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TESI DI DOTTORATO DI RICERCA

TRANSLATIONAL CONTROL IN MALIGNANT PLEURAL MESOTHELIOMA

Candidato: Stefania Oliveto

Coordinatore: Giorgio Malacarne

Tutor: Stefano Biffo

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"Se saprai ricordarmi, sarò sempre con te"

(Isabel Allende)

A te che, in silenzio,
mi hai dato la forza di andare avanti.

ACRONYMS AND ABBREVIATIONS

4E-BPs: eIF4E binding proteins

AGO: argonaute

ALL: acute lymphoblastic leukemia

BAP1: BRCA1-associated protein-1

BCL2: B-cell lymphoma 2

CDKN2A/ARF: cyclin-dependent kinase inhibitor 2A/alternative reading frame

CDS: coding sequence

CLL: chronic lymphocytic leukemia

CPEB: cytoplasmic polyadenylation element binding protein

CPI-17: C-kinase potentiated Protein phosphatase-1

CT: computed tomography

DAG: dyacilglycerol

DGCR8: DiGeorge syndrome critical region gene 8

DLBCL: diffuse large B cell lymphoma

EFL1: elongation factor-like 1

eIF6: eukaryotic initiation factor 6

ECM: extracellular matrix

EMT: epithelial-mesenchymal transition

EPP: extrapleural pneumonectomy

FACS: fluorescence-activated cell sorting

FGF: fibroblast growth factor

GCN2: general control nonderepressible-2

GEF: guanine nt exchange factor

GEO: Gene Expression Omnibus

GRB10: growth factor receptor–bound protein 10

GSK: glycogen synthase kinase-3

HCC: hepatocellular carcinoma

HGF: hepatocyte growth factor

HOXD10: homeobox D10

HRI: heme-regulated inhibitor

IRES: internal ribosome-entry site

MCL1: myeloid cell leukemia sequence 1

MPM: Malignant Pleural Mesothelioma

mTOR: mammalian target of Rapamycin

MTT: (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide

MVB: multivesicular bodies

MYPT1-PP1 δ: myosin phosphatase targeting subunit 1-protein phosphatase 1δ

NF2: neurofibromatosis type 2

NOD-SCID: non-obese diabetic-severe combined immunodeficiency

PABP: polyA binding protein

PDGF: plateled-derived growth factor

PET: positron emission tomography

PERK: protein kinase RNA-like endoplasmic reticulum kinase

PKC: protein kinase C

PKR: protein kinase RNA activated

PI3K: phosphoinositide 3-kinase

PMA: phorbol 12-myristate 13-acetate

PRAS40: proline-rich Akt substrate 40 kDa

PS: phosphatidylserine

RACK1: receptor for activated C Kinase 1

RHOC: Ras homolog gene family, member C

RISC: RNA-induced silencing complex

SBDS: Swachman Bodian Diamond Syndrome

SNP: single nucleotide polymorphism

SRL: sarcin-ricin loop

TGFβ: transforming growth factor β

Tif6: translation initiation factor 6

TOP: terminal oligopyrimidine

TRBP: TAR RNA binding protein

ULK1: Unc-51 like autophagy activating kinase 1

uORF: upstream open reading frame

UPR: unfolded protein response

UTR: untranslated region

XPO5: exportin 5

VEGF: vascular endothelial growth factor

ZEB: Zinc finger E-box-binding homeobox 1

Contents

1. ABSTRACT

Protein synthesis is a cellular process finely regulated during growth and development and its deregulation can lead to cell apoptosis or disease. Translational control is rate-limiting in cancer growth and translation initiation step is emerging as an attractive therapeutic target. eIF6 is an antiassociation factor that regulates the availability of active 80S. Its activation is driven by the RACK1/PKCβ axis, in a mTORc1 independent manner. We previously described that eIF6 haploinsufficiency causes a striking survival in the Eμ-Myc mouse lymphoma model, with lifespans extendend up to 18 months. microRNAs have been shown to regulate a wide range of biological processes destabilizing messenger RNAs and by repressing the translation of these mRNAs. Involvement of microRNAs in repression of translation suggests that they might be associated with polysomes. Here we screen for 1) eIF6 expression in human cancers and 2) association of microRNAs with polysomes in Malignant Pleural Mesothelioma (MPM). We show that MPM tumors and a MPM cell line (REN cells) contain high levels of hyperphosphorylated eIF6. Enzastaurin is a PKC beta inhibitor used in clinical trials. We prove that Enzastaurin treatment decreases eIF6 phosphorylation rate, but not eIF6 protein stability. The growth of REN, *in vivo*, and metastasis are reduced by either Enzastaurin treatment or eIF6 shRNA. Molecular analysis reveals that eIF6 manipulation affects the metabolic status of malignant mesothelioma cells. Less glycolysis and less ATP content are evident in REN cells depleted for eIF6 or treated with Enzastaurin (Anti-Warburg effect). We propose that eIF6 is necessary for Malignant Mesothelioma growth, *in vivo*, and can be targeted by kinase inhibitors. Finally we found that the MPM miRNA signature was characterized also by differential miRNAs subcellular distribution. In particular, only some miRNAs were expressed in the polysomal pool with variability in miRNAs occupancy, indicating that some miRNAs can repress translation, while others cannot. Particularly, we

evidenced that polysome-bound miRNAs present a correlation with the cell cycle pathway in REN cell, a MPM epithelioid cell line, suggesting that their polysomal localization could explain how these miRNAs may regulate cell cycle components translation.

2. INTRODUCTION

2.1 Malignant Mesothelioma

Malignant mesothelioma is a rare but highly aggressive tumour and its mortality is one of the highest associated with cancers, up to 1% (Carbone, Albelda et al. 2007). There are two major localizations of malignant mesothelioma: the pleura and peritoneum, sporadically it may also arise in the pericardium or tunica vaginalis testis (Chekol and Sun 2012).

2.1.1 Pleura: structure, functions and pathological conditions

The chest cavity surrounds the heart and lungs and comprises the ribs, associated muscles and connective tissue. This cavity is covered by the parietal pleura, which is attached to the chest wall and by a continuous parietal mesothelial cell layer. The lungs themselves are enclosed by the visceral pleura which is integral to the lung surface and which has a surface visceral mesothelial layer. The close fitting of the lungs to the inside of the chest wall means that there is a thin space between the two mesothelial layers that contains the pleural fluid and a population of pleural macrophages (Donaldson, Murphy et al. 2010). The pleural mesothelial cells derive from the mesoderm and cover the surface in an epithelial-like manner, hence the word mesothelium (Michailova and Usunoff 2006). The normal mesothelial cell layer appears glistening, smooth, and semi-transparent. Mesothelial cells may vary from a row of flattened and elongated ovoid nuclei widely separated by cytoplasm to columnar or cuboidal cells with round basal nuclei and a cuboidal luminal surface (Batra and Antony 2015). These cells have microvilli and multiple intercellular adherens junctions and focal adhesions that anchor the mesothelial cell onto the extracellular membrane via integrins (Batra and Antony 2015). The connective tissue is intersected with blood vessels, lymphatic vessels, immune cells and fibroblast-like cells. It has been shown that fibroblast-like cells may differentiate and replace

the damaged mesothelium following injury (Mutsaers, Whitaker et al. 2002; Michailova and Usunoff 2006). In the pleural cavity between the two layers there is a small amount of fluid for lubrication that reduces friction between the visceral and parietal layers during breathing. This fluid is continuously circulating and is produced by the mesothelial cells and then drained into the lymphatic circulation. The pleural mesothelium also controls several tissue functions such as regulation of fibrinolysis, trans-membrane material flux, maintenance of serosal integrity by producing growth factors (GF) and extracellular matrix (ECM) components (Agostoni and Zocchi 2007). The most common growth factors found in the pleural space are vascular endothelial growth factor (VEGF), transforming growth factor β (TGFβ), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), hepatocyte growth factor (HGF) and heparin-binding epidermal growth factor-like growth factor. Common cytokines and chemokines include interleukins IL1, IL6, IL8 and IL15, SDF-1and prostaglandins (Michailova and Usunoff 2006).

Many pathogens and carcinogens can hit pleura and cause cellular injuries; the consequent infections and inflammation led to big challenges in diagnosis and treatment (Murthy, Raja et al. 2012). Long-term inhalation of several natural and industrial fibers irritates the pleura causing chronic inflammation and scarring. Inflammation and injury result in cellular responses both from the immune system and the mesothelium itself (Cagle and Allen 2011). Cancers in the pleural cavity are often metastatic adenocarcinomas from other organs (Cagle and Allen 2011), as , stomach, breast, lung and ovary (Batra and Antony 2015), but there are also tumors arising from the pleural tissue (Cagle and Allen 2011). Solitary fibrous tumors of the pleura are mostly benign mesenchymal tumors without the standard mesothelial immunophenotype, but when malignant transformation occurs is highly difficult to distinguish it from the most common primary malignancy of pleural origin, the malignant pleural mesothelioma (Usami, Iwano et al. 2007)

2.1.2 Malignant Pleural Mesothelioma

The pleural form of malignant mesothelioma is the most common type accounting for more than 70% of all mesothelioma cases (Chen and Pace 2012). Malignant pleural mesothelioma represents a common malignant disease (Ismail-Khan, Robinson et al. 2006) arising from mesothelial cells of the pleura and showing a close relationship with previous exposure to asbestos fibers. Asbestos is the most common causative agent for MPM (Yang, Testa et al. 2008), with 80% of mesothelioma patients reporting asbestos exposure (Pass et al., 2008). However only a fraction of subjects exposed to high levels of asbestos develop MPM, suggesting that additional factors, such as genetic predisposition, may render some individuals more susceptible to asbestos carcinogenicity (Testa, Cheung et al. 2011). Recently, it has been reported that DAS, an artificial clay used as a toy and teaching material, contains a large amount of asbestos. This striking discovery changes the scenario of number of subjects exposed to asbestos fibers, showing that the presence of 30% of asbestos in DAS composition may cause exposure to a different variety of users, including teachers, artists and children. Since DAS has been used not only in Italy, it is essential that mesothelioma patients should be asked about their use of DAS, in particular if they do not report a past asbestos exposure (Silvestri, Di Benedetto et al. 2016). Wagner and colleagues were the first to describe the relationship between asbestos and MPM in 1960 when he published a series of MPM cases in asbestos mine workers from United States, Western Europe, South Africa, Japan, India, China, Australia, Indonesia and Vietnam (Porpodis, Zarogoulidis et al. 2013). This relationship is one of the clearest between a carcinogen and its associated cancer. Asbestos is a group of hydrated fibrous silicate minerals that occur in nature and is distinguished in two major groups: the white asbestos and the blue asbestos. The white asbestos include serpentines and chrysotile, instead the blue asbestos include the amphiboles, crocidolite and amosite (Yang, Testa et al. 2008). While white asbestos comprises 90% of the

world's entire asbestos consumption, the blue form is the most carcinogenic (Yang, Testa et al. 2008). Due to its remarkable heat-resistant capacities, asbestos has been called a miracle-fiber and has been extensively used in industry (Ismail-Khan, Robinson et al. 2006). The people most exposed to this carcinogen are therefore asbestos miners, plumbers, electricians, shipyard workers, construction workers and people in similar professions (Craighead 2011; Chen and Pace 2012). Since most asbestos exposure is work-related, mesothelioma is considered an occupational disease and, considering that asbestos exposure is more common in occupations with a predominantly male workforce, the incidence of MPM is higher among men than among women (5:1 ratio) (Nasreen, Khodayari et al. 2012). The risk fraction attributable to occupational asbestos exposure is lower than 40% in women and higher than 80% in men. Environmental mesotheliomas are linked either with a natural exposure in areas in the world where asbestos exists as a geological components of the soil or with neighborhood exposure in people living close to asbestos factories or mines (van Meerbeeck, Scherpereel et al. 2011). The commercial use of asbestos peaked between 1930 and 1960 (Kaufman and Pass 2008), but asbestos has been strongly restricted or banned, in several countries since then (Kao, Reid et al. 2010). Inhaled asbestos fibers accumulate in the mesothelium leading to a status of chronic inflammation and signaling activation, favoring the carcinogenic process (Yang, Rivera et al. 2010). Asbestos fibers of a certain length and width are inhaled all the way out to the alveoli. Over time, these fibers migrate out to both layers of the pleura. These fibers are like thin spears that can penetrate plasma membranes without killing the cells. Upon ingestion by macrophages and other cells the asbestos fibers become covered by iron-rich proteins and iron deposit. These ferruginous bodies may lead to increased formation of reactive oxygen species (ROS). ROS lead to cellular damage, especially DNA mutations, and have been linked to tumor progression (Wu 2006). Macrophages that try to phagocytize the asbestos fibers fail, but in the process produce more

cytokines and ROS. This is called frustrated phagocytosis and is part of a chronic inflammation in the lung and pleura (Wu 2006). Properties of the asbestos fibers and the increased ROS production during the inflammation process are thought to be some of the main biological causes of Malignant Pleural Mesothelioma. Incidence of MPM reach 100 cases/million/year in occupationally exposed populations opposed to 1 case/million/year in the general populations (Porret, Madelaine et al. 2007), although there are prominent differences in incidence of MPM reported from different countries worldwide varying from 7 per million (Japan) to 40 per million (Australia) inhabitants per year. In Europe the incidence is around 20 per million with large intercountry variation. It is logical that these differences are due to differences in historical asbestos import and consumption (Pass and Carbone 2009). All individuals who have been exposed to asbestos are considered as a population at risk. The mean latency of MPM after exposure to asbestos is around 30-40 years. The median age at diagnosis in Western countries is 69 years with an increasing fraction of patients with co-morbidities (van Meerbeeck, Scherpereel et al. 2011). Although 50 years have passed since the discovery of the first incidence of MPM, an optimal strategy has not been yet established, as the diagnosis, staging and treatment remains highly complex.

2.1.3 Pathogenesis, diagnosis and therapies of Malignant Pleural Mesothelioma

The most common symptoms of MPM are shortness of breath and pain (90%), tiredness (36%), cough (22%), sweating (22%), worry (29%) and constipation (22%) (Muers, Stephens et al. 2008). There are three major histopathological subtypes of MPM: epithelioid (60%), sarcomatous (10%) and biphasic (30%). The phenotypes are closely linked to patient survival, the median survival time has been reported to fall from 12 months for epithelioid mesotheliomas, associated with the best prognosis, to only 4 months for sarcomatoid

mesotheliomas (Pinto, Novello et al. 2013). Given that the disease is infrequent and only a few pathologists have extensive experience with mesothelioma, the diagnosis is sometimes delayed (Porpodis, Zarogoulidis et al. 2013). The clinical procedure is first imaging with chest x-ray that can show the effusion and the tumor or pleural thickening. Computed tomography (CT) can show a pleural mass and invasion. More advanced imaging techniques, such as magnetic resonance imaging and positron emission tomography (PET) can be helpful in evaluating tumour likelihood, invasiveness and staging. Staging is useful in planning surgical management however is of little importance for medical management of malignant mesothelioma (Robinson, Musk et al. 2005). Video-assisted thoracoscopy is the best biopsy technique (accuracy of 98%) and cytology, a reliable diagnostic tool for experienced cytopathologists, can offer additional tissue confirmation. Thus, several immunohistochemical panels are proposed to distinguish between sub-types of mesothelioma, secondary carcinoma and other malignant tumors metastatic to serosal membranes (Henderson, Reid et al. 2013). Calretinin is the most commonly used antibody, positive for mesothelioma with a reported specificity of 87% and sensitivity of 95%. Other useful antigens include thrombomodulin, mesothelin and cytokeratin 5 (Yaziji, Battifora et al. 2006). Molecular genetic analysis has revealed three key genetic alterations in MPM: cyclin-dependent kinase inhibitor 2A/alternative reading frame (CDKN2A/ARF), neurofibromatosis type 2 (NF2) and BRCA1-associated protein-1 (BAP1) genes. CDKN2A)/ARF gene is the most frequently inactivated tumor suppressor gene in human MPM (Musti, Kettunen et al. 2006). CDKN2A encodes $p16^{INK4a}$ whereas ARF encodes $p14^{ARF}$. $p16^{INK4a}$ controls the cell cycle via the CDK4/cyclin D retinoblastoma protein pathway, whereas $p14^{ARF}$ regulates p53 protein. The homozygous deletion of CDKN2A/ARF causes the inactivation of two major tumor suppressing pathways of retinoblastoma and p53 in the cell. It has been reported that MPM cases of epithelioid type showed ~70% of homozygous deletion of CDKN2A whereas

sarcomatoid type showed ~100% of homozygous deletion. Moreover, although p53 is the most frequently inactivated tumor suppressor genes in human malignancies, only a limited number of MPM cases show a p53 mutation. (Sekido 2013). It has been demonstrated that miR-31 is co-deleted with CDKN2A, and reintroduction of miR-31 in MPM cells shows a suppressive effect on mesothelioma cells (Ivanov, Goparaju et al. 2010). Mouse studies showed that mice deficient for *Arf*, but not $p16^{INK4a}$, were susceptible to accelerated asbestos-induced MPM (Altomare, Menges et al. 2009). Instead the inactivation of both *Arf* and $p16^{INK4a}$ may cooperate to accelerate asbestos-induced tumorigenesis *in vivo* (Altomare, Menges et al. 2011). NF2 gene encodes merlin, a tumor suppressor protein that can be inactivated not only genetically but also with other mechanisms, such as phosphorylation of CPI-17, an oncogene product that inhibits the merlin phosphatase MYPT1-PP1 δ (Thurneysen, Opitz et al. 2009). Merlin is able to regulate multiple cell signaling cascades including mTOR and the Hippo pathways, which regulate cell proliferation and growth. Furthermore, a study suggested that upregulation miR-885-3p might target NF2 (Guled, Lahti et al. 2009), however it still remains unclear how much these inactivation mechanisms are actually involved in MPM cases. To clarify the mechanism of NF2 mutation in MPM it has been developed a NF2 knockout mouse model. Asbestos exposed Nf2 (+/−) knockout mice exhibited accelerated MPM tumor formation compared with asbestos-treated wild-type (Altomare, Vaslet et al. 2005). Finally, BAP1 encodes a nuclear ubiquitin C-terminal hydrolase, a class of deubiquitinating enzymes. It has been implicated in various biologic processes including DNA damage, response and regulation of cell cycle and growth (Eletr and Wilkinson 2011). BAP1 is also involved in histone modification and its inactivation induces the impairment of global gene expression profiling. Germline mutations of BAP1 gene were detected in two families with a high incidence of MPM and some BAP1 mutations occur in the families developed other types of tumors including uveal melanoma (Testa, Cheung et al.

2011). BAP1 was also shown to be frequently mutated in uveal melanomas of the eye (Harbour, Onken et al. 2010) and germline mutation of BAP1 was identified in families carrying melanocytic tumors (Wiesner, Obenauf et al. 2011). It is an important tumor suppressor in multiple tissues and its germline mutation may have a causative role in uveal and cutaneous melanoma, mesothelioma, melanocytic BAP1-mutated atypical intradermal tumors and other cancers (Sekido 2013).

Treatment of MPM can be classified into radical procedures such as surgery and into palliative measures which consist in the removal of pleural effusions and the preventing of their recurrence in order to relieve the symptoms such as dyspnea and chest pain (Porpodis, Zarogoulidis et al. 2013). Today, once the diagnosis is made, there are no accepted or published guidelines to establish a standard surgical approach, as extrapleural pneumonectomy and pleurectomy. It is a fact that surgery is not an option for the majority of the patients due to the diffuse spreading growth of this tumor (Porpodis, Zarogoulidis et al. 2013). Moreover several factors should be taken into account concerning the choice of surgery treatment such as patient's cardiopulmonary reserve, disease stage, surgeon's experience and the extent of planned adjuvant therapy (Kaufman and Flores 2011). However, since the role of surgery as single-modality therapy in MPM remains controversial, the management of MPM consists of combinations between platinum-based chemotherapy, surgery and radiation. Similarly to surgical treatment, there is no evidence of survival benefit concerning radical radiotherapy of the hemithorax when compared to best supportive care (Porpodis, Zarogoulidis et al. 2013). Actually, multimodality strategies include EPP or pleurectomy combined with adjunctive therapies such as immunotherapy, radiotherapy and chemotherapy. However, frequently, the only choice available is palliative treatment (West and Lee 2006). In locally advanced or metastatic disease, chemotherapy improves the quality of life and induces symptomatic relief. However the tumor is generally characterized by

chemoresistance, and it has been observed that most single agents exhibit low intrinsic activity (Montanaro, Rosato et al. 2009). The current standard first line therapy for systemic treatment of advanced MPM is represented by combined chemotherapy with cisplatin and antifolate. The median response rate to chemotherapy is only 30% and its impact on overall survival (OS) is negligible. According to a recent study run by Zalcman and colleagues, a phase 3 clinical trial showed that addition of bevacizumab to pemetrexed and cisplatin improved overall survival of malignant pleural mesothelioma, with tractable toxic effects, suggesting that it should be a new acceptable treatment for MPM (Zalcman, Mazieres et al. 2015). Other approaches have been tested in MPM clinical trials, such as instillation of cytokines, antibodies, vaccines, immunogene therapy and adoptive transfer of T cell (Pinton, Manente et al. 2012).

By now, several investigations are necessary to understand, on one hand how improving the quality patients life, on the other hand trying to define new therapies.

2.2 Translation

Protein synthesis, or translation, is essential for cell growth. It is regulated by ribosomes synthesis in the nucleolus and by ribosome usage in the cytoplasm. Translation is deregulated in cancer cells (Silvera, Formenti et al. 2010; Loreni, Mancino et al. 2014). Recent works have shown that the translational machinery plays an active role in transformation and tumor malignancy, suggesting that it can be a therapeutic target (Sonenberg 2008; Ruggero 2013). Translation is the cellular process in which mRNA, previously transcribed from DNA and processed, is decoded by ribosomes to make proteins. Ribosomes are constituted by ribosomal RNA (rRNA) and structural proteins. In Eukaryotes they are formed by a small subunit (40S) and by a large subunit (60S). The joining between 40S and 60S subunits generates a

translational competent ribosome (80S). Translation can be divided in four major steps: initiation, elongation, termination and ribosomal recycling. Each of these steps is assisted by protein factors - called eukaryotic initiation factors (eIFs), eukaryotic elongation factors (eEFs) and eukaryotic termination factors (eRFs), which transiently associate with the ribosome and/or the mRNA. Translation initiation consists of the events that led up to the positioning of an elongation-competent 80S ribosome at the start codon of the mRNA. Polypeptide synthesis takes place during the elongation phases. The completed polypeptide is released after the ribosome encounters a stop codon during translation termination (Lackner and Bahler 2008). The importance and complexity of translation initiation compared to elongation and termination is further underscored by the fact that only few dedicated factors are needed for the elongation and termination processes, whereas more than 25 proteins are needed to guarantee a proper translation initiation (Holcik and Pestova 2007).

2.2.1 Cap-dependent translation

Translation initiation can be subdivided into four steps: 1) binding of the specific initiator Met-tRNA, to the small ribosomal subunit (40S), 2) binding of the formed complex to the cap structure at the 5' end of mRNA, 3) scanning of the 5'untranslated region (5'UTR) of the mRNA and start codon recognition, 4) joining of the large ribosomal subunit (60S) to generate a translation competent ribosome (80S). As physiological conditions favor the association of 40S and 60S ribosomal subunit to form complete 80S ribosomes, but only free ribosomal subunits can initiate translation, it is important that post termination ribosomes dissociate (Preiss and Hentze 2003). The eukaryotic initiation factors eIF3, eIF1, eIF1A and eIF6 are thought to promote this dissociation in eukaryotes (Holcik and Pestova 2007). As reported in Figure 1, the first step in 43S preinitiation complex formation is the assembly of a ternary complex, consisting of eIF2, methionyl tRNA (met-tRNA) and GTP. Its assembly is

stimulated by the guanine nt exchange factor (GEF) eIF2B. GTP is hydrolyzed after recognition of the AUG start codon producing eIF2 bound to GTP (Sonenberg and Hinnebusch 2007). eIF2B promotes GDP-GTP exchange to regenerate active eIF2. Binding of the ternary complex to the 40S ribosomal subunit is supported by eIF1, eIF1A and eIF3 in mammalian cells (Preiss and Hentze 2003; Holcik and Pestova 2007). The 43S preinitiation complex is ready to bind to the 5' end of the mRNA.

