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Molecular description of the role of protein synthesis in the cellular response to the treatment with the antitumoral drug Sorafenib in HCC lines.

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RESUMEN

El carcinoma hepatocellular (CHC) es reconocido como el principal cáncer primario de hígado y uno de los cánceres con mayor incidencia y mortalidad a nivel mundial. Éste se desarrolla en un contexto de enfermedad hepática crónica donde la aparición, así como la progresión de la enfermedad, está influenciada por numerosos factores de riesgo. Desafortunadamente, el alto grado de heterogeneidad genética intratumoral dificulta el desarrollo de terapias verdaderamente eficaces. Sorafenib fue el primer fármaco aprobado por la FDA (*Food and Drug Administration*) para su uso como tratamiento en pacientes avanzados de CHC. A pesar de que otras terapias hayan sido recientemente aprobadas para su uso como primera línea de tratamiento en dichos pacientes, Sorafenib sigue siendo la opción recomendada para un porcentaje significativo de ellos. Este fármaco es un inhibidor multiquinasa con efectos anti-proliferativos, anti-angiogénicos y pro-apoptóticos. Desgraciadamente, Sorafenib cuenta con unos beneficios clínicos muy limitados ya que por un lado produce numerosos efectos secundarios y por otro, el desarrollo de resistencia al tratamiento es muy frecuente en esta enfermedad. Otras terapias basadas en diferentes inhibidores multiquinasa han sido estudiadas en ensayo clínico, pero desgraciadamente, han mostrado una eficacia inferior o igual al Sorafenib, sugiriendo que la efectividad clínica del fármaco podrías estar basada en efectos adicionales a su acción inhibidora de la actividad quinasa.

El proceso de síntesis de proteínas, también conocido como traducción, consiste en la formación de nuevas cadenas polipeptídicas a partir de la información contenida en una molécula de RNA mensajero (mRNA). Dicho proceso es mediado por el ribosoma que, junto con numerosas proteínas llamadas factores de traducción, sintetizan las proteínas *de novo* en función de la demanda celular. El proceso de traducción constituye el principal punto de regulación de la expresión génica, donde los niveles del denominado complejo dependiente de cap, así como los niveles de fosforilación del factor de iniciación eIF2α (eIF2α) son los principales puntos de control. Adicionalmente, existen otros mecanismos de control menos frecuente que, junto con los anteriores, permiten a las células responder rápidamente a los cambios ambientales en un proceso conocido como reprogramación traduccional. La actividad aberrante del proceso de traducción está estrechamente vinculada a la transformación tumoral, así como a la resistencia celular a diversos tratamientos. De hecho, la hiperactivación de la actividad traduccional es considerada un sello del cáncer, donde la reprogramación traduccional contribuye a la alta plasticidad celular característica de las células tumorales.

Sorafenib inhibe una de las principales vías de regulación de la síntesis de proteínas, la ruta de las MAPKs, la cual regula la actividad del complejo dependiente de cap. Asimismo, existen evidencias de un aumento de la fosforilación de eIF2α como consecuencia de la acumulación de proteínas mal plegadas en el retículo endoplásmico (ER) inducida por Sorafenib. Sin embargo, a pesar de las

numerosas evidencias sobre la inhibición de la traducción por Sorafenib, y de los numerosos estudios que ponen en valor el proceso de síntesis de proteínas durante la transformación y progresión tumoral, el papel de la traducción en la respuesta celular al Sorafenib no ha sido estudiado con la suficiente profundidad.

Esta tesis doctoral ha tenido como objetivo detallar el efecto de Sorafenib sobre la síntesis de proteínas, así como descifrar la relevancia biológica de este proceso entre las actividades tumorogénicas del fármaco. En primer lugar, nuestros resultados indican que Sorafenib afecta a la actividad de la maquinaria traduccional a distintos niveles: por un lado, afecta a la actividad del complejo dependiente de cap y por otro, induce la fosforilación de eIF2α. En segundo lugar, destacamos que la relevancia de cada mecanismo afectado difiere a lo largo del tiempo del tratamiento. Así pues, nuestros resultados muestran que, Sorafenib inicialmente altera la actividad del complejo dependientede cap, inhibiendo de forma específica la síntesis de un subconjunto de mRNAs y que, posteriormente, esta inhibición se extiende a un mayor rango de mRNAs tras la inducción de la fosforilación de eIF2α. Finalmente, parte de nuestras investigaciones estuvieron encaminadas a descifrar la reprogramación traduccional inducida por Sorafenib. Así pues, hemos realizado un mapa de traducción que muestra: (i) los mRNAs que se traducen en un contexto en el que la síntesis global de proteínas se encuentra inhibida; (ii) los procesos celulares en los que estos mRNAs están involucrados; y por último, (iii) los mecanismos que permiten su traducción.

En conclusión, los resultados aquí presentados tratan de poner en relieve el papel de la síntesis proteica en la respuesta celular a Sorafenib. En primer lugar, basándonos en la inhibición selectiva de la traducción de mensajeros pro-tumorales como consecuencia de la desregulación de la actividad del complejo dependiente de cap, consideramos que la inhibición de la traducción posiblemente contribuye a los efectos anti-tumorogénicos del fármaco. Así pues, el uso de inhibidores de la maquinaria traduccional en combinación con Sorafenib podría resultar un enfoque terapéutico interesante para el tratamiento del CHC. En segundo lugar, proponemos que el estudio de la reprogramación traduccional inducida por Sorafenib podría abrir paso a nuevas líneas de investigación orientadas a predecir la respuesta al tratamiento, descifrar posibles mecanismos involucrados en la resistencia al fármaco y diseñar biomarcadores de pronóstico y de respuesta al tratamiento.

INTRODUCTION

Hepatocellular carcinoma

Liver cancer is the most common cancer-related death globally and the sixth most commonly diagnosed cancer worldwide. Hepatocellular carcinoma (HCC) represents the most common type of primary liver cancer and accounts for 90% of cases. This cancer occurs mainly in a context of chronic liver disease and its appearance and progression are strongly influenced by a wide range of factors. It is estimated that HCC will become the third leading cause of cancer-related death by 2030, thus, making it a global health challenge (1, 2).

Pathogenesis

HCC is a complex multistep disease that occurs in the setting of chronic liver disease, being cirrhosis -from any aetiology- the main risk factor. The contribution of different aetiologies to HCC incidence varies between countries and regions, which translates into a heterogeneous incidence worldwide. The most determinant risk factors include chronic alcohol consumption abuse, diabetes, non-alcoholic fatty liver disease, (NAFLD), non-alcoholic steatohepatitis (NASH) and infection by Hepatitis C (HCV) or Hepatitis B (HCB) viruses. The chronic impact of the above risk factors is associated with a sustained inflammatory response and fibrosis in the liver, leading to cirrhosis. This is frequently accompanied by progressive liver dysfunction and an appropriate microenvironment for the mutation burden to spread, eventually leading to HCC development. The prevalence of the risk factors for HCC varies globally and, in many cases, the risk of developing HCC is a multifactorial and includes demographic factors like age or sex, the severity and activity of underlying disease, metabolic factors, and lifestyle factors. The diagnosis of HCC occurs mainly in people aged between 60-70 years, and predominantly affects men (3:1) (3, 4) (**Figure 1 A**,**B**).
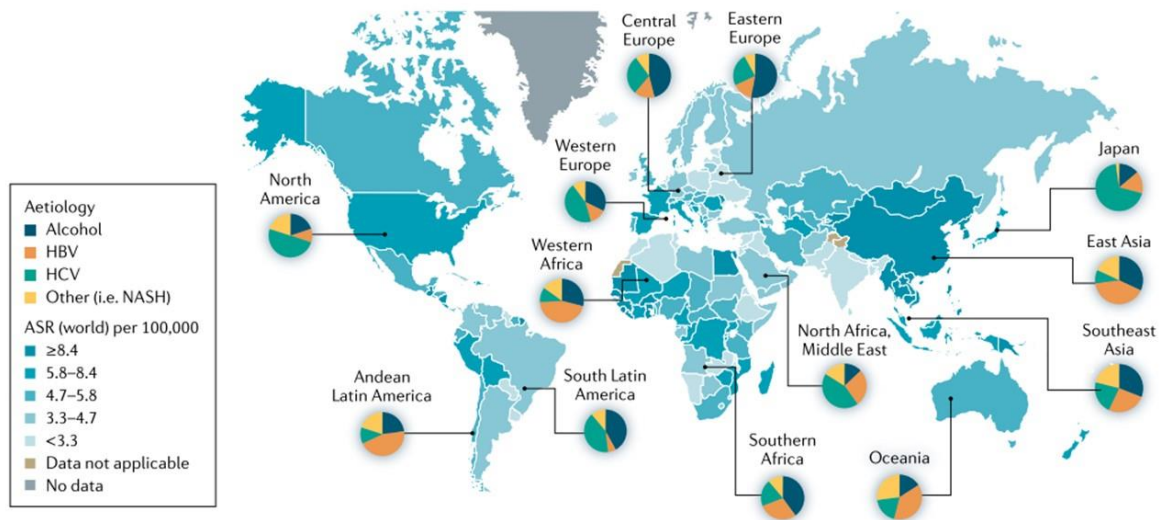
Figure 1. Aetiologies and incidence of HCC. The picture depicts the incidence and the main aetiological factors involved in hepatocarcinogenesis. ASR (age-standardized incidence rate); HBV (Hepatitis B Virus); HCV (Hepatitis C Virus) (1).

The molecular events that underlie HCC formation are very complex, with numerous intracellular signalling pathways affected that negatively impact the survival of patients in advanced stages. A high-throughput next-generation sequencing technique has enabled the identification of cancer driver genes that are frequently altered in HCC (5, 6). Telomerase reverse transcriptase (TERT) is the most frequent mutated gene observed in HCC. Other driver genes with an aberrant expression are usually found, including the tumour protein TP53 and members of Wnt-signalling pathways like CTNNB1 (β-catenin) and AXIN1. The Wnt-signalling pathway controls cell differentiation through proper regulation of cell proliferation and migration (7). However, it is still unclear whether the Wnt-signalling pathway acts as a driver or as a collaborator with other promoting factors in HCC development (8). The mutation profile impacts the clinical staging and survival of the patient in association with the progression of the underlying liver disease (9). Nowadays, numerous multi-scale studies are being conducted towards developing a multi-platform that will provide us with a better understanding of the potential molecular targets that may lead to improved therapeutic strategies and the identification of biomarkers (**Figure 2**).
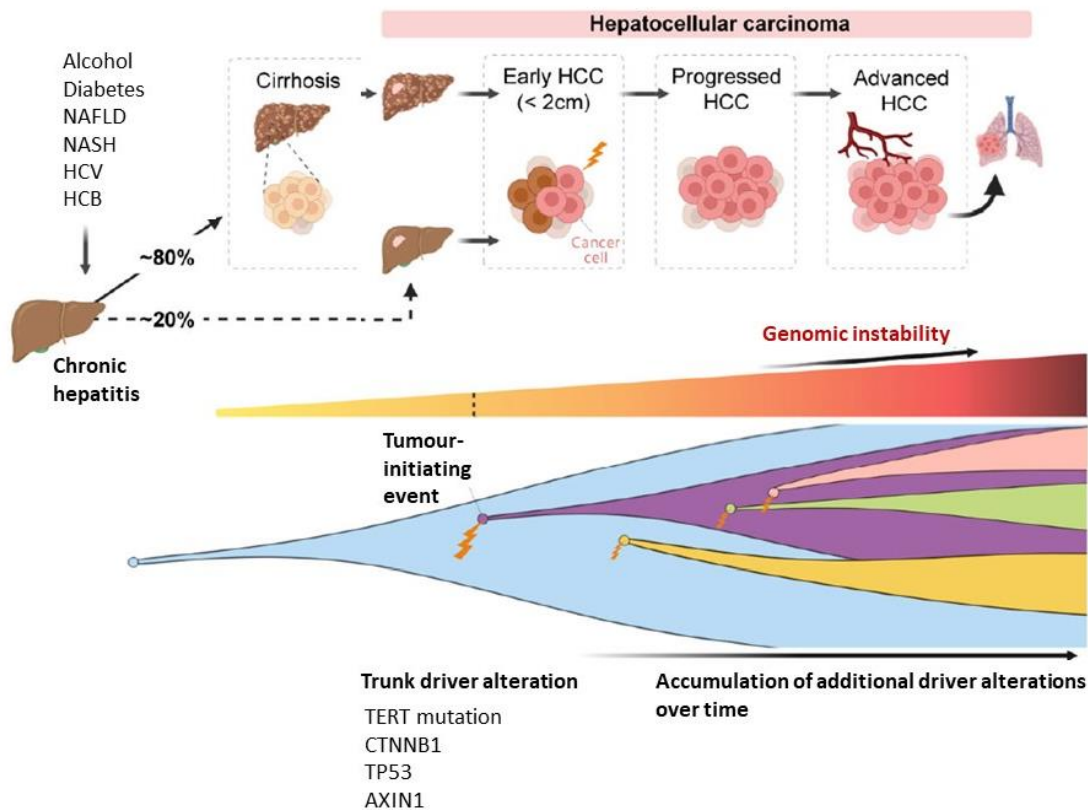
Figure 2. Molecular pathogenesis of HCC. The image depicts the appearance and progression of HCC. The development of HCC occurs mainly in a context of chronic liver disease being cirrhosis the main risk factor. Numerous risk factors are involved in the appearance of the disease like alcohol consumption, diabetes, NAFLD, NASH, HCV or HCB. Some driver mutations have been identified where TERT, TP53, CTNNB1 and AXIN1 are the most frequents for this disease. The progression of HCC is linked to accumulation of a wide variety of mutations causing genomic instability. HBV (Hepatitis B Virus); HCV (Hepatitis C Virus); NASH (Non-alcoholic steatohepatitis); NAFLD (Non-alcoholic Fatty liver disease) (Adapted from (10)).

Staging, Diagnosis and Treatment

The management of patients with HCC involves a complex decision-making process, taking into account different clinical parameters, including tumour extent, patient comorbidities and the severity of liver dysfunction. Thus, HCC management requires a multidisciplinary team approach to achieve the best outcome.

The diagnosis and staging of HCC are usually based on circulating markers, imaging criteria or tissue biopsy analysis. Nowadays, liver biopsy is used for the clarification of blurring areas of the diagnosis. However, as the risk of bleeding during biopsy is not relevant specially at advanced stages, it is strictly implemented in specific cases and under facultative indications (11). Once the HCC

diagnosis has been done, patients receive the recommended treatment according to a staging system. Currently, the Barcelona Clinic Liver Cancer (BCLC) classification is the most widely used standard staging system for HCC (12). The BCLC system subdivides HCC into five stages and provides estimated median survival periods and recommended treatment for patients at each stage. Hepatic resection and liver transplantation represent the main curative treatment options, but they are only recommended in very early (BCLC 0) and early (BCLC A) HCC stages. BCLC B stages includes patients with multifocal lesions in absence of tumour extrahepatic spread who are regularly treated with chemoembolization. Currently, systemic therapy is the only recommended treatment for advanced stages (BCLC C) and it is estimated that 50% of HCC patients will be exposed to systemic therapy in their lifespan. The survival of patients treated with systemic agents has significantly improved since 2017, and new therapeutic options are continuously being assessed. However, the survival of patients at intermediate and advanced stages remains an important challenge for scientific research. Patients in BCLC D receive palliative treatment (13) (**Figure 3**).
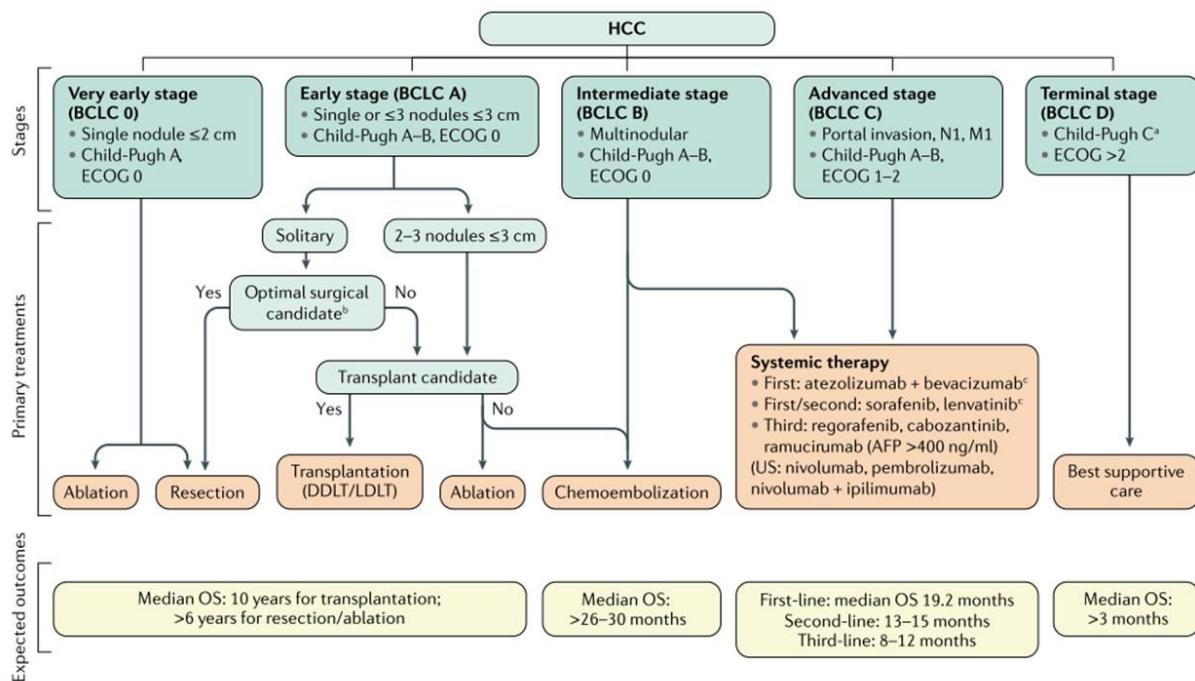
Figure 3. Barcelona Clinic Liver Cancer (BCLC) staging and treatment strategy. The BCLC system classifies the HCC patients into five groups according to different clinical parameters. Among these, size and number of nodules, invasion and the liver function. Liver function should be evaluated according to Child-Pugh classification. Each stage (BCLC 0, BCLC A, BCLC B, BCLC C, BCLC D) is linked to first line treatment recommendation. Asymptomatic patients with low tumour burden and good liver function (BCL 0/A) should be treated with local curative treatment (ablation, resection or transplantation). Asymptomatic patients with multinodular disease and adequate liver function (BCLC B) should receive chemoembolization. Finally, those patient in advance stage (BCLC C) should be treated with systemic therapies. The expected outcome is showed as median survival of each tumour stage according to the

available scientific evidence. AFP (α-fetoprotein); DDLT (deceased-donor liver transplantation); ECOG (Eastern Cooperative Oncology Group); LDLT (living-donor liver transplantation); OS (Overall survival). (Adapted from (1)).

Sorafenib

Sorafenib (Nexavar®, BAY43-9006) was approved by the FDA in 2008 as the first-line treatment for advanced HCC, and it has been the only therapeutic option in these cases for more than a decade (14-16). Although various therapeutic options have recently been approved by the FDA (16-18), Sorafenib treatment is still the appropriate treatment for a significant proportion of HCC patients despite its poor clinical benefit and the numerous adverse effects it produces (12).

Sorafenib is a small-molecule multikinase inhibitor that targets different tyrosine kinase receptors (19). It was the first targeted therapy to show efficacy in advanced HCC patients in prolonging the survival of patients with advanced-stage HCC with a median overall survival of 2-3 months (15, 20). Sorafenib blocks tumour cell proliferation by inhibiting serine/threonine kinase Raf, Raf-1 and B-Raf, leading to the inhibition of mitogen-activated protein kinase/extracellular signal-regulated kinase (ERK) signalling pathway. In addition, Sorafenib inhibits the activity of several tyrosine kinases involved in tumour angiogenesis, including vascular endothelial growth factor receptor (VEGFR) and platelet-derived growth factor receptor (PDGFR). Intriguingly, several other tyrosine kinase inhibitors (TKIs) failed to show efficacy in HCC (21-23), suggesting that mechanisms in addition to kinase inhibition contribute to the activity of Sorafenib. We and others have described that Sorafenib induces tumour apoptosis as well as the potential molecular mechanisms and the sequential events involved in the proapoptotic properties of Sorafenib (24-26).

Targeting protein synthesis in cancer

Protein synthesis, also known as translation, is the cellular process by which the information encoded in the mRNA is used to synthesize protein by the ribosomes. Its dysregulation has been linked to aberrant cell proliferation, survival, angiogenesis, alterations in the immune response, and cancer energetics (27-29). Although dysregulation of protein synthesis is usually associated with the upregulation of the signalling pathways that control this process, like the MAPKs and mTOR pathways, many translation initiation factors are frequently amplified or dysregulated in tumours as well (30-32) (**Table 1**).

Table 1. Dysregulation of factors involved in translation in human cancers. This table shows different examples of translational factors and regulators of translational process that are dysregulated in different human cancers (Adapted from (27)).

Factor	Dysregulation	Clinical impact
eIF4E	Overexpression	Decreased survival in breast, head and neck, liver, prostate, bladder and stomach cancers; correlates with disease progression and aggressive subtypes in many cancers, and with resistance to therapy.
eIF4E	Phosphorylation	Elevated in early stage of development of breast, colon, gastric and lung cancers; Increased in prostate cancer; Poor prognosis marker in non-small-cell lung cancer.
4E-BP1	Overexpression	Inversely correlated with tumour grade. Correlates with better survival in lung and prostate cancers. Correlates with absence of lymph node and distant metastases in gastric cancer.
4E-BP1	Loss	Possible responsible for loss of translational control in the majority of pancreatic tumours.
4E-BP1	Phosphorylation	Correlated with tumour grade and poor prognosis in breast, lung, ovarian and prostate cancers.
eIF4G	Increased expression	Lung, breast and cervical cancer; Correlates with poor prognosis in nasopharyngeal carcinoma.
eIF4A	Increased expression	Lung and cervical cancer
PDCD4	Decreased expression	Linked to poor prognosis in breast, lung, colon and ovarian cancers and gliomas. Inversely correlates with advanced tumour stage in renal cell carcinoma.
eIF2α	Increased expression	Lymphoma
eIF5A	Increased expression	Correlates with poor prognosis in early-onset colorectal cancer. Its overexpression correlates with invasion on non-small-cell lung cancer and hepatocellular carcinoma
eIF6	Altered expression and function	Elevated expression linked to colorectal cancer, head, neck and ovarian serous carcinoma; low expression correlates with reduced disease-free survival in ovarian serous carcinoma.
eIF3a	Increased expression	Breast, cervical, oesophageal, lung and stomach cancers-
eIF3b	Increased expression	Bladder, breast and prostate cancer
eIF3c	Increased expression	Meningioma and testicular seminoma
eIF3h	Increased expression	Breast, colon, liver and prostate cancer
eIF3I	Increased expression	Breast, head, neck, liver cancer and melanoma and neuroblastoma
eIF3m	Increased expression	Colon cancer
eIF3e	Decreased expression	Breast, lung and prostate cancers
eIF3f	Decreased expression	Breast, colon, small intestine, ovarian, pancreatic and vulval cancers and melanoma

Tumour cells are "addicted" to elevated protein synthesis rates and exhibit augmented activity of most of the components of the translation machinery and signalling pathways, whose activities converge on the translation process (33-35). High translational rate is a cancer hallmark and a general feature found throughout the tumour, thereby targeting the component of the translation machinery, which holds promise for overcoming a major hurdle associated with intra-tumour heterogeneity observed in many cancers, including the HCC (36-39).

The role of translation in cancer was discovered several years ago (40-42), but the number of publications studying how the translation plays a role in tumorigenesis has greatly increased over the last few years. Cells are able to control protein production quickly depending on the external environment, promoting or inhibiting the translation of different subsets of mRNAs in a process called translational reprograming. Thus, translation allows the tumour cell to adapt to adverse conditions by coordinating the different cell programs that lead cells to survive (43). Indeed, it has been reported that translation has a pivotal role in cell plasticity, which is defined as the ability of cells to change their phenotypes without genetic mutations in response to environmental cues, a cellular aspect that is highly increased in neoplasia (44). Taking all this into account, several strategies that target the translation process have been devised to treat different neoplasias, and some of them are currently being used in clinic, like asparaginase in acute lymphoblastic leukaemia and paediatric acute myeloid leukaemia (45).

One of the most studied translation-associated proteins in cancer is the eukaryotic translation initiation factor 4E (eIF4E). eIF4E forms a complex, called eIF4F complex, that mediates the canonical translation initiation (46). It has been reported that both the overexpression of eIF4E itself and increased levels of Phospho-eIF4E promote tumorigenesis (47-49) (**Table 1**). Additionally, an aberrant eIF4F complex assembly makes cancer cells resistant to inhibition of MAPKs or PI3K signalling pathways (50). Thereby, the purpose of the studies reported here was to build an understanding of the mechanism of the role of translation, especially the role of eIF4E, in cancer and in the antitumoral activity of Sorafenib as well as its possible implication in the therapy resistance in human liver cancer cells.

OBJECTIVES

This project was born from the collaboration between the basic research group led by Dr Jesús de la Cruz, focused on studying the synthesis and function of ribosome, and the biomedical research group led by Dr Jordi Muntané, specialized in the study of the HCC. The main objective of this thesis was to elucidate the molecular consequences that underlie the action of a Sorafenib treatment in HCC cell line models, as well as evaluate the mechanisms that could be involved in Sorafenib resistance. Overall, this work was ultimately aimed at identifying novel molecular targets to improve the therapeutic options for HCC patients.

The precise objectives of this work were to:

1. Describe the impact of Sorafenib on translation and analyse the different molecular pathways through which Sorafenib dysregulates this process, including the phosphorylation of eIF2α and the activity and levels of the cap-binding complex.

2. Assess the role of the upstream regulatory pathways controlling translation, including the mTOR and MAPKs pathways, in Sorafenib-treated cells.

3. Elaborate a translational-reprogramming map of those genes differentially translated in Sorafenib-treated cells showing the dysregulated pathways and the mechanisms that determine the particular translational behaviour upon the treatment.

RESULTS

CHAPTER 1

Molecular impact of Sorafenib on protein synthesis process in liver cancer cells

INTRODUCTION

Protein synthesis phases: Initiation, Elongation, Termination

Protein synthesis, or mRNA translation, is a fundamental biological process by which the ribosome assisted by the corresponding charged tRNAs decodes the mRNAs and synthesizes a polypeptide chain by linking amino acids according to the genetic code. In eukaryotes, translation begins when the ribosome binds to a mRNAs, recognizes its start codon (AUG) and the first amino acid encoding methionine is brought to the ribosome by the initiator transfer RNAs (Met-tRNA$_i^{Met}$). Protein synthesis can be divided into four different phases: initiation, elongation, termination, and ribosome recycling. Each phase of translation involves a different set of translation factors, and for translation to take place, these factors need to interact with the ribosome. The ribosome is composed by two subunits, 40S and 60S, that must be brought together in order to constitute an active 80S ribosome in an mRNA molecule (51-55). A general overview of the protein synthesis process is shown in **Figure 1**.
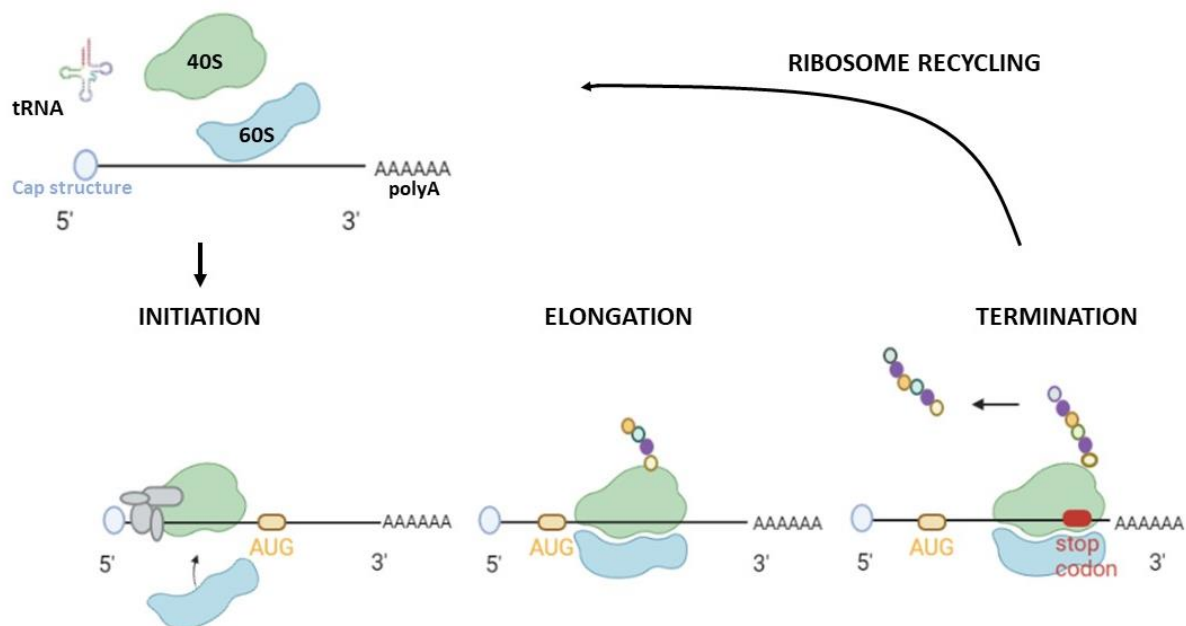
Figure 1. Translation phases. Schematic illustration showing the protein synthesis process. Translation can be divided into for phases: initiation, elongation, termination and ribosome recycling. Initiation, ribosomal subunits interact with translation initiation factors to form the active ribosome. The initiator tRNA brings the first amino acid in the polypeptide chain to bind to the start codon on mRNA. During elongation, different tRNAs bring amino acids one by one to form the nascent polypeptide chain. At the termination step, the stop codon is recognized, and the translational complex dissociated, resulting in the completion of a polypeptide. Components are recycled to re-initiate a new translation round. AUG (start codon); tRNA (transfer RNA).

For translation initiation occurs, several consecutive steps lead to the formation of three complexes: the 43S Pre-Initiation Complex (43S PIC), the subsequent formation of the 48S Pre-Initiation complex (48S PIC), and finally the active 80S ribosome (56). These complexes are assembled by the interactions of eukaryotic initiation factors (eIFs) with the ribosomal subunits. The translation factors involved in translation are summarized in **Table 1**. During translation initiation, different eukaryotic initiation factors (eIFs 1, 1A, 2, 3 and 5) interact with the small ribosomal subunit to form firstly the 43S PIC (57). The preassembled 43S PIC also contained the Met-tRNA$_i^{Met}$ bound to eIF2α-GTP in the so-called Ternary Complex (TC). The multifactorial 43S PIC is recruited to the mRNA template assisted by the cap-binding complex, consisting of the mRNA 5'-cap-binding protein 4E (eIF4E), the large scaffolding protein 4G (eIF4G) and the RNA helicase 4A (eIF4A), leading to 48S PIC assembly (58-60). In the recruitment of the 43S PIC to the mRNA, the multisubunit eIF3 factor plays a key role, possibly by forming a protein bridge to the mRNA by interacting with eIF4G (61). eIF4G also interacts with the Poly(A)-binding protein (PABP), which is associated with the mRNA poly (A) tail at 3' end of the RNA, resulting in the mRNA circularization, which stabilize the mRNA (62, 63). The mRNA circularization has also been associated with mRNA protection from degradation as well as optimizing the translation initiation process by facilitating ribosome recycling. The 48S PIC scans the 5' untranslated region (5' UTR) of the mRNA in the 5'- 3' direction to find normally the first AUG codon (a start codon). After initiation codon recognition, eIF5 and eIF5B induce the hydrolysis of the GTP bound to eIF2 (64). The hydrolysis promotes the displacement of eIF2 and the joining of a 60S subunit resulting in the assembly of an elongation-competent 80S ribosome (65, 66). Formation of a translation-competent 80S ribosome marks the end of the initiation phase and the beginning of the elongation one (**Figure 2**).
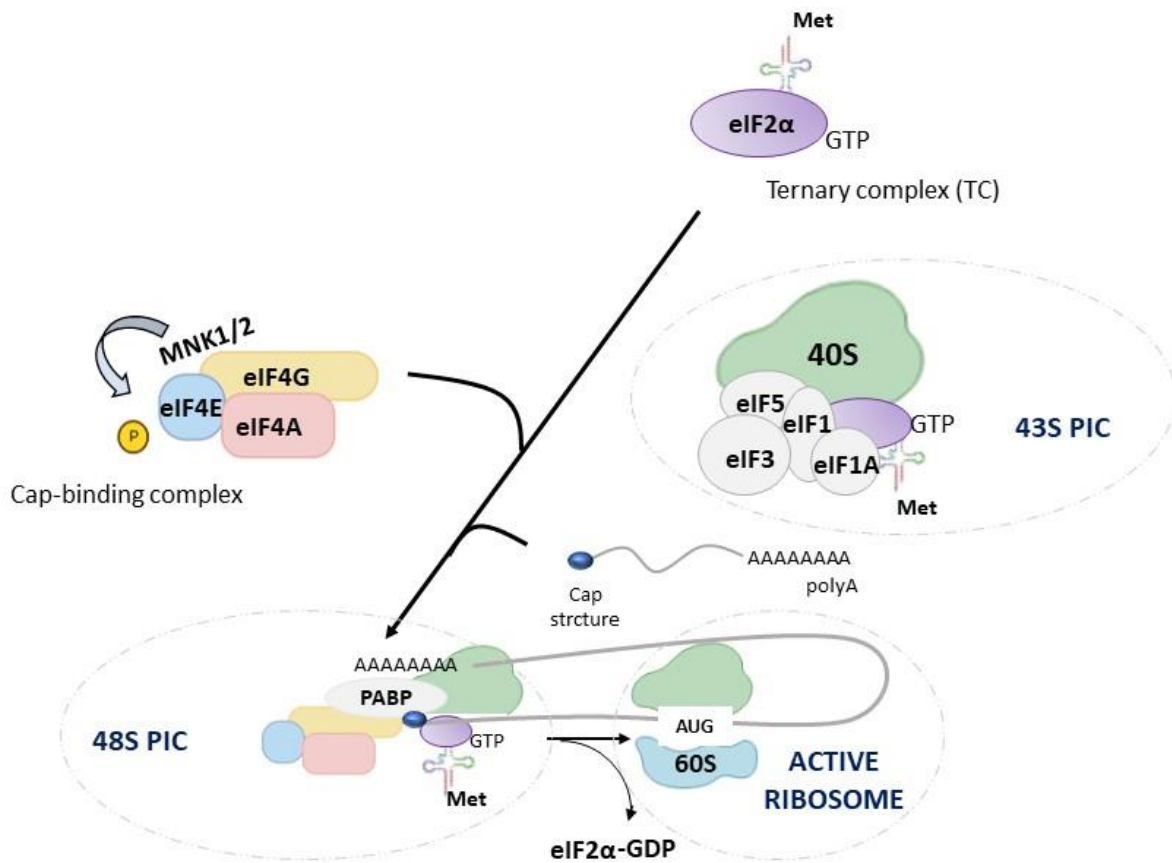
Figure 2. Translation initiation phase. Schematic illustration showing the initiation phase of the translation process. For translation taking place, three different complexes must be form: 43S Pre-Initiation complex (43S PIC), 48S Pre-Initiation complex (48S PIC) and active 80S. The 43S PIC is a large multifactorial complex composed of the 40S ribosomal subunit, the ternary complex (TC), and the eukaryotic initiation factors (eIFs) eIF1, eIF1A, eIF3 and eIF5. The TC is constituted by eIF2, which contains α-, β- and γ-subunits, the initiator methionyl tRNA and GTP. 43S PIC is recruited into the mRNA to form the 48S PIC assisted by cap-binding complex. The cap-binding complex (eIF4F) is form by the cap-binding protein eIF4E, the RNA helicase protein eIF4A and the scaffolding protein eIF4G. The eIF4F complex interacts with the poly(A)-binding protein (PABP) causing the mRNA circularization to stabilize mRNA and enhance translation. Recognition of the initiation codon AUG leads to the release of eIFs and joining of the 60S ribosomal subunit. The formation of an active and competent ribosome marks the end of translation initiation and the beginning of elongation. MNK, MAPK-interacting kinase. Met, methionine. P, phosphorylation.

Translation elongation commences with the delivery of the cognate elongating aminoacyl-tRNA to the ribosome and the subsequent peptide bond formation. During protein synthesis elongation, ribosomes move along the mRNA and decode the template nucleotide triplets to generate a protein amino acid chain. The movement of the ribosome also involves the shifting of the mRNA-tRNA complexed within the ribosome in a process known as translocation. This process occurs in a unidirectional manner by which the ribosome advances to the 3' end of mRNA. In contrast to the complex factor requirements in translation initiation, the elongation phase relies on two essential protein factors with non-overlapping function, eEF1 and eEF2 (67) (see **Table 1**). Eukaryotic

Elongation Factor 1 (eEF1) is a complex that catalyses the aminoacylated-tRNA (aa-tRNA) delivery step whereas GTPase translation elongation factor 2 (eEF2) mediates the translocation movement (68, 69). The ribosome advances along the mRNA, decoding one codon at a time until all of the codons of the open reading frame are read. Lastly, termination occurs when the ribosome reaches a stop codon. The ribosome detects that translation is finished since no tRNA molecules can recognize these codons, leading to disassembly of the translation complex and the release of the new protein (70).

Eukaryotic release factors eRF1 and eRF3 form a ternary eRF1/eRF3-guanosine triphosphate (GTP) complex that recognize the stop codon. After stop codon recognition, eRF3 induces the GTP hydrolysis and eRF1 triggers the hydrolysis of the polypeptidyl-tRNAs causing the release of the recent protein synthesized. Then, the ribosome is disassembled from mRNA assisted by the ABC-type ATPase ABCE1 to participate in a further round of translation in a process called ribosome recycling (71). ABCE1 physically interacts with the release factors to promotes its function in stop-codon recognition to dissociate the ribosome into subunits. Additionally, there exist evidence that release factors also interact with PABP facilitating the re-initiation of translation (72).

Table 1. Translation factors. This table summarize the translation factors involved in each translation phase.

Phase	Factor	Function/Activity
	eIF1	Interacts with eIF1A promoting an open, scanning-competent 43S PIC
	eIF1A	Interacts with eIF1 promoting an open, scanning-competent 43S PIC
	eIF2	Forms a ternary complex (TC) with initiator transfer RNA and GTP
	eIF3	Multiprotein complex formed by 13 non-identical subunits. It acts as scaffold for several other initiation factors. Involved in cap-dependent and cap-independent translation
	eIF4E	Forms the eIF4F complex with eIF4A and eIF4G. It recognizes and binds the cap structure
	eIF4A	Forms the eIF4F complex with eIF4E and eIF4G. An RNA DEAD-box protein
	eIF4G	Forms the eIF4F complex with eIF4E and eIF4A. A scaffolding protein
	eIF5*	GTPase-activating protein. It induces the hydrolysis of GTP bound to eIF2
Elongation	**eEF1**	Catalyzes the aminoacylated-tRNA delivery
	eEF2	GTPase involved in the ribosome translocation movement

	eRF1	Forms a complex with eRF3 and GTP. Induces the hydrolysis of the polypeptidyl-tRNAs
Termination	**eRF3**	Forms a complex with eRF1 and GTP. Induces the hydrolysis of GTP
	ABCE1	ATPase that favors the dissociation of the ribosome from the mRNA

*eIF5 was originally identified as an initiation factor but later was shown to promote translation elongation and termination (70).

Protein synthesis regulation

Protein synthesis is the most energy-consuming cellular process in the cells, and it has a major role in the regulation of gene expression (73). Cells quickly adapt to environmental cues by controlling translation. Different signalling pathways, activated by extracellular stimuli, transduce the signals into the translation machinery, modulating its activity. The activity of the translation complex can be modulated along the four translations phase, although initiation is the rate-limiting and one of the most regulated steps (51, 74).

Translation of most mRNAs occurs by a cap-dependent manner. The cap is a structure located in the 5' UTR of all nuclear-transcribed mRNAs that is added very early at the transcription elongation step (46, 60)(75). This structure is recognized by the eIF4F complex, which leads to the formation of the 48S PIC into the mRNA. Although cells are able to control cap-dependent translation by different mechanisms, two are well characterized: (i) through the phosphorylation of eIF2α which influences the levels of the TC, and (ii) through the phosphorylation of repressor proteins in 4E, which impairs the assembly of the eIF4F complex (51, 52, 74).

Translational control by eIF2α phosphorylation

A major mechanism to regulate translation at the initiation phase involves the phosphorylation of the α subunit of the heterotrimeric eIF2 at serine-51. The eIF2 (containing α-, β- and γ-subunits), combines with guanosine triphosphate (GTP) to provide Met-tRNA$_i^{Met}$ to the 43S PIC. eIF2α helps to ensure that start codon recognition is accurately recognized and when this occurs, the GTP hydrolysis is triggered by eIF5 (76). The eIF2-GDP complex has low affinity for Met-tRNA$_i^{Met}$ and leaves the ribosome together with eIF5. To facilitate the next round of translation initiation, GDP associated with eIF2α needs to be exchanged by GTP. The exchange is catalyzed by a guanine nucleotide exchange factor, eIF2B. eIF2B first removes eIF5 and then exchange GDP for GTP, leading to the TC regeneration (77). The phosphorylation of eIF2α at serine-51 increases the affinity of eIF2α-GDP for

the limiting amount of eIF2B. Thus, reduced levels of eIF2B available cause a decrease of levels of TC able to be delivered to ribosome, thereby leading to suppression of global translation at the initiation phase (69)(**Figure 3**).
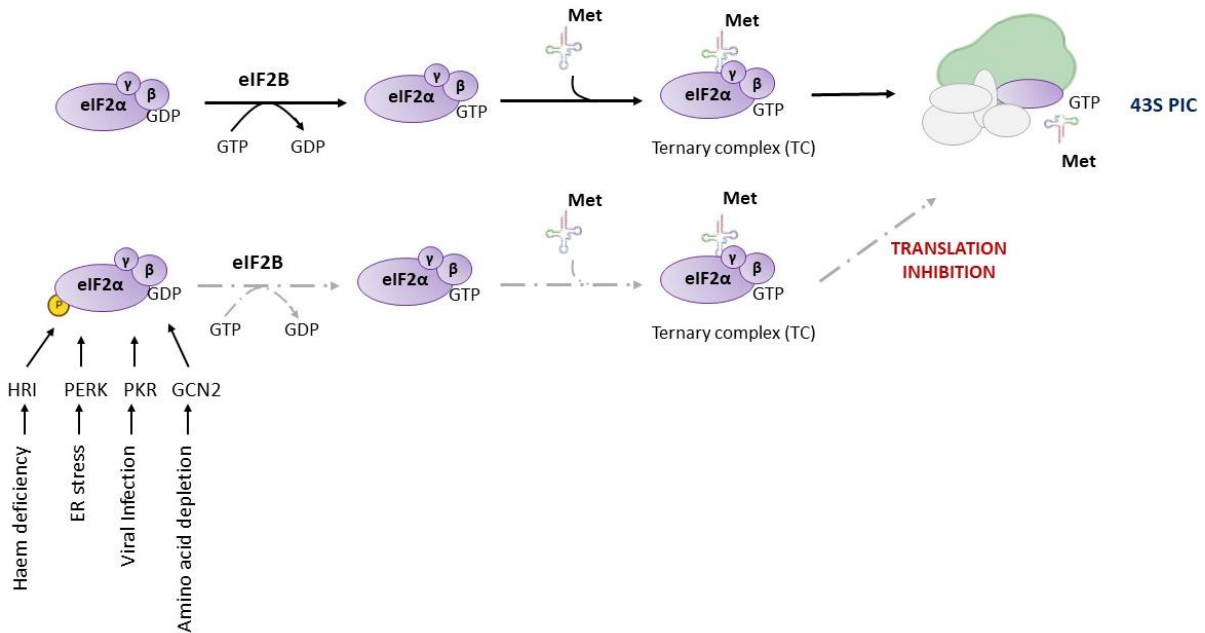
Figure 3. The phosphorylation of eIF2α drives the inhibition of translation initiation. The guanine nucleotide exchange factor eIF2B exchanges GDP for GTP, leading to the formation of the ternary complex (TC) with the initiator methionyl tRNA. Then, the 43S PIC is formed, and translation takes place. In adverse conditions, four different kinases (HRI, PERK, PKR and GCN2) phosphorylate the α subunit of the eIF2. The phosphorylation of eIF2α stabilized the interaction between eIF2B and eIF2-GDP inhibiting the nucleotide exchange and resulting in translation inhibition at the initiation phase.

The eIF2 complex regulates translation under a wide variety of stress-related signals that determine the cell fate in these conditions. The accumulation of unfolded protein in the endoplasmic reticulum (ER), amino acid depletion, hemo deficiency as well as viruses infection, induce the activation of phosphorylation of eIF2α (Phospho-eIF2α) by PKR-like ER kinase (PERK), general control non-derepressible 2 (GCN2), hemo-regulated inhibitor (HRI) and double-stranded RNA-dependent protein kinase (PKR), respectively (78-81) (**Figure 3**). When misfolded proteins are accumulated into the ER, the Unfolded Protein Response (UPR) is activated. In mammalian cells, three ER transmembrane proteins: IRE1α (Type I transmembrane protein inositol requiring 1), PERK (eukaryotic initiation factor 2α kinase), and ATF6 (activating transcription factor 6) mediate the UPR to sustain the proper folding environment in the ER. During UPR, transcription of genes encoding secretory proteins is downregulated whereas increases the removal of misfolded protein by

activation of degradation pathways (e.g., ER-associated degradation (ERAD)). Additionally, transcription of genes involved in the proper folding of proteins, like chaperones is also induced and the global protein synthesis is repressed by PERK-phosphorylated eIF2α. All these processes reduced the amount of substrates presented to the folding machinery in the ER lumen, allowing the recovery of the ER homeostasis (82-84). Although, Phospho-eIF2α causes translation inhibition, paradoxically it also stimulates the selective translation of a subset of stress-related mRNAs. These mRNAs contain several upstream Open Reading Frame (uORFs) that impede their translation in non-stress conditions (85, 86). One of these mRNAs whose translation is enhanced by Phospho-eIF2, encodes to the Transcriptional Factor 4 protein (ATF4). ATF4 acts as a basic zipper transcriptional activator of stress-related genes involved in metabolism, protection against oxidative damage, and regulation of apoptosis. The induction of the Phospho-eIF2α/ATF4 axis induced by different kinases is known as the Integrated Stress Response (ISR) (87)(**Figure 4A**).

The preferential translation of ATF4 mRNA by Phospho-eIF2α based on the presence of two uORFs within its 5' UTR has been well studied. The 5'- proximal uORF1 is a positive-acting element that enables ribosomes to reinitiate translation at a downstream coding region in the ATF4 mRNAs. When eIF2-GTP is easily accessible (nonstressed cells), ribosomes that have finished translating uORF1 continue their downstream scan and restart at uORF2, an inhibitory element that prevents ATF4 production. In stressed cells, Phospho-eIF2α that leads to lowered levels of eIF2-GTP increases the time required for scanning ribosomes to reinitiate translation. Delayed reinitiation allows the ribosome to scan through the inhibitory uORF2 and instead initiate translation into the ATF4 coding region (88) (**Figure 4B**).

ATF4 activates the transcription of a broad spectrum of target genes, including apoptosis-related genes (e.g., Bcl-2, Bim), genes involved in metabolism, in redox balance, in autophagy, and in protein synthesis. The protein product of these genes has been described to increase the aggressiveness of cancer since the initial eIF2α phosphorylation under stress-condition allows the cell survival, but the phosphorylation status maintained over time can have a deleterious effect on cell fate (89, 90). Therefore, increasing eIF2α phosphorylation could be a good strategy to treat cancer.
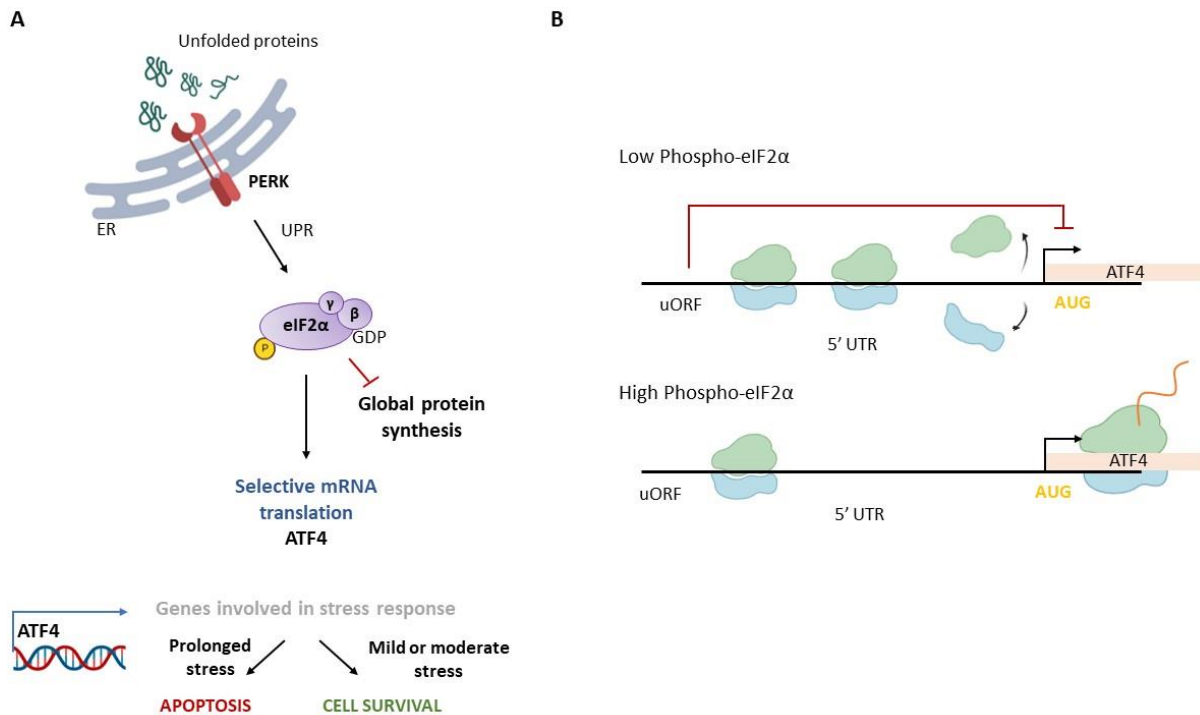
Figure 4. The phosphorylation of eIF2α inhibits global protein synthesis whereas promoting selective mRNAs translation. The schematic representation shows the protein synthesis control under the phosphorylation of eIF2α. (**A**) Unfolded proteins accumulated into endoplasmic reticulum (ER) induce the activation of the transmembrane protein eukaryotic initiation factor 2α kinase (PERK). PERK, in turn, phosphorylates eIF2α leading to the inhibition of the overall translation, while stimulating the translation of a subset of mRNAs including transcription factor 4 (ATF4). ATF4 activates transcription of a broad spectrum of genes that lead the cells to apoptosis or cell survival, depending on the time window. (**B**) The phosphorylation levels of eIF2α are linked to translation of mRNAs with several upstream open reding frames (uORFs). ATF4 mRNA contains two uORFs within its 5' UTR. uORF1 inhibits the uORF2 avoiding the appropriated translation of ATF4 (from uORF2) under low phosphorylation levels of eIF2α. Under high levels of Phospho-eIF2α, the ribosome could scan the second uORF, translating ATF4 mRNA.

Translational control by 4E-BPs

4E-Binding Proteins (4E-BPs) are a family of proteins that bind and inhibit the cap-binding protein 4E (eIF4E), which forms the eIF4F complex responsible for recruiting the ribosome to the 5'-mRNA cap structure. 4E-BPs (4E-BP1, -2 and -3 in mammals) interfere with eIF4F complex assembly by binding to the site on eIF4E that overlaps with the eIF4G motif leading to the blockage of the eIF4E-eIF4G association (91). The 4E-BPs activity is regulated by their phosphorylation in response to several stimuli like growth factors. Growth factors promote 4E-BPs phosphorylation and enhance translation, resulting in an increase in cell growth and proliferation. In contrast, nutrient depletion leads to a hypophosphorylatedstatus of 4E-BPs, driving translation inhibition and cell growth arrest.

