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Effect of 5-Fluorouracil on translational regulation in colorectal cancer cells

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TITRE

**Effect of 5-Fluorouracil on translational regulation in colorectal cancer
cells**

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Résumé en français

Le 5-Fluorouracile (5-FU) est un anti-métabolite intensément utilisé dans les traitements chimiothérapeutiques de la plupart des cancers. Il est souvent utilisé en première intention de traitement, seul ou en combinaison avec d'autres molécules. Aujourd'hui on constate un manque d'efficacité de cette molécule, notamment pour le traitement de cancers avancés qui résulte, en grande partie de la mise en place de mécanismes de résistance. Cependant, les mécanismes moléculaires de l'action de cet agent anti-cancer, son impact sur la biologie cellulaire et les processus de résistance restent encore largement à déterminer. Etant donné que le 5-FU est capable de s'incorporer dans toutes les classes d'ARN, nous avons postulé qu'il pouvait affecter la plupart des étapes de l'expression génique et en particulier la traduction puisque cette étape implique le décryptage précis d'une molécule d'ARN, l'ARNm par une autre molécule d'ARN, l'ARN ribosomique en faisant intervenir d'autres ARN comme les ARNt mais aussi des ARN régulateurs comme les petits et grand ARN non-codants.

Pour évaluer cette hypothèse, le travail conduit au cours de la thèse a consisté à caractériser les variations du « translatome » de cellules de cancers colorectaux induites par un traitement au 5-FU.

C'est ainsi que la connaissance de l'impact du 5-FU sur la régulation traductionnelle apportera non seulement des données pour mieux comprendre son mécanisme d'action et les mécanismes de mise en place de la résistance mais aussi de mieux appréhender la gestion des problèmes cliniques liés à son utilisation.

Dans le manuscrit de thèse, l'introduction présente brièvement la traduction et sa régulation et, de façon plus détaillée, l'état de la question concernant le mode d'action du 5-FU et la problématique clinique liée à son utilisation en chimiothérapie. Le rationnel, les résultats et la discussion de l'étude principale du travail de thèse sont ensuite présentés suivis de deux manuscrits d'articles en cours de soumission dans des revues internationales à comité de lecture.

Introduction

Partie I : Régulation traductionnelle chez les eucaryotes

Composants principaux de l'appareil traductionnel

La traduction est le processus qui permet de décoder les informations contenues dans les ARNm pour synthétiser les protéines. Il apparaît de plus en plus clairement que cette étape de l'expression génique est extrêmement régulée et que cette régulation joue un rôle crucial dans la physiopathologie cellulaire. En dehors des ARNm, l'appareil traductionnel actif est composé des ARNt, des ribosomes et de différents facteurs, ARN ou protéines, impliqués dans la régulation de son activité.

Chez les eucaryotes les gènes codants pour les ARNt sont transcrits par l'ARN polymérase III. Une des premières étapes de la spécificité de traduction est assurée par les ARNt et les amino-acyl transférase qui permettent d'identifier et de charger un acide aminé défini et de le positionner sur le codon correspondant au niveau de l'ARNm.

Le ribosome se compose de deux sous-unités : une petite sous-unité 40S et une grande sous-unité 60S. Chez les humains, la grande sous unité contient les ARNr 28S, 5,8S et 5S associés à 46 protéines ribosomiques ; la petite sous-unité comprend les ARNr 18S ARNr et 33 protéines

ribosomiques. Le ribosome possède une organisation fonctionnelle optimisée dans laquelle les ARN jouent un rôle crucial dans la mesure où ils portent les principales fonctions du ribosome. C'est ainsi qu'il est désormais admis que le ribosome est un ribozyme.

Le processus de traduction et sa régulation

La traduction se déroule en trois étapes : l'initiation, l'élongation et la terminaison. L'initiation est considérée comme une des étapes limitante dont la régulation impacte fortement le processus global de traduction. Sa régulation fait intervenir des facteurs trans tels que les facteurs d'initiation et des ARN non codants et des facteurs cis correspondant à des séquences localisées généralement dans les extrémités non traduites des ARNm. De nombreux exemples montrent que la dérégulation de la traduction, et en particulier de l'initiation, joue un rôle primordial dans la pathologie cancéreuse dans la réponse aux drogues utilisées en chimiothérapie.

Partie II : La traduction et les traitements des cancers

De nombreuses molécules utilisées en chimiothérapie anti-cancéreuse affectent l'expression génique au niveau de la traduction en agissant soit sur les composants de l'appareil traductionnel eux-mêmes soit sur les régulateurs. Toutefois, à l'heure actuelle les répercussions sur le phénotype cancéreux ou encore sur la réponse aux traitements restent encore peu étudiées. Parmi les drogues anti-cancer, le 5-Fluorouracil, une molécule qui s'intègre dans les ARN, est largement utilisé pour le traitement de la plupart des cancers, souvent comme traitement de première intention mais son mécanisme d'action et son impact sur la régulation de la traduction reste à élucider.

5-Fluorouracil

Depuis 1958, le 5-FU reste un traitement de référence pour de nombreuses tumeurs solides comme les cancers colorectaux et du sein. Plusieurs stratégies ont été développées pour améliorer l'efficacité du 5-FU. D'une part des stratégies permettant de moduler son efficacité en jouant sur son métabolisme, aussi bien anabolisme que catabolisme, et d'autre part en l'utilisant en combinaison avec d'autres molécules comme l'oxaliplatine ou l'irinotecan.

Mécanismes d'action de 5-FU : état des lieux

Le 5-FU cible la thymidylate synthase qui est l'enzyme clé responsable de la synthèse de novo de thymidine indispensable au métabolisme de l'ADN. Cette inhibition de l'activité de la thymidylate synthase induit une réduction de la synthèse de l'ADN et de la prolifération cellulaire.

Le 5-FU peut également être incorporé dans l'ADN, il avait donc été postulé que cette incorporation dans l'ADN était responsable de sa cytotoxicité. Cependant, le 5-FU peut également s'intégrer de façon efficace dans l'ARN et il apparaît de plus en plus clairement que c'est cette propriété qui est responsable de son effet cytotoxique. En effet, 5-FU affecte la maturation des pré-ARNm, des snARN, des ARNt et des ARNr.

RESULTATS

Effet du 5-FU sur la synthèse protéique globale

En toute première approximation et étant donné que le 5-FU est un anti-métabolite, nous avons évalué son effet sur le taux de synthèse protéique global. Pour cela nous avons mesuré les variations d'incorporation de [35S] méthionine suite au traitement de cellules HCT116 par du 5-FU. Ces expériences préliminaires montrent que le 5-FU n'induit pas de diminution majeure du taux de synthèse protéique lorsqu'il est utilisé à des doses faibles, qui néanmoins, induisent un effet cytotoxique.

Optimisation des conditions de traitement par le 5 -FU

L'impact du 5 -FU sur la viabilité cellulaire a été mesuré par différentes méthodes. Différentes doses et différents temps de traitement ont été effectués. L'objectif de ces expériences était d'établir une condition expérimentale pour laquelle la dose et le temps induisait une cytotoxicité partielle afin d'identifier les modifications 5-FU-induit du transcriptome pour des cellules sensibles mais qui avait pu résister à l'effet délétère du traitement. Une technique en point finale de la mesure de l'activité métabolique nous a permis de déterminer une IC50 à 24 h de 12,9µM.

Une technique de mesure en temps réel d'impédance (Technologie xCELLigence, ACEA) proportionnelle à la morphologie et au nombre de cellules vivantes a été utilisé pour suivre le comportement des cellules suite au traitement par le 5-FU pendant des temps allant jusqu'à 72 h. Cette technique a permis de confirmer que la viabilité d'un certain pourcentage de cellule n'était pas affectée suite à un traitement, même prolongé par 10µM. L'ensemble de ces données, complétées par des mesures de mortalité cellulaire utilisant du bleu Trypan nous ont permis de déterminer la dose et la fenêtre de temps de traitement correspondant à l'objectif fixé initialement : traitement pendant 24 h avec une dose de 10µM.

Effet du 5 -FU sur la production des ribosomes

Il est connu que le 5-FU peut perturber la maturation des ARNr et ainsi inhiber la biogenèse des ribosomes. Pour déterminer l'effet du 5-FU sur la biogenèse des ribosomes, nous avons quantifié les ARNr néosynthétisés par marquage métabolique de l'orthophosphate-³²P- et la quantité totale de ribosomes cytoplasmiques stables. Les résultats montrent que les cellules traitées par 10µM de 5-FU pendant 24 h continuent à produire des ribosomes et que cette production est suffisante pour maintenir une quantité de ribosomes cytoplasmiques jusqu'à 50-80 % de la quantité normale des ribosomes.

Effet du 5 -FU sur le transcriptome

Stratégie expérimentale : les cellules HCT116 ont été traitées pendant 24 h avec 10µM de 5-FU. Les ARNm actifs et inactifs niveau traductionnel ont ensuite été identifiés en séparant les ARNm cytoplasmiques engagés dans les polysomes de ceux non engagés par fractionnement sur gradient de saccharose et en analysant le contenu de chaque fraction par une puce Exon. Pour chaque ARN détecté grâce à la puce, le statut traduit vs non traduit a été estimé par mesure de sa distribution entre la fraction polysomique (P) et la fraction sous-polysomique (M).

Le rapport (P/M) de l'intensité du signal a été calculé pour chaque ARNm et un rapport de rapport (R2) a été calculé ((P/M (5-FU) et P/M (CTL)) qui représente la variation de statut traductionnel de chaque ARNm suite au traitement. En utilisant des seuils de $-1,3 \geq R2 \geq 1,3$, nous avons identifié 855 ARNm dont le statut traductionnel altéré. La traduction de 642 (75,08%) ARNm est augmentée tandis que celle de 213 ARNm (24,91%) est diminuée. Les résultats obtenus par cette analyse sur puce ont été validés par l'analyse en qRT-PCR de 13 ARNm.

Analyse fonctionnelle des gènes régulés en niveau traductionnel

Pour déterminer si certaines catégories fonctionnelles sont enrichies après traitement au 5-FU, nous avons effectué une analyse d'ontologie sur les 855 gènes régulés au niveau traductionnel en utilisant le programme « PANTHER GO annotation ». Nous avons étudié les processus biologiques, les fonctions moléculaires et les classes protéiques. On constate un enrichissement suivant le traitement pour les processus métaboliques primaires et pour la traduction. Cette analyse montre également un enrichissement pour les fonctions moléculaires impliquées dans les activités

de liaison de l'acide nucléique. Enfin cette analyse montre un enrichissement important pour différentes classes de facteurs de transcription.

Effet du 5-FU sur l'expression du gène HIVEP2 : impact sur le microARN miR-155

Un des gènes dont la traduction est fortement stimulée par le 5-FU est le gène HIVEP2. Il était connu que la traduction de l'ARNm d'HIVEP2 pouvait être régulée par miR-155. Nous avons donc déterminé si l'augmentation de la traduction d'HIVEP2 induite par le 5-FU était médiée, au moins en partie, par miR-155. Pour explorer l'effet de miR-155 sur la régulation de la traduction du gène HIVEP2 après le traitement au 5-FU, les cellules HCT116 ont été co-transfectées avec un vecteur d'expression codant pour la luciférase fusionnée à la séquence 3'UTR d'HIVEP2 contenant l'élément de réponse à miR-155 et un vecteur d'expression permettant l'expression de miR-155. Les résultats démontrent que la stimulation de l'expression induite par le 5-FU est bien inhibée par la surexpression de miR-155 pour la ramener à celle des cellules non-traitées. Ceci suggère que le 5-FU induit une diminution de l'expression de miR-155. En effet, par dosage de miR-155 par qRT-PCR, nous avons pu démontrer que le 5-FU induit une diminution de l'expression de miR-155. L'ensemble de ces données montrent donc que le 5-FU induit une stimulation de la traduction de HIVEP2 par un mécanisme miR-155 dépendant.

Discussion

L'ensemble des expériences conduites au cours de ce travail montrent que des doses faibles de 5-FU induisent une légère inhibition du taux global de la synthèse protéique, certes, mais qui est associée à une reprogrammation traductionnelle.

En effet, l'analyse des modifications du translatome induites par le 5-FU montre que cette drogue est capable simultanément de stimuler et d'inhiber la traduction d'un grand nombre d'ARNm qui codent pour des protéines possédant diverses fonctions. L'analyse fonctionnelle des gènes régulés par le 5-FU a permis d'identifier un groupe de gènes impliqués dans le processus de traduction. La majorité de ces gènes codent notamment pour des protéines ribosomiques (RPS15a, RPS16, RPL32 et RPL35a) ou des facteurs de traduction (eIF3E et eEF1A1). Ces gènes ont un rôle positif sur la prolifération des cellules tumorales, la progression et la résistance. Ils peuvent maintenir la survie cellulaire et inhiber la mort cellulaire dans les cellules traitées au 5-FU.

Un autre groupe de gènes dont la traduction est modulée par le 5-FU correspondent à des gènes codants pour des protéines impliquées dans le métabolisme général.

C'est ainsi que la traduction d'ARNm de plusieurs kinases est activée, ABL2, PIK3C2B et FRK. Par exemple, ABL2 est une kinase qui régule la croissance cellulaire, la survie et surtout permet le maintien de la prolifération des cellules cancéreuses dans des conditions de stress. ABL2 est activée dans les cancers du côlon et du sein.

On peut donc proposer que l'activation de l'expression de ces deux grandes fonctions activées suite au traitement par le 5-FU participent à la mise en place d'un mécanisme de défense cellulaire requise pour contrecarrer l'effet anti-prolifératif du 5-FU. Dans ces conditions expérimentales de temps et de dose, il est donc vraisemblable que l'impact de ces effets sur le devenir d'un certain pourcentage de cellules a été supérieur à ceux cytotoxique ce qui a permis la survie de ce groupe de cellules. Il sera donc intéressant d'analyser en détail les conséquences de l'expression de ces gènes sur le phénotype cellulaire et notamment sur la résistance au stress et aux agents cytotoxiques en particulier.

Par ailleurs, la traduction des gènes ING2, PPARD, PDP2 étant également stimulée, il est possible que certaines des étapes conduisant au processus de sénescence soient, en partie modulées par le 5-FU participant vraisemblablement à la dérégulation de l'équilibre entre les fonctions pro-tumorales et celles impliquées dans la suppression de tumeur comme celles médiées par les suppresseurs de tumeur GNG7, RASSF6, RREB1 et SOCS6 que l'on voit activés par le 5-FU. Enfin, un des résultats importants de ce travail est la démonstration de la stimulation de la traduction de l'ARNm du gène HIVEP2 par un mécanisme dépendant du microARN miR-155. Ceci confirme que le 5-FU est capable de moduler l'expression de microARN, comme ceci avait été démontré pour d'autres microARN. Cependant, en validant le mécanisme par lequel le 5-FU régule la traduction d'un gène clairement identifié, notre étude révèle un mécanisme de régulation traductionnelle modifié par le 5-FU qui peut affecter, et de façon coordonnée, la régulation de l'expression d'un réseau de gènes contenant les éléments de réponse à un microARN particulier. HIVEP2 code pour un facteur de transcription qui se compose de 2437 acides aminés contenant deux domaines à doigts de zinc C2H2. HIVEP2 est considéré comme un régulateur de différenciation des cellules T et dans la formation osseuse par son interaction directe avec les facteurs Smad1/4. Il peut interagir avec CLIC4 (chloride intracellular channel 4) qui est critique pour l'apoptose induite par p53 et c-Myc. HIVEP2 stabilise la phosphorylation de Smad 2/3 et donc régule la signalisation du TGF β . Dans les tissus neuronaux, l'expression d'HIVEP2 est absente dans les cellules prolifératives et abondante dans les cellules post-mitotiques. HIVEP2 est surexprimé au cours de la différenciation de gliome. L'expression d'HIVEP2 peut donc induire un arrêt de croissance et stimuler la différenciation des cellules neuronales. Son rôle n'a jamais été étudié dans le contexte du traitement par le 5-FU. Notre étude suggère donc qu'il pourrait être un acteur important de la réponse cellulaire induite par le 5-FU et notamment être un des composants dont l'expression est activée par le 5-FU et qui participe à l'arrêt de la prolifération cellulaire et éventuellement à la mise en place d'un programme de différenciation.