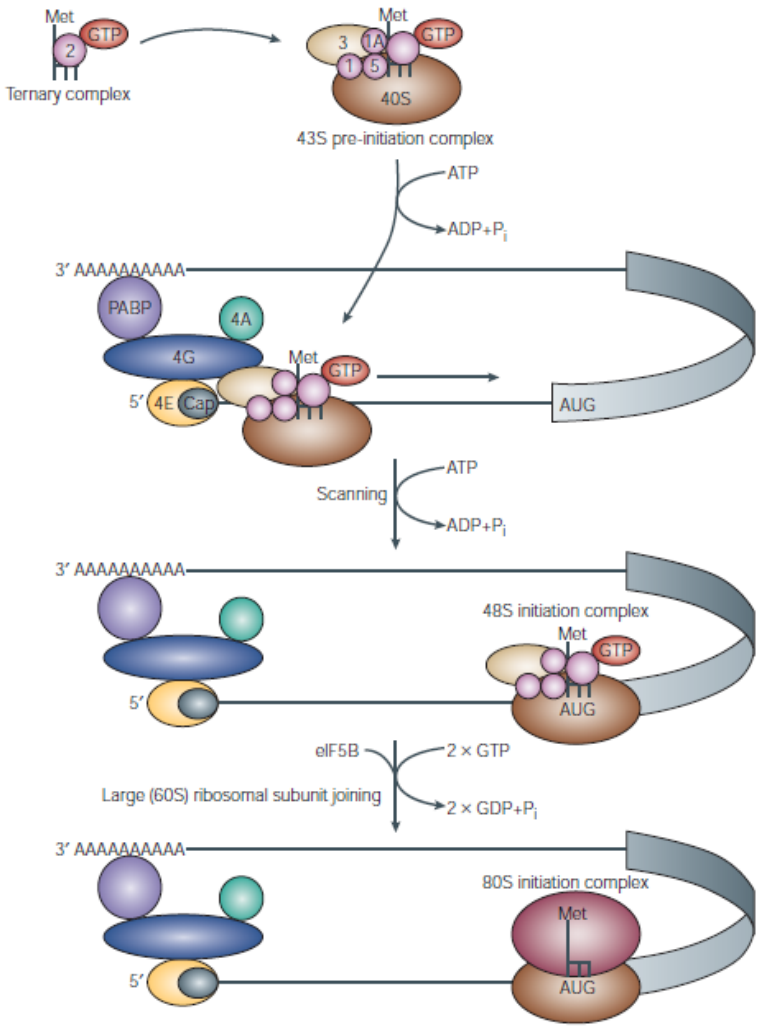


Figure 1. Cap-mediated translation initiation (Gebauer and Hentze 2004)

The eIF4F complex bounds to the 5'm^7GpppN cap structure and promotes the recruitment of the 43S preinitiation complex to the 5'end of the mRNA generating a complex called 48S.

eIF4F is composed of the cap-binding protein eIF4E, the scaffold protein eIF4G and the ATP-dependent helicase eIF4A that, assisted by eIF4B, unwinds secondary structures in the 5'UTR of the mRNA. The binding of the preinitiation complex to the mRNA involves the cooperative activities of eIF4F, eIF3, eIF4B and PABP. PABP was identified as a protein that associated with polyA tail at the 3'UTR of the mRNA. The PABP-eIF4G interaction is thought to promote a circularization of the mRNA molecule forming a closed loop. This circularization provides a possible framework by which 3'UTR-binding proteins can regulate translation initiation (Gebauer and Hentze 2004). Once assembled near the 5' end of the mRNA, the 48S complex scan along the mRNA to find the AUG starts codon. In eukaryotes, recognition of an AUG as a start codon critically depends on its surrounding sequence. The scanning process requires ATP and a study using a reconstitute mammalian translation initiation system suggests that this requirement reflects the necessity of unwinding secondary structures in the 5' UTR by the eIF4A and eIF4B RNA helicases (Pestova and Kolupaeva 2002). Furthermore eIF1 and eIF1A have been shown to play an important role in the scanning process as well as in the recognition of the corresponding initiation codon. Several events take place in order for the 60S subunit to join the 48S complex and form the 80S ribosome. Joining of the 60S ribosomal subunit to the 48S complex requires hydrolysis of two GTP molecules. First, eIF5 triggers GTP hydrolysis by eIF2, which leaves the complex thereafter in the GDP bound state together with eIF5 (Unbehaun, Borukhov et al. 2004). eIF1 and eIF3 remain associated with the complex until eIF5B, a second GTPase, binds to the 43S preinitiation complex and allows the 60S subunit to join. Finally, GTP hydrolysis in eIF5B, triggered by 60S subunit joining, results in the dissociation of eIF5B in the GDP bound form and the formation of an elongation competent 80S ribosome (Pestova, Lomakin et al. 2000).

2.2.2 The translational regulation

Protein synthesis, in comparison to the other biosynthetic processes, is the most energetically expensive process going on within the cells; therefore translation has to be highly regulated. Translational regulation is involved in the response to cellular stress (Holcik and Sonenberg 2005), in the misregulation of gene expression during cancer, in apoptosis and in development (Hinton, Coldwell et al. 2007). The need for translational control is also important for systems where transcriptional control is not possible, such as RNA viruses and reticulocytes, where the nucleus is absent. These systems provided us much of our understanding of translational regulation, e.g. reticulocytes are the most efficient cell-free protein synthesis *in vitro*. Translational regulation can be divided into global regulation of translation and mRNA specific regulation (Gebauer and Hentze 2004): global regulation affects the translation efficiency of most mRNAs through a general tuning of translation, while mRNA specific regulation affects the translation of specific mRNAs. Global regulation of translation is generally mediated through modifications of translation initiation factors that transform the information from external compartments to the cell. Initiation phase of translation is the limiting step for a given mRNA, and initiation factors act as regulators, downstream of signaling events (Sonenberg and Hinnebusch 2009). Certain mRNA can be specifically regulated, usually by proteins that bind to *cis*-regulatory sequences present in 5' and/or 3' UTRs of a given mRNA. The ribosome itself can be targeted to exhert translational regulation, and several of its protein constituents can stand posttranslational modifications (Lackner and Bahler 2008).

Regulation of ternary complex formation. One of the best studied examples of the translational downregulation is the control of the active ternary complex formation. Binding of Met-$tRNA_i^{Met}$ to the 40S subunit through the ternary complex is an essential step in translational initiation. After the exposure to stress, the α-subunit of eIF2 (eIF2α) is

phosphorylated and inhibits the exchange GDP-GTP by eIF2B, and the formation of active ternary complexes is highly reduced, downregulating global translation (Holcik and Sonenberg 2005; Oyadomari, Harding et al. 2008) (Figure 2).

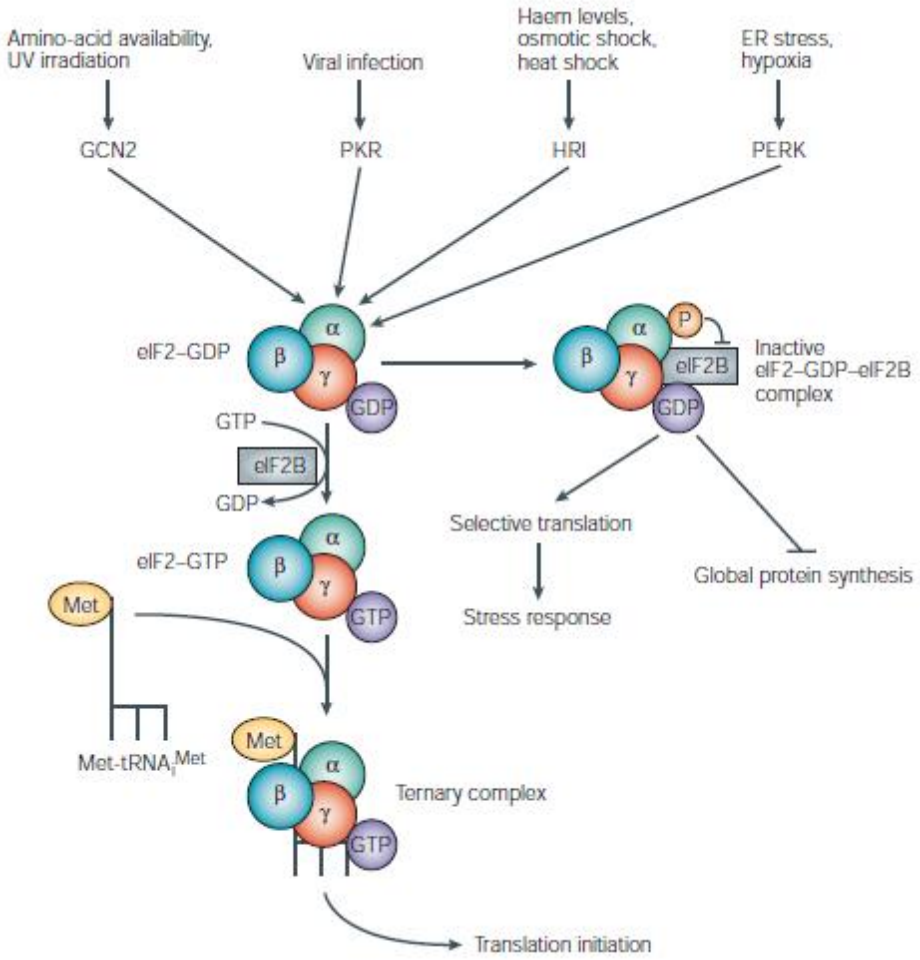


Figure 2. Integration of stress responses by the phosphorylation of eIF2α (Holcik and Sonenberg 2005).

Induction of p-eIF2α serves as an important regulator, under which general protein synthesis and cell proliferation are blocked, thus allowing cells to recuperate from stress or be eliminated if the damage is beyond repair (Koromilas 2015). Phosphorylation of eIF2α is mediated by PKR, an interferon (IFN)-inducible protein with pro-inflammatory and antiviral properties, which is activated by binding to double-stranded (ds) RNA; a family of kinases consisting of the heme-regulated inhibitor (HRI), which is activated by heme deficiency; the

endoplasmic reticulum (ER)-resident protein kinase PERK/PEK, which is activated by the accumulation of misfolded proteins in the ER; and finally the general control non-derepressible-2 (GCN2), which is activated by accumulation of uncharged tRNAs caused by amino-acid or nutrient deprivation (Chen 2007; Koromilas 2015). Phosphorylation of the α-subunit of eIF2 inhibits the GDT-GTP exchange reaction mediated by eIF2B due to a reduced dissociation of eIF2 from eIF2B. As a result, less eIF2B is available to promote GDP-GTP exchange and global translation is inhibited (Holcik and Sonenberg 2005).

Regulation of cap-dependent translation and mTOR pathway. Most of the cap-dependent translation is regulated by the pathway of the mammalian Target of Rapamycin (mTOR). mTOR is an evolutionarily conserved Ser/Thr kinase, which regulates proliferation and growth in response to cellular energy status, growth factors, hormones, and nutrient availability (Zoncu, Efeyan et al. 2011). mTOR exists in two functionally and structurally distinct protein complexes: mTOR complex 1 and 2 (mTORC1 and mTORC2). These two complexes regulate disparate cellular functions by phosphorylating distinct sets of substrates. Several substrates of mTORC1 have been identified including the eIF4E-binding proteins (4E-BPs), S6 kinases 1 and 2 (S6Ks), PRAS40, Ser/Thr kinase Ulk1 (also known as hATG1), and growth factor receptor-bound protein 10 (Grb10) (reviewed by (Caron, Ghosh et al. 2010). The function, the upstream regulators and the associated substrates of mTORC2 are less understood (Oh and Jacinto 2011). mTORC2 phosphorylates AGC kinase family members and controls cytoskeletal organization and cell survival (Guertin and Sabatini 2007; Garcia-Martinez and Alessi 2008). mTORC2 also associates with the ribosome (Zinzalla, Stracka et al. 2011) where it phosphorylates residues in nascent polypeptide chains that are important for optimal protein folding (Oh, Wu et al. 2010). mTORC1 plays a central role in the regulation of proliferation and cell growth (Ma and Blenis 2009), cellular processes that are directly proportional to translational activity. Hormones, growth factors and glucose

stimulate mTORC1, up-regulate translation and stimulate cellular growth and proliferation. Conversely, under conditions in which energy production, oxygen supply and nutrients are inadequate, mTORC1 signaling is down-regulated, resulting in inhibition of translation, reduction in cellular growth proliferation, and induction of autophagy. Rapamycin is a naturally occurring allosteric inhibitor of mTORC1 (Guertin and Sabatini 2007). mTOR inactivation, by treatment with rapamycin, mimics deprivation of nutrients, both in mammals and in yeast. Main downstream targets of mTOR kinase are eIF4E-binding proteins (4E-BPs), rpS6 kinases (S6K) and eEF2 kinase (Hay and Sonenberg 2004). 4E-BPs and S6Ks are the most extensively studied and best-understood downstream effectors of mTORC1, which have been implicated in the regulation of translation (Figure 3).

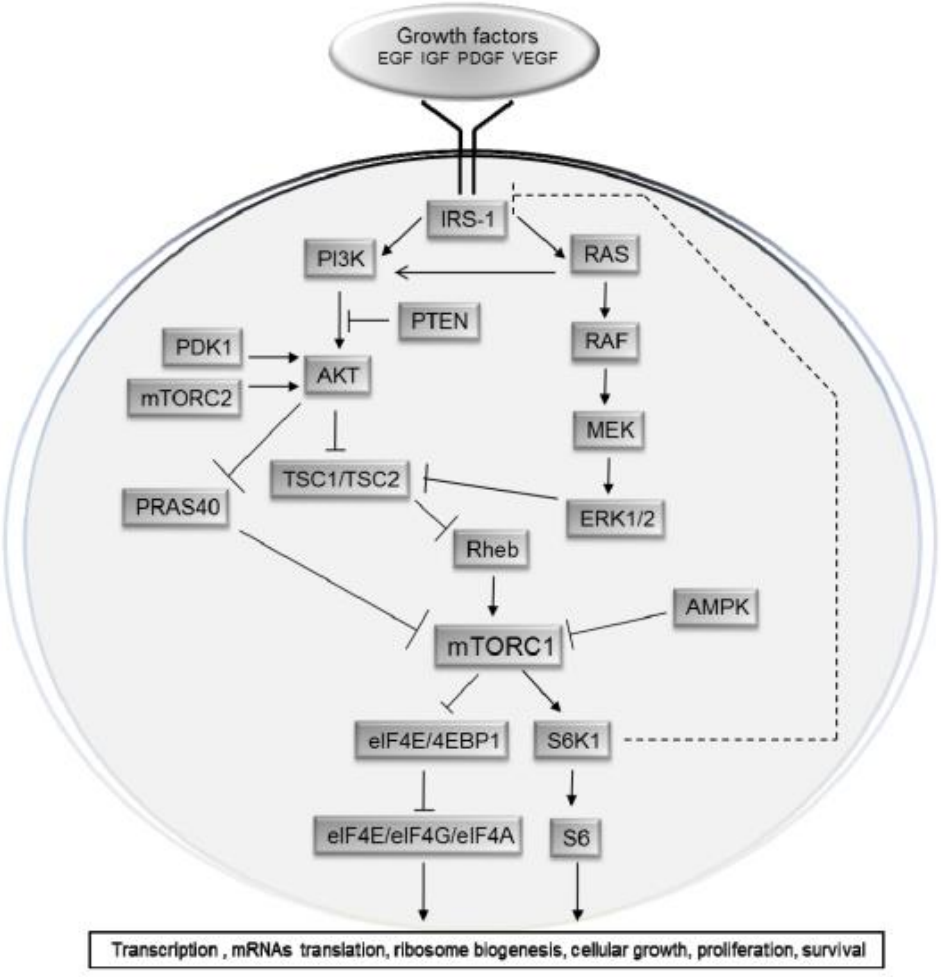


Figure 3. mTOR signaling pathway (Populo, Lopes et al. 2012)

The first step of cap-dependent translation initiation is the assembly of the eIF4F complex on the mRNA cap structure (Topisirovic and Sonenberg 2011). eIF4E binds to the 5' cap structure of eukaryotic mRNAs and provides the first contact between the translational machinery and the mRNA in de novo translation initiation. eIF4E interacts with several types of protein binding partners. It binds the scaffold protein eIF4G, which in turn, interacts with the RNA helicase eIF4A, the multisubunit eIF3 which provides the association to the 40S subunit, and the poly (A)-binding protein (PABP). The eIF4E/4G/4A complex is referred as the eIF4F complex which is thought to be of key importance in mediating normal, cap-dependent translation initiation. A second group of eIF4E binding proteins comprises low molecular mass proteins that block its interaction with eIF4G. In mammals three eIF4E binding proteins are known, 4E-BP1/2/3. 4E-BPs interferes with the assembly of the eIF4F complex by competing with eIF4G for binding to eIF4E. On activation, mTORC1 phosphorylates residues corresponding to Thr37 and Thr46 on human 4E-BP1, which act as priming sites for the phosphorylation of Ser65 and Thr70. Phosphorylation of 4E-BPs on these four residues, leads to their dissociation from eIF4E, allowing the assembly of the eIF4F complex. In addition to 4E-BPs, TOR regulates translation by activating the S6Ks (Ma and Blenis 2009). Although Drosophila expresses a single S6K protein (dS6K), mammals express two variants of S6K (S6K1 and S6K2). rpS6 was the first identified S6K substrate. Five phosphorylation sites (Ser235, Ser236, Ser240, Ser244, and Ser247 in humans and rodents) are clustered in the carboxyl terminus of rpS6 (Meyuhas 2008). It has been demonstrated that, using S6K1/S6K2 double knockout mice, both S6K1 and S6K2 isoforms contribute to the regulation of basal and inducible rpS6 phosphorylation at S235/236 and S240/244 sites (Pende, Um et al. 2004; Chauvin, Koka et al. 2014). Notably, S6K2 knockout mice display a reduction of rpS6 phosphorylation only at S235/236 while S6K1-deficient mice show no alterations (Bhattacharya, Kaphzan et al. 2012). Several studies have demonstrated that S6K1

regulates translation initiation through the phosphorylation of the cap binding complex component eIF4B at S422 (Raught, Peiretti et al. 2004). Finally, for many years it has been believed that the phosphorylation of rpS6 had an effect on the translation of a specific subset of mRNAs bearing a 5′ terminal oligopyrimidine tract (TOP). Actually, this model has been changed by studies showing that both double mutant S6K1/2 MEFs and rpS6 knockin mouse exhibit normal TOP translation (Ruvinsky, Sharon et al. 2005).

Regulation of cap independent translation (IRES)

An important mode of translational regulation during stress is the selective recruitment of mRNA through internal ribosome-entry site (IRES). The IRES directly recruits ribosomes, bypassing the requirements for the mRNA 5' cap structure and eIF4E (Johannes and Sarnow 1998; Hellen and Sarnow 2001). Expression of genes bearing IRES elements in their mRNAs is controlled by multiple molecular mechanisms, with IRES-mediated translation favored when cap-dependent translation is compromised (Komar and Hatzoglou 2011). The translation initiation of several IRES-containing mRNAs occurs predominantly during stress and apoptosis (Holcik and Sonenberg 2005). By this alternative mechanism, even if cap-dependent translation is reduced, some cellular mRNAs can be efficiently translated (Figure 4). Kozak points out that evidence cited in support of the internal initiation hypothesis is often flawed, in fact, when putative IRESs are examined more carefully, they often turn out to harbor cryptic promoters or splice sites (Kozak 2005). Nevertheless it is clear that IRES-mediated translation is used relatively frequently under both normal physiological and pathological conditions.

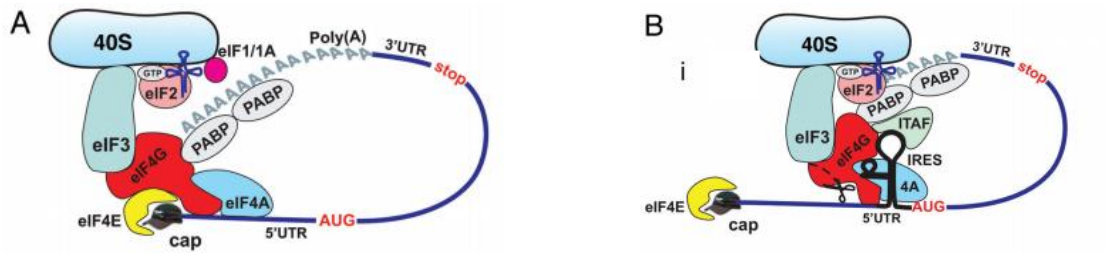


Figure 4. Cap dependent (a) versus IRES dependent (b) translation initiation (Komar and Hatzoglou 2011)

Other mechanisms of mRNA translational regulation

Of great importance in the translational mechanism are some regulatory sequences represented by upstream open reading frame (uORF), which interferes with the expression of the CDS. uORFs, particularly common in transcripts for oncogenes and growth factors, are present in 10% of mRNAs (Sachs and Geballe 2006). uORFs interpose a barrier to prevent ribosomal access to initiation codon, preventing so the translation of the downstream ORF and affecting gene expression and mRNA stability. Even if the ribosome recognizes an initiation codon and translates the uORF, it might reinitiate at a downstream AUG codon thereby overcoming the barrier, typically in conditions of reduced translation driven by impaired eIF2-GTP-Met-tRNA ternary complex formation during specific cellular stress, as amino acid deprivation and unfolded protein response (UPR) (Baird and Wek 2012). For instance, when misfolded proteins accumulate in ER, eIF2α may be phosphorylated by PERK causing a reduction in global translation and favoring reinitiation at downstream ORF (Sonenberg and Hinnebusch 2009).

Several lines of evidence indicate that mRNAs exist in an actively translated and associated with polysomes form and in a translationally repressed and associated with P-bodies state. The idea that the recruitment of mRNAs to P-bodies interferes with translation initiation is supported by the finding that inhibition of translation elongation causes P-bodies to disappear, while inhibition of translation initiation increases the size and number of P-bodies. In

mammalian cells, several proteins with established roles in translational repression localize to P-bodies: eIF4E inhibitory protein eIF4E-T, RCK/p54 and CPEB (reviewed by (Decker and Parker 2012). The exact mechanism of how mRNAs shuttle into P-bodies and become translationally repressed is yet unknown.

2.2.3 Novel concepts in translational control: regulation by microRNA

miRNA biology is associated, in the last years, to translation mechanism. microRNAs are short non coding RNA of 21-26 nt emerged as key posttranscriptional regulators of gene expression in metazoan animals, plants, and protozoa. Current studies estimate that human genome encodes hundreds of different miRNAs and that they could regulate almost the 60% of all genes (Friedman, Farh et al. 2009). In animals, miRNAs form imperfect hybrids with sequences in the mRNA 3'-untranslated region (3' UTR), with the miRNA 5'-proximal "seed" region (positions 2–8) providing most of the pairing specificity (reviewed in (Bushati and Cohen 2007; Filipowicz, Bhattacharyya et al. 2008). Until very recently, it appeared that plant miRNAs generally base-pair to mRNAs with perfect complementarity and trigger endonucleolytic mRNA cleavage by the RNA interference (RNAi) mechanism. Generally, the binding partially complementary to target mRNAs, leads to mRNA degradation and translation inhibition (Iorio and Croce 2012) recruiting the decapping and deadenylating machinery. Recently, however, some reports identify miRNAs as activator of mRNA translation during cell quiescence (Vasudevan, Tong et al. 2008; Niepmann 2009), as reported for miRNA 369-3: the direct base pairing between miRNA 369-3 and its target is required for translational upregulation after serum starvation (Vasudevan, Tong et al. 2008). Most studies affirm that miRNA mechanism acts at initiation of translation and can work as tumor suppressor or accelerating factor. The example of tumor suppressor is represented by miR-21

whose targets are PI3K and the apoptotic pathways (Loreni, Mancino et al. 2014). In addition to classical tumor suppressor and oncogene functions, miRNAs can be also implicated in cell migration and metastasis, as the highly expressed miR10-b in metastatic breast cancer that positively regulates cell migration and invasion (Ma, Teruya-Feldstein et al. 2007). miRNAs associate with Ago proteins to form RNA-induced silencing complexes (RISCs), through which they can modulate gene expression components. Components of miRISC and repressed mRNAs are enriched in processing bodies, which are cytoplasmic structures involved in the storage or degradation of translationally repressed mRNAs. Some P-bodies components are important for effective repression of protein synthesis by miRNAs. Recently, multivesicular bodies (MVBs) and endosomes were also identified as cellular organelles contributing to miRNA function or miRISC turnover (reviewed in (Fabian, Sonenberg et al. 2010). Regulation of gene expression via small RNAs and sequestration to P bodies, and interplay between miRNA translation inhibition and mRNA decay add further complexity to cellular posttranscriptional control. As 60% of genes are potential miRNA targets (Lewis, Burge et al. 2005), miRNAs could exhert their function in a dual way: a mRNA could be regulated by several miRNAs and a miRNA could target several mRNAs. Moreover, both their expression and action is cell and tissue specific, as microRNA can target different mRNAs in different cell and tissues. This implicates that the action of microRNA is not conserved, but depends from its environment. Elucidation of the molecular events behind these mechanisms is needed.