The mTORC1 kinase is known as a major 4E-BPs kinase but other kinases have been identified as enzymes able to phosphorylate 4E-BPs such us the glycogen synthase kinase 3β (GSK3β), the casein kinase 1ε (CK1ε), and the cyclin-dependent kinase 1 (CDK1)(92, 93). In growth cell condition, 4E-BPs are phosphorylated hierarchically in different residues that include threonine-37 (Thr37), threonine-46 (Thr46) that preceded phosphorylation of threonine-70 (Thr70) and serine-65 (Ser65) (94). Phosphorylation of 4E-BPs facilitates their dissociation from eIF4E which allows the eIF4E-eIF4G interaction and eIF4F complex assembly (92, 95). In turn, the hypophosphorylation status of 4E-BPs interacts strongly with eIF4E affecting the eIF4E free levels which results in an overall translation inhibition (**Figure 5**).
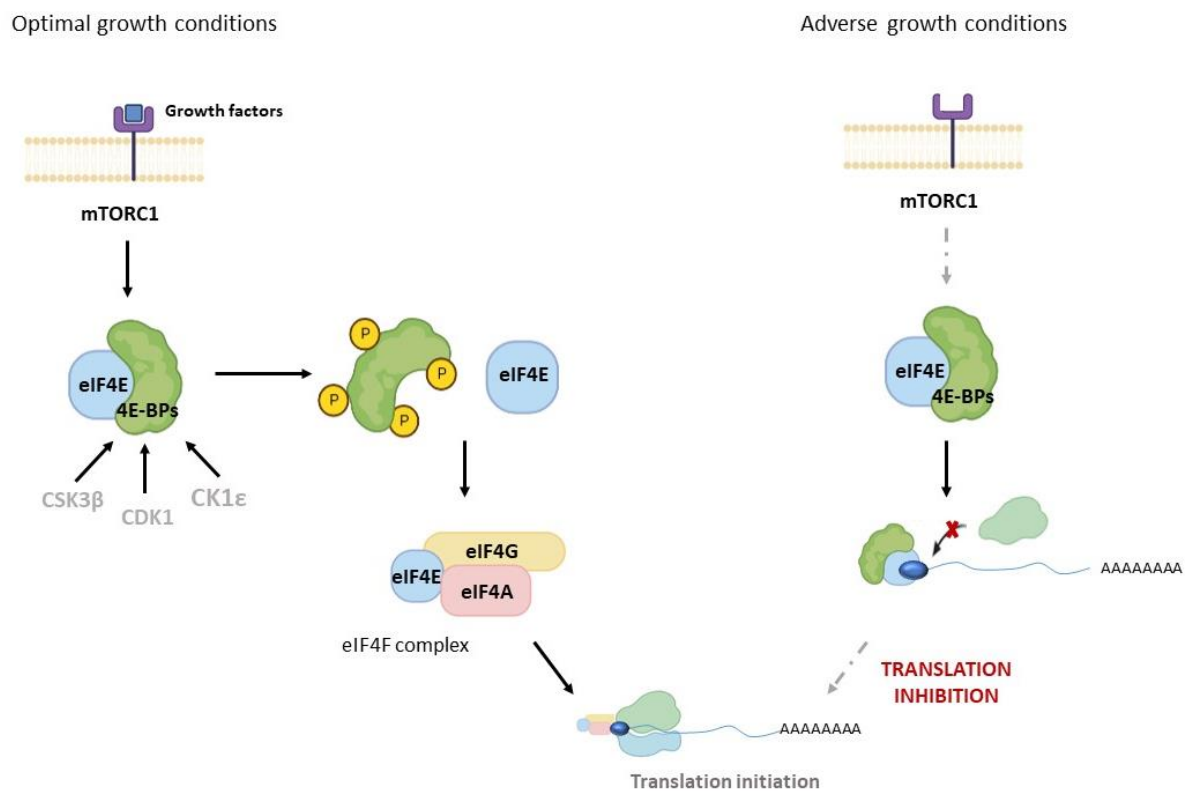
Figure 5. The repressor proteins 4E-BPs control the protein synthesis process. The schematic representation shows the regulation of translation by 4E-BPs. The activity of 4E-BPs is controlled by phosphorylation. Various kinases (CSK3β, CDK1, CK1, and mTORC1) phosphorylate 4E-BPs in a hierarchical manner, with mTORC1 being the most well-known. The phosphorylation of 4E-BPs promotes the dissociation of 4E-BPs from eIF4E. eIF4E free interacts with eIF4A and eIF4G forming the eIF4F complex, allowing translation. In contrast, the hypophosphorylated 4E-BPs bind to eIF4E and prevent the formation of the eIF4F complex, resulting in translation inhibition.

The double phosphorylation at threonine-37 and threonine-46 of 4E-BPs does not disrupt the 4E-BPs/eIF4E complex but it induces conformational changes to allow the subsequent phosphorylation at serine-65 and threonine-70 that release eIF4E and stimulate translation. Additionally, the phosphorylation of threonine-37 and threonine-46 is a prerequisite for the subsequent phosphorylation on 4E-BPs produced by mTORC1 or other kinases (94). To summarize, several kinases phosphorylate 4E-BPs relying on external signals received and the accessibility to the 4E-BPs residues, which in turn, depend on the previous activity of other kinases (96). This makes the sensitivity of the 4E-BPs phosphorylation sites differ depending on different extracellular stimuli and on different cell types (97).

Moreover, the 4E-BPs phosphorylation status has been associated with tumorigenesis, probably interfering with the levels and activity of eIF4F complex. The overexpression of non-phosphorylatable of 4E-BP1 mutant that constitutively binds to eIF4E is sufficient to suppress cellular proliferation and neoplastic growth. Indeed, the loss of 4E-BP1 expression accelerates tumorigenesis driven by p53 loss (98, 99). All this makes that the 4E-BPs function as a tumour suppressor.

RESULTS

Sorafenib inhibits global protein synthesis

The components of the translation machinery integrate almost all proto-oncogenic signals that allow the cell to proliferate. An aberrant activity of this process allows cells to proliferate even in adverse conditions because it allows cancer cells to adapt to drastic changes in the tumour environment and sustain their characteristic high proliferation. (27, 28, 44) . In this Thesis, we studied the consequences of Sorafenib on the protein synthesis process and we wondered whether the anti-proliferative, anti-angiogenic, and/or pro-apoptotic properties of Sorafenib can be explained, at least in part, by its impact on translation. To tackle this aim, a classic technique called polysome profile was firstly used to analyse the global translation status of cells treated with Sorafenib. Polysome profile provides a snapshot of translation activity. Briefly, extracts from human cell lines are loaded on sucrose gradient (10 - 50%) and are fractionated according to their molecular size by ultracentrifugation. The absorbance at 254 nm (A_{254}) is simultaneously measured and a profile is recovered. The polysome profile shows several peaks corresponding to the light cellular materials, the free 40S and 60S ribosomal subunits, the 80S peaks (constituted in turn by vacant 80S ribosome, free 40S and 60S ribosomal subunits connected by weak-interactions and monosome, ribosome engaged to the mRNA), and the different polysomes peaks (**Figure 1**; for a representative profile see **Figure 2A**). Alterations in the polysome/monosome ratio, the absence of a distinct peak of free ribosomal subunits, or specific modifications such as half-mer polysomes are all very informative since they indicate problems with the translation or ribosomal biogenesis processes.
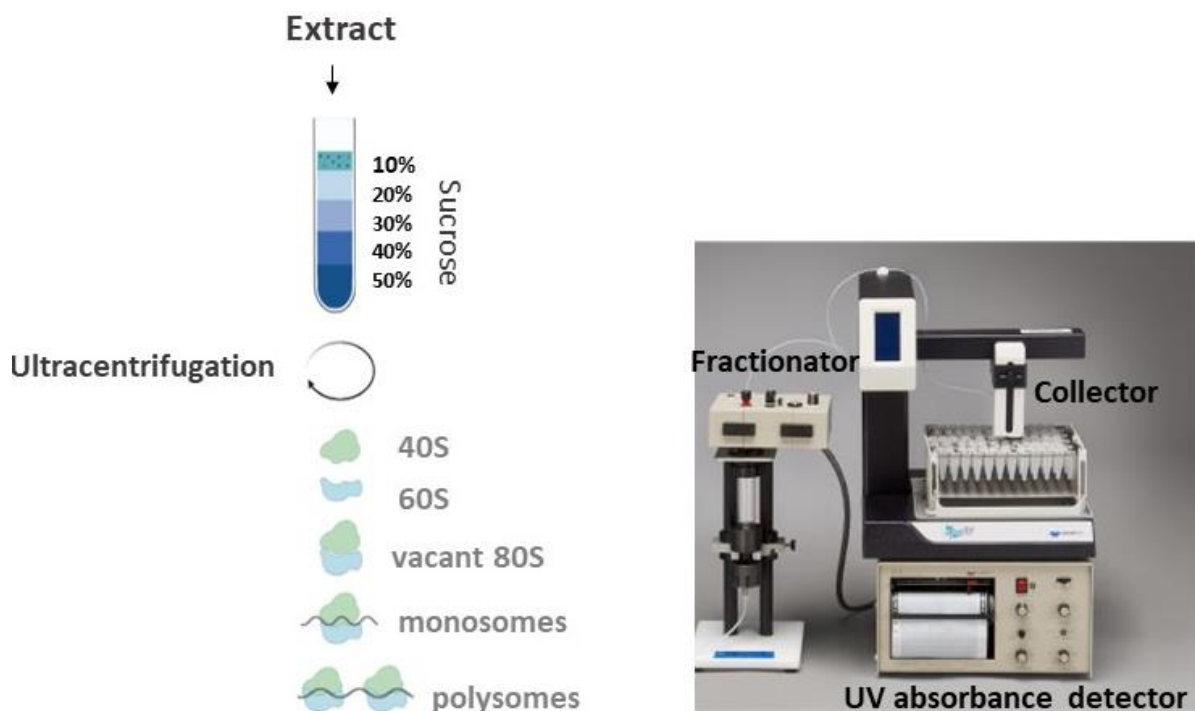
Figure 1. **Schematic representation of the sucrose gradient system used to fractionate ribosome-free and ribosome bound mRNAs.** Polysome extracts are obtained as described in the Materials and Methods section. A whole cell extract is loaded on the top of a 10 – 50% sucrose gradient. The different cellular complexes are fractionated depending on their molecular weight after centrifugation. Polysome profile is monitored by measuring the Abs_{254} using a UV detector.

In this study, the HepG2 human cell line was used to perform most of the experiments due to its proximity to primary human hepatocytes. The HepG2 cell line was an hepatoblastoma derived from the liver tissue of a 15-year-old white male hepatoblastoma, but the changes at proteomic levels are comparable to processes in HCC cells so the use of the HepG2 cell line to investigate the metabolism of anticancer drugs and tumour processes is justified (100, 101). HepG2 cells were treated with 10 µM Sorafenib, which is equivalent to that found in blood from patients during treatment. The kinetic study of polysome profile showed a characteristic profile of translation inhibition with an increase on the 80S peak, whereas those of polysomes decreased. Additionally, the obtained ratio polysome/monosome was smaller over time compared to the control conditions (DMSO-treated cells), indicating a stronger inhibition at long-term (**Figure 2**).
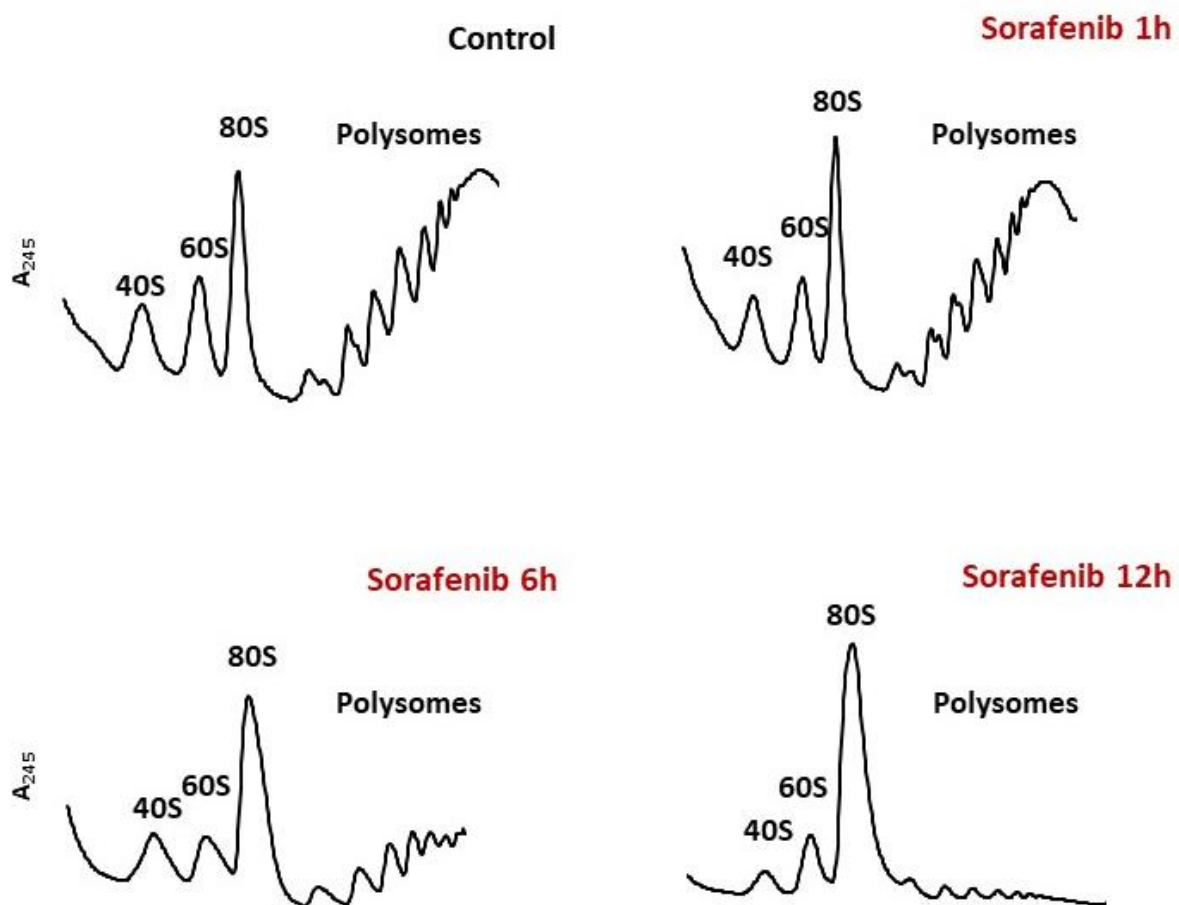
Figure 2. Global protein synthesis is inhibited by Sorafenib. Polysome analysis of HepG2 cells treated or untreated with 10 µM Sorafenib. Whole cell extracts were obtained and fractionated on sucrose gradient as described in the Materials and Methods section. The polysome profiles were recorded. A representative profile is shown.

To confirm the translation inhibition exerted by Sorafenib, we also tested the translation status in Sorafenib-treated cells by a puromycin assay that measures cell's ability to synthesize new proteins. HepG2 cells were treated with 10 µM Sorafenib for different times and puromycin was added 10 min before harvesting the cells. Puromycin is an antibiotic that inhibits protein synthesis by ribosome-catalysed incorporation into the C-terminus of elongating nascent polypeptide chains, blocking further extension and resulting in a premature termination of translation. These puromycylated chains are detected using a specific antibody raised against puromycin by western blotting. The levels of puromycylated polypeptides are proportional to the overall rate of translation. Thus, in agreement to the previous polysome profile results, we found a gradual reduction in the puromycin signal over time in cells treated with Sorafenib, indicating a clear impact of this drug on the ability of the cell to synthesize new polypeptides. Cells treated with cycloheximide, a classic translation inhibitor that interferes with the translocation step during the protein synthesis elongation, was used as experimental positive control. The densitometric analysis of the blots corresponding to three independent replicates showed a significant reduction by 30% of the overall translation at short term and by more than 50% after 4 h of 10 µM Sorafenib treatment (**Figure 3**).
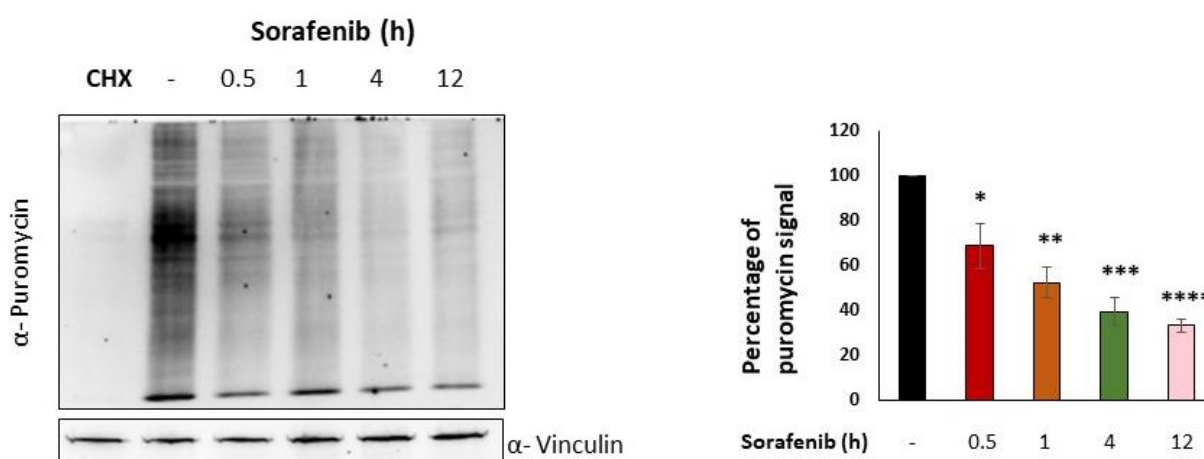
Figure 3. Time-course analysis of protein synthesis by a puromycin assay. Left panel: HepG2 cells untreated or treated with 10 µM Sorafenib were incubated with puromycin (5 µg/ ml) for 10 min before proteins extraction. Puromycin incorporation into nascent polypeptide chain was detected by western blot using a specific antibody raised against puromycin. The untreated cells condition is represented by

a - mark. Additionally, a positive control with cycloheximide (100 µM for 30 min) is shown. A single representative experiment is shown. **Right panel**: Densitometry analysis showing the percentage of the puromycin signal for each condition. The quantification is based on three independent experiments, with the control condition taken as the reference value (100%). Statistical significances were analysed by the Student's t test (* p <0.05; ** p < 0,001; *** p < 0,0001; **** p < 0,00001).

To determine whether the translation inhibition produced by Sorafenib was general or specific to HepG2 cells, we screened the global translation state in other HCC cell lines. To do so, several liver cancer cells were first selected according to their differentiation state and p53 profile (see Materials and Methods) and polysome profiles were performed for all of them after a Sorafenib treatment for 12 h. As a result, we found that Sorafenib also strongly inhibits translation in moderately and poorly differentiated liver cancer cells and in a p53-independent manner (**Figure 4**).
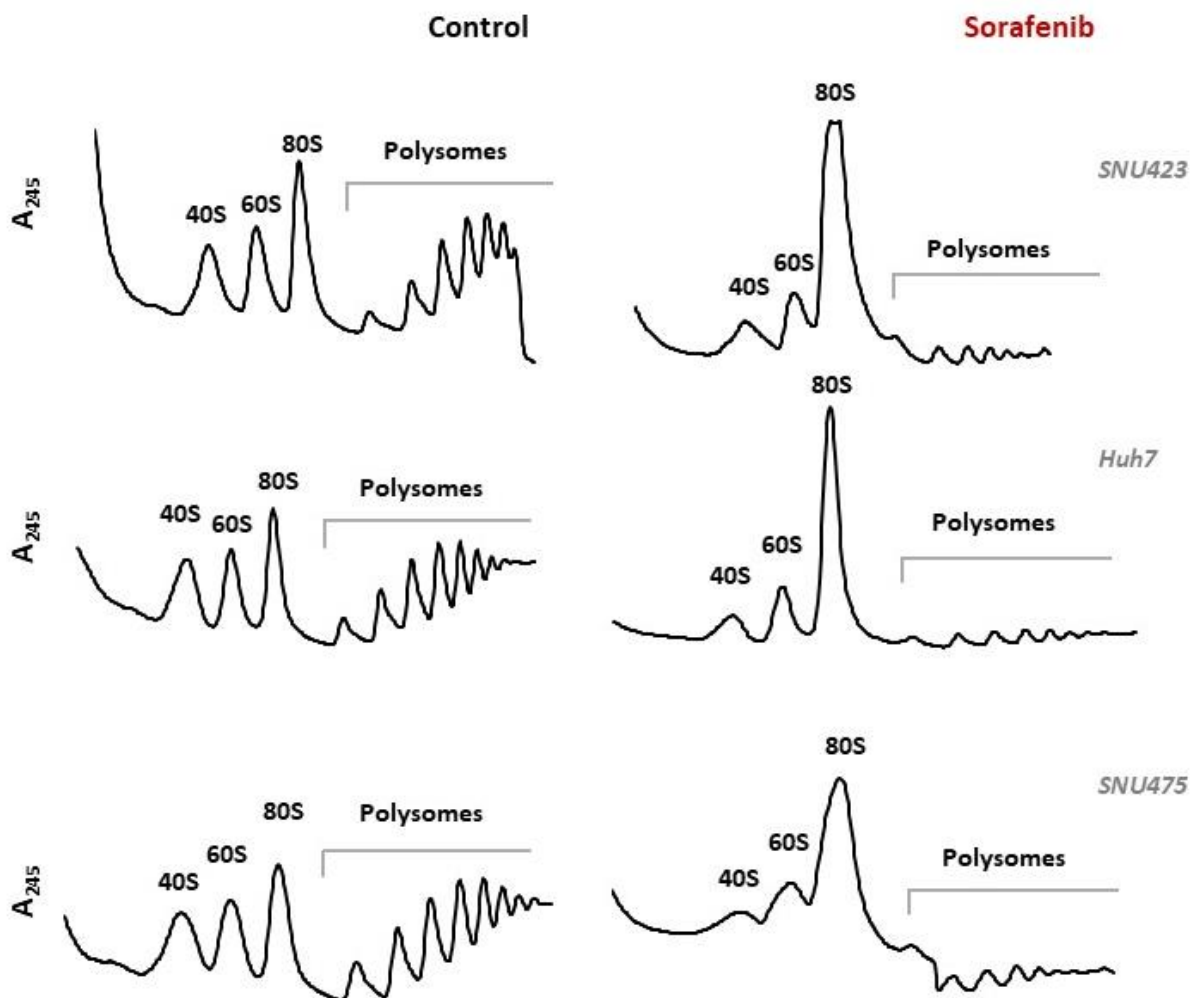
Figure 4. Translation inhibition is globally affected by Sorafenib in several HCC cell lines. Polysome profiles of Huh7, SNU423 and SNU475 cell lines untreated or treated with 10 µM Sorafenib for 12 h. Extracts were obtained and fractionated on sucrose gradient as described in the Materials and Methods section. Polysome profiles were recorded. A representative profile is shown.

Protein synthesis process includes three different phases: initiation, elongation, and termination. Cells control it mainly at the initiation phase, regulating the formation of active ribosomes (51, 74). The polysome profiles obtained for Sorafenib-treated cells suggest that the translation inhibition occurs at either the initiation or the early elongation phase, in which the ribosome cannot bind to the mRNA, or it is bound at the initiation codon but cannot translocate to the second one, respectively. To distinguish between both situations, new polysome profiles were performed in high salt sucrose gradient conditions. When NaCl is present in the sucrose gradient, a dissociation of vacant 80S (80S that are not bound to mRNAs) into free 40S and 60S ribosomal subunits occurs while monosomes (80S that are bound to mRNAs) remain unaltered. Thus, if the large 80S ribosome population is dissociated upon a NaCl treatment, the inhibition of translation takes place mainly at the initiation level. We next determined whether the 80S peak detected in Sorafenib-treated cells were vacant 80S ribosomes or monosomes; as shown in **Figure 5**, most 80S ribosomes dissociated into free ribosomal subunits. Thus, we could conclude that Sorafenib inhibits protein synthesis mainly at the initiation phase.
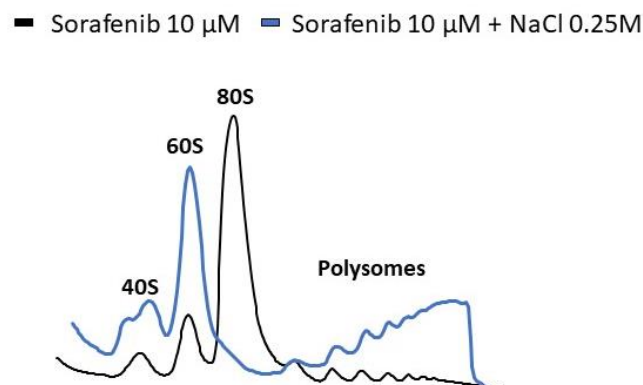
Figure 5. High-salt polysome profiles show that the inhibition of translation exerted by Sorafenib occurs mainly at the initiation phase. Polysome profile of HepG2 cells treated with 10 µM Sorafenib. Whole cell extracts were obtained and fractionated on sucrose gradient supplemented with (blue) or without (black) NaCl 0.25 M as described in the Materials and Methods section. A representative profile is shown.

In all eukaryotes, the mTORC1 complex regulates protein synthesis (102) and indeed therapies based on mTORC1 inhibitors, such as Sirolimus or Everolimus, have shown a favourable effect in reducing the incidence of HCC recurrence after liver transplantation (103). These drugs act by forming a complex with the FK binding protein (FKBP-12), which binds with high affinity to the conserved mTOR kinase. This binding interrupts the mTOR intracellular signalling pathway, affecting cellular processes such as translation (104, 105). Thus, we wondered whether the efficacy of those therapies based on mTORC1 inhibitors relied on their effect on translation, and if they were comparable to the effect produced by Sorafenib. To do so, we carried out a translational study, using polysome profile technique, of cells treated with two different concentrations of both Everolimus and Sirolimus (10 and 100 μM) for 12 h. We interestingly found that large doses of mTOR inhibitors were required to get an abnormal polysome profile. The polysome profile of cells treated with high doses of both drugs showed a dramatic increase of 80S peak and a reduction of actively translating polysome, which was not seen at low doses.(**Figure 6A**). In addition, this 80S peak was no dissociated in high salt gradient conditions, suggesting that the observed translation inhibition exerted by these drugs occurs mainly at early translation elongation phase in contrast to that of Sorafenib (**Figures 5 and 6B**). In conclusion, our findings showed a modest effect of those therapies based on mTORC1 inhibitors on overall translation. This does not rule out a role for translation in response to these therapies, but further and more specific experiments would be necessary. As regard translation, we can also conclude that Sorafenib works differently to mTOCR1 inhibitors, as they impair translation mainly at the initiation and elongation stages, respectively.
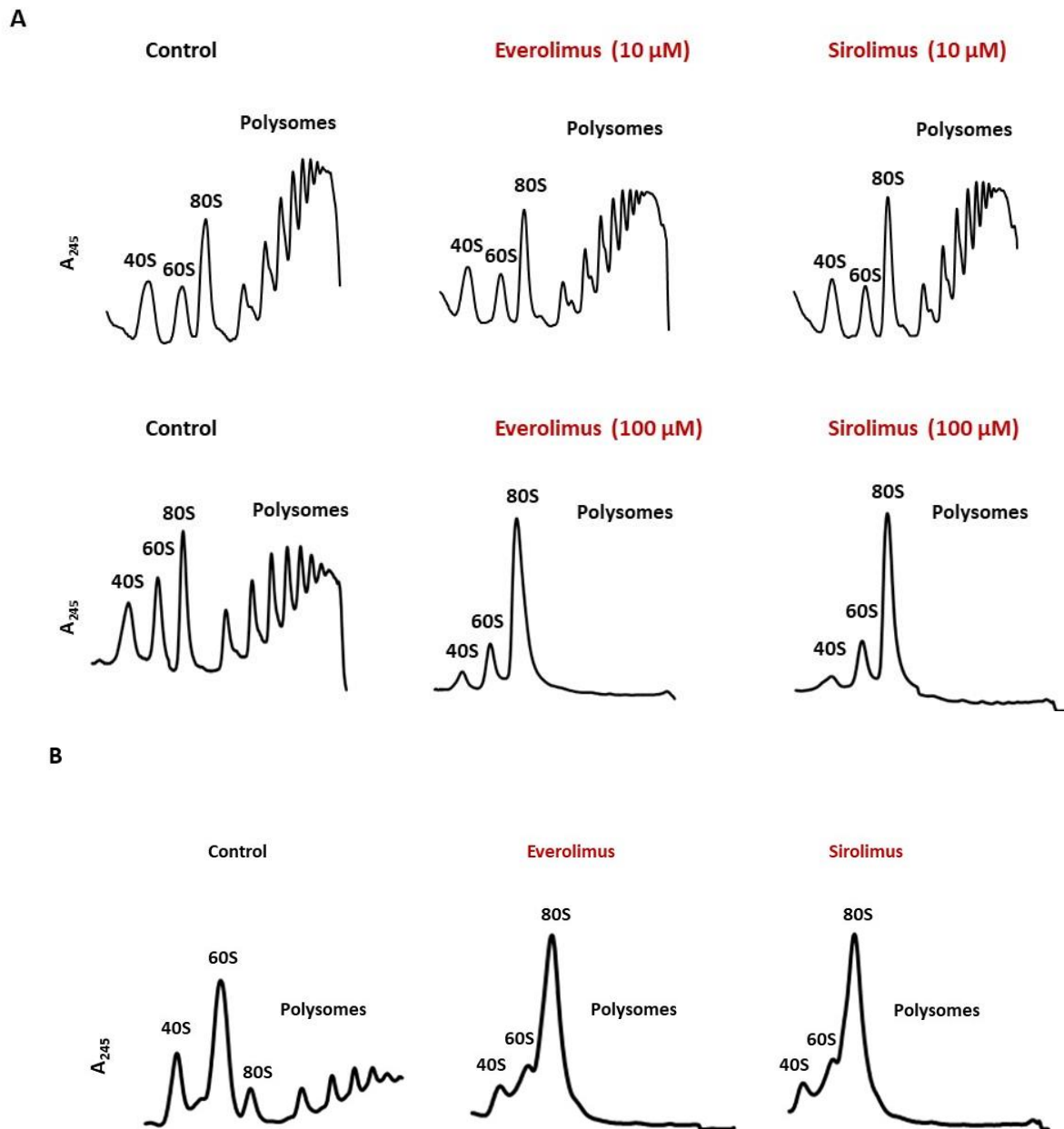
Figure 6. Therapies based on mTORC1 inhibitors affect translation at the early elongation phase using a large concentration of those inhibitors. (**A**) Polysome profile of HepG2 cells untreated or treated with Sirolimus or Everolimus for 12 h at two different concentrations (10 and 100 μM). (**B**) Polysome profile of HepG2 cells untreated or treated with 100 μM Sirolimus or Everolimus for 12 h. Whole cell extracts were obtained and fractionated on sucrose gradient supplemented with NaCl 0.25 M as described in the Materials and Methods section. A representative profile is shown.

In the last few years, ribosome biogenesis has also been highly investigated as a potential therapeutic target in cancer (106), so we wondered if this process was also affected by Sorafenib. When the biogenesis of either ribosomal subunits is affected or exist disbalance between the free ribosomal subunits, characteristics polysomes profiles are obtained with deficit of free 40S subunits,

typical of defects in the 40S ribosomal subunit biogenesis or free 60S subunits and presence of half-mer polysomes, typical of defects in the 60S ribosomal subunit biogenesis. None of these features were seen in our profiles, suggesting that ribosome biogenesis is likely not affected by Sorafenib in liver cancer cells (**Figures 2; 4**).

eIF2α phosphorylation has a central role in the translation inhibition produced by Sorafenib

We then sought to determine for which mechanisms Sorafenib is inhibiting translation initiation. One of the main canonical manners to control protein synthesis is regulating levels of the TC. The TC is a ribonucleoprotein complex formed by the charged initiator transfer RNA (Met-tRNA$_i^{Met}$), the eukaryotic initiation factor 2 (eIF2) and GTP, which is bound to the alpha subunit of eIF2 (eIF2α). This complex interacts with the 40S ribosomal subunit and other initiation factors to form the so-called 43S PIC. Initiation requires the hydrolysis of the eIF2-bound GTP, the release of pyrophosphate, and the regeneration of eIF2-bound GDP by another fresh molecule of GTP to get involved in a second initiation round (74, 77). Under stress conditions, cells use mechanisms of translational control, such as the Integrated Stress Response (ISR) to conserve energy and reprogram gene expression. During ISR, eIF2α is phosphorylated by any of four different eIF2α kinases in response to a variety of stresses: GCN2, PKR , HRI, and PERK (87). Phospho-eIF2α blocks the eIF2B-mediated exchange of eIF2-GDP to eIF2-GTP, limiting the regeneration of TC. The reduction in the TC levels leads to inhibition of global protein synthesis at initiation phase (**Figure 7A**). To assess whether Sorafenib inhibits translation initiation through the control of eIF2α phosphorylation, first, the ratio between phosphorylated eIF2α (Phospho-eIF2α) and total eIF2α was assessed after the Sorafenib treatment by western blot hybridization. As a result, we found that the Phospho-eIF2α/eIF2α ratio is low in control conditions and increases upon the Sorafenib treatment over time (**Figure 7B**).
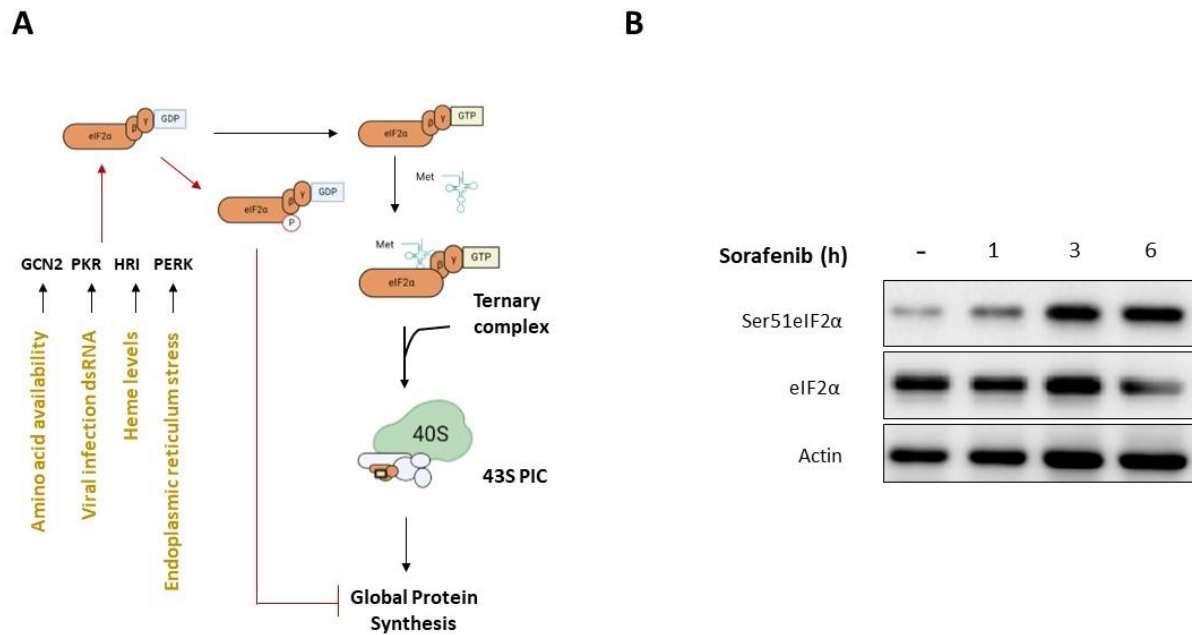
Figure 7. Sorafenib induces the phosphorylation of eIF2α. (A) Scheme of the regulation of protein synthesis by eIF2α. **(B)** Kinetics of eIF2α phosphorylation. Total protein extracts from untreated or treated HepG2 cells with 10 μM Sorafenib were obtained and analyzed by western blot. Actin was used as loading control. The untreated cells condition is represented by a – mark. The signal of total eIF2α and Phospho-eIF2α were detected using specific antibodies raised against eIF2α and Ser51eIF2α, respectively. A representative image is shown.

In previous studies, our group has reported that Sorafenib induces stress in the endoplasmic reticulum and activates the Unfolded Protein Response (UPR) mediated by PERK. This leads to an increase of eIF2α phosphorylation and an inhibition of global protein synthesis after 12 h of treatment (107). We decided to examine whether PERK activation was associated with the Sorafenib-induced eIF2α phosphorylation in a shorter period of time. Hence, we measured the phosphorylation status of eIF2α in Sorafenib-treated cells expressing a shRNA against PERK. As a result, we found significantly lower eIF2 phosphorylation levels after Sorafenib treatment compared to control cells (cells transfected with empty PLKO vector). Therefore, we concluded that an important percentage of Sorafenib-induced eIF2α phosphorylation is mediated by PERK kinase in a time-independent manner (**Figure 8A**). To test the translation status of silenced cells, the global protein synthesis state was analysed in cells with low Phospho-eIF2α/eIF2α ratio (HepG2-PLKO-shPERK) and was compared with cells with higher Phospho-eIF2α/eIF2α ratio (HepG2-PLKO) after a Sorafenib treatment by polysomes analysis. As shown in **Figure 8B**, the polysomes profiles of HepG2-PLKO-shPERK cells showed a lower translation inhibition than HepG2-PLKO after the Sorafenib treatment for 3 h. This clearly indicates that the phosphorylation eIF2α induced by PERK plays a key role in translation inhibition exerted by Sorafenib. However, the amelioration was not maintained at a long term,

suggesting that other Phospho-eIF2 independent mechanisms must be involved over time on the Sorafenib-translation inhibition.
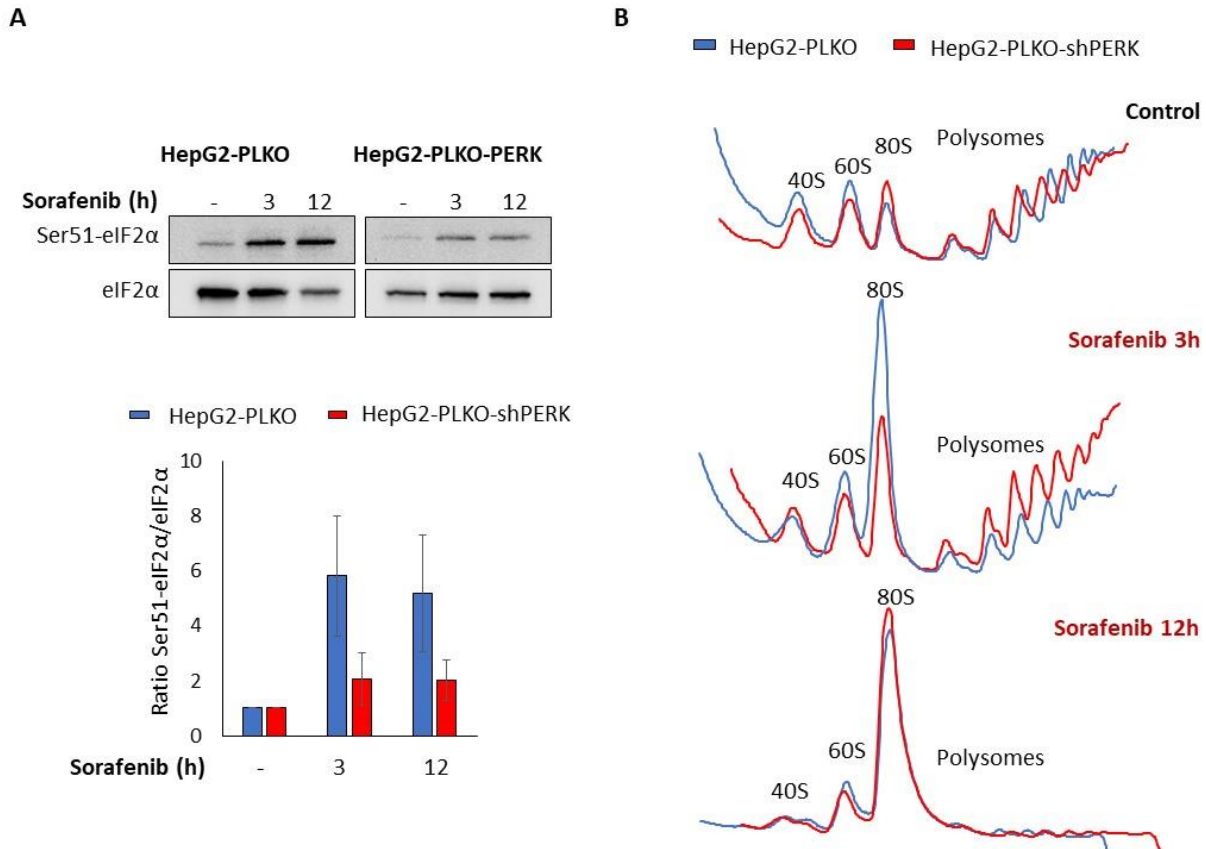
Figure 8. An important percentage of Sorafenib-induced eIF2α phosphorylation is mediated by PERK. (**A**) eIF2α phosphorylation levels in cells expressing shPERK. **Top panel**: Total protein extracts from HepG2 cells expressing shPERK or PLKO empty vector untreated or treated with 10 µM Sorafenib were obtained and analysed by western blot. The untreated cells condition is by a - mark. The signals of total and Phospho-eIF2α were detected using specific antibodies raised against eIF2α and Ser51-eIF2α, respectively. A representative image is shown. **Bottom panel**: Densitometry analysis showing ratio between phospho-eIF2α/eIF2α of three independent replicates. Data normalized to the control condition (DMSO-treated cells) (**B**) Polysome analysis of HepG2 cells expressing shPERK (red) or PLKO empty vector (blue) untreated or treated with 10 µM Sorafenib. Whole cell extracts were obtained and fractionated on sucrose gradient as described in the Materials and Methods section. Polysome profiles were recorded. A representative profile is shown.

It is well known that the PERK-eIF2α axis represents a survival pathway for cancer cells that need to adapt and overcome hypoxic stress during tumour progression (84). Phosphorylation of eIF2α causes inhibition of protein synthesis but paradoxically it stimulates the translation of a small subset of mRNAs with short upstream ORFs (uORFs). These mRNAs encode master transcriptional

regulators of the stress response such as ATF4. In turn, ATF4 triggers increased transcription of a subset of specific target genes involved in amino acid metabolism, differentiation, metastasis, angiogenesis, resistance to oxidative stress, and drug resistance (85, 88-90). We next evaluated whether translation of Phospho-eIF2α target ATF4 was increased by Sorafenib and compared with that of a housekeeping mRNA like β-actin in Sorafenib-treated cells. We found that Sorafenib treatment resulted in an increased presence of ATF4 mRNA in heavy polysome whereas the distribution of β-actin mRNA remained unaltered. The turnover of ATF4 mRNA from monosome or light polysomes to heavy polysomes indicates that its translation is enhanced by Sorafenib. We also measured the polysome profiling of ATF4 mRNA in transfected shPERK after a Sorafenib treatment and we found that the messenger was more represented in monosomes and light polysomes consistently with the phosphorylation status of eIF2α (**Figure 9**). Taken together, we conclude that Sorafenib leads to an increase of eIF2α phosphorylation through PERK activation, driving global translation inhibition. Indeed, cells expressing shRNA against PERK show reduced eIF2α phosphorylation levels after Sorafenib treatment, which is associated with an increased rate of protein synthesis compared to the wild-type cell. All these results point to the PERK/eIF2α axis as a powerful therapeutic target.
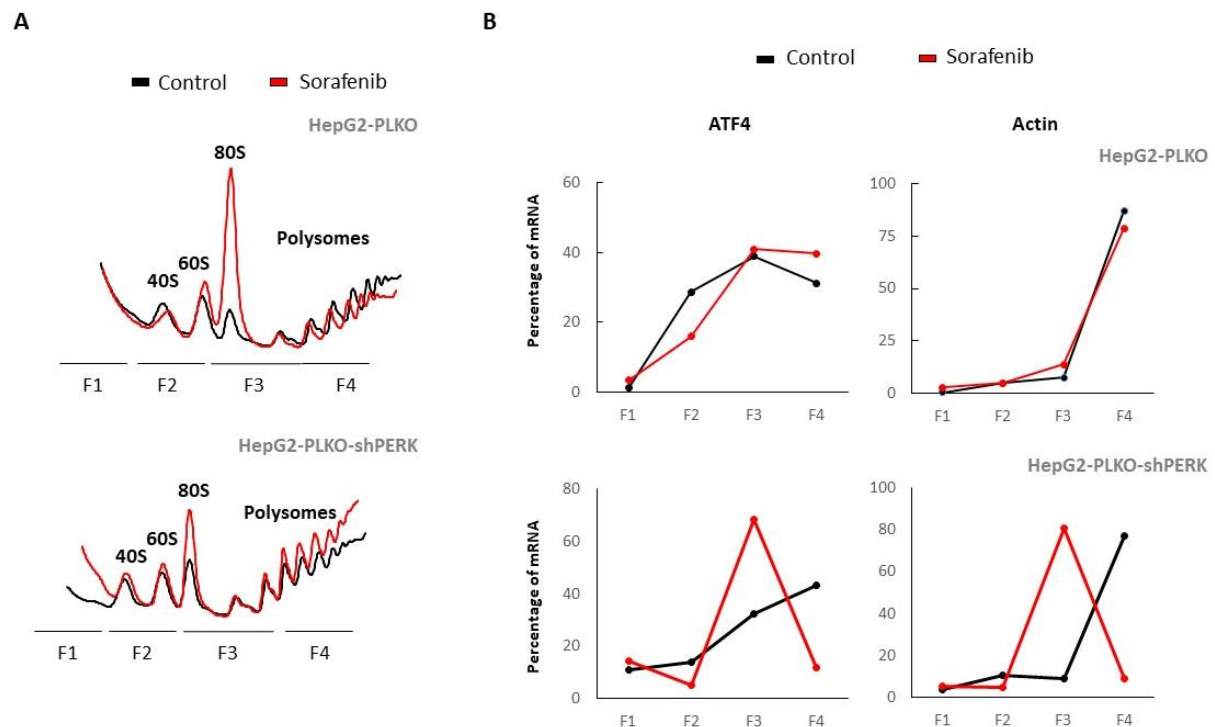
Figure 9. Sorafenib changes the turnover of ATF4 on polysome through eIF2α phosphorylation. HepG2 cells expressing shPERK (bottom) or PLKO empty vector (top) untreated or treated with 10 μM Sorafenib. Whole cell extracts were prepared and fractionated on sucrose gradient as described in the Materials

and Methods section. Polysome profiles were recorded. A representative profile is shown. Total RNA was isolated from non-translating fraction 1 [soluble fraction; F1], non-translating fraction 2 [free ribosomal subunits; F2], low translated fraction [monosome and light polysomes; F3] and high translated fraction [heavy polysomes; F4]. **(B)** The levels of ATF4 (Phospho-eIF2α target) and actin (housekeeping) mRNA were analysed by qRT-PCR for each transduced cell line. ATF4 and actin mRNA levels were standardized against a specific amount of RNA luciferase added before the RNA extraction. mRNA expression is performed as percentage of total mRNA in the polysome profile.

4E-BP has not a dominant role in the translation inhibition exerted by Sorafenib

For cap-dependent translation to take place, the formation of 48S PIC is necessary. The 48S PIC formation occurs when the 43S PIC is loaded with mRNA aided by the cap-binding complex (eIF4F complex). It is well known that cells regulate protein synthesis by modulating the activity and the levels of eIF4F complex in a wide variety of manner. One of them is by 4E-BPs family protein (4E-BP1, 4E-BP2 and 4E-BP3 in mammalian cells). 4E-BPs are major translational repressors that inhibit cap-dependent translation by binding and repressing eIF4E (91). The 4E-BPs-eIF4E interaction prevents the recruitment of eIF4G and the subsequent formation of eIF4F complex. Dissociation of 4E-BPs from eIF4E is dependent on the stepwise phosphorylation of at least four critical sites (threonine residues 37, 46 and 70 and serine residue 65), and allow eIF4G to bind eIF4E to facilitate cap-dependent translation. The hypophosphorylated form of 4E-BPs sequestrate eIF4E and prevent the interaction with eIF4G. The net result is the impairment of eIF4F complex formation and an inhibition of global translation (92, 94, 95)(**Figure 10A**). To know whether 4E-BPs mediate the translation inhibition exerted by Sorafenib, we analysed the phosphorylation levels of 4E-BP1 at its serine-65 in Sorafenib treated-cells by western blotting. Interestingly, the phosphorylation levels of 4E-BP1 remained unaltered after the Sorafenib treatment and the Phospho-4E-BP1/4E-BP1 ratio did not change as the densitometry analysis shows (**Figure 10B**).

A

B
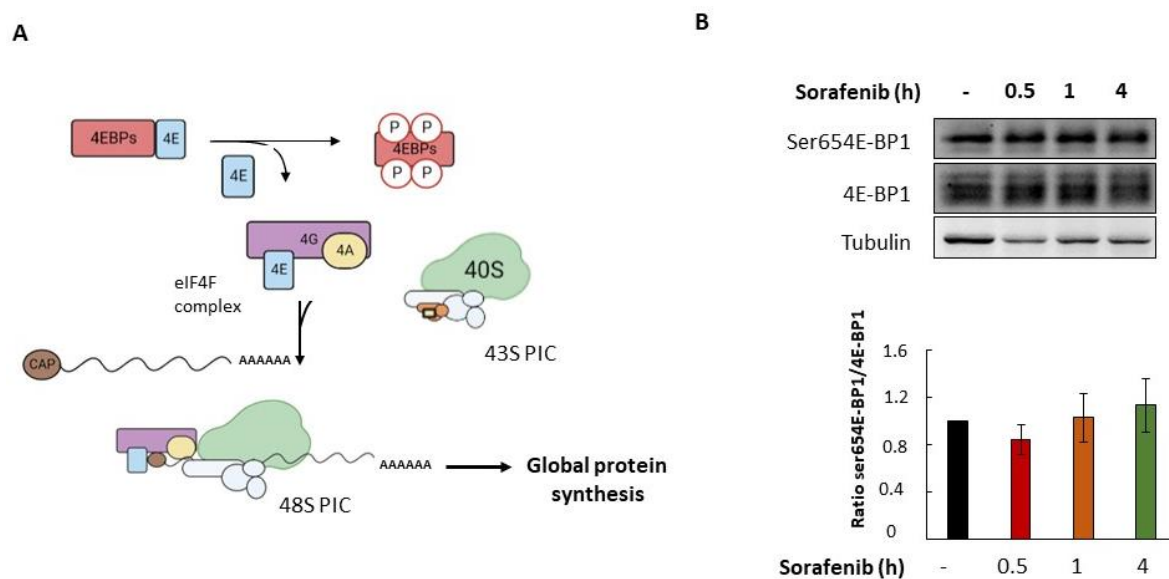
Figure 10. Phosphorylation of 4E-BP1 repressor protein is insensitive to Sorafenib treatment. (A) Simplified representation of 4E-BPs regulating global translation. **(B)** Time-course of 4E-BP1 phosphorylation at its Ser65. Top panel: Total protein extracts were obtained from HepG2 cells untreated or treated with 10 µM Sorafenib and analysed by western blotting. Tubulin was used as loading control. The untreated cells condition is represented by a - mark. The signal of total 4E-BP1 and Phospho-4E-BP1 were detected using specific antibodies raised against 4E-BP1 and Ser65-4E-BP1, respectively. A representative image is shown. Bottom panel: The densitometry analysis of the phospho-4E-BP1/total 4E-BP1 ratio based on three independent experiments is shown.

In general, the regulation of eIF4F complex is mediated by phosphorylation state of 4E-BPs which represses rate-limiting cap-dependent translation factor eIF4E as above-mentioned but other scenarios could exist. Some reporters have postulated that the combination of phosphorylations is necessary to dissociate 4E-BP from eIF4E (94, 96). Hence, to better understand the role of 4E-BPs in the Sorafenib translation inhibition, we examined the translation state in Sorafenib-treated cells after the silencing of the genes encoding for 4E-BP1 and 4E-BP2, the most common 4E-BP isoforms. We created a stable cell line expressing shRNA against the 4E repressor protein isoforms 1 and 2 (HepG2-PLKO-4EBP1/2) and control cells transfected with an empty vector (HepG2-PLKO). Then, translation was analysed by polysome profile after the Sorafenib treatment. Consistently with the previous blotting results, the global translation was not apparently ameliorated silenced in cells expressing sh4EBP1/2, suggesting that at least 4E-BP1/2 have not a predominant role in the translation initiation inhibition exerted by Sorafenib (**Figure 11**).
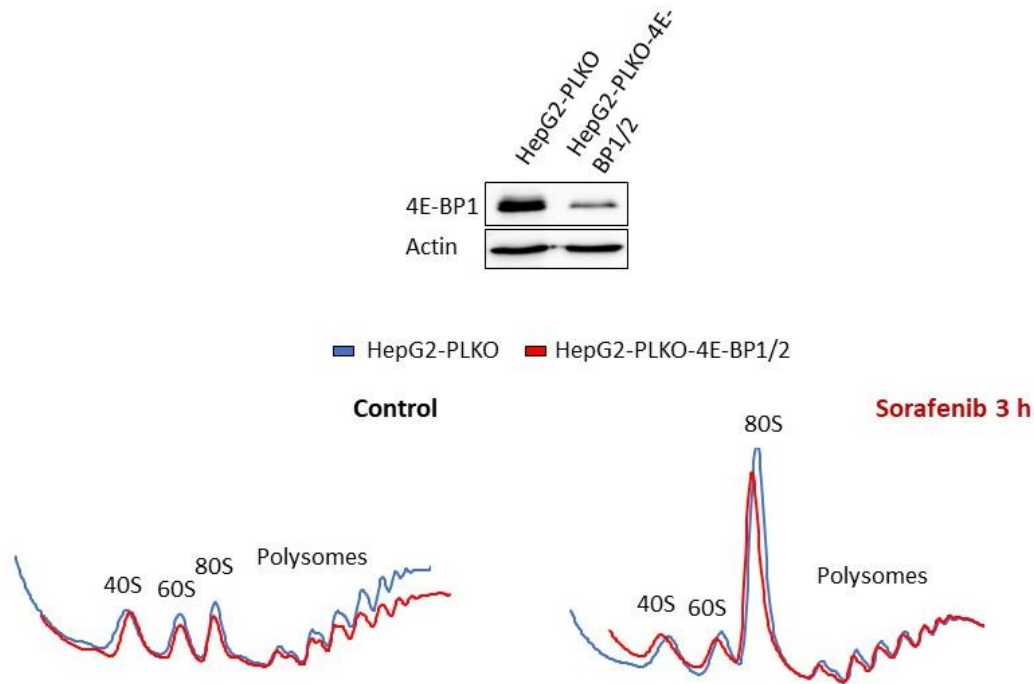
Figure 11. The 4E-BP repressor proteins 1 and 2 play no dominant role in the global translation inhibition exerted by Sorafenib. Top panel: Total protein extracts from HepG2 cells expressing sh4EBP1 and sh4EBP2 or PLKO empty vector were obtained and analysed by western blotting. The signals of total 4E-BP1 and actin were detected using specific antibodies raised against 4E-BP1 and actin, respectively. **Bottom panel**: Polysome analysis of HepG2 cells expressing sh4EBP1 and sh4EBP2 (red) or PLKO empty vector (blue) untreated or treated with 10 µM Sorafenib. Whole cell extracts were obtained and fractionated on sucrose gradient as described in the Materials and Methods section. Polysome profiles were recorded. A representative profile is shown.