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Abbreviations

5-FdUrd: 5-fluoro-2'-deoxyuridine	FUMP: Fluorouridine monophosphate
5-FU: 5-Fluorouracil	FUrd: 5-Fluorouridine
ABCE1: ATP-binding cassette	FUTP: Fluorouridine triphosphate
AGO: Argonaute proteins	FUTP: Fluorouridine triphosphate
ASOs: antisense oligonucleotides	GCN2: General control non-derepressible-2
ATC: anatomical therapeutic chemical	GIST: Gastrointestinal stromal cancer
AUC: area under the curves	GNG7: G protein gamma 7
BER: base excision repair	H ₂ O ₂ : Hydrogen peroxide
BIC: B-cell integration cluster	HCC: Hepatocellular carcinoma
CBC: cap-binding complex	HCV: Hepatic C virus
CH ₂ -THF: 5, 10-methylene-tetrahydrofolate	HDFs: Human diploid fibroblasts
CHX: Cycloheximide	HIF-1 α : Hypoxia-inducible factor-1 α
CI: Cell index	HIV-1: Human immunodeficiency virus type 1
CLIC4: Chloride intracellular channel 4 protein	I β : Interferon-binding protein 2
CML: Chronic myeloid leukemia	HRI: Heme-regulated inhibitor
CPT: Camptothecin	INFs: Interferons
CPT-11: Irinotecan	I-OHP: Oxaliplatin
CRC: Colorectal cancer	IRES: Internal ribosomal entry site
Cryo-EM: cryo-electron microscopy	ITS: internal transcribed spacer
CSN: COP9 signalosome complex	LOH: Loss of heterozygosity
CTX: Cyclophosphamide	LSU: Large subunit
CrPV: Cricket paralysis virus	LV: Leucovorin
DC: Decoding center	M: Monosome
DHF: Dihydrofolate	m ⁵ C: 5-Methylcytosine
DHFR: Dihydrofolate reductase	m ⁷ G: 7-Methylguanosine
DNA: Deoxyribonucleic acid	MBP-2: Major histocompatibility complex
DOXO: Doxorubicin	MHC class 1 binding protein 2
DPD: Dihydropyrimidine dehydrogenase	mCRC: Metastatic colorectal cancer
dTMP: Deoxythymidine monophosphate	Met-tRNA ⁱ : Initiator methionyl-tRNA
dUMP: Deoxyuridine monophosphate	MIBP1: c-myc intron 1 binding protein 1
eIF: Eukaryotic initiation factor	miRNA: microRNA
EMCV: Encephalomyocarditis virus	MMR: Mismatch mechanism
ETS: External transcribed spacer	mTOR: Mammalian target of rapamycin
FA: Folic acid	MTS: Tetrazolium agent
FC: Fold change	MTX: Methotrexate
FdUMP: Fluorodeoxyuridine monophosphate	NDV: Newcastle disease virus
FdUTP: Fluorodeoxyuridine triphosphate	OIS: Oncogene-induced senescence
FGF-2: Fibroblast growth factor	OPRT: Orotate phosphoribosyltransferase
FUDR: Fluorodeoxyuridine	
FUMP: 5-Fluorouridine monophosphate	

P: Polysome
 PABP: PolyA binding protein
 PDP2: Pyruvate dehydrogenase phosphatase catalytic subunit 2
 PD-ECGF: Platelet-derived endothelial cell
 PDT: Photodynamic therapy
 PDH: Pyruvate dehydrogenase enzyme
 PERK: PKR-like endoplasmic reticulum kinase
 PIC: Pre-initiation complex
 PKC: Protein kinase C
 PKR: Protein kinase double-stranded RNA-dependent
 PPAR δ : Peroxisome proliferator-activated receptor delta
 pre-miRNA: Precursor miRNA
 pri-miRNA: Primary miRNAs
 PRPP: 5-phosphoribosyl-1-pyrophosphate
 PRPP: Phosphoribosyl pyrophosphate
 PTC: Peptidyl transferase center

 RACK1: Receptor of activated C kinase
 rDNA: Ribosomal DNA
 RNA: Ribonucleic acid
 RNA Pol I, II and Pol III: RNA polymerase I, II and III
 ROS: Reactive oxygen species
 RP: Ribosomal protein
 RR: Ribonucleotide reductase

 RREBP1: Ras responsive element binding protein 1
 rRNA: Ribosomal RNA
 siRNA: Short interfering RNAs
 SOCS6: Cytokine signaling 6
 SSU: Small subunit

 t-BHP: Tert-butylhydroperoxide
 TC: Ternary complex
 TDG: Thymidine DNA glycosylase
 THF: Tetrahydrofolate.
 TK: Thymidine kinase
 TP: Thymidine phosphorylase
 tRNA: Transfer RNA
 TS: Thymidylate synthase
 TSER: Thymidylate synthase gene
 VEGF: Vascular endothelial growth factor

 uAUG: Upstream AUG
 UDG: Uracil DNA-glycosylase
 UDPG: Uridine diphosphoglucose
 UK: Uridine kinase
 uORF: Upstream open reading frames
 UP, UPase: uridine phosphorylase

 Ψ SI: Pseudouridine synthase I
 Ψ : Pseudouridine

INTRODUCTION

Part I

Eukaryotic translational regulation

1. Overview of core effectors of eukaryote translational apparatus

Translation is the biological process responsible for protein synthesis of all living organisms. This is a very efficient and highly regulated process. For example, it has been predicted in yeast that about 13,000 proteins can be produced per second (von der Haar 2008). Translation proceeds following three main steps: initiation, elongation and termination. Translation is performed with a high degree of precision by ribosomes, tRNAs and several translation factors.

1.1. Transfer RNA

The genes coding for transfer RNAs (tRNAs) are transcribed by RNA Polymerase III (Paule and White 2000) to give rise to a precursor (pre-tRNA) that is processed in the nucleoplasm and for some part within nucleoli (Bertrand, Houser-Scott et al. 1998). The mature tRNA adopts a characteristic 3D conformation of a cloverleaf (Fig. 1) (Kowal, Kohrer et al. 2001).

The first step of protein synthesis selectivity to translate the genetic code occurs when specific amino acids are attached to specific mature tRNAs containing a defined anticodon sequence to form amino-acyl-tRNAs. This is performed by specific aminoacyl-tRNA synthetases. Addition of the amino acid occurs at adenosine residue of the ACC highly conserved sequence of the tRNA (Fig 1). Anticodon formed by sequence arrangement of nucleotides 34, 35 and 36 is localized at the opposite position. Transcripts of tRNAs undergo extensive post-transcriptional modifications necessary for their structure and ultimately their function (El Yacoubi, Bailly et al. 2012). For example dihydrouridine is one of the chemical modifications allowing a better flexibility and dynamic motion ability of tRNAs (Dalluge, Hashizume et al. 1996). Pseudouridine (Ψ) is another chemical modification that plays a crucial role in tRNA stability (Durant and Davis 1999). tRNAs base modifications are required for translation accuracy. Modifications of the anticodon nucleosides are important for the stringency of codon-anticodon recognition (Yarian, Townsend et al. 2002). As a consequence, alterations of tRNAs chemical modifications and of the enzymes responsible of these modifications have been shown to be involved in human pathologies such as diabetes type 2, neurological disorders and cancers (Torres, Batlle et al. 2014). For example, NSUN2 has been reported to be overexpressed in breast and colon human tumors (Frye and Watt 2006; Torres, Batlle et al. 2014). NSUN2 is a methyltransferase that catalyzes methylcytosine (m^5C) formation at different positions of many tRNAs, but in particularly at position 34 of anticodon of tRNA^{Leu} which is critical for translational accuracy.

1.2. The ribosome

The ribosome is the machinery that decodes the mRNA and converts the coding sequence into a protein. This machinery has been identified in the 1960s and many efforts have been made since then to elucidate its structure, the molecular mechanisms of protein synthesis and the modes of regulation of its activity. Structure and composition of ribosomes have been resolved for several prokaryotes and archae such as *Thermus thermophilus* (Yusupov, Yusupova et al. 2001), *Haloarcula marismortui* (Ban, Nissen et al. 2000). The first atomic model of eukaryotic 80S ribosome has been reported for *Saccharomyces cerevisiae* using x-ray crystal data resolved at a 3.0 Å resolution. This model that contains all eukaryotic specific proteins and the universally conserved ones points out two different ribosomes in the 40S rotation degree (Ben-Shem, Garreau de Loubresse et al. 2011). Recently, ribosome structures of *Drosophila melanogaster* and *Homo sapiens* have been determined using resolution density maps processed from cryo-electron microscopy (cryo-EM) images for the 80S ribosome in a complex with eEF2, tRNA in E site and Stm1-like proteins (Anger, Armache et al. 2013). The ribosome is a large macromolecular complex made of RNAs and proteins. In eukaryotes, the functional ribosome (80S) is composed of a small subunit (SSU) of 40S and a large subunit (LSU) of 60S. The ribosome contains four ribosomal RNA (rRNA): 18S, 5.8S, 5S and 25S/28S for yeast and higher eukaryotes respectively and 79 ribosomal proteins (RPs). In humans, the 60S subunit contains the 28S, 5.8S and 5S rRNAs and 46 RPs, while the 40S subunit is composed of the 18S rRNA and 33 RPs (Fig. 2) (Graifer and Karpova 2012).

Eukaryotic ribosomes biogenesis occurs mainly within nucleoli. It requires a highly coordinated regulation of the three RNA polymerases (RNA Pol I, II and III) to transcribe the rDNAs genes (by RNA Pol I and RNA Pol III) and the genes coding for ribosomal proteins (RNA Pol II). The pre-rRNAs undergo many maturation reactions to produce mature rRNAs that are assembled with ribosomal proteins to form pre-40S and pre-60S that are then exported to the cytoplasm. More than 200 transiently associated factors are involved in assembly, maturation, quality-control and export (Fig. 3) (Lempiainen and Shore 2009).

All the biogenesis steps lead to a mature ribosome that is functionally organized into two subunits, each involved in specific functions. The 40S subunit contains the mRNA path and the decoding center (DC) where the codon-anticodon interaction is controlled, and the binding sites for several translational eukaryotic initiation factors (eIFs). The 60S subunit contains the peptidyl transferase center (PTC) responsible for catalyzing the peptide bond formation. The 60S subunit contains also the exit tunnel involved in co-translational protein folding and modifications (Graifer and Karpova 2012). For protein synthesis the 80S ribosome is organized following three main sites (Fig. 4): (i) the peptidyl-tRNA binding site (P site), is the first to be occupied by the initiator tRNA_i^(Met) that carries a methionine (Met) and can hybridize with the mRNA start codon; (ii) the aminoacyl-tRNA binding site (A site), is the second site to be filled by a tRNA that carries the amino acid corresponding to the second codon of the mRNA; (iii) the exist site (E site) for deacyl-tRNA exit resulting from peptide bound formation of the amino acid with the nascent peptide. The

peptide bond formation is highly dynamic and requires several movements of the ribosome. During each elongation cycle only two binding sites are occupied. Peptidyl bond forms when the peptidyl-tRNA in P site transfer the peptide chain to the aminoacyl-tRNA in A site. Next, the new peptidyl-tRNA translocates from A to P site, while the deacyltRNA translocates from P site to E site to exit the ribosome when a new aminoacyl-tRNA binds to A site (Melnikov, Ben-Shem et al. 2012).

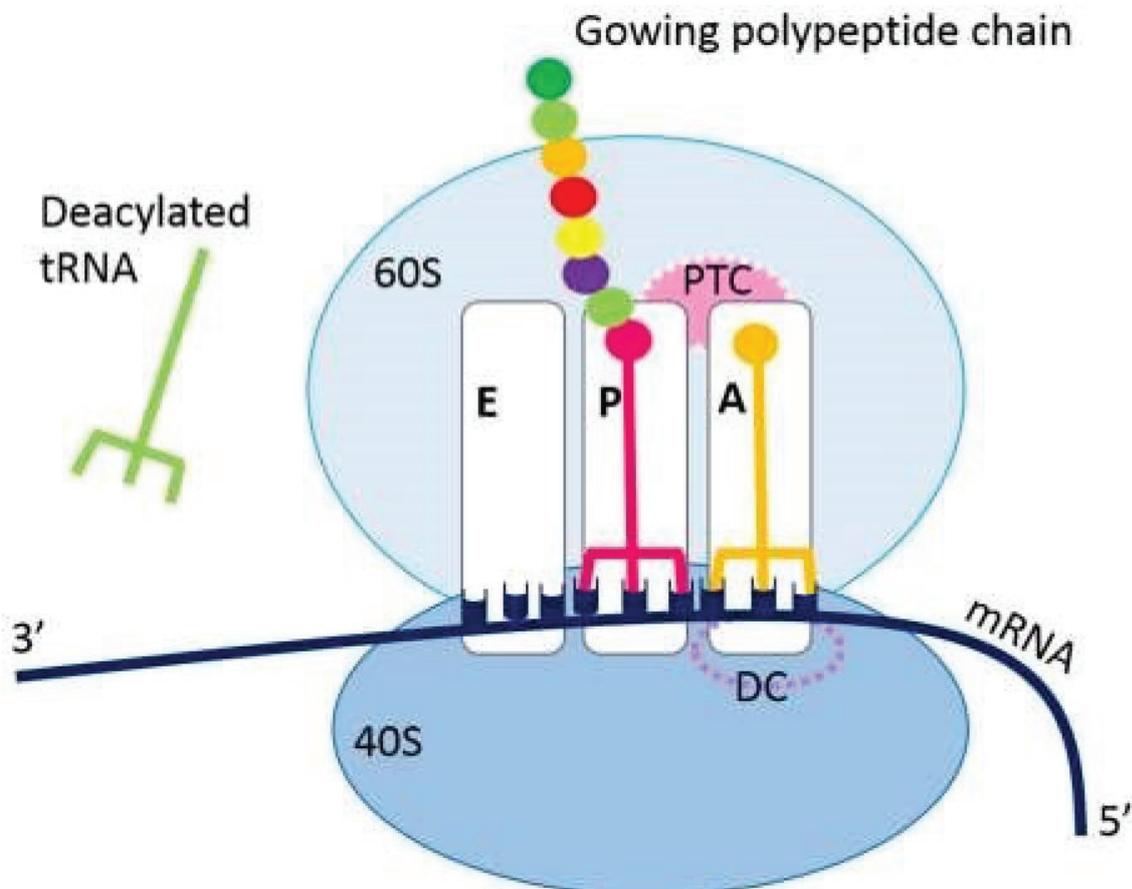


Figure 4. The ribosome structure. The ribosome consists of the small subunit (40S) and the large subunit (60S). Three tRNA binding sites are known: the site A- site occupied by aminoacyl-tRNA, the P- site for the peptidyl tRNA and the E- site that contains the empty tRNA. DC, decoding center; PTC, peptidyl transferase center.

Ribosomal RNA

Ribosomal RNA (rRNA) transcripts could represent up to 50 % of the total nascent RNAs in the cell. In humans, the genes coding for the 28S, 18S and 5.8S are organized in tandem repeat units. About 400 copies of these rDNA genes are distributed on the 5 acrocentric chromosomes, 13, 14, 15, 21 and 22. Each unit exhibits 43-kb that contains 13-kb of transcribed region and 30-kb of intergenic spacers. The 43-kb rDNA unit is transcribed by RNA Pol I into a large 47S rRNA precursor. This primary rRNA contains internal transcribed spacers (ITS) that separate the 28S, 18S and 5.8S sequences, in addition to external transcribed spacers (ETS) at each end of the transcript. This pre-rRNA undergoes many methylation and pseudouridylation reactions that are carried out by snoRNPs and by enzymatic complexes containing fibrillarin and dyskerin

respectively. ITS and ETS are removed from the 47S pre-rRNA by several endonucleases and exonucleases to produce the three mature rRNAs: 28S, 18S and 5.8S (Fig. 5).

The 5S rDNA tandem repeat unit contains 2.2 kb and is transcribed by the RNA Pol III (Srivastava and Schlessinger 1991; Stults, Killen et al. 2008).