2.3 Eukaryotic Initiation Factor 6 (eIF6)

Eukaryotic Initiation Factor 6 is an evolutionary conserved protein. The primary sequence shows two main features: 1) the protein is 245 aa long and it is 77% identical between humans and yeast (Biffo, Sanvito et al. 1997), 2) eIF6 primary sequence is evolutionarily

unique, with no conserved motifs. eIF6 structure has been solved, according to X-ray data: it is a rigid protein organized with a cyclic fold, called pentein or star-like structure, formed by 5 stretches of α/β subdomains arrayed about a five-fold axis of psuedosymmetry (Groft, Beckmann et al. 2000). The structure encloses a cavity that contains sixteen well-ordered water molecules, with limited degree of motility (Figure 5).

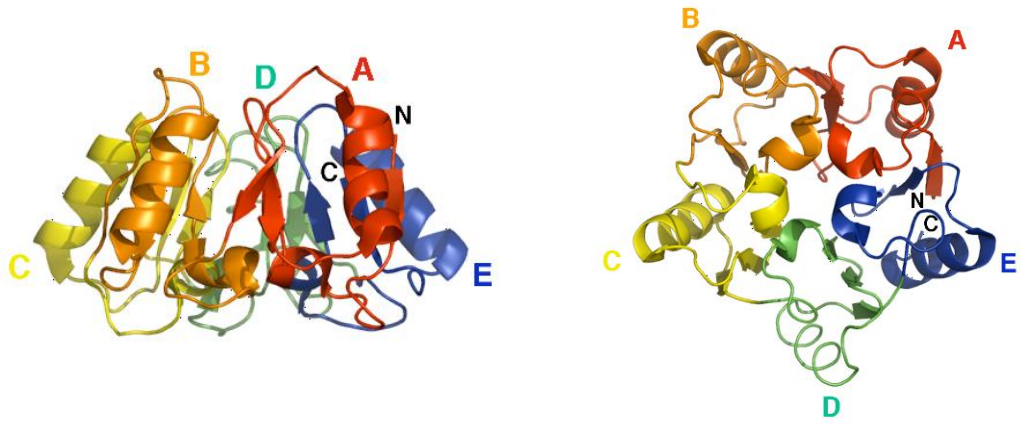


Figure 5. Structure of eIF6. The protein has a unique star-like structure known as pentein, which is formed by five quasi identical subdomains (A–E) (Groft, Beckmann et al. 2000).

Structural data have identified that eIF6 binds to intersubunit space of the large ribosomal subunit (Klinge, Voigts-Hoffmann et al. 2011). It is able to interact with the hydrophobic C-terminal chain of the ribosomal protein L23 (rpL23). The sarcin-ricine loop and rpL24 also contribute to the interaction of eIF6 with the 60S subunit. Since the steric hindrance, it prevents binding between 60S and 40S.

The semiconserved C-terminal tail seems a candidate region for eIF6 regulation and transport due to its flexibility. The C-terminus of eIF6 is characterized by the presence of many phosphorylation sites that are highly conserved in mammalian cells (Figure 6).

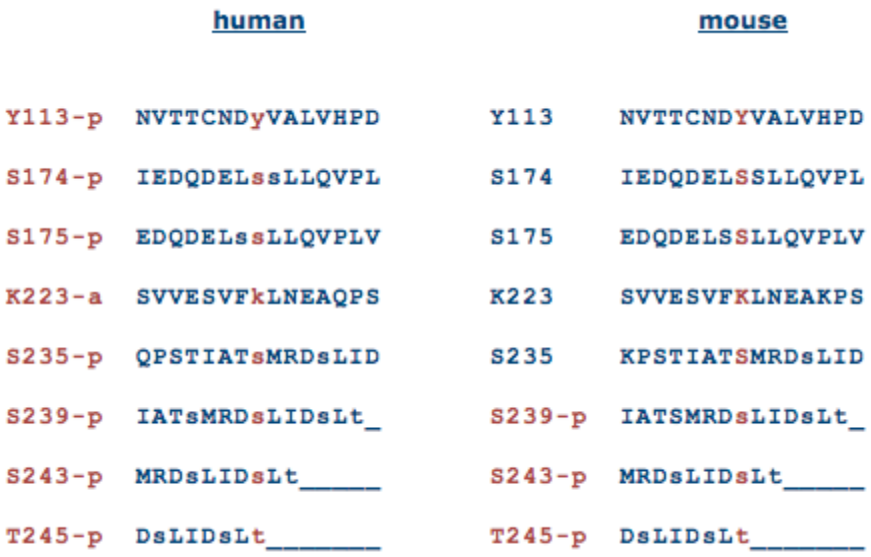


Figure 6. eIF6 phosphorylation sites (http://www.phosphosite.org**)**

The phosphorylation sites Ser174 and Ser175 are located at the accessible surface of eIF6, not involved in the interaction with the 60S subunit. Also the flexible C-terminal sequence that contains Ser235 phosphorylation site is located at the outer surface of eIF6 (Gartmann, Blau et al. 2010). Mutation of the yeast homologues of eIF6, called Tif6p, at Serine-174 to Alanine reduced phosphorylation drastically and caused loss of cell viability and growth. When both Ser-174 and Ser-175 were mutated to alanine, phosphorylation of Tif6p was abolished. Furthermore, while wild-type Tif6p was distributed both in nuclei and the cytoplasm of yeast cells, the mutant Tif6p, containing Ser174Ala and Ser175Ala, became a constitutively nuclear protein (Basu, Si et al. 2003). Several studies have shown that eIF6 has a dual function: it is necessary for the maturation of 60S ribosomal subunit in the nucleus and possesses a ribosomal antiassociation activity (Miluzio, Beugnet et al. 2009), and it is involved in translation in the cytoplasm (reviewed by Biffo et al., A. Parsyan ed., 2014).

Data collected through genomic sequencing projects reveal that evidences for eIF6 gene duplication do not exist, suggesting a strong evolutionary pressure for control of the protein

concentration. Human eIF6 gene is constitutively expressed *in vitro*, but modulated *in vivo*, since protein level *in vivo* are variable among different organs. Studies on levels of eIF6 in several metazoan tissues show that the protein is expressed at low level in muscle and high in brain. Furthermore, eIF6 is particularly expressed in stem cells or in cycling cells, but undetectable in some postmitotic cells (Donadini, Giodini et al. 2001).

2.3.1 eIF6 on ribosome biogenesis and antiassociation activity

Ribosome biogenesis is a very important process that occurs in the nucleolus and leads to the production of large and small mature ribosomal subunits and to their export to the cytoplasm. The small and large subunit are separately processed and exported, although they derived from the same rRNA precursor (47S in mammals). The large subunit matures through intermediate steps known as 90S-66S-pre60S-60S. Several lines of direct and indirect evidences support the necessity of eIF6 in ribosome biogenesis. Deletion of the yeast homolog Tif6 leads to a loss of the 60S ribosomal subunit that can be rescued by the ectopic expression of human eIF6 (Sanvito, Piatti et al. 1999; Si and Maitra 1999; Brina, Grosso et al. 2011). Moreover biochemical evidences converge to the role of eIF6 in ribosome biogenesis. The protein is identified in molecular complexes from 66S to mature 60S. In agreement with this finding, a pool of eIF6 is localized in the nucleolus of both yeast and mammalian cells (Sanvito, Vivoli et al. 2000). The molecular mechanism by which eIF6 regulates 60S biogenesis is not completely clear. rRNA pulse-chain has shown that yeast cells depleted of eIF6 have defective pre-RNA processing. This causes the reduced formation of mature 25S and 5.8S rRNA relative to 18S rRNA, which may account for the selective deficit of 60S ribosomal subunit. Thus, eIF6 acts in biogenesis of 60S subunit, rather than in its stabilization

(Basu, Si et al. 2001; Woolford and Baserga 2013). However, our knowledge of the eIF6 function in the biogenesis of 60S subunit is not clear and requires further studies.

Moreover, eIF6 has a relevant biochemical activity, preventing binding of 40S and 60S in the absence of mRNA and thus avoiding an accumulation of inactive 80S subunit. In this way eIF6 is able to keep the small and large subunit available for initiation of translation (reviewed by Biffo et al., A. Parsyan ed. 2014). eIF6 was initially identified on the basis of its antiassociation activity in calf liver (Valenzuela, Chaudhuri et al. 1982) and wheat germ (Russell and Spremulli 1979), but it cannot dissociate preformed 80S complexes. It has been published the crystal structure of the 60S ribosomal subunit in complex with eIF6 in *Tetraymena termophyla* (Klinge, Voigts-Hoffmann et al. 2011). The structure reveals interactions between eukaryotic specific ribosomal proteins in the stabilization of the active site. The site of the eIF6 binding to 60S was mapped to the 40S-60S interface, close to sarcin-ricin loop (SRL) and ribosomal protein rpL23 e rpL24, where it would prevent binding of the 40S subunit. It is rationale to speculate that the antiassociation activity of eIF6, as observed *in vitro*, is relevant for translational control *in vivo* (Biffo et al., A. Parsyan ed. 2014). Furthermore, although eIF6 is dispensable for translation in vitro, low concentrations of eIF6 have a slight stimulatory effect on translation, whereas higher concentrations inhibit it (Russell and Spremulli 1979).

2.3.2 eIF6 and translation

Ceci et al showed that eIF6 is able to repress translation after binding to 60S ribosomal subunit and similar observations were made with eIF6 bound to 50S (Benelli, Marzi et al. 2009). Furthermore, mammalian and yeast eIF6 have common properties, such as the mainly cytoplasmic localization, which correlates with a role of eIF6 in the control of translation.

Since the binding of eIF6 to the large ribosomal subunits is able to affect translational initiation, it is possible to assume that there is a mechanism that favors its release leading to dissociation of eIF6 from the 60S ribosomal subunit. Two models for eIF6 release have been proposed: 1) 60S, bound to eIF6, is translocated from the nucleus to the cytoplasm. Here, the interaction between the Swachman-Bodian-Diamond Syndrome protein (SBDS) and the GTPase Efl1p with the 60S subunit leads to an allosteric change of 60S mediating the release of eIF6 (Wong, Traynor et al. 2011). This mechanism is relevant during the maturation step of the 60S subunit (Bussiere, Hashem et al. 2012). 2) Release of eIF6 is mediated by RACK/PKC complex. RACK1 acts as a scaffold receptor protein for active PKC and binds to the small ribosomal subunit (Ceci, Gaviraghi et al. 2003; Volta, Beugnet et al. 2013). Activated PKC translocates from endomembrane to the small subunit, comes in vicinity with eIF6 bound to 60S subunit and catalyzes the phosphorylation of eIF6 on Ser235 and its subsequent release (Brina, Grosso et al. 2011) (Figure 7).

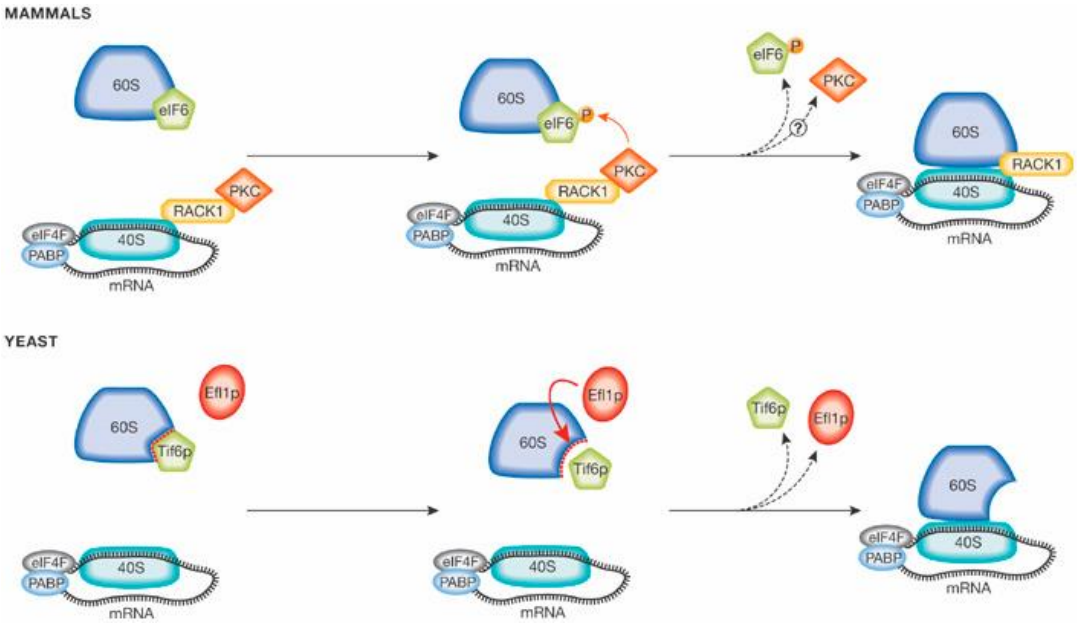


Figure 7. The two models of eIF6 release that regulate the interaction of the two ribosomal subunits.

Mouse model of eIF6 haploinsufficiency evidence that eIF6 is critical for translation initiation (Gandin, Miluzio et al. 2008). eIF6-null mice are embryonic lethal in mammals, but heterozygous mice, presenting a 50% of the eIF6 protein level, are viable. This reduction of the protein affects the cytoplasmic pool, and not the nuclear levels, leading to a proper biogenesis of the 60S ribosomal subunit. This confirm the notion that the function of the protein is cytoplasmic, translation related, and not nuclear, ribosomal biogenesis-related. The analysis of polysomal profiles of eIF6 heterozygous mice shows an increase in the 80S peak and a decrease in polysomes, confirming the role of eIF6 in initiation of translation. All mouse tissues of heterozygous mice have levels of the eIF6 protein reduced of 50% compared to the wild-type controls. The liver of eIF6 heterozygous mice shows an accumulation of inactive 80S complexes, and hepatocytes have normal level of translation but are not able to regulate its response to insulin. Thus full levels of eIF6 are necessary to perform the translation program induced by insulin of the cell, *in vivo*. The expression of eIF6 is rate limiting for tissue growth, as mice haploinsufficient for eIF6 have smaller livers than wild-type and reduced white fat mass. The deficit in insulin-stimulated translation occurring in eIF6+/– cells correlates with a high insulin sensitivity in tissues. Hepatocytes, fibroblasts and adipocytes from heterozygous eIF6 cells show a delayed G1-to-S phase progression but are normal in size, and have normal apoptosis and senescence (Gandin, Miluzio et al. 2008).

2.3.3 eIF6 and cancer

The research into the role in of eIF6 in cancer is still in the twilight. In tumors oncogenic pathways that promote tumor development and cellular transformation are hyperactivated and deregulation in translational control are endpoint of these these pathways (Silvera, Formenti et al. 2010). eIF6 is overexpressed in several cancer types, such as head and neck cancer (Rosso

et al., 2004), lung metastasis (Martin, Sanz et al. 2008), acute promyelocitic leukemia (Harris, Ozpolat et al. 2004) and malignant mesothelioma (Biffo, Sanvito et al. 1997). The mechanism that explains the eIF6 overexpression in cancer is unclear. eIF6 overexpression may reflect an increased demand for the protein by proliferating cancer cells, and not its role in etiology and cancer development. Cells with halved level of eIF6 protein show a reduction in MYC or HRAS-mediated oncogenic transformation (Gandin, Miluzio et al. 2008). MYC-induced lymphomagenesis is reduced in murine lymphoma with reduced eIF6 levels resulting in prolonged tumor free survival in the absence of negative side effects (Miluzio, Beugnet et al. 2011). Mutation of eIF6 in the PKC consensus site Ser235 reduces the rate of transformation, suggesting its role in tumorigenesis (Miluzio, Beugnet et al. 2011). The most relevant information related to the regulation of the eIF6 activity by signaling are: 1) eif6 is hyperphosphorylated in cancer cells, in the C-terminus at Ser235, Ser239 and Thr243 (Ceci, Gaviraghi et al. 2003; Dephoure, Zhou et al. 2008); 2) mutation of Ser235 to Ala reduces translation and tumorigenesis (Gandin, Miluzio et al. 2008; Miluzio, Beugnet et al. 2011); 3) eIF6 activity is independent from mTORC1 activation but essential for growth factor and insulin activation (Gandin, Miluzio et al. 2008); 4) eIF6 interacts with RACK1 (Ceci, Gaviraghi et al. 2003; Guo, Wang et al. 2011), which is able to affect translation (Volta, Beugnet et al. 2013). The PKC isoform that binds RACK1 is PKCβ, and only the PKCβII isoform show a higher affinity for RACK1 receptor (Stebbins and Mochly-Rosen 2001). Moreover PKCβ inhibition reduces translation not affecting mTORC1 targets (Grosso, Volta et al. 2008), suggesting a role for the PKC axis in the regulation of translation. However it is possible that eIF6 activity is affected by mTORC2, upstream of several PKCs (Hagiwara, Cornu et al. 2012). These data suggest a role of eIf6 as a modulator of tumorigenesis and tumor growth.

2.4 MicroRNAs

microRNAs are endogenous, small non-coding single-stranded RNAs of ~22 nucleotides in length, found in both plants and animals. They act as negative regulators of gene expression in several cellular processes and, in mammals, they are able to control the activity of more than 60% of all protein-coding genes (Friedman, Farh et al. 2009). miRNAs regulate protein synthesis by base-pairing to target mRNAs. In animals, miRNAs form imperfect hybrids with sequences in the 3'UTR of mRNA, with the miRNA 5'-proximal "seed" region (positions 2–8) providing most of the pairing specificity. In contrast plant miRNAs base-pair to mRNAs with perfect complementarity and trigger mRNA cleavage by the RNA interference (RNAi) mechanism (Filipowicz, Bhattacharyya et al. 2008; Bartel 2009). This is the typical strategy used by miRNAs to reduce the translation and stability of mRNAs, including those of genes that mediate processes in tumorigenesis, such as cell cycle regulation, inflammation, stress response, differentiation, apoptosis and invasion (Iorio and Croce 2012).

miRNAs were originally shown to be important in timing of larval development in *C. Elegans* , leading to the identification of the best known miRNAs lin-4 and let-7. Initial understanding of miRNA-mRNA target recognition came from observations of sequence complementarity of the lin-4 RNA to multiple conserved sites within the lin-14 3'UTR; molecular genetic analysis showed that this complementarity was required for the repression of lin-14 by lin-4 (Lee, Feinbaum et al. 1993; Wightman, Ha et al. 1993; Reinhart, Slack et al. 2000).

microRNA biogenesis is divided into two main processing steps that take place in the nucleus and in the cytoplasm: primary microRNAs are first processed into the nucleus by RNAse III Drosha, associated to the double stranded RNA-binding protein DGCR8 (DiGeorge syndrome critical region gene 8; Pasha in flies) known as the microprocessor complex, that generates a ~70 nucleotides precursor miRNA products, which fold into stable secondary stem-loop

structures. The latter are recognized by the Ran-GTP-dependent transporter Exportin 5, which mediates the translocation to the cytoplasm. Here Dicer, a RNAse III enzyme, associated to TRBP (TAR RNA-binding protein) and Argonaute proteins (AGO1-4), cleave the miRNA precursor hairpin and generate a transitory miRNA/miRNA* duplex (also named respectively miR-3p/miR-5p), which includes the mature miRNA guide, selected by thermodynamic properties, and the complementary passenger strand, usually subjected to degradation. This duplex is then loaded into the miRNA-associated RNA induced silencing complex (RISC or miRISC), including the mature single-stranded miRNA molecule and AGO proteins, where the mature miRNA could regulate gene expression, binding through partial complementarity to target messenger RNAs (mRNAs) and leading to translation inhibition or mRNA degradation, depending on the sequence complementarity between the miRNA and the target mRNA (reviewed by (Iorio and Croce 2012). Recent reports have tried to clarify the complex mechanisms regulating miRNA function on target mRNAs: microRNAs mainly recognize complementary sequences in the 3´ UTR of their target mRNAs, but recent studies have reported that they may also bind to the 5´ UTR or the open reading frame (Lytle, Yario et al. 2007; Orom, Nielsen et al. 2008; Moretti, Thermann et al. 2010). Sites located in coding regions appear to be less robust than those in the 3'UTR (Gu, Jin et al. 2009) and, surprisingly, miRNAs can upregulate translation upon growth arrest conditions (Henke, Goergen et al. 2008; Orom, Nielsen et al. 2008; Vasudevan, Tong et al. 2008) (Figure 8). Moreover it has been evidenced that the mature form of microRNAs may also be localized in the nucleus (Hwang, Wentzel et al. 2007).

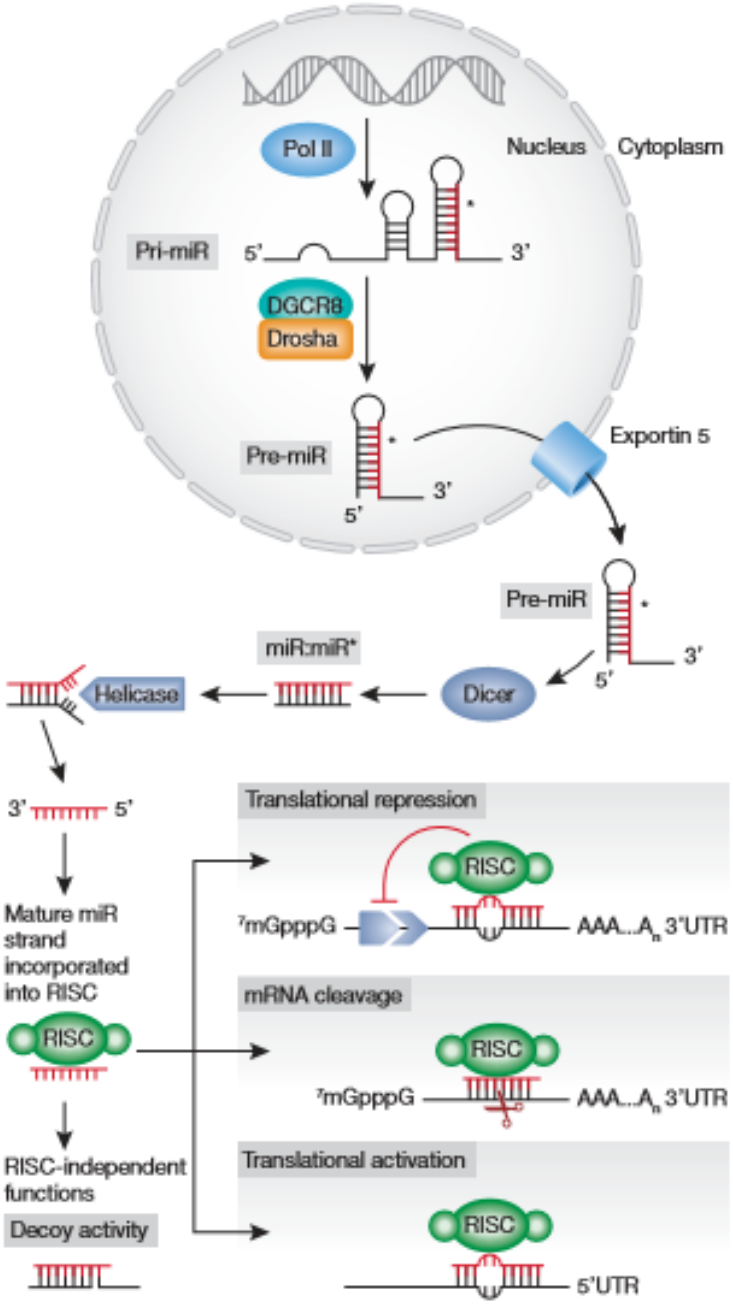


Figura 8. microRNAs biogenesis and function (Iorio and Croce 2012)

miRNAs interact with their mRNA targets via base-pairing. The most important requirement is a contiguous and perfect Watson-Crick base-pairing of the seed region of the miRNA, the 5' nucleotides 2–8, guide for the base-pairing. However, functional miRNA sites that contain bulged nucleotides or mismatches in the seed region have also been identified as shown for

the Lin-41 mRNA targeted by let-7 miRNA in *Caenorhabditis elegans* (Vella et al., 2004). Moreover miRNA-mRNA duplexes containing mismatches and bulges in the central region (miRNA positions 10–12) could prevent endonucleolytic cleavage of mRNA. AU-rich sequence context and structural accessibility of the sites could improve their efficacy (Bartel 2009). Multiple sites, for the same or different miRNAs, are required for effective repression, and when the sites are close to each other, they tend to act cooperatively (Grimson, Farh et al. 2007).