MATERIALS AND METHODS

Cell lines, culture conditions and treatments

Four different liver cancer cells were used in this study. All were obtained from the American Type Culture Collection (ATCC/LGC Standards, S.L.U., Spain). They are listed in **Table 1** where some features are also shown.

Table 1. Lives cancer cell lines

Cell line	Differentiation	Origin	p53 profile
HepG2	Well-differentiated	Hepatoblastoma	Wild type
Huh7	Well-differentiated	Hepatocellular carcinoma	Point mutant in 220 codon
SNU423	Moderately differentiated	Hepatocellular carcinoma	Deleted mutant 126-132
SNU475	Poorly differentiated	Hepatocellular carcinoma	Point mutant in 785 codon

Cells were cultured in minimal essential medium (MEM) with Earle's balanced salts with L-glutamine (ref E15-825, PAA Laboratories Inc, Toronto, Canada) supplemented with 10% fetal bovine serum (FBS, F7524, Sigma Aldrich, Lot No. 022M3395, endotoxin < 0.2 EU/ml), 1% sodium pyruvate (ref. S11-003, PAA Laboratories Inc), 1% non-essential amino acids (ref. M11-003, PAA Laboratories Inc) and penicillin-streptomycin solution (100 U/ml-100 µg/ml; ref. P11-010, PAA Laboratories Inc). Cells were grown in culture flasks at 37 °C in a humidified incubator with 5% CO_2 until reaching a density of 100,000 cells/cm^2. Absence of mycoplasma species was tested routinely.

The different drugs used in this study are shown in **Table 2**. Sorafenib, Sirolimus and Everolimus were dissolved in DMSO (Dimethyl sulfoxide) as a 10- and 100-mM stock solution. Cycloheximide and puromycin were dissolved in water as a 10 mg/ml stock solution. All drugs were aliquoted and stored at - 20 °C. Treatments were performed minimum 24 h after plating.

Table 2. Drugs and reagents

Drug	Commercial reference
Sorafenib tosylate	FS10808, Carbosynth
Sirolimus	37094, Sigma-Aldrich
Everolimus	FE23209, Carbosynth Limited
Cyloheximide	C7698, Sigma-Aldrich
Puromycin dyhydrochloride	NP09203, Carbosynth

Polysome analysis and sucrose gradient fractionation

The protocol for polysome preparation of liver cancer cells was adapted from that we routinely employ for yeast cells. Briefly, cells were grown to 70% confluency in 100 mm dishes as above described. Normally, two dishes were used per condition assayed. Before harvesting the cells, 200 µg/ml cycloheximide was added and incubated for 5 min at 37 °C. Each dish was then placed on ice, the media aspirated and the cultures washed twice with PBS without Ca^{2+} and Mg^{2+} containing 200 µg/ml cycloheximide. Then, 600 µl of lysis buffer (10 mM Tris-HCl, pH7.4, 150 mM NaCl, 10 mM $MgCl_2$, 200 µg/ml cycloheximide, 2 mM DTT, 0.5% NP40) was added to one dish, cells were scraped, and transferred to the second dish. After scraping the cells corresponding to the second dish, the whole juice was transferred to a 1.5 ml-Eppendorf tube. The tubes were incubated at 4 °C with gentle end-over-end rotation for 10 min and then centrifuged at 16,000 $x\,g$ for 8 min at 4 °C in a refrigerated microfuge. The corresponding supernatants were recovered and the A_{260} measured using a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific). About 11 A_{260} units were layered on top of 10-50% (w/v) sucrose gradients prepared in a buffer containing 50 mM Tris-acetate, pH 7.5, 50 mM NH_4Cl, 12 mM $MgCl_2$, and 1 mM DTT. The gradients were centrifuged at 260110 x g (39.000 rpm) in a Beckman SW41 rotor at 4 °C for 2 h 45 min. To dissociate the vacant 80S ribosomes, high-salt conditions were achieved by adding NaCl to a final concentration of 0.25 M in the gradients. Gradient analysis was performed with an ISCO UA-6 system (ISCO, Inc. Lincoln) equipped to continuously monitor the A_{254}. When required, fractions of 1 ml were collected from the gradients. RNA was extracted from each fraction and analysed as described above.

Protein extraction and western blot analysis

Protein extracts were obtained by lysing cell pellets at 100 °C for 10 min in 2x Laemmli buffer (125 mM HCl-Tris, pH 6.8, 4% SDS, 0.02% bromophenol blue, 20% glycerol, 200 mM DTT). Cellular extracts were then sonicated in a Bioruptor (Diagenode, Belgium) for 1 min at high intensity. Protein extracts were subjected to 10% SDS-PAGE and transferred to nitrocellulose membranes (Amersham^{TM} Protran® 0.45 µm, GE Healthcare). The membranes were blocked for 1 h with 5% Bovine serum albumin (BSA) in TTBS (15 mM HCl-Tris, pH 7.5, 200 mM NaCl, 0.2 M NaCl, 0.1%(v/v) Tween-20), followed by incubation with primary antibody at 4 °C overnight. Antibodies used are shown in **Table 3**.

Table 3. Antibodies used in this study

Antibody	Dilution	Origin	Commercial Reference
Phospho-4E-BP1(Ser65)	1:2000	Rabbit	#9459, Cell Signaling
4E-BP	1:2000	Rabbit	#9452, Cell Signaling
Phospho-eIF2α(Ser51)	1:2000	Rabbit	#9721, Cell Signaling
eIF2α	1: 2000	Rabbit	#5324, Cell Signaling
Beta-Actin	1:1000	Mouse	ab8224, Abcam
Alpha-Tubulin	1:1000	Rabbit	ab52866, Abcam
Puromycin	1:10.000	Mouse	Mabe343, Sigma-Aldrich

After washings with TTBS buffer, the membranes were incubated with HRP-conjugated secondary antibody (Bio-Rad) at a 1:5000 dilution for 1 h at room temperature. Proteins were detected using an enhanced chemiluminescence detection kit (Super-Signal West Pico, Pierce) in a ChemiDoc™ Touch Imaging System (Bio-Rad) and the relative intensity value quantified with the Image Lab software provided with this system.

Puromycin assay

The puromycin assay relies on the incorporation of puromycin into nascent polypeptide and its subsequent detection with a monoclonal antibody against the antibiotic. About 8×10^5 HepG2 cells were seeded in 6-multi-well for each experimental condition. Puromycin (5 µg/ml) was added 10 min before harvesting the cells. Cells were collected and protein content was analyzed by western blotting. Details regarding protein extraction and processing procedures for the immunoblot analysis are described in the previous section.

Mammalian lentiviral shRNAs and viral infection

The small hairpin RNA (shRNA) vector for human PERK was purchased from the Sigma-Aldrich (Clone ID: TRCN0000262381). This vector is based on the Sigma/TRC MISSION pLKO.1 vector but modified to confer puromycin resistance. HepG2 cells were seeded in 96-multi well reaching 80% of confluency for transduction. A 24 h later, cells were treated with polybrene (8 µg/ml) and transduced with lentiviral particles. Viral particles-containing medium was removed and replaced with fresh, pre-warmed complete culture medium. The day after, puromycin (2 µg/ml) was added for selection of transduced cells. Appropriated concentration of puromycin was selected based on a kill curve

experiment previously performed. Non-transduced control cells were also exposed to puromycin. Puromycin containing-medium was replaced every 3 days until cells of control experimental condition die. Several puromycin resistant clones were tested by western blot to determine which one provided the optimal degree of gene knockdown. The small hairpin RNA (shRNA) vectors for human 4E-BP1 and 4E-BP2 were created in our lab. To do so, shRNA sequences were cloned into PLKO.1 vector with *Age*I and *Eco*RI enzymes. Competent *E. coli* cells were transformed

and a screening for positive clones was carried out. To confirm the identity of shRNA construct, the candidates were sequenced. The sequences of both the shRNA and the primers used to sequencing are listed in **Table 4**. The packaging of lentiviruses was done used 293T-lentiX cells. Cells were seeded in 100 mm dishes and transfected using GeneJuice with the envelope and packaging plasmids, pVSV-G and pCMV-dR8.91, respectively, and 4EBP1 and 4EBP2 shRNA constructs. A 48h later, lentiviral particles-containing supernatant was collected and centrifuged at 30.000 rpm in a Beckman SW41 rotor at 4 °C for 2 h. The supernatant was discarded, the pellet resuspended in 100 µl of PBS and aliquoted for storage at - 80 °C. HepG2 cells were infected as described before.

Table 4. Sequence of shRNAs and oligonucleotides used for sequencing

Primer	Forward (5' - 3')	Reverse (5' - 3')
4EBP1	CCGGCGGTGAAGAGTCACAGTTTGACTCGA	AATTCAAAAACGGTGAAGAGTCACAGTTTG
shRNA	GTCAAACTGTGACTCTTCACCGTTTTTG	ACTCGAGTCAAACTGTGACTCTTCACCG
4EBP2	CCGGGCTGTATTTCTGTAGAGCTAACT	AATTCAAAAAGCTGTATTTCTGTAGAGCTAAC
shRNA	CGAGTTAGCTCTACAGAAATACAGCTTTTTG	TCGAGTTAGCTCTACAGAAATACAGC
PLKO.1	CAAGGCTGTTAGAGAGATAATTGGA	

RNA isolation from sucrose gradient fractions, RT-PCR and qPCR

Firstly, 100 µl of each sucrose gradient fraction were taken to form the sample that represent whole RNA in the profile. Then, the fractions were pooled together according to the experimental design and a commercial RNA luciferase was added to each one. A treatment with proteinase K (37,5 µl 10 % SDS, 7.5 µl 0.5 EDTA and 4 µl 20 mg/ml proteinase K per 1 ml) was performed for 1h at 50 °C. Equal volume of phenol acidic:choroform:isoamyl alcohol (25:24:1, v/v) was added to the sucrose fractions and samples were mixed during 30 s and centrifuge 10 min at maximum speed at 4 °C. Approximately 80% of the aqueous phase was place in a new tube and an equal volume of chloroform was added. After mixing for 30 s using a vortex, samples were centrifuged 10 min at maximum speed at 4 °C. Again, 80% of the aqueous phase was place in a new tube and the RNA was precipitated using 1:10 of 3 M sodium acetate, pH 5.2 and 1.5 volumes of absolute ethanol. The mix was incubated

overnight at - 20 °C. Samples were then centrifuged at maximum speed for 30 min at 4 °C, the pellet was washed with 70 % ethanol and finally, was resuspended in RNase-free water.

Equal volumes of RNA samples were treated with 1 µl of DNase I (Promega) following the manufacturer's instructions. RNA was then reverse transcribed using SuperScript™ III First Strand Synthesis for RT-PCR also according to the manufacturer's instruction (Invitrogen, USA) and random hexamer primers (Roche, Switzerland). RT-qPCR was performed using SYBR® Green Premix Ex Taq™ 2X (Takara, Japan) and primer specifics of each transcript. Primer pair used for the RT-qPCR are shown in **Table 5**. The data were processed normalizing to the whole profile RNA and using RNA luciferase as an external control. The percentage of mRNA was calculated, and the date were expressed as the mean ± the standard deviation.

Table 5. Primers used for RT-qPCR

Primer	Forward (5' - 3')	Reverse (5' - 3')
ATF4	CAC TAG GTA CCG CCA GAA GA	AAT CCG CCC TCT CTT TTA GA
ACTB	TCC CTG GAG AAG AGC TAC GA	AGG AAG GAA GGC TGG AAG AG
28S rRNA	CAA AGC GGG TGG TAA ACT CC	TTC ACG CCC TCT TGA ACT CT
Luciferase	ATC GGG AAG CGA CCA ACG CC	GTC GGG AAG ACC TGC CAC GC

CHAPTER 2

Role of eIF4E in the cellular response to Sorafenib

INTRODUCTION

Signalling pathways controlling protein synthesis: MAPKs and mTOR pathways

Multiple signalling pathways converge on the translational machinery to regulate its function in response to various stimuli, including environmental stresses, extracellular stimuli and intracellular cues. Regarding translational control, both Mitogen-Activated Protein Kinases (MAPKs) and Phosphoinositide 3-kinase (PI3K)/AKT pathways are the best understood signalling pathways. Both routes downstream control the activity of many translation factors, modulating the activity of the translation machinery and regulating translation homeostasis. In human cells, dysregulation of these pathways is linked to pathogenesis events, including cancer (27-29, 108-110).

MAPKs pathways

The mitogen-activated protein kinases (MAPKs) are Serine/Threonine kinases that regulate many essential processes, including gene expression, mitosis, metabolism, motility, survival, apoptosis, and differentiation. In mammalian cells, three MAPK families have been described: ERK1/2, Jun N-terminal protein kinase (JNK) and p38 kinase (111). These are differentially activated depending on the signalling context, and, as a result, they phosphorylate a repertoire of distinct targets. These include proteins directly implicated in the regulation of mRNA translation, such as p90 ribosomal S6 kinase (RSK) and the MAPK interacting kinases (MNKs) (112, 113).

The MNKs (MNK1 and MNK2) are targeted by both ERK1/2 and p38 MAPKs pathways. MNK1 (MAPK-interacting kinase 1) is strongly activated by growth factors, cellular stresses, and inflammatory cytokines via ERK1/2 and/or the p38 MAPK, whereas MNK2 has a relatively high basal activity that is hardly affected by changes in MAPK activity (114, 115). Both MNK1 and MNK2 suffer alternative splicing, resulting in two protein isoforms, MNK1a, MNK1b and MNK2a, MNK2b, respectively. The four different proteins share the N-terminal domain responsible for the interaction with eIF4G. In contrast, the C-terminal domain, which has a MAPK-binding site, is just present in MNK1a and MNK2a, meaning that those are more sensitive to MAPKs' activity with respect to each isoform. In turn, MNK1a binds to both ERK1/2 and p38 MAPK whereas MNK2a associates only with ERK1/2 (116, 117). Although most published studies have reported that ERK1/2 and p38 MAPKs function upstream of MNKs, other works have proposed a downstream activation of p38 MAPKs by MNK2a, resulting in a positive regulatory loop (118, 119).

The cap-binding protein eIF4E, is the most well-known substrate of MNKs (120)(121). MNK phosphorylates eIF4E at its serine 209 downstream of MAPKs pathways and requires the interaction with the scaffolding protein eIF4G to phosphorylate it properly (119, 122, 123)(**Figure 1**). Through

the phosphorylation of eIF4E, MNKs regulate the translation process, influencing in cellular growth and proliferation (121). Although the interaction of MNKs with eIF4G could be involved in translational control, the effect of eIF4E phosphorylation on translation has been more deeply investigated (113). Most work postulate that eIF4E phosphorylation increases its affinity for the cap structure and enhances the formation of a more stable eIF4F complex. In agreement with that, it has been reported that Phospho-eIF4E is positively correlated with increased protein synthesis, cell cycle progression and cell proliferation (124, 125). Nevertheless, some publications support that eIF4E phosphorylation markedly reduces its affinity for capped mRNA and decreases global mRNA translation rate (126, 127). Thus, the biological significance of eIF4E phosphorylation remains a bit controversial.
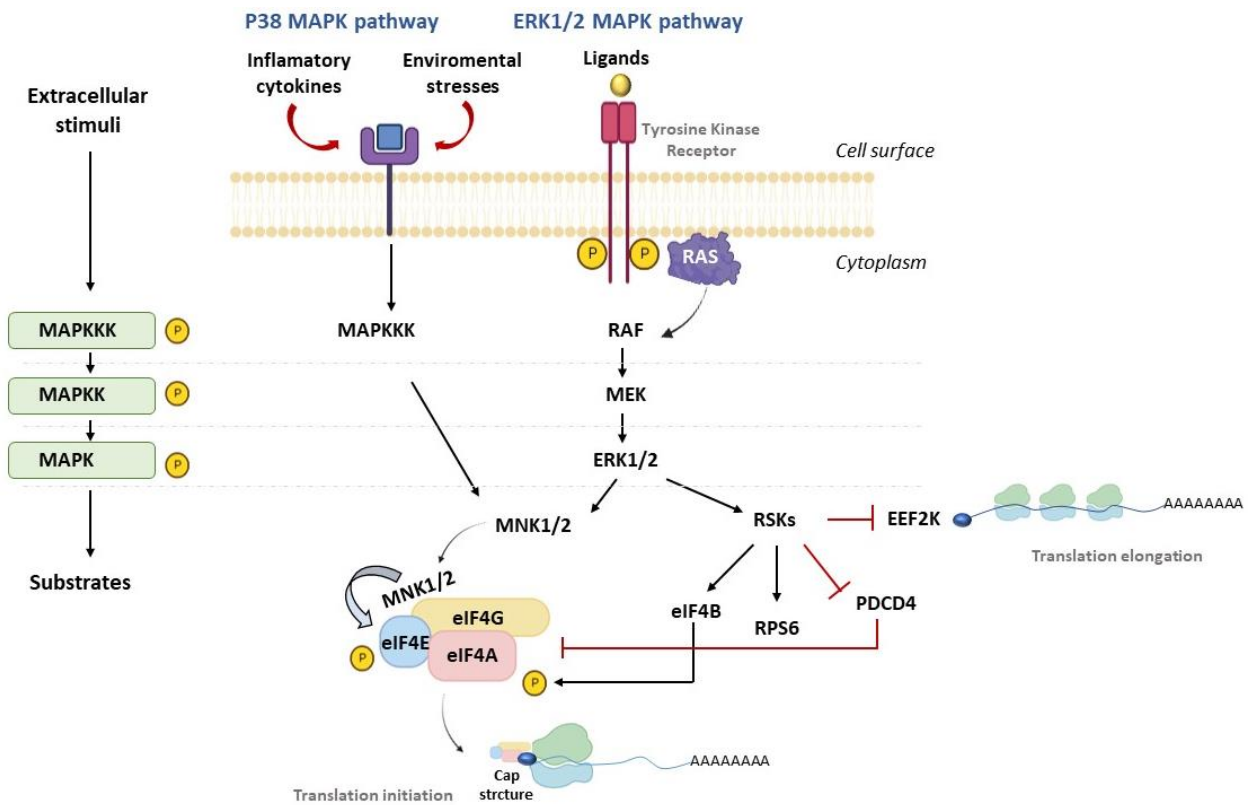
Figure 1. MAPKs signalling pathways control the protein synthesis process. The illustration depicts the two major MAPK signalling pathways involved in translation control: p38 MAPKs and ERK1/2 signalling pathways. In response to extracellular stimuli, protein receptors transduce the signal into the cell and activate a cascade of protein kinases. Kinases are phosphorylated (MAPKKK) and, in turn, phosphorylate kinases downstream (MAPKK and MAPK) regulating the activity of a broad variety of substrates like MNK1/2 (MAPK-interacting kinase 1 and 2). MNK1/2 modulates protein synthesis by interacting with eIF4G and phosphorylating eIF4E. Besides, ERK1/2 controls translation through the phosphorylation of RSK (p90 ribosomal S6 kinase), that phosphorylates a broad variety of proteins like eEF2K (eEF2 kinase), eIF4B or PDCD4 (Programmed Death Cell 4). Black arrows indicate activation. Red segments indicate inhibition.

The role of MNKs in the selective mRNAs translation has been extensively studied during the last few years. Although the translation of a specific subset of transcripts has been linked to the MNKs activity, the molecular mechanism by which this occurs is still poorly understood (128). Among these mRNAs are found those encoding the cytokines IL17 and pro-inflammatory TNFα, the anti-apoptotic protein Mcl-1, ribosomal proteins S19 (eS19) and L32 (eL32), proteins involved in cell proliferation (e.g., CDK2, CDK8, HIF-1α), proteins involved in cell cycle progression (e.g., Cyclin A, B, D1 and D3), cancer-related protein like VEGF, and transcription factors (e.g., CHOP and β-catenin) (49, 129). Several mRNAs in this pool are known as "eIF4E-sensitive mRNAs," whose translation is promoted by both Phospho- and total eIF4E levels, implying that some MNKs-target mRNAs are translated in an eIF4E-dependent manner. Besides, the MNKs activity has been associated with the translation mediated by the Internal Ribosomal Entry Sites (IRES) (126). IRESs are sequences located in the 5' untranslated region (5' UTR) of some mRNAs allowing translation without being necessary the cap structure recognition (130, 131). Although the mechanisms used to enhance IRES-mediated translation by MNKs are not well understood, they seem to be eIF4E independent.

Over the past years, there have been a significant number of publications exploring the function of MNKs in cancer. Despite of MNKs are not essential for cell viability, its activity has been linked to cell transformation and cancer progression, especially in those with the KRAS gene mutated (121). MNKs isoforms are overexpressed in several types of cancer and their high expression levels are associated with worse prognosis. Their oncogenic functions have been associated with the phosphorylation of eIF4E and translation of the subset of mRNAs above mentioned (128, 132). Thus, hyperactivation of MNKs/eIF4E axis positively correlates with an increased protein synthesis, especially of those mRNAs involved in cell growth, cell proliferation, cell hypertrophy, transformation, and metastasis (50, 124, 125). As a result, pharmacological inhibitors directed against MNKs may provide an effective anti-tumour strategy, indeed, some of them, alone or in combination, are currently in clinical trials (28, 133-136).

The MAPKs pathways also controls protein synthesis by regulating the RSKs activity. ERK1/2, but not p38 MAPKs, phosphorylates RSKs which in turn phosphorylate some component of the translation machinery apparatus (113, 137, 138). RSKs are members of a Serine/Threonine kinases family that specifically phosphorylates S6 ribosomal protein (RPS6, eS6) on its serine 235 and 236 and provides an alternative linking to MAPK signalling and mTOR/S6K-independent pathway of controlling mRNA translation. Other RSKs readouts shared with the mTOR pathway are eIF4B and the kinase of eEF2 (eEF2K), involved in translation initiation and elongation, respectively (109). eIF4B is involved in the formation of 48 PIC and its phosphorylation is correlated with an increased translation

whereas eEF2K phosphorylates and inhibits the translation elongation factor 2, resulting in translation inhibition. The phosphorylation of eEF2K impairs its activity, so upon the MNKs activation, the activity of eEF2K is inhibited. Additionally, RSKs also promote translation by stimulating the RNA helicase activity of eIF4A by phosphorylation and inhibition of its repressor protein, programmed death cell 4 (PDCD4)(139-141) (**Figure 1**).

mTOR

A Serine/Threonine kinase called Mammalian Target of Rapamycin (mTOR) exists as two physically and functionally distinct complexes called mTOR complex 1 (mTORC1) and complex 2 (mTORC2)(142). Whereas mTORC1 affects cellular proliferation, growth, protein synthesis, metabolic programs, and autophagy, mTORC2 is mainly involved in cell survival and cytoskeletal reorganization (99, 102, 143, 144). Moreover, mTORC2 has been implicated in degradation of newly synthesized proteins and has been found associated with the ribosome (145). In addition to different functional and structural distinctions, mTORC1 and mTORC2 are differentially sensitive to the drug known as rapamycin, which is a naturally occurring allosteric inhibitor of mTOR. mTORC1 is sensitive to rapamycin, whereas the activity of mTORC2 is unaffected by this drug (146-148).

mTORC1 controls the translational process by regulating the activity of the eIF4F complex according to extracellular and intracellular growth cues (149, 150). Under favourable growth conditions, mTORC1 promotes assembly of the eIF4F complex, which leads to the recruitment of the ribosome to the 5' cap structure. The activation of mTORC1 pathway leads to phosphorylation of 4E repressor proteins (4E-BPs) in multiple sites, reducing the affinity of 4E-BPs to its target eIF4E. This allows the eIF4E-eIF4G interaction and the assembly of eIF4F complex (30, 102, 149) (**Figure 2**). Although the control of the phosphorylation status of 4E-BPs by mTORC1 is a classic regulatory pathway of translation initiation, cells do not respond equally to the inhibition of mTORC1. One possible explanation for this comes from findings that the levels of expression of 4E-BPs respective to eIF4E determine the response of cells to mTOR inhibitors (151). Given that the eIF4E/4E-BPs ratio differs between cell types, this fact provides a likely explanation for the broad differences on susceptibility of cancer cells to mTOR inhibitors. Additionally, 4E-BPs can also be phosphorylated by other kinases like GSK3β and CK1ε (92, 94, 96).
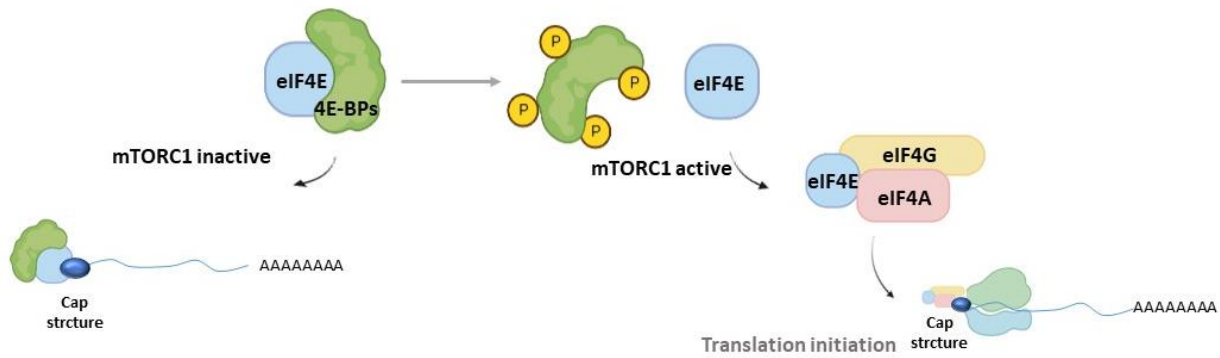
Figure 2. mTORC1 controls translation initiation through 4E-BPs. When mTORC1 is active, phosphorylates the 4E repressor proteins (4E-BPs) in four different residues. The hierarchical phosphorylation of 4E-BPs promotes its dissociation from the 4E-BPs-eIF4E complex. Thus, eIF4E free interacts with eIF4A and eIF4G to form the eIF4F complex resulting in translation initiation. However, hypophosphorylated 4E-BPs bind to eIF4E and impede the eIF4G-eIF4E interaction, resulting in the inhibition of translation initiation.

mTORC1 also regulates protein synthesis by phosphorylation of other substrates implicated in the translational process such as S6K and eIF4G (152-154). S6 kinases (S6K1 and S6K2) regulates the phosphorylation of multiple components of the translational machinery including RPS6 in five different residues (in human cells, serine-235, -236, -240, S244 and -247), eIF4B, eEF2K and PDCD4 (149). S6Ks promotes translation by stimulating the activity of the elongation factor 2 (eEF2) and the helicase activity of eIF4A through the phosphorylation of eF4B. As RSKs, S6Ks phosphorylates and inactivates the eEF2 kinase and phosphorylates and induces the degradation of the MAPK-target PDCD4 (139, 155) (**Figure 2**).

mTORC1 activity has been linked to the specific translation of a subset of mRNAs. The underlying mechanisms of this remain elusive, but a well-known group of mRNAs particularly sensitive to mTORC1 inhibitors harbours terminal oligopyrimidine (TOP) tract at their 5' ends, called TOP mRNAs (102, 156). These mRNAs encode for component of the translational apparatus, such as ribosomal proteins, translation elongation factors like eEF2 and poly(A)-binding protein (PABP). Recent studies using ribosome profiling showed that mTORC1 stimulates the translation of TOP mRNAs and TOP-like pyrimidine-rich translational element (PRTE) motifs through phosphorylation and inactivation of 4E-BPs (102). Additionally, mTORC1 promotes translation of mRNAs that encode mitochondrial protein as well as some of the known "eIF4E-sentitive" mRNAs such as Cyclin D1 or Ornithine decarboxylase (ODC)(157, 158) (**Figure 3**).
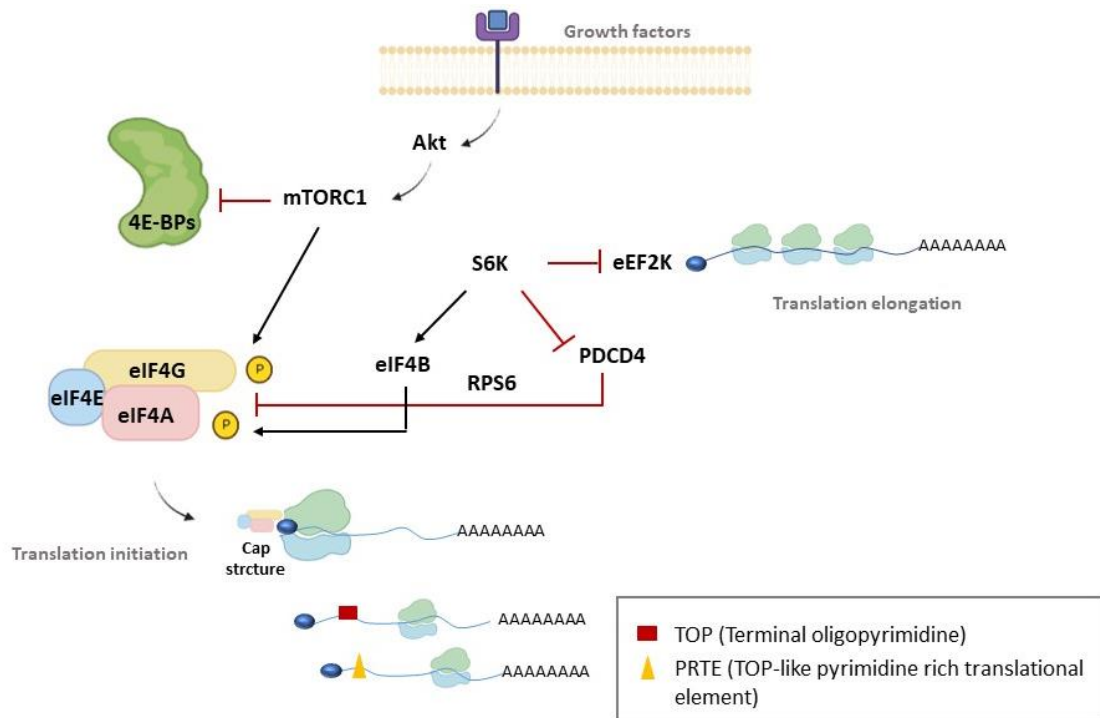
Figure 3. Akt/mTOR signalling pathway controls the protein synthesis process. The illustration depicts how mTORC1 regulates protein synthesis through the phosphorylation of a wide range of proteins. In optimal cell growth conditions, mTORC1 is phosphorylated and activated by Akt. mTORC1 phosphorylates different proteins involved in translation, such as 4E-BPs (4E-binding proteins) and S6K (S6 kinase). In turn, S6K phosphorylates eIF4B (eukaryotic Initiation Factor 4B), RPS6 (ribosomal protein S6) and eEF2K (eEF2 kinase), allowing translation initiation. Additionally, mTORC1 activation has been linked to enhanced translation of a subset of mRNAs characterized by having TOP and PRTE elements in their 5'UTRs. Black arrows indicate activation. Red segments indicate inhibition.

The cap-binding complex: eukaryotic translation initiation factors 4A, 4E and 4G and their relation with cancer

The cap-binding complex eIF4F contains the scaffolding protein eIF4G, the helicase eIF4A and the cap interaction protein eIF4E. The activity and levels of this complex are regulated by transcriptional and post-transcriptional mechanisms.

c-Myc is the best characterized transcription factor that regulates the expression levels of eIF4E, eIF4A and eIF4G. c-Myc promotes the transcription of eIF4F components and is itself a translational target of the cap-binding complex. These results reveal a regulatory feedforward loop in which c-Myc raises eIF4F levels, which in turn encourages c-Myc mRNA translation (27, 159, 160). c-Myc belong to the Myc family (including c-Myc, N-Myc, and L-Myc) which is known as the "super-transcription factor" because regulates transcription of at least 15% of the whole human genome.

Myc proteins control a wide variety of cellular processes that include ribosome biogenesis, protein translation, cell-cycle progression, metabolism, cell proliferation, differentiation, and survival (161, 162). The translation of Myc genes occurs mainly in a cap-dependent manner but when overall protein synthesis is compromised, an IRES located in the 5'UTR of their mRNAs allow their translation (163, 164). c-Myc is an oncogene dysregulated in many human cancers and it has been demonstrated that c-Myc translation enhanced by Phospho-eIF4E has an important role in oncogenesis (165, 166) (**Figure 4**).
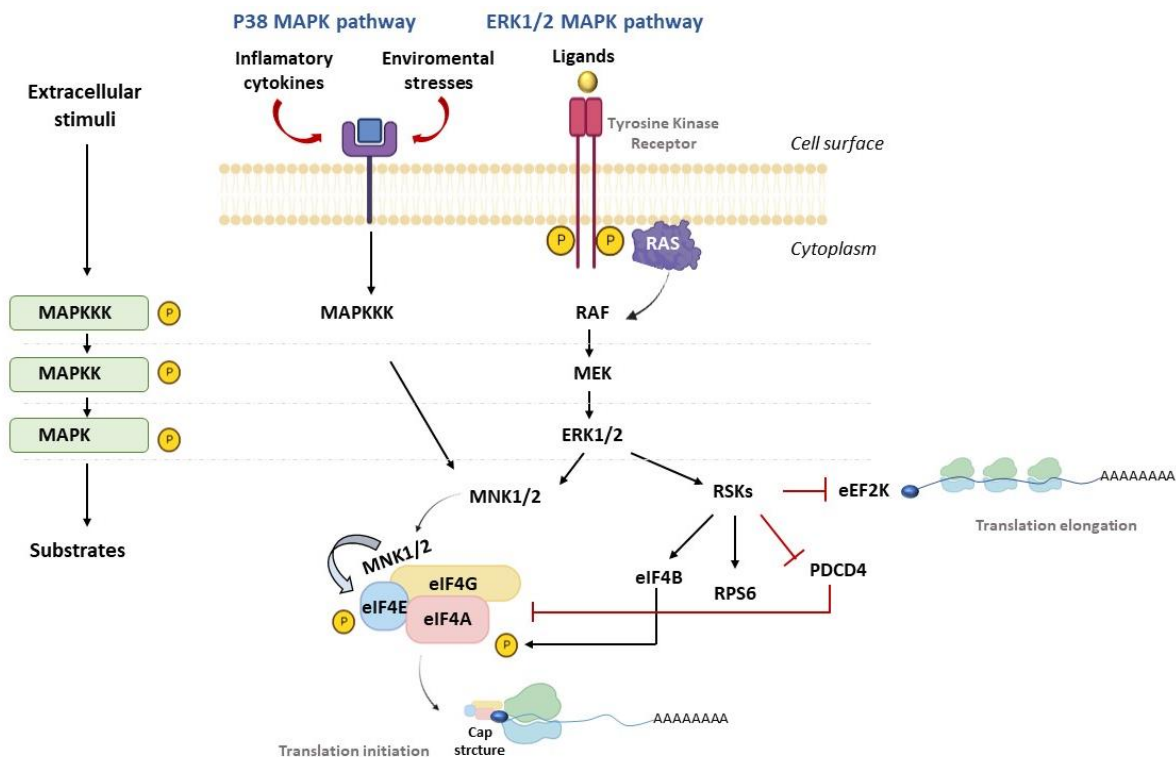
Figure 4. The cap-binding complex modulates translation. The illustration depicts selective mRNA translation linked to cap-binding complex activity. (a) Both phosphorylation and total levels of eIF4E are associated with the translation of specific groups of mRNAs. Those called eIF4E-sensitive mRNAs include transcripts whose translation is enhanced by either the phospho- or total levels of eIF4E. Several features in their sequence have been identified, including TOP and PRTE motifs found in ribosomal proteins (RPs), whose translation appears to be more sensitive to mTORC1 activity. Furthermore, translation of long and structured mRNAs has been linked to both phospho- and total eIF4E levels, such as VEGFA or Cyclin D1. (b) The RNA helicase eIF4A enhances the translation of mRNAs with a structured 5' UTR, for example, those with G/C enrichment in their 5' UTR. (c) The scaffolding protein eIF4G mediates the translation of mRNAs with a specific type of IRES element in their 5' UTR. IRES elements can be present in cap- and uncapped mRNAs (d) c-Myc is a transcription factor that can be translated in both a cap-dependent and an IRES-dependent manner. It is known as an eIF4E-sensitive mRNA whose translation is enhanced by the cap-binding complex. c-Myc, in turn, activates the transcription of eIF4E, eIF4A, and eIF4G, forming a feed-forward positive loop.

Post-transcriptional mechanisms that regulate the activity of the cap-binding complex include the phosphorylation of both eIF4E and the repressor proteins of the different components of this complex. As described in the previous section, two main pathways regulate the activity of eIF4F complex through the phosphorylation of different translation factors. eIF4E is the main limiting factor in the assembly of the eIF4F complex, whose activity is not only regulated through phosphorylation but also through repressor proteins (46, 113). The translation initiation factor 4E is a 25-kDa protein that serves to initiate cap-dependent translation via mRNA cap-binding. In human cells, there exist three members in the eIF4E family (eIF4E1, eIF4E2 and eIF43), but only eIF4E1 and eIF4E2 have the ability to interact with the cap structure (167-169). eIF4E1 is the most abundant isoform and mediates translation in optimal condition whereas eIF4E2 seems to mediate translation under low-oxygen conditions, opening up a new perspective for eIF4E2 modulating translation in tumour hypoxia (170). It is believed that eIF4E3 has not the cap-binding ability, but recent findings have shown that eIF4E3 does indeed interacts with the cap structure in an atypical manner, allowing translation occurs. It has been reported that the reduction of eIF4E1 phosphorylation after MNKs inhibition leads to upregulation of eIF4E3, suggesting that MNKs could be involved in a balance between eIF4E1 and eIF4E3 to control translation (132, 171). Therefore, when eIF4E1 function is compromised, eIF4E3 can replace its activity (172)(**Figure 5**).

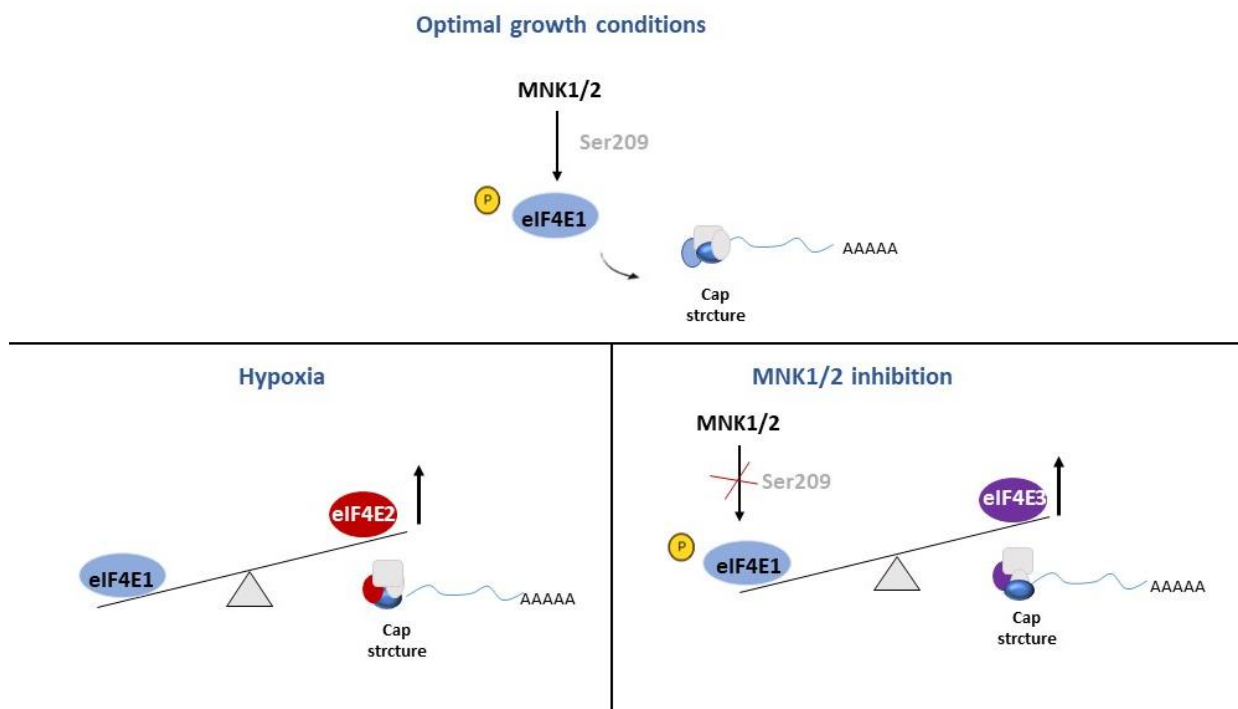**Figure 5. In mammalian cells, there exist three isoforms of eIF4E.** The illustration shows the three different isoforms of eIF4E (eIF4E1, eIF4E2, and eIF43). eIF4E1 is the most abundant isoform under physiological conditions. Its activity is regulated by MNK1/2, which phosphorylates its serine 209. Under low oxygen conditions, eIF4E2 is upregulated and forms an alternative cap-binding complex, promoting the translation of specific mRNAs associated with hypoxia. eIF4E3 is upregulated when the activity of MNK1/2 is impaired. Increased levels of eIF4E2 and eIF4E3 compared to those of eIF4E1 under specific conditions lead to the formation of an alternative cap-binding complex.

The role of eIF4E in cancer has been deeply studied in the last few years, both the total levels of eIF4E and its phosphorylation status has been associated with cancer development and progression (32, 47-49, 165, 173, 174). Indeed, the overexpression of eIF4E itself has been observed in many human tumours including HCC and is associated with poor prognosis (175). Additionally, phosphorylation status of eIF4E appears to confer resistance to cellular stress, including oxidative stress, starvation, and cytotoxic stress (50, 176, 177). As a result, there is currently a lot of interest in the potential role of inhibiting eIF4E and/or its phosphorylation as a therapeutic target against cancer.

Phosphorylated form and total levels of eIF4E have been linked to enhanced translation of a subset of mRNA. However, the underlaying mechanism for this mRNA selective translation is not completely understood. In general, the eIF4E-sensitive mRNAs have long and structured 5' UTR like VEGFA or Cyclin D1 mRNAs. Furthermore, some features within their 5'UTR have been discovered, such as the 5' terminal oligopyrimidine (5' TOP) motif and the pyrimidine-rich translational element (PRTE), whose translation has been reported to be more sensitive to mTORC1 activity than ribosomal proteins (RPs) or translation factors (102, 178). Other studies have reported that the haploinsufficiency of eIF4E also causes the downregulation of translation of mRNAs with cytosine-enriched regulator of translation (CERT) in their 5' UTR (48, 178). All these elements make the mRNAs sensitive to changes in the levels of phosphorylated and/or total eIF4E. In addition, the interaction between eIF4E and other translation machinery components also determines selective mRNA translation. For example, the long and structured 5' UTRs render them critically dependent of the unwinding activity of eIF4A subunit of the eIF4F complex (178-180).

eIF4A is a canonical DEAD-box helicase protein that exhibits RNA-dependent ATPase and ATP-dependent bidirectional helicase activities. In mammals, there are three isoforms of eIF4A: eIF4A, eIF4AII and eIF4III. Both eIF4AI and eIF4AII but not eIF4III are involved in the control of translation initiation. The eIF4A RNA helicase has been implicated in the translation of mRNAs with long and complex 5' UTRs and also with those that contain 12-nucleotide (CGG)4 motif (140, 178, 179, 181). The activity of this factor is mainly controlled by its repressor protein PDCD4 whose activity is

controlled by RSKs and S6Ks. Under optimal conditions, PDCD4 is phosphorylated by both RSKs and S6Ks, resulting in the release of eIF4A from the inhibitory PDCD4-eIF4A complex, allowing cap-dependent translation (139). PDCD4 also contains an mRNA binding domain through which it binds to the IRES sequences of several mRNAs and avoids the formation of 48S PIC, resulting in its translation inhibition (141). Thus, PDCD4 acts as a repressor protein of eIF4A and inhibitor of translation of specific mRNAs containing IRES. The eIF4A helicase activity is also modulated by eIF4B which in turn is controlled by both mTORC1 and MAPKs signalling pathways. It has been published that eIF4B interacts with eIF3 and promotes eIF4A helicase activity increasing translation (109, 155, 182, 183). eIF4H, a homologous protein of eIF4B, is another modulator of eIF4A and it is thought that both proteins eIF4H and eIF4B increase translation rate by allowing eIF4A to unwind long and more stable RNA duplexes (184, 185)(**Figure 6A**). The expression of eIF4A itself has not been deeply investigated in tumours but it has been recently published that eIF4A overexpression has an important role in pancreatic ductal adenocarcinoma (PDA) and its inhibition could represent an interesting therapeutic target (186). In fact, Silvestrol, an eIF4A inhibitor, has been demonstrated to be an interesting anticancer drug since it negatively impacts survival pathways and angiogenesis by inhibiting translation of malignancy-related mRNAs like VEGFA or BCL2 (28, 133, 187-190) (**Figure 6B** and **6B**).
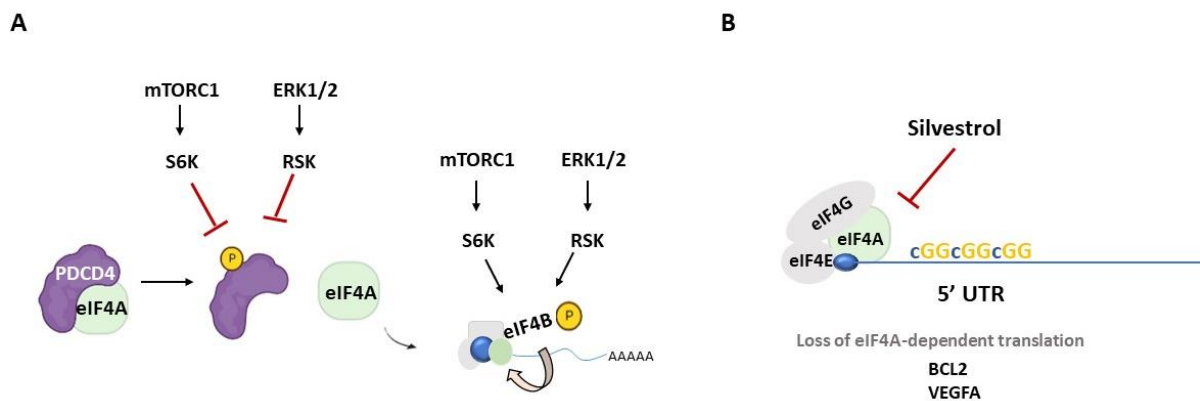
Figure 6. The activity of eIF4A is controlled by mTORC1 and MAPKs pathways. (**A**) In optimal growth conditions, both mTORC1 and ERK1/2 phosphorylate S6K and p90 RSK, respectively. S6K and RSK phosphorylate PDCD4, causing its degradation. PDCD4 is an eIF4A repressor protein that, in adverse conditions, sequestrates eIF4A, avoiding the eIF4F assembly. The activity of eIF4A is also stimulated by eIF4B, whose activity is controlled by S6K and RSK. (**B**) Silvestrol, an eIF4A inhibitor, specifically inhibits the translation of those mRNAs with a 12-nucleotide (CGG)4 motif, which includes mRNAs encoding oncoproteins such as VEGFA and BCL2.

The third component of the eIF4F complex is the scaffolding protein eIF4G which has an important role in both cap-dependent and independent translation. eIF4G recruits different initiation factors to the cap structure and bridges the 5' UTR of mRNAs with the polyadenylated 3' UTRs via interaction with polyA binding protein (PABP). The eIF4G:PABP interaction induces mRNA circularization and enhances mRNA translation initiation and mRNA stability (62). In mammalian cells two functional eIF4G homologs are known, termed eIF4GI and eIF4GII (also called DAP5) which share a 46% identity. Whereas eIF4GI interacts with eIF4A and eIF4E to form the eIF4F complex, DAP5 is unable to bind to eIF4E but interacts with eIF3 (191). This highlights an alternative cap-dependent mechanism of translation when eIF4GI is impaired. eIF4GI is also involved in IRES-dependent translation although the underlying mechanisms of this fact are poorly understood (**Figure 4C** and **7**). IRES were identified in picornaviruses, but many cellular mRNAs also use these elements as regulator of translation initiation when cap-dependent translation is compromised. It is estimated that about 10% of all cellular mRNAs contain IRES, among them those of the Myc family (163, 192). They are classified into 4 groups which differ in the requirements of trans-acting and canonical factors. Few trans-acting factors have been identified, but it is hypothesized that a combinatorial code of RNA-binding trans-acting factors and canonical translation factors exist that form a complex under different cellular conditions allowing specific IRES translation (130, 164, 178). eIF4G has been associated with the translation of IRES type 2 which includes encephalomyocarditis virus (EMCV) IRES. Other findings have suggested that eIF4G could have a role in the translation of poliovirus IRES (type 1) together with eIF4A. In contrast, hepatitis C virus (HCV) IRES seems to require eIF2 and eIF3 but not eIF4G (193, 194).

eIF4GI overexpression has been associated with enhanced selective mRNA translation of genes involved in cell survival and DNA damage response (195). Some of those mRNAs have not had an identified IRES element in their sequences, so other features must exist to favour their translation. For these cases, the eIF4E-eIF4G interaction could have a pivotal role, resulting in disrupting the protein-protein interaction between eIF4E and eIF4G and providing an interesting possible therapeutic approach (196-198).
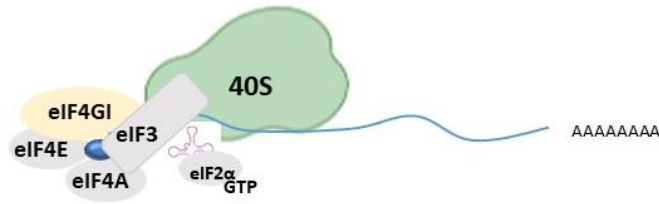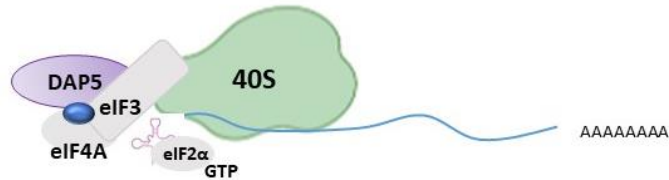
Figure 7. Scaffolding protein eIF4G is implicated in non-canonical translation initiation. In mammalian cells, two isoforms of eIF4G have been described (eIF4GI and eIF4GII or DAP5). eIF4G1 interacts with eIF4E and eIF4A, forming the canonical cap-binding complex. The cap-binding complex recognizes the cap structure located in the 5'UTR of most mRNAs, allowing cap-dependent translation to take place. DAP5 does not have the ability to bind to eIF4E but is able to interact directly with eIF3, forming an alternative cap-dependent complex. Both eIF4GI and DAP5 have been implicated in IRES-dependent translation, allowing translation without cap-structure recognition.

RESULTS

Sorafenib is described as a multikinase inhibitor that targets the MAPK pathways and deregulates the Akt-mTOR pathway as well, although the latter one does not seem to play a dominant role in the first cellular response to Sorafenib (24). In this Thesis work, we sought to figure out the relevance of the MAPK inhibition by Sorafenib, with the ultimately aim of figuring out the selective mRNA translation promoted by this drug.

Sorafenib downregulates MAPKs pathway leading to reduction of eIF4E phosphorylation status

Two main signalling pathways transduce the external stimuli to the translational machinery: PI3K-mTORC1 and Ras-MAPK-MNKs. Both pathways regulate the activity of different translation factors, modulating protein synthesis in this way (108, 110, 182). We wanted to evaluate whether either of one, or both of them, mediate the protein synthesis inhibition produced by Sorafenib in HepG2 cells. To do so, HepG2 cells were treated with the mTORC1 inhibitor Sirolimus, the MNK1 inhibitor 4-Amino-5-(4-fluoroanilino)-pyrazolo[3,4-d]pyrimidine and the tyrosine-kinase inhibitor Sorafenib during 1 h and their impact on translation were assessed. We measured the phosphorylation status of some mTORC1 targets and some components of the MAPKs pathway by western blot hybridization. In basal conditions, HepG2 cells displayed increased phosphorylation in mTORC1 targets 4EBP1 and RPS6, the MNKs target eIF4E, and the MAPKs pathway component ERK1/2 (**Figure 11**). These results are in agreement with optimal growth conditions in the absence of inhibitors and with a large translational rate observed by polysome profile experiments (**Figure 2A**). The MNK1 inhibitor resulted in a clear dephosphorylation of eIF4E, while a mild increase in the phosphorylation of ERK1/2 in their Thr202/Tyr204 was observed, consistent with being downstream and upstream MNK1 effectors, respectively. ERK1/2 phosphorylates MNK1 in response to stimuli and the inhibition of MNK1 drives the hyperactivation of ERK1/2 as a mechanism to counteract the suppression (199). The inhibition of mTORC1 with Sirolimus caused a huge reduction at the phosphorylation status of RPS6 in the double Ser235/236 phosphorylation and a modest decrease in its phosphorylation at Ser240/244. Interestingly, despite of the clear reduction in the phosphorylation levels of RPS6, the time and the concentration of Sirolimus used were not enough to alter the phosphorylation status of 4E-BP at its Thr37/46. In turn, Sorafenib strongly abrogated the phosphorylation of eIF4E and ERK1/2, and a clear reduction of the phosphorylation status of RPS6 at Ser235/236 was also observed. Additionally, hyperphosphorylation of ERK1/2 induced by the MNK1 inhibitor was abolished by Sorafenib, suggesting that Sorafenib acts upstream MNK1. In contrasts, well-known mTOR targets, Ser240/244-RPS6 and Thr37/46-4EBP1, remained unaffected, which is consistently with the results showed in chapter 1 (see **Figure 9** and **11**). Altogether, we conclude that

Sorafenib, early after its treatment, switches off completely the MAPK kinase signalling pathway, altering translation machinery activity through the reduction in the phosphorylation at Ser209 of eIF4E.

