **20**(9): p. 1431-1439.

97. Ørom, U.A., F.C. Nielsen, and A.H. Lund, *MicroRNA-10a binds the 5' UTR of ribosomal protein mRNAs and enhances their translation.* Molecular cell, 2008. **30**(4): p. 460-471.
98. Espinosa, C.E.S. and F.J. Slack, *Cancer issue: The role of microRNAs in cancer.* The Yale journal of biology and medicine, 2006. **79**(3-4): p. 131.
99. Hammond, S.M., *An overview of microRNAs.* Advanced drug delivery reviews, 2015. **87**: p. 3-14.
100. Yamakuchi, M., M. Ferlito, and C.J. Lowenstein, *miR-34a repression of SIRT1 regulates apoptosis.* Proceedings of the National Academy of Sciences, 2008. **105**(36): p. 13421-13426.
101. Chang, T.-C., et al., *Widespread microRNA repression by Myc contributes to tumorigenesis.* Nature genetics, 2008. **40**(1): p. 43-50.
102. Han, H., et al., *A c-Myc-MicroRNA functional feedback loop affects hepatocarcinogenesis.* Hepatology, 2013. **57**(6): p. 2378-2389.
103. Lewis, K.A., H.R. Jordan, and T.O. Tollefsbol, *Effects of SAHA and EGCG on growth potentiation of triple-negative breast cancer cells.* Cancers, 2019. **11**(1): p. 23.
104. Hanahan, D. and R.A. Weinberg, *Hallmarks of cancer: the next generation.* cell, 2011. **144**(5): p. 646-674.
105. Huang, X. and J. Lyu, *Tumor suppressor function of miR-483-3p on breast cancer via targeting of the cyclin E1 gene.* Experimental and therapeutic medicine, 2018. **16**(3): p. 2615-2620.
106. Xie, D., et al., *MicroRNA-424 serves an anti-oncogenic role by targeting cyclin-dependent kinase 1 in breast cancer cells.* Oncology reports, 2018. **40**(6): p. 3416-3426.
107. Wang, J., et al., *miR-424-5p regulates cell proliferation, migration and invasion by targeting doublecortin-like kinase 1 in basal-like breast cancer.* Biomedicine & Pharmacotherapy, 2018. **102**: p. 147-152.
108. Tsouko, E., et al., *miR-200a inhibits migration of triple-negative breast cancer cells through direct repression of the EPHA2 oncogene.* Carcinogenesis, 2015. **36**(9): p. 1051-1060.
109. Sossey-Alaoui, K., et al., *The Kindlin-2 regulation of epithelial-to-mesenchymal transition in breast cancer metastasis is mediated through miR-200b.* Scientific reports, 2018. **8**(1): p. 1-13.
110. Sabit, H., et al., *Triple Negative Breast Cancer in the Era of miRNA.* Critical reviews in oncology/hematology, 2020: p. 103196.
111. Carter, J.V., et al., *The microRNA-200 family acts as an oncogene in colorectal cancer by inhibiting the tumor suppressor RASSF2.* Oncology letters, 2019. **18**(4): p. 3994-4007.
112. Hu, W., et al., *Functional miRNAs in breast cancer drug resistance.* Oncotargets and therapy, 2018. **11**: p. 1529.