The functional role of rRNAs has been definitely revealed after atomic resolution of ribosomal structure. It has been demonstrated that, within the mature ribosome, the peptidyl transferase center (PTC) contains only RNA and no proteins. Thus, rRNAs exhibit ribozyme activity since they catalyze peptide bond formation from the aminoacyl-tRNA and the peptidyl-tRNA (Nissen, Hansen et al. 2000; Steitz and Moore 2003; Moore and Steitz 2011).

Ribosomal proteins

RPs represent two-thirds to one-half of the ribosome mass in eukaryotes and prokaryotes respectively (Siekevitz and Zamecnik 1981). Genes coding for ribosomal proteins are transcribed by RNA Pol II, mRNA translated within cytoplasm and RPs are imported into the nucleoli to be assembled with rRNAs. RPs from the small ribosomal subunit are called S1, S2, S3, up to S31; except for one ribosomal protein in human which is called RACK1 (Receptor of Activated C Kinase). RPs from the large subunit are called L1, L2, L3, up to L44; except P1/P2, P0 and LP1/P2, LP0 in yeast and human respectively (Fig. 2) (Ban, Beckmann et al. 2014).

Although rRNA activity is crucial for ribosome catalytic activity, RPs play very important and complementary roles for the translation. RPs are indispensable for an efficient translation, they are crucial for stabilizing many rRNAs sites, the interaction between the two subunits and sustaining the optimal configuration of ribosome (Fig. 6). For example, many RPs are essential for mRNA interaction with the ribosome (S7, S11 and RACK1) (Brodersen and Nissen 2005). RACK1 is a multifunctional ribosomal protein located on the 40S subunit close to the E site. It acts as an activating platform for many kinases and mRNA binding proteins such as protein kinase C and Scp160p, respectively (Nilsson, Sengupta et al. 2004). Additionally, RACK1 can regulate mRNA translation *via* interacting with miRISC complex and mediating miRNA translation repression (Jannot, Bajan et al. 2011).

RPs can participate in extra-ribosomal activities that can control the balance of ribosomal components through the auto-regulation of RPs synthesis. In human cells, S13 regulates the level of its own mRNA by binding to the first intron of its transcript leading to splicing inhibition. Furthermore, RPs extra-functions support the nucleolar stress and abnormal ribosomal synthesis (Warner and McIntosh 2009). For example, L23 can activate P53 *via* its binding to MDM2 and L23 overexpression results in apoptosis or cell cycle arrest (Dai, Zeng et al. 2004). Interestingly, certain RPs genes display differential expression that is related to particular pathologies such as cancer. The mRNA transcripts coding for RP S3, S6, S8, S12, L5 and P0 display elevated levels in both colon tumors and polyps (Pogue-Geile, Geiser et al. 1991). However, the role played by this specific differential overexpression in the physiological and pathological process remains elusive until present, it is not clear whether pathological deregulation of RP genes expression result of an alteration of ribosomal and/or extraribosomal RP functions. However, the discovery of

multifunctional potential of RPs allows supporting the diversity of physio-pathological processes that occur as a result of RP genes expression alterations.

2. Translational process and main features of its different modes of regulation

The translational process proceeds following three main steps: initiation, elongation and termination. The whole process is orchestrated by several trans- and cis-acting regulators to ensure the efficiency and the accuracy. A global view of translation initiation is presented in (Fig. 7).

2.1. Translational initiation and its regulation

One of the first identified feature of eukaryotic translational regulation is mediated by the presence of a particular structure named the Cap, at the 5' extremity of mRNAs synthesized by RNA pol II. The eukaryotic mRNA capping is the addition of a 7-methylguanosine (m^7G) to the first transcribed nucleotide at the 5' end by forming an unusual 5'-5' triphosphate linkage (Shatkin 1976). Capping synthesis occurs at early stage after transcription and is catalyzed by RNA triphosphatase, RNA guanylyltransferase and RNA (guanine-7) methyltransferase enzymes (Venkatesan, Gershowitz et al. 1980; Shuman 2001). The cap structure interacts with many proteins such as the nuclear cap-binding complex (CBC), which affects the transcription process through recruiting many transcriptional factors to promoters and elongation factors. CBC is also involved in mRNA splicing through a direct interaction with U4/U6.U5, the snRNP components of the spliceosome. In addition, CBC can compete with decapping enzymes and participates in mRNA stability (Gonatopoulos-Pournatzis and Cowling 2014). The cap structure has also an important influence on mRNA transcript stability. A study carried in HeLa cells has demonstrated that uncapped mRNAs were totally degraded by exoribonucleases, while the capped mRNAs were stable due to the cap structure that protect them from degradation (Murthy, Park et al. 1991).

Two main specific enzymes catalyze Cap hydrolysis. The first one is the Dcp2 enzyme that attacks only the capped mRNAs, while the second one is the DcpS, which functions on free Cap structures of degraded mRNAs (Arribas-Layton, Wu et al. 2013).

In eukaryotes, a large number of mRNAs initiate their translation by a Cap-dependent mechanism, which depends on the recognition of m^7G cap structure at the 5' end of the mRNA. This process is highly organized and requires the functional coordination of at least 12 eukaryotic initiation factors (eIFs) (Sonenberg and Hinnebusch 2009).

The first step of Cap-dependent translation initiation is the assembly of a ternary complex (TC) that consists of the initiator methionyl-tRNA ($Met-tRNA_i$), eIF2 and GTP (Fig. 7) (Valasek 2012).

Then, initiation factors eIFs 1, 1A, 3 and 5 work together to induce recruitment of the TC to a free 40S subunit to give the 43S pre-initiation complex (PIC) that displays an "open" conformation (Passmore, Schmeing et al. 2007; Sonenberg and Hinnebusch 2009; Liljas 2013). Another possibility is the assembly of eIFs 1, 3 and 5 with TC to form a large multifactor complex

(MFC), together with eIF1A they will binds to 40S and form 43S PIC complex (Asano, Clayton et al. 2000).

The eIF3 is necessary for binding the ternary complex to the 40S to form the PIC complex (Dong and Zhang 2006; Hinnebusch 2006). Additionally, eIF3 interacts directly with the mRNA (Dong and Zhang 2006). Together, eIF1 and 1A promote a conformation change that converts the closed empty PIC into an open scanning competent form. This form opens the latch of the mRNA channel resulting in mRNA loading and impedes the lock of this latch at non-AUG codons. However, neither eIF1 nor eIF1A is able alone to perform this conformational change (Passmore, Schmeing et al. 2007; Martin-Marcos, Nanda et al. 2013).

Importantly, Cap structure through its 5'-5' bond can impede mRNA entering an abnormal PIC that lacks some of its essential factors (Mitchell, Walker et al. 2010). Intervention of Cap structure in translational regulation is the main reflect of its ability to be recognized by eIF4E. The interaction between eIF4E and cap structure is highly controlled by phosphatidylinositol-3 kinase PI3K/AKT, RAS/mitogen activated protein kinase (MAPK) and the mammalian target of rapamycin (mTOR) signaling pathways. eIF4E is activated by mTOR that phosphorylates and dissociates its binding protein 4E-BP1. mTOR is activated by the main cellular survival PI3K/AKT signaling pathway and by RAS/MAPK signaling. These pathways are involved in tumorigenesis mainly by modulating Cap dependent translation through eIF4E activation (Silvera, Formenti et al. 2010). Furthermore, it has been demonstrated that mTOR interacts directly with eIF3 and controls its association with eIF4 (Harris, Chi et al. 2006).

The interaction between the mRNA and the translational machinery is mediated by eIF4F complex that consists of the 4E Cap binding subunit, the 4A subunit, an RNA helicase, and the 4G subunit a scaffold protein together with eIF3, eIF4B and the poly (A)-binding protein (PABP). These factors promote mRNA recruitment to the 43S PIC to produce the 48S, the initiation intermediate that will be able to scan the 5'mRNA UTR to find the AUG initiator codon (Martineau, Derry et al. 2008). During 5'UTR scanning, eIF4B factor enhances strongly the RNA helicase activity of eIF4A, which alone exhibits an insufficient activity (Rozen, Edery et al. 1990; Jaramillo, Dever et al. 1991). Stimulating eIF4A activity results in unwinding the secondary structures within 5'UTR of the mRNA in an ATP-dependent reaction (Hinnebusch 2014).

Identification of the AUG initiator codon could be performed by the nature of the surrounding nucleotides as determined by the Kozak's rules. According to these rules, the AUG codon is surrounded by a specific nucleotide context called the Kozak consensus sequence. It has been shown that -3 and +4 nucleotide positions (relatively to AUG) has an important effect on translation initiation frequency. The optimal Kozak context has an (A) in the -3 position and a (G) in the position +4. In case of unfavorable context, the scanning complex will bypass the AUG and eventually initiate translation at the next AUG codon, therefore modulating the initiation of translation (Kozak 1986).

After initiator codon recognition, eIF5B joins the complex allowing recruitment of the 60S subunit to form the 80S initiation complex. During these two steps, several eIFs are released. eIF5

(a GTPase protein) mediates conversion of eIF2 to its GDP-bound state resulting in PIC “closed” conformation and release of eIF2.GDP and eIF5 (Nanda, Saini et al. 2013). Then, eIF2.GDP will be recycled by eIF2B that regenerates an active eIF2.GTP for a new translation round (Kimball 1999; Das and Maitra 2001). Finally, eIF5B and eIF1A are released allowing formation of the first peptide bond and elongation cycles (Fringer, Acker et al. 2007).

An upstream open reading frame (uORF) can be presented within the 5'UTR of mRNA when an inframe stop codon is found after an upstream AUG (uAUG) and before the main AUG (Fig. 8). Occurrence of uORF is frequent since it has been shown that half of the proteins coding genes have such uORFs (Calvo, Pagliarini et al. 2009). uORFs affect translation efficiency and downstream expression of the main ORF. Furthermore, presence of uORFs is correlated with 30% to 80% reduction in protein expression (Calvo, Pagliarini et al. 2009).

When uAUG is recognized, the scanning ribosome translates the uORF and when it reaches stop codon of the uORF, multiple fates are possible: (i) the ribosome can stay on the mRNA, resume scanning and reinitiate at the main ORF's AUG; (ii) it can stall and leave the mRNA without translating the main ORF resulting in reduced protein expression (Morris and Geballe 2000); (iii) in the absence of upstream stop codon, the ribosome can continue scanning to produce an extended protein (Ingolia 2014). However, the scanning ribosome can bypass the uAUG (probably if this uAUG displays a poor Kozak context) and initiate translation from the distal AUG of the main ORF according to a mechanism called leaky scanning (Fig. 9) (Morris and Geballe 2000).

Leaky scanning mechanism has been shown to modulate translation rate of important proteins according to environmental modifications. For example, protein kinase C (PKC) is a serine/threonine kinase involved in cell growth regulation. A novel protein kinase C isoform, the PKC η has been reported to act as an anti-apoptotic protein and its expression was correlated with drug resistance (Rotem-Dai, Oberkovitz et al. 2009). The 5'UTR of PKC η contains two conserved uORFs that regulate its translation. During normal conditions, the two uORFs suppress protein expression in growing breast cancer cells (MCF-7). While under stress conditions (amino acid starvation), they increase protein expression PKC η through leaky scanning that allows ribosome to reach the main ORF (Fig. 9). Experiments using mutated uORFs have demonstrated a de-repression of luciferase reporter regardless of the presence or absence of physiological stress conditions and/or the expression GCN2 kinase that phosphorylates eIF2, the ratelimiting factor for the first step of initiation (Raveh-Amit, Maissel et al. 2009).

Other functional elements within the 5'UTR can also regulate translation under stress conditions. These elements are called internal ribosomal entry sites (IRESs). They allow protein synthesis to be continued in a subset of mRNAs whereas initiation does not require the 5'Cap structure. IRESs can bind directly to the 40S ribosomal subunit in a complete or partial absence of initiation factors (Thompson 2012).

Two major initiation factors, eIF2 and eIF4 contribute importantly to the regulation of translation initiation. eIF2 is responsible of ternary complex formation that subsequently will bind to the ribosomal subunit to form the 43S complex. This step depends on eIF2 recycling and

phosphorylation. While, eIF4F mediates the association of mRNA to form the 48S initiation complex.

Additionally, the polyA binding protein (PABP) is implicated in translation initiation and it can be considered as a translation initiation factor like. Furthermore, miRNAs can also regulate the translation of targeted mRNAs through their 5' or 3' UTRs.

The eukaryote translation initiation factor eIF2

eIF2 is a heterotrimeric factor that consists of three subunits α , β and γ . It has an essential role in recognizing the unique initiator Met-tRNA_i and binding it to P site on the 40S subunit (Valasek 2012). As illustrated in (Fig. 10), eIF2 can binds GTP and the Met-tRNA_i forming the ternary complex (TC) that consists of Met-tRNA_i.eIF2.GTP. Then, eIF2 promotes the TC to bind to 40S subunit (Shin, Kim et al. 2011). After start codon recognition, eIF5 interacts with eIF2 β subunit and induces a conformational modification of eIF2 leading to activation of the GTPase activity of eIF2 γ subunit. Together, these events result in eIF2.GDP release. Then, eIF2B binds to eIF2.GDP and regenerates the active eIF2.GTP ready to enter a new translation round (Kimball 1999; Das and Maitra 2001).

The phosphorylated (p-eIF2) and unphosphorylated eIF2 availability levels has different consequences on protein synthesis process. eIF2 can be phosphorylated during stress conditions by several kinases such as PKR and CGN4. p-eIF2 inhibits eIF2B and causes a global and generally strong reduction in translation. However, p-eIF2 can enhance the translation of specific mRNAs as an adaptive response to stress, such as CHOP and ATF4 through leaky scanning or translation re-initiation, respectively (Wek, Jiang et al. 2006). In normal conditions, the uORF of CHOP mRNA represses its translation. The induction of eIF2 phosphorylation under stress conditions allows the ribosome to bypass the inhibitory sequence of uORF and translate CHOP (Palam, Baird et al. 2011). The ATF4 has two uORFs, the first uORF1 is considered as a positive-acting element that permits ribosome to resume scanning and reinitiate at the downstream uORF2. Whereas, uORF2 inhibits the downstream translation of ATF4 coding region. In stressed cells, both p-eIF2 and the reduced eIF2.GDP recycling increase the time for ribosome scanning and allow the ribosome to bypass the uORF2 and reinitiate translation at the main ORF (Vattem and Wek 2004).

The unphosphorylated eIF2 leads to translation increase and tumor development. In NIH 3T3 murine cells, a form eIF2 α that cannot be phosphorylated has been produced by mutated its unique phosphorylation site serine 51 into alanine leading to appearance of malignant transformation properties (Donze, Jagus et al. 1995). Moreover, inactivation of PKR kinase, responsible of eIF2 α phosphorylation, increases unphosphorylated eIF2 α levels and enhances proliferation in NIH 3T3 cells (Meurs, Galabru et al. 1993). Finally, cells expressing mutated eIF2 α or inactive PKR had a tumorigenic capacity and were able to develop large tumors after their injection into nude mice (Meurs, Galabru et al. 1993; Donze, Jagus et al. 1995).

eIF2 α phosphorylation plays also an important role in response to certain anticancer therapies. For example, Bortezomib is proteasome inhibitor used to treat multiple myeloma cells. It has been reported that a fraction of cells can survive and become quiescent under Bortezomib. These surviving cells showed a reduction in eIF2 α phosphorylation. Therefore, inhibition of eIF2 α dephosphorylation by specific phosphatase inhibitor such as Salubrinal can sensitize cells to Bortezomib treatment (Schewe and AguirreGhiso 2009). Together, these studies suggest that

deregulation of eIF2 activity either directly or indirectly through one of its phosphorylating kinases can contribute to cancer initiation, progression, chemosensitivity and resistance.