It has been reported that the inactivation of mTORC1 results in a feedback activation of ERK1/2. In agreement with this, Sirolimus treatment induced an increase in the phosphorylation status of eIF4E that was completely abrogated when cells were treated simultaneously with Sorafenib. However, the Sirolimus treatment combined with MNK1 inhibitor had only a modest impact on phosphorylation status of eIF4E, in clear contrast with the treatment with Sirolimus and Sorafenib simultaneously. Furthermore, the total protein levels remained unaffected indicating that the treatments only altered the phosphorylation status of the studied proteins (**Figure 12**).
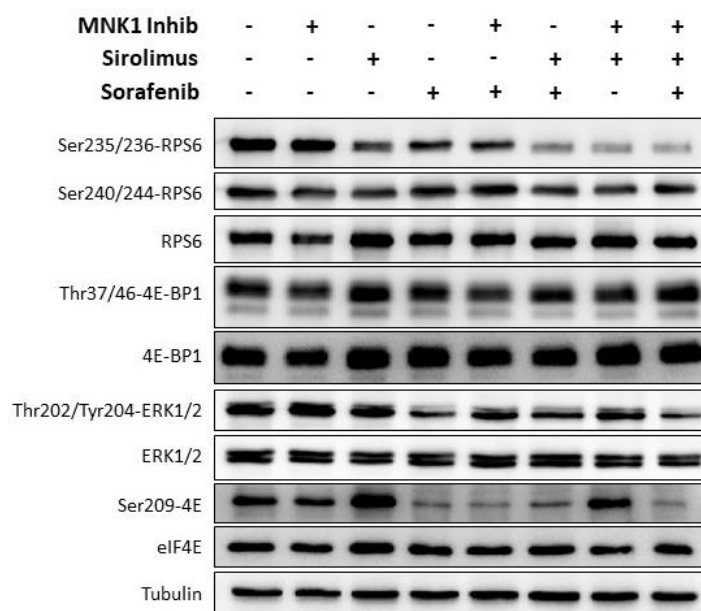
Figure 12. Sorafenib downregulates MAPKs pathways and reduces phospho-eIF4E/eIF4E ratio. Total protein extracts from HepG2 cells untreated or treated with 20 µM 4-amino-5-(4-fluoroanilino)-pyrazolo[3,4-d]pyrimidin (a MNK1 inhibitor), 100 nM Sirolimus and 10 µM Sorafenib for 1 h were obtained and analysed by western blotting as described in the Materials and Methods section. The absence of the drug is represented by a - mark. To test the mTOR activity the Phospho-S6/S6 and the Phospho-4E-BP1/4E-BP1 ratio were analysed using specific antibodies raised against Ser240/244-S6, RPS6, Thr37/46-4E-BP1 and 4E-BP1, respectively. To test MAPKs pathway were analysed the phospho-ERK1/2/ERK1/2 and the phospho-eIF4E/eIF4E ratio using specific antibodies raised against Thr202/Tyr204-ERK1/2, ERK1/2, Ser209-eIF4E and eIF4E, respectively. Both pathways, mTORC1 and MAPKs, share the phosphorylation sites in RPS6 at Ser235/236. Tubulin was used as a loading control. A representative image is shown.

Focused on the MAPKs pathway, we wanted to evaluate the effect on the phosphorylation levels of ERK1/2 and eIF4E over time. Thus, we measured the total and phosphorylated levels of these

proteins by western blot hybridization upon a time course of Sorafenib treatment (**Figure 13A**). Then, the phospho-eIF4E/eIF4E and phospho-ERK1/2/ERK1/2 ratio were calculated. The Ser235/236-RPS6/RPS6 and Ser240/244-RPS6/RPS6 ratios were also assessed (**Figure 13B**). The double phosphorylation of RPS6 at Ser240/244 has been reported to be an exclusive read-out of mTORC1; in contrast, the Ser235/236-RPS6 phosphorylation seems to be produced by both the MAPKs and mTORC1 pathways (137). As shown in **Figure 13,** a clear decrease of phosphorylation levels of eIF4E, ERK1/2 and in the double phosphorylation of RPS6 at Ser235/236 was observed but Ser240/244-RPS6 remained unaffected (**Figure 13A** and **13B**). In terms of translation, these data point to the MAPKs, rather than the mTOR pathway, as playing a dominant role in the Sorafenib response.
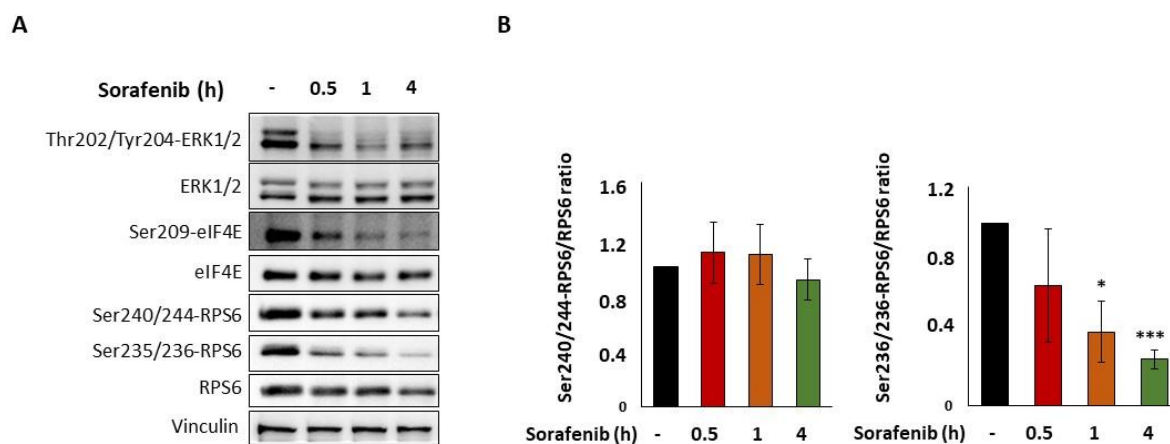
Figure 13. Sorafenib switches off MAPK signalling pathway leading to reduction of the eIF4E phosphorylation. Time-course of ERK1/2 and eIF4E phosphorylation. (**A**) Total protein extracts from HepG2 cells untreated or treated with 10 µM Sorafenib were obtained and analysed by western blotting as described in the Materials and Methods section. Vinculin was used as a loading control. The untreated cells condition is represented by a - mark. The signals of total and Phospho-ERK1/2 were detected using specific antibodies raised against ERK1/2 and Thr202/Tyr204-ERK1/2, respectively. The signal of total and Phospho-eIF4E were detected using specific antibodies raised against eIF4E and Ser209-eIF4E, respectively. The signal of total and Phospho-RPS6 were detected using specific antibodies raised against RPS6 and Ser240/244-RPS6 and Ser235/236-RPS5, respectively. A representative image is shown. (**B**) Densitometric analysis of the Ser240/244-RPS6/RPS6 and Ser235/236-RPS6/RPS6 ratio based on three independent replicates are shown. Statistical significances were analysed by the Student's t test (* $p < 0,05$; *** $p < 0,0001$); Error bar: SD.

The cap-binding complex modulates translation in response to Sorafenib treatment

The eIF4E has been reported to be the limiting initiation factor for mRNA translation (46, 52, 74, 113). The phosphorylation levels of eIF4E have been associated with translation of a set of mRNAs involved in different aspects of cancer cells and drug resistance. Cyclin D1 (involved in G1/S cell cycle

transition), c-Myc (a transcription factor involved in a broad cellular functions including cell proliferation), Mcl-1 (an anti-apoptotic protein) and Vascular Endothelial Growth Factor A (VEGFA, which induces proliferation and migration of vascular endothelial cells, and is essential for both physiological and pathological angiogenesis) have been reported as Phospho-eIF4E targets (32, 165, 177). The hyperphosphorylation status of eIF4E is linked to enhanced translation of those eIF4E mRNAs targets so we wondered whether the anti-proliferative, anti-angiogenesis and pro-apoptotic properties of Sorafenib could be explained, at least in part, by the downregulation of translation of these pro-tumoral transcripts. To test this, we analysed the translation of these Phospho-eIF4E targets after the Sorafenib treatment in HepG2 cells. Firstly, polysome profiles of untreated and Sorafenib-treated cells for 1 h were performed and then, two RNA populations from the fractionation were obtained, a low-translated fraction and high a translated fraction (**Figure 14A**). The abundance of mRNAs of the various Phospho-eIF4E targets in polysomes was assessed in two ways: (1) the percentage of mRNA, which compares the amount of mRNAs in the polysome fraction to its amount in the low-translated fraction, and (2) the relative mRNA levels, which divide the amount of mRNAs in the polysome by its total cellular mRNA. Additionally, the translation of several ribosomal proteins (RPs) which are well-known mTORC1 targets was also analysed (102). Our findings showed that the percentage of the mRNAs of Cyclin D1 and VEGFA but not of c-Myc and Mcl-1 was significantly reduced after the Sorafenib treatment compared to the control condition. However, the c-Myc mRNAs relative level in polysome was clearly reduced by Sorafenib. Unexpectedly, an increase in the VEGFA relative mRNA level in polysome was found, in contrast to the previous results. Moreover, it must be pointed out that the translation of Mcl-1 does not seem to be affected in spite of the fact that its translation has been closely linked to the phosphorylation of eIF4E (49). Regarding the translation of RPs, no differences were observed after the Sorafenib treatment, whereas the Sirolimus treatment that was used as the control condition for this experiment, resulted in a notable reduction of RPs translation. The absence of significance in Sirolimus treatment is probably explained by the high variability of our results in the control conditions (**Figure 14B**).

We next wondered whether Sorafenib only affected translation or whether the total amount of these mRNAs was also affected. Thus, we analysed their total cellular mRNA levels after Sorafenib treatment by RT-PCR. Intriguingly, we found that the VEGFA mRNA level was significantly reduced compared to the control whereas those of Cyclin D1, c-Myc and Mcl-1 were unaffected. Consistently with the VEGFA translational data, in which we observed a decrease in the percentage of VEGFA mRNA in polysomes in Sorafenib-treated cells, we also found an increase when the data were normalized to total cellular mRNA since Sorafenib also reduces the total cellular VEGFA mRNA levels (**Figure 13C**). Therefore, we conclude that Sorafenib specifically inhibits the translation of a specific

subset of mRNAs that are Phospho-eIF4E targets, including Cyclin D1 and c-Myc but also alters the translation and mRNA concentration of other ones, such as that of VEGFA.
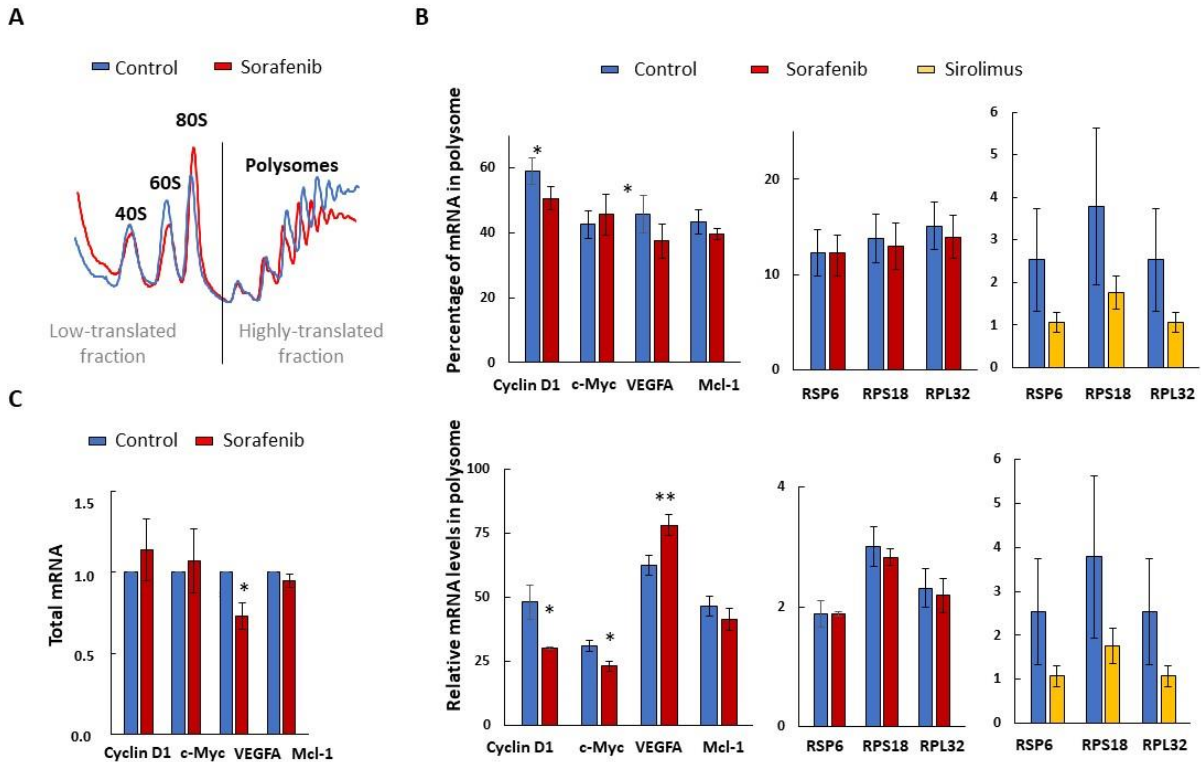
Figure 14. Translation of the eIF4E-sensitive mRNAs. (A) Polysome profile of HepG2 cells treated with Sorafenib. Whole cell extracts from HepG2 cells untreated (blue) or treated (red) with 10 µM Sorafenib for 1 h were prepared and fractionated on sucrose gradient as described in the Materials and Methods section. Polysome profiles were recorded. Total RNA was isolated from low translated fraction [soluble fraction, free ribosomal subunits and monosomes] and from high translated fraction [heavy polysomes]. **(B)** Histograms of the percentages of mRNAs in polysomes are shown. The analysis includes cells treated with 100 nM Sirolimus for 1 h. The levels of eIF4E-sensitive mRNAs (Cyclin D1, c-Myc, VEGFA, Mcl-1) and well-known mTORC1 targets (RPS6, RPS18, RPL32) were analysed by RT-PCR. **(C)** Histograms of the mRNAs relative levels in polysomes are shown. The levels of eIF4E-sensitive (Cyclin D1, c-Myc, VEGFA, Mcl-1) and well-known mTORs-targets (RPS6, RPS18, RPL32) mRNAs were analysed by RT-PCR in total cellular and in polysomes RNAs. The relative levels were calculated by the polysome mRNA/total RNA ratio. **(D)** Histogram of the total cellular RNA levels is shown. The levels of eIF4E-sensitive mRNAs (Cyclin D1, c-Myc, VEGFA, Mcl-1) were analysed by RT-PCR in total cellular RNAs. The mRNA levels were standardized against actin and normalized to the control condition. Analysis was based on four and three independent experiments in Sorafenib- and Sirolimus-treated cells, respectively. Statistical significances were analysed by the Student's t test (* $p < 0.05$) Error bar: SD.

The total protein levels of Cyclin D1 and c-Myc were analysed after a Sorafenib treatment by western blot hybridization to check whether the reduction of translation we observed corresponds to a decrease in the total protein levels. A strong reduction in c-Myc and Cyclin D1 protein levels were

found and was higher over time. Indeed, after 4 h of sorafenib treatment, the Cyclin D1 protein level was practically lacking (**Figure 15**). We also wanted to analyse VEGFA protein level but unfortunately, we could not find an appropriate antibody against this protein so far. In conclusion, Sorafenib suppresses the translation of pro-tumoral mRNAs such as Cyclin D1, c-Myc and VEGFA, consistent with the downregulation of the eIF4E activity associated to the inhibition of the MAPK signalling pathway. In addition, the reduction in the translation levels correlates with a decrease in their protein levels which could drive, at least in part, to the anti-angiogenic and anti-proliferative effects of Sorafenib.
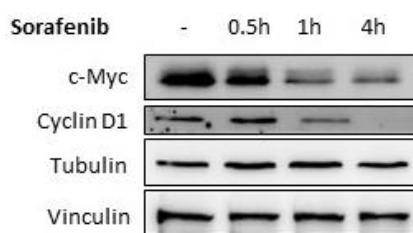
Figure 15. Time-course of eIF4E-target proteins levels. Total protein extracts from HepG2 cells untreated or treated with 10 µM Sorafenib were obtained at the indicated times and analysed by western blotting as described in the Materials and Methods section. Vinculin and Tubulin were used as loading controls. The untreated cells condition is represented by a - mark. The signal of c-Myc, Cyclin D1, Tubulin and Vinculin were detected using specific antibodies. A representative image is shown for each protein.

As it has been described in the Chapter 1 of this Thesis, protein synthesis is clearly inhibited 3 h after a Sorafenib treatment. This inhibition is not completely explained by the phosphorylation of eIF2α since the polysome profile of Sorafenib-treated cells with lower phosphorylated levels of eIF2α was only partially restored, implying that another mechanism may also be contributing. It is known that the phosphorylation of eIF4E is associated with translation of specific mRNAs but it does not directly have a huge impact on global protein synthesis (48, 49). However, it is known that the phosphorylation status of eIF4E controls expression of the transcription factor c-Myc. c-Myc promotes transcription of several components of the translation machinery including eIF4E, eIF4A and eIF4G, stablishing a regulatory positive feedforward loop by which c-Myc increases eIF4F levels, which in turn promotes the translation of c-Myc mRNA (27, 159, 160). In agreement with that, we speculated that Sorafenib also affects globally to translation through downregulation of c-Myc. To test this hypothesis, the protein levels of the component of eIF4F complex, eIF4A, eIF4E and eIF4G, were analysed after a Sorafenib treatment by western blot hybridization. As shown in **Figure 16,** a

long-term Sorafenib treatment (12 h) caused a clear reduction of both eIF4A and eIF4G although eIF4E levels remained apparently unaltered (**Figure 16A**). The levels of eIF4A and eIF4G proteins were then subjected to a kinetic study to test whether or not either protein responded differently to Sorafenib. Interestingly, we found that the protein levels of the eIF4A were more sensitive to Sorafenib treatment at shorter times than the levels of eIF4G, implying that Sorafenib acts more selectively on eIF4A, most likely via other mechanisms that do not simultaneously affect the eIF4G levels (**Figure 16B**).
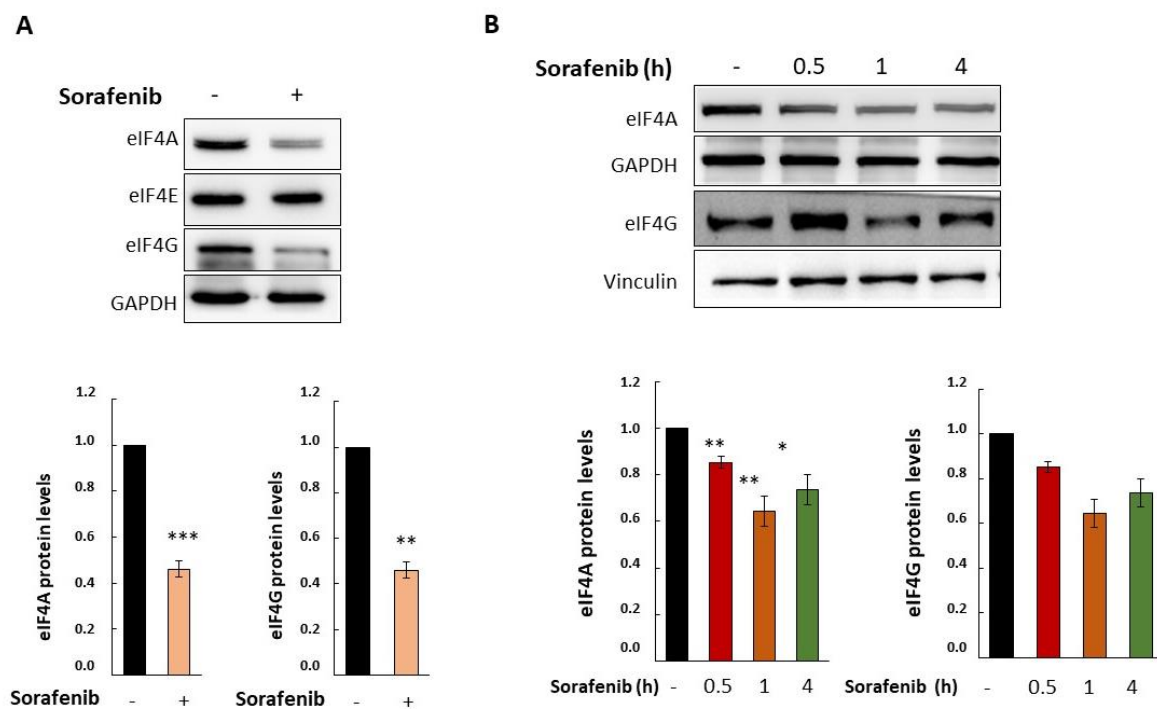
Figure 16. Levels of the cap-binding complex components upon a Sorafenib treatment. (A) Levels of the cap-binding complex components after the Sorafenib treatment. **Top panel**: Total protein extracts from HepG2 cells untreated or treated with 10 µM Sorafenib for 12 h were obtained and analysed by western blotting as described in the Materials and Methods section. The untreated cells condition is represented by a - mark. GAPDH was used as loading control. The signal of eIF4A, eIF4E, eIF4G and GAPDH were detected using specific antibodies. A representative image is shown. **Bottom panel**: The densitometric analysis based on three independent replicates is shown. **(B)** Time-course of the levels of eIF4A and eIF4G after a Sorafenib treatment. Top panel: Total protein extracts from HepG2 cells untreated or treated with 10 µM Sorafenib were obtained and analysed by western blotting as described in the Materials and Methods section. GAPDH and Vinculin were used as loading control. Bottom panel: The densitometric analysis based on three independent replicates is shown. The untreated cells condition is represented by a - mark. Statistical significances were analysed by the Student's t test (* $p < 0,05$; ** $p < 0,001$; *** $p < 0,0001$); Error bar: SD.

Some canonical translation factors such as eIF4G or eIF4A has been also associated with IRES-mediated translation (163, 164, 194). IRESs allow the mRNAs to be translated by a cap-independent manner which is relevant when the cap-dependent scanning mechanism of translation initiation is compromised (79, 130, 178). We next considered whether IRES-mediated translation is promoted by Sorafenib. To study this, a bicistronic reporter plasmid based on a dual luciferase assay to measure IRES-mediated translation versus cap-dependent translation was used. HepG2 cells were transfected with a rLuciferase-POLIRES-fLuciferase bicistronic vector. Translation of the Renilla luciferase (rLuciferase) is cap-dependent whereas the translation of Firefly luciferase (fLuciferase) is directed by the poliovirus IRES. Upon measuring both luciferase activities sequentially from the same sample, the fLuciferase/rLuciferase ratio was calculated and normalized to the control condition, whose ratio was arbitrarily set to one. As a result, the analysis of the ratio revealed a significant increase of IRES-dependent translation after 3 h of Sorafenib treatment. Intriguingly, the translation ratio decreased at long-term treatment (**Figure 17**). These data are consistent with changes in eIF4A and eIF4G protein levels over time, implying that changes in the proportion of the eIF4F complex components could be associated with IRES-dependent translation.
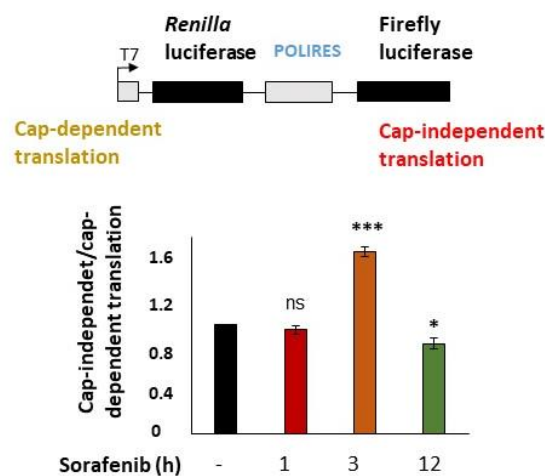
Figure 17. Sorafenib switches IRES-mediated translation and cap-dependent translation consistent with the changes in eIF4A and eIF4G levels. Top panel: Diagram of the bicistronic reporter (rLuciferase-IRES-ffLuciferase which mediates the cap-dependent translation of *Renilla* luciferase and IRES-dependent translation of firefly luciferase). **Bottom panel**: HepG2 cells were transfected with the reporter for 48 h and treated with 10 µM Sorafenib for the indicated times. Firefly and *Renilla* luciferase assays were performed for each experimental condition. Data are expressed from the Firefly luciferase signal divided by *Renilla* luciferase signal both generated from the same vector. The control condition is taken as the reference value (see the Materials and Methods section). The quantification is based on four separate experiments. Statistical significances were analysed by the Student's t test (* $p < 0.05$; ** $p < 0,001$; *** $p < 0,0001$; **** $p < 0,00001$; ns: not significant); Error bar: SD.

Role of MNKs in the Sorafenib-response

The MNK1 is the most common kinase of eIF4E. It is known that MNK1 controls the eIF4E activity by phosphorylation on its serine 209 downstream of both the MEK/ERK and p38 MAPK pathways (112, 115, 119, 120, 123). The laboratory of Dr Martín has described two isoforms of MNK1 in human cells, MNK1a and MNK1b. The MNK1b is a spliced variant of MNK1a that presents different features in its C-terminus and has higher basal eIF4E kinase activity. Moreover, its activity is poorly upstream controlled by ERK or p38 MAPKs (117). In contrast, the capacity of MNK1a to phosphorylate eIF4E has been described to be highly dependent on both pathways ERK1/2 and p38 MAPK. The physiological function of MNK1 remains practically unknown, but the necessity of interaction with eIF4G to phosphorylate eIF4E is well-characterized. eIF4G acts as a scaffolding protein for eIF4E and MNKs, allowing the phosphorylation of eIF4E when both are positioned in close proximity (122). Regarding translation, some reports support a positive role of MNK1 in translation based on the fact that its overexpression clearly increases the phosphorylation levels of eIF4E, which seem to encourage the translation efficiency (124, 125). However, another report postulated that the overexpression of MNK1 causes a reduction in the amount of cap-dependent translation, pointing to a detrimental role for MNK1 in the translational control (126, 127).

A number of studies have stablished the importance of the MNK1-eIF4E axis in the progression of several tumours. In these tumours, the downregulation of MNK1 or the suppression of eIF4E phosphorylation seems to be favourable for their progression (32, 47, 125, 134-136). To better understand the role of MNK1 on the response to Sorafenib, we overexpressed both MNK1 isoforms, MNK1a and MNK1b, and evaluated the phosphorylation status of eIF4E and cell proliferation. To do so, we first transfected HepG2 cells with pcDNA3-MNK1a-Flag and pcDNA3-MNK1b-Flag plasmids, kindly provided by Dr Martín (200). We then evaluated cell proliferation after a 12 h of Sorafenib treatment. Those cells overexpressing MNK1a but not MNK1b showed a slightly lower effect on cell proliferation following the Sorafenib treatment compared to control cells with endogenous levels of both proteins. Nevertheless, this was so slight that it was not significant (**Figure 18A**). Furthermore, we wanted to look at the phosphorylation status of eIF4E in cells overexpressing both MNK1 isoforms because the role of MNK1 in cell proliferation has been linked to their ability to phosphorylate eIF4E. Consistently with the previous result, we observed that the phosphorylation status of eIF4E was mildly increased in cells overexpressing MNK1a but not MNK1b compared with cells expressing endogenous levels of both proteins in Sorafenib-treated cells (**Figure 18B**). It must be noted that the overexpression of both isoforms of MNK1 seems not to significantly increase the phosphorylation levels of eIF4E in control conditions where basal eIF4E phosphorylation levels are normally high. In

short, our findings do not discard a role for MNKs in the response to Sorafenib but demonstrate that the overexpression of both isoforms of MNK1 is not enough to counteract the Sorafenib-MAPK inhibition in the experimental conditions used. Firstly, the MNK1 overexpression was not enough to abrogate the cell proliferation inhibition induced by Sorafenib and secondly, this seems to be related to the inability to sustain the phosphorylation levels of eIF4E following the Sorafenib treatment. At this point, we next decided to analyse the role of MNKs in cells upon Sorafenib treatment at lower doses.
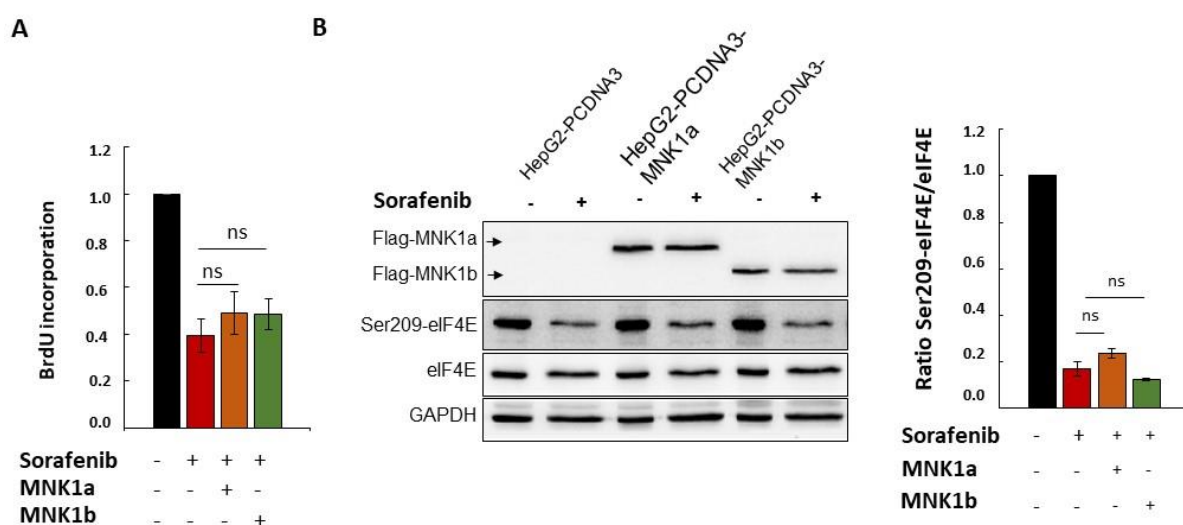
Figure 18. Role of Mnk1 on sorafenib response. HepG2 cells transfected with PCDNA3, PCDN3-MNK1a-Flag and PCDNA3-MNK1b-Flag during 48 h. **(A)** HepG2 cells untreated or treated with 10 µM Sorafenib for 12 h were obtained and prepared to analyse cell proliferation as described in the Materials and Methods section. **(B) Left panel:** Total protein extracts from transfected cells untreated or treated with 10 µM Sorafenib for 1 h were obtained and analysed by western blotting as described in the Materials and Methods section. GAPDH was used as a loading control. The signals of MNK1a and MNK1b were detected using specific antibody raised against the Flag epitope. Total and phospho-eIF4E were detected using eIF4E and Ser209-eIF4E antibodies, respectively. The untreated- and treated-cells condition are represented by a - and + marks, respectively. **Right panel**: The densitometric analysis of the phospho-eIF4E/eIF4E ratio based on three independent replicates is shown. The untreated cell condition a is represented by a - mark. The overexpression is indicated by a + mark. Data were normalized to the control condition. Statistical significances were analysed by the Student's t test (ns, not significant); Error bar: SD.

Hence, we performed a western blot hybridization to check the phosphorylation levels of eIF4E upon a treatment with 1 µM Sorafenib. We observed that control cells with endogenous levels of MNK1a and MNK1b showed a reduction of the eIF4E phosphorylation status by approximately 20% after the treatment, in clear contrast with cells overexpressing MNK1a, whose phosphorylation levels

of eIF4E were increased. Interestingly, the overexpression of MNK1b had no apparent effect on the phosphorylation status of eIF4E (**Figure 19**). Therefore, these observations support our previous assumption that the overexpression of both isoforms of MNK1 was insufficient to overcome the inhibitory effect of Sorafenib at a high dose (10 µM). However, at lower concentrations (1 µM), the overexpression of MNK1a but not MNK1b, seems to be able to counteract the inhibitory effect of Sorafenib.
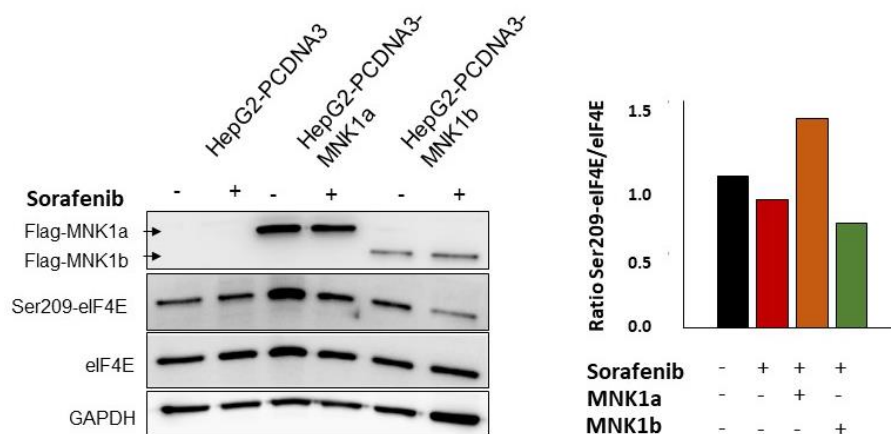
Figure 19. The eIF4E phosphorylation levels are insensitive to lower sorafenib doses after MNK1a overexpression. HepG2 cells transfected with PCDNA3, PCDN3-MNK1a-Flag and PCDNA3-MNK1b-Flag during 48 h. **Top panel**: Total protein extracts from cells untreated or treated with 1 µM Sorafenib for 1 h were obtained and analysed by western blotting as described in the Materials and Methods section. The signals of exogenous MNK1a and MNK1b were detected using antibody raised against the Flag epitope. GAPDH was used as loading control. The treated- and untreated-cells condition are represented by a + and - marks, respectively. **Bottom panel**: The densitometry analysis of the Phospho-eIF4E/eIF4E ratio based on two independent replicates is shown. The overexpression is indicated by a + mark.

Role of the eIF4E phosphorylation in Sorafenib-response

The overexpression of eIF4E by itself has been shown to contribute directly to cellular transformation and, prognostically, eIF4E overexpression has also been shown to correlate with a poorer outcome in a variety of human cancers, including HCC. Recent data have highlighted the importance of eIF4E phosphorylation at its serine 209 in transformation due to the induction of translation of subset of cancer-promoting mRNAs (32, 47, 49, 165, 174-176). In a previous section, we have demonstrated that Sorafenib reduces the phosphorylation status of eIF4E; concomitantly Sorafenib decreases translation of Phospho-eIF4E mRNAs targets like Cyclin D1 and c-Myc. We hypothesized that this decrease in Cyclin D1 and c-Myc translation might be due to a drop in

Phospho-eIF4E. To check this hypothesis, we overexpressed a phosphomimetic isoform of eIF4E (eIF4E-S209D) and evaluated the protein levels of Phospho-eIF4E mRNAs targets after the standard Sorafenib treatment. The phosphomimetic mutant simulates a constitutively active version of eIF4E allowing us to study the role of the eIF4E phosphorylation in Sorafenib-treated cells. In addition, we evaluated the protein levels of Cyclin D1 and c-Myc in cells overexpressing the wild-type isoform to rule out the effect of eIF4E overexpression by itself.

The plasmid pLPCX-eIF4E-S209D was a generous gift from Dr Aasen (Molecular Pathology, Hospital Universitari Vall d'Hebron, Vall d'Hebron Institu de Recerca, VHIR) while the pLPCX-eIF4E-WT was obtained by site-directed mutagenesis as described in the Materials and Methods section (176). HepG2 cells were transfected with both plasmids, which contain the c-Myc tag that allows the detection of exogenous proteins by western-blot hybridization. Then, the protein levels of Cyclin D1 and c-Myc were evaluated after a kinetics of a 10 µM Sorafenib treatment in non-transfected- and transfected-cells.

Figure 20. The phosphomimetic isoform of eIF4E restore partially the eIF4-target proteins levels. HepG2 cells transfected with pLPCX-eIF4E-WT and pLPCX-eIF4ES209D during 48 h. Total protein extracts from cells untreated or treated with 10 µM Sorafenib for 1 h were obtained and analysed by western blotting as described in the Materials and Methods section. **Top panel**: The signal of exogenous c-Myc-eIF4E-WT and c-Myc-eIF4E-S209D were detected using specific antibody against raised c-Myc. GAPDH was used as a loading control. The control condition is represented by a - mark. The untransfected cells condition is also shown. **Bottom panel**: The same total protein extracts from transfected HepG2 cells were used to analyse the levels of c-Myc and Cyclin D1. The signals of Cyclin D1 and c-Myc were detected using specific antibodies. GAPDH was used as a loading control. The untreated cells condition is represented by a - mark.

HepG2 cells overexpressing eIF4E-S209D showed practically unaltered Cyclin D1 protein levels compared with cells overexpressing the wild type isoform (eIF4E-WT) or cells with endogenous levels of Phospho-eIF4E. Curiously, differences between eIF4E-S209D and eIF4-EWT in the c-Myc protein levels were not so clear, although apparently they were partially restored compared with non-transfected cells after 1 h of Sorafenib treatment (**Figure 15** and **20**). Therefore, our findings indicate that the Cyclin D1 downregulation seems to be the result of the decrease of eIF4E phosphorylation status, as a clear link could be observed between the phosphorylation status of eIF4E and protein levels of Cyclin D1. In contrast, no such clear relation between c-Myc protein levels and the phosphorylation status of eIF4E was observed. Consequently, we draw the conclusion that c-Myc expression in sorafenib-treated cells exhibits a lower phospho-eIF4E dependence; its downregulation appears to be the result of dysregulation of numerous mechanisms controlling its protein level. other factors must be involved in the c-Myc downregulation.

Cyclin D1 is a member of the highly conserved cyclin family whose protein abundance is characterised by a dramatic periodicity throughout the cell cycle. It forms a complex with CDKs (Cyclin-dependent kinases) whose activity is required for cell cycle G1/S transition (201, 202). Our data showed that overexpression of eIF4E-S209D restored the levels of Cyclin D1 in Sorafenib-treated cells, suggesting that its downregulation is dependent on Phospho-eIF4E. Thus, we next decided to analyse the cell cycle progression in Sorafenib-treated cells after eIF4E-S209D or eIF4E-WT overexpression. Flow cytometry was carried out to analyse the effect of constitutively active isoform of eIF4E on cell cycle. Cell cycle of HepG2 cells transfected with both pLPCX-eIF4E-S209D and pLPCX-eIF4E-WT was analysed and compared with non-transfected cells after a Sorafenib treatment. Non-transfected cells treated with Sorafenib showed an increase in S-phase cell numbers of approximately 10%. In agreement with this increase, a significant reduction in the cells number in the G2-phase was observed as well. Intriguingly, the cell cycle arrest exerted by Sorafenib seems to be Cyclin D1-independent since it is involved in the G1/S cell phase transition and our results showed an accumulation of cells in S phase instead of G1. Thereby, our findings were apparently more concomitant with cells subjected to damage driving cell cycle arrest in S phase, for instance, cells subjected to DNA damage. Curiously, Sorafenib-induced cell cycle arrest was not observed in cells overexpressing eIF4E-S209D as well as eIF4E-WT (**Figure 21**). Hence, we conclude that eIF4E could mediate the Sorafenib-induced cell cycle arrest, although in a Cyclin D1-independent manner.
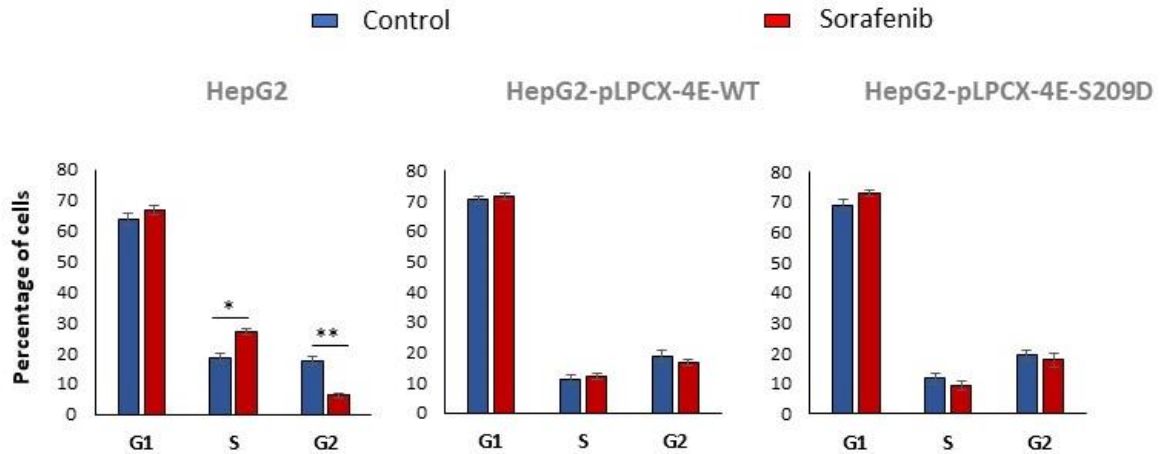
Figure 21. Sorafenib does not induce a cell cycle arrest in cells overexpressing either eIF4E-WT or eIF4ES209D. Histograms of the percentage of cells in each cell cycle phase based on three independent experiments are shown. HepG2 cells were untransfected or transfected with pLPCX-eIF4E-WT and pLPCX-eIF4ES209D during 48 h. Whole cell extracts from HepG2 cells untreated or treated with 10 μM Sorafenib for 12h were obtained and prepared to analyse cell cycle by flow cytometry as described in the Materials and Methods section. Statistical significances were analysed by the Student's t test (* *p* <0.05; ** *p* < 0,001); Error bar: SD.

Taken all into account, we conclude that the phosphorylation of eIF4E at its serine 209 seems to play an important role in the Sorafenib response, regulating the translation of pro-tumoral genes like Cyclin D1. More interestingly, we found that the overexpression of either mutated or wildtype versions of eIF4E suppressed the cell cycle arrest exerted by Sorafenib. All these observations point out that eIF4E plays a more complex role in the Sorafenib response in liver cancer cells than we initially suspected.

MATERIALS AND METHODS

Cell lines, culture conditions and treatments

The HepG2 cell line was used in this study. Some features of this line are collected in **Table 1,** shown in the Materials and Methods section of the Chapter 1 of this Thesis book. HepG2 cells were cultured and maintained as was routinely done (see the Materials and Methods section of Chapter 1).

The different drugs used in this study are shown in **Table 6**. Sorafenib, Sirolimus and 4-amino-5-(4-fluoroanilino)-pyrazolo[3,4-d]pyrimidin (Mnk1 inhibitor) were dissolved in DMSO (Dimethyl sulfoxide) as 10 mM, 100 µM and 20 mM stock solution, respectively. Cycloheximide was dissolved in water as a 10 mg/ml stock solution. All drugs were aliquoted and stored at - 20 °C. Treatments were performed minimum 24 h after plating.

Table 6. Drugs and reagents

Drugs	Commercial reference
Sorafenib tosylate	FS10808, Carbosynth
Sirolimus	37094, Sigma-Aldrich
Mnk1 inhibitor	454861 5MG, Sigma-Aldrich
Cyloheximide	C7698, Sigma-Aldrich

Protein extraction and western blot analysis

Protein extracts were obtained as described in the Materials and Methods section of Chapter 1. They were subjected to 10% SDS-PAGE and transferred to nitrocellulose membranes (Amersham™ Protran® 0.45 µm, GE Healthcare). The membranes were blocked for 1 h with 5% Bovine serum albumin (BSA) in TTBS buffer (15 mM HCl-Tris, pH 7.5, 200 mM NaCl, 0.2 M NaCl, 0.1% (v/v) Tween-20), followed by incubation with a primary antibody at 4 °C overnight. Antibodies used in the experiments of this chapter are shown in **Table 7**.

Table 7. Antibodies used in this study

Antibodies	Dilution	Origin	Reference
Cyclin D1	1:1000	Rabbit	Sc-718, Sta Cruz Biotechnology
c-Myc (D84C12)	1:1000	Rabbit	#5605, Cell Signaling
eIF4A (C32B4)	1:2000	Rabbit	#2016, Cell Signaling
eIF4E	1:2000	Rabbit	#9742, Cell Signaling
eIF4G(C45A4)	1:2000	Rabbit	#2469, Cell Signaling
ERK1/2	1:2000	Rabbit	#9102, Cell Signaling

4E-BP	1:2000	Rabbit	#9452, Cell Signaling
Phospho-eIF4E(Ser209)	1:4000	Rabbit	#9741, Cell Signaling
Phospho-ERK1/2(Thr202/Tyr204)	1:2000	Rabbit	#4370, Cell Signaling
Phospho-4E-BP(Thr37/46)	1:2000	Rabbit	#9459, Cell Signaling
Phospho-RPS6(Ser235/236)	1:5000	Rabbit	#2211, Cell Signaling
Phospho-RPS6(Ser240/244)	1:5000	Rabbit	#5364, Cell Signaling
RPS6	1:5000	Rabbit	#2217, Cell Signaling
Tubulin	1:1000	Rabbit	ab52866, Abcam
GAPDH	1:1000	Mouse	Sc-47724, Santa Cruz Biotechnology
Vinculin	1:1000	Mouse	Sc-25336, Santa Cruz Biotechnology
Flag	1:1000	Mouse	F3165, Sigma-Aldrich
c-Myc (9E10)	1:1000	Mouse	sc-40, Santa Cruz Biotechnology

After washings with TTBS buffer, the membranes were incubated with a HRP-conjugated secondary antibody (Bio-Rad) at a 1:5000 dilution for 1 h at room temperature. Proteins were detected using an enhanced chemiluminescence detection kit (Super-Signal West Pico, Pierce) in a ChemiDoc™ Touch Imaging System (Bio-Rad) and the relative intensity value quantified with the Image Lab software provided with this system.

Polysome profile and RNA isolation from sucrose gradient fractions

The protocol for polysome preparation liver cancer cells and fractionation has been describe in the Materials and Methods of Chapter 1. The samples collected from the gradients were stored at -80 °C until to be used. For RNA extraction, firstly, 100 µl of each sucrose gradient fraction were taken from the sample to form the sample representing the total RNA content in the profile. Then, the fractions were pooled together according to the experimental design and a commercial RNA luciferase was added to each one. A treatment with a proteinase K solution (37,5 µl 10 % SDS, 7.5 µl 0.5 EDTA and 4 µl 20 mg/ml proteinase K per 1 ml) was performed for 1 h at 50 °C. Equal volume of phenol acidic:choroform:isoamyl alcohol (25:24:1, v/v) was added to the sucrose fractions and samples were mixed during 30 s and centrifuge 10 min at maximum speed at 4 °C. Approximately 80% of the aqueous phase was place in a new tube and an equal volume of chloroform was added. After mixing for 30 s using a vortex, samples were centrifuged 10 min at maximum speed at 4 °C. Again, 80% of the aqueous phase was place in a new tube and the RNA was precipitated using 1:10 of 3M sodium acetate, pH 5.2 and 1.5 volumes of absolute ethanol. The mix was incubated overnight

at - 20 °C. Samples were then centrifuged at maximum speed for 30 min at 4 °C, the pellet was washed with 70% ethanol and finally, was resuspended in RNase-free water.

RT-PCR and qPCR

Equal volumes of RNA samples from the polysome profile were treated with 1 µl of DNase I (Promega) following the manufacturer's instructions. RNA was then reverse transcribed using SuperScript™ III First Strand Synthesis for RT-PCR, also according to the manufacturer's instruction (Invitrogen, USA) using random hexamer primers (Roche, Switzerland). RT-qPCR was performed using SYBR® Green Premix Ex Taq™ 2X (Takara, Japan) and primer specifics of each transcript. Primer pairs used for the RT-qPCR are shown in **Table 8**. The data were processed normalizing to the whole profile RNA. The percentage and the relative amount of mRNA were calculated. The actin mRNA was used as the reference mRNA. The data were expressed as the mean ± the standard deviation (SD).

Table 8. Primers used for RT-qPCR

Primers	Forward (5' - 3')	Reverse (5' - 3')
Cyclin D1	GTGAAGTTCATTTCCAATCCG	GGTCACACTTGATCACTCTGG
c-Myc	GGAGGAGACATGGTGAACCAG	AGGAGGCCAGCTTCTCTGAGA
Mcl-1	GCTTCAGTCTCGGAACATGAC	CTTATGGCTCTGAGATGGGC
VEGFA	CCATCCAATCGAGACCCTGG	CTCCAGGCCCTCGTCATTG
RPS6	TGTCCGCCTGCTACTGAGTAA	GCAACCACGAACTGATTTTCTC
RPS18	GCGGGAGAACTCACTGAGG	CGTGGATTCTGCATAATGGTGAT
RPL32	GCCCAAGATCGTCAAAAAGAGA	TCCGCCAGTTACGCTTAATTT
B-actin	TCCCTGGAGAAGAGCTACGA	AGGAAGGAAGGCTGGAAGAG

Total RNA extraction

About 8×10^5 HepG2 cells were seeded in 6-multi well plates for each experimental condition. Total RNA was extracted from each sample using a RNeasy mini kit according to the manufacturer's instructions (QIAGEN, Hilden, Germany). RNA was then stored at - 80 °C before further analyses. Then, 1 µg of RNA was treated with 1 µl of DNase I (Promega) also following the manufacturer's instructions. Finally, RT-PCR and qPCR were performed as described in the previous section. Primers used are shown in **Table 8**.

Plasmid constructs and DNA transfection

About $7x10^5$ HepG2 cells were seeded in 6-multi well plates for each experimental condition. A period of 24 h later, cells were transfected with 2 µg of each plasmid using Lipofectamine™ 2000 (Invitrogen™) according to the manufacturer's instruction. Cell transfection was performed in serum-free medium in the absence of antibiotics. A 6 h later, lipofectamine containing-medium was removed and replaced by fresh, pre-warmed completed culture medium. Further analyses were performed 48 h post-transfection. Plasmid constructs used are listed in **Table 9**.

Table 9. Plasmid used in this work

Plasmids	Reference
Flag-pcDNA3-Mnk1a	Provided by Dr Elena Martín (Department of Biochemistry-Research, IRYCIS-Hospital Ramón y Cajal)
Flag-pCDNA3-Mnk1b	Provided by Dr Elena Martín (Department of Biochemistry-Research, IRYCIS-Hospital Ramón y Cajal)
pLPCX-S209D-eIF4E	Provided by Dr Trond Aassen (Molecular Pathology, Hospital Universitari Vall d'Hebron, Vall d'Hebron Institu de Recerca, VHIR)
pLPCX-WT-eIF4E	Our lab
pcDNA3-RLUC-POLIRES-FLUC	#45642, Addgene

pLPCX-WT-eIF4E plasmid construct was generated by PCR-mediated mutagenesis using pLPCX-S209D-eIF4E plasmid provided by Dr Trond Aasen. The PCR setting was chosen according to the instructions of High-fidelity DNA Polymerase (NEB#M0515). The PCR product was sequenced to confirm the change of the mutant to the wild type of codon. Primers used to generate the mutation and to sequence the final product are listed in **Table 10**.

Table 10. Primer used for site directed mutagenesis and for plasmid sequencing

Primers	Forward	Reverse
eIF4E-WT	CTACTAAGAGCGGCTCCACCACTAAAAATAG	CTATTTTTAGTGGTGGAGCCGCTCTTAGTAG
pLPCX	AGCTCGTTTAGTGAACCGTCAGATC	ACCTACAGGTGGGGTCTTTCATTCCC

IRES and cap-dependent translation analysis

To measure the IRES-mediated translation *versus* cap-dependent translation, HepG2 cells were seed in a 6-multi well plate and transfected with the dual-luciferase reporter plasmid (T7-rLuciferase-

POLIRES-ffLuciferase) described in **Table 10**. Luciferase activities were measured using Dual-Glo® Luciferase Assay Sistem (Promega, USA). Firefly and *Renilla* luminescence levels were measured at room temperature with a CLARIOstal 1.20 microplate reader (BMG Labtech, Germany) adjusted to readtype endpoint and default setting. The *Renilla*/firefly ratio was calculated for each condition and normalized to the control. The assays were done in quadruplicate and the data expressed as the mean ± the standard deviation (SD).

Cell proliferation assay

The measurement of bromodeoxyuridine (BrdU) incorporation was done using a commercial kit (ref. 11 647229001, Roche Diagnostics, Germany). Briefly, $3x10^4$ HepG2 cells were seeded in 96-well plates. Two hours before cell harvesting, 20 µl of 10 µM BrdU was added to the cultures. DNA was denaturalized with 200 µl FixDenat solution included in the commercial kit for 30 min at room temperature. FixDenat solution was removed and then cells were incubated with 100 µl of monoclonal anti-BrdU antibody HRP conjugated for 90 min at room temperature. Afterward, cells were washed with PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) and incubated with 100 µl revealing solution for 15 min at room temperature. The A_{370} was measured using an Infinite 200 PRO microplate reader (Tecan, Switzerland).