2.4.1 microRNAs and translation

Several studies tried to clarify the mechanisms of protein synthesis suppression by microRNAs. These studies showed that miRNAs could inhibit translation of target mRNAs or facilitate their deadenylation and subsequent degradation. All miRNA-mRNA interactions seem to downregulate gene expression at post-trancriptional level, but the scale of regulation vary and depends on the specific miRNA-mRNA target combination. Whether this event is due by accessibility of the mRNA to miRNAs or by other factors is unknown (Maroney et al., 2006). How do miRNAs regulate gene expression? Early analysis indicated that regulation was at the level of translation: the abundance of the regulated mRNA does not change, but the abundance of proteins encoded by these mRNAs was reduced (reviewed by (Maroney, Yu et al. 2006). The first question in clarifying the mechanism of translational repression by miRNAs is to determine whether miRNAs suppress the initiation of translation or act at the postinitiation stage. Lin-4 was discovered in *C. elegans*, and causes inhibition of translation of lin-14 without a reduction in mRNA levels or a shift in polysomes, leading to the conclusion that miRNAs could inhibit mRNA translation at the elongation step of the translation process (Olsen and Ambros 1999). In other experimental models several results denoted defects in the control of translation initiation and mRNA stability. miRNA-mediated repression of

translation initiation was observed in HeLa cells. Analysis of mRNA levels was unsuccessfull to detect pronounced degradation of mRNAs targeted by miRNA, demonstrating that translation was inhibited (Pillai, Bhattacharyya et al. 2005). Furthermore, let-7 targeted mRNAs shifted to lighter fractions of polysomal sucrose density gradients, an event that is indicative of repressed translation at the initiation step, caused by a defect in ribosome recruitment to the mRNA (Ding and Grosshans 2009). Moreover miRNAs which target mRNAs are present in the polysomes fraction, although the proteins encoded by those target mRNAs were not detectable. Since ribosomes already initiated translation of mRNAs present in the polysomes fraction, it was concluded that targets were silenced at the post-initiation stage. This model was supported by the observation that miRNAs silencing occurred in the absence of the 5′-cap structure (Petersen, Bordeleau et al. 2006). Several groups have reported that mRNAs that lack a functional 5'-cap structure, or that present a cap-independent translation, are refractory to a miRNA-mediated translational repression (Wang, Love et al. 2006; Mathonnet, Fabian et al. 2007). All these studies concluded that the miRNA-mediated translation inhibition takes place at the initiation step and this is due to the interference with the cap recognition process. This is further supported by the findings that miRNAs failed to inhibit IRES-dependent translation or translation from ApppG-capped mRNAs. Moreover, several studies evidence that miRNAs translational repression is due to the inhibition of the 80S complex assembly: miRNAs might affect 60S joining. eIF6 protein, associated with the 60S ribosomal subunit, coimmunoprecipitated with the AGO2-Dicer-TRBP complex. Depletion of eIF6 from either human cells or *C. elegans* partially alleviated the inhibition of let-7 or lin-4 miRNA targets, leading to the conclusion that miRISC association with eIF6 disrupts polysomes formation by inhibiting 80S complex assembly (Chendrimada, Finn et al. 2007). However, the validity of these results was not confirmed because depletion of eIF6 from *Drosophyla* cells had no striking effect on miRNA-mediated repression (Eulalio,

Huntzinger et al. 2008). The interaction of PABP with the eIF4G of the eIF4F complex led to an increase in cap-dependent translation of mRNAs (Kahvejian, Svitkin et al. 2005). Thus, miRNA-mediated mRNA deadenylation causes a reduction in translation initiation. The role of the poly(A) tail in miRNA-mediated translational repression is in open debate: both the 5' cap and poly(A) tail were required for mRNA translational repression by a miRNA mimic in HeLa cells (Humphreys, Westman et al. 2005) but no substantial difference in the repression between capped poly(A)− and poly(A)+ mRNAs was noted by others (Meister, Landthaler et al. 2004). And again, numerous studies concluded that miRNAs could inhibit translation also at postinitiation steps. This conclusion was based from polysomal sedimentation analyses. Indeed the most used method to establish the step at which translation is blocked is the measurement of the location of mRNAs across a polysomal sucrose gradient. Recent studies in mammalian cells have displayed that miRNAs repressed mRNAs showed the same mRNA distribution pattern across poly-ribosomes compared with non-repressed mRNAs (Nottrott, Simard et al. 2006; Gu, Jin et al. 2009). Same results were reached by studies in *C.Elegans* (Seggerson, Tang et al. 2002). These results led to models of post-initiation inhibition of mRNA translation, including cotranslation protein degradation (Nottrott, Simard et al. 2006), increased premature termination (ribosomal dropoff) (Petersen, Bordeleau et al. 2006), and impaired elongation (Gu, Jin et al. 2009). Another method to distinguish initiation and post-initiation inhibition relied on checking whether internal ribosome entry sequence (IRES)-containing mRNAs were resistant to miRNA-mediated repression (Sonenberg and Hinnebusch 2009). Like the polysomal profile experiments, contradictory results were reported by different groups. Some studies demonstrated that IRES-initiated translation was still subject to miRNA-mediated repression, therefore excluding eIF4E-cap recognition as a potential target for miRNA function. Other studies concluded that microRNAs inhibit target mRNA translation at the initiation step (reviewed by Gu and Kay 2010).

2.4.2 microRNAs and cancer

The first evidence of the involvement of microRNAs in human cancers derived from studies on chronic lymphocytic leukemia (CLL). Croce et al. discovered that a region of the chromosome 13 contains two microRNAs, miR-15a and miR-16-1, expressed in the same polycistronic RNA. This is the first evidence that microRNAs could be involved in the pathogenesis of cancers, and study of a large collection of CLLs displayed that the 69% of CLLs showed knock-down of miR-16-1 and miR-15a (Iorio and Croce 2012). They mapped all the known microRNA genes and found that many of them are placed in regions of the genome involved in chromosomal alterations, such as amplification or deletion (Calin, Sevignani et al. 2004). After these observations, all known microRNA genes have been mapped and several platforms have been developed. This results helpful to study the global expression of microRNA genes in normal and diseased tissues and establishes whether microRNA profiling could be used for tumor classification, diagnosis and prognosis (Calin and Croce 2006). microRNA profiles can distinguish not only between cancerous and normal tissues identifying also tissues of origin, but they can also discriminate different subtypes of a particular cancer or specific oncogenic abnormalities. Moreover, microRNA profiling can also predict disease outcome or response to therapy, such as miR-155 overexpression and let-7a downregulation were able to predict poor disease outcome in lung cancer (Yanaihara, Caplen et al. 2006; Caramuta, Egyhazi et al. 2010). Finally, but not less important, it is possible evaluating miRNA expression to predict the response to specific drugs since it might be useful for an accurate selection of patients potentially responsive to a specific therapy, as for miR-21 expression in the response of chemotherapy in pancreatic cancer (Giovannetti, Funel et al. 2010) and adenocarcinoma (Schetter, Leung et al. 2008).

MicroRNA expression, like the expression of other cancer associated genes, can be altered by chromosomal amplification or deletion, promoter methylation, and transcription factor

activation. Many cancer cells have genetic alterations that are microRNA mechanism-specific: altered target binding site, processing and post-transcriptional editing. Binding site variation in the 3'UTR of the target mRNA is a common feature of cancer cells (Ziebarth, Bhattacharya et al. 2012); mutations and single nucleotide polymorphisms (SNPs) have been identified, and also deletions of 3'UTRs during mRNA splicing in cancer cells, rendering mRNAs insensitive to regulation by microRNAs (Sun, Yan et al. 2009). Alterations in the microRNA processing machinery are reported in cancer cells. Mutations that impair the efficiency of the microRNA processing machinery have been identified, and they affect the levels of mature microRNAs in the cell, as for instance mutations in exportin-5 (XPO5) lead to trapping of pre-microRNAs in the nucleus, preventing further microRNA processing (Melo, Moutinho et al. 2010).

The complex program of cancer to elude the treatment relies on the communication between multiple cell types. The six main features of cancer progression are self-sufficiency in growth signals, apoptosis evasion, insensivity to anti-growth signals, angiogenesis, unlimited replicative potential, tissue invasion and metastasis (Hanahan and Weinberg 2000). Dysregulated microRNAs may function as tumor suppressors or as oncogenes in cancer by targeting each one of these processes (Figure 9).

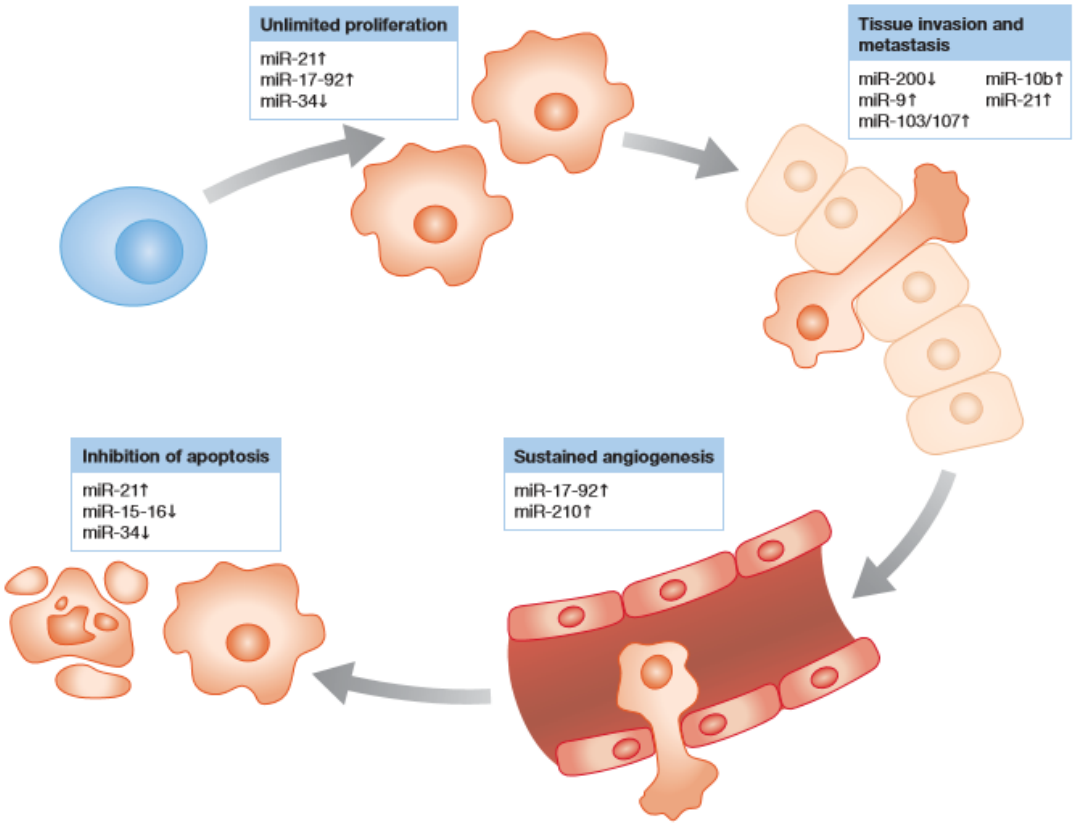


Figure 9. microRNAs targeting the hallmarks of cancer (Iorio and Croce 2012)

Gain-of-function approaches have shown that miRNAs act as tumor suppressors targeting oncoproteins with crucial roles in various cancer pathways, such as BCL2 targeted by miR-15a–miR-16-1 (Cimmino, Calin et al. 2005), myeloid cell leukemia sequence 1 BCl-2-related, MCL1, targeted by miR-29 (Garzon, Heaphy et al. 2009), RAS and MYC regulated by let-7 (Johnson, Grosshans et al. 2005; Sampson, Rong et al. 2007). To assess the biological effects of oncogenic miRNAs, frequently overexpressed in cancer cells, in vitro silencing, using antisense oligonucleotides, are helpful. This approach was used in breast cancer model (Iorio, Ferracin et al. 2005), colon cancer (Schetter, Leung et al. 2008) and glioblastomas (Ciafre, Galardi et al. 2005) in which miR-21 is overexpressed. Blocking miR-21 expression in these cell lines determined an increase on activation of caspases and apoptosis. Several miRNAs are reported showing oncogenic or tumor suppressor role, and among them, microRNAs in

clusters, which are expressed together and show functional cooperation, result of great importance. The oncogenic miR-17-92 microRNAs cluster induces lymphomagenesis in a B-cell-specific transgenic mouse model (Sandhu, Fassan et al. 2013), and miR-19b, miR-20a, and miR-92 from this cluster, along with miR-26a and miR-223, promote T cell acute lymphoblastic leukemia (ALL) development in mouse models (Mavrakis, Van Der Meulen et al. 2011). Several studies reported that mice deficient for miR-17-92 cluster die after birth with a ventricular septal defect and lung hypoplasia (Ventura, Young et al. 2008). Instead, the deletion of the complete miR-17-92 cluster slows down Myc-induced oncogenesis (Mu, Han et al. 2009). In contrast, miR-155 overexpression in the lymphoid compartment was sufficient to induce cancer without any other cooperative mutation or Myc expression, suggesting that the dysregulation of a single miRNA can lead to malignancy (Iorio and Croce 2012). In addition to classical tumour suppressor or oncogene functions, miRNAs have been implicated also in cell migration and metastasis. The overexpression of miR-10b in metastatic breast cancer regulates cell invasion and migration, overexpressed miR-10b in non-metastatic breast cancer cells initiates invasion and metastasis. It was demonstrated that these effects are mediated by direct targeting of HOXD10 by miR-10b, improving the overexpression of the pro-metastatic gene RHOC (Tian, Luo et al. 2010). Other relevant examples are miR-126 and miR-335 which act as negative regulators of tumor invasion and metastasis in human breast and lung cancer (Tavazoie, Alarcon et al. 2008). It has been observed that primary tumors and metastasis from the same tissue show a similar pattern of microRNAs expression (Rosenfeld, Aharonov et al. 2008). miRNA profiling is a more accurate classifier than mRNA profiling, and thus has the potential to elucidate one of the most challenging issues in cancer diagnostic: the origin of metastasis of unknown primary tumors (Iorio and Croce 2012). Instead, miR-34a, lost in several tumors and involved into the p53 mediated network (He, He et al. 2007), inhibits migration and invasion downregulating MET expression in human HCC cells (Li, Fu

et al. 2009). By the way, as part of their role in shaping the fate of a cell, microRNAs are also fundamental in the control of EMT. Some microRNAs, such as the miR-200 family and miR-34a, are protectors of the epithelial phenotype, and their downregulation during EMT enhance targets as ZEB1 and ZEB2, mesenchymal specific (Hao, Zhang et al. 2014). And again, positive correlation of miR-138 and EMT has uncovered its role in driving the process through many targets including Vimentin, ZEB2 and epigenetic regulators such as EZH2 (Liu, Wang et al. 2011). Similarly, it has been shown that miR-155 is able to repress TGFβ-induced EMT, and depletion of this microRNA can suppress EMT in a mouse model (Kong, Yang et al. 2008). Currently, there are multiple clinical trials that are assessing the correlation between miRNAs expression and cancer diagnosis and prognosis. Due to the pleiotropic effects of miRNAs, they have been an attractive way for patients diagnosis evaluation and prognosis. Moreover, miRNAs, because of their size, are highly stable and resistant to RNAses and thus have a higher level of stability than mRNA (Price and Chen 2014). Expression profiles of many miRNAs derived from tumor tissues have been shown to be useful in prognosis and diagnosis of the patients. In this context miRNAs expression profiles can be used to classify various types of cancers, or poorly differentiated tumors, better that mRNA profile. For example, seven miRNAs, miR-15b, miR-23a, miR-133a, miR-150, miR-197, miR-497 and miR-548b-5p, were decreased in the serum of patients with astrocytomas in advanced stage, and the miRNAs signature could distinguish between normal and cancer patients (Yang, Wang et al. 2013). Furthermore, several studies have identified stable miRNAs in human serum or plasma and circulating miRNAs serve as diagnostic or prognostic indicators. Differential expression of circulating miRNAs has been showed in patients of many types of cancers, including breast cancer, multiple myeloma, prostate cancer, gastric cancer, colon cancer, pancreatic cancer, diffuse large B-cell lymphoma, lung cancer, squamous cell carcinoma and ovarian cancer (Price and Chen 2014).

Here we report a list of known microRNAs with biological role in Malignant Pleural Mesothelioma.

MicroRNA	Expression change in MPM *vs.* normal tissue		Activity		Experimentally validated function(s)
	Cells	Tumours	*In vitro*	*In vivo*	
miR-29c-5p	Down	N.D.	√		Mimic inhibits growth and migration; targets *DNMT1/3A*
miR-31	Down	Down	√		Mimic inhibits growth and migration; targets *PPP6C*
Let-7a	N.D.	N.D.	√		Induced by EphrinA1; inhibits RAS
miR-34b/c	Down	Down	√	√	Induced by p53 in response to cell stress; mimics inhibit MPM cell growth; inhibitors increase proliferation in mesothelial cells
miR-15a/b	Down	Down	√		Inhibits growth of MPM cells
miR-16	Down	Down	√	√	Tumour suppressor functions; downregulates cell cycle and anti-apoptotic genes *CCND1* and *BCL2*
miR-126	Down	Down	√	√	Mimic inhibits respiration; Induced by oxidative stress; alters metabolism; targets IRS1
miR-205			√		Involved in EMT, affects migration; targets *ZEB1* and *ZEB2*
miR-145	Down		√	√	Inhibits clonogenicity and migration, sensitizes to pemetrexed; *regulates OCT4*
miR-1	N.D.	Down	√		Growth inhibition

MPM, malignant pleural mesothelioma; EMT, epithelial-to-mesenchymal transition; N.D., not determined.

Table 2. MicroRNAs with biological activity in MPM (modified from (Reid 2015)

In conclusion, understanding how miRNAs regulate target genes in cancer initiation, progression, metastasis, relapse, and drug response and resistance is a notable point in the comprehension of the miRNAs related cancer biology.

2.5 Protein Kinase C β and Enzastaurin

Protein kinase C (PKC) is a family of phospholipid dependent serine/threonine kinases that function in numerous cell types, differ in their structure, cofactor requirement and substrates specificity. Based on their biochemical properties and activation characteristics, this protein family can be further classified into three subfamilies: conventional or classic PKC isozymes (cPKCs; α, βI, βII, and γ), novel or nonclassic PKC isozymes (nPKCs; δ, ε, η, and θ), and atypical PKC isozymes (aPKCs; ζ, ι, and λ). Conventional PKCs are calcium dependent and

activated by both phosphatidylserine (PS) and DAG, novel PKCs are calcium independent and regulated by DAG and PS, and finally, atypical PKCs are calcium-independent and do not require DAG for activation, although PS can regulate their activity (Griner and Kazanietz 2007; Steinberg 2008). Phorbol esthers, such as phorbol 12-myristate 13-acetate (PMA) stimulates conventional (α, βI, βII, γ) and novel (δ, ε, η, θ) PKC by mimicking the activating ligand DAG. Typically, kinases exert their signaling activity either directly via a phosphorylation of the final effector protein or indirectly via modulation of intermediate factors. As part of a vast cellular system, PKCs are critically involved in the signaling of vital physiological responses, including inflammatory, autoimmune responses, tumor progression, and cardiovascular functions. They are cytosolic enzymes, although, once activated, they translocate to the cell membrane using the membrane anchoring receptors for activated C-kinases (RACK) (reviewed by (Marengo, De Ciucis et al. 2011). The discovery of PKC, as the phorbol ester "receptor", has led to a strong interest in the contribution of these kinases to tumorigenesis and tumor progression (Kikkawa, Takai et al. 1982). In cancer cells, PKC isozymes are involved in cell proliferation, survival, invasion, migration, apoptosis and angiogenesis, through their increased or decreased participation in various cellular signaling pathways. Particularly, PKCβ overexpression contributes in several ways to tumor formation and is involved in tumor host mechanisms such as inflammation and angiogenesis in breast cancer and in retinal tissue. Elevated expression of PKCβ seems to be an early event in colon cancer development and transgenic overexpression of PKCβII in the intestine induces hyper-proliferation and an invasive phenotype in epithelial cells. In patients with diffuse large B-cell lymphoma, PKCβ is one of the most overexpressed genes while the loss of PKCβ expression has been observed in melanoma cell lines (Marengo, De Ciucis et al. 2011).

Enzastaurin (LY317615.HCl) is an ATP-competitive and oral selective inhibitor of protein kinase C (PKC) β. It inhibits kinase activity by competing with ATP for the enzyme's ATP

binding site. It was initially developed as a selective inhibitor of PKCβ, with a 50% inhibitory concentration (IC50) of 6 nmol/l. Enzastaurin also inhibits other PKC isoforms at higher concentrations (IC50 values calculated from a 10-point curve from filter-binding assays run at 30 mmol/l ATP: PKCβ 0.006 mmol/l, PKCα 0.039 mmol/l, PKCγ 0.083 mmol/l, and PKCε 0.110 mmol/l). Enzastaurin at low concentration, in a range of 1 to 4 μM, is able to suppress cell proliferation, and induce apoptosis, of various tumor cells such as colon carcinoma, glioblastoma, non–small cell lung cancer, breast cancer, leukemia, prostate cancer. In addition, Enzastaurin treatment can reduce the phosphorylation of glycogen synthase kinase (GSK)3βSer9, which has been linked to both PKCβ activity and AKT activity. Furthermore, Enzastaurin suppresses the phosphorylation of ribosomal protein S6 Ser240/244 and of AKT Thr308, suggesting that Enzastaurin also affects the AKT pathway (Figura 10). Initially Enzastaurin has been developed as an antiangiogenic agent, later it has been demonstrated that in xenograft models oral administration of enzastaurin at a dose of 75 mg/kg has a potent antitumor effects in multiple human cancer cell lines. (Graff, McNulty et al. 2005; Ma and Rosen 2007).

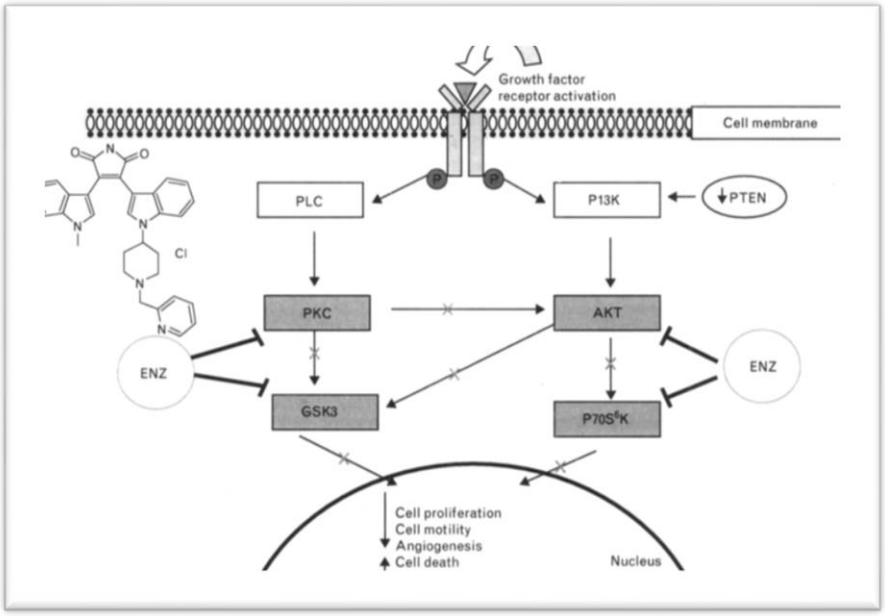


Figure 10. Schematic of Enzastaurin activity

In clinical studies, Enzastaurin was well tolerated and has shown encouraging activity in a variety of tumors. Phase I studies showed that Enzastaurin is well tolerated at the recommended dose of 525 mg/day with few clinically significant grade 3 or 4 toxicities; evidence of early activity was seen with significant stable disease (Carducci, Musib et al. 2006). A recent open-label, single-arm, phase II study of Enzastaurin investigated whether Enzastaurin has activity in patients with grade 1 or 2 follicular lymphoma and showed that Enzastaurin was well tolerated with mostly grade 1 or 2 toxicities (Schwartzberg, Hermann et al. 2014).

Phase III study compared the efficacy and safety of Enzastaurin versus lomustine in patients with recurrent glioblastoma. Enzastaurin was well tolerated and had a better hematologic toxicity profile, but did not have superior efficacy compared with lomustine (Wick, Puduvalli et al. 2010). Moreover a phase III study of Enzastaurin in patients with high-risk diffuse large B cell lymphoma (DLBCL) showed that Enzastaurin did not improve disease-free survival, event-free survival, or overall survival in patients with high-risk DLBCL (Crump et al., 2013, *http://hdl.handle.net/1854/LU-5792451).* To date, exploring Enzastaurin as a monotherapy in the prevention of relapse in patients with DLBCL failed to show a statistically significant increase compared to placebo in disease-free survival.