113. Cataldo, A., et al., *miR-302b enhances breast cancer cell sensitivity to cisplatin by regulating E2F1 and the cellular DNA damage response.* Oncotarget, 2016. **7**(1): p. 786.
114. Pogribny, I.P., et al., *Alterations of microRNAs and their targets are associated with acquired resistance of MCF-7 breast cancer cells to cisplatin.* International journal of cancer, 2010. **127**(8): p. 1785-1794.
115. Lu, X., et al., *MiR-129-5p sensitizes the response of Her-2 positive breast cancer to trastuzumab by reducing Rps6.* Cellular Physiology and Biochemistry, 2017. **44**(6): p. 2346-2356.
116. Liu, M., et al., *MicroRNA-5195-3p enhances the chemosensitivity of triple-negative breast cancer to paclitaxel by downregulating EIF4A2.* Cellular & molecular biology letters, 2019. **24**(1): p. 1-11.
117. Qattan, A., *Novel miRNA Targets and Therapies in the Triple-Negative Breast Cancer Microenvironment: An Emerging Hope for a Challenging Disease.* International Journal of Molecular Sciences, 2020. **21**(23): p. 8905.
118. Tormo, E., et al., *The miRNA-449 family mediates doxorubicin resistance in triple-negative breast cancer by regulating cell cycle factors.* Scientific reports, 2019. **9**(1): p. 1-14.
119. Yin, H., et al., *Delivery of anti-miRNA for triple-negative breast cancer therapy using RNA nanoparticles targeting stem cell marker CD133.* Molecular Therapy, 2019. **27**(7): p. 1252-1261.
120. Jeansonne, D., et al., *Anti-tumoral effects of miR-3189-3p in glioblastoma.* Journal of Biological Chemistry, 2015. **290**(13): p. 8067-8080.
121. Bian, Y., et al., *miR-3189-3p mimics enhance the effects of S100A4 siRNA on the inhibition of proliferation and migration of gastric cancer cells by targeting CFL2.* International journal of molecular sciences, 2018. **19**(1): p. 236.
122. Jones, M., et al., *Growth differentiation factor-15 encodes a novel microRNA 3189 that functions as a potent regulator of cell death.* Cell Death & Differentiation, 2015. **22**(10): p. 1641-1653.
123. Schuller, A.P. and R. Green, *Roadblocks and resolutions in eukaryotic translation.* Nature Reviews Molecular Cell Biology, 2018. **19**(8): p. 526-541.
124. Jackson, R.J., C.U.T. Hellen, and T.V. Pestova, *The mechanism of eukaryotic translation initiation and principles of its regulation.* Nature Reviews Molecular Cell Biology, 2010. **11**(2): p. 113-127.
125. Passmore, L.A., et al., *The eukaryotic translation initiation factors eIF1 and eIF1A induce an open conformation of the 40S ribosome.* Molecular cell, 2007. **26**(1): p. 41-50.

126. Pestova, T.V. and V.G. Kolupaeva, *The roles of individual eukaryotic translation initiation factors in ribosomal scanning and initiation codon selection.* Genes & development, 2002. **16**(22): p. 2906-2922.
127. Pisarev, A.V., et al., *Ribosomal position and contacts of mRNA in eukaryotic translation initiation complexes.* The EMBO journal, 2008. **27**(11): p. 1609-1621.
128. Pisarev, A.V., et al., *Specific functional interactions of nucleotides at key-3 and+ 4 positions flanking the initiation codon with components of the mammalian 48S translation initiation complex.* Genes & development, 2006. **20**(5): p. 624-636.
129. Kapp, L.D. and J.R. Lorsch, *GTP-dependent recognition of the methionine moiety on initiator tRNA by translation factor eIF2.* Journal of molecular biology, 2004. **335**(4): p. 923-936.
130. Pestova, T.V., et al., *The joining of ribosomal subunits in eukaryotes requires eIF5B.* Nature, 2000. **403**(6767): p. 332-335.
131. Unbehaun, A., et al., *Release of initiation factors from 48S complexes during ribosomal subunit joining and the link between establishment of codon-anticodon base-pairing and hydrolysis of eIF2-bound GTP.* Genes & development, 2004. **18**(24): p. 3078-3093.
132. Sharma, D.K., et al., *Role of eukaryotic initiation factors during cellular stress and cancer progression.* Journal of nucleic acids, 2016. **2016**.
133. Tahmasebi, S., et al., *Translation deregulation in human disease.* Nature Reviews Molecular Cell Biology, 2018. **19**(12): p. 791-807.
134. Wek, R., H.-Y. Jiang, and T. Anthony, *Coping with stress: eIF2 kinases and translational control.* Biochemical Society Transactions, 2006. **34**(1): p. 7-11.
135. Kahvejian, A., et al., *Mammalian poly (A)-binding protein is a eukaryotic translation initiation factor, which acts via multiple mechanisms.* Genes & development, 2005. **19**(1): p. 104-113.
136. Wakiyama, M., H. Imataka, and N. Sonenberg, *Interaction of eIF4G with poly (A)-binding protein stimulates translation and is critical for Xenopus oocyte maturation.* Current Biology, 2000. **10**(18): p. 1147-1150.
137. Ali, M.U., et al., *Eukaryotic translation initiation factors and cancer.* Tumor Biology, 2017. **39**(6): p. 1010428317709805.
138. Musa, J., et al., *Eukaryotic initiation factor 4E-binding protein 1 (4E-BP1): a master regulator of mRNA translation involved in tumorigenesis.* Oncogene, 2016. **35**(36): p. 4675-4688.
139. Rousseau, D., et al., *The eIF4E-binding proteins 1 and 2 are negative regulators of cell growth.* Oncogene, 1996. **13**(11): p. 2415-2420.