Cell cycle analysis

Cell cycle progression was assessed by flow cytometric analysis. For this, HepG2 cells were seeded in a 6-well plate. The day of the harvest, cells were trypsinised and fixed in 70% ethanol in PBS buffer, overnight at 4 °C. After this, cells were resuspended in PBS buffer and incubated with 0.5 mg/ml RNase A for 1 h at 37 °C. Propidium Iodine (PI) was added to a final concentration of 0.05 mg/ml for 20 min at room temperature. Finally, cells were filtered to avoid the aggregation and cell cycle progression was assessed in a FACSCantoTM Flow Cytometer and analyzed using the FACSDiva software (BD Biosciences, Switzerland).

CHAPTER 3

Translational reprogramming induced by Sorafenib

INTRODUCTION

Sorafenib, as described in this Thesis work, has a strong inhibitory effect on translation, endangering overall protein production. However, in general, repression of global protein synthesis is often accompanied with a selective translation of a subset of mRNAs encoding protein that are essential for cell survival and stress recovery. Cells have different mechanisms that control the selective translation of mRNAs depending on the internal signals and environmental cues that, eventually, determine the protein concentration and the cellular context. Malignant cells have often completely dysregulated some of these control translation mechanisms leading to tumour formation, metastatic spread, and therapy resistance (27, 28, 44, 79). In this work, we tried to get clues on the molecular bases that underlie the cellular resistance to Sorafenib at the translational level by identifying those mRNAs preferentially translated after a Sorafenib treatment and distinguishing those that could be involved in the cellular resistance to this drug.

Translation reprogramming in stress response

The tumour environment is hostile, characterized by limited oxygen and nutrients availability. The tumour cells are able to survive by rewiring cellular functions through translational and epigenetic alterations in a process called "cellular plasticity". The cellular plasticity can be defined as the ability of cells to change their phenotypes without genetic mutations in response to environmental cues. Over past years, numerous publications have associated cellular plasticity, especially the translational reprogramming, to resistance to classical chemotherapeutic agents (44). The translation control, in contrast to transcription, provides cells with the capacity to adapt quickly to environmental changes. Indeed, numerous mRNAs encoding proteins that govern cancer cell plasticity have alternative translation mechanisms when overall translation is compromised (43). Among these mechanisms, there are examples involving secondary mRNA structures, mRNA methylation, presence of upstream open reading frames (uORFs), and the presence of internal ribosomal entry sites (IRES) within the 5' UTR of the mRNAs (28, 178, 203). All these features have been extensively studied in relation to translational control responses, although further research is needed to fully understand their precise mechanisms.

In previous sections of this work, some of the different mechanisms controlling translation have been described. Among these, sequences located within the 5' UTR such as uORFs, whose translation is strongly dependent on the phosphorylation of eIF2α (see **Chapter 1**) or IRES elements, which allow cap-independent translation (see **Chapter 2**). On one hand, uORFs represent an important alternative translation initiation site, which are found in nearly 50% of all human genes; they typically act to repress translation of the downstream main ORF. These elements allow the translation of the main ORF of mRNAs only under stress conditions, enabling an appropriate, but temporally limited response

while cells recover a homeostatic balance (85, 86). Several oncogenes are enriched in uORFs within their 5' UTR, such as the negative p53 regulator MDM2, CDC274 encoding PD-L1 (204), and numerous tyrosine kinase genes (203). Although translation of mRNAs containing uORFs is linked to the phosphorylation status of eIF2α, other translation initiation factors play also important roles. For instance, it has been demonstrated that eIF5B promotes the efficient translation of CDC275 under ISR (205, 206) as well as it has been proposed that the alternative translation initiation factor, called eIF2A, plays a role in the translation of the main downstream ORF from some cancer-related genes under stress-conditions (207). Although the selective translation of these mRNAs by uORFs remains mechanistically unknown, its association with tumorigenesis is well known (**Figure 1A**). On the other hand, IRES elements are sequences located in a subset of mRNAs that acquire particular structures. These structures allow the recruitment and the assembly of the initiating ribosome without recognizing the cap-structure. Both canonical translation factors and non-canonical IRES *trans*-activating factors (ITAFs) are involved in IRES-mediated translation (130, 163, 164, 178, 192). IRESs can be classified into different groups according to the factors required for their translation. However, how mechanistically the IRES-mediated translation occurs is neither well understood. Numerous reports have shown the relation between eIF4GI and eIF3 with IRES-mediated translation, but it has to be noted that the activation of cellular IRESs also depend on the expression and subcellular location of specific ITAFs under stress conditions. Under homeostatic conditions, IRES elements precisely regulate translation initiation of a selective group of transcripts, limiting their protein levels and maintaining the appropriate composition of the expressed proteome. These structures have been detected in several key proangiogenic, hypoxia, and survival mRNAs, whose translation increase under stress conditions, highlighting their importance in tumorigenesis (28, 187, 203) (**Figure 1B**).
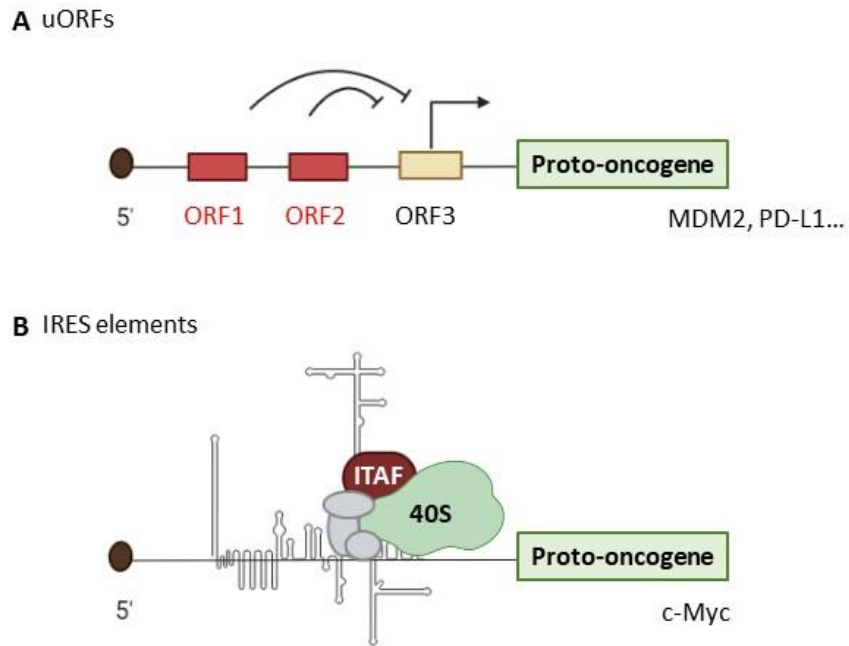
A uORFs

B IRES elements

Figure 1. mRNA regulatory elements involved in translational control and in oncogenic programs. The image depicts two different alternative mechanisms of translation initiation operating under stress conditions. (**A**) uORFs and (**B**) IRES elements. MDM2 (murine double minute 2); PD-L1 (Programmed death ligand 1); ITAF (IRES *trans*-acting factor).

Additionally, it has been reported that when the assembly of eIF4F is impaired, an isoform of eIF4G, known as DAP5, forms an alternative cap-binding complex, allowing selective translation of a subset of capped-mRNAs. DAP5 is unable to interact with eIF4E but bind to eIF3, specifically with the eIF3d subunit, promoting the translation of approximately 20% of capped mRNAs (191). Furthermore, it was found that DAP5 can also promote IRES-dependent translation under stress conditions. It is still possible that distinct mRNAs can use both eIF4G/eIF4E and DAP5/eIF3 complexes equally well under the same physiological conditions and that one or both of these mechanisms are used depending on the cell type and the environmental conditions. Different DAP5/eIF3 target-mRNAs have been reported, including genes encoding proteins that promote cell survival upon stress (208). However, further research into the functional role of DAP5 in translation will be key to understanding how cells use one or another cap-dependent mechanism of translation according to external and internal changes and what their relevance is during tumorigenesis.

Sequence features/motifs located into the mRNAs that determine translation

Sequence-specific RNA elements in the UTRs of genes have been functionally associated with altered translational efficiency in response to pro-tumorigenic signals. As above described, the

Akt/mTORC1 pathway controls cell growth by remodelling the translatome under stress condition, driving tumour formation and malignancy. The mTORC1 activity is linked to the translation of selective mRNAs that contain 5' TOP sequences or 5' TOP like PRTEs (102, 156, 209). The 5' TOP motif is characterized by an invariable cytidine proximal to the cap followed by an uninterrupted stretch of 4-15 pyrimidines, whereas the PRTE is a sequence located at a variable position within the 5' UTR characterized by an invariant uridine at position 6, flanked by pyrimidines (**Figure 2A**). These sequences have been identified in mRNAs of ribosomal proteins, translation factors and key regulators of metastasis (210, 211). All these mRNAs, whose translation is highly controlled by mTORC1, have been linked to the pro-tumoral activity of this pathway by coordinating cancer cell growth and invasion (99).

Sequence within the 3' UTR have also been linked to the selective translational regulation of pro-oncogenic processes. One notable motif is the cytoplasmic polyadenylation element (CPE: 5'-$UUUUA_{1-2}U$-3') which seems to modulate the translation of several pro-tumoral mRNAs (212). The CPE element is recognized and bound by RNA binding proteins named CPE-binding proteins (CPEBs) that can act as either translation activators or repressors. In human cells, four different CPEB proteins have been identified (CPEB1, 2, 3, and 4). CPEB4 has been described to promote translation of a subset mRNAs during ISR. Thus, the PERK-induced eIF2α phosphorylation leads to inhibition of global protein synthesis while promoting the translation of uORF-containing transcripts. In parallel, CPEB4 activates translation of CPE-regulated mRNAs in an uORF-independent manner. Although the precise mechanisms of 3'UTR-driven translational control are neither well understood, CPEB4 represents a second temporal wave of translational activation following ER stress (213). Some of the CPEB4-targets encode translation factors and genes involved in cell migration and metastasis, reason why CPEB4 has been proposed to be involved in tumorigenesis (214)(**Figure 2A** and **2B**). Indeed, high expression levels of CPEB4 have been observed in several tumours, including glioblastoma and ductal adenocarcinoma (215).
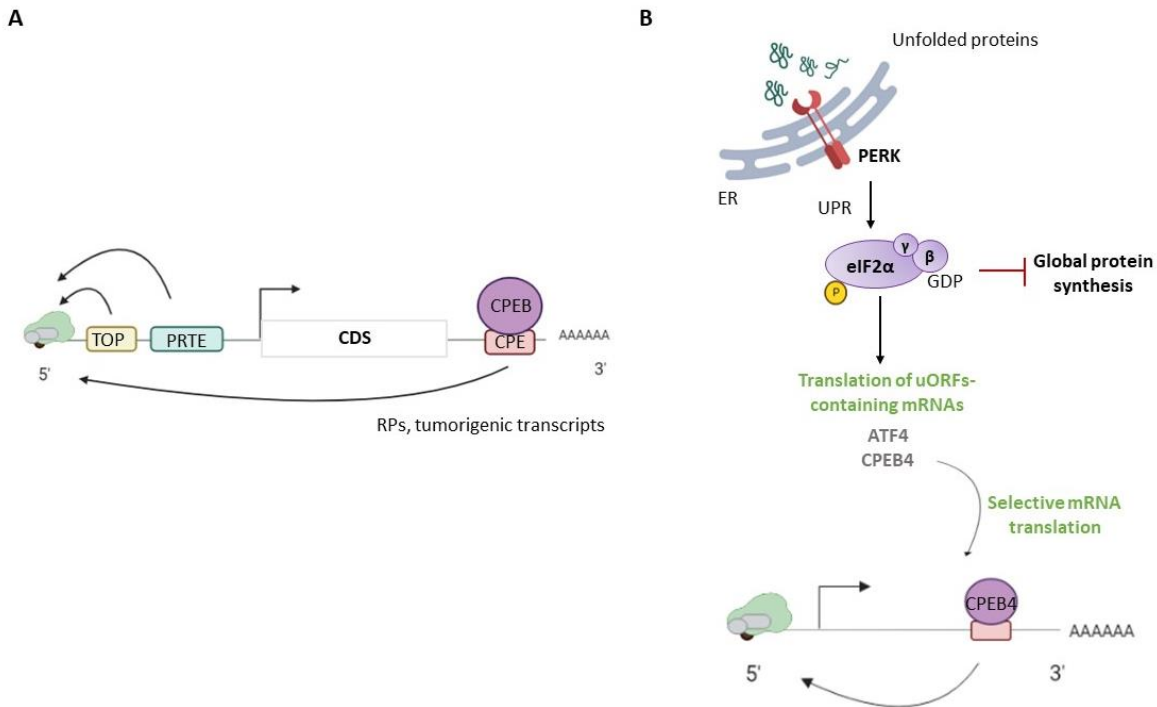
A

B

Figure 2. Specific sequence motifs into the 5' UTR and 3' UTR of mRNAs involved in translational control. (A) The image depicts the sequence motifs within the 5' UTR, including the 5'TOP and PRTE motifs, and the CPE element located in the 3' UTR of certain mRNAs. **(B)** Illustration of the translational control circuit during UPR. The combined action of uORFs and CPEB4 defines two temporal waves of translational activation following PERK activation. CPEB4 recognizes and binds to CPE-elements in a subset of mRNAs, influencing their translation.

Another well characterized *cis*-regulatory element of mRNAs is the AU-rich element (ARE) located as well in the 3' UTR of labile short-lived mRNAs. This element is characterised not only by an enrichment in A and U but also by their patterns, repeats and context. This element is recognised and bound by RNA-binding proteins (RBPs), regulating different cellular aspects of the mRNA, including translation (216, 217). AREs are contained in 5-8% of human mRNAs coding factors involves in cell proliferation, differentiation, signal transduction, and metabolism (218) (219). An aberrant expression and deregulated activity of different ARE-RBPs have been described in different cancers (220). The HuR (Human antigen R) protein is the most well-known player in binding ARE-containing mRNAs whose role in carcinogenesis has been identified (221). HuR enhances cell proliferation, increases cell survival and local angiogenesis, promotes evasion of immune recognition, and facilitates cancer cell invasion and metastasis by promoting the translation or enhancing the stability of numerous mRNA targets, including a subset that encodes

pro-survival proteins such as c-Myc, XIAP, MCL1, and SIRT1. (222, 223,224,225). HuR has been found overexpressed in various cancer, including liver cancer (226).

RNA modifications involved in translational control

In eukaryotic cells, the N^6-methyladenosine (m^6A) methylation is the most abundant and classical mRNA modification. It has been reported that this modification is involved in mRNA translational control, mRNA stability, and splicing regulating in this way the cell metabolism, differentiation, and growth (227, 228). The m^6A methylation occurs in a dynamic and reversible manner, depending on the action of different types of enzymes (229, 230). Two RNA methyltransferase called "writers", METTL3 and METTL14, are responsible for the vast majority of m^6A modifications. METTL3 and METTL14 form a heteromeric complex where METTL3 is the catalytic subunit and METTL14 is required for RNA binding (231). In general, m^6A modifications are recognised in a sequence-independent manner and bound by proteins named "readers". In mammalian cells, five readers proteins have been identified (YTHDC1, YTHDC2, YTHDF1, YTHDF2, and YTHDF3), which play important roles in the regulation of nuclear processes such as transcription, splicing, and mRNA export, as well as cytoplasmic functions like translation and mRNA degradation (232). Finally, m^6A methylation can be removed by proteins called "erasers" of which FTO and ALKH5 are the best characterized (233-235)(**Figure 3**).
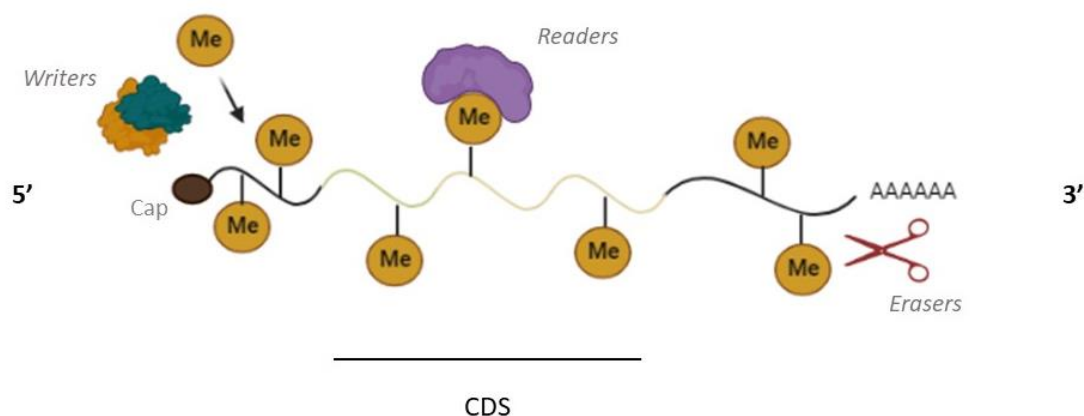
Figure 3. m^6A mRNA modification. The m^6A methylation status of mRNAs depends on the activity of two different types of proteins: those that add the methyl group (*writers*) and those that remove it (*erasers*). Another group of proteins, called *readers*, recognise the m^6A modification. The m^6A modification can occur in any part of the mRNAs (5' UTR, CDS, or 3' UTR), influencing various aspects of their fates. CDS (coding sequence); Me (Methylation).

The publications highlighting the role of m^{6}A modifications in translational controls have increased during the last few years. However, further investigations must be done to clarify the molecular mechanism underlined. Currently, it is known that the influence of the m^{6}A modification on translation depends basically on the specific location of the m^{6}A within the mRNAs (5' UTR, CDS or 3' UTR regions). Three distinct mechanisms have been proposed. The first one involved the canonical m^{6}A reader YTHDF1 which seems to interact and recruit eIF3 to the 3' UTR region of target mRNAs, enhancing their translation (**Figure 4A**). The second proposed mechanism involves the direct binding of m^{6}A-containing mRNAs in their 5' UTRs to eIF3, enhancing the translation of these mRNAs. In this model, the m^{6}A-mediated translation initiation does not require eIF4E, thus providing an alternative mode to promote translation initiation when eIF4E is impaired (236). It must be noticed that only few mRNAs containing m^{6}A in their 5' UTRs have been identified, suggesting that this mechanism is just limited to a small subset of m^{6}A-containing mRNAs. Nevertheless, under stress conditions, the m^{6}A methylation in this region is notably increased, potentially enhancing the translation of a major group of mRNAs in response to stress (237) (**Figure 4B**). The third mechanism involves the direct translation activation by METTL3. The translation of mRNAs with m^{6}A modification within their 3'UTRs, is enhanced by the interaction between METLL3 at the 3' UTR and eIF3 present in the 5' proximal part of the same mRNA, resulting in mRNA circularization (**Figure 4C**). Some studies have demonstrated that the METTL3-eIF3 interaction enhances translation of certain mRNAs which are involved in oncogenic transformation. Indeed, depletion of METTL3 seems to inhibit tumorigenesis, pointing this protein as a potential therapeutic target (238, 239).
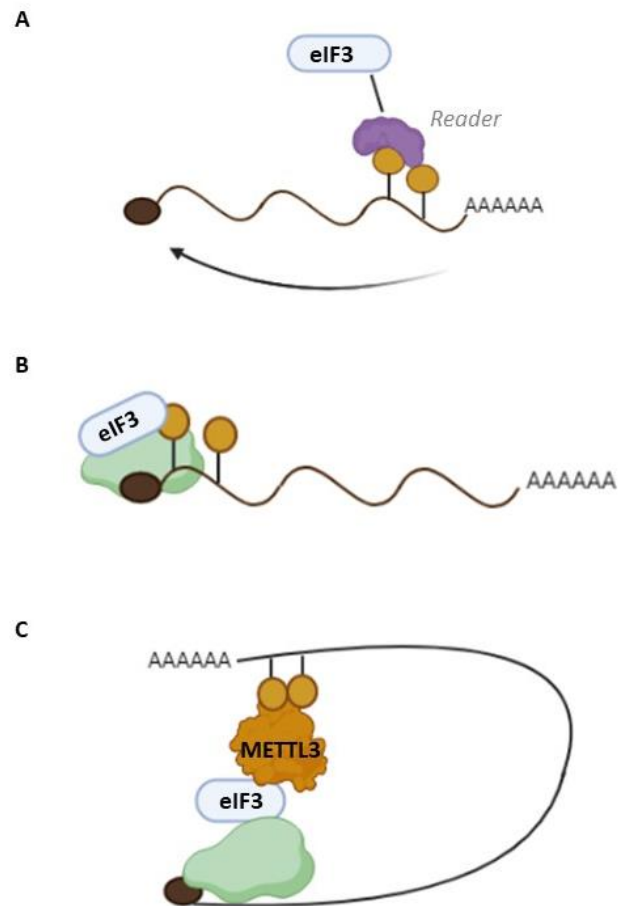
Figure 4. Translation is influenced by m^{6}A mRNA methylation. The image depicts how m^{6}A methylation is involved in translational control. (**A**) Methylation within the 3' UTR promotes the interaction of m^{6}A reader proteins with eIF3, thereby enhancing translation. (**B**) Methylation within the 5' UTR allows the direct interaction of eIF3 with the mRNA, forming a 43S PIC in an eIF4F-independent manner. (**C**) The interaction of the writer METTL3 with a m^{6}A modification within the 3' UTR and eIF3 at the 5' proximal part of the same mRNA promotes mRNA circularization.

The studies to be addressed here are intended to identify the common features that determine the behaviour of those mRNAs whose translation changes under Sorafenib treatment. This will allow us describing the new cellular context established after the Sorafenib treatment from a translational point of view.

RESULTS

Sorafenib is an inhibitor of several kinases involved in tumour cell proliferation and angiogenesis (24, 25). Sorafenib-inhibited targets drive cell cycle arrest, apoptosis, and reduction of angiogenesis; eventually, malignant cells also alter their gene expression programs to adapt, grow, and survive in response to the Sorafenib-induced stress. In this work, we sought to identify the mechanisms involved in translational control that drive cells to a new cellular context and those that could mediate the cellular resistance to Sorafenib.

Sorafenib causes transcriptional changes and translational reprograming in liver cancer cells. For many decades, transcriptional regulation has been the major focus of cancer biologists, whereas translational control has remained on a secondary level. However, the findings highlighting the role of translation in cancer have exponentially increased in the last years; indeed, the dysregulation of translation is nowadays considered a hallmark of the disease. Controlling the activity of the translation machinery allows cells to respond quicker to environmental changes than transcription. Thus, cancer cells control protein synthesis, which impacts on the selective translation control of specific subsets of mRNAs, allowing them to survive in adverse conditions. Indeed, protein synthesis control has been strongly linked to chemotherapy resistance. Therefore, we sought to identify mRNAs that overall change their translation status in Sorafenib-treated *versus* non-treated cells. With this aim, we performed a polysome profiling approach to identify those mRNAs present in polysomes in both Sorafenib-treated and untreated cells. Briefly, extracts from HepG2 cells untreated or treated with 10 µM Sorafenib for 12 h were obtained and polysome fractionated. Samples were differentially collected in two pools: the low-translated fraction (V1) and the high-translated fraction (V2) as depicted in **Figure 22**. The V1 fraction corresponds to mRNAs not bound to ribosomes, or bound only to monosomes, while the V2 fraction contains mRNAs with two or more engaged ribosomes. RNA was isolated from the pools, and the mRNA, purified using oligo(dT) paramagnetic beads, was prepared for RNA Ilumina Sequencing (see the Materials and Methods section for details). The appropriately normalized data were used to estimate the translation ratio by calculating the logarithm of V2/V1. We assume that those mRNAs with a positive ratio correspond with actively translated genes since they show a higher proportion of mRNAs in the polysome fraction than the average. In contrast, those mRNAs with a negative ratio would be expected for those poorly-translated mRNAs as they are more enriched in the low-translated fraction than the average. In parallel, we validated the result of the global translational analysis by measuring the specific translation status of some randomly chosen mRNAs by RT-PCR in each condition. From the data obtained by the qPCR, we calculated the percentage of mRNA in each polysome fraction, and we compared it with the result of the global analysis. Validating our results, we observed that those mRNAs whose percentage in the polysome fraction was smaller in Sorafenib-treated cells than in

control cells, showed a lower logarithm V2/V1 in Sorafenib-treated cells than in control cells (**Supplementary Figure 1**).
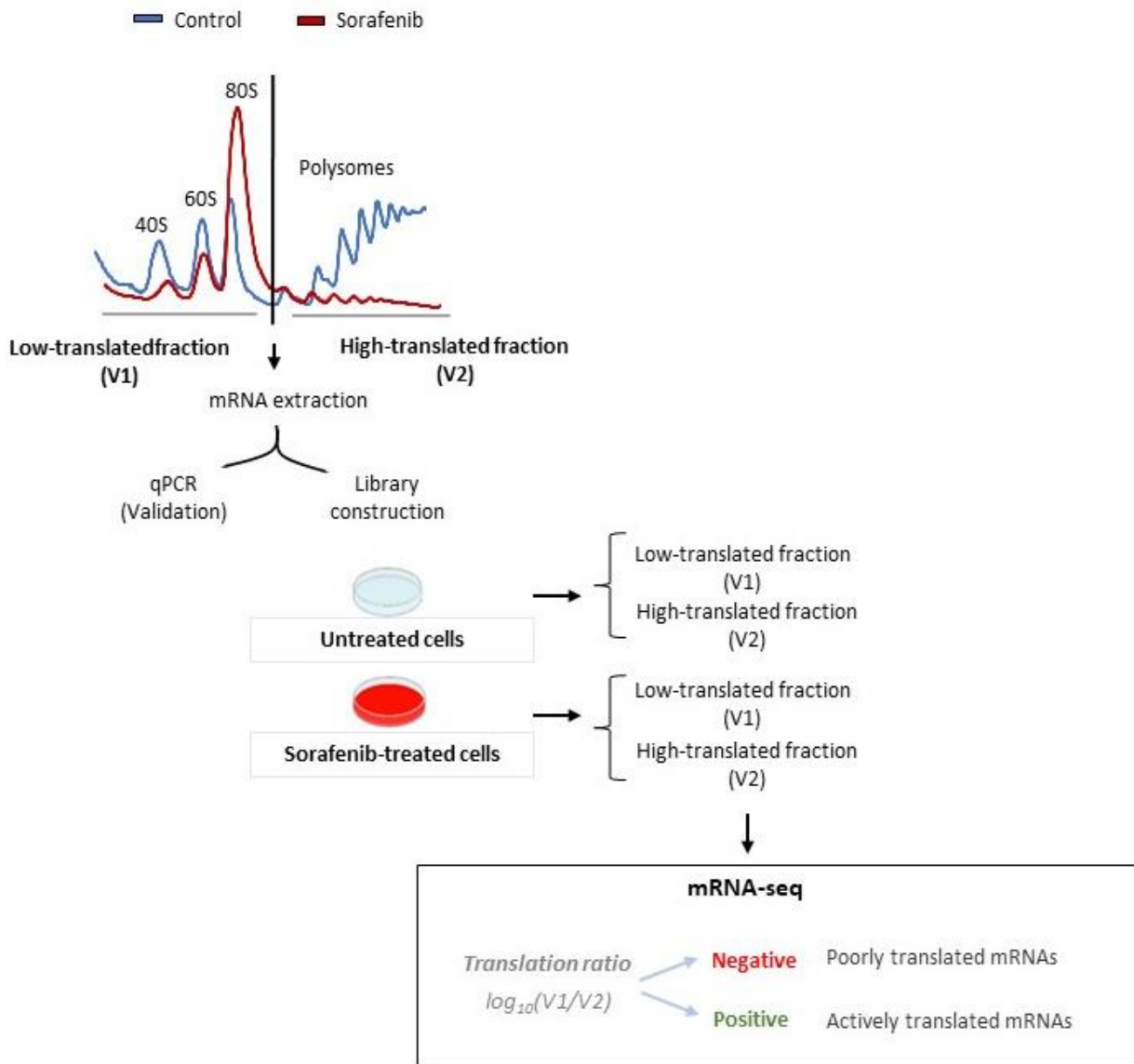
Figure 22. Experimental design of our translational study. The image depicts the process to obtain and create the different mRNA libraries used in our translational study. The mRNA contained in both V1 and V2 fractions from untreated and Sorafenib-treated cells were obtained from three independent experiments. The amount and quality of mRNAs were measured before creating the four different libraries, which corresponded to the low-translated fraction and high-translated fraction of each cellular condition. The obtained data were normalized and used to calculate the translation ratio. Typical polysome profiles of the control condition (untreated cells) and of 10 µM Sorafenib-treated cells for 12 h are shown in blue and red, respectively.

We then normalized the data from Sorafenib-treated cells to the control condition, allowing us to define eight populations of significant genes (always using a *p*-value lower than 0.05) (**Supplementary Figure 2**). The first two groups correspond to genes that, regardless of their translational status in the other condition, are actively translated in both untreated and Sorafenib-treated cells, since they showed a positive translational ratio in each condition. Applying the same approach, we identified another two groups of genes corresponding to those that are poorly translated in each condition; they showed a negative translational ratio in untreated as well as Sorafenib-treated cells, independently of their status in the other condition. Then, we combined these results from untreated and Sorafenib-treated cells in order to identify: (1) those mRNAs whose translation status remains unaffected following the Sorafenib treatment. These mRNAs have a positive translation ratio in both conditions at the same time, indicating that they are well translated mRNAs in physiological conditions but are subjected to mechanisms that allow active translation in a context of overall translation inhibition exerted by Sorafenib; (2) those mRNAs that were poorly translated in both conditions simultaneously because they showed a negative translation ratio in both untreated and Sorafenib-treated cells; 3) those mRNAs that are actively translated in response to Sorafenib-induced stress. This group is constituted by those mRNAs were poorly translated in control condition, showing a negative translation ratio in this one, but actively translated following the treatment, showing positive translation ratio; and (4) finally, those mRNAs that are actively translated in physiological conditions, but whose translation is strongly inhibited by Sorafenib; They showed a positive translational ratio in untreated cells but a negative ratio after the treatment. In the **Figure 23** are showed the different genes populations identified in this analysis.
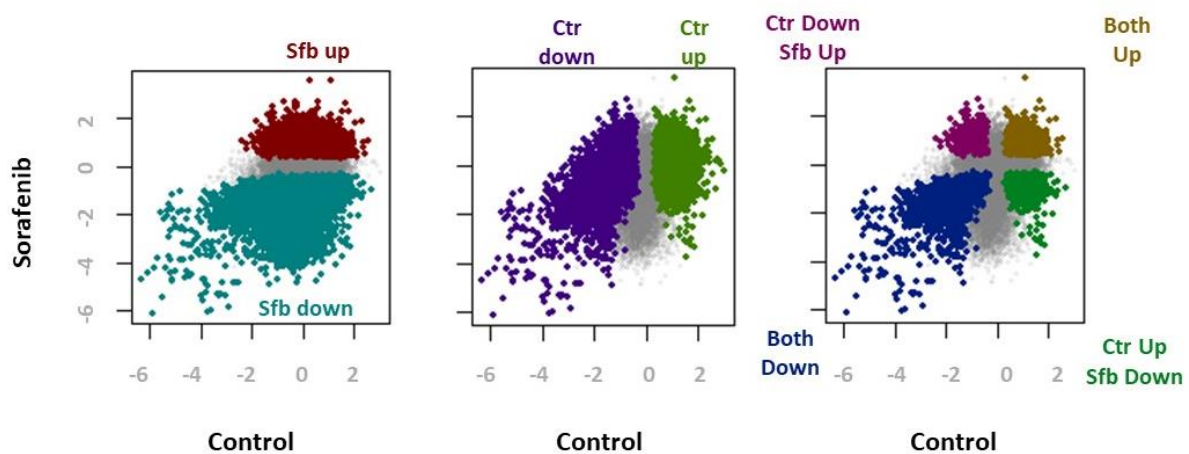
Figure 23. Translation inhibition exerted by Sorafenib resulted in a translation reprograming. Eight different gene populations were arbitrarily defined according to the values of the translation ratio (log2(V2/V1)) in both untreated (Ctr) and Sorafenib-treated (Sfb) cells. Figures represent the translation ratio in the control condition (X axis) and the translation ratio in Sorafenib-treated cells (Y axis) with data properly normalized. **Left panel**: Sfb up and Sfb down correspond to those genes with a positive and negative translation ratio, respectively, in Sorafenib-treated cells, independently of their ratio in the control condition. **Middle panel**: Ctr down and Ctr up correspond to those genes with a positive and negative translation ratio, respectively, in untreated cells, independently of their ratio in the Sorafenib-treated condition. **Right panel**: Ctr down/Sfb up shows the set of genes that has a negative translation ratio in control cells but a positive one in Sorafenib-treated cells. Ctr up/Sfb down shows those genes with a positive translation ratio in the control but a negative one in Sorafenib-treated cells. Both up and Both down categories represent those genes that simultaneously have a positive or negative translation ratio in both conditions.

The number of significant genes actively translated in both conditions was quite similar: 2593 genes showed a positive translation ratio following the treatment, whereas 2950 were found in control cells. Consistent with the translation inhibition exerted by Sorafenib, we found that the number of poorly significant translated mRNAs in Sorafenib treated cells was much higher than those poorly translated in control condition, as we found 5584 genes versus 3237 genes with a negative translation ratio in Sorafenib-treated and untreated cells, respectively. Curiously, we observed that 458 genes significantly actively translated in control were significantly poorly translated following the treatment, suggesting that a particular mechanism controlling their translation is selectively affected by Sorafenib or features common in these mRNAs make them more sensitive to the inhibitory effect of Sorafenib. Focusing only on the significant genes actively translated in Sorafenib-treated cells, we found that 765 genes also showed a positive translation ratio in the control condition, suggesting that the translation of these genes is still maintained after the treatment. Secondly, we found that 1365 genes showed not significant variance in control conditions. And finally, our findings showed that 463 genes were actively translated in Sorafenib-treated cells but poorly translated in the control condition. **Figure 24** summarises all these results.

	Sorafenib			
	DOWN	**NSV**	**UP**	**Sum**
DOWN	1080	1694	463	3237
NSV	4046	10150	1365	15561
UP	458	1727	765	2950
Sum	5584	13571	2593	21748

Control (row label on the left side)

Figure 24. Number of genes in each translation population. The table shows the number of genes found in each defined gene set in our polysome profiling experiment. UP: Significantly positive translation ratio. DOWN: Significantly negative translation ratio. NSV: Not significantly variance.

Next, we sought to identify intrinsic features on all these different groups of mRNAs that could explain their respective behaviours after the Sorafenib treatment; we speculated that these features would give insights into the possible mechanisms that specifically control their translation in each condition.

Sorafenib enhances alternative translation initiation mechanisms

To be able to identify those features that could be involved in the Sorafenib-induced translational reprogramming, we performed an overrepresentation analysis (ORA) in the eight defined categories according to the translation ratio in both control and Sorafenib conditions. Then, we searched for certain features in mRNAs that have previously been reported to be implicated in stress-related translation regulation: (i) presence of uORFs; (ii) presence of IRES elements within their 5' UTR; (iii) enrichment of AU-elements (AREs) in their 3' UTR; and (iv) enrichment of both DAP5 and CPEB4 gene targets (28, 178, 203).

The presence of uORFs represents an alternative mechanism of translation initiation for mRNAs when the global overall translation is impaired. The translation of uORFs-containing mRNAs is strongly linked to phosphorylation of eIF2α that occurs during UPR (85, 86). Consistently, with the PERK-induced eIF2 phosphorylation, we found that mRNAs with uORFs in their 5' UTR were enriched in the category with a positive translation ratio in Sorafenib-treated cells (p-value: 5.54×10^{-60}), suggesting that they are being actively translated. Curiously, we also found an enrichment in these genes in control condition, although with a lower p-value (p-value: 1.83×10^{-41}), implying than some of those uORFs-containing mRNAs are also actively translated in control condition. Interestingly, during UPR, another branch is activated, mediated by CPEB4, that also modulates protein synthesis. CPEB4 is an RBP that recognizes and binds to the CPE element within the 3' UTR of a subset of mRNAs, enhancing their translation (212, 215). Thus, we next wondered whether Sorafenib promoted the translation of CPEB4-target mRNAs because of the ER stress induced by this drug. We found that those mRNAs identified as CPEB4 target genes showed a similar behaviour to those uORFs-containing mRNAs, but with a much lower p-value; thus, the gene set corresponding to actively translated mRNAs was enriched in CPEB4-target genes in Sorafenib-treated (p-value: 1.52×10^{-08}) as well as untreated cells (p-value: 8.24×10^{-10}). Taken all together, our findings suggest that both types of mRNAs are translated in both conditions although the presence of uORFs and the CPE element could enhance the translation of those that are poorly translated in control conditions whereas allowing the translation of others to continue.

Following our study, we investigated the behaviour of those mRNAs that contain an ARE element since this is recognized by RBPs mediating the rapid turnover of mRNAs according to the cellular conditions (216, 217, 225). Interestingly, our results showed an enrichment of ARE-containing mRNAs only in the group of genes corresponding to those actively translated following the treatment (*p*-value 4.54 x 10^{-54}) but not in the control condition (*p*-value 1.59 x 10^{-08}) (**Figure 23**). This observation suggests that AREs can play an important role in optimising translation in response to Sorafenib-induced cellular stress.

	Both Up	Both Down	Control Up Sorafenib Down	Control Down Sorafenib Up	Control Up	Control Down	Sorafenib Up	Sorafenib Down
uORF	2.51E-36	9.98E-01	9.17E-02	5.49E-02	1.83E-41	9.30E-01	5.54E-60	1.00E+00
CPEB4	1.93E-03	7.99E-01	1.31E-04	4.57E-05	8.24E-10	5.91E-03	1.52E-08	1.00E+00
ARE	1.36E-15	1.00E+00	1.00E+00	4.21E-03	1.59E-08	1.00E+00	4.54E-54	1.00E+00
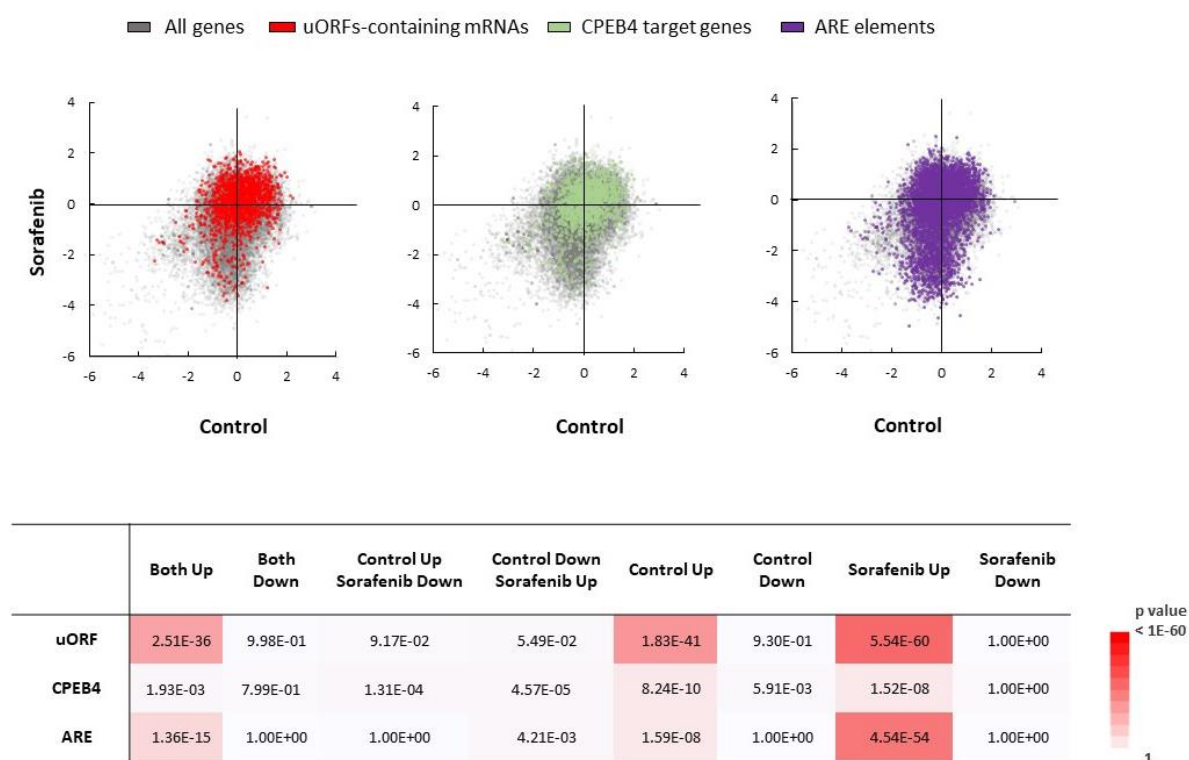
Figure 25: Sequences within the 5' and 3' UTRs appear to play roles in the selective mRNA translation during Sorafenib treatment. The figure shows the overrepresentation analysis (ORA) of genes belonging to each category defined according to the significant translation ratio in the control and Sorafenib conditions. **Top panel:** Three scatter plots showing the translation ratio for control conditions (X axis) and for Sorafenib-treated cells (Y axis) of uORF-containing mRNAs (red), CPEB4-target genes (green), and ARE-containing mRNAs (purple). Each scatter plot overlapped with those of all genes. **Bottom panel:** Table showing the *p*-values for each category for the different studied elements. The intensity of the red colour represents the level of significance of the values.

Our previous study showed that Sorafenib negatively affects the cap-binding complex, which mediates the translation of majority capped-mRNAs. However, mammalian cells have other

alternative cap-dependent mechanisms, like those mediated by DAP5, that allow translation of a subset of capped mRNAs under certain physiological conditions. DAP5 was first described for its role in mediating IRES-dependent translation, however, a substantial body of evidence reveals that it also mediates the translation of a subset of capped mRNAs in response to stress (191, 208). Therefore, we decided to examine how DAP5-targets genes respond to the Sorafenib treatment. As a result, we found that DAP5-targets genes were significantly enriched in the gene set corresponding to actively translated in Sorafenib treated cells (*p*-value 3.71 x 10 $^{-30}$). Interestingly, our finding showed that DAP5-target genes were also enriched in those mRNAs with are poorly translated in control conditions but actively translated following the treatment, suggesting that their translation is enhanced by the drug (*p*-value 4.11 x 10 $^{-27}$). Surprisingly, we found that translation of DAP5-target genes that were actively translated in control conditions, were inhibited by Sorafenib since our data showed an enrichment of those genes in the category corresponding to mRNAs with a positive translation ratio in control conditions and negative in Sorafenib treated cells (*p*-value 7.00 x 10 $^{-15}$). After this observation, we defined a new set of genes combining these data. Thus, we established a set of genes so-called "opposite direction" that contains mRNAs with a positive translation ratio in the control condition but a negative one in Sorafenib-treated cells, as well as those with a negative translation ratio in the control condition but a positive one in Sorafenib-treated cells. Consistently with the previous result, we observed that this new category was highly enriched in DAP5-target genes (*p*-value 7.00 x 10 $^{-41}$) (**Figure 26**). Taken all together, our findings strongly suggest that DAP5 is involved in translation control following the Sorafenib treatment. Although it likely enhances the translation of a subset of mRNAs, in parallel, it negatively affects the translation of another group of mRNAs.

Additionally, because the association of DAP5 with IRES-dependent translation (208), we anticipated the possibility that IRES-containing mRNA may have a similar effect on DAP5-target genes with respect to the translational behaviour in response to Sorafenib. As shown in **Figure 5**, we observed that those genes containing IRES elements showed a tendency very similar to that observed for DAP5-target genes, although with a much lower *p-value*; the *p-value* corresponding to the set of genes that behave in the opposite direction was 9.97 x 10^{-06}). However, only the 16% of those IRES-containing mRNAs were also identified as DAP5-target genes, suggesting that other mechanisms are involved in translation via IRES, although without ruling out a role for DAP5 in this process.
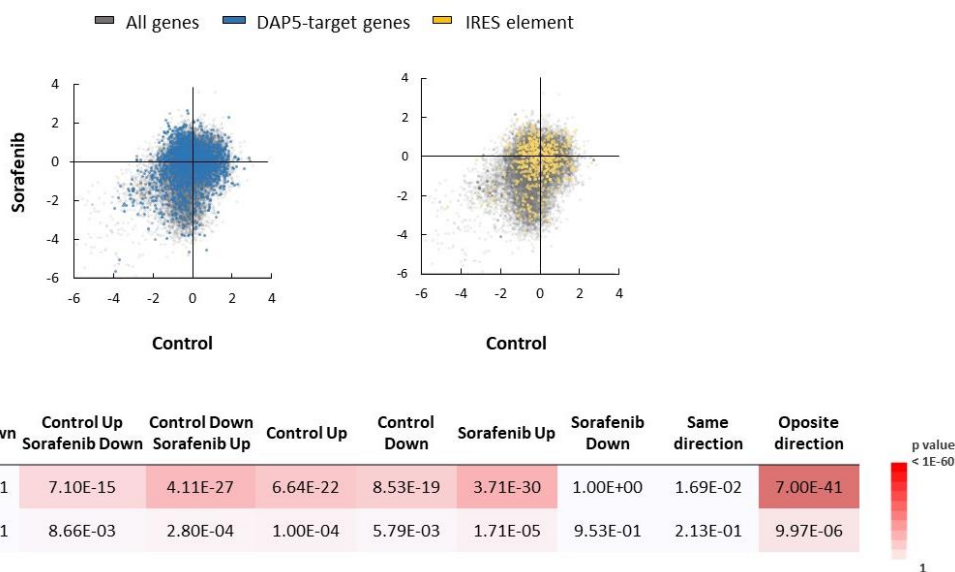
Figure 26. Sorafenib dysregulates the translation of DAP5-target genes. The figure shows ORA of genes belonging to each category defined according to their translation ratio in control and Sorafenib conditions. Additionally, two more categories were defined, including genes that behave in the same direction (negative or positive translation ratio in both conditions) and genes that behave in the opposite direction (negative translation ratio in the control condition but positive in Sorafenib-treated cells, and *vice versa*). **Top panel**: Scatter plots showing the translation ratio for control condition (X axis) and for Sorafenib treated cells (Y axis) of DAP5-target genes (blue) and IRES-containing mRNAs (yellow). Each plot overlapped with those of all genes. **Bottom panel**: Table showing the *p*-values for each category. The intensity of the red colour represents the level of significance of the values.

We then wondered whether some intrinsic characteristics of mRNAs, such as the G+C content, the length of the whole transcript, and the length of the coding sequence (CDS) as well as those of the 5' UTR and 3' UTR, could also be influencing their translation status in response to Sorafenib. The ORA analysis showed no significant differences in the G+C content nor in the length of the 5' and 3' UTR, but we observed that the length of the whole transcript as well as the CDS of those actively translated genes in Sorafenib-treated cells, were significantly longer than the average. In parallel, we observed that the length of both the whole transcript and CDS was significantly shorter than the average in the control condition. In the **Figure 27A**, this difference is more remarkable when comparing the gene set corresponding to those actively translated in Sorafenib treated cells and poorly translated in control conditions (group 5) with those corresponding to mRNAs poorly translated in Sorafenib treated cells and actively translated in control conditions (group 4).

In order to study this correlation in more depth, we represented the length of the CDS of the genes *versus* the translation ratio in both the control and Sorafenib-treated groups. In the case of the control condition, we found an inverse correlation of the CDS length with the translation ratio

whereas in Sorafenib-treated cells, we interestingly found the opposite result, thus, genes showed a positive correlation of the CDS length with translation ratio (**Figure 27B**). We interpret this observation as meaning that the translation of longer mRNAs seems to be more resistant to the inhibitory effect of Sorafenib, whereas the translation of the shortest ones, which are preferentially translated in control conditions, is strongly inhibited following the treatment.

Intriguingly, we detected a specific population of genes, that we called "hypersensitive genes to Sorafenib". This gene set did not show any correlation between their CDS length and their translation ratio (**Figure 27C**). This led us to an interesting question: is there a mechanism(s) specifically impaired by Sorafenib, or a mRNA feature that makes them especially sensitive to the drug? In addressing this question, we found an intriguing relationship between the absence of the m^6A modifications in the mRNA sequences and the translation status of this set of genes.
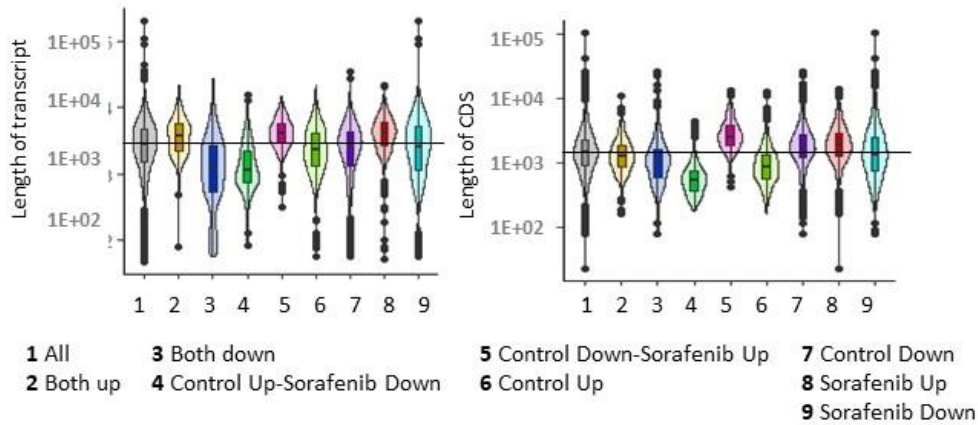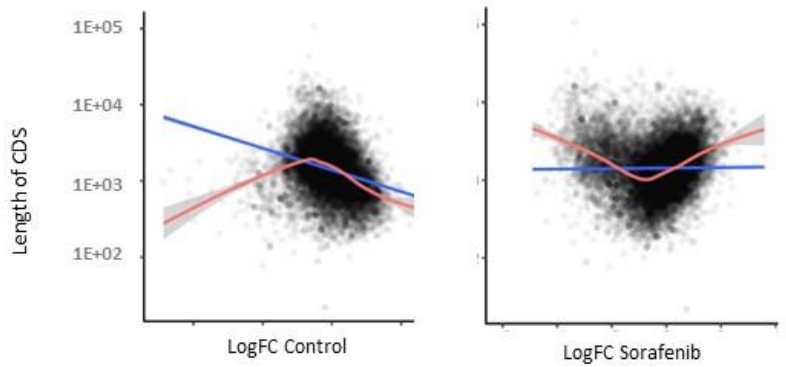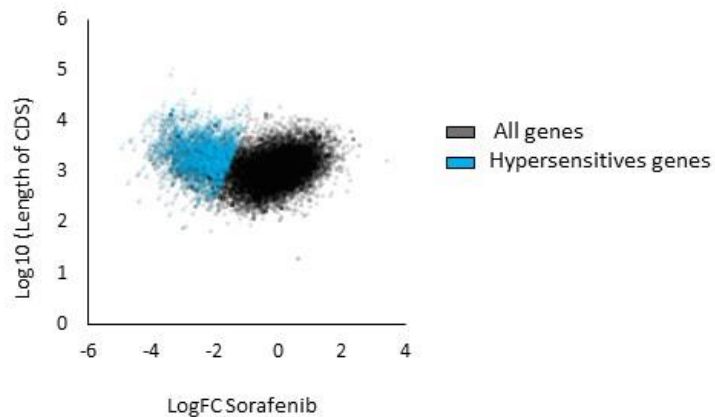
Figure 27. Translation of longer genes is, in general, more resistant to the inhibition exerted by Sorafenib (**A**) The figures show the average length (Y axis) of whole transcripts (left) and only the CDS region (right) in each defined gene population (X axis). (**B**) The figure shows the length of the CDS (Y axis) plotted against the translation ratio in control (left) and Sorafenib-treated cells (right). (**C**) Representation of the length of transcript (Y axis) against the V2/V1 ratio in Sorafenib treated cells. The scatter plot, corresponding to all genes (grey), overlapped with that for the hypersensitive genes to Sorafenib population (blue).

Translation of no methylated mRNAs is more sensitive to Sorafenib

N^6-methyladenosine (m^{6}A) is the most prevalent post-transcriptional RNA modification on eukaryote mRNAs, which is dynamically regulated (230). The methylation grade of mRNA in human cells is determined by the activity of methyltransferases called "writers, which catalyse the methylation, and demethylases called erasers which remove it. Additionally, these modifications are recognized by readers proteins that bind to m^{6}A modifications independent on the RNA sequence (240). The m^{6}A mRNA modification influences in diverse biological processes in mammalians cells including translation (227). Thus, we investigated the translation status of those methylated mRNAs and those not methylated in response to Sorafenib.