2.6 Aim and main conclusions of the work

Protein synthesis is a vital cellular process regulated during growth and development. Its deregulation can lead to cell apoptosis or disease. Molecular mechanisms and signaling pathways which control mRNA translation and the protein synthetic machinery are constituted by steps potentially involved in tumorigenesis, pointing them as novel druggable targets for cancer therapy. Translational control is rate-limiting in cancer growth and translation initiation step is emerging as an attractive therapeutic target. Drugs targeting the mTOR pathway, such as rapalogs, are used in cancer treatment, and explicate their action impairing eIF4F formation. Unfortunately not all cancer cells are sensitive to rapalogs. In this work we searched for initiation factors that are rate limiting for translation and controlled by growth factors activation, but not by mTOR. It has been shown that eukaryotic Initiation Factor 6 (eIF6) is a limiting factor in tumorigenesis, *in vivo*, regulating the availability of free 60S subunit, and that inhibition of translation initiation is the earliest molecular event affected by miRNAs that play an important role in gene regulatory networks. eIF6 activity is regulated by Protein Kinase C isoform β (PKCβII). Most tumor cells overexpress both eIF6 and PKCβII. Here, we observed that Malignant Pleural Mesothelioma (MPM) shows high levels of phosphorylated eIF6 and that PKCβ inhibitor Enzastaurin (Ely-Lilly) induces eIF6 dephosphorylation in time-dependent manner. Treatment of mesothelioma cells, with either Enzastaurin or shRNA for eIF6 reduces cell growth, *in vitro*, and impaired tumor growth and metastasis formation, *in vivo*. Furthermore, molecular analysis reveals that eIF6 manipulation affects the metabolic status of malignant mesothelioma cells, resulting in less glycolysis and less ATP content after depletion of eIF6 or Enzastaurin treatment. In addition, since miRNAs may inhibit the translation of target mRNAs at the initiation stage of protein synthesis or at the postinitiation phase, we performed a sucrose density gradient analysis of MPM cells allowing mRNAs to be separated, based on the number of polysomes associated. To identify

the localization of miRNAs in RNA subpopulations, we analyzed miRNAs distribution both in monosomes and active polysomes and we found that the miRNA signature was characterized also by differential miRNAs distribution. In particular, only some miRNAs were expressed in the polysomal pool with variability in miRNAs occupancy, indicating that some miRNAs can repress translation, while others cannot.

3. MATERIALS AND METHODS

3.1 Mice

All experiments involving mice were performed in accordance with italian national regulations. Experimental protocols were reviewed by local Institutional Animal Care and Use Committees (IACUC form sk481). Eight-week old immunocompromised NOD-SCID mice (Charles River Laboratories) were used for detecting tumor growth after intraperitoneal (i.p.) injection of REN cells, as indicated.

3.2 Cell lines and lentiviral vectors

For this study we used different MPM cell lines: REN cells for Epithelioid subtype, MM98 for Sarcomatous subtype and MSTO-211H for Biphasic subtype. Met-5A (ATCC® CRL--9444™) are SV40 immortalized non-tumorigenic mesothelial cells. REN and MM98 cells were grown in DMEM (Lonza), MSTO-211H were grown in RPMI1640 (Lonza) and Met-5A cells in Medium 199 (Life Tech). All media were supplemented with 10% FBS (Fetal Bovine Serum) and 1% penicillin, streptomycin, L-glutamine, and all cells were maintained at 37°C and 5% CO_2. For Western Blotting analysis, normal human primary mesothelial cells were used as Non-tumoral control. Cells were purchased from Cambridge Bioscience (Cambridge, UK) and maintained according to manufacturer instructions, up to 3 passages. Densitometric analysis was performed by ImageJ software.

MPM cells were stably infected with either one constitutive lentiviral vector carrying scramble ShRNA, used as control, or one carrying eIF6 ShRNA. Lentiviral vectors, pGIPZ Lentiviral ShRNA, were provided by Open Biosystem. Specifically, mature antisense sequences of constitutive shRNA of eIF6 were: 5'-AGCTTCCTACTAGCACCTG-3' (V3LMM_421640; GIPZ eIF6 shRNA: RMM4532-EG16418). After lentiviral infection, REN

cells were selected with puromycin (1µg/ml) for 48 hours, expanded and treated with Enzastaurin as specifically described.

3.3 Antibodies and reagents

The following antibodies were used: rabbit polyclonal antibodies against eIF6, rpS6, phospho-rpS6 (Ser240/244), total 4EBP1 (Cell Signaling), P-PKCβII (Cell Signaling); goat polyclonal anti-rpL28 and anti-PKCβII (Santa Cruz) rabbit polyclonal anti-VEGFA (Abcam); mouse monoclonal antibodies against β-Actin (Sigma), PKCβ (BD-Bioscience) RACK1 IgM (BD Transduction Laboratories). Biotin was obtained from Pierce, EuroClone (EZ-LINK NHS-LC-BIOTIN). Lambda Protein Phosphatase (Lambda PP) was provided by NEB. Enzastaurin was provided by Eli Lilly and Company (Indianapolis, USA). eIF6 recombinant protein was produced in E. Coli by simultaneous co-expression with chaperones, followed by affinity chromatography and size exclusion chromatography (SEC; GE Healthcare), according to (Pesce, Minici et al. 2015).

3.4 RNA extraction and real time RT-PCR

Total RNA was extracted with TRIzol reagent (Invitrogen). After treatment of total RNA with RQ1 RNase-free DNase (Promega), reverse transcription was performed with MMLV reverse transcriptase enzyme (Promega) according to the manufacturer's instructions. Reverse transcribed complementary DNA (100 ng) was amplified with the appropriate primers. Taqman probes specific for eIF6 (Hs00158272_m1) and 18S rRNA as an internal standard, were used. Target mRNA quantification by quantitative reverse-transcriptase PCR using ΔΔCt-method using Taqman Universal PCR Master Mix (4304437; Life Technologies) was performed on an ABIPRISM 7900HT Sequence Detection System (Applied Biosystems). Results are represented as means ± SD of at least three independent experiments.

For total, subpolysomal and polysomal RNA extractions from sucrose gradient aliquotes, the sucrose fractions were divided in two. The pulled fractions were used for subpolysomal and light and heavy polysomal RNA. Afterward, samples were incubated with proteinase K and SDS 1% for 1 h at 37°C. RNA was extracted by phenol/chloroform/isoamyilic acid method. The same defined amounts of synthehtic RNA spike-ins (osa-miR-414, ath-miR-159a, cel-miR-248) are added to all samples during preparation, to normalize the measurement of the RNA samples.

3.5 Cell proliferation, cell cycle and cell death analysis

Proliferation rate of MPM cells was analyzed by MTT test: briefly, cells were plated in 96 wells plates at different concentration, and assayed after 24, 48 and 72 hours. MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) was added and left on cells for 3 hours at 37° C and 5% CO_2. The resulting intracellular purple formazan was solubilized with SDS and quantified by spectrophotometer at λ= 550/650 nm. Cell cycle analysis was performed on G1 synchronized REN cells. Cells were starved in DMEM without FBS for 12 hours, and then in PBS plus 0,5 mM $MgCl_2$, 10 mM D-Glucose, 1mM $CaCl_2$ for 3 hours. At the indicated time points, cells were fixed, stained with propidium iodide (PI) and acquired on a BD FACS CANTO II flow cytometer. Cell cycle analysis was performed using the FCS Express software (BD). Cell death detection was performed using APC-Annexin V (BioLegend). Each experiment was done at least in triplicate.

3.6 Polysomal profile

Growing cells were lysed using a glass douncer in 50 mM Tris HCl pH 7.8, 240 mM KCl, 10 mM $MgSO_4$, 5mM DTT, 250 mM sucrose, 2% Triton X-100, 90 µg/ml cicloheximide, 30U/ml RNasin. After centrifugation at 39000 r.p.m. for 3 hours at 4 °C, the equivalent of two-hundred micrograms of RNA was loaded on a 15-55% sucrose gradient dissolved in 25 mM

Tris HCl pH 7.4, 25 mM NaCl, 5 mM MgCl2, 1 mM DTT and spun at 260.000 g for 3h30min with SW41Ti swing rotor (Beckman Coulter). The gradient was then analyzed by continuous flow absorbance at 254 nm, recorded by BioLogic LP software (BioRad). Peaks for 40S, 60S, 80S and polysomes were quantified. For dissociation studies, total extracts of REN cells were incubated 2 minutes at 37 °C, with 5µg of recombinant eIF6 protein or matched controls (PBS; denatured protein), and separated on a 7-45% sucrose gradient. Extracts containing up to 200 micrograms of RNA were loaded on a 7–45% (w/v) sucrose gradient containing 50 mM Tris-acetate pH 7.5, 50 mM NH_4Cl, 12 mM $MgCl_2$ and 1 mM DTT, and centrifuged in a Beckman SW41 Ti rotor for 3h30min at 260.000 g. The gradient was analyzed as above. In addition, individual fractions were collected. Fractions were precipitated with 10% trichloroacetic acid (TCA), separated on SDS-PAGE and analyzed by Western blot.

For microRNAs profiling on polysomal profile of REN cell, they were lysed using a glass douncer in 50 mM Tris HCl pH 7.8, 240 mM KCl, 10 mM $MgSO_4$, 5mM DTT, 250 mM sucrose, 2% Triton X-100, 90 µg/ml cicloheximide, 30U/ml RNasin ± 30mM EDTA. Following clearing, RNA was loaded on a 10-50% sucrose gradient dissolved in 25 mM Tris HCl pH 7.4, 25 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT ± 30mM EDTA, and spun at 260.000 g for 3h30min with SW41Ti swing rotor (Beckman Coulter). The gradient was then analyzed by continuous flow absorbance at 254 nm, recorded by BioLogic LP software (BioRad). Peaks for 40S, 60S, 80S and polysomes were quantified. Fractions of 1 ml were collected.

3.7 Datamining

Datasets were retrieved by GEO databases. The affy package was then used to carry out RMA based normalization. Quantitation of target genes was performed by setting expression thresholds at upper one/third. Calculation was performed as follows: original set of microarray data was retrieved from GSE2549. The dataset contains 54 MMP patients.

Samples without follow-up survival were discarded, obtaining 42 patients. Expression data on eIF6 and PRKCB were retrieved. Retrieved values ranged for eIF6 from 106 (min) to 468 (max), and for PRKCB from 62 (min) to 403 (max). Assuming that eIF6 hyperphosphorylation and overexpression were linked, we calculated the combined expression by multiplying the eIF6 x PRKCB values. Samples which gave a result in the first quartile of combined expression (practically with values above 1.5 fold the average expression of eIF6 and PRKCB) were compared to the others with the null hypothesis that combined eIF6-PRKCB expression had no effect on survival. Statistical analysis was performed by a paired t-test.

3.8 Immunohistochemistry

Immunohistochemical and histological analysis were performed on paraffine-embedded human mesothelioma tissues (provided by Hospital Dall'Angelo, Pathology, Venice, Italy). Immunoistochemistry (IHC) for eIF6 and calretinin were done using the Vectastain Elite ABC kit (Vector), as previously described (Sanvito, Vivoli et al. 2000; Gandin, Miluzio et al. 2008; Carbone, Ly et al. 2012). Some sections were counterstained with Hematoxylin-Eosin (H&E).

3.9 Two-dimensional (2D) gel electrophoresis

Protein extracts of REN, Met-5A and tumor samples, in all described conditions were examined in 2D gel electrophoresis. Samples were lysed in SDS-free RIPA buffer and proteins were precipitated with 10% TCA. Pellets were resuspended in 2-D buffer (7 M Urea, 2 M Thiourea, 50 mM DTT and 4% CHAPS) and 100 μg of proteins were isoelectrofocused. The first dimension was performed on Ready Strip IPG (pH 3.9–5.1; Biorad). For the reduction/alkylation step, the strips were incubated with re-equlibration buffer (50 mM Tris-HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, bromophenol blue) plus 10 mg/ml of DTT and re-equilibration buffer plus 45 mg/ml of iodoacetamide, respectively. Then, the strips

were subjected to SDS/PAGE for the second dimension. Proteins were transferred on PVDF membrane and subsequently incubated with eIF6 monoclonal antibodies. The signal was detected with an anti-mouse secondary antibody and ECL substrate kit (GE Healthcare). Each experimental sample was run at least twice, and at least three different biological replicates were analyzed.

3.10 Measurements of lactate secretion and ATP content

REN cells were plated at $2x10^5$ cells/well in 12-well dishes in high-glucose medium for 24 hr. Cells were switched to serum-free high-glucose (4,5 g/L)/high insulin (100 nM) medium for 4 hr. Lactate secreted into the medium was measured using a fluorogenic assay, Lactate Assay Kit (Biovision). Average of fluorescent intensity was calculated for each condition replicates. Values were normalized to protein content obtained from the same wells. For ATP measurements, cells were lysed in ice-cold ATP buffer (20 mM Tris, pH 7.5, 0.5% Nonidet P-40, 25 mM NaCl, 2.5 mM EDTA) for 5 min. Lysates were centrifuged at 13000 g for 30 min. Proteins were quantitated by BCA analysis. Luminometric determination of ATP was assayed using the ATP-determination kit (Molecular Probes).

3.11 microRNAs profiling

RNAs were processed with the nanoString nCounter system (nanoString, Seattle,Washington, USA) in the Nucleic Acid Shared Resource of The Ohio State University. By probes hybridization reaction, the miRNA panel can detect 699 endogenous miRNAs (with 654 probes), 5 housekeeping transcripts and 3 Spike-in RNA, small RNA molecules used as control. After hybridization, probes in excess are removed and samples are immobilized on a nCounter Cartridge. The cartridge is placed in a Digital Analyzer instrument and data are collected.

3.12 Statistical analysis

Each experiment was repeated at least three times, as biological replicates; means and standard deviations between different experiments were calculated. Statistical p-values obtained by Student *t*-test were indicated: three asterisks *** for p-values less than 0.001, two asterisks ** for p-values less than 0.01 and one asterisk * for p-values less than 0.05.

4. RESULTS

4.1 eIF6 and Malignant Pleural Mesothelioma

4.1.1 eIF6 is a marker of aggressive Malignant Pleural Mesothelioma (MPM)

To study whether eIF6 protein was expressed in malignant pleural mesothelioma (MPM), we performed an immunohistochemistry staining on 24 human MPM samples, using an anti-eIF6 polyclonal antibody (Biffo, Sanvito et al. 1997). Of these, 19 were epithelial, 3 sarcomatous, and 2 biphasic, as reported in Table 1.

Patients Number/Sex	Age(years)	Therapy	Survival(months)	Histotype
1. Male	55	PI+CT+RT	11	EP
2. Male	58	PPE+CT+RT	9	EP
3. Male	59	TP+CT+RT	42	EP
4. Male	60	PT	15	EP
5. Male	60	CT+RT	42	EP
6. Male	63	TP+CT+RT	38	EP
7. Male	64	PPE	3	S
8. Male	65	PPE+CT	13	EP
9. Male	67	PPE+CT	9	EP
10. Male	68	PPE+CT	10	EP
11. Male	68	PPE+CT	9	EP
12. Male	69	PI+CT+RT	16	EP
13. Male	70	TP+CT+RT	38	EP
14. Male	71	TP+CT+RT	25	B
15. Male	71	TP+CT	17	EP
16. Male	71	TP+CT+RT	25	B
17. Male	72	PPE	8	EP
18. Male	75	TP+CT	4	EP
19. Male	76	CT+RT	13	EP
20. Male	76	TP+CT	14	EP
21. Male	80	NO	3	S
22. Female	68	CT	6	S
23. Female	75	CT+RT	8	EP
24. Female	77	TP+CT	26	EP

Histotypes: **EP:** Epithelioid **B:** Biphasic **S:** Sarcomatous

Therapy: **TP:** Total Pleurectomy **PPE:** Pleuropneumonectomy **CT:** Chemotherapy **RT:** Radiotherapy

Table 1. Human Malignant Pleural Mesothelioma Cases used for Immunohystochemistry analysis.

Representative stainings of epithelioid and biphasic histotypes of MPM are shown in Figure 11. Human epithelioid biopsies showed widespread mesothelioma infiltration that presented, with different prevalence, epithelial and connective components. Tumor components were characterized by islands or tubular formations. Biphasic histotypes showed both spindle-shaped cells, typical of sarcomatoid subtype, and epithelial areas. In all analyzed cases, eIF6 was expressed at high levels both in the nucleoli (black arrows) and in the cytoplasm of MPM cells. Nucleoli were enlarged, suggesting abnormal ribosome biogenesis. By using calretinin as a diagnostic marker for MPM, we confirmed that eIF6 overexpression was limited to tumor cells. Conversely, both eIF6 and calretinin are less expressed in non-tumoral pleural biopsies (Fig. 11).

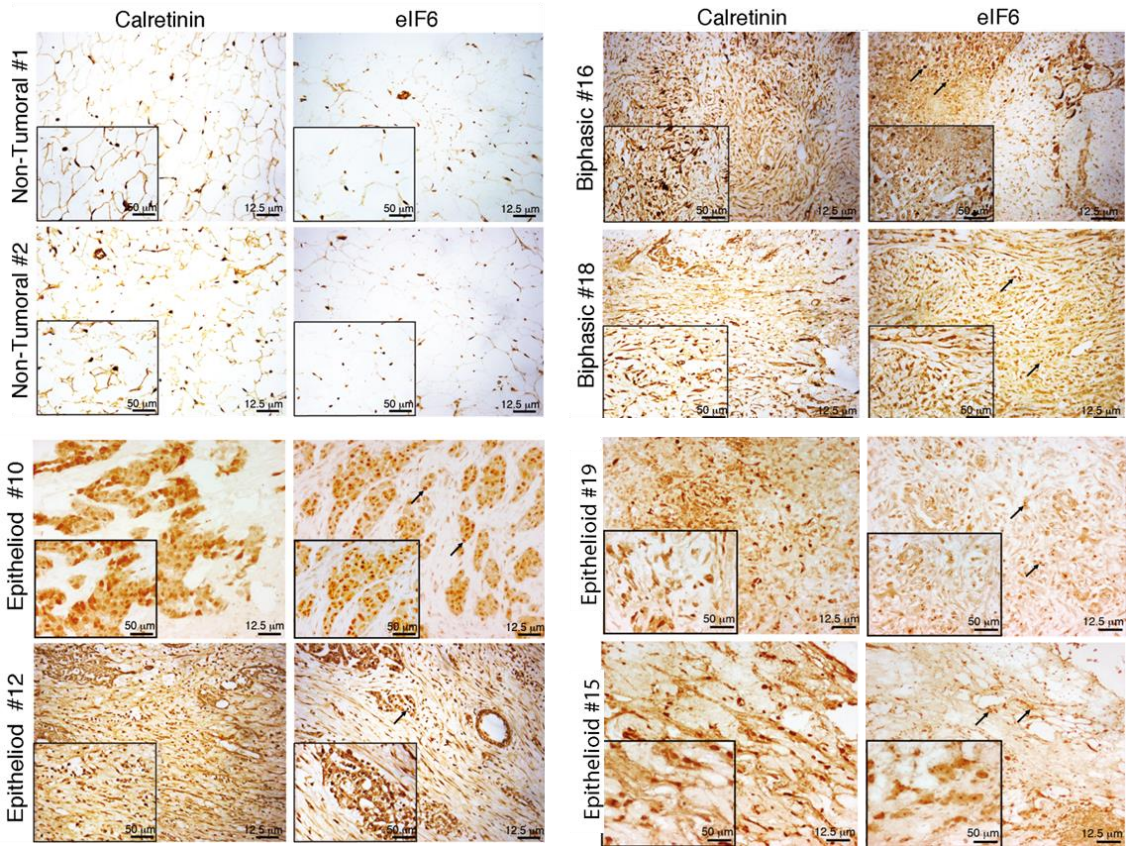


Figure 11. **eIF6 is expressed at high levels in Malignant Pleural Mesothelioma tissues.** IHC stainings on representative human non-tumoral samples and on biopsies of epithelial and biphasic malignant pleural mesothelioma biopsies show that eIF6, marked in brown, is highly expressed both in the nucleoli, as indicated by black arrows, and in the cytoplasm of tumor cells; we used Calretinin as a positive marker of MPM tumors.

Next, we evaluated both eIF6 expression and phosphorylation on human MPM epithelial tumors. First, we confirmed by Western Blot analysis that eIF6 overexpression is a constitutive feature of MPM (Fig. 12 A). Second, 2-D electrophoresis on a pool of three tumoral samples displayed 3 well-focused spots compatible with eIF6 phosphorylation sites. Tumors treated with phosphatase showed a single focused spot (Fig. 12 B).

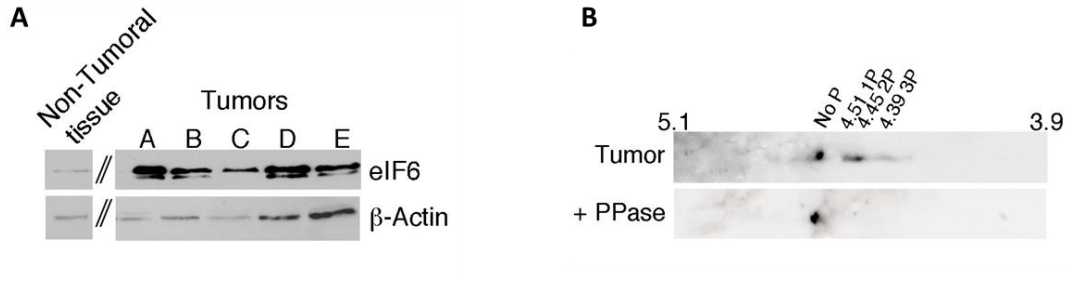


Figure 12. eIF6 is overexpressed and hyperphosphorylated in MPM tumors. A) Western Blot analysis of different human biopsies of malignant pleural mesothelioma, indicated as A-E, displays that eIF6 protein levels are higher in tumor samples compared to non tumoral ones. We used actin as loading control. **B)** Bidimensional electrophoresis performed on a pool of three tumoral extracts indicates that eIF6 is hyperphosphorylated in MPM cells. We can see the phosphorylation sites of the protein as focused spots. Predicted eIF6 isoelectric points are indicated. The same samples, treated with PPase as control, show a single focused spots.

We data-mined eIF6 mRNA levels from MPM microarray studies. Data showed that 35/42 MPM patient datasets expressed higher levels of eIF6 mRNA in tumor samples. However, no relationship between eIF6 mRNA levels at time of analysis and survival was observed. eIF6 can be phosphorylated by the RAS/PKC pathway. We data-mined on mesothelioma datasets the expression of PKCβ (PRKCB), the favoured RACK1 partner. Combined expression of PRKCB and eIF6 was then used to evaluate survival. Strikingly, high eIF6/high PRKCB expression correlated with lower survival, $p \leq 0.005$ (Fig. 13). In conclusion, the combination of eIF6 expression and phosphorylation correlates with negative survival, raising the question whether its inhibition may be beneficial.

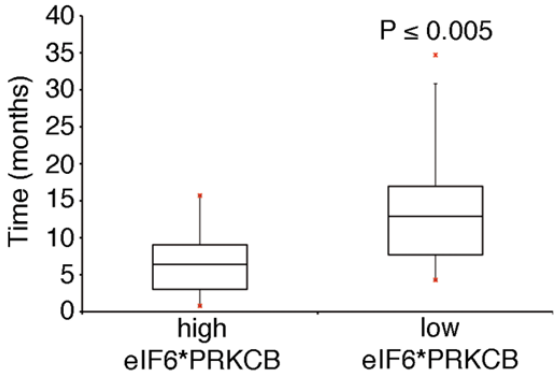


Figure 13. High co-expression of eIF6 and PKCβ is associated to lower survival of MPM patients. We used data of PRKCB and eIF6 expression derived bu mesothelioma datasets to evaluate survival of MPM patients. High eIF6/high PKCβ expression correlates with lower survival.

4.1.2 eIF6 hyperphosphorylation in REN, a MPM cell line

We analyzed the expression and phosphorylation of eIF6 in the epithelial MPM cell line, REN, and compared it to the expression of eIF6 in non-tumorigenic Met-5A mesothelial cells. We observed that REN cells show an increase of both eIF6 protein levels (Fig. 14A) and of mRNA levels (Fig. 14B), indicating that it is highly expressed in MPM cells. In Fig 14C we analyze the phosphorylation pattern of REN and MET-5A cells, showing that MPM cells display more focused spots than mesothelial cell line. This means that eIF6 is hyperphosphorylated in REN cells, but not in mesothelial MET-5A cells.