The eukaryote translation initiation factor eIF4F

eIF4F plays a major role in translational regulation, it binds to mRNA and stimulates the ribosomal 40S subunit loading onto mRNA (Fig. 11). eIF4F is a heterotrimeric complex that consists of eIF4E, eIF4A and eIF4G. eIF4E is a Cap binding protein that recognizes the m⁷G of Cap structure of mRNA during Cap-dependent translation initiation (Pestova and Kolupaeva 2002). eIF4A is a DEAD-box RNA helicase that can unwind the secondary structures of the 5'UTR and ensure PIC binding close to the Cap structure. eIF4G exhibits a scaffold function and acts as a temporary platform that binds 4E, 4A subunits, PABP, eIF3 and mRNA to stimulate its binding to PIC (Valasek 2012) (Fig. 11). The formation of eIF4F complex depends on eIF4E availability, eIF4E binding proteins (4E-BPs) bind to eIF4E causing repression of Cap dependent translation initiation. However, the phosphorylation of 4E-BPs leads to their dissociation from eIF4E and releases repression. PI3K-AKT and MAPK/ERK pathways mainly regulate the 4E-BP phosphorylation. The activation of these pathways lead to the downstream activation of mTOR, which phosphorylates the eIF4E binding proteins (4E-BPs). Importantly, eIF4E can acts as an oncogene and it has been reported to be upregulated in many human cancers such as prostate cancer (Furic, Rong et al. 2010; Jia, Polunovsky et al. 2012). A recent study has analyzed the expression of phosphorylated eIF4E (p-eIF4E) in 380 patient tumors of 17 cancers types. The tissue microarrays of these samples revealed that phosphorylated eIF4E (p-eIF4E) expression was significantly increased in tumor tissues compared to normal ones. Furthermore, p-eIF4E has a higher expression in early stages than advanced stages in colorectal cancers suggesting the implication of eIF4E in early stages of tumorigenesis (Fan, Ramalingam et al. 2009). Therefore, eIF4E is considered as an attractive target for anticancer drugs and eIF4E-specific antisense oligonucleotides (ASOs) have been developed to target *eIF4E* mRNA expression.

Indeed, eIF4E ASOs decreasing expression of eIF4E in human tumor xenografts inhibited the growth of tumor cells (Graff, Konicek et al. 2007). Recently, it has been demonstrated that eIF4E expression could be downregulated by miR-497 that targets its 3'UTR. miR-497 was frequently downregulated in gastric cancers and associated with aggressive pathologies. Interestingly, eIF4E and miR-497 has shown a reverse correlation in gastric cancers suggesting the possibility of eIF4E targeting through its miR-497 (Li, Jin et al. 2014). These results emphasize on the emerging role of eIF4E as a target for anti-cancer drugs.

The poly (A) binding proteins (PABP)

The majority of eukaryotic mRNAs undergo a polyadenylation process, which consists of adding a long tail of adenosines to the 3' end of the nascent mRNA (Proudfoot 2011). This process is necessary for mRNA stability, mRNA export to the cytoplasm and efficiency of translation (Mangus, Evans et al. 2003). The poly (A) tail protects the 3' end of an mRNA from ribonuclease attack and the subsequent degradation (Kuraishi, Sun et al. 2000). It is added by poly (A) binding

complex that contains the poly (A) polymerase and additional poly (A) binding proteins ; the PABPs cover the nascent poly (A) tail and control its final size. When the mature mRNA gets its poly (A) tail, it exits nucleus to reach the cytoplasm and the PABPs can assist the mRNA export through interacting with certain nucleoporins (Mangus, Evans et al. 2003).

The main role of polyA tail in translational regulation is due to PABPs which stimulate the formation of an mRNA closed loop through interaction between 5' Cap and 3' PABPs (Fig. 11). Through the closed loop arrangement and the cooperative interactions among PABPs, eIF4G and eIF4E promotes 40S recruitment and initiate translation (Tomek and Wollenhaupt 2012).

The effect of PABP on translation initiation was studied using PABP depleted extracts of Krebs-2 mammalian cells. Depletion of PABP strongly reduced the formation of 80S initiation complex and the capacity of cellular extracts to translate mRNAs that harbor or not a polyA. However, the addition of PABP to depleted extracts exhibited recovered translation inhibition and stimulated the formation of 80S initiation complex (Kahvejian, Svitkin et al. 2005). These results suggest that PABP mediates initiation through stimulating the formation of 80S initiation complex either through polyA or through its own interaction with other factors. Interestingly, PABP depletion impaired the interaction between eIF4E and Cap structure. To investigate the importance of eIF4G/PABP interaction in stimulating translation, a mutant PABP unable to bind eIF4G was supplemented to depleted extracts. Although, the mutant PABP had the same affinity to polyA than the wild type, it could not restore the translation while the wild type did (Kahvejian, Svitkin et al. 2005). PABP can stimulate translation by stimulating the recruitment of initiation factors to mRNA. However, PABP and polyA can promote miRNA translation repression by facilitating miRISC association on the targeted mRNA. Then, PABP leaves the translationally repressed mRNA (Moretti, Kaiser et al. 2012). Therefore, microRNAs can directly induce deadenylation resulting in subsequent mRNA degradation (Wu, Fan et al. 2006; Braun, Huntzinger et al. 2011).

microRNAs

microRNAs (miRNAs) are single-strand non-coding small RNAs consisting of about 22 nucleotides. They bind to different mRNA targets and regulate their expression. The miRNAs genes exist either as unique units or as gene clusters in the genome (Stroynowska-Czerwinska, Fiszler et al. 2014). miRNAs of a cluster are transcribed together as one primary transcript such as miR-17-92 cluster that contains miR-17-5p, miR-17-3p, miR-18a, miR-19a, miR-20a, miR-19b-1 and miR-92a-1 (Bonauer and Dimmeler 2009). miRNAs are transcribed by RNA Pol II and they are generated from non-coding regions of transcripts or from the intergenic regions (Stroynowska-Czerwinska, Fiszler et al. 2014).

miRNAs biogenesis starts in the nucleus, when RNA Pol II transcribes the miRNA genes into primary miRNAs (pri-miRNAs) (Fig. 12). A pri-miRNA displays a hairpin structure and has both a Cap and a poly (A) tail. Together, Drosha enzyme (which is a member of RNase III family) and DGCR8 process the pri-miRNA into ~ 60 nucleotides precursor miRNA (pre-miRNA) with a stem-loop structure (Stroynowska-Czerwinska, Fiszler et al. 2014). Then, exportin complex transports the pre-miRNA to the cytoplasm where a cytoplasmic endonuclease, Dicer, cleaves the stem-loop to form a miRNA/miRNA* duplex of ~ 22 nucleotides (Fig. 12) (Stroynowska-Czerwinska, Fiszler et al. 2014). The stable strand is called the “guide strand” and the other strand the “passenger strand” which is supposed to be degraded later. The duplex is loaded into ribonucleoprotein complex called RISC (for RNA induced silencing complex), which contains the Argonaute proteins (AGO). The strand “passenger” or miRNA* will be removed and degraded.

miRNA regulates gene expression by two main different mechanisms. If the miRNA has imperfect match with the 3'UTR of the mRNA target, translation will be repressed. On the contrary, if the mRNA target has a perfect match, the mRNA will be degraded (Fig. 12) (Stroynowska-Czerwinska, Fiszler et al. 2014). miRNAs are implicated in many human diseases such as Parkinson, Alzheimer, haematological and solid tumors (Faraoni, Antonetti et al. 2009). Many studies have reported different miRNAs expression profiles in tumor cells. Many miRNAs were downregulated in cancer cells and can act as tumor suppressor genes like let-7 micro RNA (Manikandan, Aarthi et al. 2008). In contrast, some miRNAs showed enhanced expression in cancers and are considered as oncogenes, such as the miR-17-92 cluster and the miR-155 that have been reported to be upregulated in B-cell lymphoma (Manikandan, Aarthi et al. 2008; Faraoni, Antonetti et al. 2009).

The miR-155: a prototypic example

miR-155 is a non-protein coding transcript encoded by *BIC* gene (B-cell integration cluster). miR-155 is a multifunctional microRNA molecule that affects a wide range of physiological processes. It is involved in inflammation, haematopoiesis, (Faraoni, Antonetti et al. 2009), and functions as a regulator of the immune system (Vigorito, Kohlhaas et al. 2013). Additionally, miR-155 is involved in various pathologies including cardiovascular diseases, viral infections and different types of cancers. miR-155 is considered as an oncomiR because many studies have shown that miR-155 dysregulation was associated with different cancers including lymphoma, leukemia (Faraoni, Antonetti et al. 2009), breast, lung and colon tumors (Volinia, Calin et al. 2006).

A study on 89 breast cancer patients revealed that the miR-155 has increased levels in serum compared to that of the healthy individuals (Roth, Rack et al. 2010). Another study has demonstrated that hypoxia induced expression of miR-155 in lung cancer cells. Increase of miR-155 expression protected cells from radiotherapy. miR-155 inhibition enhances the sensitivity of hypoxic lung cancer cells to radiotherapy. These results suggest that miR-155 can mediate radio-resistance (Babar, Czocho et al. 2011). Moreover, inhibition of miR-155 can increase significantly the effect of cisplatin in lung cancer cells. Indeed, it has been proposed that miR-155 downregulation leads to upregulation of Apaf-1 (Apoptotic protease activity factor 1, a miR-155 predicted target) that activates apoptosis (Zang, Zhong et al. 2012). Recently, it has been indicated that overexpression of miR-155 could produce miR-155 dependent-lymphoma in mice. The delivery of anti-miR encapsulated nanoparticles resulted in miR-155 inhibition and growth delay of pre-B cell tumor cells in mice. These results suggested a potential therapeutic option for lymphomas (Babar, Cheng et al. 2012).

Recently, a study performed on Mutu I cells found that miR-155 can directly target the 3' untranslated region of HIVEP2 (Yin, McBride et al. 2008; Yin, Wang et al. 2010).

HIVEP2

Human immunodeficiency virus type I enhancer binding protein 2 (HIVEP2) is a transcription factor that contains two zinc fingers. It is also called schnurri-2, MBP2 for MHC enhancer binding protein 2 (Shukla and Yuspa 2010), MIBP1 for c-myc intron binding protein 1 as it represses the transcription of c-myc (Fukuda, Yamasaki et al. 2002). HIVEP2 is required for bone remodeling and the differentiation of T cells and neural cells (Shukla and Yuspa 2010). HIVEP2 is also involved in TGF- β signaling via the stabilization of Smad2 and Smad3 proteins (Shukla, Malik et al. 2009). To identify the primary transcription targets of HIVEP2, a microarray was performed on the human embryonic kidney cell line HEK293 that overexpressed HIVEP2. This analysis showed that HIVEP2 down-regulates NF- κ B pathway via its binding with NF- κ B site and these results were confirmed by reporter assays (Iwashita, Fukuchi et al. 2012). Interestingly, HIVEP2 transcription was found to be induced in human vascular endothelial cells

during hypoxia (Manalo, Rowan et al. 2005). This observation suggest the implication of HIVEP2 in cellular stress response.

HIVEP2 is highly expressed in post-mitotic neurons (Fukuda, Yamasaki et al. 2002). A study performed on glioma patients samples found that HIVEP2 expression has the tendency to decrease in the gliomas advanced tumor stages. However, transfection of glioma cells with HIVEP2 has stimulated their differentiation and inhibited their proliferation (Sun, Chen et al. 2014).

HIVEP2 role in immature T cells development was investigated using HIVEP2-deficient mice. Experiments showed that HIVEP2 disruption blocked the positive selection of thymocytes responsible for their differentiation (Takagi, Harada et al. 2001). Similarly, *in vivo* experiments demonstrated that HIVEP2 is critical for the function of natural killer cells. Interestingly, deficient-HIVEP2 mice developed T cell lymphoma (Yamashita, Iwamura et al. 2012).

Finally, very few studies investigated the expression of HIVEP2 in patients' tumors. A study on breast cancer tumors found that HIVEP2 expression in patients' samples was frequently down regulated (Fujii, Gabrielson et al. 2005). While HIVEP2 expression was found to be upregulated in chronic lymphocytic leukemia patients with 11q23 deletion (Aalto, El-Rifa et al. 2001). Similarly, the regulation of HIVEP2 expression is poorly studied. Recently, it has been demonstrated that miR-155 regulates the expression of HIVEP2 via its 3'UTR (Yin, McBride et al. 2008; Yin, Wang et al. 2010).

Altogether, these observations suggest that HIVEP2 contributes to the pathogenesis of different types of cancers through a mechanism that remains to be clarified.

Cellular stress and translational regulation

Many different environmental stresses can affect gene expression regulation. It appears that a part of the stress-induced gene expression response is essential to protect cells from macromolecular damages enabling them to survive. The different elements of the translational machinery can be affected by stresses allowing an adaptive response to the stress (Liu and Qian 2014). However, stress-induced response varies according to severity of stress, cell type and stress type (Fulda, Gorman et al. 2010). Stresses include various physiological and environmental conditions such as temperature changes, ultraviolet irradiation, exposures to drugs and toxins, viral infections, nutrient deprivation, hypoxia and oxidative stress. In general, the stresses alter global cell translation, which is repressed in the majority of conditions to save cellular energy (Holcik and Sonenberg 2005). Most studies available to analyze the effects of stresses on translational regulation have focused on translation initiation, which is considered as the rate-limiting step for translational regulation. The main effect of stresses occurs at two steps: the ternary complex formation that contains eIF2 α and the recognition of cap structure by eIF4E cap-binding protein (Donnelly, Gorman et al. 2013).

The phosphorylation status of eIF2 α is very important for translation initiation because it is responsible for the availability of the ternary complex (eIF2 α -GTP-Met-tRNA_i) (Fig. 7) (Fig.

10). At present at least four kinases have been identified to phosphorylate eIF2 α in a response to different stress stimuli, PKR-like endoplasmic reticulum kinase (PERK), protein kinase double-stranded RNA-dependent (PKR), general control non-derepressible-2 (GCN2) and heme-regulated inhibitor (HRI) (Donnelly, Gorman et al. 2013). Many studies have shown that inhibition of translation initiation following different types of stresses result from the phosphorylation of eIF2 α (David Ron 2007; Donnelly, Gorman et al. 2013).

For example in nutrients limitation such as glucose depletion, the phosphorylation of eIF2 α at serine 51 inhibits the exchange of GTP for GDP and abolishes the formation of the ternary complex. Consequently, the phosphorylated eIF2 α causes a rapid reduction in global translation through the inhibition of translation initiation (Ashe, De Long et al. 2000; Baird and Wek 2012). However, the phosphorylation of eIF2 α by GCN2 kinase can lead to an increase of translation of selective mRNAs involved in stress response such as GCN4 in yeast. This is due to a regulation of translational re-initiation as a result of low ternary complex concentration (Dever, Feng et al. 1992).

In viral infections, the PKR kinase plays an important role in antiviral defense. A recent study performed in HeLa cells reported that infection with newcastle disease virus (NDV) induces PKR activation that phosphorylates eIF2 α leading to impair viral replication (Zhang, Sun et al. 2014). However, it seems that this is not the case for all viruses, since certain viruses can develop a translational resistance to eIF2 α phosphorylation. For example a study performed in murine embryonic fibroblasts demonstrated that the infection with alphaviruses Sindbis (SV) triggers PKR leading to total eIF2 α phosphorylation. In spite of that, viral translation is not repressed because of a hairpin loop structure in the viral RNA that can overcome the eIF2 α phosphorylation (Ventoso, Sanz et al. 2006).

Another well documented example of modulation of translation initiation by an eIF2-dependent mechanism has been identified following oxidative stress. Under oxidative stress, the phosphorylation status of eIF2 α controls cell survival and destiny. The impaired eIF2 α phosphorylation or the knockdown of eIF2 α kinases (GCN2 and PERK) can sensitize tumor cells to death induced by hydrogen peroxide (Rajesh, Krishnamoorthy et al. 2015). Furthermore, the oxidative stress caused by doxorubicin can induce a premature senescence in eIF2 α deficient tumor cells (Rajesh, Papadakis et al. 2013).