To do so, we used the complete list of m^{6}A-modified mRNAs described by Liu *et al.* (241) in order to identify those described methylated mRNAs in our analysis. Firstly, we found that most mRNAs from our analysis had at least one identified m^{6}A-methylation in its sequence and only around the 3% of the genes were not present in the m^{6}A-modified mRNA database. Secondly, to examine whether the presence of m^{6}A in their sequence could influence on their translation, we performed an enrichment analysis by ORA to analyse the translation of those non-methylated genes. As a result, we found that those apparently non m6A genes were highly enriched among the genes poorly translated following the treatment (*p*-value = 1.952 x 10^{-39}), whereas those m6A methylated mRNAs were significantly enriched in genes actively translated in both control and Sorafenib-treated cells. More interestingly, our data revealed that those Sorafenib hypersensitive genes were significantly enriched in non m^{6}A-methylated (*p*-value = 7.82 x 10^{-26}) (**Figure 28**). These data are consistent with the idea that m^{6}A methylation could influence the translation status of mRNAs, and we hypothesize that that will determine the response to the Sorafenib treatment. This assumption is based on: (1) those m6A methylated mRNAs show a correlation between their translation status and their CDS length, suggesting that their translation is subjected to the different mechanisms controlling translation triggered in response to Sorafenib and otherwise (2) the translation of those non-methylated mRNAs seems to be unresponsive to any characteristic involved in translational control breaking out the general tendency. Thus, in consideration of these data, we propose that it may be interesting to induce mRNA demethylation to make cells much more sensitive to Sorafenib by hindering translation for a broader range of mRNAs.
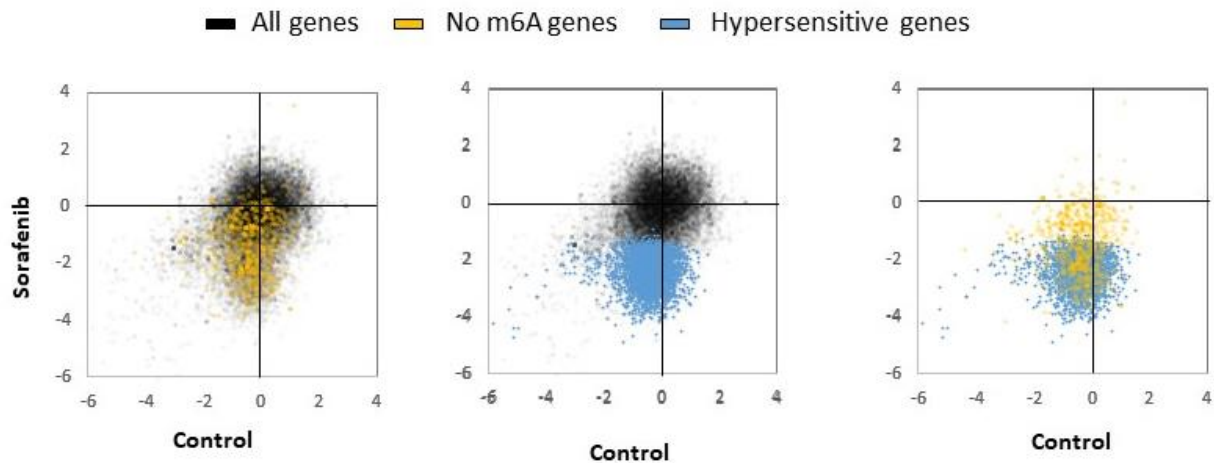
Figure 28. The Sorafenib hypersensitive gene population is highly enriched in non m^{6}A-methylated mRNAs. Left: Scatter plot overlapping the translation ratio of all genes (grey) with that of non m^{6}A-methylated mRNAs (yellow). **Middle:** Scatter plot overlapping the translation ratio of all genes (grey) with that of mRNAs hypersensitive to Sorafenib (blue). **Right:** Scatter plot overlapping the translation ratio of non m^{6}A-methylated mRNAs (yellow) and mRNAs hypersensitive to Sorafenib (blue). Non m^{6}A-methylated mRNAs were obtained from the dataset listed in Liu *et al.*

It is stablished that the METTL3-METTL14 complex is the main responsible for the m^{6}A mRNA methylation (231). Moreover, it has been reported that the methylase METTL3 is also involved in translational control independent on its methylase activity (239). On the one hand, it has been reported that the depletion of METTL3 causes a repression of overall protein synthesis and, on the other hand, Choe et al reported few years ago that METTL3 promoted translation of a subset of mRNAs with oncogenic properties. Thus, we asked whether Sorafenib affected the translation of METTL3 target genes.

To answer this question, we used two distinct dataset that reported on METTL3 target genes; one of them was published by Choe et al., (239) while the other one was published by Babieri et al., (242). When we compared our findings to those of Choe et al., we discovered that mRNAs whose translation appears to be mediated by METTL3 were significantly enriched among actively translated mRNAs in control conditions (*p*-value 1.03 x 10^{-114}). Moreover, we also observed an enrichment in the group of genes corresponding to those actively translated in both conditions simultaneously (*p*-value 6.04 x 10^{-28}). Interestingly, we did not find a significant p-value for those poorly translated mRNA in any conditions and just a low significant p-value for those mRNA actively translated in Sorafenib-treated cells (*p*-value 8 x 10^{-04}) (**Figure 29A**). In this line, when Babieri database was used for comparison, we also found that the actively translated genes in control conditions (*p*-value 2.15

x 10^{-31}) as well as in both conditions simultaneously (*p*-value 4.36 x 10^{-15}) were enriched in METTL3-target genes. However, this analysis also showed that those mRNAs actively translated following the treatment were much more significantly enriched in METTL3 target genes (*p*-value 3.65 x 10^{-48}) (**Figure 29B**). The apparently observed difference, although not mutually exclusive, from both analyses could depend on the experimental design of each one, like the cell line used. Nonetheless, our data suggest that the drug has no effect on the translation of METTL3 target genes, so we propose that silencing METTL3 could make cells more sensitive to Sorafenib, based on: (1) METTL3 is the main responsible for m^{6}A methylation, so silencing it could result in a lower number of m6A methylated mRNAs, which, consistent with the previous idea, would make mRNA translation more susceptible to the treatment (2) METTL3 plays a direct role in translational control independently of its methylase activity thus its silencing would drive down regulation of specific subset of mRNAs involved in tumorigenesis. It must be noted that the role of METTL3 in translation seems to be very complex and is not fully understood so further experiments must be done to address the assumption above.
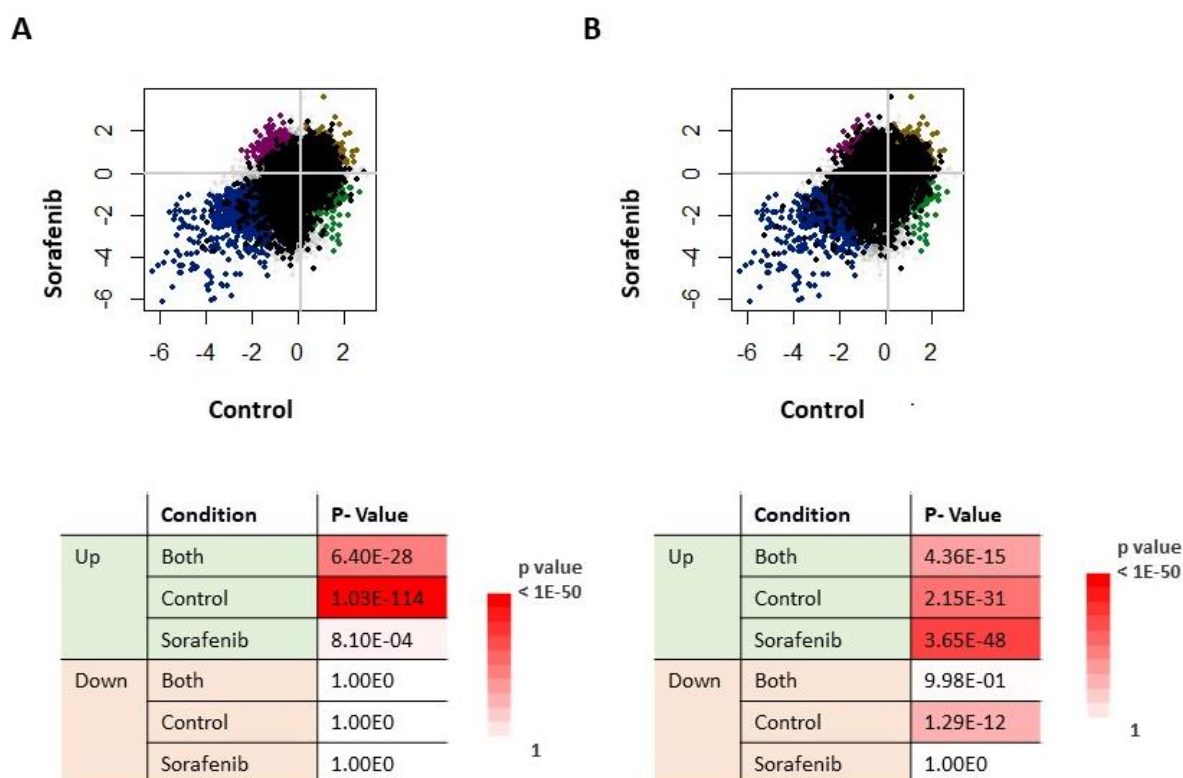
Figure 29. Translation of METTL3-target genes seems to remain unaffected by Sorafenib. Top panel: Scatter plot overlapping all genes with the METTL3-target gene population described by Choe *et al.* (**A**) or Babieri *et al.* (**B**). **Bottom panel**: Table showing the *p*-value of the enrichment in METTL3-target genes for each category of our study. The intensity of the red colour represents the level of significance of the values.

Sorafenib induces translational reprogramming that brings cells to a new cellular context

To better understand the biological consequences of the translational changes induced by Sorafenib, we analysed the categories enriched either in poorly translated mRNAs or highly translated in mRNAs after the Sorafenib treatment. To this, we performed an enrichment analysis using the REACTOME database. As a result, we first found that the most significant categories enriched in poorly translated mRNAs were related with electron transport chain, including ATP synthesis (q-value = 7.27 x 10^{-16}); respiratory electron transport (q-value = 1.79 x 10^{-12}); and TCA cycle and respiratory electron transport (q-value = 5.75 x 10^{-10}). Besides targeting genes important for mitochondrial activity, our data also showed categories related with collagen biosynthesis significantly downregulated following the treatment. Among these REACTOME terms with a q value < 1x10^{-10} we found collagen chain trimerization (q-value = 4 x 10^{-10}); extracellular matrix organization (q-value = 7.70 x 10^{-11}); and collagen biosynthesis (q-value = 9.02 x 10^{-10}). We then wondered whether the most significant downregulated-Sorafenib categories work properly in optimal conditions. Thus, we performed the same enrichment analysis for untreated cells, and we found that these categories were significantly upregulated in control conditions, although some of them were not found significantly dysregulated in any sense (**Figure 30**).

Figure 30. REACTOME enrichment analysis of significantly poorly translated genes following the Sorafenib treatment. The figure shows an enrichment analysis using the REACTOME database. In red are shown the most significant categories (q-value < 1 x 10^{-10}) enriched in poorly translated genes and the gene enrichment in Sorafenib-treated cells. Additionally, in blue is shown with these categories with their respective q-value and the enrichment actively translated gene in the control condition. The q-value were transformed into -$\log_{10}$.

Next, we investigated which were the most significant upregulated categories following the Sorafenib treatment and, more interestingly, whether some of the previously discussed features controlling translation were present in the up-regulated genes. Thus, applying the approach describe above, we discovered among the most significant over-represented REACTOME terms categories (q value $< 1 \times 10^{-10}$) those associated with: (i) RNA processes, including the terms of RNA metabolism (q-value 2.54 x 10^{-28}) processing of capped intron-containing pre-mRNA (q-value 1.27 x 10^{-19}) and mRNA splicing (q-value 3.29 x 10^{-12}); (ii) signalling pathways like signalling by Rho GTPases (q-value 2.05 x 10^{-26}); (iii) cell cycle such as cell cycle category (q-values 1.92 x 10^{-15}), M phase (q-value 1.92 x 10^{11}) and mitotic metaphase and anaphase (q-value 9.64 x 10^{-19}); (iv) membrane trafficking, including membrane trafficking category (q-value 2.28 x 10^{-14}) and vesicle mediated transport (q-value 3.65 x 10^{-11}); and (v) cellular response to stress including, post-translational protein modification (q-value 1.90 x 10^{-14}), SUMOylation (q-value 9.62 x 10^{-10}) and IRE1α-dependent chaperone activation (q-value 3.56 x 10^{-10}). We then considered the status of these categories in the control condition, observing that those categories associated with the RNA process, cell cycle, and post-translational protein modification were also significantly up-regulated in this one (q-value < 1 x 10-5). Although it should be noted that the q-value for the enrichment in poorly translated genes was higher for all of them. In terms of Rho GTPase signalling, the transport of mature transcript to cytoplasm and SUMOylation categories did not show a significant enrichment in actively or poorly translated genes in control conditions, except for transport of mature transcript to cytoplasm, which did (q-value 7.66 x 10^{-06}) (**Figure 31**). These findings suggest that the translational reprograming induced by Sorafenib drives a shift in the working sense of cellular processes, as well as triggers additional ones. As a result, we believe that the cell fate will ultimately be determined by the combined activity of the Sorafenib-deregulated categories. Although, the biological relevance of each one remains to be investigated.
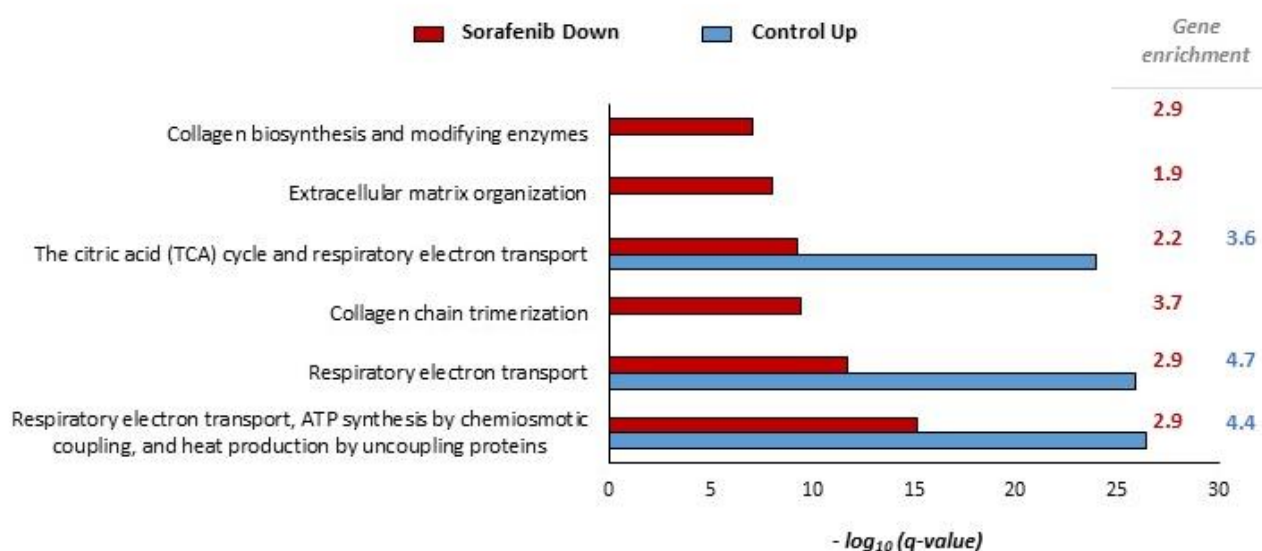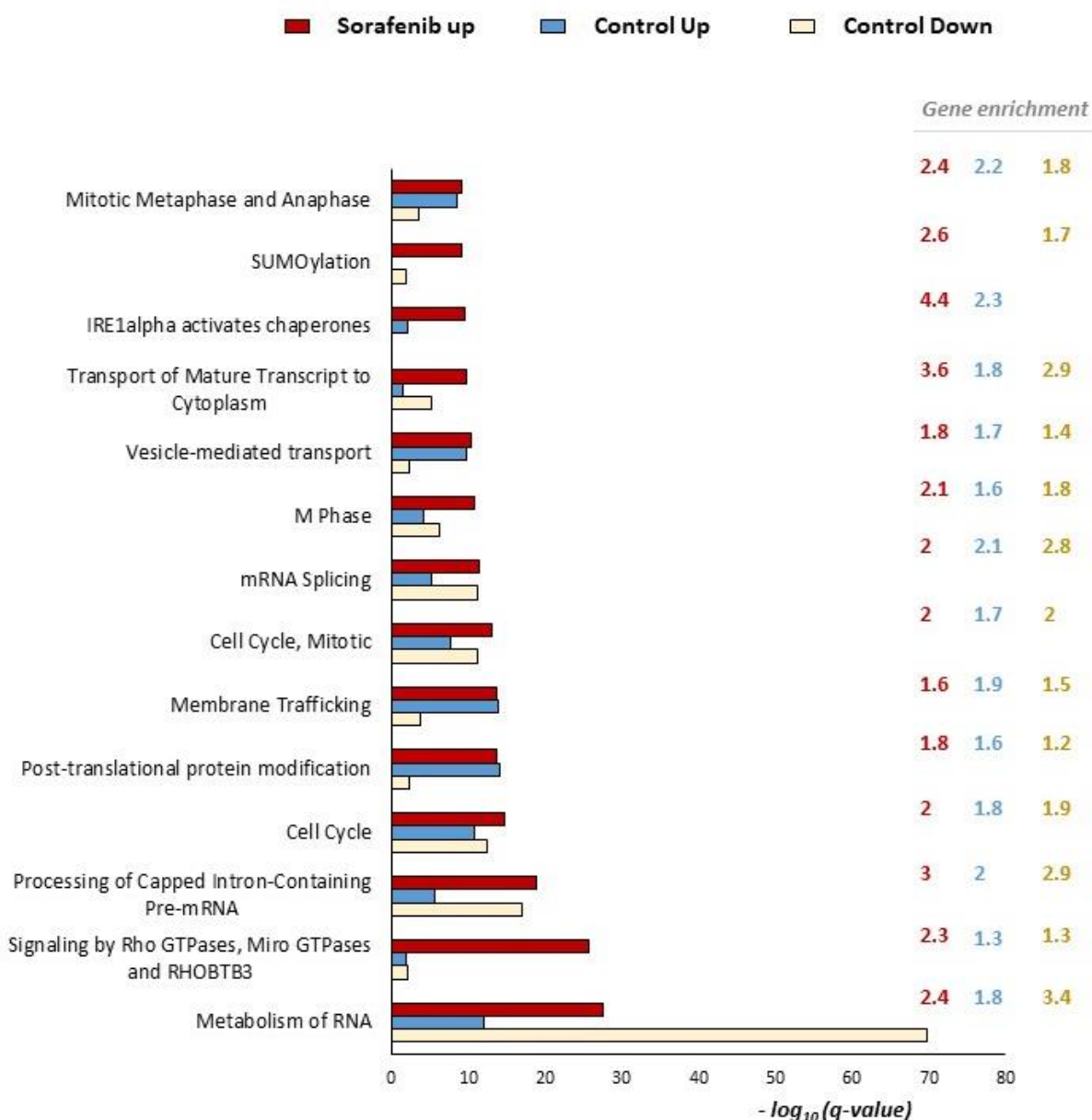
Figure 31. REACTOME enrichment analysis of significantly actively translated genes following the Sorafenib treatment. The figure shows an enrichment analysis using the REACTOME database. The red bars represent the most significant categories (*q-value* < 1 x 10^{-10}) enriched in actively translated genes and the gene enrichment in Sorafenib-treated cells. Additionally, the figure shows the q-value and the gene enrichment of these categories in actively translated (blue) ad poorly translated (yellow) genes in the control condition. The q-value were transformed into -log$_{10}$.

To study the relevance of the previously discussed features involved in translational control in Sorafenib-induced translational reprograming, we examined the presence of these features in the gene set corresponding to each up-regulated category. We found that DAP5-target genes as well as ARE-containing mRNAs were the most abundant features in those up-regulated processes. Indeed,

the DAP5-target genes seem to be especially important in those RNA-related categories, including RNA metabolism and processing of capped mRNAs, whereas ARE elements are likely more important in the translation of another gene set like signalling by Rho GTPAse (**Figure 32**).
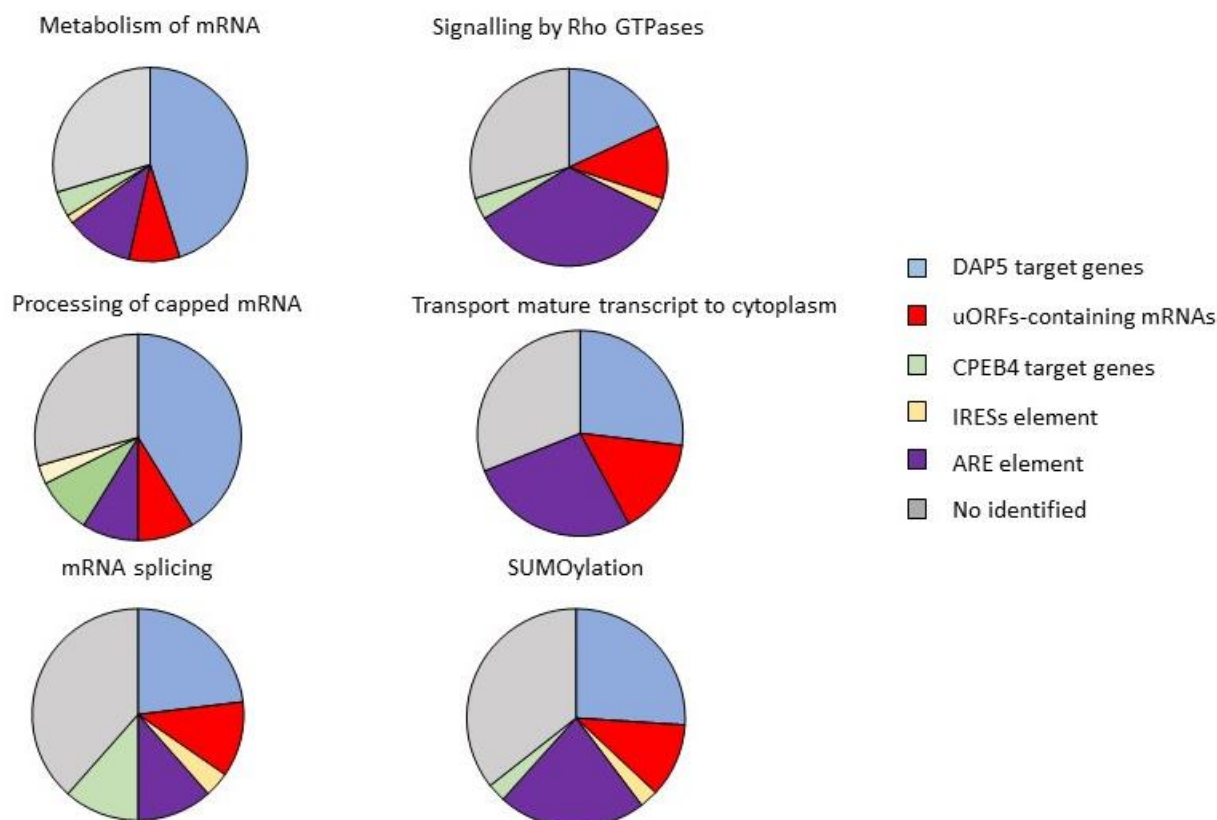
Figure 32. Features involved in translational control that were highly represented in those REACTOME categories up-regulated in Sorafenib-treated cells. Each category is represented by a different pie chart. Each colour indicates the percentage of genes for each feature analysed. In grey are shown the percentage of genes where any of the analysed features were not identified.

In addition, to further explore the relevance of these features in translational control, we compared the fold change of those genes with some features identified in their sequence with those in which any of the features mentioned above were not found. In this case, we thought that the transport of mature transcripts to the cytoplasm category would provide us better insights into the presence of certain trans-acting features in the mRNAs and their selective translation; it was the only one significantly enriched in actively translated genes in Sorafenib-treated cells whereas it was significantly enriched in poorly translated genes in the control condition. Additionally, there was no significant enrichment in actively translated genes in the control condition, implying that this

category does not play a pivotal role in control condition but likely does in response to the treatment. Following this approach, we classified the genes of the category into different subgroups: DAP5-target genes and non DAP5-target genes, uORFs-containing mRNAs and mRNAs lacking uORFS, and ARE-containing mRNAs and those that do not, genes containing AREs and genes lacking AREs. We then compared the translation ratios of these two subgroups, resulting in the finding that the translation of DAP5-target genes as well as ARE-containing mRNAs was significantly more enhanced than that of their respective counterparts. Interestingly, the translation ratio similar to those uORFs-containing mRNAs showed a similar ratio to those without uORFs in their sequence (**Figure 33A**). These results suggest that the presence of these sequences is likely to be strongly linked to promoting the translation of specific mRNAs. We then sought additional evidence of this fact by individually examining the translation ratios of genes that were poorly translated in the control condition but whose translation was promoted following treatment. Consistent with our assumption, we found that 8 out of the 10 genes in this category that met this criterion consistently have been described as DAP5-target genes or ARE-containing mRNAs (**Figure 33B**).

A
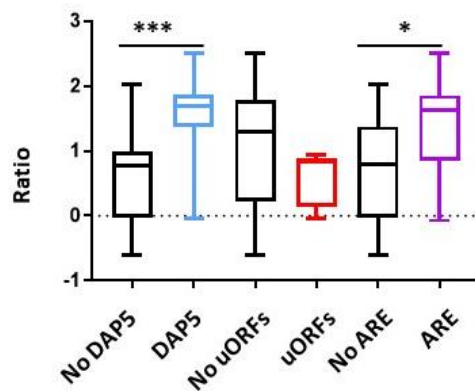
B

Genes	p Value	LogFC	P Value	LogFC	Ratio	DAP5	ARE element
AAAS	0,00022162	-1,070	0,02011083	0,712	1,782	FALSE	FALSE
CPSF1	0,00403103	-0,732	3,57E-06	1,280	2,021	FALSE	FALSE
NUP107	9,42E-07	-1,148	0,00675044	0,665	1,813	TRUE	TRUE
NUP133	0,02515527	-0,520	0,00176326	0,775	1,295	TRUE	FALSE
NUP160	0,00153484	-0,774	3,65E-05	1,100	1,874	TRUE	TRUE
NUP205	0,01365335	-0,551	1,76E-05	1,075	1,626	TRUE	FALSE
NUP210	0,01394232	-0,700	0,00078996	1,042	1,741	TRUE	TRUE
NUP93	0,00533206	-0,548	0,01470672	0,494	1,042	TRUE	FALSE
NUP98	0,00169889	-0,561	1,40E-10	1,280	1,841	TRUE	FALSE
TPR	0,00180337	-1,056	8,31E-05	1,445	2,500	TRUE	TRUE

Figure 33 DAP5-target and ARE-containing mRNAs were present among the group with a higher fold change in Sorafenib-treated cells. (A) Boxplot representing the fold change of genes in each defined gene group. **(B)** The table shows common genes related to nucleo-cytoplasmic mRNA export that were significantly up-regulated in Sorafenib-treated cells but significantly down-regulated in the control conditions. FC: Fold Change; Ratio represents LogFC of genes for Sorafenib-treated cells *versus* LogFC for untreated cells. True: identified as DAP5-target genes of ARE-containing mRNA. False: not identified.

Here, we have demonstrated that Sorafenib induces translational reprogramming through the dysregulation of pathways whose activities converge in translation machinery. This drives cells a new cellular context from a translational point of view, where the mitochondrial function and the collagen biosynthesis processes are negatively impaired, and categories related to RNA processes, signalling by Rho GTPases, cell cycle, membrane, and cellular response to stress were significantly upregulated. Additionally, our result showed an enrichment of different features involved in translational control present in those actively translated genes, suggesting that they can mediate their translation in response to Sorafenib-induced stress. It must be noted that these assumptions are based exclusively on the presence of these features into the mRNA sequences. Even so, we speculated that cis-acting elements controlling translation, as well as those proteins involved in its recognition, determine the translation status of different mRNAs, which ultimately plays an important role in the Sorafenib-driven cellular context.

MATERIALS AND METHODS

Cell lines, culture conditions and treatments

The HepG2 cell line was used in this study. Some features of this line are collected in Table 1, shown in the Materials and Methods section of the Chapter 1 of this Thesis book. HepG2 cell line was routinely cultured and maintained as described in the Materials and Methods section of Chapter 1. A mycoplasma test was done before the experiment.

Sorafenib was dissolved in DMSO and added to plates to reach a final concentration of 10 µM. The treatments were performed within 24 h after plating. Control cells were treated with the vehicle DMSO. The experiment was performed 12 h after adding the treatments.

Polysome fractionation

Polysome profile analysis was carried out using cell extract of cells treated either with the vehicle (DMSO) or with 10 µM Sorafenib for 12 h and further treated with 0.2 mg/ml cycloheximide for 5 min at 37 °C. Then cells were lysed and polysome profile were fractionated on a sucrose gradient as described in the Materials and Methods section of Chapter 1.

mRNA library preparation

Libraries were prepared using the RNA collected from the polysome profile of cells treated either with DMSO or with Sorafenib. Input samples from the low-translated fraction (V1) and highly-translated fraction (V2) was collected as described in the Materials and Methods section of Chapter 1. Low-translated fraction represents the RNA present either in the soluble fraction or peaks corresponding to 40S, 60S ribosomal subunits and 80S ribosomes. The highly translated-fraction depicts the mRNAs bound to more than one ribosome, forming polysomes. The concentration and quality of RNAs obtained as described in the Materials and Methods section of Chapter 1 were assessed with Qubit (Qubit™ DNA HS assay) and a 2100 Bioanalyzed Nano Chip (Agilent Technologies Genomics, USA), respectively. The RNA Integrity Number (RIN) values were > 9 in all the RNA samples. Polyadenylated RNA was isolated from total RNA samples using NEBNext Oligo d(T)25 beads (New England Biolabs, USA) according to the manufacturer's instructions. The same RNA concentration for each sample was used to create the libraries in order to be processed by Ilumina Sequencing. Three independent experiments were carried out for the sequencing analysis.

RNA sequencing and data analyses

RNA sequencing was performed with a NextSeq500 Mid-Output and 2x75 pb length parameters (paired-end). RNA-Seq data were first filtered using the FASTQ Toolkit v1.0.0 program and then analysed using the BaseSpace Onsite v3.22.91.158 from Illumina. Only genes that were upregulated or downregulated with a *p*-value < 0.5 and [log$_2$(fold changes)] $\geq \pm 0.5$ were selected. Data were normalized using *z-score*. The log$_{10}$(V1/V2) was applied to calculate the translation ratio for each gene in both the control and Sorafenib-treated conditions. A positive ratio reflects a higher proportion of mRNA in the highly-translated fraction (V2) than the average. A negative value corresponds to those mRNAs with an enrichment in low-translated fraction (V1). Applying this criterio, we identified two set of genes for each condition: (i) gene set enriched in mRNAs with a positive translation ratio and (ii) gene set enriched in mRNAs with a negative ratio.

The previous data were combined to identify the changes in the translational behaviour of mRNAs in response to the Sorafenib treatment. Four additional categories were identified: (i) a gene set enriched in genes with a positive translation ratio in both conditions (ii) a gene set enriched in genes with a negative translation ratio in both conditions (iii) gene set enriched in genes with a positive ratio in Sorafenib-treated and a negative ratio in untreated cells and, (iv) a gene set enriched in genes with a negative ratio in Sorafenib-treated and positive in untreated cells.

Over-representation analysis (ORA)

To identify the mRNA features present in the different categories described, an Over-Representation Analysis (ORA) was performed using Fisher´s exact test, selecting the genes with a *p*-value lower than 0.001 in the RNA-Seq data, either in each condition or in both conditions simultaneously. Different databases were used for the enrichment analysis (208, 239, 241-246). A REACTOME database (https://reactome.org) was performed to investigate either activated or inhibited pathways of the differentially expressed genes in both untreated and Sorafenib-treated cells.

RT-PCR and qPCR analysis

To validate the results of the mRNA-seq analysis, the relative RNA abundance of selected mRNAs, chosen randomly, was quantified by RT-qPCR in each fraction from both conditions untreated and Sorafenib-treated cells. Thus, equal volumes of RNA samples were treated with 1 µl of DNase I (Promega) following the manufacturer's instructions. RNA was then reverse transcribed using SuperScript™ III First Strand Synthesis for RT-PCR, also according to the manufacturer's instruction (Invitrogen, USA) using random hexamer primers (Roche, Switzerland). RT-qPCR was performed using SYBR® Green Premix Ex Taq™ 2X (Takara, Japan) and primer specifics of each transcript. Primer pairs used for the RT-qPCR are shown in **Table 1**. The data were processed normalizing to the whole profile RNA. The RNA luciferase, added to each sample before the RNA extraction, was used as an external control. The percentage of mRNA was calculated, and the data were expressed as the mean ± the standard deviation (SD).

Table 1. Primers used for RT-qPCR

Primers	Forward (5' - 3')	Reverse (5' - 3')
BOP1	CTG ATT CAC CAG CTG AGC C	GAC GCC ACC AAC AGG AAG
eIF4E	CTA CTA AGA GCG GCT CCA CC	GGT TCA GCT CCC AAA TCT CG
eIF4E2	TGA AAG ATG ATG ACA GTG GGG A	CTGATTCTTGTCTCGTTCCGT
NDUFA7	AAC TCA GCC TCC TCC CAA G	CTC TCT GCT GGC TTG CCT
RAD51	GCA CTG GAA CTT CTT GAG CA	GTT GTT TTC ATT AAG GGC ACT CC
SDHD	CCT CTG CTT TGT CAT GCC AT	GGC AAC CCC ATT AAC TCA CC
Luciferase	ATC CGG AAG CGA CCA ACG CC	GTC GGG AAG ACC TGC CAC GC

Statistical analysis

Statistical analyses were performed with the Prism 6.01 software (GraphPad). Data were generated from several repeats (at least three ones) of different biological replicates (at least three ones). Mean ± S.D. were represented in the different graphs. To determine significance, the Student's test for unpaired samples with confidence interval of 95% were computed. Significance between conditions were indicated with the symbols *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$. Regression plots and determination of Pearson coefficients and *p*-value were performed using the R software.

DISCUSSION

Sorafenib inhibits global protein synthesis in a mTORC1-independent manner

HCC has a high and heterogeneous incidence, ranking sixth among all cancers. Additionally, it is one of the most mortal cancers, posting the second-highest mortality rate worldwide. Paradoxically, despite its high incidence and mortality, the therapeutic options for HCC patients are limited. Only HCC patients diagnosed during the first stages, including the very early, early, and intermediate stages (following the BCLC system), can receive curative treatments such as liver transplantation, tumour resection, or chemoembolization. For those patients who are unfortunately diagnosed in advanced stages, where cancer dissemination has occurred and the symptoms have appeared, systemic pharmacotherapy is the appropriate treatment, but with low clinical benefits. Sorafenib was the first drug approved by the FDA for the treatment of advanced HCC. This is an multikinase inhibitor with anti-proliferative, anti-angiogenic and pro-apoptotic properties that poorly but significantly improves the overall survival and time to progression of advanced HCC patients. Other tyrosine kinase inhibitors have been studied in clinical trials for their use as first-line treatment of HCC but have not outperformed Sorafenib. This reveals that other mechanisms, independent on multikinase inhibition, are pivotal in its anti-cancer activity, explaining why it has been the only available treatment for more than a decade. However, the low clinical efficacy as well as the numerous side effects associated with the treatment argue for the need to improve this therapeutic option. Sorafenib was originally developed as a specific C-Raf and B-Raf inhibitor, but different tyrosine kinase receptors like PDGFR or VEGFR have also been identified as Sorafenib's targets. These receptors activate different signalling pathways, including the RAF/RAF/MEK/ERK cascade, which controls cell growth and proliferation. Several years ago, it was demonstrated that Sorafenib decreased the phosphorylation status of MEK1/2 and ERK1/2 in different HCC cell lines (24). Indeed, the partial tumour regression induced by Sorafenib observed in different *in vivo* experiments was reported to be associated to downregulation of the activity of RAF/RAF/MEK/ERK cascade (25). However, the use of MEK inhibitors to treat advanced HCC in monotherapy like Trametinib or Selumetinib did not show good results in clinical trials, falling into phase II due to a lack of adequate anti-tumour activity (247). This observation suggests that the impact of Sorafenib on other signalling pathways or cellular processes is essential for its antitumor properties.

RAF/RAF/MEK/ERK is the major MAPK signalling pathway that regulates a variety of essential cellular processes that contribute to cell survival and growth, including protein synthesis. The protein synthesis process, also known as translation, is highly hyperactivated in cancer cells. In this way, malignant cells are able to produce all the materials needed to support their high activity. Additionally, the altered activity of the translation machinery allows cancer cells to quickly adapt to

adverse environmental conditions through a wide variety of mechanisms. Despite the clear link between the MAPK signalling pathway and the translation process, little is known about the role of protein synthesis in the Sorafenib response. The inhibition of global protein synthesis by Sorafenib was reported several years ago (248). However, it was not until 2018 that the first publication, focused on describing the impact of Sorafenib on protein synthesis was published (249). This gave rise to new questions and doubts about how Sorafenib mechanistically impairs translation and the cellular consequences derived of this impairment. Consistent with the previous publications, we found that Sorafenib strongly inhibits translation in different liver cancer cells. Polysome profiles of Sorafenib-treated cells showed an increase in the 80S peak while the number of polysomes decreased. This profile was not exclusive to HCC cell lines, since we obtained similar profiles for other malignancies cell upon a Sorafenib treatment like the 5637 cell line from the human bladder (data not shown). This result was further confirmed by a puromycin assay of Sorafenib-treated cells that showed a gradual decrease in the cellular translation status over time compared to an untreated control. Additionally, polysome profiles in high salt gradient conditions demonstrated that the inhibition exerted by Sorafenib happened mainly at the initiation phase. These profiles showed a notable decrease in the 80S peak because the dissociation of ribosomal subunits maintained together by weak interactions. Thus, we conclude that Sorafenib inhibits protein synthesis preferentially at the initiation phase, resulting in an accumulation of free ribosomal subunits that weakly interact, forming the so-called vacant 80S ribosomes and avoiding the formation of active ribosomes bound to mRNAs.

A well-known pathway that controls translation is that regulated by the mTORC1 kinase (102). This protein complex is activated downstream of growth factor receptors that drive cell proliferation and growth under optimal environmental conditions. The binding of multiple growth factors and cytokines to their receptors causes the activation of PI3K, which in turn activates Akt that finally leads to the activation of mTORC1. The PI3K/Akt/mTORC1 pathway is one of the most commonly altered pathways in HCC patients (5). Indeed, therapies based on mTORC1 inhibitors like Sirolimus or Everolimus have been studied in clinical trials for HCC treatment. Everolimus has been shown in preclinical HCC models to significantly reduce cell proliferation in liver cancer cells and tumour progression (250, 251). However, no significant differences were observed compared to placebo groups in clinical trials (252). Sirolimus, also known as Rapamycin, has not shown clinical benefits in the combination therapy with Sorafenib for advanced HCC but seems to produce a favourable effect in reducing the incidence of HCC recurrence after liver transplantation (253, 254). Despite the fact that these drugs are targets of the mTORC1 complex, our findings revealed no changes in the polysome profiles of HCC cells treated with them at the concentrations used. High doses of these

mTOR inhibitors, where a strong effect on cellular viability was observed, were required to see an impairment in overall translation. Besides, we have also shown that the translation inhibition exerted by Everolimus and Sirolimus occurs mainly at the early elongation phase instead of initiation (255). Currently, the exact role of mTORC1 in the response to the Sorafenib treatment is a bit controversial. It does not seem to play a dominant role in the Sorafenib response but its hyperactivation has been associated with acquired resistance to this drug in HCC. Thus, the combined treatment of Sorafenib with the Akt inhibitor MK-2206 has been suggested that could overcome the Sorafenib resistance (256). This hyperactivation has also been identified in other malignancies like the Acute Myeloid Leukemia (AML), where the combined treatment of the mTORC1 inhibitor, Gedatolisib, and Sorafenib seemed to efficiently block colony formation, and in addition decrease cell proliferation, induce apoptosis, and block tumour growth *in vivo* (257). In conclusion, the different impact on the overall translation process by mTORC1 inhibitors and Sorafenib suggests that Sorafenib inhibits translation independently of mTORC1.

Sorafenib hierarchically affects different pathways controlling translation

Cell controls the protein synthesis process mainly at the initiation stage. The two most well-known mechanisms involved in translational control at this level include (1) those that regulate the levels of TC, which are in turn determined by the phosphorylation status of eIF2, and (2) those that regulate the activity and levels of the eIF4F complex.

The TC, formed by eIF2, Met-tRNA$^{Met}_i$ and GTP, interacts with the small ribosomal subunit and different translation initiation factors to constitute the 43S PIC. Under stress conditions, the levels of this complex decrease as a consequence of the phosphorylation of eIF2 at the conserved serine-51 of its α subunits. Increased Phospho-eIF2 levels cause a decrease in TC levels, resulting in an inhibition of cap-dependent translation at the initiation stage. We and others have reported that Sorafenib induces ER stress, resulting in the activation of the PERK kinase, which downstream phosphorylates eIF2α at its serine-51 (107). In agreement, we observed that the levels of Phospho-eIF2α were associated with enhanced translation of a selective group of mRNAs, including ATF4. ATF4 is one of the principals Phospho-eIF2α targets, which in turn activates the expression of different genes in response to stress. Thus, consistently with the increased levels of Sorafenib-induced Phospho-eIF2α, we demonstrated that the translation of ATF4 mRNA was also enhanced by this drug. Interestingly, we observed that PERK-silenced cells showed lower levels of Phospho-eIF2α as well as a partial recovery of the translation status after 3 h of Sorafenib treatment. Moreover, these cells also showed a completely different translation pattern of ATF4 from non-transfected cells in response to

Sorafenib. In non-transfected cells, the majority of ATF4 mRNA was found in the heavy polysome fraction upon the Sorafenib treatment, whereas in PERK-silenced cells, it was found in the low-polysome fraction. These results highlight the relevance of the eIF2α phosphorylation in translational control during Sorafenib treatment. These findings are consistent with previously published results by Rahmani M, (248), who demonstrated that the silencing of PERK caused a substantial decrease in the levels of Phospho-eIF2α. Although more interestingly, they demonstrated that PERK-silenced cells showed lower levels of cell death compared than PERK-expressing cells. Taken together, our results strongly suggest that the induction of phosphorylation of eIF2α makes cells more sensitive to Sorafenib-mediated lethality through its role in translation. However, the partial recovery of translation observed in PERK-silenced cells further suggests that another mechanism(s) controlling translation must be involved in the inhibition of translation initiation exerted by Sorafenib in HCC cells.

The family of 4E-BPs are repressor proteins whose phosphorylation status determines the formation of the eIF4F complex, which is involved in the cap-dependent translation of most mRNAs. The phosphorylation of 4E-BPs can be produced by different kinases, although mTORC1 is the main responsible and best characterized one. In optimal growth conditions, the different 4E-BPs kinases phosphorylate it in four different residues, including serine-65, threonine-37, threonine-46, threonine-70 and serine-65. These phosphorylations occur in a hierarchical manner, being the phosphorylation in threonine-37 and threonine-46 a prerequisite for the subsequent ones. The phosphorylation status of 4E-BP influences its ability to bind to eIF4E, which caused a block of cap-dependent translation at the initiation phase. 4E-BPs have been proposed to be involved in translation inhibition exerted by Sorafenib (249). However, our findings clearly show that 4E-BPs was not the responsible for the translation inhibition observed upon the Sorafenib treatment at least in HepG2 cells, relying on: (1) the phosphorylation status of 4E-BP1 in the three different residues studied, as well as its total levels, are unaffected in our conditions. Consistently, the phospho-4E-BP1/4E-BP1 ratio remained constant after Sorafenib treatment. (2) Regardless of the phosphorylation status of 4E-BPs, we generated a stable cell line with no expression of 4E-BP1 and 4E-BP2 that displayed an apparently similar inhibition degree of translation as normal cells upon the Sorafenib treatment. Thus, if 4E-BPs had been responsible for the translation inhibition exerted by Sorafenib, the silencing of the two major 4E-BP forms would have very likely resulted in a minor impact on translation. (3) The phosphorylation levels of 4E-BPs are strongly linked to the mTORC1 activity, whose activity seems to be unaffected directly by Sorafenib. In fact, the phosphorylation status of another readout of mTOR, RPS6, whose phosphorylation at its serine-240 and serine-244 is

just dependent on mTORC1 activity, did not show alteration in Sorafenib-treated cells in our conditions.

Apart from mTORC1, the RAS/MEK/ERK signalling pathway also controls translation by regulating the activity of different translation initiation factors and ribosomal proteins. Indeed, some of these proteins are also mTORC1 targets, including RPS6 at its serine-235 and serine-236 (109, 113). The RAS/MEK/ERK cascade phosphorylates MNKs and RSKs, which in turn phosphorylate the translation initiation factors eIF4E and the ribosomal protein RPS6, respectively (112). Thus, the decrease in the phosphorylation levels of RPS6 at serine-235 and serine-236 that we have observed could be explained by Sorafenib-induced RAS/MEK/ERK switching off. Supporting this idea, we also found that the phosphorylation status of ERK1/2 and the cap-binding protein eIF4E were notably reduced. Additionally, although the treatment with Sirolimus caused an increase in the levels of Phosho-eIF4E, the combined treatment with Sorafenib completely suppressed. In contrast, the inhibition of eIF4E phosphorylation observed upon the MNK1 inhibitor treatment, although a bit lower than that exerted by Sorafenib, was not maintained after the combined treatment with Sirolimus. These observations are consistent with the idea previously discussed. On one hand, Sorafenib switches off the RAS/RAF/MEK/ERK signalling pathway, leading to a reduction in the phosphorylation levels of eIF4E. This suppression occurs at a very early stage of the Sorafenib treatment, even before the initiation of the phosphorylation of eIF2α. Thus, we speculate that the low levels of phosphorylation of eIF4E mediate the first response to Sorafenib, modulating the activity of the translational machinery. On the other hand, the hyperphosphorylation status of eIF4E upon Sirolimus treatment highlights the crosstalk between pathways controlling translation, thus, the inhibition of mTORC1 by Sirolimus drives an increase in the activity of MNK1 to counteract this inhibition. Therefore, we hypothesize that the translational apparatus serves as a unifying factor that could encourages the aberrant expression of the PI3K/Akt/mTORC1 pathway as a consequence of the inhibition of MAPKs, resulting in resistance to the Sorafenib treatment.

Sorafenib selectively inhibits translation of pro-tumoral mRNAs consistently with the inhibition of eIF4E phosphorylation.

The cap-binding protein eIF4E has been recognized as the limiting initiation factor for mRNA translation (259). It forms the eIF4F complex together with the RNA helicase eIF4A and the scaffolding protein eIF4G, that recognizes and binds to the cap structure, an N^7-methylated guanosine linked to the first nucleotide of a mRNA via a 5'-5' triphosphate linkage. The activity of eIF4E is regulated by its phosphorylation in its serine-209 which is enhanced in optimal growth conditions. The total levels of

eIF4E as well as its phosphorylation status have been deeply linked to tumorigenesis. Indeed, high expression levels of eIF4E has been closely correlated with tumour malignance and poor prognosis in HCC (47, 175). Despite the fact that the total eIF4E protein levels as well as its phosphorylation status have been described as not leading to a huge impact on overall translation, their tumorigenic activities have been associated with their role in translation. Both Phospho-eIF4E and total protein eIF4E influence the translation of a selective group of mRNAs encoding pro-tumoral proteins (32, 48, 49, 165, 174, 260). The underlying mechanism based on the selective translation by eIF4E remains unclear. However, some Phospho-eIF4E targets have been identified, including Cyclin D1, involved in cell cycle progression, c-Myc, a transcription factor that controls the expression of a broad spectrum of genes, VEGFA, involved in angiogenesis, and Mcl-1, an anti-apoptotic protein (48, 261). Sorafenib has long been known to inhibit the phosphorylation status of eIF4E. Indeed, it has been hypothesized that Sorafenib-induced cell death was caused by a decrease in Mcl-1 translation due to the inhibition of eIF4E phosphorylation (177). In this thesis work, we have focused on describing the inhibitory effect of Sorafenib on the phosphorylation of eIF4E and its implications for translation. Thus, we individually studied the translation status of some of the described Phospho-eIF4E targets by analysing their distribution along polysome profiles. The results obtained were analysed in two different ways:

(1) On the one hand, the percentage of each mRNA was measured for each polysome profile fraction. The percentage corresponding to heavy (assumed as highly-translated) fractions in untreated and treated conditions was compared, resulting that those mRNAs with a lower percentage in Sorafenib conditions than in control ones, are mRNAs that are being translationally inhibited by Sorafenib.

(2) On the other hand, the relative mRNA levels were calculated by the division of mRNA amount in highly-translated fraction respect to total amount of this mRNA. In this case, we also took into account the stationary mRNA levels upon Sorafenib treatment.

Our finding showed that Sorafenib selectively inhibits the translation of a subset of mRNAs, including cyclin D1, c-Myc, and VEGFA, whereas the translation status of mRNAs for several ribosomal proteins that are well-known mTORC1-target remained unaltered. Besides, VEGFA is also downregulated at the transcriptional level, showing significantly lower total mRNA levels in Sorafenib-treated cells compared to the control. Thus, we observed a decrease in the percentage of VEGFA mRNA in the highly-translated fraction in Sorafenib-treated cells but a higher amount of its relative levels, as a consequence of the reduction of its total mRNA levels. In agreement with these findings, we observed reduced protein levels of cyclin D1 and c-Myc by western blot analysis of whole

extracts of cells treated with Sorafenib. Unexpectedly, we did not find that the translation status of Mcl-1 was affected at very early stage of Sorafenib treatment. However, we could not rule out the possibility that the translation of Mcl-1 was inhibited by a longer period of treatment as we and others have demonstrated that Sorafenib reduced Mcl-1 levels protein at long-term (25, 107). This result highlights the complex role of eIF4E controlling translation. On the one hand, it has been described a set of mRNAs called "eIF4E-sensitive mRNAs" whose translation is highly enhanced by eIF4E overexpression. Additionally, the translation of some of these messengers is also enhanced by an increase on the phosphorylation levels of eIF4E, including cyclin D1, c-Myc, VEGFA, and Mcl-1. These eIF4E-sensitive mRNAs often contain extensive secondary structure in their 5' UTRs. However, their translation is also influenced by the activity of the eIF4A helicase, resulting in a set of mRNAs whose translation is dependent on the activity of both translation initiation factors (179). On the other hand, eIF4E works in the nucleus but causing an impact on translation. eIF4E has been described to be involved in the nucleus-cytoplasmic transport of a set of mRNAs where the phosphorylation status of eIF4E seems to promote this activity (47, 262). Additionally, eIF4E itself promotes the expression of the capping-machinery, increasing the global levels of some m^7G-capped mRNAs called eIF4E-dependent capping RNAs (260). In summary, we speculate that the suppression of eIF4E phosphorylation by Sorafenib drives the selective translation inhibition of some of those "eIF4E-sensitive mRNAs", including cyclin D1, c-Myc and VEGFA. On the other hand, the many mechanisms by which eIF4E can facilitate selective mRNA translation may have diverse consequences on the translation state of its targets.

The components of the eIF4F complex are differentially impaired by Sorafenib.

The levels of Phospho-eIF4E have also been indirectly linked to the levels of the eIF4F complex in a c-Myc-dependent manner. Thus, the eIF4F complex promotes translation of c-Myc, which in turn, regulates the expression of a wide variety of target genes, including eIF4E itself, eIF4A and eIF4G (160). Thus, we hypothesize that downregulated Phospho-eIF4E levels may contribute to the translation inhibition observed upon Sorafenib treatment at long term. Following this idea, we measured the levels of the eIF4F component upon Sorafenib treatment over time. To our surprise, we found that the levels of the three different components of the complex were differentially affected. First, the levels of eIF4A were remarkably reduced at short times upon the treatment while the eIF4G levels were only affected at a long-term. In contrast, the levels of eIF4E were sustained over time, showing that Sorafenib only downregulates its phosphorylation status. These findings suggest that c-Myc-independent mechanisms must be involved in the downregulation of cap-binding complex levels, especially those that affect the RNA helicase eIF4A. It is well known that an imbalance

between the different components of cap-binding complex impairs cap-dependent translation and promotes cap-independent translation of a mRNA subset, including the IRES-mediated ones (79, 178, 192). Therefore, we performed a kinetic study to measure the IRES-dependent translation *versus* the cap-dependent upon a Sorafenib treatment. The result showed changes in the IRES-dependent translation/cap-dependent translation ratio over time. Interestingly, we observed that the translation mediated by IRES of the reporter used was significantly increased after 3 h of Sorafenib treatment but decreased even to lower levels that those found in the control situation at longer period of time. The change in the ratio correlated with the differential dysregulation of eIF4A and eIF4G levels; indeed, sustained levels of eIF4G and decreased eIF4A levels have been associated with an increase of IRES-mediated translation to the detriment of the cap-dependent one. Thus, we could conclude that:

(1) Dysregulation of the cap-binding complex activity by Sorafenib is the result of the combined activity of different mechanisms whose signals converge in the eIF4F complex. One of these mechanisms could come from the downregulation of c-Myc.

(2) The reduced levels of cap-binding complex may also explain the translation inhibition observed upon Sorafenib treatment, together with the induction of phosphorylation of eIF2α.

(3) The differential impairment on the levels of eIF4F components seems to reduce the activity of cap-dependent translation while increase the activity of cap-independent mechanisms such as translation via IRES.

Phospho-eIF4E mediates translation inhibition of specific mRNAs and its overexpression abrogates the cell cycle arrest induced by Sorafenib.

The tumorigenic properties of Phospho-eIF4E have been associated with its role in the selective mRNAs translation of those encoding proteins involved in cell cycle, growth, and invasion (49). In human cells, MNKs are considered to be the main kinases that phosphorylate eIF4E *in vivo* at its serine-209. Their activity is regulated downstream by RAF/RAS/MEK/ERK that results in the phosphorylation of eIF4E. Two different MNK isoforms have been identified, MNK1 and MNK2. Both MNK1 and MNK2 have the ability to phosphorylate eIF4E, however, they differ in their sensitivity to the ERK signalling activity. Whereas MNK1 is highly activated downstream ERK, MNK2 is responsible for basal phosphorylation of eIF4E. In addition, both isoforms suffer alternative splicing, generating MNK1a, MNK1b and MNK2a and MNK2b, respectively (120, 121, 263, 264).