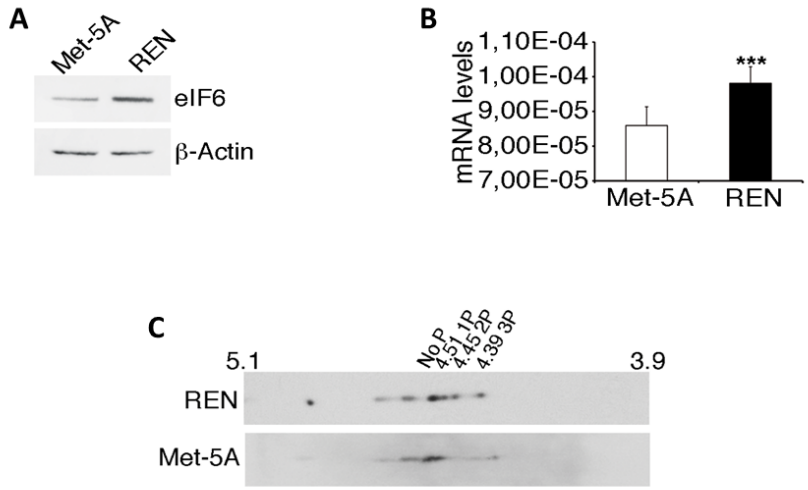


Figure 14. eIF6 is overexpressed and hyperphosphorylated in epithelioid REN cells. A) Western Blot analysis shows that eIF6 is overexpressed in REN cells compared to non-tumorigenic Met-5A cells. **B)** Real-Time PCR confirms that eIF6 mRNA levels are increased in REN cells. **C)** Representative 2-D gel electrophoresis on REN and Met-5A cells shows that REN cells display more focused spots than the MET-5A cells, therefore eIF6 is hypershosphorylated in REN cells, but not in non tumorigenic cells.

Phosphorylation of eIF6 occurs downstream of RACK1/PKC activation. PKCβ is the preferential partner of RACK1 (Ceci, Gaviraghi et al. 2003). Enzastaurin is a specific PKCβ inhibitor that has been used in clinical trials for treating B-cell malignancies, i.e. (Schwartzberg, Hermann et al. 2014). Enzastaurin (1μM) was administered to REN cells, in growing conditions. Cells were lysed at 24 hours, 48 hours and 72 hours post-treatment and the degree of eIF6 phosphorylation was analyzed by 2-D electrophoresis, followed by Western Blot analysis. Growing REN cells showed 4 well-focused spots compatible with eIF6 phosphorylation state. Cells treated with 1μM Enzastaurin, up to 48 hours, showed 3 spots compatible with 1-2 phosphate groups. Long-term treatment (72 hours) of Enzastaurin augmented dephosphorylation of eIF6. Finally, cell lysates treated with phosphatase showed a single focused spot (Fig. 15A). Enzastaurin did not affect the stability of both eIF6 and PKCβ, but the latters are more expressed in REN cells compared to non-tumoral Met-5A cells (Fig. 15C). It was recently reported that Enzastaurin affects the phosphorylation of the downstream

target of mTORc1 kinase, 4E-BP1, the main mediator of cap-dependent translation (Dumstorf, Konicek et al. 2010). However, in MPM both 4E-BP1 and rpS6 were phosphorylated in the presence of Enzastaurin (Fig. 15B), indicating that mTORc1 kinase is not inhibited by Enzastaurin and remains activated.

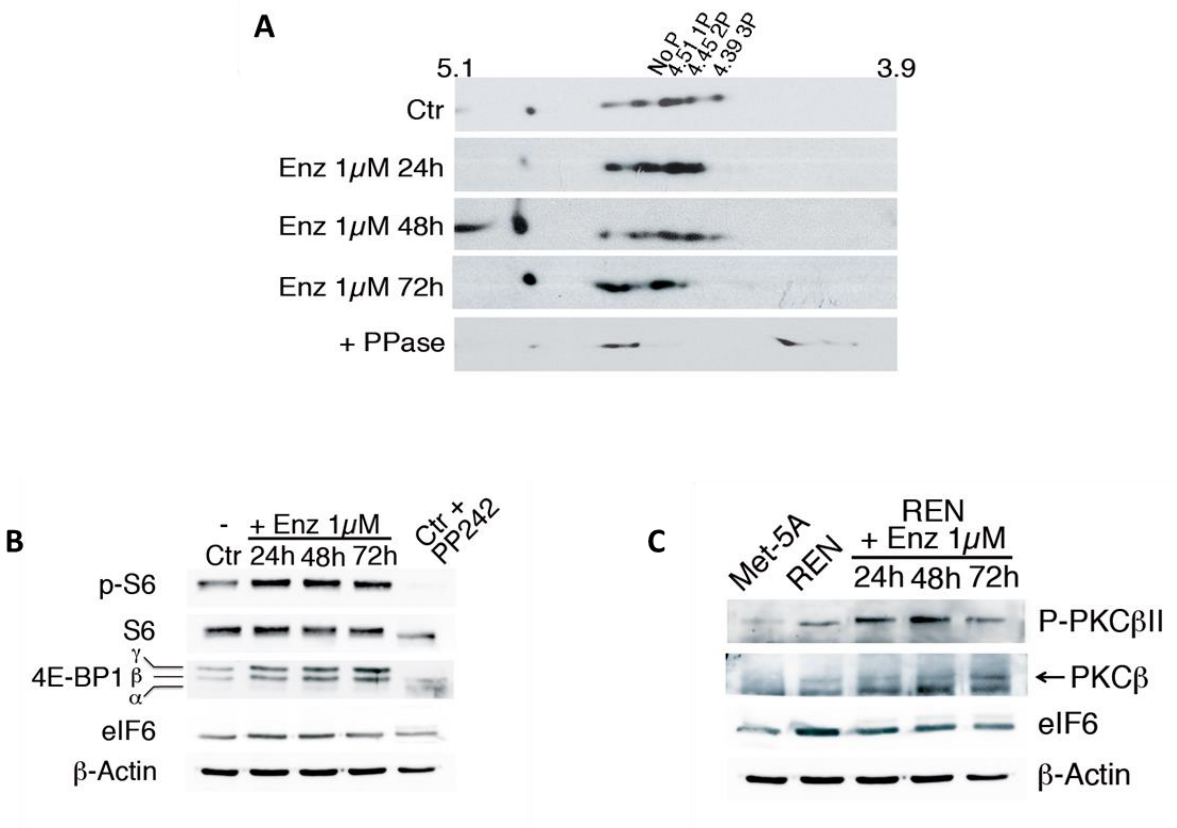
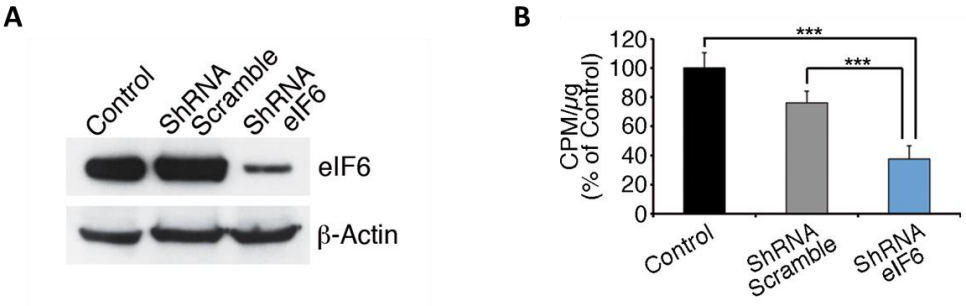


Figure 15. eIF6 hyperphosphorylation in REN cells is sensitive to Enzastaurin treatment, not affecting mTOR pathway. A) Representative 2-D gel electrophoresis on REN cells treated with 1μM Enzastaurin for 24, 48 and 72 hours shows that eIF6 phosphorylation is sensitive to Enzastaurin treatment, in a time dependent manner. Lambda PPase is used as positive control of unphosphorylation state. **B)** Representative Western Blot analysis on REN cells treated with Enzastaurin at different time points indicates that mTORc1 kinase is activated: phosphorylation of rpS6 and 4E-BP1 are equivalent in control cells and upon drug treatment. PP242 treatment is used as control of mTORc1 inactivation. **C)** Western Blot analysis shows that eIF6 and PKCβ are overexpressed in REN cells compared to non tumoral Met-5A cells. Proteins levels are similar upon Enzastaurin treatment in all considered times**.**

In conclusion, the MPM cell line REN has eIF6 PKC-dependent hyperphosphorylation and can be used to investigate the effects of eIF6 depletion and dephosphorylation.

4.1.3 eIF6 antiassociation activity is important for recycling inactive 80S

eIF6 acts in the regulation of translation initiation. We performed a methionine incorporation assay and polysomal profiles on REN cells, to analyze whether eIF6 levels can affect initiation. REN cells were previously infected with lentivirus carrying siRNAscramble as an internal control and siRNA eIF6. We showed a representative experiment performed on REN cells with either normal eIF6 protein levels (control and ShRNA Scramble), or reduced ones (ShRNA eIF6). Western Blot analysis displays that eIF6 protein expression has been reduced of ~ 80%, as indicated in Fig.16A. eIF6 depletion caused a significant reduction of newly synthesized proteins, as shown in Fig. 16B, indicating that eIF6 protein level is able to affect protein synthesis. Moreover, as we can see in fig 16C, polysomal profile indicate that the reduction of eIF6 protein level led to a slight decrease of polysomes accompanied by 80S accumulation. This means that eIF6 level is able to affect translation, particulatly the initiation step. Ratios of 80S/polysomes in each graph are also indicated.



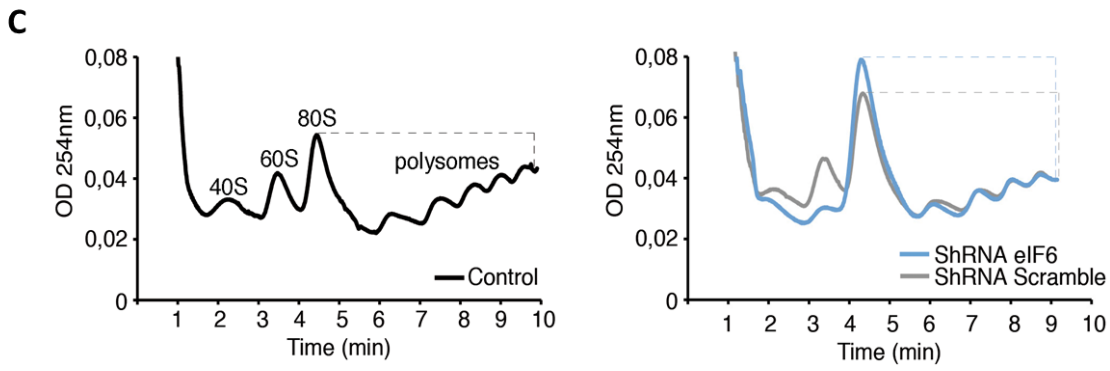
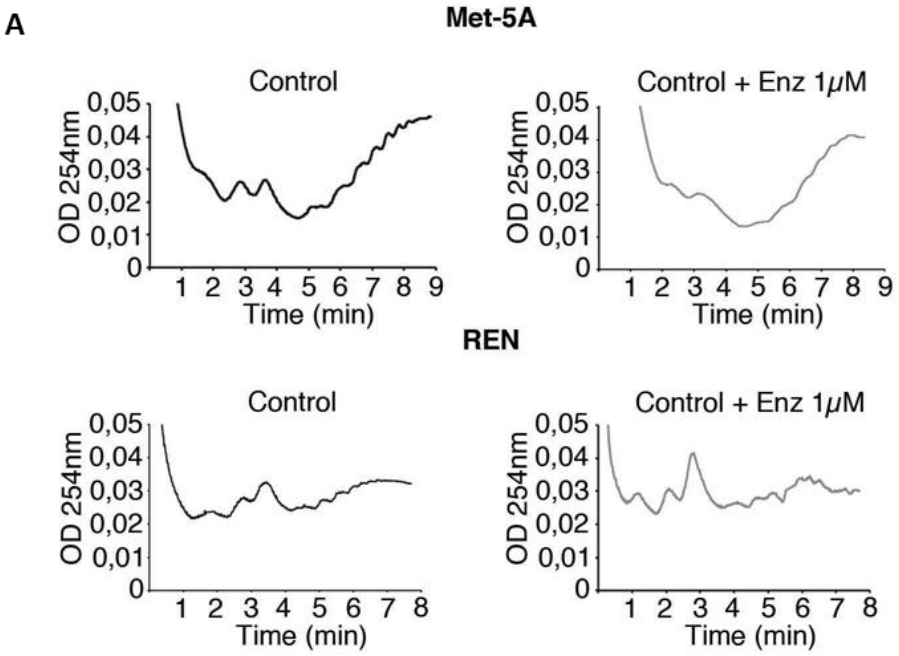


Figure 16. eIF6 depletion modulates protein synthesis in REN cells. A) Western Blot analysis on REN cells for eIF6 expression in all considered conditions show a reduction of ~80% of protein level. Data are normalized on β-Actin. **B)** Mean of three independent Methionine incorporation experiments indicates that eIF6 reduction affects protein synthesis of REN cells. **C)** Polysomal profiles show that partial depletion of eIF6 causes 80S accumulation and reduced translation.

Since Enzastaurin modulated eIF6 activity, we performed polysomal profiles and Methionine incorporation assay on REN cells, after treatment with the drug. We observed that Enzastaurin caused an increase of 80S peak in REN cells, but not in Met-5A (Fig. 17A), indicating a defect in the initiation of translation. We showed also that Enzastaurin determines a severe reduced protein synthesis (Fig. 17B), as we described also for eIF6 partial depletion.



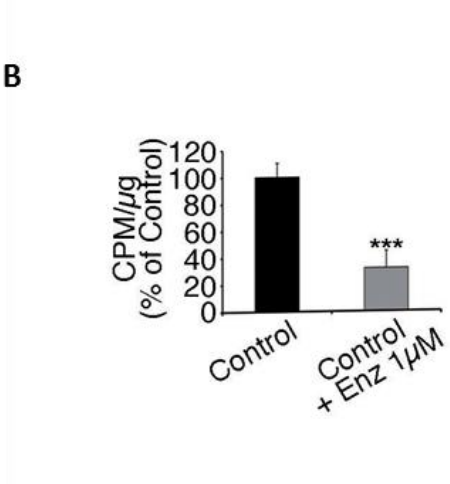


Figure17. Enzastaurin impairs translation and causes a reduction of protein synthesis. A) Representative polysomal profile on REN and Met-5A cells shows that Enzastaurin causes 80S accumulation and therefore reduced translation. **B)** Protein synthesis is significantly impaired upon Enzastaurin treatment, showed by reduction of methionine incorporation after drug treatment.

The limited amount of translational inhibition by eIF6 depletion, *in vivo*, is in line with the fact that eIF6 is not strictly necessary for translation, but is rate-limiting for oncogene-induced protein synthesis (Miluzio, Beugnet et al. 2011). Next, we analysed in an ex-vivo experiment the requirement for eIF6 on 80S cancer ribosomes, examining the effect of the recombinant eIF6 on protein synthesis, by translational profile approach. Therefore, we prepared polysomes extracts from REN cells and added recombinant eIF6 protein. Figure 18 shows that eIF6 addition can dissociate inactive 80S, as shown by the drop in the 80S peak and the simultaneous increase of free 60S, evidenced by the accumulation of 60S peak. We recovered all fractions derived from polysomal profiles in order to analyze proteins distribution by Western Blotting. We show that exogenous eIF6 was detected both on soluble and 60S fractions, but not in polysomal fractions, consistent with the dissociation data.

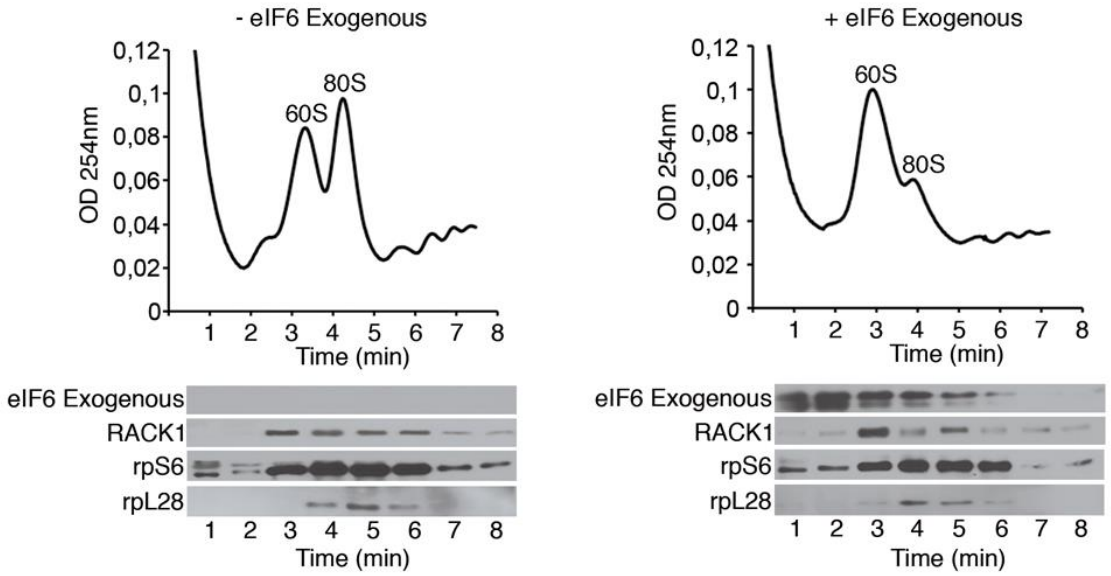


Figure 18. eIF6 causes the dissociation of inactive 80S. We added 5 µg of recombinant biotinylated eIF6 protein to polysomes extracts of REN cells and we show that exogenous eIF6 determines the dissociation of inactive 80S, in MPM cell line. Western Blot analysis on recovered fractions derived from polysomal profiles exhibits the distribution of indicated proteins, evidencing that exogenous eIF6 is present in soluble and 60S fractions.

Taken together our data indicate that eIF6 activity in cancer cells is necessary for keeping ribosomes dissociated, and for initiating new protein synthesis. We wondered whether this activity is important for tumor growth.

4.1.4 eIF6 reduction and dephosphorylation slow cell growth in cultured cells

Established that eIF6 is hyperexpressed and hyperphosphorylated in MPM and in the REN cell line, we asked whether its depletion or dephosphorylation affected growth. We analysed MPM cells growth at 24, 48 and 72 hours after plating and upon eIF6 depletion (Fig. 19). MTT assay revealed that the proliferation rate of eIF6 depleted cells was slightly reduced compared to the control, *in vitro.*

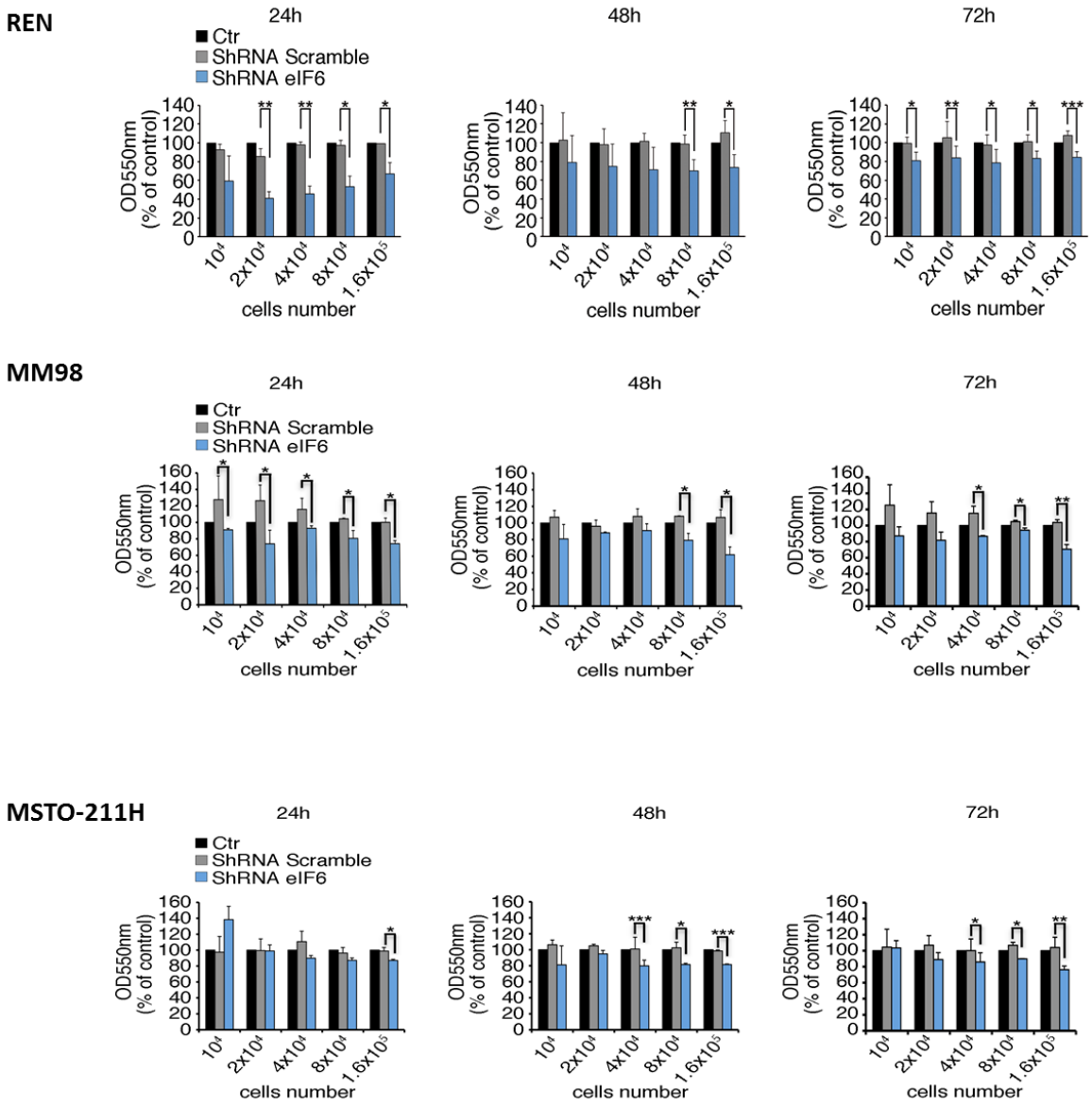


Figure 19. Partial depletion of eIF6 affects proliferation of MPM cell lines. We analysed the proliferation rate of 3 different MPM cell lines, representative of MPM histological subtypes, after transduction with shRNA scramble and shRNA eIF6 vectors. MTT assay reveals that eIF6 depletion impairs proliferation in all cell lines considered, REN (epithelioid), MM98 (sarcomatous) and MSTO-211H (biphasic).

In parallel, we performed a MTT Assay on REN cells, treated with 1μM, 5μM and 10μM of Enzastaurin and we measured the proliferation rate at the indicated time points (Fig. 20A). Enzastaurin reduced REN growth, indicating its cytostatic effect. The effect was more evident in low-serum conditions. eIF6 protein levels were very similar at all time points upon Enzastaurin treatment at the indicated concentrations (Fig. 20B).

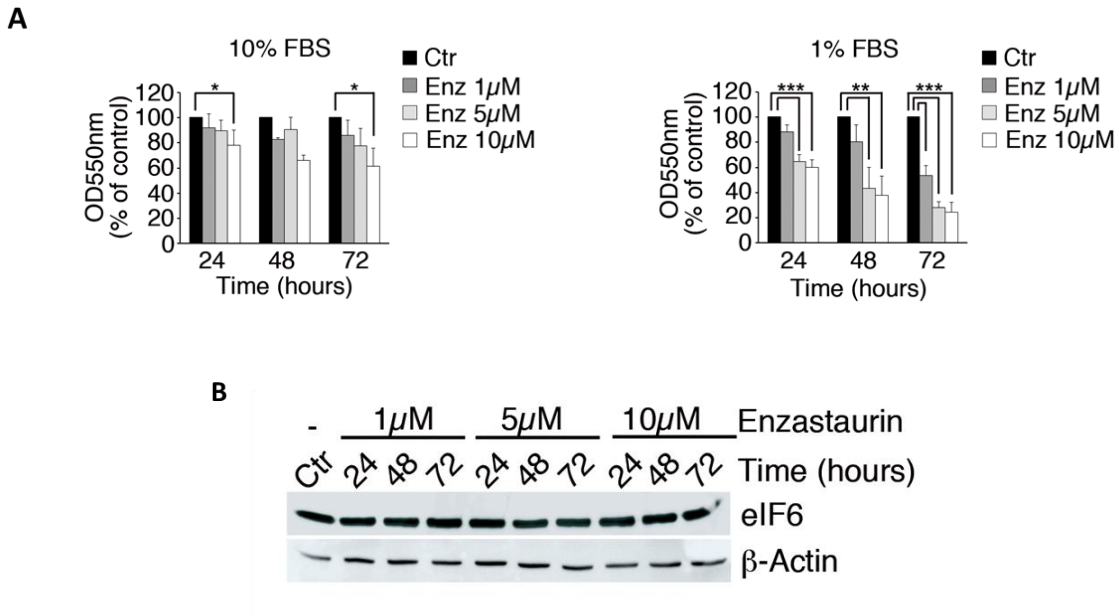


Figure 20. Enzastaurin treatment impairs proliferation of REN cells. A) MTT assay on REN cells treated with 1μM, 5μM and 10 μM of Enzastaurin shows that high doses and long-term treatment with Enzastaurin reduce REN cell growth, indicating its cytostatic effect that becomes stronger under serum deprivation. B) Western Blot analysis reveals that Enzastaurin does not affect eIF6 protein levels even at high concentrations.