The phosphorylation of the cap binding protein eIF4E via MAPK signaling promotes its binding to cap structure (Wang, Flynn et al. 1998). Diverse stresses conditions can affect differentially the phosphorylation state of eIF4E and thereby the cap-dependent initiation. Treatment of Chinese hamster ovary cells with either arsenite or sorbitol, which induces chemical or osmotic stress, respectively induced an inhibition of protein synthesis correlated with increase of 4F-BP1 binding to eIF4E leading to a loss of eIF4F complex (Patel, McLeod et al. 2002). Consistent with that finding, several studies reported the activation of cap-independent translation initiation mechanism during hypoxia for some mRNAs that contain IRESs such as HIF-1 α and VEGF whereas the cap-dependent mechanism was inhibited (Stein, Itin et al. 1998; Lang, Kappel et al. 2002). However, modification of eIF4E phosphorylation status is not always key for translational initiation inhibition. In smooth muscle cells, the hydrogen peroxide (H₂O₂) stimulated

the phosphorylation of eIF4E as an essential event of the oxidative stress response. In these conditions the global protein synthesis was not significantly changed however the translation of a subset of mRNAs crucial for cell survival was induced (Rao 2000). Another study performed in *Xenopus* kidney cells showed that anisomycin or heat shock increased the phosphorylation of eIF4E, the formation of eIF4F complex and its association with PABP in spite of severe inhibition of protein synthesis which probably resulted from eIF2 α phosphorylation (Fraser, Pain et al. 1999). Other translational elements such as tRNA, rRNA and miRNAs were also found to be affected by stress conditions. The effect of oxidative stress on tRNAs was studied in yeast. Bioinformatic and biological analyses demonstrated that stress induced high tRNA modifications level in particularly at the wobble position, which resulted in selective mRNA translation (Chan, Pang et al. 2012). UV-irradiation could damage specific sites of pyrimidine nucleotides in the 28S rRNA 3'end, which is involved in translation elongation and inhibited protein synthesis (Iordanov, Pribnow et al. 1998). Finally, stress can also affect the regulation of miRNAs. Hypoxic stress inhibited the processing of selective miRNAs in an EGFR dependent manner in different cancer cell lines (Shen, Xia et al. 2013). Altogether, cellular stresses can alter the translation process by affecting the different elements of its machinery.

Cap-independent translation

Cap-dependent translation initiation was considered for a long time as the unique mechanism that could initiate mRNAs translation. However, it has been observed that the uncapped picornavirus mRNAs were translated efficiently whereas the cellular cap-dependent translation was completely inhibited (Prats and Prats 2002). Late in the 1980s, Studies on the expression of viral genes have allowed to point out that viral mRNAs can be translated in an alternative cap-independent initiation mechanism that use specific mRNA regions called internal ribosome entry site (IRES) (Prats and Prats 2002). The IRES structure allows the small ribosomal subunit 40S to be recruited directly to the mRNA without the need of eIF4E cap binding protein (Prats and Prats 2002). Viral IRES can be classified into 4 groups according to their relative location according to the start codon and the factors they require (Kieft 2008). The first group contains IRES that can bind directly the 40S without any additional factors even without the initiator tRNA, the cricket paralysis virus (CrPV) IRES belongs to this group. The second group of IRESs such as Hepatic C virus (HCV) need eIF2, eIF3 and the initiator tRNA (Kieft 2008). The IRES of the third and the fourth groups require additional factors called IRES trans-activating factors (ITAFs). IRES of these groups are found in encephalomyocarditis virus (EMCV) and rhinovirus, respectively (Kieft 2008).

Similar to viral IRES, the first eukaryotic IRES was discovered in the immunoglobulin heavy chain binding protein (Bip) mRNA, which can be translated in poliovirus infected-cells in spite of the severe inhibition of the cap-dependent translation (Macejak and Sarnow 1991; Prats and Prats 2002). Following this first discovery, many eukaryotic IRESs have been reported in mammalian mRNAs such as those coding for hypoxia-inducible factor-1 α (HIF-1 α), vascular

endothelial growth factor (VEGF), and fibroblast growth factor (FGF-2) (Lang, Kappel et al. 2002; Prats and Prats 2002). A public database of IRESs (www.iresite.org) has been developed by Morkejs and colleagues. It supplies important biological and experimental information about IRESs such as their origin, sequence and structure (Mokrejs, Vopalensky et al. 2006; Mokrejs, Masek et al. 2010). There is 68 viral IRESs and 115 eukaryotic cellular IRESs experimentally validated. At present although the mRNA transcripts that contain IRES structures have similar properties within their 5'UTR such as the length, GC % content and the number of uAUGs, the prediction of cellular IRES is still impossible by bioinformatics analysis (Baird, Turcotte et al. 2006).

Cap-independent translation initiation keeps a low translational level of mRNA transcripts containing IRES structures under normal conditions when cap-dependent translation is efficient (Komar and Hatzoglou 2011). However, cap-independent translation initiation allows the translation of several mRNAs containing IRESs when cap-dependent translation is suppressed during mitosis, apoptosis and stress conditions such as hypoxia and drugs exposure (Holcik and Sonenberg 2005; Komar and Hatzoglou 2011). Hypoxia is a common cellular stress that inhibits the overall protein synthesis, even though translation of the main regulator of hypoxia, HIF-1 α , was not affected in hypoxic tumor cells. This is due to the presence of an IRES in its 5'UTR. (Lang, Kappel et al. 2002). Hypoxia can also induce VEGF that secures the oxygen supply. Interestingly, VEGF 5'UTR also contains an IRES structure that maintains its translation during hypoxia (Huez, Creancier et al. 1998) (Stein, Itin et al. 1998). A study in transgenic mice has investigated the activity of two VEGF IRES in ischemic muscles using bi-cistronic reporter vectors. The two IRES allowed an efficient translation of VEGF, whereas the cap-dependent translation was repressed by hypoxia in ischemic muscles (Bornes, Prado-Lourenco et al. 2007). In metastatic cancer cells, hypoxia inhibited VEGF-C transcription and cap-dependent translation. However, hypoxia induced VEGF-C translation via an IRES dependent translation initiation mechanism and this activation was independent of the hypoxic factor HIF-1 α (Morfoisse, Kuchnio et al. 2014). Other experiments in in vivo models demonstrated that hypoxia induced FGF2 translation which was associated with a strongly FGF2 IRES-activity. Gene silencing experiments showed that this translation activation involved a complex crosstalk between HIF-1 α and FGF2 (Conte, Riant et al. 2008). Additionally, hyperglycemia can inhibit the global translation. However, Experiments on diabetic mice showed that high glucose levels stimulated the translation of FGF-2 in an IRES-dependent mechanism leading to abnormal proliferation that resulted in more pathological complications (Teshima-Kondo, Kondo et al. 2004). Altogether, cap-independent translation initiation mechanism can secure the production of specific proteins under unfavorable conditions when cap-dependent mechanism is suppressed.

2.2. Translation elongation

Elongation is a dynamic process involving several ribosome movements mediated by elongation factors and requiring GTP hydrolysis. After start codon recognition and codon anticodon base pairing, the elongation phase begins while the initiator tRNA is still in the P-site

of the 80S ribosome (Proud 1994; Dever and Green 2012). Elongation cycles proceed in three stages: tRNA delivery, formation of peptide bond and tRNA translocation. This mechanism requires the three functional A, P and E ribosome sites (Proud 1994). As illustrated in (Fig. 13) tRNA delivery is catalyzed by eEF1, whereas aminoacyl tRNA enters the ribosome at the A (aminoacyl) site, as a ternary complex (eEF1.GTP.aminoacyl tRNA) and is followed by GTP hydrolysis. Next, peptidyl bond is formed between the amino group of aminoacyl tRNA in A site and the carbonyl of peptidyl-tRNA in P site. This reaction does not require any GTP hydrolysis and results in transferring the amino acid from P site to A site to form the polypeptide (Merrick 1992). The 3' end of the new peptidyl-tRNA (which was the previous aminoacyl tRNA) moves from A site to P site, while its anticodon end is still base paired with the mRNA codon in the A site. The 3' end of the deacylated tRNA is shifted into the E site and at the same time its anticodon remains in P site (Julian, Konevega et al. 2008). This hybrid sites (A/P and P/E) are associated with conformational change in rRNAs to give up a pre-translocation ribosome, to which the eEF2.GTP complex shows high affinity. eEF2 provides the mechanical energy required for translocation through the hydrolysis of its GTP (Kaul, Pattan et al. 2011). Then, translocation takes place and the mRNA moves one codon to place a new codon in the A site. The ribosome undergoes conformational changes and switches to form the post-translocation state leading to eEF2.GDP dissociation (Dever and Green 2012). Together, these events results in a complete placement of the peptidyl tRNA in P site and the unacylated tRNA in the E site. The ribosome is then ready for another elongation cycle (Fig. 13) (Proud 1994; Wilson and Nierhaus 2006).

2.3. Translation termination

When termination occurs the last peptidyl tRNA is in the P site and the stop codon is in the A site. A model of four steps that implicates eRF1 and eRF3 factors has been proposed for termination in eukaryotes (Fig. 14) (Alkalaeva, Pisarev et al. 2006). eRF3 is a GTP binding protein that can form a stable complex with eRF1 (Frolova, Le Goff et al. 1996), which can recognize the three stop codons (UAA, UAG and UGA) when one of them joins the decoding center (Song, Mugnier et al. 2000). At the beginning, the eRF1, eRF3 and GTP binds to the pre-termination complex possibly as a ternary complex. This binding could result in either conformational changes, or translocation of peptidyl tRNA anticodon arm from P site to E site and the stop codon on mRNA from A site to P site (Fig. 14). Next, eRF3.GTP could be hydrolyzed resulting in either eRF3.GDP release or change the interaction with eRF1. Finally, eRF1 could induce hydrolysis of polypeptide release, followed by eRF1 and eRF3 dissociation from the ribosome (Alkalaeva, Pisarev et al. 2006). After termination, the 80S ribosome is still bound to mRNA. The ABCE1 protein (ATP-binding cassette) stimulates the dissociation of the post-termination complex into free 60S and mRNA- tRNA bound 40S complex (Pisarev, Skabkin et al. 2010) (Fig. 14). Therefore, there are two possibility after termination, either reinitiate the translation on the same mRNA or eject the deacylated tRNA and mRNA from the 40S to start a new translation cycle (Rajkowitsch, Vilela et al. 2004).

Part II

Translation and cancer therapy

3. Translation and chemotherapy

A wide range of chemotherapeutic anticancer drugs are used to treat cancers, these drugs have different effects on cellular processes and components. It appears more and more clearly proposed that these drugs could have an impact on both translation process and ribosome biogenesis.

The effect of three platinum anticancer drugs on mRNA translation has been studied using an *in vitro* translation system. An mRNA that encodes a green fluorescent protein (GFP) were transcribed *in vitro* and treated for one hour with cisplatin, oxaliplatin and carboplatin. Results have shown that cisplatin could inhibit the translation of GFP-mRNA. Oxaliplatin had a weak and insignificant effect, while carboplatin did not affect GFP-mRNA translation (Becker, Weiss et al. 2014). Furthermore, it has reported that cisplatin could inhibit translation at initiation step. It could impair the formation of 80S ribosome and prevent the 60S subunit from joining the 40S in the pre-initiation complex (PIC). Therefore, cisplatin arrested translation initiation and caused PIC accumulation (Rosenberg and Sato 1993).

Camptothecin is a topoisomerase inhibitor that reported to impair pre-rRNA processing. It inhibits the particular step that generates the 28S, but this effect is reversible within five minutes after camptothecin remove (Wu, Kumar et al. 1971).

The inhibitory effect of 36 chemotherapeutic drugs on ribosome biogenesis has been performed in human fibrosarcoma cells. The results showed that 20 drugs can inhibit ribosome biogenesis either at transcriptional step or/and at rRNA processing step. For example, doxorubicin and oxaliplatin can inhibit ribosome biogenesis at transcriptional level. While flavopiridol and 5-fluorouracil (5-FU) can inhibit ribosome biogenesis at early and lately steps of rRNA processing respectively (Burger, Muhl et al. 2010).

Interestingly, the 5-FU has proposed to affect both rRNA synthesis and translation. A study has treated the partially hepatectomized rats by 5-FU and the liver RNAs were radioactive labelled. Experiments have demonstrated that 5-FU did not affect the synthesis of poly (A) RNAs. While 5-FU caused 60% reduction of rRNA synthesis, in addition to the presence of an abnormal 38S pre-rRNA. Surprisingly, the poly(A) RNAs from 5-FU treated animals have stimulated the translation by 240% compared to poly(A) RNAs from non-treated animals using a wheat germ translation system (Carrico and Glazer 1979). These observations demonstrate that different anticancer drugs have the capacity to affect mRNA translation. Even though, the effect of anticancer drugs on translation regulation has not been well appreciated.

4. Chemotherapeutic treatments: the 5-FU based prototype

Chemotherapy is one of the major mode of treatment for cancers. Together with surgery and radiation, they are considered as the most common options of cancer treatments. There are different ways of applying the chemotherapies. In primary treatment, chemotherapeutic drugs are used either alone or in combination with other agents. While in the adjuvant chemotherapy, the drugs are combined with surgery or radiotherapy. Whereas, the neo-adjuvant chemotherapy includes the administration of the drug before the surgery.

The commonly used chemotherapeutic drugs can be classified into five major classes of drugs according to anatomical therapeutic chemical (ATC) criteria (L01) (WHO Collaborating Centre for Drug Statistics Methodology. Oslo 2010), (Missailidis 2008):

1. **Alkylating agents:** These agents act by crosslinking DNA and prevent the RNA synthesis form its DNA template, such as busulfan and cyclophosphamide.
2. **Antimetabolites:** They affect the biosynthesis of nucleic acids by their ability to substitute the normal metabolites and blocking the enzymes implicated in nucleic acid synthesis. There are different categories of antimetabolites. The most important are the pyrimidine analogs that contain 5-Fluorouracil, capecitabine and gemcitabine. In addition to the purine analogs such as mercaptopurine and the antifolates analogs like methotrexate.
3. **Antimicrotubule agents (Plant alkaloids and other natural products):** They can affect microtubule dynamics and destroy mitotic spindle leading to mitotic block. Therefore, they prevent cell division and cause metaphase arrest and apoptosis. Taxanes are an important category that consists of different compounds such as paclitaxel and docetaxel. Taxanes can stabilize microtubules and suppress depolymerization resulting in cell death.
4. **Antitumour antibiotics:** They exert their antitumor effects by intercalating to DNA or by stabilizing the complexes of topoisomerases I and II with DNA. Many categories of antibiotics are used as anticancer such as the actinomycines that intercalate to DNA, the anthracyclines like the doxorubicin that inhibits topoisomerase II and irinotecan from the Camptothecins that inhibit topoisomerase I.
5. **Others neoplastic drugs:** This class contains all the other drugs that cannot be classified in the previous classes like the platinum compounds that considered alkylating-like agents. They can form adducts with DNA, leading to DNA synthesis inhibition and saturate the capacity of repairing platinum adducts of DNA in the cell. The most important used platinum compounds are the oxaliplatin, cisplatin, and carboplatin.

In addition to chemotherapy, many other therapies are currently in use alone or in combination with chemotherapy. The hormonal therapy is used to treat for different cancers such as Tamoxifen, which is largely used in breast cancer treatments (Missailidis 2008). The Photodynamic therapy (PDT) that depends on the photochemical reaction of the photosensitizer

to generate reactive oxygen species (ROS) which will cause cell death, Photofrin is a photosensitizing agent used to treat high grade dysplasia in Barrett's esophagus (Gray and Fullarton 2013).

New strategies have been developed by using immunotherapy that intends to directly or indirectly use the immune system against tumor. The immunotherapy can use specific monoclonal antibodies against common cancer antigens such as cetuximab that targets epidermal growth factor receptor (EGFR) and used in metastatic colorectal cancer (mCRC) treatments (Missailidis 2008). Major advanced in therapeutic approaches have been recently introduced concerning the targeted therapy. Targeted therapy can use small-molecule tyrosine kinase inhibitors such as Imatinib "Gleevec", which is mainly used to treat chronic myeloid leukemia (CML) and gastrointestinal stromal cancer (GIST) (Iqbal 2014). A supplementary therapeutic option is lately emerged called gene therapy. It aims to regulate gene expression by using short interfering RNAs (siRNA) or microRNA (miRNAs) that target the key functional genes in cancer (Gandhi, Tekade et al. 2014).

5. The 5-Fluorouracil

5-Fluorouracil (5-FU) is an anticancer drug widely used since 1957. It is considered as a key drug in chemotherapeutic treatments for a large range of solid tumors such as breast, head and neck, gastric and colorectal cancers (Labianca, Beretta et al. 2004; Garcia, Carrasco et al. 2011; Somani, Goyal et al. 2011). 5-FU has a heterocyclic aromatic organic structure similar to pyrimidine's molecules found in DNAs and RNAs. It is considered as a uracil analogue the hydrogen being replaced by a fluorine atom in position C5 (Fig. 15). This structure enables 5-FU to incorporate into DNA and RNA, and alters their metabolisms. Several strategies have been developed to improve 5-FU efficiency, combination with other drugs and/or monoclonal antibodies, biochemical modulation of its biodisponibility and by modulation of its metabolism.