A large body of evidences supports the idea that the MNK1/eIF4E axis is involved in cell transformation and progression (32), mainly through a role in translational control (125, 174). Our data, discussed above, point to Phospho-eIF4E mediating some of the anti-tumorogenic properties of Sorafenib, mainly through selectively inhibiting mRNA translation. To understand the role of Phospho-eIF4E in the cellular response to Sorafenib, we studied the role of MNK1 in response to the Sorafenib treatment. Moreover, we also investigated the effect of impairing the eIF4E phosphorylation by itself during the Sorafenib treatment.

Regarding the role of MNK1, we analysed the contribution of both MNK1 variants, MNK1a and MNK1b in the translation response to Sorafenib. MNK1b is a spliced variant of MNK1a that results in a shorter protein. In addition to their different lengths, the kinase activities of both MNK1 isoforms seem to be differently regulated, as the activity of MNK1a is dependent on ERK whereas that of MNK1b seems to sustain the basal phosphorylated levels of eIF4E (264). Additionally, both MNK1a and MNK1b have been associated with oncogenic cellular transformation, although neither is essential for cell viability (265, 266). In this work, we have overexpressed both MNK1a and MNK1b, and then analysed the impact of such overexpression on cell proliferation. As a result, we observed a lower impact of Sorafenib on cell proliferation in cells overexpressing either MNK1a or MNK1b, although this impact was not entirely significant. The oncogenic properties of MNK1 have been linked to its ability to phosphorylate eIF4E (128, 132). Thus, we also analysed the phosphorylation status of eIF4E upon the Sorafenib treatment in these conditions. Consistently, we observed a tendency in the recovery of eIF4E phosphorylation status after the overexpression of MNK1a but not MNK1b in Sorafenib-treated cells. However, this recovery was neither significant. Thus, we speculated that the inhibitory effect of the MAPK pathway by Sorafenib is much higher than the positive effect of MNK1 overexpression. In fact, it was insufficient to reverse the effects of the drug, thus, resulting on no significant differences on the phosphorylation status of eIF4E. Following this idea, we decided to reanalyse the role of MNK1, but in cells treated with a lower concentration of Sorafenib. Therefore, we treated cells with 1 µM Sorafenib and we measured the phosphorylation levels of eIF4E in normal cells as well as cells overexpressing eitherMNK1 variants. We observed that the Phospho-eIF4E levels were reduced by 20% in normal cells and only the overexpression of MNK1a, but once again not that of MNK1b, was able to suppress the inhibition exerted by Sorafenib. So, we conclude that the overexpression of MNKs is not enough to suppress the anti-proliferative effect of Sorafenib and we suggest that is related with this inability to phosphorylate eIF4E.

Focusing on the role of eIF4E phosphorylation during the Sorafenib response, we evaluated the overexpression of an eIF4E phosphomimetic mutant (eIF4E-S209D) in Sorafenib-treated cells. This

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eIF4E version simulates a constitutive phosphorylated form of eIF4E, independently of the treatment of Sorafenib. As a result, the overexpression of eIF4E-S209D but not that of a wild-type eIF4E version seems to suppress the downregulation of cyclin D1 protein levels following 1 h of Sorafenib treatment. This highlights the influence of Phospho-eIF4E in the translation status of cyclin D1. In contrast, the overexpression of both eIF4E versions caused different effect on the c-Myc protein levels. Thus, while the overexpression of eIF4E-S209D seems to slightly restore the protein levels of c-Myc only after 1 h of Sorafenib, that of wild-type version of eIF4E levels seems to keep them high. Any of them were however able to maintain the levels of c-Myc at long term. These results highlight the complex role of eIF4E and the activity of eIF4F in translational control. Firstly, our findings showed that Sorafenib inhibits the translation of cyclin D1, c-Myc, and VEGFA, which correlated with suppression of eIF4E phosphorylation upon the treatment. However, the overexpression of a constitutive phosphorylated form of eIF4E just had a mild impact on the protein levels of cyclin D1 but not a clear one on c-Myc. Even so, the overexpression of the wild-type version of eIF4E seemed to have a higher effect on c-Myc protein levels. This lack of a clear relationship between c-Myc protein levels and Phospho-eIF4E can be explained by the multiple ways of controlling selectively c-Myc translation. Thus, we conclude that the translation status of Cyclin D1 is strongly linked to the phosphorylation status of eIF4E whereas the translation levels of c-Myc are influenced by Phospho- and total eIF4E but also by other translation factors and/or regulatory events, likely also affected by the treatment.

We and others have reported that Sorafenib suppresses cell proliferation, showing reduced BrdU incorporation in Sorafenib-treated cells compared to control cells (267, 268). Consistently with previous publications, we showed that Sorafenib induced cell cycle arrest in HepG2 cells. In a first instance, we speculated that the Sorafenib-induced cell cycle arrest may be mediated by downregulation of cyclin D1. This protein plays an important role in cell cycle progression, especially in determining the G1/S phase transition. However, our results revealed that Sorafenib induces cell cycle arrest mainly by blocking the transition from S to G2 phase, thus, showing accumulation of cells in the S phase, concomitant with a reduction of cells in the G2 phase. Then, the regulation of cyclin D1 seems not to be responsible for the Sorafenib-induced cell cycle arrest, as its downregulation would delay the G1/S phase transition, causing an accumulation of cell in G1 phase. Unexpectedly, the overexpression of both eIF4E-S209D and wild-type eIF4E suppressed the cell-cycle arrest induced by Sorafenib. Indeed, these cells showed cell cycle profiles similar to those of control cells. We conclude that Sorafenib induce a cell-cycle arrest that occurs in a cyclin D1-independent manner.

Taken together, we propose a model that shows the relevance of the translation machinery on the anti-tumorigenic properties of Sorafenib. On the one hand, we showed that Sorafenib impairs the phosphorylation of eIF4E. This leads to the selective translation inhibition of pro-tumoral mRNAs like cyclin D1. Fairly later, Sorafenib negatively affect the accumulation of eIF4A, which might surely affect the assembly of the eIF4F complex. Then, the reduced levels of eIF4F complex might likely contribute to a global translation inhibition, which is also reinforced by PERK-induced phosphorylation of eIF2α (**Figure 1**). The overexpression of either a phosphomimetic eIF4E-S209D or a wild-type eIF4E factor is able to suppress the cell cycle arrest induced by Sorafenib, perhaps as the result of the partial reconstitution of eIF4F complex.

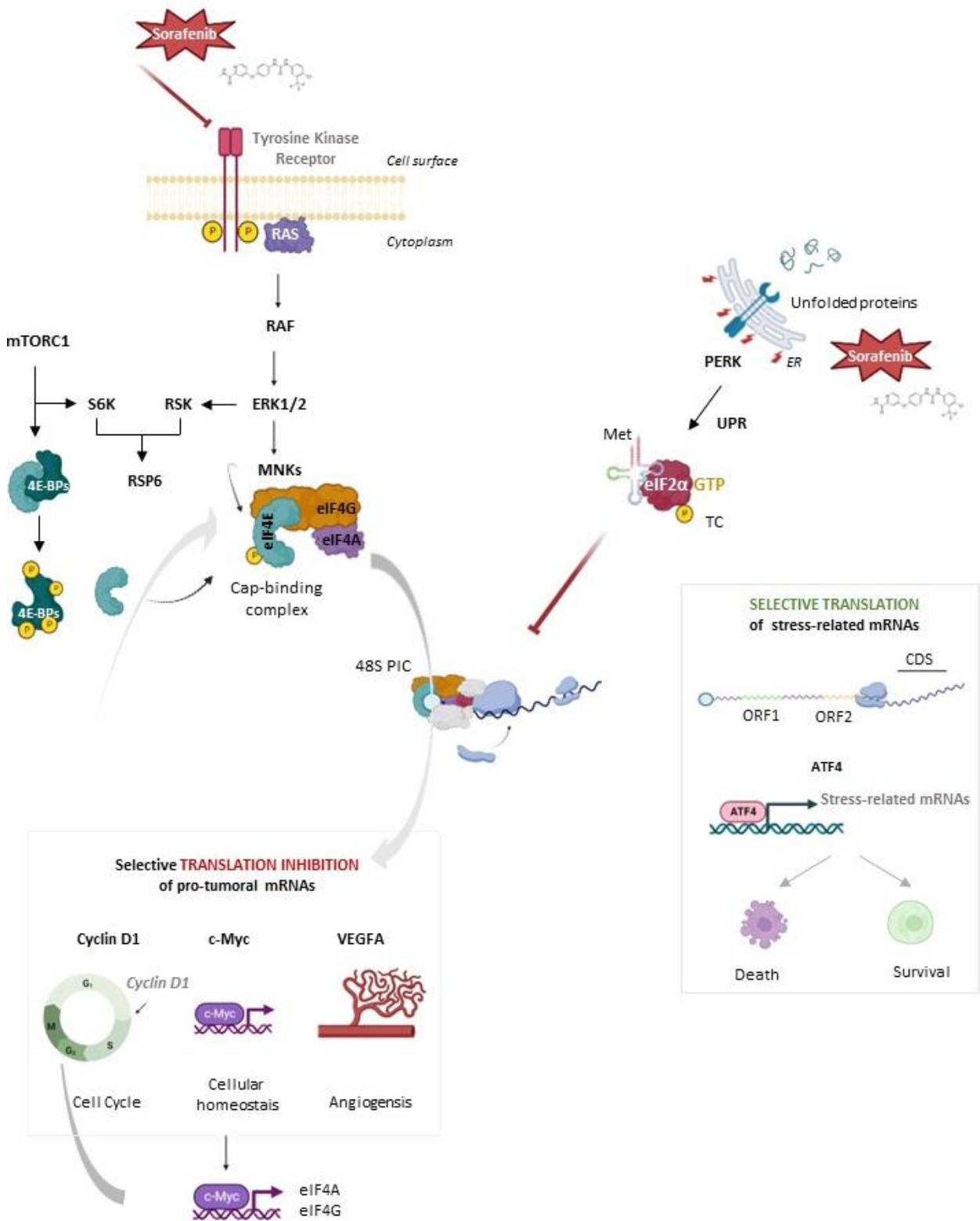**Figure 1. Sorafenib hierarchically affects different signalling pathways controlling protein synthesis, which leads to dysregulated activity of the translation machinery.** The image depicts the signalling pathways whose signals converge into the translational apparatus and are dysregulated in Sorafenib treated cells. Sorafenib inhibits tyrosine kinase receptors, switching off the MAPKs pathway.

Downregulated activity of MAPKs causes a reduction in the phosphorylation levels of eIF4E, resulting in the selective translation inhibition of a subset of mRNAs involved in cell cycle progression, cellular homeostasis, and angiogenesis. In parallel, Sorafenib induces ER stress, triggering the UPR. PERK-induced eIF2α phosphorylation inhibits overall translation and stimulates translation of a subset of mRNAs such as ATF4. The phosphorylation levels of eIF2 over time determine the cell fate: death or survival. Additionally, Sorafenib could break the feedforward loop that links c-Myc and the cap-binding complex, contributing to the translation inhibition exerted by Sorafenib over the long term. The image also represents how the translational apparatus can serve as a unifying factor that could encourage the aberrant activation of the mTOR pathway as a consequence of inhibition of MAPKs.

The selective translational control allows cells to quickly respond to environmental stress

The aberrant assembly of eIF4F complex as well as the phosphorylation of eIF2α, rapidly modulate the mRNA translation. Firstly, they cause a selective inhibition of a specific subset of mRNAs that eventually drives a global repression of protein synthesis. Secondly, this is often accompanied by selective mRNA translation of mRNAs encoding proteins essential for cell survival and stress recovery (27, 28, 43, 79). Thus, we considered that deciphering the mechanisms underlying the Sorafenib-induced translational reprogramming may provide us with a better understanding of how Sorafenib works and ultimately lead to the development of novel therapeutic strategies for HCC.

So far, the analyses aimed at understanding how Sorafenib works have been based on transcriptomic analysis by mRNA-seq and microarray. Both of them only provide information about the mRNA levels that, although useful information, does not always correspond with the final protein concentration (24, 268). The translational analysis shown here completes previous studies on the molecular mechanism of action of Sorafenib and provides a more accurate view of the Sorafenib-driven cellular context.

The translational study carried out in this project is based on the analysis of the mRNA distribution along the polysome profile, assuming that those mRNAs with ribosomes bound (present in the polysomes fraction) are being translated. On the one hand, we measured the amount of mRNA in the fraction corresponding to low-translated fraction (mRNAs with no ribosome bound or just one; V1) and those corresponding to highly-translated fraction (polysomes fraction; V2). Later, we calculated the logarithm of the V2/V1 ratio for control cells and Sorafenib-treated cells. Finally, we established that those mRNAs with negative ratio correspond to those that are poorly translated, whereas those with a positive ratio correspond to actively translated mRNAs. On the other hand, we compared the V2/V1 ratio obtained for control and Sorafenib-treated cells to get an idea of how their translation change after the treatment. Applying this approach, we described eight populations of genes. Firstly, we identified those corresponding to (i) mRNAs actively translated in physiological

conditions (untreated cells); (ii) mRNAs actively translated in Sorafenib-treated cells; (iii) mRNAs poorly translated in physiological conditions; and (iv) mRNAs poorly translated in Sorafenib-treated cells. Secondly, we combined the previous data to identify (v) those mRNAs actively translated in both conditions simultaneously, meaning that their translation is maintained upon Sorafenib treatment; (vi) those mRNAs poorly translated in both conditions simultaneously; (vii) those mRNAs actively translated in physiological conditions but poorly translated in Sorafenib-treated cells, meaning that their translation is inhibited upon the treatment; and finally (viii) those mRNAs poorly translated in physiological conditions but whose translation is promoted by Sorafenib.

In a first approach, we observed that the number of genes significantly actively translated in Sorafenib-treated cells was notably lower (2593) than those significantly poorly translated (5584). In contrast, we observed that the number of genes significantly actively (2950) and poorly (3237) translated was similar in control conditions, although the number of genes significantly poorly translated was much lower compared to treated cells. This result was consistent with the Sorafenib-induced protein synthesis repression, showing a much higher number of poorly translated genes following the treatment even after properly normalizing the data.

The Sorafenib-induced cellular stress triggers mechanisms controlling translation thereby dynamically reprograming the types of mRNAs that are translated and driving cellular adaptation. Here, we have reported that the translational reprograming induced by Sorafenib is mediated in part by the activated signals downstream ER stress. Thus, in parallel to the increased levels of Phospho-eIF2α, our data also showed an enrichment in uORFs-containing mRNA in the gene set corresponding to actively translated mRNAs in Sorafenib-treated cells. Additionally, we also observed that those mRNAs identified as target of the RNA binding protein (RBP) CPEB4, whose activity is also downstream increased downstream ER stress (213), were enriched in this same gene set. Curiously, we found an enrichment of both uORFs-containing mRNAs as well as CPEB4 target genes in those mRNAs actively translated in both conditions simultaneously. Thereby, we speculated that either upstream ORFs located into the 5' UTR and the CPE element in the 3' UTR recognized by CPEB4 could allow translation of subset of mRNA, which are translated in control cells, following the treatment. These data are concomitant with the evidence pointing out that Sorafenib induces ER stress and inhibits translation mainly by PERK-induced eIF2α, at least at short-term. Additionally, we also detected that mRNAs actively translated following the treatment were enriched in AU-containing mRNAs, which apparently have a similar behaviour that uORFs-containing mRNAs. This element is located into the 3' UTR and is recognized and bound by several RBPs, stabilizing, and mediating its translation in response to stress (216-218, 220). Curiously, some of these AU-containing mRNAs are

c-Myc and Mcl-1, whose translation has been strongly linked to Phospho-eIF4E. This finding supports our hypothesis: the different effects of Sorafenib on the translational status of different mRNAs can be explained by the multiple mechanisms that can control their translation and their importance of each one of them in response to Sorafenib-induced stress.

Interestingly, our finding revealed that Sorafenib also impairs the activity and assembly of eIF4F complex, which eventually could contribute, together with Phospho-eIF2α, to a repression of overall translation. Many reports support the fact that the altered activity of eIF4F complex often stimulates the activity of alternative cap-binding complexes or even those that mediate cap-independent translation (27, 28, 203). Consistently with this, we found that mRNAs, whose translation have been linked to the activity of alternative cap-binding complex formed by DAP5, were enriched in those gene actively translated following the Sorafenib treatment. DAP5, a factor unable to interact with eIF4E, mediates the cap-dependent translation by forming an alternative cap-binding complex (208). Interestingly, we observed that DAP5-target mRNAs were enriched in the gene set corresponding to those mRNA poorly translated in control conditions but were actively translated following the treatment, as well as those that were actively translated in control conditions but whose translation was inhibited in Sorafenib treated cells. This finding clearly indicates that Sorafenib inhibits the translation of a specific set of DAP5-target mRNAs but additionally promotes the translation of another one. In this regard, it is interesting to note that DAP5 mediates translation of capped mRNAs by forming a complex with the translation factor eIF3 but also, it has been recently reported that enhances translation of the main ORF of those uORFs containing-mRNAs with long and unstructured 5' UTR, assisted by eIF4A (269). Therefore, we speculate that the opposite behaviour with respect to translation of those DAP5-target mRNAs is due to the different mechanisms by which DAP5 is controlling their translation.

The role of DAP5 in translation was firstly linked to IRES-mediated translation, a structure located into the 5' UTR which allow the cap-independent translation when cap-dependent translation is impaired. Indeed, the tumorigenic activity of DAP5 was firstly associated with its role in controlling the IRES-mediated translation, since this structure has been identified in mRNAs encoding protumoral proteins, including the anti-apoptotic proteins Bcl-2 or the proto-oncogene c-Myc (164, 192, 270). However, it has been recently reported that DAP5 mediate translation of different subset of capped mRNAs which do not contain IRES element and could also determine the cell fate (191). In fact, our finding reveals that the behaviour of IRES-containing mRNAs was similar to mRNAs as DAP5-target genes, although with a much lower p value. However, only the 16% percentage of those IRES-containing mRNAs were identified as target of DAP5, suggesting that a different set of translation

factors mediate their IRES-dependent translation. Additionally, our finding also revealed that the role of IRES-mediated translation is likely much relevant in the Sorafenib-induced translational reprograming at earlier time of treatment (3 h), where the ratio of IRES-dependent translation versus cap-dependent translation is significantly higher following the treatment.

There are different mechanisms that control selective mRNA translation that are likely employed by the cells in response to the sorafenib-induced stress. Here we have reported some of them, including uORFs, IRES, regulatory elements located into the 3' UTR recognized by RBPs and alternative cap-dependent complexes like those formed by DAP5. Surprisingly, looking for new characteristics that could be involved in translational controls, we found that Sorafenib more selectively affects the shorter genes whereas the translation of the longer ones seems to be more resistant to the inhibitory effect of Sorafenib. As a result, we observed a clear correlation between the length of the CDS and the translation status. Thus, following the Sorafenib treatment, the translation of shorter, mRNAs which are actively translated in control conditions, are clearly affected than the longer one. Unexpectedly, our results allowed us to identify a gene population that we called "hypersensitive to Sorafenib" which showed a translation negatively affected by the treatment, independently their CDS length, breaking the overall tendency.

Combined Sorafenib treatment with mRNA demethylase could make more sensitive to the drug

Finding the mechanism underlying this selective translation inhibition would provide us with a better understanding of the mechanism of action of Sorafenib, which eventually could be used to enhance the drug's effect. Thus, focusing on the common features present in these hypersensitive mRNAs, we found that this gene population was enriched in genes with no m^{6}A modification in their sequence. The m^{6}A modification is the most common epitranscriptomic modification on mammalians mRNAs, which is involved in different cellular aspects, including protein synthesis (230, 234). According to the high prevalence of this mRNA modification, we found that the majority of mRNAs in our analysis contained at least one m^{6}A modification. In addition, the m^{6}A methylated mRNAs showed a clear correlation between their length of CDS and their translation status. Interestingly, we found that the translation of the small percentage of non-methylated mRNAs (approximately 3%) was strongly inhibited, regardless of their CDS length. Thus, we thought that the mRNA methylation grade could be influencing the translational behaviour of mRNAs in response to Sorafenib.

The m^{6}A methylation status of mRNA changes dynamically depending on the cellular physiology as well as the environmental changes (230). This is determined by the activity of methylases and demethylases, which work in parallel to modulate the methylation pattern of m^{6}A which influences in different aspects of the mRNA, including its translation (240, 271-273). Various mechanisms have been proposed to explain the role of m^{6}A modification in translation, however, they are not fully understood. A large body of evidence points out an important role for the mRNA methylase METTL3 in translation control. Indeed, the activity of METTL3 has been linked to the translation of selective group of mRNAs encoding proteins with pro-tumoral activities and, interestingly, this seems to occur independently on its methylase activity (238, 239, 242). At this point we wondered whether inhibiting the activity of METTL3 could reduce the amount of m^{6}A methylated mRNAs such that, according to our hypothesis, this could make their translation more sensitive to the inhibitory effect of Sorafenib. Following this idea, firstly we investigated the translation status of those mRNAs identified as METTL3 targets. To do so, we used different databases that reported on METTL3 targets (239, 242). Surprisingly, we found that those mRNAs with a positive translation rate in Sorafenib-treated cells were enriched in METTL3 targets. Thus, we conclude that the activity of METTL3 seems to remain unaffected by the treatment based on: (i) the m^{6}A methylated mRNAs correspond with METTL3 targets; and (ii) those mRNAs with a positive translation ratio (meaning that they are actively translated following the treatment) were enriched in METTL3 target genes. Therefore, we speculated that inhibiting METTL3 could result in a high amount of non-methylated mRNAs which in turn would make their translation more susceptible to Sorafenib. However, it must be noted that METTL3 plays a role in translation independently on its methylase activity. Thereby, further investigation would be essential to figure out the impact of inhibiting METTL3 on the translation of a selective group of mRNAs since the depletion of METTL3 seems to enhance the resistance to Sorafenib *in vivo* assays, which has been associated with selective translation mRNAs allowing cell survival (274).

Based on our result, we propose that the induction of mRNA demethylation could represent an interesting therapeutic approach by enhancing the translation-related inhibitory effect of Sorafenib in HCC. Additionally, it has recently been reported that that HCC patients harbour a distinct m^{6}A regulator modification pattern, contributing to the heterogeneity and diversity of HCC (275, 276). Thus, evaluating m^{6}A gene signature and stablishing a m^{6}A score for individual tumours could provide us with a new strategy for prognosis prediction and treatment response.

Sorafenib-driven cellular context through the translational reprograming

To elucidate the cellular context driven by the Sorafenib-induced translational reprograming, we applied an enrichment analysis using REACTOME database. In agreement with previous studies reporting that Sorafenib impairs mitochondrial activity (26, 268, 277), we found that categories associated with the electron transport chain were negatively affected with a high significant *q-value*. In contrast, these categories were found significantly enriched in actively translated genes in control cells, implying that this process is actively working in optimal conditions but is negatively dysregulated following the treatment from a translational point of view. Thereby, our findings suggest that Sorafenib could also impair the mitochondrial activity through the translation inhibition of different components working in the electron transport chains. Additionally, we also detected that those categories associated with collagen biosynthesis were also significantly enriched in genes poorly translated. Collagen is the main component of the extracellular matrix (ECM), and its remodelling influences different aspects of cancer, including angiogenesis and migration (278-280). Indeed, enzymes involved in collagen processing have been found to be increased in a variety of cancers, including HCC. As Sorafenib seems to negatively affect collagen biosynthesis, we speculate that this could contribute to the anti-tumorigenic activities of Sorafenib since this process has been proposed as a possible therapeutic target (281, 282). Furthermore, the variety of collagens is valuable as a biomarker in the diagnosis (283). Thus, the generated fragment of collagen forms by impaired the collagen biosynthesis could be detected in peripheral blood and with further investigation could be used as a predictor of treatment response like is used for other cancers (284, 285).

In parallel, the translational reprogramming also leads to the activation of cellular processes that, in combination with the downregulated processes, determine the cell fate. Regarding the upregulated categories, we differentiated among those that were significantly enriched in actively translated genes in Sorafenib-treated cells, but (i) they were also significantly enriched in control conditions, including those categories associated with cell cycle, membrane trafficking, and post-translational protein modifications (ii) those that were significantly enriched in actively translated genes but much more significantly enriched in poorly translated mRNAs in control conditions. Among these, we found categories associated with RNA metabolism. In contrast to the ones above mentioned, although these categories worked in control conditions, the translation of a higher number of genes involved in them was inhibited following the treatment that likely resulted in switching their working sense; and (iii) those categories were not significantly enriched in actively translated genes in control conditions, including signalling by Rho GTPases, Transport of Mature

transcript to Cytoplasm and SUMOylation. This last one corresponds with those categories that were activated in response to the treatment.

We then wondered whether some of the features mentioned above had driven the translation of these mRNAs. As first approach, we looked for these characteristics in the mRNAs corresponding to the second and third groups of categories. As a result, we found a high number of DAP5 target mRNAs, ARE-containing mRNAs, and a lesser number of uORFs-containing mRNAs. The number of DAP5-target genes seems to be important especially in those categories linked to mRNA metabolism. However, the number of ARE-containing mRNAs raise notably in Signalling by Rho GTPase, transport mature transcript to cytoplasm and SUMOylation, though can be still observe a high number of DAP5 target genes. Then, we raised the question: How determinant is the presence of these characteristics in the mRNA for their translation? In order to answer this question, we focused on the single category significantly enriched in actively translated genes in Sorafenib treated cells and significantly enriched in poorly translated in control conditions, implying that this process is especially triggered in response to the treatment. We firstly compared the fold change of translation ratio between those mRNAs identified as DAP5-target genes *versus* non DAP5-target genes, those ARE-containing mRNAs *versus* those that do not and those uORFs-containing mRNAs and those that do not. As a result, we observed that the translation of those mRNAs identified as DAP5 target genes as well as those containing an ARE element in their structure was much more increased compared with their respective groups. Secondly, we observed that those mRNAs with a negative translation ratio in control conditions and a positive one in Sorafenib-treated cells were identified as DAP5-target genes or ARE-containing-mRNAs. We conclude that the presence of different features into the mRNAs could influence into their translational behaviour in response to Sorafenib (**Figure 2**). However, it must be highlighted that, likely, it is the combination of different features as well as the proteins involved in their recognition that ultimately determine the translation status of each mRNA.
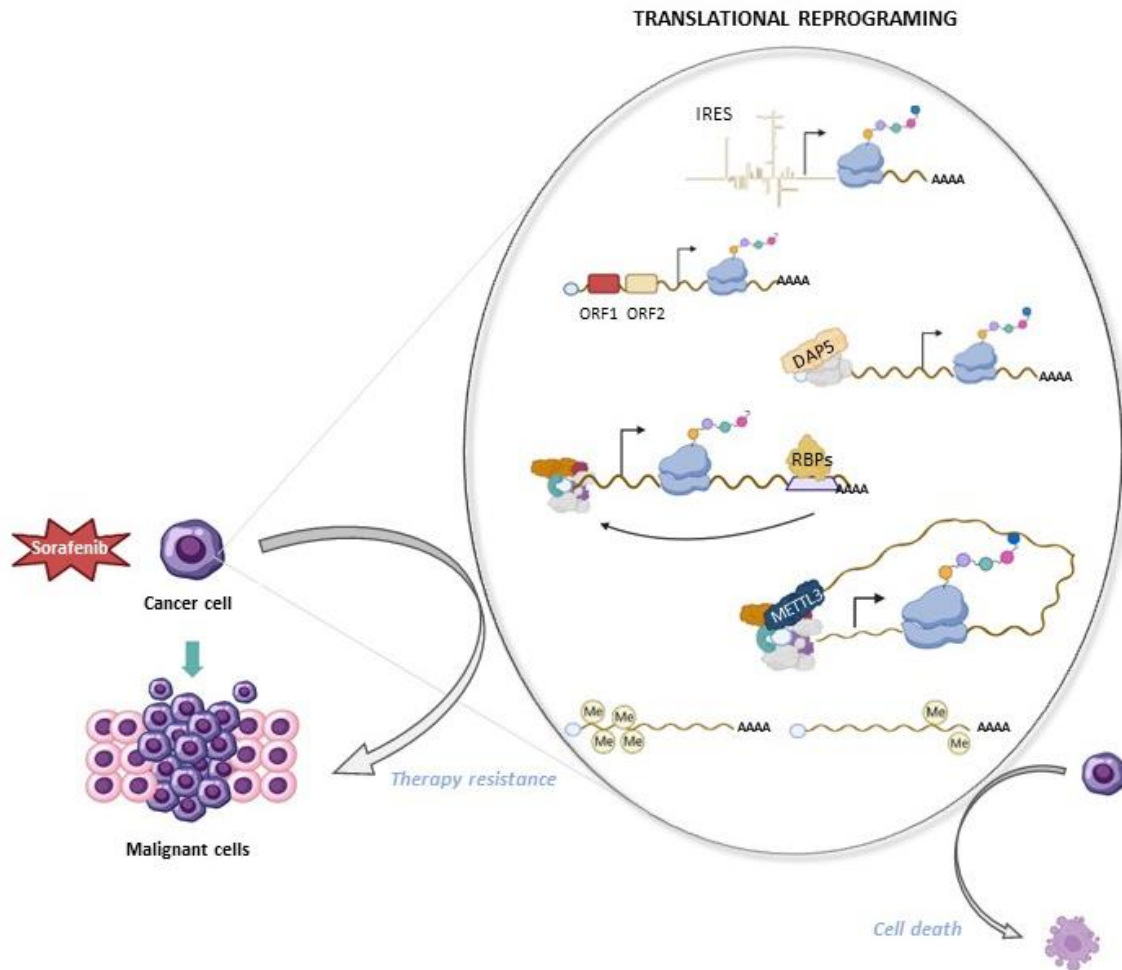
Figure 2. Sorafenib-induced translational reprograming. The image depicts the molecular consequences of the effect of Sorafenib on the translation process. Sorafenib impairs the protein synthesis production, depriving cell all they materials needed to growth and proliferate. In parallel, alternative translation mechanisms allow translation of different subset of mRNAs in response to the Sorafenib-induced stress. The mRNA methylation degree and the position of these methylation also influence in the translational behavior of mRNAs. The balance between the different mechanisms controlling translation influence in the final cellular fate: death or survival.

CONCLUSIONS

1. Sorafenib strongly inhibits translation in different HCC cell lines models mainly at the initiation stage and blocks the progression of the cell cycle.

2. Sorafenib hierarchically affects different mechanisms controlling protein synthesis, which drive the inhibition of translation The PERK-induced eIF2α phosphorylation is particularly significant. Additionally, it promotes translation by alternative cap-dependent and cap-independent mechanisms.

3. Sorafenib switched off the MAPKs pathways, causing a reduction in the phosphorylation levels of the cap-binding protein eIF4E. This reduction is accompanied by a reduced translation of a specific set of mRNAs, encoding pro-tumoral proteins such as Cyclin D1, c-Myc and VEGFA.

4. Sorafenib also negatively affects the protein levels of the other two components of the cap-binding complex, the RNA helicase eIF4A and the scaffolding protein eIF4G.

5. The overexpression of a constitutively active isoform of eIF4E (eIF4E-S209D) demonstrates that the translation inhibition of Cyclin D1 is strongly linked to the phosphorylated levels of eIF4E. The overexpression of eIF4E-S209D but not of a wild-type eIF4E isoform (eIF4E-WT) restores the protein levels of Cyclin D1 in Sorafenib-treated cells. Overexpression of both eIF4E isoforms suppresses the Sorafenib-induced cell cycle arrest, which seems to be independent on Cyclin D1. The relationship between eIF4E and the translation status of c-Myc is not as clear, suggesting that c-Myc protein level regulation is subjected to more than one mechanism.

6. The overexpression of MNK1 is not enough to suppress the inhibitory effect of Sorafenib on the MAPKs pathway, likely because this overexpression does not restore the phosphorylation levels of eIF4E in Sorafenib-treated cells.

7. Sorafenib leads to a translation reprograming, affecting differentially the translation of a specific mRNA subset. There exists a strong correlation between the CDS length and the mRNA translation status, with the translation of longer ones showing more resistance to the inhibitory effect of Sorafenib.he length and the presence of AU-rich sequences within the mRNAs are also important elements mediating such reprograming.

8. We have identified a set of genes that we named "hypersensitive genes to Sorafenib" as those strongly inhibited by Sorafenib despite their length. This set is significantly enriched in m^6A non-methylated genes.

9. The Sorafenib-induced translational reprograming enhances the translation of mRNAs associated with mRNA export from the nucleus to the cytoplasm, signalling by Rho-GTPases and protein SUMOylation. In contrast, the mitochondrial function-related categories and those related to collagen biosynthesis are enriched among the poorly translated genes.

FUTURE PERSPECTIVES

HCC constitutes a serious health problem worldwide. Developing new and truly efficacious strategies for treating patients in advanced stages of HCC is an absolutely required therapeutic challenge. Currently, Sorafenib remains the best option for an important proportion of advanced HCC patients. However, this drug has limited clinical benefits due to several side effects as well as the often appearance of resistance to the treatment, which has been linked to its high non-specificity. Thus, studies aimed at understanding the molecular action of the Sorafenib treatment and the underlying mechanisms of therapy resistance are key to improve and increase the therapeutic options for these patients. In this line, in this Thesis work, we highlight the role of translation in the cellular response to Sorafenib and we propose that some of its properties could be explained, at least in part, by its impairment on the translation process.

Obviously, many questions remain to be answered, and further steps in this direction are obligated. Even so, the results shown here point to the possibility that the regulation of protein synthesis could be an interesting therapeutic option. Thereby, we propose that targeting against the cap-binding complex activity could inhibit the translation of pro-tumoral mRNAs, including VEGFA, one of the main targets used to design HCC therapies. This could be really interesting, as a high translation rate is a homogeneous characteristic along tumours, overcoming the intratumoral heterogeneity issue. Additionally, it has been reported that HCC patients show a high expression of phosphorylated eIF4E, which also show a homogeneous distribution into the tumour, supporting our suggestion.

However, it must be noted that the translation status of the different mRNAs is ultimately determined by the combined activity of multiple mechanisms controlling translation. Thus, different subsets of mRNAs are translated based on the harmony exerted between the various systems involved in translational control, which ultimately determine the cell fate: death or survival. Thus, we propose that investigating and designing a translational profile for HCC patients could provide us with better tools to design and improve their therapy.

Finally, we found that the m^6A methylation grade of mRNAs could influence the inhibitory effect of Sorafenib on translation. Thus, analysing the m^6A methylation pattern in patients responding to Sorafenib treatment *versus* those that do not, could give us clues about the relevance of this modification during the therapy. Indeed, our next aims are focused on the role of m^6A methylation in the Sorafenib-response. Thus, our future experiments are intended at testing whether inducing m^6A mRNA demethylation could make cells from HCC cell lines more sensitive to the Sorafenib-treatment.

BIBLIOGRAPHY

1. J. M. Llovet *et al.*, Hepatocellular carcinoma. *Nature Reviews Disease Primers* **7** (2021).

2. L. Rahib *et al.*, Projecting cancer incidence and deaths to 2030: the unexpected burden of thyroid, liver, and pancreas cancers in the United States. *Cancer Res* **74**, 2913-2921 (2014).

3. C. Global Burden of Disease Liver Cancer *et al.*, The Burden of Primary Liver Cancer and Underlying Etiologies From 1990 to 2015 at the Global, Regional, and National Level: Results From the Global Burden of Disease Study 2015. *JAMA Oncol* **3**, 1683-1691 (2017).

4. T. M. Arndt Vogel, Gonzalo Sapisochin, Riad Salem, Anna Saborowski, Hepatocellular carcinoma. *Lancet* **400**, 1345-1362 (2022).

5. K. Schulze *et al.*, Exome sequencing of hepatocellular carcinomas identifies new mutational signatures and potential therapeutic targets. *Nat Genet* **47**, 505-511 (2015).

6. w. b. e. Cancer Genome Atlas Research Network. Electronic address, N. Cancer Genome Atlas Research, Comprehensive and Integrative Genomic Characterization of Hepatocellular Carcinoma. *Cell* **169**, 1327-1341 e1323 (2017).

7. J. Liu *et al.*, Wnt/beta-catenin signalling: function, biological mechanisms, and therapeutic opportunities. *Signal Transduct Target Ther* **7**, 3 (2022).

8. E. Kim *et al.*, Promotion of growth factor signaling as a critical function of beta-catenin during HCC progression. *Nat Commun* **10**, 1909 (2019).

9. J. M. Llovet, R. Montal, D. Sia, R. S. Finn, Molecular therapies and precision medicine for hepatocellular carcinoma. *Nat Rev Clin Oncol* **15**, 599-616 (2018).

10. J. M. Llovet *et al.*, Molecular pathogenesis and systemic therapies for hepatocellular carcinoma. *Nat Cancer* **3**, 386-401 (2022).

11. e. e. e. European Association for the Study of the Liver. Electronic address, L. European Association for the Study of the, EASL Clinical Practice Guidelines: Management of hepatocellular carcinoma. *J Hepatol* **69**, 182-236 (2018).

12. M. Reig *et al.*, BCLC strategy for prognosis prediction and treatment recommendation: The 2022 update. *J Hepatol* **76**, 681-693 (2022).

13. T. T. Ghaziani, R. Dhanasekaran, Recent Progress in Systemic Therapy for Hepatocellular Cancer (HCC). *Curr Treat Options Gastroenterol* **19**, 351-368 (2021).

14. J. M. Llovet, J. Bruix, Molecular targeted therapies in hepatocellular carcinoma. *Hepatology* **48**, 1312-1327 (2008).

15. M. D. Josep M. Llovet, Sergio Ricci, M.D., Vincenzo Mazzaferro, M.D., *et al.*, Sorafenib in Advanced Hepatocellular carcinoma. *New England Journal of Medicine* (2008).

16. A. L. Cheng *et al.*, Updated efficacy and safety data from IMbrave150: Atezolizumab plus bevacizumab vs. sorafenib for unresectable hepatocellular carcinoma. *J Hepatol* **76**, 862-873 (2022).

17. M. Arndt Vogel *et al.*, Lenvatinib versus sorafenib for first-line treatment of unresectable hepatocellular carcinoma: patient-reported outcomes from a randomised, open-label, non-inferiority, phase 3 trial. *Lancet* https://doi.org/10.1016/S2468-1253(21)00110-2 (2021).

18. R. S. Finn *et al.*, Atezolizumab plus Bevacizumab in Unresectable Hepatocellular Carcinoma. *N Engl J Med* **382**, 1894-1905 (2020).

19. S. M. Wilhelm *et al.*, BAY 43-9006 exhibits broad spectrum oral antitumor activity and targets the RAF/MEK/ERK pathway and receptor tyrosine kinases involved in tumor progression and angiogenesis. *Cancer Res* **64**, 7099-7109 (2004).

20. A. L. Cheng *et al.*, Efficacy and safety of sorafenib in patients in the Asia-Pacific region with advanced hepatocellular carcinoma: a phase III randomised, double-blind, placebo-controlled trial. *Lancet Oncol* **10**, 25-34 (2009).

21. A.-L. C. Masatoshi Kudo, Joong-Won Park, Jae Hyung Park, Po-Chin Liang, Hisashi Hidaka , Namiki Izumi, Jeong Heo, Youn Jae Lee, I-Shyan Sheen, Chang-Fang Chiu, Hitoshi Arioka, Satoshi Morita , Yasuaki Arai M, Orantinib versus placebo combined with transcatheter arterial chemoembolisation in patients with unresectable hepatocellular carcinoma (ORIENTAL): a randomised, double-blind, placebo-controlled, multicentre, phase 3 study. *Lancet* (2018).

22. T. M. Ghassan K. Abou-Alfa, Ann-Lii Cheng, Anthony B. El-Khoueiry, Lorenza Rimassa, Baek-Yeol Ryoo, Irfan Cicin, Philippe Merle, YenHsun Chen, Joong-Won Park, Jean-Frederic Blanc, Luigi Bolondi, Heinz-Josef Klümpen, Stephen L. Chan, Vittorina Zagon, Tiziana Pressiani, Min-Hee Ryu, Alan P. Venook, Colin Hessel, Anne E. Borgman-Hagey, Gisela Schwab, and Robin K. Kelley., Cabozantinib in Patients with Advanced and Progressing Hepatocellular Carcinoma. *The New England Journal of Medicine* (2018).

23. J. Bruix *et al.*, Regorafenib for patients with hepatocellular carcinoma who progressed on sorafenib treatment (RESORCE): a randomised, double-blind, placebo-controlled, phase 3 trial. *Lancet* **389**, 56-66 (2017).

24. M. Cervello *et al.*, Molecular mechanisms of sorafenib action in liver cancer cells. *Cell Cycle* **11**, 2843-2855 (2012).

25. L. Liu *et al.*, Sorafenib blocks the RAF/MEK/ERK pathway, inhibits tumor angiogenesis, and induces tumor cell apoptosis in hepatocellular carcinoma model PLC/PRF/5. *Cancer Res* **66**, 11851-11858 (2006).

26. M. A. Rodríguez-Hernández *et al.*, Dose-dependent regulation of mitochondrial function and cell death pathway by sorafenib in liver cancer cells. *Biochem Pharmacol* **176**, 113902 (2020).

27. M. Bhat *et al.*, Targeting the translation machinery in cancer. *Nat Rev Drug Discov* **14**, 261-278 (2015).

28. D. K.-O. Joanna R Kovalski, Davide Ruggero, Protein synthesis control in cancer:selectivity and therapeutic targeting. *EMBO J* (2022).

29. M. M. F Loreni, S Biffo, Translation factors and ribosomal proteins control tumor onset and progression: how? *Oncogene* (2014).

30. M. C. Andrew C Hsieh, Ornella Zollo, Cole Davis, Morris E Feldman, Joseph R Testa, Oded Meyuhas, Kevan M Shokat, Davide Ruggero, Genetic Dissection of the Oncogenic mTOR Pathway Reveals Druggable Addiction to Translational COntrol via 4EBP-eIF4E. *Cancer Cell* (2010).

31. M. Dimri, A. Satyanarayana, Molecular Signaling Pathways and Therapeutic Targets in Hepatocellular Carcinoma. *Cancers (Basel)* **12** (2020).

32. L. Furic *et al.*, eIF4E phosphorylation promotes tumorigenesis and is associated with prostate cancer progression. *Proceedings of the National Academy of Sciences* **107**, 14134-14139 (2010).

33. S. Lee, J. Rauch, W. Kolch, Targeting MAPK Signaling in Cancer: Mechanisms of Drug Resistance and Sensitivity. *International Journal of Molecular Sciences* **21**, 1102 (2020).

34. M. Sinkala, P. Nkhoma, N. Mulder, D. P. Martin, Integrated molecular characterisation of the MAPK pathways in human cancers reveals pharmacologically vulnerable mutations and gene dependencies. *Communications Biology* **4** (2021).

35. R. Liu *et al.*, PI3K/AKT pathway as a key link modulates the multidrug resistance of cancers. *Cell Death & Disease* **11** (2020).

36. D. Hanahan, Robert, Hallmarks of Cancer: The Next Generation. *Cell* **144**, 646-674 (2011).

37. P. H. D. Nguyen *et al.*, Intratumoural immune heterogeneity as a hallmark of tumour evolution and progression in hepatocellular carcinoma. *Nature Communications* **12** (2021).

38. Q. Zhang, Y. Lou, X.-L. Bai, T.-B. Liang, Intratumoral heterogeneity of hepatocellular carcinoma: From single-cell to population-based studies. *World Journal of Gastroenterology* **26**, 3720-3736 (2020).

39. S. Ramon Y Cajal *et al.*, Beyond molecular tumor heterogeneity: protein synthesis takes control. *Oncogene* **37**, 2490-2501 (2018).

40. A. E. Koromilas, S. Roy, G. N. Barber, M. G. Katze, N. Sonenberg, Malignant transformation by a mutant of the IFN-inducible dsRNA-dependent protein kinase. *Science* **257**, 1685-1689 (1992).

41. N. N. Nupponen *et al.*, Amplification and Overexpression of p40 Subunit of Eukaryotic Translation Initiation Factor 3 in Breast and Prostate Cancer. *The American Journal of Pathology* **154**, 1777-1783 (1999).

42. D. R. Scoles, W. H. Yong, Y. Qin, K. Wawrowsky, S. M. Pulst, Schwannomin inhibits tumorigenesis through direct interaction with the eukaryotic initiation factor subunit c (eIF3c). *Hum Mol Genet* **15**, 1059-1070 (2006).

43. L. Fabbri, A. Chakraborty, C. Robert, S. Vagner, The plasticity of mRNA translation during cancer progression and therapy resistance. *Nature Reviews Cancer* **21**, 558-577 (2021).

44. L. J. Lee *et al.*, Cancer Plasticity: The Role of mRNA Translation. *Trends in Cancer* **7**, 134-145 (2021).

45. L. Maese, R. E. Rau, Current Use of Asparaginase in Acute Lymphoblastic Leukemia/Lymphoblastic Lymphoma. *Frontiers in Pediatrics* **10** (2022).

46. A. Yanagiya *et al.*, Translational Homeostasis via the mRNA Cap-Binding Protein, eIF4E. *Molecular Cell* **46**, 847-858 (2012).

47. I. Topisirovic, M. Ruiz-Gutierrez, K. L. Borden, Phosphorylation of the Eukaryotic Translation Initiation Factor eIF4E Contributes to Its Transformation and mRNA Transport Activities. *Cancer Res* **64**, 23 (2004).

48. Morgan *et al.*, Differential Requirements for eIF4E Dose in Normal Development and Cancer. *Cell* **162**, 59-71 (2015).

49. H.-G. Wendel *et al.*, Dissecting eIF4E action in tumorigenesis. *Genes & Development* **21**, 000.002-000 (2007).

50. P. A. Geter *et al.*, Hyperactive mTOR and MNK1 phosphorylation of eIF4E confer tamoxifen resistance and estrogen independence through selective mRNA translation reprogramming. *Genes & Development* **31**, 2235-2249 (2017).

51. F. Gebauer, M. W. Hentze, Molecular mechanisms of translational control. *Nature Reviews Molecular Cell Biology* **5**, 827-835 (2004).

52. T. V. Pestova *et al.*, Molecular mechanisms of translation initiation in eukaryotes. *Proceedings of the National Academy of Sciences* **98**, 7029-7036 (2001).

53. L. M. Dedkova, S. M. Hecht, Expanding the Scope of Protein Synthesis Using Modified Ribosomes. *Journal of the American Chemical Society* **141**, 6430-6447 (2019).

54. T. A. Thanaraj, P. Argos, Ribosome-mediated translational pause and protein domain organization. *Protein Science* **5**, 1594-1612 (1996).

55. V. Ramakrishnan, Ribosome Structure and the Mechanism of Translation. *Cell* **108**, 557-572 (2002).

56. A. P. Schuller, R. Green, Roadblocks and resolutions in eukaryotic translation. *Nature Reviews Molecular Cell Biology* **19**, 526-541 (2018).

57. M. Sokabe, C. S. Fraser, Human eukaryotic initiation factor 2 (eIF2)-GTP-Met-tRNAi ternary complex and eIF3 stabilize the 43 S preinitiation complex. *J Biol Chem* **289**, 31827-31836 (2014).

58. J. Marcotrigiano *et al.*, A Conserved HEAT Domain within eIF4G Directs Assembly of the Translation Initiation Machinery. *Molecular Cell* **7**, 193-203 (2001).

59. J. Marcotrigiano, A.-C. Gingras, N. Sonenberg, S. K. Burley, Cocrystal Structure of the Messenger RNA 5' Cap-Binding Protein (eIF4E) Bound to 7-methyl-GDP. *Cell* **89**, 951-961 (1997).

60. A. Haghighat, N. Sonenberg, eIF4G Dramatically Enhances the Binding of eIF4E to the mRNA 5'-Cap Structure. *Journal of Biological Chemistry* **272**, 21677-21680 (1997).

61. A. K. Lefebvre *et al.*, Translation Initiation Factor eIF4G-1 Binds to eIF3 through the eIF3e Subunit. *Journal of Biological Chemistry* **281**, 22917-22932 (2006).

62. A. W. B. Craig, A. Haghighat, A. T. K. Yu, N. Sonenberg, Interaction of polyadenylate-binding protein with the eIF4G homologue PAIP enhances translation. *Nature* **392**, 520-523 (1998).

63. S. Z. Tarun, A. B. Sachs, Association of the yeast poly(A) tail binding protein with translation initiation factor eIF-4G. *The EMBO Journal* **15**, 7168-7177 (1996).

64. T. K. Katsura Asano, Lon Phan, Graham D.Pavitt and AlanG.Hinnebusch, Conserved bipartite motifs in yeast eIF5 and eIF2Bε,GTPase-activating and GDP–GTP exchange factors intranslation initiation, mediate binding to theircommon substrate eIF2. *EMBO J* 10.1093/emboj/18 (1999).

65. R. Majumdar, U. Maitra, Regulation of GTP hydrolysis prior to ribosomal AUG selection during eukaryotic translation initiation. *The EMBO Journal* **24**, 3737-3746 (2005).

66. A. Unbehaun, S. I. Borukhov, C. U. T. Hellen, T. V. Pestova, Release of initiation factors from 48S complexes during ribosomal subunit joining and the link between establishment of codon-anticodon base-pairing and hydrolysis of eIF2-bound GTP. *Genes & Development* **18**, 3078-3093 (2004).

67. L. L. a. G. S. Benjin Xu1, Functions and Regulation of Translation Elongation Factors. *Frontiers in Molecular Biosciences* (2022).

68. D. Susorov *et al.*, Eukaryotic translation elongation factor 2 (eEF2) catalyzes reverse translocation of the eukaryotic ribosome. *Journal of Biological Chemistry* **293**, 5220-5229 (2018).

69. G. R. Andersen *et al.*, Structural Basis for Nucleotide Exchange and Competition with tRNA in the Yeast Elongation Factor Complex eEF1A:eEF1Bα. *Molecular Cell* **6**, 1261-1266 (2000).

70. A. P. Schuller, C. C.-C. Wu, T. E. Dever, A. R. Buskirk, R. Green, eIF5A Functions Globally in Translation Elongation and Termination. *Molecular Cell* **66**, 194-205.e195 (2017).

71. T. Becker *et al.*, Structural basis of highly conserved ribosome recycling in eukaryotes and archaea. *Nature* **482**, 501-506 (2012).

72. T. M. Alexandr Ivanov, Boris Eliseev, Lahari Yeramala,, D. S. Elizaveta Sokolova, Alexey Shuvalov, Christiane Schaffitzel and, E. Alkalaeva, PABP enhances release factor recruitment and stop codon recognition during translation termination *Nucleic Acids Res* (2016).

73. F. Buttgereit, M. D. Brand, A hierarchy of ATP-consuming processes in mammalian cells. *Biochemical Journal* **312**, 163-167 (1995).

74. N. Sonenberg, A. G. Hinnebusch, Regulation of Translation Initiation in Eukaryotes: Mechanisms and Biological Targets. *Cell* **136**, 731-745 (2009).

75. G. B. R. a. S.-H. C. Anand Ramanathan, mRNA capping: biological functions and applications. *Nucleic Acids Res* (2016).

76. S. G. Anil Thakur, Anil K. Vijjamarri and Alan G. Hinnebusch, eIF2 interactions with mRNA control accurate start codon selection by the translation preinitiation complex. *Nucleic Acids Res* (2020).

77. Y. Z. Martin D. Jennings, Sarah S. Mohammad-Qureshi, David Bennett, and Graham D. Pavitt, eIF2B promotes eIF5 dissociation fromeIF2•GDP to facilitate guanine nucleotideexchange for translation initiation. *Genes & Development* 10.1093/emboj/18 (2013).

78. D. Scheuner *et al.*, Translational Control Is Required for the Unfolded Protein Response and In Vivo Glucose Homeostasis. *Molecular Cell* **7**, 1165-1176 (2001).

79. S. Yamasaki, P. Anderson, Reprogramming mRNA translation during stress. *Curr Opin Cell Biol* **20**, 222-226 (2008).

80. I. N. Heather P. Harding, Yuhong Zhang, Huiqing Zeng,Ron Wek,† Matthieu Schapira, and David Ron, Regulated Translation Initiation Controls Stress-Induced Gene Expression in Mammalian Cells. *Molecular Cell* (2000).

81. H. P. Harding, Y. Zhang, D. Ron, Protein translation and folding are coupled by an endoplasmic-reticulum-resident kinase. *Nature* **397**, 271-274 (1999).

82. K. Ma, K. M. Vattem, R. C. Wek, Dimerization and Release of Molecular Chaperone Inhibition Facilitate Activation of Eukaryotic Initiation Factor-2 Kinase in Response to Endoplasmic Reticulum Stress. *Journal of Biological Chemistry* **277**, 18728-18735 (2002).

83. B. F. Teske *et al.*, The eIF2 kinase PERK and the integrated stress response facilitate activation of ATF6 during endoplasmic reticulum stress. *Mol Biol Cell* **22**, 4390-4405 (2011).

84. H. P. Harding, Y. Zhang, A. Bertolotti, H. Zeng, D. D Ron, PERK Is Essential for Translational Regulation and Cell Survival during the Unfolded Protein Response. *Molecular cell* (2000).

85. N. A. Paolini *et al.*, Ribosome profiling uncovers selective mRNA translation associated with eIF2 phosphorylation in erythroid progenitors. *PLoS One* **13**, e0193790 (2018).

86. S. E. Calvo, D. J. Pagliarini, V. K. Mootha, Upstream open reading frames cause widespread reduction of protein expression and are polymorphic among humans. *Proceedings of the National Academy of Sciences* **106**, 7507-7512 (2009).

87.   K. Pakos-Zebrucka *et al.*, The integrated stress response. *EMBO reports* **17**, 1374-1395 (2016).

88.   K. M. Vattem, R. C. Wek, Reinitiation involving upstream ORFs regulates ATF4 mRNA translation in mammalian cells. *Proceedings of the National Academy of Sciences* **101**, 11269-11274 (2004).