Furthermore, we performed FACS analysis on synchronous REN cells, with normal or depleted eIF6 protein levels, and/or treated with 1μM Enzastaurin. Data confirmed that eIF6 reduction impaired G1/S progression and caused a reduced number of cycling cells in G2/M phase. Similar results were obtained with Enzastaurin treatment (Fig. 21).

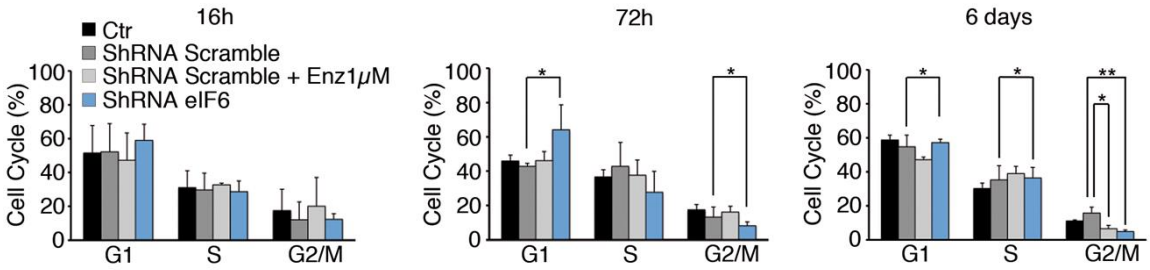


Figure 21. eIF6 depletion and Enzastaurin treatment impairs G1/S progression in REN cells. Cell cycle analysis of synchronous REN cells, with normal or depleted eIF6 protein levels, and treated with 1μM Enzastaurin reveals that eIF6 reduction impairs G1/S progression in synchronised REN cells and causes a reduced number of cycling cells in G2/M phase, at considered time points.

Finally, we quantitated the apoptotic rate of all these cells after 72 hours of treatment: we found that the percentage of cell death was similar in all considered condition (Fig. 22A, B)

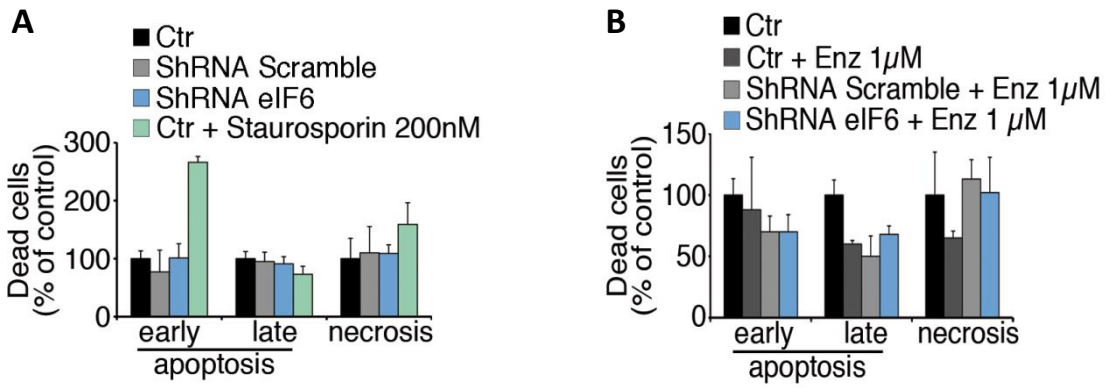


Figure 22. Depletion of eIF6 and Enzastaurin treatment do not affect apoptosis rate in REN cells. A) We analyze apoptotic rate of REN cells after transduction with siRNA scramble and siRNA eIF6 vectors. FACS analysis shows that the apoptotic rate is similar in all considered conditions. We used Staurosporin as positive control. **B)** Analysis of cell cycle of REN cells with normal and reduced level of eIF6 protein treated with Enzastaurin displays similar apoptotic rate in all evidenced conditions.
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In conclusion, both shRNA for eIF6 or Enzastaurin treatment slightly reduce proliferation in cultured REN cells*, in vitro*.

4.1.5 eIF6 depletion and Enzastaurin administration have an antitumoral effect*, in vivo*

Then, we addressed the role of eIF6 activity and Enzastaurin, *in vivo*. We developed a murine MPM model by injecting REN cells into immunocompromised NOD-SCID mice. We injected i.p. 10 millions cells/mouse with either wt eIF6 or eIF6 depleted cells. A group of control mice was treated with Enzastaurin: administration (75 mg/Kg) was performed by gavage twice/daily, starting at day 7 after injection and suspending it after 5 weeks. Mice were sacrificed 60 days after cells injection and tumor mass was analyzed. By autopsy, we measured the weight of total body, tumor mass, spleen and diaphragm and we scored for

developed metastasis and hemorrhage (Fig. 23). Mice injected with REN cells depleted of eIF6 showed reduced tumor mass weight, indicating that the amount of eIF6 is a limiting factor for cellular growth, *in vivo*. These mice also revealed less metastasis, since the diaphragm weight was reduced. Enzastaurin administration provided also a protective effect against tumor growth: indeed, tumor mass was strongly reduced, metastases were limited to diaphragm and hemorrhage was mild.

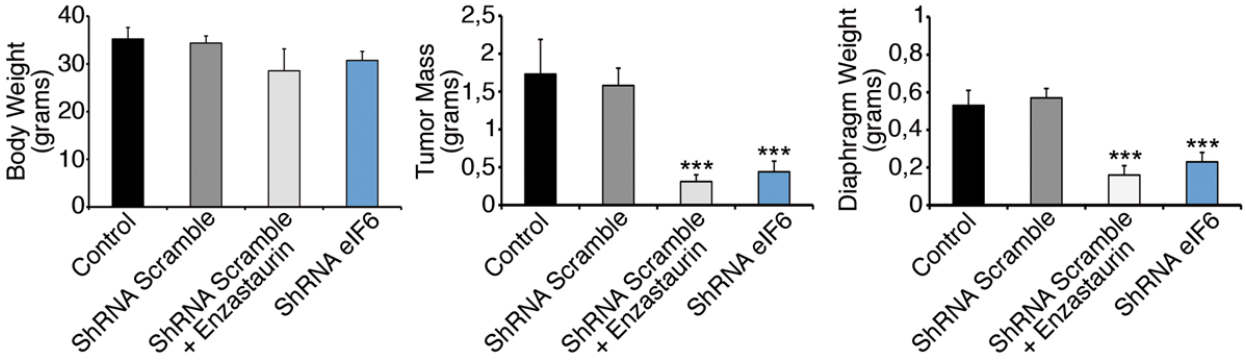


Figure 23. eIF6 depletion and Enzastaurin administration reduce tumor growth, *in vivo.* REN cells with either wt or depleted eIF6 protein are injected (i.p.) in NOD-SCID mice. A cohort of control mice were treated with Enzastaurin (75mg/kg) twice daily for 5 weeks. Mice were sacrified two months after tumoral cells injection. We can see that eIF6 depletion and Enzastaurin administration reduce tumor masses weight and diaphragm metastasis

Both Enzastaurin-treated tumors and shRNA eIF6 tumors recovered from NOD-SCID mice showed less CD31 and VEGFA-positive cells, indicating reduced angiogenesis and close correlation with diminished solid tumor growth and metastasis (Fig.24).

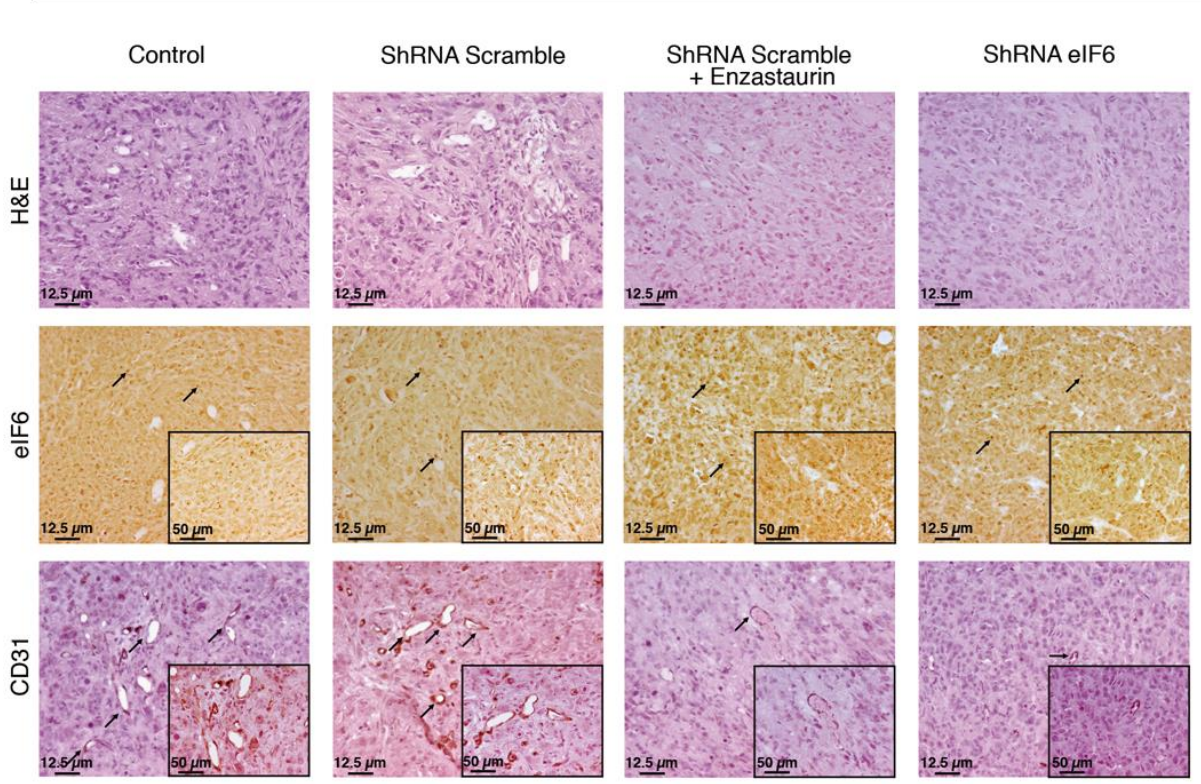


Figure 24. Reduced expression of eIF6 and Enzastaurin administration cause a decrease of angiogenesis. IHC stainings of tumors recovered from NOD-SCID mice. Tissue morphology is evidenced with Hematoxylin and Eosin staining; eIF6 is overexpressed both in the nucleoli (black arrows) and in the cytosol of tumoral cells; staining for CD31 (black arrows) reveals positive vessels; neo-angiogenesis is diminished both in eIF6 depleted conditions and upon drug administration. Scale bar is indicated.

In addition, we recently found that VEGFs genes are translationally controlled by eIF6 levels: we observed that 50% of eIF6 protein significantly reduces VEGFγ and VEGFβ expression. (Brina, Miluzio et al. 2015). These findings may be in agreement with the protective role of eIF6 depletion and/or inactivation by Enzastaurin in neo-angiogenesis and metastasis development.

Taken together, these data suggest that both inhibition of eIF6 expression and eIF6 phosphorylation is effective *in vivo*, or that eIF6 is potentially targetable by Enzaustarin.

4.1.6 eIF6 depletion and Enzastaurin cause metabolic changes of cancer cells

The protective role of reduced eIF6 and Enzastaurin administration *in vivo*, compared to the modest effects on cell growth *in vitro* raises the question of whether this effect could be linked to metabolic changes of tumoral REN cells. This selective effect would be consistent with the limited effect of eIF6 depletion on basal translation. A screening for eIF6-regulated mRNAs showed several transcription factors involved in metabolism (Brina, Miluzio et al. 2015). Here, we show that acute depletion of eIF6 by lentiviral ShRNA on REN cells, and Enzastaurin treatment led to a reduction of lactate secretion, an index of glycolytic flux, that became significant after 72 hours from lentiviral infection and drug treatment (Fig. 25A). In both cases ATP production was significantly reduced in each considered time (Fig. 25B).

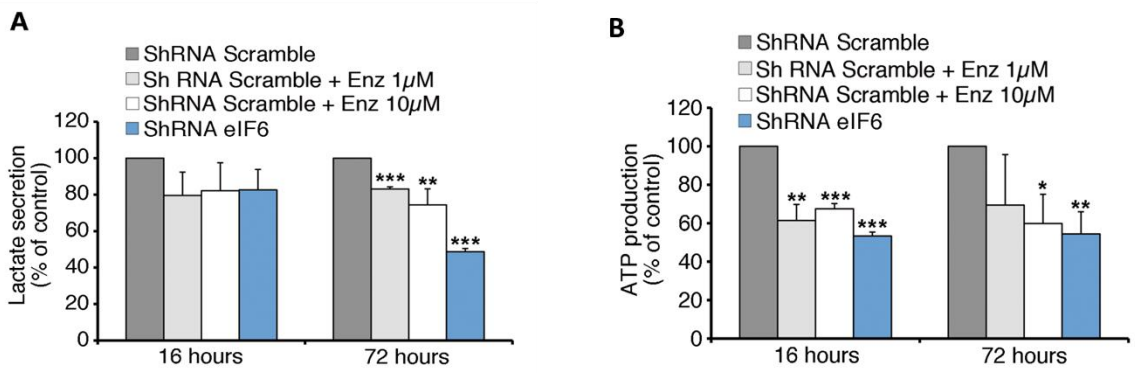


Figure 25 eIF6 depletion and Enzastaurin decrease glycolysis and ATP levels of cancer cells. A) We measured lactate secretion into REN cells surnatant, an index of glycolytic flux, and we see that it is significantly reduced in eIF6 depleted cell. **B)** We show that ATP content depends on eIF6 levels and Enzastaurin treatment, acute depletion of eIF6 and Enzastaurin treatment lead to a reduction of ATP levels of REN cells.

In summary eIF6 activity is required for a glycolytic switch that may account for its need for tumor growth *in vivo* (Fig.26).

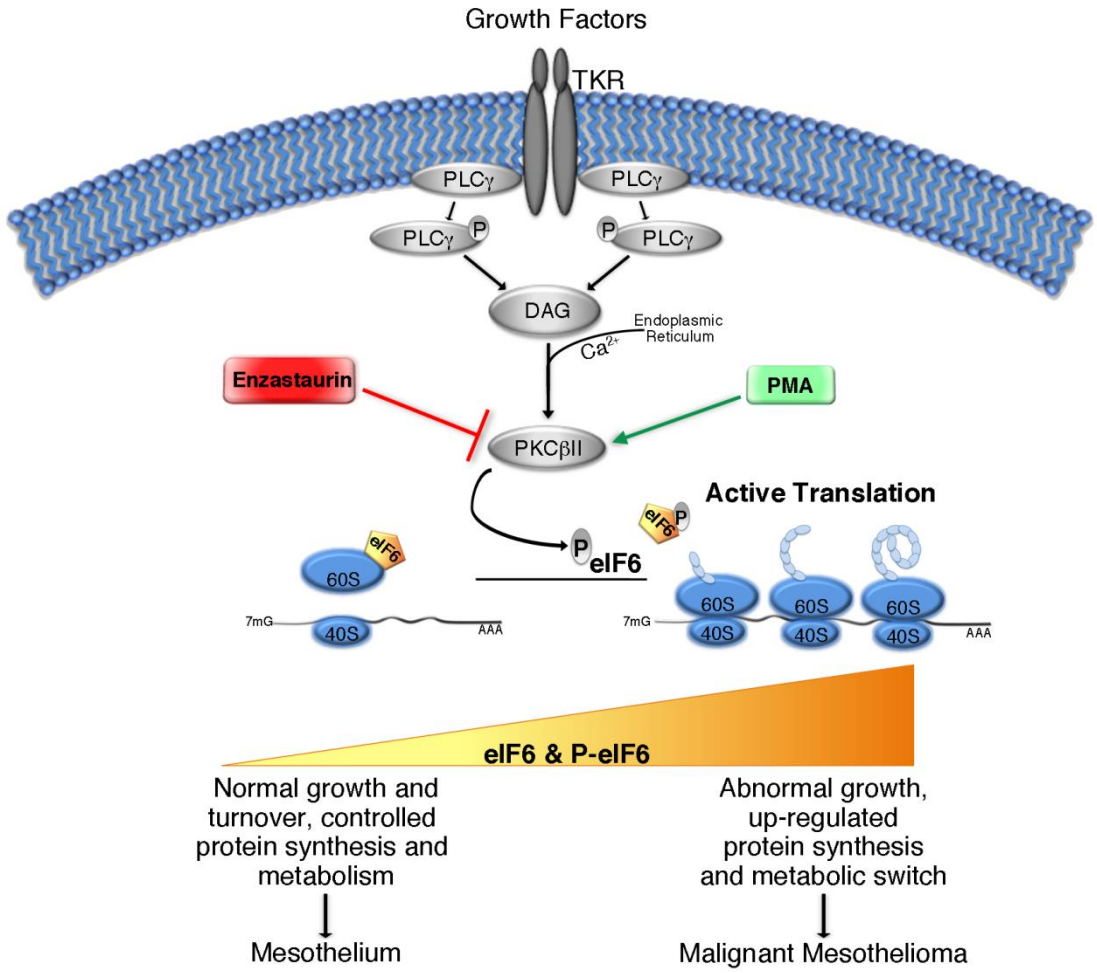


Figure 26. Simplified graphical summary of eIF6 activity in Malignant Pleural Mesothelioma. The translational rate increases during tumorigenesis. eIF6 expression and its activity could modulate protein synthesis, cell growth and metabolic status: in malignant mesothelioma, contribution of both eIF6 hyperexpression and eIF6 hyperphosphorylation improves protein synthesis, aerobic glycolysis and impaired cellular growth, giving rise to tumor development and malignancy.

4.2 microRNAs subcellular distribution in MPM

4.2.1 microRNAs association with polysomes define the subcellular distribution of miRNAs

We hypothesize that miRNAs localization is essential to mediate oncogenic effect. In order to investigate which mRNAs are translationally regulated, we studied miRNAs association with polysomes using a density gradient sedimentation followed by high-throughput analysis of microRNAs in different fractions of the gradient, as depicted by Molotski and Soen. This

protocol extends the method of profiling mRNA association with polysomes (Arava 2003; Hendrickson, Hogan et al. 2009; Melamed, Eliyahu et al. 2009) to make it applicable to microRNAs (Molotski and Soen 2012). Performing polysomal profiles on REN cells, we collected the ribosome free (unbound) fraction into one pool and the lighter and heavier polysomal fractions into another two pools (Fig.27). We then isolated RNA from each pool and measured the levels of 799 miRNAs by nanoString nCounter system (nanoString, Seattle, Washington, USA). Profiling of miRNAs distribution in the translational machinery of REN cells indicates that 8% of miRNAs analysed are expressed in the polysomal pool. Particularly some miRNAs present a different expression between subpolysomal and polysomal subsets (Figure 28). We hypothesize that miRNAs localization is essential to mediate their oncogenic function.

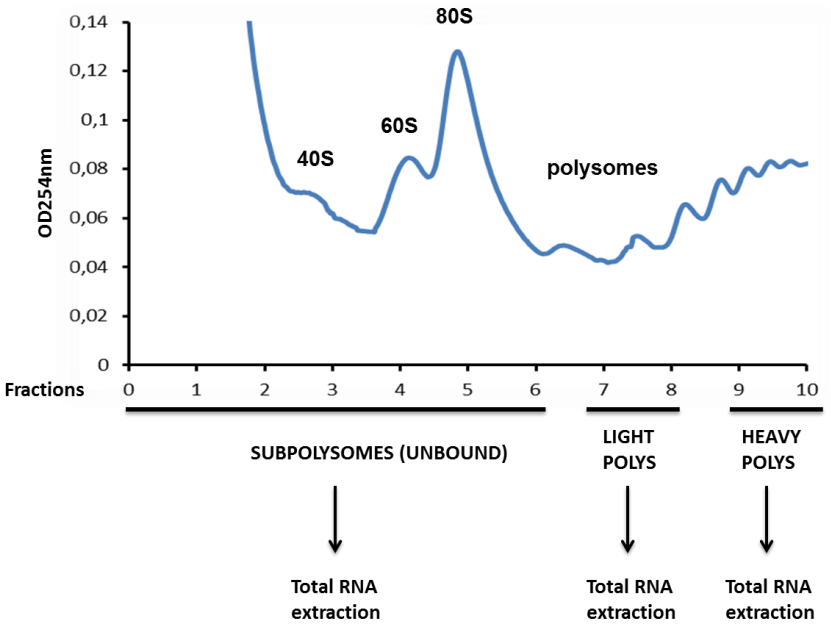


Figure 27. Schematic of approach: analysis of microRNAs association with polysomes. We performed a polysomal profile and collected ribosome free (subpolysomes) and polysomes fractions. We pooled fractions as indicated, and isolated total RNA from each pool. RNA extracted has been processed by nCounter System for miRNA profiling analysis.

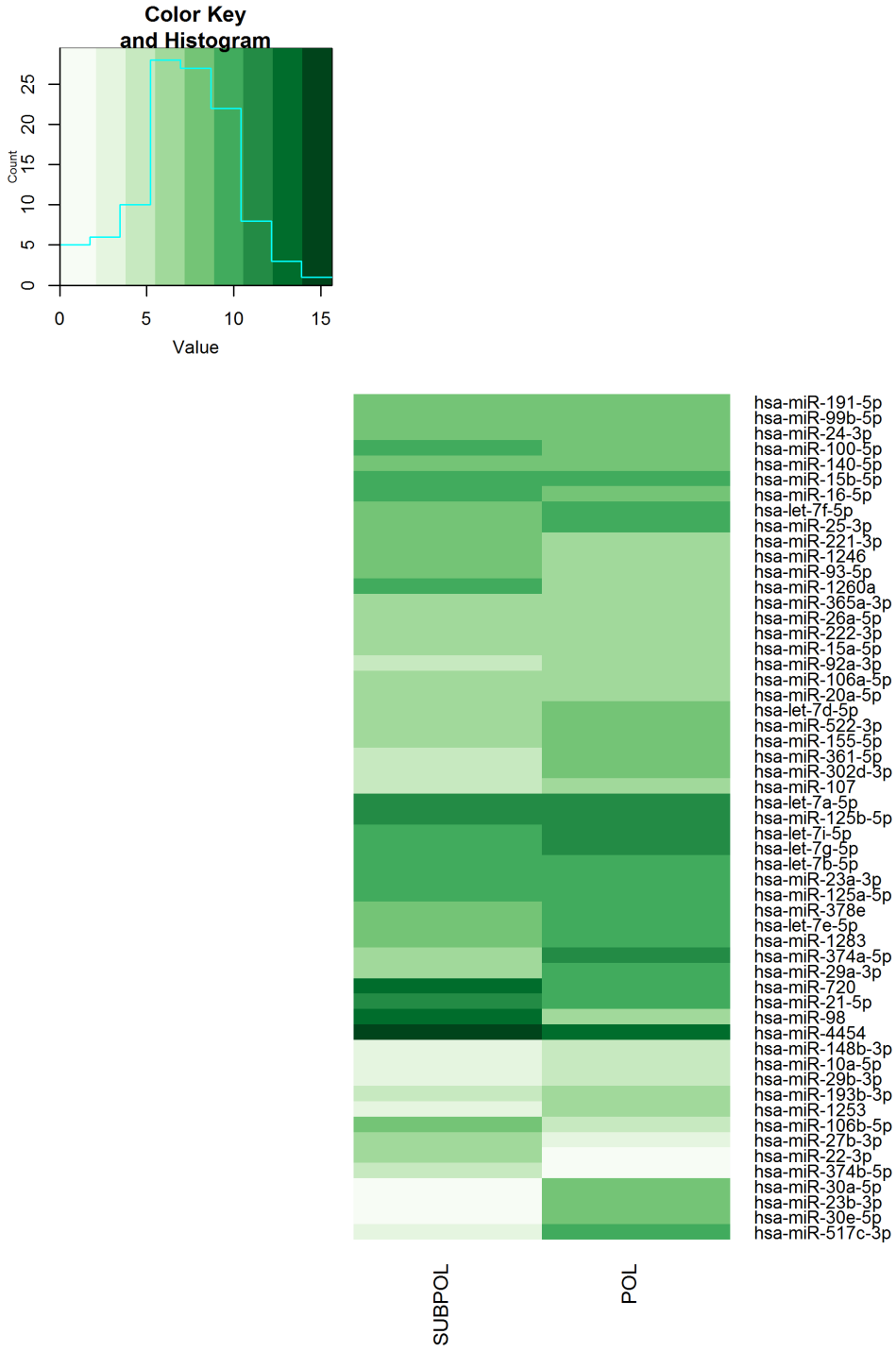


Figure 28. Subcellular distribution of microRNAs in REN cells. Heatmap of microRNAs expressed in subpolysomal and polysomal fractions, in REN cells. We show that only 8% of miRNAs analysed are expressed in the polysomal pool and that some miRNAs present a different expression between subpolysomal and polysomal subsets.