140. Rojo, F., et al., *4E-binding protein 1, a cell signaling hallmark in breast cancer that correlates with pathologic grade and prognosis.* Clinical Cancer Research, 2007. **13**(1): p. 81-89.
141. Zhou, X., et al., *Activation of the Akt/mammalian target of rapamycin/4E-BP1 pathway by ErbB2 overexpression predicts tumor progression in breast cancers.* Clinical Cancer Research, 2004. **10**(20): p. 6779-6788.
142. Nishikawa, M., et al. *Significance of 4E-binding protein 1 as a therapeutic target for invasive urothelial carcinoma of the bladder*. in *Urologic Oncology: Seminars and Original Investigations*. 2015. Elsevier.
143. Chao, M.-W., et al., *eIF4E binding protein 1 expression is associated with clinical survival outcomes in colorectal cancer.* Oncotarget, 2015. **6**(27): p. 24092.
144. Salehi, Z. and F. Mashayekhi, *Expression of the eukaryotic translation initiation factor 4E (eIF4E) and 4E-BP1 in esophageal cancer.* Clinical biochemistry, 2006. **39**(4): p. 404-409.
145. Mueller, S., et al., *PTEN promoter methylation and activation of the PI3K/Akt/mTOR pathway in pediatric gliomas and influence on clinical outcome.* Neuro-oncology, 2012. **14**(9): p. 1146-1152.
146. Roh, M.S., et al., *Phosphorylated 4E-binding protein 1 expression is associated with poor prognosis in small-cell lung cancer.* Virchows Archiv, 2015. **467**(6): p. 667-673.
147. No, J.H., et al., *Activation of mTOR signaling pathway associated with adverse prognostic factors of epithelial ovarian cancer.* Gynecologic oncology, 2011. **121**(1): p. 8-12.
148. Graff, J.R., et al., *eIF4E activation is commonly elevated in advanced human prostate cancers and significantly related to reduced patient survival.* Cancer research, 2009. **69**(9): p. 3866-3873.
149. De Benedetti, A. and J.R. Graff, *eIF-4E expression and its role in malignancies and metastases.* Oncogene, 2004. **23**(18): p. 3189-3199.
150. Graff, J.R., et al., *Targeting the eukaryotic translation initiation factor 4E for cancer therapy.* Cancer research, 2008. **68**(3): p. 631-634.
151. Braunstein, S., et al., *A hypoxia-controlled cap-dependent to cap-independent translation switch in breast cancer.* Molecular cell, 2007. **28**(3): p. 501-512.
152. Sriram, A., J. Bohlen, and A.A. Teleman, *Translation acrobatics: how cancer cells exploit alternate modes of translational initiation.* EMBO reports, 2018. **19**(10): p. e45947.
153. Johannes, G. and P. Sarnow, *Cap-independent polysomal association of natural mRNAs encoding c-myc, BiP, and eIF4G conferred by internal ribosome entry sites.* Rna, 1998. **4**(12): p. 1500-1513.