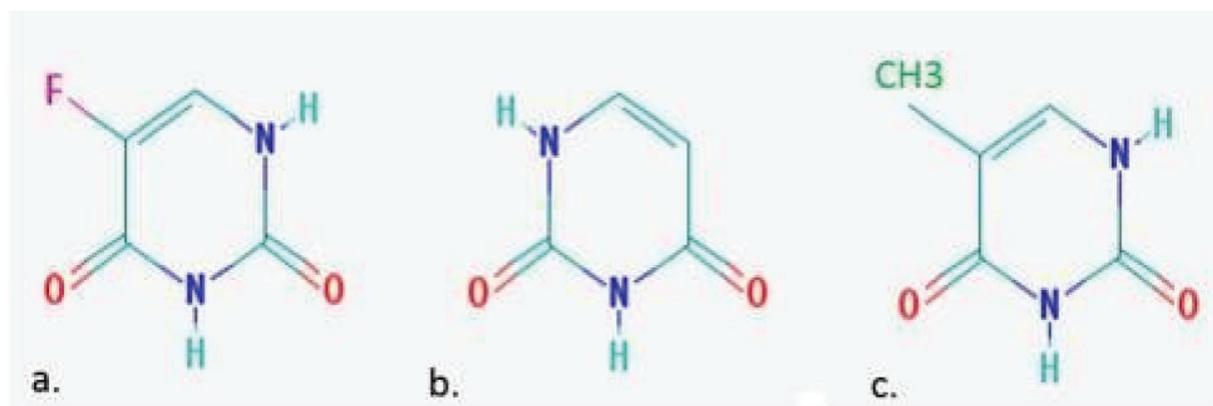


Figure. 15. Chemical structure of 5-Fluorouracil, Uracil and Thymine. a. 5-Fluorouracil. b. Uracil or 2, 4-Dihydropyrimidine. c. Thymine or 5-methyluracil. Adapted from (NCBI: PubChem Compound on line).

5.1. 5-FU based chemotherapeutic combination treatments

5-FU treatment is a first line treatment for many types of cancers, either used as a single agent or combined with other drugs. Combination with other drugs improves 5-FU response rate, overall survival, and reduce its toxicity (Table. 1). Oxaliplatin (I-OHP) and irinotecan (CPT-11) have been introduced into 5-Fluorouracil and folinic acid (5-FU/LV) treatment protocols. Many different combinations of chemotherapeutic regimens are worldwide administrated such as 5-FU/LV with oxaliplatin (FOLFOX), or irinotecan (FOLFIRI), or with both oxaliplatin and irinotecan (FOLFOXIRI). Recently, these regimens have be improved by adding the targeted therapies like monoclonal antibodies and tyrosine kinase inhibitors, particularly for the advanced and metastatic forms of cancer (Edwards, Chadda et al. 2012).

Table. 1. Summary of different 5-FU protocols used as fist-line treatment in clinical studies.

Patient's Cancer	5-FU combined chemotherapy	Combination advantages
Advanced CRC	5-FU / calcium folinate + Irinotecan	Response increase (Douillard, Cunningham et al. 2000)
mCRC	5-FU/±LV + Oxaliplatin	Improve response rate and anti-tumor efficacy (Giacchetti, Perpoint et al. 2000)
Oesophageal cancer	FOLFOX	FOLFOX regime is more efficient than 5-FU plus cisplatin treatment (The surgery is unsuitable for these tumors, while 5-FU combined treatment is possible) (Conroy, Galais et al. 2014)
mCRC	Chronomodulation 5-FU/LV + Oxaliplatin + Irinotecan (chronoIFLO)	Tolerable regime, efficacy in stopping tumor progression (Gholam, Giacchetti et al. 2006)
Advanced CRC	5-FU/LV + Irinotecan + Bevacizumab (FOLFIRI-B)	Efficiency of combination chemotherapy regime confirmation (Analysis of 29 studies :3502 patients), (Petrelli, Borgonovo et al. 2013)
mCRC (KRAS wild type)	FOLFIRI + Cetuximab	Significant improvement in clinical outcome and overall survival(Lang, Kohne et al. 2013)
mCRC	FOLFIRI or FOLFOX6 + Cetuximab	Significant improvement in clinical outcome and overall survival (Ocvirk, Brodowicz et al. 2010)
mCRC	5-FU + Oxaliplatin + Irinotecan + Cetuximab	Response rates improvement (Akhtar, Chandel et al. 2014)

mCRC	IFL + Bevacizumab, FOLFOX, FOLFIRI	Similar excellent efficiency in phase III of trials for the three regimes (Kelly and Goldberg 2005)
Head and neck carcinoma	Docetaxel + Cisplatin + 5-FU	This regime is more efficient than cisplatin plus 5-FU (Posner, Hershock et al. 2007)

5.2. 5-FU biodistribution biomedical modulation

Several methods have been investigated to modulate 5-FU, modulation strategies include decrease 5FU degradation, increase its activation, and reducing 5-FU toxicity with retaining the antitumor effects.

Dihydropyrimidine dehydrogenase (DPD) inhibition. A large amount of the subjected 5-FU is rapidly deactivated in the liver by the rate-limiting DPD enzyme. The formulation UFT consists of uracil and the oral active pro-drug of 5-FU (Ftorafur) have been used. Uracil and Ftorafur compete with 5-FU for DPD enzyme resulting in DPD saturation increase of 5-FU half-life time and drug availability (Longley, Harkin et al. 2003).

Capectabine. Capectabine is an oral fluoropyrimidine 5-FU prodrug. It is metabolized into 5'DFUR in the liver, then converted into 5-FU by thymidine phosphorylase (TP) and uridine phosphorylase (UP) enzymes which are highly activated in tumor cells. As a result, more 5-FU is generated in tumor cells leading to additional 5-FU exposure in tumoral cells than healthy cells (Mazzaferro, Bouchemal et al. 2013).

Leucovorin (LV). Leucovorin or fonilnic acid (FA) is a 5-FU metabolism modulator since it is a source of CH₂THF, the cofactor in thymidylate synthase (TS) reaction. LV is metabolized into CH₂THF keeping by that the CH₂THF intra-cellular level and stabilizing the TS-FdUMP complex. The increase in TS inhibition results in enhancing the therapeutic efficacy of 5-FU (Machover, Ulusakarya et al. 2008).

Interferons. Interferons (INFs) are cytokines with anti-proliferative activity and immunomodulatory effects. INFs can modulate 5-FU through enhancing its metabolism. It has been demonstrated that INFs treatment increases the TP and UP enzymes levels in colon cancer cells leading to increasing 5-FU conversion into FdUMP and FdUR (Eda, Fujimoto et al. 1993; Braybrooke, Propper et al. 2000). However, it seems that there is no difference in overall survival between treatment with 5-FU or 5-FU/LV without INFs versus plus INFs (Wolmark, Bryant et al. 1998).

Methotrexate (MTX). Methotrexate is an antimetabolite antimetafolate drug, it inhibits the dihydrofolate reductase (DHFR) that reduces dihydrofolate (DHF) to tetrahydrofolate (THF). THF is the precursor of CH₂THF, the cofactor enzyme in thymidine production, a reaction catalyzed by TS. THF is also necessary in purine and nucleic acid synthesis. Therefore, MTX can inhibit thymidine and purine synthesis. MTX application before 5-FU treatment increases its anti-cancer effects. Indeed, the anti-purine effect of MTX leads to an accumulation of the Phosphoribosyl pyrophosphate (PRPP). The PRPP is a cofactor in the 5FU rate-limiting reaction that is catabolized by orotate phosphoribosyltransferase (OPRT). In this reaction, OPRT converts 5-FU into FUMP

that subsequently incorporates into RNA. Therefore, MTX reinforces 5FU effects through enhancing its incorporation into RNA (Cadman, Heimer et al. 1981).

Uridine. As exposed above 5-FU cytotoxicity is mainly due to its capacity to be integrated within RNA. Treatment with a high dose of 5-FU followed by delayed uridine administration allows to rescue from 5FU toxicity with retaining the anti-tumor effect. In vivo experiments on colon tumors-bearing mice have shown that delayed administration of uridine has protected healthy cells against lethal 5-FU doses and decreased the hematological 5-FU toxicity, while the anti-tumor effect and drug sensitivity were not affected by uridine administration (Peters, van Dijk et al. 1988). Similar results have been obtained using a uridine precursor called uridine diphosphoglucose (UDPG). Administration of UDPG after 5-FU treatment resulted in therapeutic efficacy increasing consistently with a reduction of 5-FU toxic side effects (Codacci-Pisanelli, Kralovanszky et al. 1997). Clinical investigations on patients with advanced colorectal cancer showed that 5-FU combination with delayed uridine favored recovery from bone marrow suppression. These findings highlights the positive rescue effects of uridine administration during 5-FU treatment (van Groeningen, Peters et al. 1993).

5.3. Metabolism of 5-FU

The anti-cancer activity of 5-FU is determined by many enzymes that are involved in its metabolism. The activities and the quantity of some of them are used to predict the response to 5-FU. Both anabolic and catabolic processes are important in 5-FU activation. As the majority of 5-FU is rapidly eliminated by catabolism in the liver, the rested available amount of 5-FU for anabolism is responsible for anti-cancer cytotoxic effects.

Catabolism of 5-FU. Dihydropyrimidine dehydrogenase (DPD), also known as dihydrouracil dehydrogenase, is a rate-limiting enzyme of pyrimidine catabolism. DPD is highly active in the liver and catabolizes more than 85% of 5-FU into inactive metabolites. DPD activity varies between normal and tumor tissues: in colorectal cancer cells, it has been demonstrated that DPD activity is correlated with mRNA expression and protein level. Moreover, the enzyme activity is inversely correlated with 5-FU concentrations. In contrast, healthy cells did not show the same correlation of mRNA expression, protein level and 5-FU concentration. This selective correlation in cancer cells indicates that DPD activity can be used to predict 5-FU concentrations (Tanaka-Nozaki, Onda et al. 2001). Therefore, it has been proposed that DPD activity in tumor cells determines the actual delivered dose of 5-FU and modifies its efficacy. These findings emphasize the role of DPD enzyme as one of the main factor impacting 5-FU sensitivity. A study on patients with lung adenocarcinoma demonstrated this inverse correlation between DPD expression and overall survival. Whereas patients with low intratumoral DPD expression levels were sensitive to 5-FU, patients with high tumors DPD expression displayed a worse prognosis even than non-treated patients with 5-FU (Shintani, Inoue et al. 2011). These observations show the necessity to estimate intra-tumoral DPD expression to adjust the 5-FU dose and estimate its efficacy.

While low DPD expression improves the 5-FU sensitivity as explained previously, DPD deficiency can threaten the life because of reducing drug elimination and increasing the half time

of 5-FU up to 10 folds. As a consequence, the prolonged exposure to the drug leads to severe toxicity and can cause death in extreme cases. This DPD deficiency is due to genetic variations of the *DPYD* gene that encodes this enzyme. A complete deficiency causes a mental retardation in childhood while partial deficiency manifests as severe toxicity after the administration of standard dose of 5-FU. Many genetic variations have been reported to contribute to DPD deficiency, such as the splice site mutation IVS14+1 G>A (base change in exon 14) that reduces significantly the enzyme activity (van Kuilenburg, Muller et al. 2001; van Kuilenburg 2004).

Furthermore, overexpression of DPD has been shown to be involved in acquired 5-FU resistance. DPD expression was estimated in 5-FU sensitive and resistant mice with colorectal cancer xenografts. Experimental data indicated the upregulation of DPD expression in resistant 5-FU mice comparing to sensitive and non-treated animals. This DPD upregulation (mRNA and protein) was not only detected in tumors but also in the livers of resistant mice (Li, Dong et al. 2013). All these studies show the critical role of DPD enzyme in 5-FU sensitivity, toxicity and resistance. Thus, DPD seems to be one of the primary determinants of 5-FU response.

Anabolism of 5-FU. To be efficient of the treatment in humans, 5-FU needs to be transformed to active metabolites to be integrated within DNA and RNA. Activation of 5-FU proceeds following two main pathways with three main active metabolites: fluorodeoxyuridine monophosphate (FdUMP), fluorodeoxyuridine triphosphate (FdUTP) and fluorouridine triphosphate (FUTP). The first described pathway involves the 5-FU conversion by thymidine phosphorylase (TP) into fluorodeoxyuridine (FdUR), which is subsequently phosphorylated by thymidine kinase (TK) to FdUMP that can either inhibit the thymidylate synthase (TS) or can be converted to FdUTP and incorporates into DNA. In the second pathway, 5-FU is converted into fluorouridine monophosphate (FUMP) either directly by orotate phosphoribosyltransferase (ORPT) or indirectly by uridine phosphorylase (UP) and uridine kinase (UK). Next, the FUMP is phosphorylated to the active fluorouridine triphosphate (FUTP) that will incorporate into the RNAs. Another possibility is to convert FUMP by ribonucleotide reductase (RR) into FdUDP to join the first pathway (Fig. 16) (Longley, Harkin et al. 2003).

Thymidine phosphorylase. Thymidine phosphorylase (TP) is also known as platelet-derived endothelial cell growth factor (PD-ECGF). It is widely expressed in human tissues with abnormally elevated expression reported in tumor cells comparing to the surrounding normal cells. TP catalyzes the reversible synthesis of thymidine and controls its homeostasis in the plasma. As a key enzyme involved in pyrimidines metabolism, it converts the pyrimidine analogue 5-FU into 5-fluoro-2'-deoxyuridine (5FdUrd). This is the first step of metabolic activation of 5-FU to deoxyribonucleotide that will result in DNA synthesis inhibition later. Experiments performed in human CRC demonstrated that TP overexpression enhances 5-FU cytotoxicity with high observed TP activity-cell sensitivity association. This sensitivity can be reduced by TP inhibition (Evrard, Cuq et al. 1999). In agreement with this observation, a study on CRC patients treated with 5-FU based chemotherapy showed that high expression of TP could increase the curative effect of fluorouracil drugs (Ye and Zhang 2013). While the complete TP function loss due to an unspliced pre-mRNA, is associated with fluoropyrimidine based chemotherapy acquired resistance in leukemia cell lines (Stark, Bram et al. 2011).

TP plays a dual role in tumor development and therapy. In one side, it is frequently upregulated in many tumors and can induce tumor growth, angiogenesis and migration. On the other side, its activity is essential to activate fluoropyrimidine based therapies. Therefore, it is necessary to retain the TP activity to ensure the activation reaction of 5-FU.

Orotate phosphoribosyltransferase. The orotate phosphoribosyltransferase (OPRT) metabolizes 5-FU to 5-fluorouridine monophosphate (FUMP) in the presence of 5-phosphoribosyl-1pyrophosphate (PRPP) as a cofactor. This is the major step in 5-FU activation mechanism, because it is the only direct reaction to produce FUMP.

Interestingly, it has been demonstrated that OPRT overexpression enhanced cell sensitivity to 5-FU, but it did not affect cell proliferation (Yasumatsu, Nakashima et al. 2012). In addition a reduced protein level was observed in 5-FU resistant cell line derived from a gastric cancer cell line. This observation indicates that OPRT expression decrease is involved in resistance to 5-FU (Tsutani, Yoshida et al. 2008).

The expression ratio of OPRT/DPD can be used as a predictive parameter of 5-FU response. DPD is responsible of 5-FU inactivation that results in loss of cytotoxicity, and OPRT activates 5-FU and determines cell sensitivity. According to this statement, a study showed a strong correlation between a high ORPT/DPD expression ratio and the sensitivity to 5-FU and prolonged survival in patients with metastatic colorectal cancer (Ichikawa, Uetake et al. 2003).