4.2.2 microRNAs exhibit different ratios of association with polysomes

The measurement of miRNAs polysomal association does not indicate the amount of microRNA in polysomes relative to its total amount in terms of quantitation, and the tendency of microRNAs to associate with the translational pool remains unknown as for the factors that influence this association. Our analysis reveals that five miRNAs are enriched on polysomes fractions. In order to try to give a measure of polysomal association, we calculated the microRNA polysome occupancy that denotes microRNA preferences for low, medium, or high association with polysomes. miRNAs occupancy is calculated by dividing each miRNA normalized level in the polysomal pool by the sum of its normalized levels in all the three pools (see fig. 27). We asked if miRNAs occupancy depends from miRNAs expression level in REN cells. Performing a correlation analysis, we found that miRNAs occupancy does not correlate with miRNAs expression level in REN cells, and that the bulk of miRNAs shows a medium occupancy (figure 29).

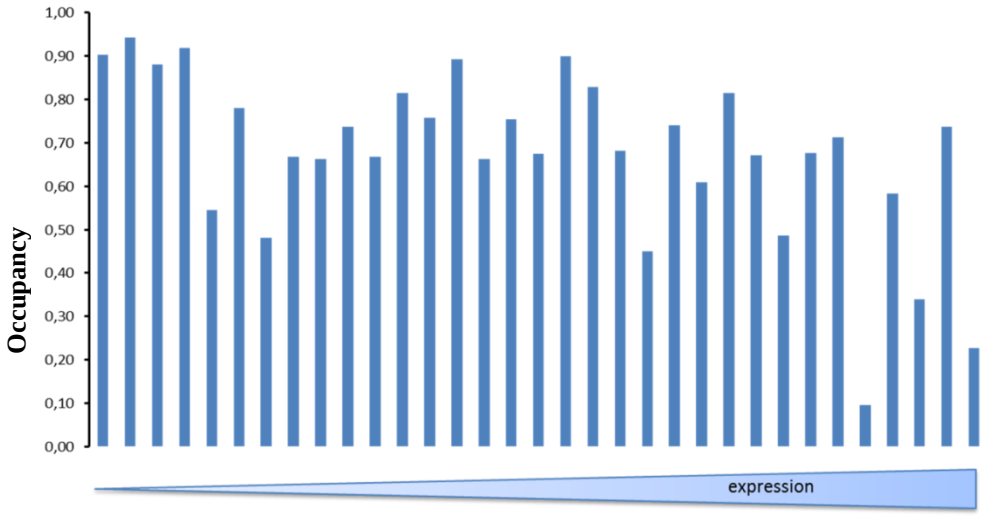


Figure 29. miRNAs occupancy does not correlate with miRNAs expression level in REN cells Correlation analysis of miRNAs occupancy and expression levels in REN cells reveals that the bulk of miRNAs shows a medium occupancy, calculated by dividing each miRNA normalized level in the polysomal pool by the sum of its normalized levels in all the three pools. There is not a correlation between occupancy and expression levels in REN cells.

As the results obtained could be caused by random cosedimentation of polysomes and other rapidly sedimenting structures, extracts were pretreated with 30 mM EDTA, to dissociate ribosomes into subunits. As expected, this treatment disrupted the polysomes and resulted in a corresponding accumulation of 40S and 60S ribosomal subunits, as illustrated in figure 30.

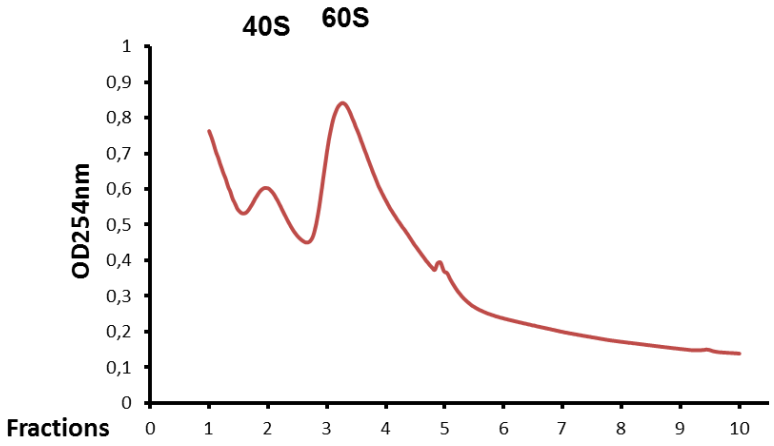


Figure 30. EDTA treatment causes the dissociation of ribosomes in single monosomes. Polysomal profile of REN cells after EDTA treatment: polysomes are completely distrupted and there is an accumulation of 40S and 60S ribosome subunits

Concomitant with polysomes disaggregation, few miRNAs results EDTA insensitive, but most miRNAs are sensitive to EDTA treatment and shifted from heavy fractions to the top of the gradient, indicating that sedimentation of the miRNAs in the polysomal regions of gradients was not a result high molecular weight particle (figure 31).

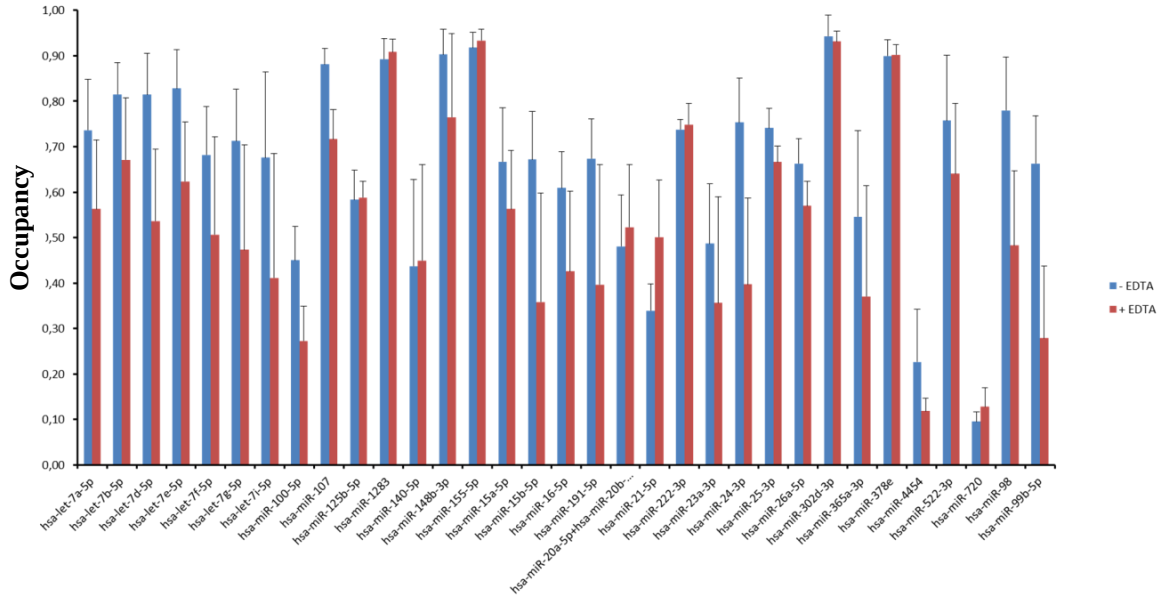


Figure 31. miRNAs occupancy reflects cosedimentation with polysomes in REN cells. After EDTA treatment we calculated miRNAs occupancy and we show most miRNAs are sensitive to EDTA treatment, although few miRNAs are insensitive

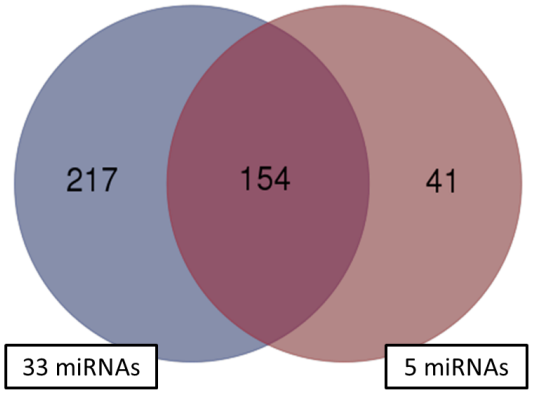
Particularly, five miRNAs, let-7g-5p, miR-15b-5p, miR-16-5p, miR-191-5p and miR-24-3p show a high statistically significative difference of occupancy, EDTA-dependent. We hypothesize that the enrichment of miRNAs on polysomes could reflect their cosedimentation with actively translated mRNAs.

4.2.3 Cell cycle pathways are related to miRNAs which are associated to polysomes

We asked if the five polysomes associated miRNAs, and EDTA-sensitive, are related to common pathways or not. We relied to a bioinformatics analysis to disclose if all five miRNAs polysomes associated are able to target genes governing the same pathway. To this end we used miRSystem database [*http://mirsystem.cgm.ntu.edu.tw/*] to perform a functional annotation of all miRNAs expressed in the polysomal pool compared to the 5 polysome-bound miRNAs. This study reveals that 217 pathways are specific for all miRNAs and 41 pathways are specific for the polysome-bound subset (Figure 32A). As shown in figure 32B,

the 41 pathways indicated are specifically targeted by miRNAs that result associated to polysomes. Notably, it is evident that several components of cell cycle pathway are related to these microRNAs enriched on polysomes.

A



B

PATHWAY	P-VALUE
REGULATION_OF_RETINOBLASTOMA_PROTEIN	3,80E-06
PROGESTERONE-MEDIATED_OOCYTE_MATURATION	2,59E-04
LYSOSOME	7,55E-04
RACCYCD_PATHWAY	3,90E-04
T_CELL_RECEPTOR_SIGNALING_PATHWAY	7,30E-05
ACUTE_MYELOID_LEUKEMIA	4,72E-04
PANCREATIC_CANCER	1,54E-03
GLYCEROPHOSPHOLIPID_METABOLISM	1,11E-02
MAPK_PATHWAY	2,82E-04
G0_AND_EARLY_G1	3,23E-04
E2F_TRANSCRIPTION_FACTOR_NETWORK	4,11E-04
G1_PATHWAY	5,54E-04
CHRONIC_MYELOID_LEUKEMIA	7,88E-03
B_CELL_RECEPTOR_SIGNALING_PATHWAY	8,86E-03
SMALL_CELL_LUNG_CANCER	1,42E-02
ATR_SIGNALING_PATHWAY	1,36E-02
G2_M_CHECKPOINTS	7,14E-02
IL3-MEDIATED_SIGNALING_EVENTS	2,55E-02
TCR_SIGNALING	6,67E-02
CYCLIN_A_CDK2-ASSOCIATED_EVENTS_AT_S_PHASE_ENTRY	1,92E-02
CYCLIN_E_ASSOCIATED_EVENTS_DURING_G1_S_TRANSITION	1,82E-02
SIGNALING_BY_FGFR	4,36E-02
DOWNSTREAM_TCR_SIGNALING	1,02E-01
SHIGELLOSIS	4,93E-02
SIGNALING_BY_EGFR	8,40E-02
DOWNSTREAM_SIGNAL_TRANSDUCTION	1,22E-01
P53-INDEPENDENT_DNA_DAMAGE_RESPONSE	1,02E-01
P53-INDEPENDENT_G1_S_DNA_DAMAGE_CHECKPOINT	1,02E-01
UBIQUITIN_MEDIATED_DEGRADATION_OF_PHOSPHORYLATED_CDC25A	1,02E-01
DOWNSTREAM_SIGNALING_OF_ACTIVATED_FGFR	1,37E-01
LEUKOCYTE_TRANSENDOTHELIAL_MIGRATION	2,27E-01
VIP_PATHWAY	1,18E-01
ACTIVATION_OF_ATR_IN_RESPONSE_TO_REPLICATION_STRESS	1,74E-01
GENERATION_OF_SECOND_MESSENGER_MOLECULES	1,98E-01
CLASS_I_PI3K_SIGNALING_EVENTS	2,18E-01
N-CADHERIN_SIGNALING_EVENTS	1,69E-01

Figure 32. Pathway prediction related to polysomes associated miRNAs indicates correlation with the cell cycle. A) Using miRSystem database *[http://mirsystem.cgm.ntu.edu.tw/]*, the functional annotation of miRNAs expressed in the polysomal pool compared to 5 polysome-bound miRNAs reveals that 217 pathways are specific for all miRNAs and 41 pathways are specific for the polysome-bound subset. **B)** We evidenced pathways targeted by the 5 miRNAs bound to polysomes and we releaved that cell cycle components (marked in green) are related to miRNAs polysomes associated

Reid et al. reported that expression of the miR-15 family was consistently downregulated in MPM tumour specimens and cell lines. Growth inhibition caused by miR-16 correlated with downregulation of target genes including Bcl-2 and CCND1 (Reid, Pel et al. 2013). The miR-15/16 family is downregulated and has tumour suppressor function in MPM. Taken together these data suggest that miRNAs polysomes localization could mediate their oncogenic or tumor suppressor function, regulating the expression of genes involved in cell cycle or tumorigenesis.

5 DISCUSSION

Translation is a cellular process finely regulated during growth and development and it is deregulated in cancer cells. It has been shown that eukaryotic Initiation Factor 6 (eIF6) is a limiting factor in tumorigenesis, *in vivo*, regulating the availability of active 80S subunit, and that it is a key mediator in miRNA-mediated translational repression. It has been reported that it can associate with miRISC and that eIF6 knockdown abrogates miRNA-mediated regulation of target protein (Chendrimada, Finn et al. 2007). In this thesis we developed two research lines, with a common relevant factor, the translational regulation in malignant pleural Mesothelioma. We analyze the role of eIF6 in MPM growth and propose it as a new target of kinase inhibitors, and the subcellular distribution of microRNAs, particularly their association with actively translating polysomes.

5.1 eIF6 in Malignant Pleural Mesothelioma

We show that eIF6 is overexpressed and activated in malignant pleural mesothelioma, and that inhibition of its activity or phosphorylation reduces tumor burden and tumor growth. Our data further establish the deregulation of the translational machinery in mesothelioma cells, suggesting that this tumor is peculiar in its capability to sustain translation, being insensitive to inhibition of the mTOR pathway (Ou, Moon et al. 2015). We will discuss our findings according to three lines: 1) the relevance for malignant mesothelioma, 2) the significance and feasibility to target eIF6, and 3) the molecular mechanism which may account for the increased eIF6 expression in mesothelioma. Malignant mesothelioma is distinguished into three morphological phenotypes, epithelial, sarcomatous and biphasic. Most tumors arise in the pleura and are epidemiologically linked to asbestos exposure. However, peritoneal mesothelioma also occurs, it is very rare and does not correlate with asbestos exposure

(Carbone, Ly et al. 2012). Malignant mesotheliomas originate as polyclonal tumors (Comertpay, Pastorino et al. 2014). Genetic analysis of abnormalities has displayed an heterogeneous mutational landscape with three predominating lesions, CDKN2A, NF2, BAP1 (Sekido 2013). Thus, Malignant Pleural Mesothelioma can be classifying as a highly heterogeneous cancer. What is not heterogeneous is the (non) response to therapy. MPM is generally found to be resistant to conventional forms of therapy, such as cisplatinum and pemetrexed combination chemotherapy (Belli, Fennell et al. 2009). Therefore, tumor heterogeneity is the most relevant obstacle for applying targeted therapies to mesothelioma and conventional therapies do not work. Since the components of the translational apparatus integrate different oncogenic pathways, targeting its components may overcome the difficult of tumor heterogeneity. Moreover, tumoral cells exhibit an increase of the translational machinery suggesting "addiction" to high protein synthesis (Ruggero 2013). The fact that eIF6 is overexpressed and hyperphosphorylated in "MPM" suggests that it may be a good target.

There is substantial body of evidence that eIF6 is rate-limiting for cancer cells. First, overexpression of eIF6 is a driver of cancer. Enlargement of eIF6 containing nucleoli is a feature of aggressive colorectal tumors. Soft agar assay of $eIF6^{+/-}$ mouse embryonic fibroblasts transduced with dominant negative p53 tumor suppressor plus H-rasV12 or with Myc plus H-rasV12 display a 70% reduction in transformed colonies, compared to the $eIF6^{+/+}$ mouse fibroblasts (Gandin, Miluzio et al. 2008). The tumorigenic potential of eIF6 is evident in a mouse model of lymphomagenesis, *in vivo*. In this model, expression of the Myc oncogene under the control of the enhancer of IgH (Eμ-Myc) in the B cells drives a lethal lymphoma, similar to B-cell lymphomas, with a median survival of only 4 months. Eμ-Myc/ $eIF6^{+/-}$ mice show increased survival, and do not have overt negative phenotypes. Even in the $p53^{-/-}$ genotype, where p53 deletion accelerates lymphomagenesis due to suppression of

apoptosis (Post, Quintas-Cardama et al. 2010), eIF6 depletion delays tumor development. Moreover, eIF6 is amplified in breast luminal cancers (Gatza, Silva et al. 2014). The phosphorylation of Ser235 residue on eIF6 is important for cancer development and transformation. eIF6 is controlled by the RACK1/PKCβ axis and led to initiation of translation. PRKCB is a target in lymphomas, and is expressed in mesotheliomas (Faoro, Loganathan et al. 2008). In our lab it has been showed that inhibition of eIF6 phosphorylation by genetic inactivation of Ser235 is a strategy to block eIF6 activity. Since eIF6 phosphorylation is driven by the PKCβ axis, we proceeded to inhibit its activity with Enzastaurin (LY317615). Enzastaurin is an FDA approved potent and selective inhibitor of PKCβ; it exerts its antitumor effects by indirectly blocking tumor induced angiogenesis and by suppressing tumor cell proliferation and inducing apoptosis (Schwartzberg, Hermann et al. 2014). Here we display that reducing eIF6 levels or treating cells with Enzastaurin, reduces MPM cell line growth, *in vitro*, and angiogenesis and tumor development *in vivo*, in an immunocompromised murine model. Questions are still open: in the long run eIF6 inhibition by Enzastaurin may not be effective because eIF6 has multiple phosphorylation sites in its C-terminus, yet poorly characterized. Alternative strategies may be therefore required. Then, we have shown that eIF6 activity in cancer is necessary for dissociating inactive 80S subunits. In this context, several point mutations of eIF6 change the efficiency of eIF6 binding to 60S. Recent work from (Pesce, Minici et al. 2015) has led to the development of an antiassociation assay which may be used for screening inhibitors of eIF6 function, by small compounds chemical libraries. Similar approaches have been successful for other translation factors like eIF4G. Cancer cells are able to alter and reprogram their metabolism to acquire advantage for developing tumor. This alteration, called Warburg effect, consists of an increase in aerobic glycolysis, in conditions of high oxygen tension, and gives rise to augmented lactate production and ATP generation. Moreover, as well as producing more energy, tumor cells

increase lipids synthesis to build membranes during oncogenesis. In our lab, we had developed a transgenic mouse model where eIF6 heterozygous mice had approximately 50% of reduction of eIF6 protein. In this *in vivo* model, we found that eIF6 translational activity directs lipogenic program through the upregulation of enzymes involved in cholesterol and fatty acid synthesis. Specifically, the efficient translation of Fatty Acid Synthase (FASN), the key-player of de novo lipogenesis, is strictly correlated to eIF6 protein levels (Brina, Miluzio et al. 2015). In this thesis, we found that eIF6 depletion and its inactivation by Enzastaurin treatment significantly impair lactate and ATP production in MPM cells. Therefore, all these metabolic effects could partial justify the anti-cancer role of eIF6 inhibition. These data are intriguing and are in agreement with the observation that mutation of eIF6 Ser235 to Ala greatly reduces cancer growth *in vivo*, more efficiently than *in vitro.* Since the effects of eIF6 depletion on polysomal accumulation are significant, but modest, we expect that specific mRNAs might be regulated by eIF6 activity, at the translational level in REN cells. It will be of particular interest applying to mesothelioma tissues novel technologies as ribosome profiling, in order to isolate them. In conclusion of the first part of this work, we suggest that modulation of eIF6 levels and activity may lead to a therapeutical strategy in tumor therapy, especially where eIF4E inhibition by rapalogs is not effective, as in MPM.

5.2 microRNAs in MPM

Several studies demonstrated that miRNA expression in MPM is highly variable (Truini, Coco et al. 2014). Early studies profiling the microRNAs expression in MPM identified many changes which affect the reduced response to apoptotic signals, rates of metabolism and proliferation, enhanced migration and invasion (Guled, Lahti et al. 2009; Busacca, Germano et al. 2010). Particularly, it has been demonstrated that overexpression of miR-29c-5p,

downregulated in MPM cell lines, is able to inhibit proliferation and invasion of MPM cell lines, in vitro (Pass, Goparaju et al. 2010). Moreover miR-31 expression is reduced in MPM cell lines, simultaneously with deletion of the CDKN2A gene (Ivanov, Goparaju et al. 2010). Re-expressing miR-31 led to reduced proliferation, migration and invasion by cell cycle arrest. Another miRNA with inhibitory effect on proliferation in MPM is let-7a that acts attenuating RAS signaling (Johnson, Grosshans et al. 2005); its tumor suppressor functions was demonstrated in lung cancer. The miR-15 family has also been shown to be downregulated in MPM (Aqeilan, Calin et al. 2010), regulating cell cycle and anti-apoptotic genes. The members of this family (miR-15a, miR-15b, miR-16 and miR-195) were found at significantly lower level in MPM samples (Reid, Pel et al. 2013). First, because the association of eIf6 with miRISC has been seen and it has been demonstrated that it is able to disrupt polysomes formation through the inhibition of 80S complex assembly and,second, since the knockdown of eIF6 abrogates miRNA-mediated regulation of target protein and mRNA levels (Chendrimada, Finn et al. 2007), we decided to identify the localization of miRNAs in RNA subpopulations in MPM. The last 5 years has seen a rapid advance in the study of the role of microRNAs in MPM biology, linking them to MPM growth, invasion, migration and drug resistance, but the actual knowledge led to conflicting data. Analysis of sublocalization of miRNAs could therefore give us an idea of how the little RNAs regulate their target genes. We have shown that, at steady state, not all miRNAs expressed in exponentially REN cells cosediment with polyribosomes, but only 8%; in addition most of them are sensitive to EDTA presence. The observed localization of miRNAs on polysomes is consistent with several previous studies. The mRNA targets of the members of the miRNA family in *Caenorhabditis elegans* were shown to be in polysomes (Seggerson, Tang et al. 2002), and subsequent studies showed that the bulk of miRNAs in *Drosophila melanogaster* cells and in *C. elegans* sediment with ribosomes (Caudy, Ketting et al. 2003). Today, it is

unclear whether microRNAs have distinct tendencies for associating with polysomes and whether these tendencies are influenced by properties of the microRNA or the cellular context. In our work we measure polysome occupancy of microRNAs, that represents the high, medium or low preference of a miRNAs to associate with polysomes, and we showed that the bulk of miRNAs have a medium occupancy. We don't know, actually, if the observed preference of individual microRNAs for association with polysomes is similar in all MPM cell lines, and if it is independent of the abundance of the microRNAs. Previous work in HeLa cells reported copurification with polysomes of three randomly chosen microRNAs (let-7, mir-21, and miR-16) and hypothesized that association with polysomes is general for most microRNAs (Maroney et al. 2006). Molotoski and Soen showed, instead, that the degree of microRNA association with polysomes is microRNA-specific (Molotski and Soen 2012). Here we showed also that there is not a correlation between miRNA occupancy and their cellular expression level, and we evidenced 5 miRNAs (let-7g-5p; miR-15b-5p; miR-16-5p; miR-191-5p; miR-24-3p) that show a significative difference of occupancy after treatment with EDTA, suggesting that this cosedimentation could result from the association of miRNAs with their target, and not with the RNPs. In particular, analyzing the common predicted target genes of these 5 miRNAs we evidence that the cell cycle pathways are related to miRNAs which are associated to polysomes, confirming the study performed by Reid et al, which link miR-15 family to Bcl2 and anti-apoptotic genes in MPM. Since the significance of association of microRNAs with polysomes has been hypothesized to reflect involvement in translation repression, we can hypothesize that the evidenced miRNAs could regulate translation of cell cycle genes binding mRNA actively translated in MPM. Further studies are required to address this question.

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