154. Balvay, L., et al., *Structural and functional diversity of viral IRESes.* Biochimica et Biophysica Acta (BBA)-Gene Regulatory Mechanisms, 2009. **1789**(9-10): p. 542-557.
155. Sarnow, P., *Viral internal ribosome entry site elements: novel ribosome-RNA complexes and roles in viral pathogenesis.* Journal of virology, 2003. **77**(5): p. 2801-2806.
156. Stoneley, M. and A.E. Willis, *Cellular internal ribosome entry segments: structures, trans-acting factors and regulation of gene expression.* Oncogene, 2004. **23**(18): p. 3200-3207.
157. Gebauer, F. and M.W. Hentze, *IRES unplugged.* Science, 2016. **351**(6270): p. 228-228.
158. Weingarten-Gabbay, S., et al., *Systematic discovery of cap-independent translation sequences in human and viral genomes.* Science, 2016. **351**(6270).
159. Godet, A.-C., et al., *IRES trans-acting factors, key actors of the stress response.* International journal of molecular sciences, 2019. **20**(4): p. 924.
160. Huez, I., et al., *Two independent internal ribosome entry sites are involved in translation initiation of vascular endothelial growth factor mRNA.* Molecular and cellular biology, 1998. **18**(11): p. 6178-6190.
161. Nanbru, C., et al., *Alternative translation of the proto-oncogene c-mycby an internal ribosome entry site.* Journal of Biological Chemistry, 1997. **272**(51): p. 32061-32066.
162. Vagner, S., et al., *Alternative translation of human fibroblast growth factor 2 mRNA occurs by internal entry of ribosomes.* Molecular and cellular biology, 1995. **15**(1): p. 35-44.
163. Kwan, T. and S.R. Thompson, *Noncanonical translation initiation in eukaryotes.* Cold Spring Harbor perspectives in biology, 2019. **11**(4): p. a032672.
164. Yang, Y. and Z. Wang, *IRES-mediated cap-independent translation, a path leading to hidden proteome.* Journal of molecular cell biology, 2019. **11**(10): p. 911-919.
165. Sawicka, K., et al., *Polypyrimidine-tract-binding protein: a multifunctional RNA-binding protein.* Biochemical Society Transactions, 2008. **36**(4): p. 641-647.
166. King, H.A., L.C. Cobbold, and A.E. Willis, *The role of IRES trans-acting factors in regulating translation initiation*. 2010, Portland Press Ltd.
167. Thakor, N., et al., *Cellular mRNA recruits the ribosome via eIF3-PABP bridge to initiate internal translation.* RNA biology, 2017. **14**(5): p. 553-567.
168. Spriggs, K.A., et al., *Canonical initiation factor requirements of the Myc family of internal ribosome entry segments.* Molecular and cellular biology, 2009. **29**(6): p. 1565-1574.

169. Walters, B. and S.R. Thompson, *Cap-Independent Translational Control of Carcinogenesis.* Frontiers in Oncology, 2016. **6**(128).
170. Blau, L., et al., *Aberrant expression of c-Jun in glioblastoma by internal ribosome entry site (IRES)-mediated translational activation.* Proceedings of the National Academy of Sciences, 2012. **109**(42): p. E2875-E2884.
171. Lee, Y., et al., *Molecular mechanisms driving mRNA degradation by m6A modification.* Trends in Genetics, 2020. **36**(3): p. 177-188.
172. Meyer, K.D., et al., *Comprehensive analysis of mRNA methylation reveals enrichment in 3' UTRs and near stop codons.* Cell, 2012. **149**(7): p. 1635-1646.
173. Meyer, K.D., et al., *5' UTR m6A promotes cap-independent translation.* Cell, 2015. **163**(4): p. 999-1010.
174. Niu, Y., et al., *N6-methyl-adenosine (m6A) in RNA: an old modification with a novel epigenetic function.* Genomics, proteomics & bioinformatics, 2013. **11**(1): p. 8-17.
175. Coots, R.A., et al., *m6A facilitates eIF4F-independent mRNA translation.* Molecular cell, 2017. **68**(3): p. 504-514. e7.
176. Mitchell, S.F. and R. Parker, *Modifications on translation initiation.* Cell, 2015. **163**(4): p. 796-798.
177. Ke, S., et al., *m6A mRNA modifications are deposited in nascent pre-mRNA and are not required for splicing but do specify cytoplasmic turnover.* Genes & development, 2017. **31**(10): p. 990-1006.
178. Dominissini, D., et al., *Topology of the human and mouse m 6 A RNA methylomes revealed by m 6 A-seq.* Nature, 2012. **485**(7397): p. 201-206.
179. Zhu, W., et al., *Detection of N6-methyladenosine modification residues.* International journal of molecular medicine, 2019. **43**(6): p. 2267-2278.
180. Schöller, E., et al., *Interactions, localization, and phosphorylation of the m6A generating METTL3–METTL14–WTAP complex.* Rna, 2018. **24**(4): p. 499-512.
181. Śledź, P. and M. Jinek, *Structural insights into the molecular mechanism of the m6A writer complex.* elife, 2016. **5**: p. e18434.
182. Wang, P., K.A. Doxtader, and Y. Nam, *Structural basis for cooperative function of Mettl3 and Mettl14 methyltransferases.* Molecular cell, 2016. **63**(2): p. 306-317.
183. Ping, X.-L., et al., *Mammalian WTAP is a regulatory subunit of the RNA N6-methyladenosine methyltransferase.* Cell research, 2014. **24**(2): p. 177-189.
184. Patil, D.P., et al., *m 6 A RNA methylation promotes XIST-mediated transcriptional repression.* Nature, 2016. **537**(7620): p. 369-373.
185. Schwartz, S., et al., *Perturbation of m6A writers reveals two distinct classes of mRNA methylation at internal and 5' sites.* Cell reports, 2014. **8**(1): p. 284-296.