89.   I. M. N. Wortel, L. T. Van Der Meer, M. S. Kilberg, F. N. Van Leeuwen, Surviving Stress: Modulation of ATF4-Mediated Stress Responses in Normal and Malignant Cells. *Trends in Endocrinology & Metabolism* **28**, 794-806 (2017).

90.   J. Bohlen *et al.*, DENR promotes translation reinitiation via ribosome recycling to drive expression of oncogenes including ATF4. *Nature Communications* **11** (2020).

91.   A. Pause *et al.*, Insulin-dependent stimulation of protein synthesis by phosphorylation of a regulator of 5'-cap function. *Nature* **371**, 762-767 (1994).

92.   A. C. Gingras *et al.*, Regulation of 4E-BP1 phosphorylation: a novel two-step mechanism. *Genes & Development* **13**, 1422-1437 (1999).

93.   L. Beretta, A. C. Gingras, Y. V. Svitkin, M. N. Hall, N. Sonenberg, Rapamycin blocks the phosphorylation of 4E-BP1 and inhibits cap-dependent initiation of translation. *The EMBO Journal* **15**, 658-664 (1996).

94.   A.-C. Gingras *et al.*, Hierarchical phosphorylation of the translation inhibitor 4E-BP1. *Genes & Development* **15**, 2852-2864 (2001).

95.   T. E. Harris *et al.*, mTOR-dependent stimulation of the association of eIF4G and eIF3 by insulin. *The EMBO Journal* **25**, 1659-1668 (2006).

96.   Raphael Bo¨hm, Stefan Imseng, Roman P. Jakob, Michael N. Hall, S. H. Timm Maier, The dynamic mechanism of 4E-BP1 recognition and phosphorylation by mTORC1. *Molecular cell* (2021).

97.   O. K. a. V. K. Rachel Ruoff, Cell type-specific control of protein synthesis and proliferation by FGF-dependent signaling to the translation repressor 4E-BP. *PNAS* (2016).

98.   Q.-B. She *et al.*, 4E-BP1 Is a Key Effector of the Oncogenic Activation of the AKT and ERK Signaling Pathways that Integrates Their Function in Tumors. *Cancer Cell* **18**, 39-51 (2010).

99.   R. J. O. Dowling *et al.*, mTORC1-Mediated Cell Proliferation, But Not Cell Growth, Controlled by the 4E-BPs. *Science* **328**, 1172-1176 (2010).

100.   V. A. Arzumanian, O. I. Kiseleva, E. V. Poverennaya, The Curious Case of the HepG2 Cell Line: 40 Years of Expertise. *Int J Mol Sci* **22** (2021).

101.   D. López-Terrada, S. W. Cheung, M. J. Finegold, B. B. Knowles, Hep G2 is a hepatoblastoma-derived cell line. *Hum Pathol* **40**, 1512-1515 (2009).

102.   C. C. Thoreen *et al.*, A unifying model for mTORC1-mediated regulation of mRNA translation. *Nature* **485**, 109-113 (2012).

103.   Yan Xiangyu *et al.*, Sirolimus or Everolimus Improves Survival After Liver Transplantation for Hepatocellular Carcinoma: A Systematic Review and Meta-Analysis. *Liver Transplantation* (2022).

104.   S. Romano *et al.*, The Emerging Role of Large Immunophilin FK506 Binding Protein 51 in Cancer. *Current Medicinal Chemistry* **18**, 5424-5429 (2011).

105.   C. J. Sabers *et al.*, Isolation of a Protein Target of the FKBP12-Rapamycin Complex in Mammalian Cells. *Journal of Biological Chemistry* **270**, 815-822 (1995).

106. J. Pelletier, G. Thomas, S. Volarevic, Ribosome biogenesis in cancer: new players and therapeutic avenues. *Nat Rev Cancer* **18**, 51-63 (2018).

107. M. A. Rodríguez-Hernández *et al.*, Molecular characterization of autophagic and apoptotic signaling induced by sorafenib in liver cancer cells. *Journal of Cellular Physiology* **234**, 692-708 (2019).

108. P. Song, F. Yang, H. Jin, X. Wang, The regulation of protein translation and its implications for cancer. *Signal Transduction and Targeted Therapy* **6** (2021).

109. D. Shahbazian *et al.*, The mTOR/PI3K and MAPK pathways converge on eIF4B to control its phosphorylation and activity. *The EMBO Journal* **25**, 2781-2791 (2006).

110. I. Topisirovic, N. Sonenberg, mRNA Translation and Energy Metabolism in Cancer: The Role of the MAPK and mTORC1 Pathways. *Cold Spring Harbor Symposia on Quantitative Biology* **76**, 355-367 (2011).

111. P. P. Roux, J. Blenis, ERK and p38 MAPK-Activated Protein Kinases: a Family of Protein Kinases with Diverse Biological Functions. *Microbiology and Molecular Biology Reviews* **68**, 320-344 (2004).

112. M. Cargnello, P. P. Roux, Activation and Function of the MAPKs and Their Substrates, the MAPK-Activated Protein Kinases. *Microbiology and Molecular Biology Reviews* **75**, 50-83 (2011).

113. I. T. Philippe P. Roux, Signaling Pathways Involved in the Regulation of mRNA Translation. *Molecular and Cellular Biology* (2018).

114. G. C. Scheper, N. A. Morrice, M. Kleijn, C. G. Proud, The Mitogen-Activated Protein Kinase Signal-Integrating Kinase Mnk2 Is a Eukaryotic Initiation Factor 4E Kinase with High Levels of Basal Activity in Mammalian Cells. *Molecular and Cellular Biology* **21**, 743-754 (2001).

115. R. Fukunag, T. Hunter, MNK1, a new MAP kinase-activated protein kinase, isolated by a novel expression screening method for identifying protein kinase substrates. *EMBO J* 10.1093/emboj/16 (1997).

116. J. L. Parra, M. Buxadé, C. G. Proud, Features of the Catalytic Domains and C Termini of the MAPK Signal-integrating Kinases Mnk1 and Mnk2 Determine Their Differing Activities and Regulatory Properties. *Journal of Biological Chemistry* **280**, 37623-37633 (2005).

117. A. O'Loghlen, V. M. González, T. Jurado , M. Salinas, M. E. Martín, Characterization of the activity of human MAP kinase-interacting kinase Mnk1b. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research* (2007).

118. Avraham Maimon *et al.*, Mnk2 Alternative Splicing Modulates the p38-MAPK Pathway and Impacts Ras-Induced Transformation. *Cell Reports* (2014).

119. S. Joshi, Mnk kinase pathway: Cellular functions and biological outcomes. *World Journal of Biological Chemistry* **5**, 321 (2014).

120. A. J. Waskiewicz *et al.*, Phosphorylation of the Cap-Binding Protein Eukaryotic Translation Initiation Factor 4E by Protein Kinase Mnk1 In Vivo. *Molecular and Cellular Biology* **19**, 1871-1880 (1999).

121. T. Ueda, R. Watanabe-Fukunaga, H. Fukuyama, S. Nagata, R. Fukunaga, Mnk2 and Mnk1 Are Essential for Constitutive and Inducible Phosphorylation of Eukaryotic Initiation Factor 4E but Not for Cell Growth or Development. *Molecular and Cellular Biology* **24**, 6539-6549 (2004).

122. S. Pyronnet, Human eukaryotic translation initiation factor 4G (eIF4G)

recruits Mnk1 to phosphorylate eIF4E. *The EMBO Journal* **18**, 270-279 (1999).

123. M. Shveygert, C. Kaiser, S. S. Bradrick, M. Gromeier, Regulation of Eukaryotic Initiation Factor 4E (eIF4E) Phosphorylation by Mitogen-Activated Protein Kinase Occurs through Modulation of Mnk1-eIF4G Interaction. *Molecular and Cellular Biology* **30**, 5160-5167 (2010).

124. P. E. D. Lachance, M. Miron, B. Raught, N. Sonenberg, P. Lasko, Phosphorylation of Eukaryotic Translation Initiation Factor 4E Is Critical for Growth. *Molecular and Cellular Biology* **22**, 1656-1663 (2002).

125. Andrea Bianchini *et al.*, Phosphorylation of eIF4E by MNKs supports protein synthesis, cell cycle progression and proliferation in prostate cancer cells. *Carcinogenesis* (2008).

126. U. Knauf, C. Tschopp, H. Gram, Negative Regulation of Protein Translation by Mitogen-Activated Protein Kinase-Interacting Kinases 1 and 2. *Molecular and Cellular Biology* **21**, 5500-5511 (2001).

127. G. C. Scheper *et al.*, Phosphorylation of Eukaryotic Initiation Factor 4E Markedly Reduces Its Affinity for Capped mRNA. *Journal of Biological Chemistry* **277**, 3303-3309 (2002).

128. N. L. Korneeva, A. Song, H. Gram, M. A. Edens, R. E. Rhoads, Inhibition of Mitogen-activated Protein Kinase (MAPK)-interacting Kinase (MNK) Preferentially Affects Translation of mRNAs Containing Both a 5'-Terminal Cap and Hairpin. *Journal of Biological Chemistry* **291**, 3455-3467 (2016).

129. S. Joshi, L. C. Platanias, Mnk kinases in cytokine signaling and regulation of cytokine responses. *Biomolecular Concepts* **3** (2012).

130. R. J. Jackson, The Current Status of Vertebrate Cellular mRNA IRESs. *Cold Spring Harbor Perspectives in Biology* **5**, a011569-a011569 (2013).

131. Hiroshi Yamamoto, Anett Unbehaun, C. M. T. Spahn, Ribosomal Chamber Music: Toward an Understanding of IRES Mechanisms. *Trends Biochemical Sciences* (2017).

132. A. L. Landon *et al.*, MNKs act as a regulatory switch for eIF4E1 and eIF4E3 driven mRNA translation in DLBCL. *Nature Communications* **5**, 5413 (2014).

133. M. Bhat *et al.*, Targeting the translation machinery in cancer. *Nature Reviews Drug Discovery* **14**, 261-278 (2015).

134. N. Hay, Mnk earmarks eIF4E for cancer therapy. *Proceedings of the National Academy of Sciences* **107**, 13975-13976 (2010).

135. S. Lim *et al.*, Targeting of the MNK–eIF4E axis in blast crisis chronic myeloid leukemia inhibits leukemia stem cell function. *Proceedings of the National Academy of Sciences* **110**, E2298-E2307 (2013).

136. S. A. Prabhu, O. Moussa, W. H. Miller, S. V. Del Rincón, The MNK1/2-eIF4E Axis as a Potential Therapeutic Target in Melanoma. *International Journal of Molecular Sciences* **21**, 4055 (2020).

137. P. P. Roux *et al.*, RAS/ERK signaling promotes site-specific ribosomal protein S6 phosphorylation via RSK and stimulates cap-dependent translation. *J Biol Chem* **282**, 14056-14064 (2007).

138. A. Astanehe *et al.*, MKNK1 is a YB-1 target gene responsible for imparting trastuzumab resistance and can be blocked by RSK inhibition. *Oncogene* **31**, 4434-4446 (2012).

139. H.-S. Yang *et al.*, The Transformation Suppressor Pdcd4 Is a Novel Eukaryotic Translation Initiation Factor 4A Binding Protein That Inhibits Translation.

Molecular and Cellular Biology **23**, 26-37 (2003).

140. A. Parsyan *et al.*, mRNA helicases: the tacticians of translational control. *Nature Reviews Molecular Cell Biology* **12**, 235-245 (2011).

141. U. Liwak *et al.*, Tumor suppressor PDCD4 represses internal ribosome entry site-mediated translation of antiapoptotic proteins and is regulated by S6 kinase 2. *Mol Cell Biol* **32**, 1818-1829 (2012).

142. M. Laplante, David, mTOR Signaling in Growth Control and Disease. *Cell* **149**, 274-293 (2012).

143. Y. Park, A. Reyna-Neyra, L. Philippe, C. C. Thoreen, mTORC1 Balances Cellular Amino Acid Supply with Demand for Protein Synthesis through Post-transcriptional Control of ATF4. *Cell Rep* **19**, 1083-1090 (2017).

144. S. Ricciardi *et al.*, The Translational Machinery of Human CD4(+) T Cells Is Poised for Activation and Controls the Switch from Quiescence to Metabolic Remodeling. *Cell Metab* **28**, 895-906 e895 (2018).

145. K. Masui *et al.*, mTOR Complex 2 Controls Glycolytic Metabolism in Glioblastoma through FoxO Acetylation and Upregulation of c-Myc. *Cell Metabolism* **18**, 726-739 (2013).

146. E. Jacinto *et al.*, Mammalian TOR complex 2 controls the actin cytoskeleton and is rapamycin insensitive. *Nature Cell Biology* **6**, 1122-1128 (2004).

147. D. S. Dos *et al.*, Rictor, a Novel Binding Partner of mTOR, Defines a Rapamycin-Insensitive and Raptor-Independent Pathway that Regulates the Cytoskeleton. *Current Biology* **14**, 1296-1302 (2004).

148. M. Shimobayashi, M. N. Hall, Making new contacts: the mTOR network in metabolism and signalling crosstalk. *Nature Reviews Molecular Cell Biology* **15**, 155-162 (2014).

149. M. K. Holz, B. A. Ballif, S. P. Gygi, J. Blenis, mTOR and S6K1 Mediate Assembly of the Translation Preinitiation Complex through Dynamic Protein Interchange and Ordered Phosphorylation Events. *Cell* **123**, 569-580 (2005).

150. V. Gandin *et al.*, mTORC1 and CK2 coordinate ternary and eIF4F complex assembly. *Nature Communications* **7**, 11127 (2016).

151. S. Grosso *et al.*, Sensitivity of global translation to mTOR inhibition in REN cells depends on the equilibrium between eIF4E and 4E-BP1. *PLoS One* **6**, e29136 (2011).

152. E. J. Brown *et al.*, Control of p70 S6 kinase by kinase activity of FRAP in vivo. *Nature* **377**, 441-446 (1995).

153. P. E. Burnett, R. K. Barrow, N. A. Cohen, S. H. Snyder, D. M. Sabatini, RAFT1 phosphorylation of the translational regulators p70 S6 kinase and 4E-BP1. *Proceedings of the National Academy of Sciences* **95**, 1432-1437 (1998).

154. B. Raught, Serum-stimulated, rapamycin-sensitive phosphorylation sites in the eukaryotic translation initiation factor 4GI. *The EMBO Journal* **19**, 434-444 (2000).

155. M. D. Dennis, L. S. Jefferson, S. R. Kimball, Role of p70S6K1-mediated Phosphorylation of eIF4B and PDCD4 Proteins in the Regulation of Protein Synthesis. *Journal of Biological Chemistry* **287**, 42890-42899 (2012).

156. J.-J. Jia *et al.*, mTORC1 promotes TOP mRNA translation through site-specific phosphorylation of LARP1. *Nucleic Acids Research* **49**, 3461-3489 (2021).

157. O. Larsson *et al.*, Distinct perturbation of the translatome by the antidiabetic drug

metformin. *Proceedings of the National Academy of Sciences* **109**, 8977-8982 (2012).

158. M. Morita *et al.*, mTORC1 Controls Mitochondrial Activity and Biogenesis through 4E-BP-Dependent Translational Regulation. *Cell Metabolism* **18**, 698-711 (2013).

159. R. M. Jones *et al.*, An essential E box in the promoter of the gene encoding the mRNA cap-binding protein (eukaryotic initiation factor 4E) is a target for activation by c-myc. *Molecular and Cellular Biology* **16**, 4754-4764 (1996).

160. C.-J. Lin, R. Cencic, J. R. Mills, F. Robert, J. Pelletier, c-Myc and eIF4F are components of a feedforward loop that links transcription and translation. *Cancer research* (2008).

161. S. Adhikary, M. Eilers, Transcriptional regulation and transformation by Myc proteins. *Nature Reviews Molecular Cell Biology* **6**, 635-645 (2005).

162. A. Menssen, H. Hermeking, Characterization of the c-MYC-regulated transcriptome by SAGE: Identification and analysis of c-MYC target genes. *Proceedings of the National Academy of Sciences* **99**, 6274-6279 (2002).

163. M. Stoneley *et al.*, c-Myc Protein Synthesis Is Initiated from the Internal Ribosome Entry Segment during Apoptosis. *Molecular and Cellular Biology* **20**, 1162-1169 (2000).

164. K. A. Spriggs *et al.*, Canonical Initiation Factor Requirements of the Myc Family of Internal Ribosome Entry Segments. *Molecular and Cellular Biology* **29**, 1565-1574 (2009).

165. H. Ruan *et al.*, eIF4E S209 phosphorylation licenses myc- and stress-driven oncogenesis. *eLife* **9** (2020).

166. K. Singh *et al.*, c-MYC regulates mRNA translation efficiency and start-site selection in lymphoma. *Journal of Experimental Medicine* **216**, 1509-1524 (2019).

167. E. Rom *et al.*, Cloning and Characterization of 4EHP, a Novel Mammalian eIF4E-related Cap-binding Protein. *Journal of Biological Chemistry* **273**, 13104-13109 (1998).

168. B. Joshi, A. Cameron, R. Jagus, Characterization of mammalian eIF4E-family members. *European Journal of Biochemistry* **271**, 2189-2203 (2004).

169. F. Gebauer, Versatility of the translational machinery during stress: changing partners to keep dancing. *Cell Research* **22**, 1634-1636 (2012).

170. J. Uniacke *et al.*, An oxygen-regulated switch in the protein synthesis machinery. *Nature* **486**, 126-129 (2012).

171. J.J *et al.*, Systemic Reprogramming of Translation Efficiencies on Oxygen Stimulus. *Cell Reports* **14**, 1293-1300 (2016).

172. M. J. Osborne *et al.*, eIF4E3 acts as a tumor suppressor by utilizing an atypical mode of methyl-7-guanosine cap recognition. *Proceedings of the National Academy of Sciences* **110**, 3877-3882 (2013).

173. Y. Mamane *et al.*, Epigenetic Activation of a Subset of mRNAs by eIF4E Explains Its Effects on Cell Proliferation. *PLoS ONE* **2**, e242 (2007).

174. N. Robichaud *et al.*, Phosphorylation of eIF4E promotes EMT and metastasis via translational control of SNAIL and MMP-3. *Oncogene* **34**, 2032-2042 (2015).

175. X.-L. Wang, Detection of eukaryotic translation initiation factor 4E and its clinical significance in hepatocellular

carcinoma. *World Journal of Gastroenterology* **18**, 2540 (2012).

176. A. Martínez *et al.*, Phosphorylation of eIF4E Confers Resistance to Cellular Stress and DNA-Damaging Agents through an Interaction with 4E-T: A Rationale for Novel Therapeutic Approaches. *PLOS ONE* **10**, e0123352 (2015).

177. T. Liu *et al.*, eIF4E promotes tumorigenesis and modulates chemosensitivity to cisplatin in esophageal squamous cell carcinoma. *Oncotarget* **7**, 66851-66864 (2016).

178. K. Leppek, R. Das, M. Barna, Functional 5' UTR mRNA structures in eukaryotic translation regulation and how to find them. *Nat Rev Mol Cell Biol* **19**, 158-174 (2018).

179. K. Feoktistova, E. Tuvshintogs, A. Do, C. S. Fraser, Human eIF4E promotes mRNA restructuring by stimulating eIF4A helicase activity. *Proceedings of the National Academy of Sciences* **110**, 13339-13344 (2013).

180. Lorenzo Calviello *et al.*, DDX3 depletion represses translation of mRNAs with complex 5' UTRs. *Nucleic Acids Res* (2021).

181. C. A. Rubio *et al.*, Transcriptome-wide characterization of the eIF4A signature highlights plasticity in translation regulation. *Genome Biology* **15** (2014).

182. D. Shahbazian, A. Parsyan, E. Petroulakis, J. Hershey, N. Sonenberg, eIF4B controls survival and proliferation and is regulated by proto-oncogenic signaling pathways. *Cell Cycle* **9**, 4106-4109 (2010).

183. N. D. Sen, F. Zhou, M. S. Harris, N. T. Ingolia, A. G. Hinnebusch, eIF4B stimulates translation of long mRNAs with structured 5' UTRs and low closed-loop potential but weak dependence on eIF4G. *Proceedings of the National Academy of Sciences* **113**, 10464-10472 (2016).

184. G. W. Rogers, N. J. Richter, W. F. Lima, W. C. Merrick, Modulation of the Helicase Activity of eIF4A by eIF4B, eIF4H, and eIF4F. *Journal of Biological Chemistry* **276**, 30914-30922 (2001).

185. A. Z. Andreou, U. Harms, D. Klostermeier, eIF4B stimulates eIF4A ATPase and unwinding activities by direct interaction through its 7-repeats region. *RNA Biology* **14**, 113-123 (2017).

186. K. Chan *et al.*, eIF4A supports an oncogenic translation program in pancreatic ductal adenocarcinoma. *Nat Commun* **10**, 5151 (2019).

187. A. L. Wolfe *et al.*, RNA G-quadruplexes cause eIF4A-dependent oncogene translation in cancer. *Nature* **513**, 65-70 (2014).

188. A. Modelska *et al.*, The malignant phenotype in breast cancer is driven by eIF4A1-mediated changes in the translational landscape. *Cell Death & Disease* **6**, e1603-e1603 (2015).

189. M. Moustafa-Kamal *et al.*, The mTORC1/S6K/PDCD4/eIF4A Axis Determines Outcome of Mitotic Arrest. *Cell Reports* **33**, 108230 (2020).

190. J. J. D. Ho *et al.*, Proteomics reveal cap-dependent translation inhibitors remodel the translation machinery and translatome. *Cell Reports* (2021).

191. V. Volta *et al.*, A DAP5/eIF3d alternate mRNA translation mechanism promotes differentiation and immune suppression by human regulatory T cells. *Nature Communications* **12** (2021).

192. C. Thoma, G. Bergamini, B. Galy, P. Hundsdoerfer, M. W. Hentze, Enhancement of IRES-Mediated Translation of the c-myc and BiP mRNAs by the Poly(A) Tail Is Independent of Intact eIF4G and PABP. *Molecular Cell* (2004).

193. Stephen M. Lewis *et al.*, The eIF4G homolog DAP5/p97 supports the translation of select mRNAs during endoplasmic reticulum stress. *Nucleic Acids Research* (2008).

194. S. De Breyne, Y. Yu, A. Unbehaun, T. V. Pestova, C. U. T. Hellen, Direct functional interaction of initiation factor eIF4G with type 1 internal ribosomal entry sites. *Proceedings of the National Academy of Sciences* **106**, 9197-9202 (2009).

195. M. Badura, S. Braunstein, J. Zavadil, R. J. Schneider, DNA damage and eIF4G1 in breast cancer cells reprogram translation for survival and DNA repair mRNAs. *Proceedings of the National Academy of Sciences* **109**, 18767-18772 (2012).

196. M. Altmann, A novel inhibitor of cap-dependent translation initiation in yeast: p20 competes with eIF4G for binding to eIF4E. *The EMBO Journal* **16**, 1114-1121 (1997).

197. N. J. Moerke *et al.*, Small-Molecule Inhibition of the Interaction between the Translation Initiation Factors eIF4E and eIF4G. *Cell* **128**, 257-267 (2007).

198. N. Sekiyama *et al.*, Molecular mechanism of the dual activity of 4EGI-1: Dissociating eIF4G from eIF4E but stabilizing the binding of unphosphorylated 4E-BP1. *Proceedings of the National Academy of Sciences* **112**, E4036-E4045 (2015).

199. M. C. Mendoza, E. E. Er, J. Blenis, The Ras-ERK and PI3K-mTOR pathways: cross-talk and compensation. *Trends in Biochemical Sciences* **36**, 320-328 (2011).

200. A. O'Loghlen *et al.*, Identification and molecular characterization of Mnk1b, a splice variant of human MAP kinase-interacting kinase Mnk1. *Experimental Cell Research* (2004).

201. M. Hatakeyama, J. A. Brill, G. R. Fink, R. A. Weinberg, Collaboration of G1 cyclins in the functional inactivation of the retinoblastoma protein. *Genes & Development* **8**, 1759-1771 (1994).

202. J. W. Harbour, R. X. Luo, A. D. Santi, A. A. Postigo, D. C. Dean, Cdk Phosphorylation Triggers Sequential Intramolecular Interactions that Progressively Block Rb Functions as Cells Move through G1. *Cell* **98**, 859-869 (1999).

203. Y. Xu, D. Ruggero, The Role of Translation Control in Tumorigenesis and Its Therapeutic Implications. *Annual Review of Cancer Biology* **4**, 437-457 (2020).

204. Y. Xu *et al.*, Translation control of the immune checkpoint in cancer and its therapeutic targeting. *Nature Medicine* **25**, 301-311 (2019).

205. Q. Li *et al.*, eIF5B regulates the expression of PD-L1 in prostate cancer cells by interacting with Wig1. *BMC Cancer* **21** (2021).

206. S. Suresh *et al.*, eIF5B drives integrated stress response-dependent translation of PD-L1 in lung cancer. *Nature Cancer*, 533-545 (2020).

207. A. Sendoel *et al.*, Translation from unconventional 5' start sites drives tumour initiation. *Nature* **541**, 494-499 (2017).

208. C. De La Parra *et al.*, A widespread alternate form of cap-dependent mRNA translation initiation. *Nature Communications* **9** (2018).

209. B. D. Fonseca *et al.*, La-related Protein 1 (LARP1) Represses Terminal Oligopyrimidine (TOP) mRNA Translation Downstream of mTOR Complex 1 (mTORC1). *Journal of Biological Chemistry* **290**, 15996-16020 (2015).

210. S. Levy, D. Avni, N. Hariharan, R. P. Perry, O. Meyuhas, Oligopyrimidine tract at the 5' end of mammalian ribosomal protein mRNAs is required for their translational control. *Proceedings of the National*

Academy of Sciences **88**, 3319-3323 (1991).

211. John, Melissa, A. Lodi, Sabrina, D. Ruggero, Protein and Nucleotide Biosynthesis Are Coupled by a Single Rate-Limiting Enzyme, PRPS2, to Drive Cancer. *Cell* **157**, 1088-1103 (2014).

212. E. Villanueva *et al.*, Translational reprogramming in tumour cells can generate oncoselectivity in viral therapies. *Nature Communications* **8**, 14833 (2017).

213. C. Maillo *et al.*, Circadian- and UPR-dependent control of CPEB4 mediates a translational response to counteract hepatic steatosis under ER stress. *Nature Cell Biology* **19**, 94-105 (2017).

214. I. Novoa, J. Gallego, P. G. Ferreira, R. Mendez, Mitotic cell-cycle progression is regulated by CPEB1 and CPEB4-dependent translational control. *Nature Cell Biology* **12**, 447-456 (2010).

215. E. Ortiz-Zapater *et al.*, Key contribution of CPEB4-mediated translational control to cancer progression. *Nature Medicine* **18**, 83-90 (2012).

216. S. M. García-Mauriño *et al.*, RNA Binding Protein Regulation and Cross-Talk in the Control of AU-rich mRNA Fate. *Front Mol Biosci* **4**, 71 (2017).

217. H. Otsuka, A. Fukao, Y. Funakami, K. E. Duncan, T. Fujiwara, Emerging Evidence of Translational Control by AU-Rich Element-Binding Proteins. *Frontiers in Genetics* **10** (2019).

218. C. Barreau, L. Paillard, H. B. Osborne, AU-rich elements and associated factors: are there unifying principles? *Nucleic Acids Res* **33**, 7138-7150 (2005).

219. E. Hitti *et al.*, Systematic Analysis of AU-Rich Element Expression in Cancer Reveals Common Functional Clusters Regulated by Key RNA-Binding Proteins. *Cancer Research* **76**, 4068-4080 (2016).

220. K. S. A. Khabar, Hallmarks of cancer and <scp>AU</scp> -rich elements. *WIREs RNA* **8**, e1368 (2017).

221. X. Wu *et al.*, Targeting the interaction between RNA-binding protein HuR and FOXQ1 suppresses breast cancer invasion and metastasis. *Communications Biology* **3** (2020).

222. K. Abdelmohsen *et al.*, Phosphorylation of HuR by Chk2 regulates SIRT1 expression. *Mol Cell* **25**, 543-557 (2007).

223. D. Durie *et al.*, RNA-binding protein HuR mediates cytoprotection through stimulation of XIAP translation. *Oncogene* **30**, 1460-1469 (2011).

224. H. H. Kim *et al.*, HuR recruits let-7/RISC to repress c-Myc expression. *Genes & Development* **23**, 1743-1748 (2009).

225. B. Pereira, M. Billaud, R. Almeida, RNA-Binding Proteins in Cancer: Old Players and New Actors. *Trends in Cancer* **3**, 506-528 (2017).

226. S. Lachiondo-Ortega *et al.*, Hu Antigen R (HuR) Protein Structure, Function and Regulation in Hepatobiliary Tumors. *Cancers* **14**, 2666 (2022).

227. K. D. Meyer, S. R. Jaffrey, The dynamic epitranscriptome: N6-methyladenosine and gene expression control. *Nature Reviews Molecular Cell Biology* **15**, 313-326 (2014).

228. I. A. Roundtree, M. E. Evans, T. Pan, C. He, Dynamic RNA Modifications in Gene Expression Regulation. *Cell* **169**, 1187-1200 (2017).

229. S. Zaccara, R. J. Ries, S. R. Jaffrey, Reading, writing and erasing mRNA methylation. *Nature Reviews Molecular Cell Biology* **20**, 608-624 (2019).

230. H. Sun *et al.*, m6Am-seq reveals the dynamic m6Am methylation in the human transcriptome. *Nature Communications* **12**, 4778 (2021).

231. J. Liu *et al.*, A METTL3-METTL14 complex mediates mammalian nuclear RNA N6-adenosine methylation. *Nat Chem Biol* **10**, 93-95 (2014).

232. Y. Mao *et al.*, m6A in mRNA coding regions promotes translation via the RNA helicase-containing YTHDC2. *Nature Communications* **10** (2019).

233. G. Jia *et al.*, N6-methyladenosine in nuclear RNA is a major substrate of the obesity-associated FTO. *Nat Chem Biol* **7**, 885-887 (2011).

234. D. Dominissini *et al.*, Topology of the human and mouse m6A RNA methylomes revealed by m6A-seq. *Nature* **485**, 201-206 (2012).

235. G. Zheng *et al.*, ALKBH5 is a mammalian RNA demethylase that impacts RNA metabolism and mouse fertility. *Mol Cell* **49**, 18-29 (2013).

236. K. D. Meyer *et al.*, 5' UTR m(6)A Promotes Cap-Independent Translation. *Cell* **163**, 999-1010 (2015).

237. J. Zhou *et al.*, Dynamic m(6)A mRNA methylation directs translational control of heat shock response. *Nature* **526**, 591-594 (2015).

238. S. Lin, J. Choe, P. Du, R. Triboulet, R. I. Gregory, The m(6)A Methyltransferase METTL3 Promotes Translation in Human Cancer Cells. *Mol Cell* **62**, 335-345 (2016).

239. J. Choe *et al.*, mRNA circularization by METTL3-eIF3h enhances translation and promotes oncogenesis. *Nature* **561**, 556-560 (2018).

240. S. Zaccara, R. J. Ries, S. R. Jaffrey, Reading, writing and erasing mRNA methylation. *Nat Rev Mol Cell Biol* **20**, 608-624 (2019).

241. S. Liu, A. Zhu, C. He, M. Chen, REPIC: a database for exploring the N6-methyladenosine methylome. *Genome Biology* **21**, 100 (2020).

242. I. Barbieri *et al.*, Promoter-bound METTL3 maintains myeloid leukaemia by m(6)A-dependent translation control. *Nature* **552**, 126-131 (2017).

243. T. Bakheet, E. Hitti, K. S. A. Khabar, ARED-Plus: an updated and expanded database of AU-rich element-containing mRNAs and pre-mRNAs. *Nucleic acids research* **46**, D218-D220 (2018).

244. E. Pérez-Guijarro *et al.*, Lineage-specific roles of the cytoplasmic polyadenylation factor CPEB4 in the regulation of melanoma drivers. *Nature Communications* **7**, 13418 (2016).

245. A. Scholz *et al.*, uORF-Tools—Workflow for the determination of translation-regulatory upstream open reading frames. *PLOS ONE* **14**, e0222459 (2019).

246. J. Zhao *et al.*, IRESbase: A Comprehensive Database of Experimentally Validated Internal Ribosome Entry Sites. *Genomics Proteomics Bioinformatics* **18**, 129-139 (2020).

247. W. Hou *et al.*, The MEK inhibitors enhance the efficacy of sorafenib against hepatocellular carcinoma cells through reducing p-ERK rebound. *Translational Cancer Research* **8**, 1224-1232 (2019).

248. M. Rahmani *et al.*, The kinase inhibitor sorafenib induces cell death through a process involving induction of endoplasmic reticulum stress. *Mol Cell Biol* **27**, 5499-5513 (2007).

249. C. Sauzay *et al.*, Protein biosynthesis, a target of sorafenib, interferes with the unfolded protein response (UPR) and

ferroptosis in hepatocellular carcinoma cells. *Oncotarget* **9**, 8400-8414 (2018).

250. A. Villanueva *et al.*, Pivotal role of mTOR signaling in hepatocellular carcinoma. *Gastroenterology* **135**, 1972-1983, 1983.e1971-1911 (2008).

251. M. S. Matter, T. Decaens, J. B. Andersen, S. S. Thorgeirsson, Targeting the mTOR pathway in hepatocellular carcinoma: Current state and future trends. *Journal of Hepatology* **60**, 855-865 (2014).

252. A. X. Zhu *et al.*, SEARCH: a phase III, randomized, double-blind, placebo-controlled trial of sorafenib plus erlotinib in patients with advanced hepatocellular carcinoma. *J Clin Oncol* **33**, 559-566 (2015).

253. T. C. Gangadhar *et al.*, Two drug interaction studies of sirolimus in combination with sorafenib or sunitinib in patients with advanced malignancies. *Clin Cancer Res* **17**, 1956-1963 (2011).

254. Y. Zhao, Y. Liu, L. Zhou, G. S. Du, Q. He, Trends of rapamycin in survival benefits of liver transplantation for hepatocellular carcinoma. *World J Gastrointest Surg* **13**, 953-966 (2021).

255. E. Navarro-Villarán *et al.*, Molecular Pathways Leading to Induction of Cell Death and Anti-Proliferative Properties by Tacrolimus and mTOR Inhibitors in Liver Cancer Cells. *Cell Physiol Biochem* **54**, 457-473 (2020).

256. K. F. Chen *et al.*, Activation of phosphatidylinositol 3-kinase/Akt signaling pathway mediates acquired resistance to sorafenib in hepatocellular carcinoma cells. *J Pharmacol Exp Ther* **337**, 155-161 (2011).

257. O. Lindblad *et al.*, Aberrant activation of the PI3K/mTOR pathway promotes resistance to sorafenib in AML. *Oncogene* **35**, 5119-5131 (2016).

258. M. C. Brown, M. Gromeier, MNK Controls mTORC1:Substrate Association through Regulation of TELO2 Binding with mTORC1. *Cell Reports* **18**, 1444-1457 (2017).

259. R. Duncan, S. C. Milburn, J. W. Hershey, Regulated phosphorylation and low abundance of HeLa cell initiation factor eIF-4F suggest a role in translational control. Heat shock effects on eIF-4F. *J Biol Chem* **262**, 380-388 (1987).

260. B. Culjkovic-Kraljacic *et al.*, The eukaryotic translation initiation factor eIF4E elevates steady-state m^{7}G capping of coding and noncoding transcripts. *Proceedings of the National Academy of Sciences* **117**, 26773-26783 (2020).

261. K. Leppek, R. Das, M. Barna, Functional 5' UTR mRNA structures in eukaryotic translation regulation and how to find them. *Nature Reviews Molecular Cell Biology* **19**, 158-174 (2018).

262. B. Culjkovic-Kraljacic, A. Baguet, L. Volpon, A. Amri, Katherine L. B. Borden, The Oncogene eIF4E Reprograms the Nuclear Pore Complex to Promote mRNA Export and Oncogenic Transformation. *Cell Reports* **2**, 207-215 (2012).

263. A. O'Loghlen *et al.*, Identification and molecular characterization of Mnk1b, a splice variant of human MAP kinase-interacting kinase Mnk1. *Exp Cell Res* **299**, 343-355 (2004).

264. A. O'Loghlen, V. M. González, T. Jurado, M. Salinas, M. E. Martín, Characterization of the activity of human MAP kinase-interacting kinase Mnk1b. *Biochim Biophys Acta* **1773**, 1416-1427 (2007).

265. C. Pinto-Díez *et al.*, Increased expression of MNK1b, the spliced isoform of MNK1, predicts poor prognosis and is associated with triple-negative breast cancer. *Oncotarget* **9**, 13501-13516 (2018).

266. X.-Y. Ke *et al.*, MNK1 and MNK2 enforce expression of E2F1, FOXM1, and WEE1 to drive soft tissue sarcoma. *Oncogene* **40**, 1851-1867 (2021).

267. J. P. Plastaras *et al.*, Cell cycle dependent and schedule-dependent antitumor effects of sorafenib combined with radiation. *Cancer Res* **67**, 9443-9454 (2007).

268. L. Contreras, A. Rodríguez-Gil, J. Muntané, J. de la Cruz, Broad Transcriptomic Impact of Sorafenib and Its Relation to the Antitumoral Properties in Liver Cancer Cells. *Cancers (Basel)* **14** (2022).

269. R. Weber *et al.*, DAP5 enables main ORF translation on mRNAs with structured and uORF-containing 5' leaders. *Nature Communications* **13** (2022).

270. L. Marash *et al.*, DAP5 Promotes Cap-Independent Translation of Bcl-2 and CDK1 to Facilitate Cell Survival during Mitosis. *Molecular Cell* **30**, 447-459 (2008).

271. Y. Feng *et al.*, METTL3/METTL14 Transactivation and m6A-Dependent TGF-β1 Translation in Activated Kupffer Cells. *Cellular and Molecular Gastroenterology and Hepatology* **12**, 839-856 (2021).

272. Y. Mao *et al.*, m6A in mRNA coding regions promotes translation via the RNA helicase-containing YTHDC2. *Nature Communications* **10**, 5332 (2019).

273. J. Liu *et al.*, N (6)-methyladenosine of chromosome-associated regulatory RNA regulates chromatin state and transcription. *Science* **367**, 580-586 (2020).

274. Z. Lin *et al.*, RNA m 6 A methylation regulates sorafenib resistance in liver cancer through FOXO 3-mediated autophagy. *The EMBO Journal* **39** (2020).

275. X. Shen *et al.*, The m6A methylation landscape stratifies hepatocellular carcinoma into 3 subtypes with distinct metabolic characteristics. *Cancer Biology and Medicine* **17**, 937-952 (2020).

276. T. Yin, L. Zhao, S. Yao, Comprehensive characterization of m6A methylation and its impact on prognosis, genome instability, and tumor microenvironment in hepatocellular carcinoma. *BMC Medical Genomics* **15** (2022).

277. F. Ek *et al.*, Sorafenib and nitazoxanide disrupt mitochondrial function and inhibit regrowth capacity in three-dimensional models of hepatocellular and colorectal carcinoma. *Scientific Reports* **12**, 8943 (2022).

278. A. Stejskalová *et al.*, Collagen I triggers directional migration, invasion and matrix remodeling of stroma cells in a 3D spheroid model of endometriosis. *Scientific Reports* **11**, 4115 (2021).

279. G. Luthria *et al.*, In vivo microscopy reveals macrophage polarization locally promotes coherent microtubule dynamics in migrating cancer cells. *Nat Commun* **11**, 3521 (2020).

280. N. U. B. Hansen *et al.*, Tissue turnover of collagen type I, III and elastin is elevated in the PCLS model of IPF and can be restored back to vehicle levels using a phosphodiesterase inhibitor. *Respiratory Research* **17** (2016).

281. H.-P. Ma *et al.*, Collagen 1A1 (COL1A1) Is a Reliable Biomarker and Putative Therapeutic Target for Hepatocellular Carcinogenesis and Metastasis. *Cancers* **11**, 786 (2019).

282. R. Shi *et al.*, Targeting type I collagen for cancer treatment. *Int J Cancer* **151**, 665-683 (2022).

283. A. M. Attallah *et al.*, Diagnostic role of collagen-III and matrix metalloproteinase-1 for early detection of hepatocellular

carcinoma. *Br J Biomed Sci* **77**, 58-63 (2020).

284. S. Nurmenniemi *et al.*, Type I and III collagen degradation products in serum predict patient survival in head and neck squamous cell carcinoma. *Oral Oncol* **48**, 136-140 (2012).

285. K. Song *et al.*, Collagen Remodeling along Cancer Progression Providing a Novel Opportunity for Cancer Diagnosis and Treatment. *International Journal of Molecular Sciences* **23**, 10509 (2022).
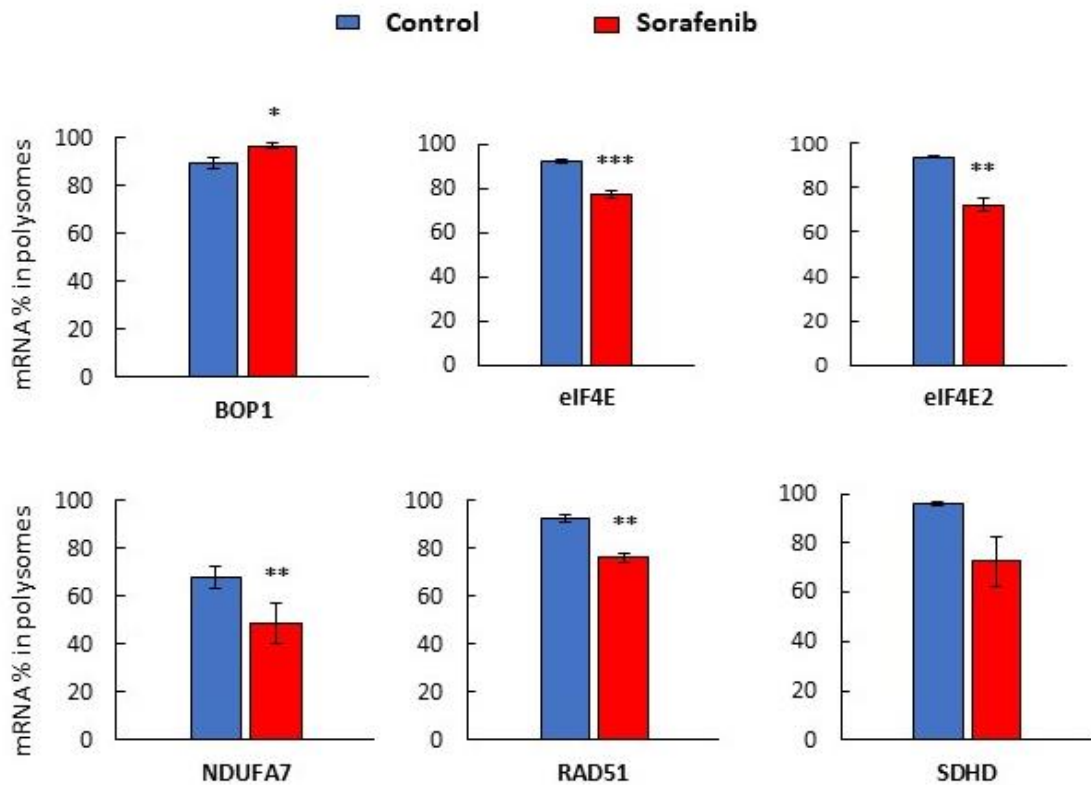
APPENDIX

SUPLEMENTARY FIGURES

A

Genes	Control	Sorafenib
BOP1	0,18	1,38
eIF4E	0,67	0,09
eIF4E2	1,07	-0,58
NDUFA7	1,15	-1,07
RAD51	1,36	0,5
SDHD	1,23	0,36

B

Supplementary Figure 1. Validation of the mRNA-seq results. The image depicts tge translation status of randomly chosen mRNAs following the Sorafenib treatment. (**A**) Translation ratio obtained from the mRNA-seq of polysome profiling. Translation ratio was calculated from $\log_{10}$(V2/V1) for untreated and Sorafenib treated cells. The table collects mRNAs whose translation ratio increased (green) or decreased (red) following the Sorafenib treatment. (**B**) Percentage of mRNAs in the polysome fraction in Sorafenib treated cells (red) *versus* control cells (blue). Statistical significances were analysed by the Student's t test (* $p < 0,05$; (** $p < 0,01$ *** $p < 0,0001$); Error bar: SD.
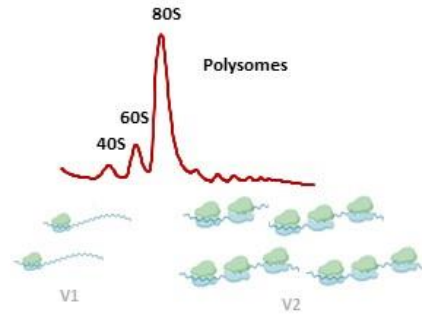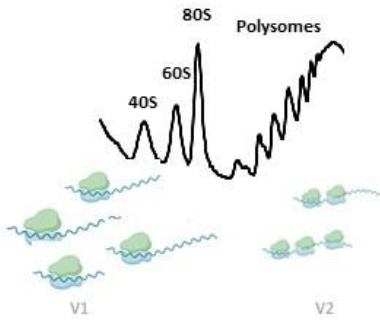
Translated-mRNAs in CONTROL conditions
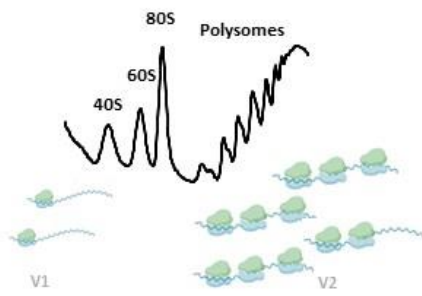
(a) **Positive V2/V1 ratio in control cells**, independently the V2/V1 ratio in Sorafenib-treated cells.
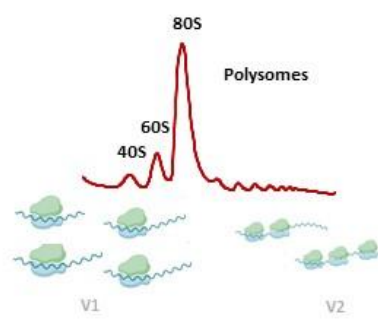
Translated-mRNAs in SORAFENIB conditions

(b) **Positive V2/V1 ratio in Sorafenib-treated cells**, independently the V2/V1 ratio in control cells.
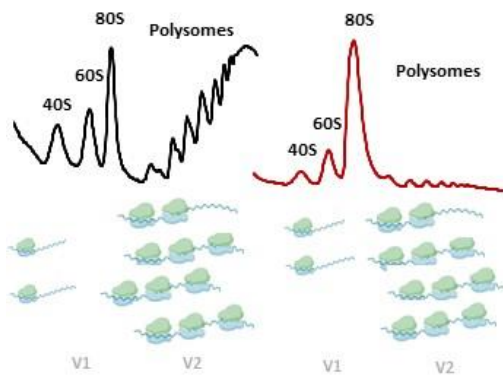
Poorly translated-mRNAs in CONTROL conditions

(c) **Negative V2/V1 ratio in control cells**, independently the V2/V1 ratio in Sorafenib-treated cells.

Poorly translated-mRNAs in SORAFENIB conditions
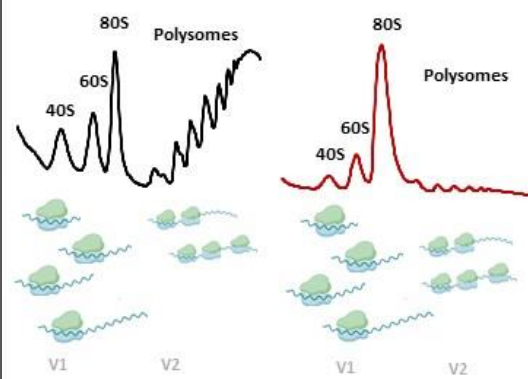
(d) **Negative V2/V1 ratio in Sorafenib-treated cells**, independently the V2/V1 ratio in control cells.
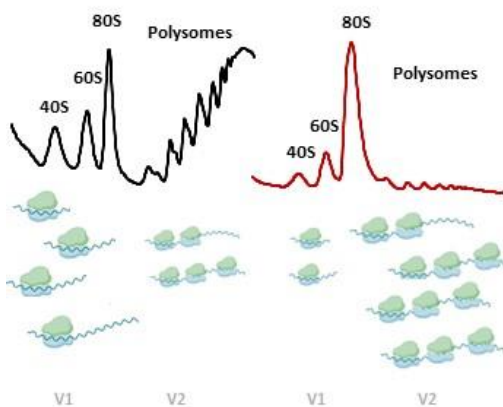
MANTAINED mRNA translation

(e) **Positive V2/V1 ratio in both** conditions simultaneously.
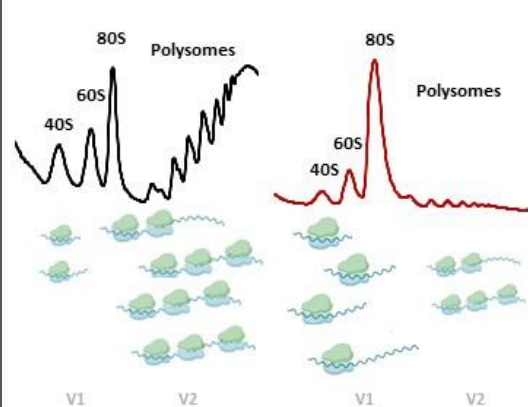
No translated mRNAs

(f) **Negative V2/V1 ratio in both** conditions simultaneously.

ENHANCED mRNA translation

(e) **Negative V2/V1 ratio** in the **control** condition, but, **positive** in Sorafenib-treated cells.

INHIBITED mRNA translation

(g) **Positive V2/V1 ratio** in the **control** condition, but, **negative** in Sorafenib-treated cells.

Supplementary Figure 2. Gene populations identified from mRNA-seq analysis of polysomes profiles of untreated and Sorafenib treated cells. A schematic representation of the eight different sets of genes identified according to their translation status in either control cells, treated cells or combining their translation status in both simultaneously.

ABBREVIATIONS

A

aa-tRNA aminoacil-tRNA

ABCE1: ATP Binding Cassette Subfamily Member 1

AKT AKT Serine/Threonine Kinase

AML Acute Myeloid Leukemia

AREs AU- rich Elements

ATF4 Activating Transcription Factor 4

ATF6 Activating Transcription Factor 6

ATP Adenosine Trifosfato

B

Bcl-2 B-cell lymphoma 2

BCLC Barcelona Clinic Liver Cancer

Bim Bcl-2-like 11

C

CDKs Cyclin-Dependent Kinases

CDS Coding Sequence

CERT Cytosine-Enriched Regulator of Translation

CHOP C/EBP Homologous Protein

CHX Cycloheximide

CK1ε Casein Kinase 1ε

CPE Cytoplasmic Polyadenylation Element

CPEB CPE Binding

CTNNB1 Catenin-Associated Protein Beta 1

D

DMSO Dimethyl Sulfoxide

E

eEFs eukaryotic Elongation Factors

eEF2K eukaryotic Elongation Factor 2 Kinase

eIFs eukaryotic Initiation Factors

ER Endoplasmic Reticulum

ERAD ER-Associated Degradation

eRFs eukaryotic Release Factors

ERK Extracellular signal-Regulated Kinase

4E-BPs 4E Binding Proteins

F

FDA Food and Drug Administration

FKBP FK Binding Protein

GAPDH Glyceraldehyde-3-phosphate Deshydrogenase

GCN2 general control nonderepressible 2

GDP Guanosine diphosphate

GSK3β Glycogen Synthase Kinase 3β

GTP Guanosine triphosphate

H

HBV Hepatitis B Virus

HCV Hepatitis C Virus

HIF-1α Hypoxia-inducible factor-1

HRI Hemo-Regulated Inhibitor

HuR Human antigen R

I

IRES Internal Ribosomal Entry Site

IRE1α Type I transmembrane protein Inositol Requiring 1

ISR Integrated Stress Response

ITAFs: IRES Trans Activating Factors

J

JNK c-Jun N-terminal Kinase

M

m^{6}A N^6-methyladenosine methylation

MAPK Mitogen-Activated Protein Kinase

MAPKK MAPK kinase (also known as MEK)

MAPKKK MAPK Kinase Kinase

MDM2 Murine Double Minute 2

Met Metionine

MNKs MAPK interacting protein kinases

mTOR mammalian Target Of Rapamycin

N

NAFLD Non-Alcoholic Fatty Liver Disease

NASH Non-Alcoholic Steatohepatitis

O

ODC Ornithine Decarboxylase

P

PABP polyA Binding Protein

PDA Pancreatic Ductal Adenocarcinoma

PDCD4 Programmed Death Cell 4

PDGFR Platelet-derived Growth Factor Receptor

PD-L1 Programmed Death Ligand 1

PERK PKR-like ER Kinase

PIC Pre-Initiation Complex

PI3K Phosphoinositide 3-Kinases (PI3Ks)

PKR Protein Kinase R

PRTE TOP-like Pyrimidine-rich Translational Element

R

RBP RNA Binding Protein

RSK 90 kDa Ribosomal S6 Kinase

S

SIRT1 Sirtuin 1

T

TC Ternary Complex

TCA Tricarboxylic Acid

TERT Telomerase Reverse Transcriptase

TKI Tirosin Kinase Inhibitor

TOP Terminal Oligopyrimidine

TP53 Tmour Protein 53

U

uORFs upstream Open Reading Frame

UPR Unfolded Protein Response

UTR Untranslated Region

V

VEGFA Vascular Endothelial Growth Factor ligand A

VEGFR Vascular Endothelial Growth Factor Receptor

X

XIAP X-linked Inhibitor of Apoptosis Protein

XIAP X-linked Inhibitor of Apoptosis Protein