Uridine phosphorylase. Uridine phosphorylase (UP or UPase) is an important enzyme in pyrimidine salvage that uses uracil, uridine and 5-FU as its preferable substrates. It can catalyze reversibly the uridine to uracil, maintaining the homeostatic regulation of uridine and its concentrations in both plasma and tissues. It contributes to the intracellular activation of 5-FU by converting it into 5Fluoruridine (FUrd). Subsequently, this FUrd will be phosphorylated into FUMP and then exerts the antitumor effects of 5-FU. *UPase* gene is transcriptionally regulated by the tumor suppressor gene *P53* (Zhang, Cao et al. 2001). It is also reported that some cytokins (such as TNF- α or IFN- γ) increase the UPase enzyme activity, and enhance tumor cells sensitivity and 5-FU cytotoxicity (Eda, Fujimoto et al. 1993). It has been demonstrated that UPase enzyme is expressed in normal and tumoral tissues, and to be highly activated in many human solid tumors such as breast, head-neck and colorectal cancers (Liu, Cao et al. 1998). This elevated expression in tumors appears to be a therapeutic advantage for patients, whereas the *UPase* gene knockout in mice resulted in reduced cellular sensitivity to 5-FU concomitantly with a significant increase in the IC₅₀ of 5-FU (Cao, Russell et al. 2002).

5.4. Mechanisms of action of 5-FU

In 1957, Heidelberger introduced tumor inhibitory properties of 5-FU (Heidelberger, Chaudhuri et al. 1957). Since this time, 5-FU has been used in chemotherapy particularly to treat colon and breast cancer patients. The concept of 5-FU usage was derived from the fact that uracil take up of tumor liver cells was more elevated than that of the surrounding normal cells. For this reason, it was anticipated that the drug could produce its anti-tumor cytotoxic effects simply through its preferential consumption by the tumor without the need for a specific targeting (Rutman, Cantarow et al. 1954).

From the first usage of 5-FU in clinic, several mechanisms of action have been proposed. Mechanisms involving an interference with DNA metabolism and interference with RNA metabolism. Surprisingly, as exposed below, it appears more and more clearly that the cytotoxic effect of 5-FU is due to its interference with RNA metabolism rather than that of the DNA.

5.4.1. DNA dependent mechanism of 5-FU cytotoxicity

The inhibition of thymidylate synthase (TS)

Comparison of 5-FU and uracil, shows that both fluorine and hydrogen atoms are similar in size. Moreover, the fluorine-carbon bond is more stable than carbon-hydrogen bond. Fluorine atom of 5-FU has the same position in the pyrimidine ring than that of the methyl group in thymine (Fig. 15). Taking these similarities into account it was supposed that 5-FU would inhibit the conversion of uracil to thymine by the thymidylate synthase (TS) resulting in DNA synthesis inhibition.

Indeed, among the deoxyribonucleotides, Thymine is the only one that form that forms in the cell starting from deoxynucleoside monophosphate (Mathews 2014). TS has been identified first as the main target of 5-FU because it is the enzyme responsible of *de novo* source of thymidine. To produce thymidine, TS modifies the pyrimidine nucleoside deoxyuridine monophosphate (dUMP) by reducing one carbon to a methyl group to produce deoxythymidine monophosphate (dTMP) in the presence of 5, 10-methylenetetrahydrofolate (CH₂-THF) as a methyl donor (Fig. 17).

The 5-FU active metabolite, the FdUMP, inhibits TS reaction by forming a stable ternary complex TS-FdUMP-CH₂THF. The stability of this complex depends on the CH₂-THF availability. Remarkably, an absence of CH₂-THF leads to unstable complex and poor TS inhibition (Peters, Backus et al. 2002).

As TS is the main target of 5-FU, it has been suggested that 5-FU efficacy was dependent on TS expression and that an inverse relation between TS and its inhibitor would play a major role in sensitivity or resistance of 5-FU based-chemotherapies. It has been demonstrated that a decreased TS mRNA and protein level was associated with improved 5-FU response in CRC patients. On the contrary patients with elevated TS mRNA and protein expression levels did not respond to 5-FU based treatment (Leichman, Lenz et al. 1997). Moreover, increased TS mRNA and protein expressions was observed in a colon cancer cell line that became resistant after long term 5-FU exposure. However, no increase in binding affinity between TS and FdUMP was

detected in these resistant cells even though it was supposed that elevated TS expression could be one of the 5-FU resistant mechanisms (Copur, Aiba et al. 1995).

It was also demonstrated that weak 5-FU response could be correlated with a variation of TS expression in patients. It has been shown that thymidylate synthase gene (*TSER*) expression can be affected by a polymorphism in the 5' untranslated region. This region contains either two or three tandem repeat sequences called TSER*2 and TSER*3 respectively. The triple tandem repeats induced a TS expression higher than the double repeats (Horie, Aiba et al. 1995). Consistent with the observation that TS level can modulate 5-FU response it has been shown that homozygous (TSER*2/TSER*2) and heterozygous (TSER*2/TSER*3) patients showed a higher response to 5-FU than homozygous patients (TSER*3/TSER*3) (Marsh, McKay et al. 2001).

However, several other studies showed that 5-FU response could not be simply elevated by the level of TS expression and that other mechanisms account for the effects of 5-FU. One study performed with metastatic CRC samples indicated that both TS expression levels and patients response could also be dependent of 5-FU administration protocols (Aschele, Debernardis et al. 2002). A 'lack of correlation' between TS level and the outcome of 5-FU treatment was also indicated by a study made on CRC tissues showing that improved response rates were observed with high TS expression (Johnston, Benson et al. 2003). Altogether, these studies confirmed that TS expression could not be used as a simple predictor for 5FU response or survival.

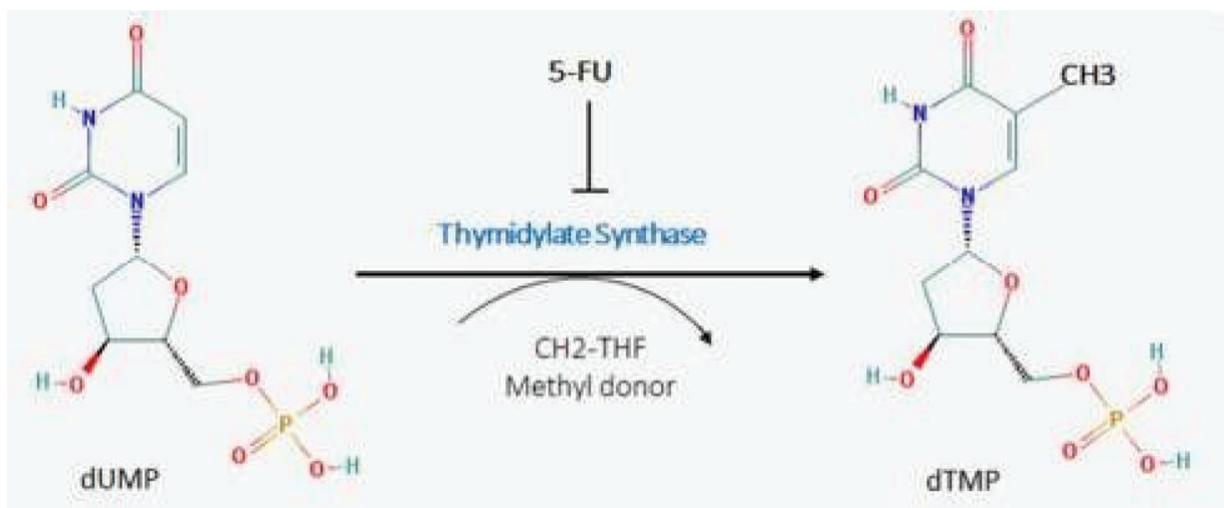


Figure. 17. The thymidylate synthase reaction. TS converts the dUMP to dTMP in the presence of CH₂THF as a cofactor. This reaction is inhibited by 5-FU metabolic derivative FdUMP. dUMP; Deoxyuridine monophosphate, dTMP; Thymidine Monophosphate or 5-Methyl-dUMP, CH₂-THF; 5,10-methylenetetrahydrofolate, FdUMP; 5-fluorodeoxyuridine monophosphate (Chemical structures of dUMP and dTMP are adapted from NCBI: PubChem online).

The 5-FU incorporation in genomic DNA

As shown in (Fig. 17) dUMP is the substrate of TS to synthesize dTMPs that is then phosphorylated to form dTTPs. The 5-FU metabolite FdUMP inactivates TS by that will be phosphorylated later to blocking the methylation of uracil in position 5' due to the presence of a fluorine atom in this position. Inhibition of TS results in the following consequences:

1-Decease in dTTP level leading to inhibition of DNA replication.

2-Accumulation of dUMP, the TS substrate, resulting in abnormal high levels of dUTP.

In controlled "normal" conditions uracil cannot be incorporated into DNA because of UTPase that maintains low dUTP levels. In addition, if there is any misincorporated uracil, uracil-DNA glycosylase (UDG) will remove it. Following 5-FU treatment, the increased concentrations of dUTP and FdUTP can overcome the dUTPase that hydrolyses dUTP to dUMP. As consequence, dUTP and FdUTP can be mis-incorporated into DNA instead of dTTP. The misincorporated uracil or 5-FU nucleotides will be recognized and removed. If the dTTP pool remains low, another dUTP or FdUTP will fill the gaps of excised nucleotides and so on. Misincorporation and remove cycles will cause DNA damage and cell death (Longley, Harkin et al. 2003). Detection of 5-FU DNA-incorporation performed *in vivo* confirmed the potential of 5-FU to be incorporated into DNA. A study was carried out with bone marrow of mice with mammary carcinoma and treated with 5-FU labeled with (³H). Measurements showed a significant amount of incorporated 5FU into DNA. The mechanism of incorporation was confirmed by competition experiments. With FdUTP competes with Thymidine complementation decreased 5-FU incorporated into DNA and reduced the inhibition of DNA synthesis (Sawyer, Stolfi et al. 1984).

Other experiments performed to measure the incorporation of 5-FU in human breast cancer cells (MCF-7) using (³H) 5-FU and (³²P) demonstrated that the rate of incorporation of 5-FU in RNA was higher than that in DNA. Measurements of *de novo* synthesized DNA by (³²P) labeling showed, as expected, an inhibition in DNA synthesis compared to non-treated cells (Major, Egan et al. 1982).

From these results it appeared that the amount of 5-FU incorporated into DNA could not, *per se*, be sufficient to explain 5-FU cytotoxicity. It was therefore suggested that 5-FU effect could be due to an interference with DNA repair mechanisms.

To investigate the relation between 5-FU cytotoxicity and DNA damage a first type of experiments was performed to determine whether 5-FU toxicity could be due to DNA fragmentation. Human colon adenocarcinoma cells (WiDr) were treated with either 5-FU or Methotrexate (Mtx) alone or by 5-FU combination protocol (Mtx followed by 5-FU). Mtx is known to prevent DNA fragmentation. These experiments showed that although 5-FU induced a DNA fragmentation there was no correlation between DNA fragmentation and the level of toxicity (Lonn and Lonn 1986). The role of DNA repair mechanisms in 5-FU cytotoxicity was then investigated.

It has been shown that the different levels of DNA repair enzymes can modulate 5-FU cytotoxicity. For example, Thymidine DNA glycosylase (TDG) overexpression renders cells more sensitive to 5-FU. TDG is an UDG that can excise incorporated 5-FU efficiently resulting in the permanent DNA strand break accumulation and cell death. In contrast, TDG loss results in

enhanced effective DNA repair and decreased DNA strand breaks leading to 5-FU resistance (Kunz, Focke et al. 2009).

Conversely, it has been reported that 5-FU cytotoxicity is not affected by another mechanism DNA repair system, the DNA mismatch mechanism (MMR). Indeed HCT116 cells which are deficient with MMR and their corresponding MMR-proficient HCT116+chr3 cell line have similar sensitivity to 5-FU and also to other anti-tumor drugs (de las Alas, Aebi et al. 1997; Fischer, Baerenfaller et al. 2007).

Recent study on human colon and breast cancer cells has demonstrated that the initiation of base excision repair (BER) mechanism by UNGs enzymes (UNG1, UNG2, SMUG1, TDG and MBD4) is the main mechanism of 5-FU-DNA repair, MMR mechanism being is limited to specific excision of 5-FU opposite guanine (FU: G). However, Knockdown of BER or MMR enzymes as well as inhibition of DNA damage response proteins by different molecular inhibitors did not affect overall 5-FU cytotoxicity.

Altogether these studies showed that inhibition of TS activity, mis-incorporation into DNA and stimulation of DNA repair mechanisms to some extent cannot account for the major cytotoxic effect of 5FU. In the course of these experiments however, it has been found that 5-FU incorporates within RNA with a rate 3000 to 15000 fold higher to that within DNA (Pettersen, Visnes et al. 2011).

5.4.2. The evidence for 5-FU RNA-based cytotoxicity

From all the data reported above suggesting that 5-FU effect was due to its interference with DNA metabolism, it was proposed that thymidine addition might overwhelm the 5-FU effects and protect cells from “thymine less death” and remediate cytotoxicity. On the contrary, if 5-FU effect was due to incorporation into RNA then uridine could relieve this effect.

Radioactive labeling in HCT116 cells treated with 10 μ M 5-FU showed a 5-FU RNA incorporation. Uridine treatment decreased FU-RNA incorporation by 42-60% and a strong inhibition of cytotoxic effect, which was not observed after pre-treatment by thymidine (Geoffroy, Allegra et al. 1994).

Another experiment performed in vivo confirmed that the cytotoxic effect of 5-FU was essentially due to its incorporation within RNA rather than within DNA. 5-FU treatment of BDF1 mice induced apoptosis in colon crypts, thymidine treatment did not reduced apoptosis whereas uridine application after 2h of 5-FU treatment was able to greatly reduce apoptosis and 5-FU incorporation into RNA. To verify that thymidine was actually able to relieve the inhibition of TS enzyme in these experimental conditions, Tomudex (a TS specific inhibitor) was administrated and induced apoptosis. As expected, thymidine addition relieved Tomudex TS inhibition and its apoptotic effect whereas uridine addition had no effect on cell death (Pritchard, Watson et al. 1997). Thymidine failure strongly suggested that TS inhibition and its consequences were not the major cause of 5-FU anti-tumor effects.

Furthermore, experiments performed in yeasts under 5-FU treatment demonstrated more elevated growth rates in the presence of UMP than in the presence of dTMP. In addition to that,

culture density with 5-FU and UMP was approximately two times the density of culture with 5-FU and dTMP (Hoskins and Scott Butler 2007).

In conclusion, because uridine but not thymidine can relieve 5-FU induced cytotoxicity strongly supported the notion that 5-FU cytotoxicity was due to its interference with RNA metabolism and function rather than that of DNA metabolism, as proposed initially.

5.4.3. RNA dependent mechanism of 5-FU cytotoxicity

As mentioned above, 5-FU metabolite (FUTP) can incorporate into RNA and this capacity is very probably responsible for the 5-FU induced cytotoxic effects. However, knowing that 5-FU can be potentially incorporated within all RNA species, the RNA-based mode of action of 5-FU needs to be determined.

5.4.3.1. 5-FU effects on pre-mRNA maturation

5-FU could substitute residues in mRNA precursor and this substitution could disturb splicing. In HeLa cells, the splicing of the mRNA precursor of β -globin produces well-known intermediates. Therefore, it was used to study the effect of 5-FU substituted transcripts on splicing fidelity. The β -globin pre-mRNAs were generated in a SP6 polymerase transcription system. 5-FU incorporation into transcripts were produced by replacing UTP with FUTP. Three new RNA species resulted from the 5-FU-substituted pre-mRNA in a standard splicing assay.

These results indicate that 5-FU substitution in pre-mRNAs can directly alter splicing fidelity. Moreover, at the concentration of IC₅₀, 5-FU can take the place of only 2% of uracil residues, while 84% of replacement is needed to detect a defective splicing. Hence, β -globin mRNA precursor requires large amount of 5-FU substituted nucleotides to alter splicing products, other transcripts may be more sensible to substituted 5-FU and need less substitution (Doong and Dolnick 1988).

Another proposed possibility is that 5-FU substituted mRNA can induce truncated proteins and translational errors. The mRNA template of thymidylate synthase TS was subjected to 100% substitution of uracil by 5-FU. The comparison of TS wild type and 5-FU substituted mRNA transcripts revealed an alteration in secondary structure of 5-FU-mRNA. Even though, the produced proteins had the same catalytic activity for both mRNAs and no evidence for translational miscoding (Takimoto, Voeller et al. 1993). However, if 5-FU incorporation into mRNA template is not sufficient by itself to introduce alteration in splicing and gene products, then it may be produce its affects through the other 5-FU effects on splicing.