186. Yue, Y., et al., *VIRMA mediates preferential m 6 A mRNA methylation in 3' UTR and near stop codon and associates with alternative polyadenylation.* Cell discovery, 2018. **4**(1): p. 1-17.
187. Mauer, J., et al., *Reversible methylation of m 6 A m in the 5' cap controls mRNA stability.* Nature, 2017. **541**(7637): p. 371-375.
188. Li, Z., et al., *FTO plays an oncogenic role in acute myeloid leukemia as a N6-methyladenosine RNA demethylase.* Cancer cell, 2017. **31**(1): p. 127-141.
189. Niu, Y., et al., *RNA N6-methyladenosine demethylase FTO promotes breast tumor progression through inhibiting BNIP3.* Molecular cancer, 2019. **18**(1): p. 1-16.
190. Bartosovic, M., et al., *N6-methyladenosine demethylase FTO targets pre-mRNAs and regulates alternative splicing and 3'-end processing.* Nucleic acids research, 2017. **45**(19): p. 11356-11370.
191. Xiao, W., et al., *Nuclear m6A reader YTHDC1 regulates mRNA splicing.* Molecular cell, 2016. **61**(4): p. 507-519.
192. Yang, Y., et al., *Dynamic transcriptomic m 6 A decoration: writers, erasers, readers and functions in RNA metabolism.* Cell research, 2018. **28**(6): p. 616-624.
193. Liu, T., et al., *The m6A reader YTHDF1 promotes ovarian cancer progression via augmenting EIF3C translation.* Nucleic acids research, 2020. **48**(7): p. 3816-3831.
194. Han, S.H. and J. Choe, *Diverse molecular functions of m 6 A mRNA modification in cancer.* Experimental & molecular medicine, 2020. **52**(5): p. 738-749.
195. Choe, J., et al., *mRNA circularization by METTL3–eIF3h enhances translation and promotes oncogenesis.* Nature, 2018. **561**(7724): p. 556-560.
196. Lin, S., et al., *The m6A methyltransferase METTL3 promotes translation in human cancer cells.* Molecular cell, 2016. **62**(3): p. 335-345.
197. Chen, M., et al., *RNA N6-methyladenosine methyltransferase-like 3 promotes liver cancer progression through YTHDF2-dependent posttranscriptional silencing of SOCS2.* Hepatology, 2018. **67**(6): p. 2254-2270.
198. Paris, J., et al., *Targeting the RNA m6A reader YTHDF2 selectively compromises cancer stem cells in acute myeloid leukemia.* Cell stem cell, 2019. **25**(1): p. 137-148. e6.
199. Eletto, D., et al., *Inhibition of SNAP25 expression by HIV-1 Tat involves the activity of mir-128a.* Journal of cellular physiology, 2008. **216**(3): p. 764-770.
200. Kadri, F., et al., *HIV-1-Tat protein inhibits SC35-mediated tau exon 10 inclusion through up-regulation of DYRK1A kinase.* Journal of Biological Chemistry, 2015. **290**(52): p. 30931-30946.

201. Pacifici, M., et al., *Cerebrospinal fluid miRNA profile in HIV-encephalitis.* Journal of cellular physiology, 2013. **228**(5): p. 1070-1075.
202. Chen, H., H. Liu, and G. Qing, *Targeting oncogenic Myc as a strategy for cancer treatment.* Signal transduction and targeted therapy, 2018. **3**(1): p. 1-7.
203. Horiuchi, D., et al., *MYC pathway activation in triple-negative breast cancer is synthetic lethal with CDK inhibition.* Journal of Experimental Medicine, 2012. **209**(4): p. 679-696.
204. Miller, D.M., et al., *c-Myc and cancer metabolism*. 2012, AACR.
205. Sabnis, H.S., R.R. Somasagara, and K.D. Bunting, *Targeting MYC dependence by metabolic inhibitors in cancer.* Genes, 2017. **8**(4): p. 114.
206. Agarwal, V., et al., *Predicting effective microRNA target sites in mammalian mRNAs.* elife, 2015. **4**: p. e05005.
207. Qin, X., B. Jiang, and Y. Zhang, *4E-BP1, a multifactor regulated multifunctional protein.* Cell cycle, 2016. **15**(6): p. 781-786.
208. Licursi, M., et al., *Promotion of viral internal ribosomal entry site-mediated translation under amino acid starvation.* Journal of general virology, 2012. **93**(5): p. 951-962.
209. Créancier, L., et al., *c-myc Internal ribosome entry site activity is developmentally controlled and subjected to a strong translational repression in adult transgenic mice.* Molecular and Cellular Biology, 2001. **21**(5): p. 1833-1840.
210. Yang, D.-D., et al., *METTL3 promotes the progression of gastric cancer via targeting the MYC pathway.* Frontiers in oncology, 2020. **10**: p. 115.
211. Yuan, Y., et al., *The M6A methyltransferase METTL3 promotes the development and progression of prostate carcinoma via mediating MYC methylation.* Journal of Cancer, 2020. **11**(12): p. 3588.
212. Scheffer, D., et al., *Active mixture of serum-circulating small molecules selectively inhibits proliferation and triggers apoptosis in cancer cells via induction of ER stress.* Cellular signalling, 2020. **65**: p. 109426.
213. Dang, C.V., *MYC on the path to cancer.* Cell, 2012. **149**(1): p. 22-35.
214. Allen-Petersen, B.L. and R.C. Sears, *Mission possible: advances in MYC therapeutic targeting in cancer.* BioDrugs, 2019. **33**(5): p. 539-553.
215. Borodovsky, A., et al., *Generation of stable PDX derived cell lines using conditional reprogramming.* Molecular cancer, 2017. **16**(1): p. 1-7.
216. Farrell, A.S. and R.C. Sears, *MYC degradation.* Cold Spring Harbor perspectives in medicine, 2014. **4**(3): p. a014365.
217. Hann, S.R. and R.N. Eisenman, *Proteins encoded by the human c-myc oncogene: differential expression in neoplastic cells.* Molecular and cellular biology, 1984. **4**(11): p. 2486-2497.

218. Alain, T., et al., *eIF4E/4E-BP ratio predicts the efficacy of mTOR targeted therapies.* Cancer research, 2012. **72**(24): p. 6468-6476.
219. Hsu, H.-S., et al., *The 4E-BP1/eIF4E ratio is a determinant for rapamycin response in esophageal cancer cells.* The Journal of thoracic and cardiovascular surgery, 2015. **149**(1): p. 378-385.
220. Hinnebusch, A.G., *Translational homeostasis via eIF4E and 4E-BP1.* Molecular cell, 2012. **46**(6): p. 717-719.
221. Furic, L., et al., *eIF4E phosphorylation promotes tumorigenesis and is associated with prostate cancer progression.* Proceedings of the National Academy of Sciences, 2010. **107**(32): p. 14134-14139.
222. Shveygert, M., et al., *Regulation of eukaryotic initiation factor 4E (eIF4E) phosphorylation by mitogen-activated protein kinase occurs through modulation of Mnk1-eIF4G interaction.* Molecular and cellular biology, 2010. **30**(21): p. 5160-5167.
223. Yang, X., W. Zhong, and R. Cao, *Phosphorylation of the mRNA cap-binding protein eIF4E and cancer.* Cellular Signalling, 2020: p. 109689.
224. Batool, A., et al., *Eukaryotic Initiation Factor 4E phosphorylation acts a switch for its binding to 4E-BP1 and mRNA cap assembly.* Biochemical and biophysical research communications, 2020. **527**(2): p. 489-495.
225. Minich, W.B., et al., *Chromatographic resolution of in vivo phosphorylated and nonphosphorylated eukaryotic translation initiation factor eIF-4E: increased cap affinity of the phosphorylated form.* Proceedings of the National Academy of Sciences, 1994. **91**(16): p. 7668-7672.
226. Shibata, S., et al., *Effect of mRNA cap structure on eIF-4E phosphorylation and cap binding analyses using Ser209-mutated eIF-4Es.* Biochemical and biophysical research communications, 1998. **247**(2): p. 213-216.
227. Lei, M., et al., *Translation and functional roles of circular RNAs in human cancer.* Molecular cancer, 2020. **19**(1): p. 1-9.
228. Lanning, N.J., et al., *Metabolic profiling of triple-negative breast cancer cells reveals metabolic vulnerabilities.* Cancer & metabolism, 2017. **5**(1): p. 1-14.
229. Lunetti, P., et al., *Metabolic reprogramming in breast cancer results in distinct mitochondrial bioenergetics between luminal and basal subtypes.* The FEBS journal, 2019. **286**(4): p. 688-709.
230. Wittrup, A. and J. Lieberman, *Knocking down disease: a progress report on siRNA therapeutics.* Nature Reviews Genetics, 2015. **16**(9): p. 543-552.
231. Golan, T., et al., *RNAi therapy targeting KRAS in combination with chemotherapy for locally advanced pancreatic cancer patients.* Oncotarget, 2015. **6**(27): p. 24560.
232. Yu, A.-M., et al., *RNA therapy: Are we using the right molecules?* Pharmacology & therapeutics, 2019. **196**: p. 91-104.

LIST OF PUBLICATIONS

- Sterol metabolism modulates susceptibility to HIV-1 Infection.

Saulle I, Ibba SV, **Vittori C**, Fenizia C, Mercurio V, Vichi F, Caputo SL, Trabattoni D, Clerici M, Biasin M. AIDS. 2020 Sep 1;34(11):1593-1602. doi: 10.1097/QAD.0000000000002591. PMID: 32501835

- Human papillomavirus in spermatozoa is efficiently removed by washing: a suitable approach for assisted reproduction.

Fenizia C*, **Vittori C***, Oneta M, Parrilla B, Granata A, Ibba S, Biasin M, Clerici M, Trabattoni D, Savasi V. . Reprod Biomed Online. 2020 May;40(5):693-699. doi: 10.1016/j.rbmo.2020.01.030. Epub 2020 Feb 14. PMID: 32295745

*Authors contributed equally

- Isolation, Transfection, and Culture of Primary Human Monocytes.

Plaisance-Bonstaff K, Faia C, Wyczechowska D, Jeansonne D, **Vittori C**, Peruzzi F.J Vis Exp. 2019 Dec 16;(154):10.3791/59967. doi: 10.3791/59967.PMID: 31885371

- Evaluation of salivary and plasma microRNA expression in patients with Sjögren's syndrome, and correlations with clinical and ultrasonographic outcomes.

Talotta R, Mercurio V, Bongiovanni S, **Vittori C**, Boccassini L, Rigamonti F, Batticciotto A, Atzeni F, Trabattoni D, Sarzi-Puttini P, Biasin M. Clin Exp Rheumatol. 2019 May-Jun;37 Suppl 118(3):70-77. Epub 2019 Jan 10.PMID: 30652678

- Endoplasmic Reticulum Associated Aminopeptidase 2 (ERAP2) Is Released in the Secretome of Activated MDMs and Reduces *in vitro* HIV-1 Infection.

Saulle I, Ibba SV, Torretta E, **Vittori C**, Fenizia C, Piancone F, Minisci D, Lori EM, Trabattoni D, Gelfi C, Clerici M, Biasin M. Front Immunol. 2019 Jul 16;10:1648. doi: 10.3389/fimmu.2019.01648. eCollection 2019.PMID: 31379846

RESEARCH INTEGRITY DECLARATION

Results reported in my PhD thesis comply with the four fundamental principles of research integrity of The European Code of Conduct for Research Integrity (ALLEA, Berlin, 2018):

- Reliability in ensuring the quality of research, reflected in the design, the methodology, the analysis and the use of resources;
- Honesty in developing, undertaking, reviewing, reporting and communicating research in a transparent, fair, full and unbiased way;
- Respect for colleagues, research participants, society, ecosystems, cultural heritage and the environment;
- Accountability for the research from idea to publication, for its management and organization, for training, supervision and mentoring, and for its wider impacts.

This thesis was evaluated by three independent Reviewers

- **Hamid Boulares,** Louisiana State University Health Sciences Center (Department of Pharmacology and Experimental Therapeutics), New Orleans, USA

- **Marilena V. Iorio**, Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy

- **Alessandra Cataldo**, Fondazione IRCCS Istituto Nazionale dei Tumori, (Research Department, Molecular Targeting Unit), Milan, Italy