Splicing process is performed by a ribonucleoprotein complex called, spliceosome (Fig. 18). The spliceosome contains different proteins and five small nuclear RNAs (U1, U2, U4, U5 and U6 snRNA) which form together the small nuclear ribonucleoproteins snRNPs. The U nomenclature of snRNAs simply comes from U, which means uracil. Therefore, uracil enriched snRNAs represent privileged target for 5-FU substitution.

A study performed on mouse sarcoma cells S-180 reported a decrease in U1 turnover after 48h of treatment with (10 μ M) and alteration in secondary structure of U4 and U6 snRNA (Armstrong, Takimoto et al. 1986). In yeast endogenous U2 was depleted leading to spliceosome inhibition. U2 was reconstituted with a total replacement of uracil by 5-FU to give up FU2. This FU2 was not able to reintroduce any splicing activity. However, splicing was partially recovered by reducing 5-FU content of FU2 and achieved 60% of its normal activity at 25% of uracil substitution. Moreover, FU2 could compete with normal U2 and cause pre-mRNA degradation probably by forming inactive complex or by poor interaction with splicing factors (Lenz, Manno et al. 1994). A complementary study performed in HeLa cells showed that splicing inhibition by FU2 is due to 5-FU incorporation in pseudouridylation site, which resulted in U2 pseudouridine lake and functional loss (Zhao and Yu 2007).

In contrast, the reconstitution of FU6 did not inhibit splicing, FU6 could restore its splicing functions but at a very high concentration. This could be explained by a reduction in FU6 binding capacity. If 5-FU incorporation occurred in U6-U4 binding region (a region of six U-A base pairs) that would decrease U6-U4 stability and induce their disassociation. Therefore, a very high concentration of U6 could overcome destabilization effect of 5-FU (Lenz, Manno et al. 1994).

5.4.3.2. The 5-FU effects on rRNAs

Ribosomal RNAs are the most abundant RNAs in the cell. rRNAs are essential for the overall structure of the ribosomes and they display ribozyme activity responsible for proteins synthesis. rRNAs are produced as precursors that is subjected to many processing steps to produce the final mature sequences. In yeast, the primary transcript contains the 18S, 5.8S and 25S rRNAs separated by internal transcribed spacers (ITS) and flanked by 5' and 3' external spacers (ETS). In mammals, the primary transcript contains the 28S, 18S and 5.8S separated also by spacers (Fig. 5). All the ITSs and ETSs are removed out during rRNA processing. Any disruption of intermediates steps will impede rRNA maturation.

A gene ontology study carried out in yeast to analyze the genomic profile under different anticancer drugs has been demonstrated that 5-FU sensitivity is associated with enrichment in genes involved in ribosome biogenesis and rRNA processing. These results suggested that 5-FU incorporation into RNA would disturb the rRNA synthesis and maturation (Giaever, Flaherty et al. 2004). Another Genome wide brought evidence that 5-FU treatment in yeast impaires rRNA processing. The internal transcribed spacers (ITS1 and ITS2) of the pre-rRNA were inefficiently removed leading to accumulation of mis-processed rRNA product that contains fragments of both spacers (Lum, Armour et al. 2004).

To further characterize the effect of 5-FU on rRNA, a study on fibrosarcoma cells using ³²P labelling has demonstrated that 5-FU can inhibit the maturation of 18S and 28S rRNAs. This experiment revealed an accumulation of 47S rRNA precursor, while in non-treated cells the 47S was undetectable. This high level of 47S precursor shows that 5-FU does not inhibit Pol I transcription but rather induces a blockage of 47S processing. To get more insight on how 5-FU can affect rRNA processing, 5-FU treatment was combined with favopiridol. This combination was reported to enhance significantly the reduction of 28S levels, while neither 5-FU alone nor

flavopiridol alone can cause a total 28S inhibition (Burger, Muhl et al. 2010). Thus, these two drugs synergistically affected rRNA processing. As 5-FU was showed to be unable to block the 3' end processing of a pre-rRNA (Ghoshal and Jacob 1994), it was proposed that flavopiridol could inhibit the 3' end processing of 28S while 5-FU could inhibit 5' end formation leading to a decreased 28S level.

Recent study confirmed the effect of 5-FU on the ITS2 processing. In addition to that, the genome wide screening data was analyzed after eliminating all genes known to be sensitive to all types of cellular stress except 5-FU. Therefore, only genes that had specific respond to 5-FU were identified. Again, genes with rRNA processing functions were found to be induced by 5-FU, such as *cgr1*, which is involved in pre-rRNA processing for the ribosomal large subunit 60S; and *Rrp45* which is involved in 5.8 rRNA maturation (Mojardin, Botet et al. 2013).

The rRNA maturation defects can be consistent also with 5-FU inhibiting exosome activity. A polyadenylated intermediate form of 27S rRNA which is normally degraded by the exosome accumulated after 5-FU treatment in yeast cells with wild type Rrp6 exosome. This accumulation was increased in deficient Rrp6 strain suggesting that 5-FU can interfere with exosome ability to degrade this poly (A) form of rRNA (Fang, Hoskins et al. 2004).

5.4.3.3. The 5-FU effects on tRNAs

The tRNAs are highly transcribed in the cell by RNA polymerase III (Pol III), the pre-tRNA will be submitted to several processing and splicing steps to convert into mature tRNA. During maturation, a tRNA undergoes many post-transcriptional nucleotide modifications such as uridine modifications which consist mostly of pseudouridine, dihydrouridine and 5-methyluridine (m^5U) formation. These modifications play an important role in tRNA stability structure. As 5-FU can replace uridine it is very probable that 5-FU might affect tRNA structure and function.

In *E.coli* treated with 5-FU, the tRNA had 85% of its uridine substituted by 5-FU. Surprisingly this 5-FU tRNA was fully functional as well as the natural tRNA (Kaiser, Jacobson et al. 1969).

Experiments performed in mice showed that formation of pseudouridine and dihydrouridine was reduced after 5-FU treatment, but this inhibition was greater than the amount of 5-FU incorporated into tRNA. Indeed, it has been shown that modifications of pseudouridilation was mainly due to enzyme inhibition rather than to a direct effect of 5-FU substitution (Tseng, Medina et al. 1978).

One of these enzymes is the pseudouridine synthase I (Ψ SI) which converts certain uridine residues into pseudouridines. Experiments in *E. coli* were carried out to determine the 5-FU effects on Ψ SI enzyme activity. The study found that 5-FU-tRNA binds tightly to Ψ SI through a specific link contains a single FUMP in the position 39 of the 5-FU-tRNA. This binding forms the stable covalent Ψ SI-5FU-tRNA complex and leads to effective inhibition of the enzyme activity (Huang, Pookanjanatavip et al. 1998). Additionally, a genome screening performed in yeast identified many mutated genes involved in tRNA maturation as 5-FU targets (Gustavsson and Ronne 2008).

From these studies, it can be concluded that 5-FU substitution does not affect the tRNA function itself but in contrast, it can affects the post-transcriptional modification, tRNA stability

and structure. However, the contribution of these 5-FU induced alterations of tRNA metabolism on 5-FU-induced cytotoxicity remains to be clarified.

5.4.3.4. The 5-FU effects on microRNAs

MicroRNAs (miRNAs) are small non-coding RNA molecules that consist of approximately 22 nucleotides that are now recognized as important players of gene expression regulation. They are predicted to negatively regulate expression of more than 60% of coding genes at the post-transcriptional level. miRNA regulatory function is mediated by their hybridization within the 3'UTR region of their mRNA targets. Generally complete base pairing interaction between miRNA and the target results in mRNA degradation, while imperfect base pairing inhibits translation process of the targeted mRNA (Friedman, Farh et al. 2009).

Effect of 5-FU on miRNAs expression has been reported for the first time in a study on long-term cultured clones of human colon cancer cells HT29 and HCT116. Cells were treated with 10 μ M 5-FU for 6 day and then subjected to microarray analysis. This demonstrated a 5-FU deregulation of miRNAs expression (Rossi, Bonmassar et al. 2007). Another miRNA microarray analysis has been performed on colorectal cancer cells HCT116 and HCT8 after 24h of 5-FU treatment. Again, it was shown that many miRNAs were deregulated by 5-FU. Further investigation demonstrated that the expression of these identified miRNAs was associated with drug resistant and/or poor prognosis. The miR-92a, which is encoded by *miR-17-92* gene cluster, has an elevated expression in CRC cells and was down regulated by 5-FU (Zhou, Zhou et al. 2010). The *miR-17-92* cluster have oncogenic function and is up regulated by *cMyc*. The expression of this miRNA cluster was also decreased after 5-FU treatment in KM12C colon cancer cells confirming that 5-FU can affect expression of specific miRNAs (Zhao, Ooyama et al. 2008). A miRNA expression profile in nasopharyngeal carcinoma (NPC) showed a decrease in the number of expressed miRNAs after 5-FU treatment. This number was greatly reduced after 5-FU combination therapy with Cisplatin. On the other hand, many of the expressed miRNAs in 5-FU treatment had tumor suppressive functions and were not expressed in the non-treated cells (Zhang and Li 2012).

A genome wide screening performed on breast cancer cells MCF-7 has revealed that 5-FU treatment for 48h with a very low dose altered the miRNAs expression profile. As well, the experiments on certain miRNAs expression showed a dose-response relationship with 5-FU revealing a specific sensitivity to the drug. Gene function analysis demonstrated that more than a half of the 5-FU deregulated miRNAs have target genes that are involved in oncogenesis, metastasis and apoptosis. Moreover, a group of microRNAs linked to P53 have been identified. It is known that 5-FU induces P53 expression and an up regulation of some of its target genes. Therefore, it is possible that the effects of 5-FU on P53 and its downstream targets are mediated, at least in part, by an effect of 5-FU on miRNAs (Shah, Pan et al. 2011).

Altogether these observations show that 5-FU alters miRNAs expression albeit but a mechanism that is still unclear. Furthermore, this 5-FU/miRNAs relationship strongly suggests that miRNAs can play a potential role in mediating 5-FU effects on genes expression at the post-transcriptional levels.

5.4.3.5. The 5-FU effects on RNA surveillance machinery

The exosome is one of the major actor of RNA quality control. It is a multi-protein complex consisting of nine protein subunits that provide a structural platform for the additional subunits with catalytic activities that are essential for exosome function, such as Rrp6 that exhibits a hydrolytic exoribonuclease activity. In the nucleus, the exosome is responsible for the precise processing and maturation of RNAs precursors or the degradation of non-coding RNAs. In the cytoplasm, it contributes to RNA decay (Fig. 19) (Schneider and Tollervey 2013).

Initial evidences of 5-FU targeting exosome components come from a recent genome-wide screening performed in yeast. Four exosome subunits (Rrp6, Rrp41, CDC21 and MAK21) as potential 5FU targets have been identified (Lum, Armour et al. 2004). It has been shown in HeLa cells that 5-FU stabilizes hRrp6 substrates, and Rrp6 depletion renders cells more sensitive to 5-FU. These results strongly supported the notion that Rrp6 is a 5-FU target (Kammler, Lykke-Andersen et al. 2008). Furthermore, it has been determined that 5-FU affects directly RRP6 association with the other multimolecular complexes. In addition, *in vivo* experiments demonstrated that an Rrp6 could not destroy efficiently rRNA when these RNAs have incorporated 5-FU. Finally, it was shown that mRNA synthesized in vitro with 5FU resisted degradation by Rrp6 whereas the normal mRNA was efficiently digested (Silverstein, Gonzalez de Valdivia et al. 2011). These findings allow to conclude that 5-FU impairs RNA decay by disturbing Rrp6 exosome subunit function and by incorporation of 5-FU into RNAs and make them less sensitive to exosome degradation.

RESULTS

1. The effect of different chemotherapeutic drugs on protein synthesis

We have evaluate whether 5-FU as an antimetabolite drug impacts the global rate of protein synthesis and whether this effect is similar to other types of drugs (i.e. genotoxic agents such as camptothecin and doxorubicin) used in chemotherapeutic treatments. For this, effect of oxaliplatin and 5FU, two drugs intensely used for treatment of colorectal cancers was evaluated on HCT116 cells protein synthesis. In parallel, effect of camptothecin and doxorubicin, two drugs used for breast cancer treatment was evaluated on MCF7 cells protein synthesis. As shown in Fig.1, HCT116 were treated during 4h and 24h with either 200 μ M of oxaliplatin or 100 μ M 5FU. One hour before the end of the treatment time, cells were labelled with [35S] methionine. As shown in Fig. 1B, after 4h of treatment with both drugs, the global rate of protein synthesis was very similar to that of the non-treated cells. Conversely 24h of treatment induces decrease, although slight, of global protein synthesis rate in both 5FU and oxaliplatin treated cells compared to non-treated cells (Fig. 20).

MCF7 cells were treated for 3h, 6h and 24h. Like for the experiment presented above, cells were incubated with [35S]methionine one hour before analysis. Cycloheximide (CHX), a well characterized inhibitor of protein synthesis was used as a control. As shown in (Fig.21) 3h and 6h of treatment with with 1 μ M of camptothecin (CPT) or with 2 μ g/ml of doxorubicin (DOXO) did not induced a visible decrease of protein synthesis rate. However, 24 h of treatment with CPT induces a marked inhibition of protein synthesis that is even more pronounced when DOXO is used (Fig. 21).

Although performed with different cell lines these experiments show that oxaliplatin and 5FU had little inhibitory effect on HCT116 cells when used at 200 μ M and 100 μ M respectively even after a long period of treatment of 24 h. In addition, although performed in a different cell line, other types of drugs CPT and DOXO induces a marked decrease of protein synthesis after 24h of treatment.

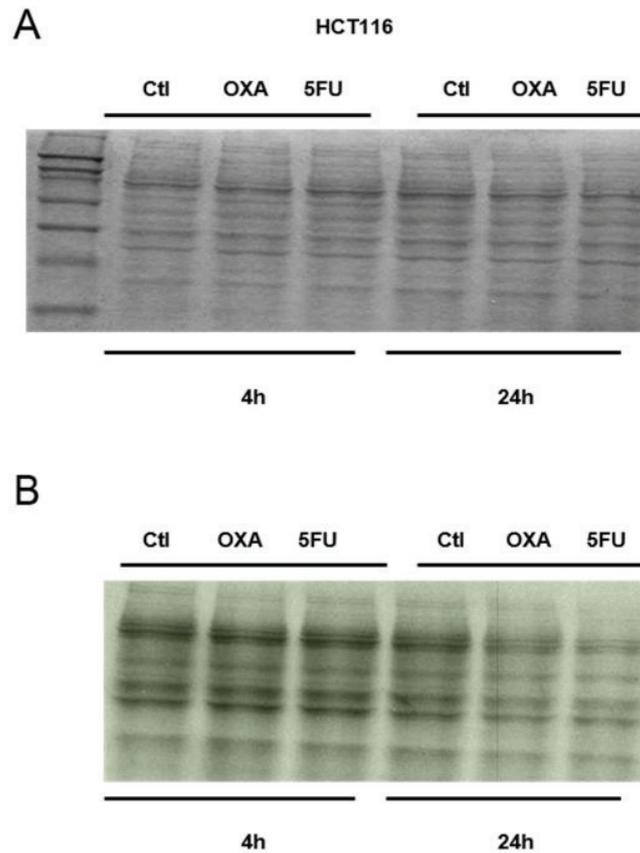


Figure. 20. *Effect of oxaliplatin and 5FU on protein synthesis of the colon cancer cell line HCT116.* cells were treated for 2h with (200 μ M) oxaliplatin then removed and replaced with fresh medium, and with (100 μ M) 5FU (continuous treatment). Cells were grown in methionine/cysteine-free medium for 30min, labeled with 100 μ Ci/ml [35 S] methionine for 60 min (5FU was presented during metabolic labeling). *A.* SDS-polyacrylamide gel electrophoresis with Coomassie based stain, using 15 μ g of protein per lane. *B.* The correspond silver staining profile for whole cell proteins visualized by autoradiography (total exposure time 3h) in SDS- polyacrylamide gel shows total labeled proteins during the last hour of 4h and 24h of treatment.

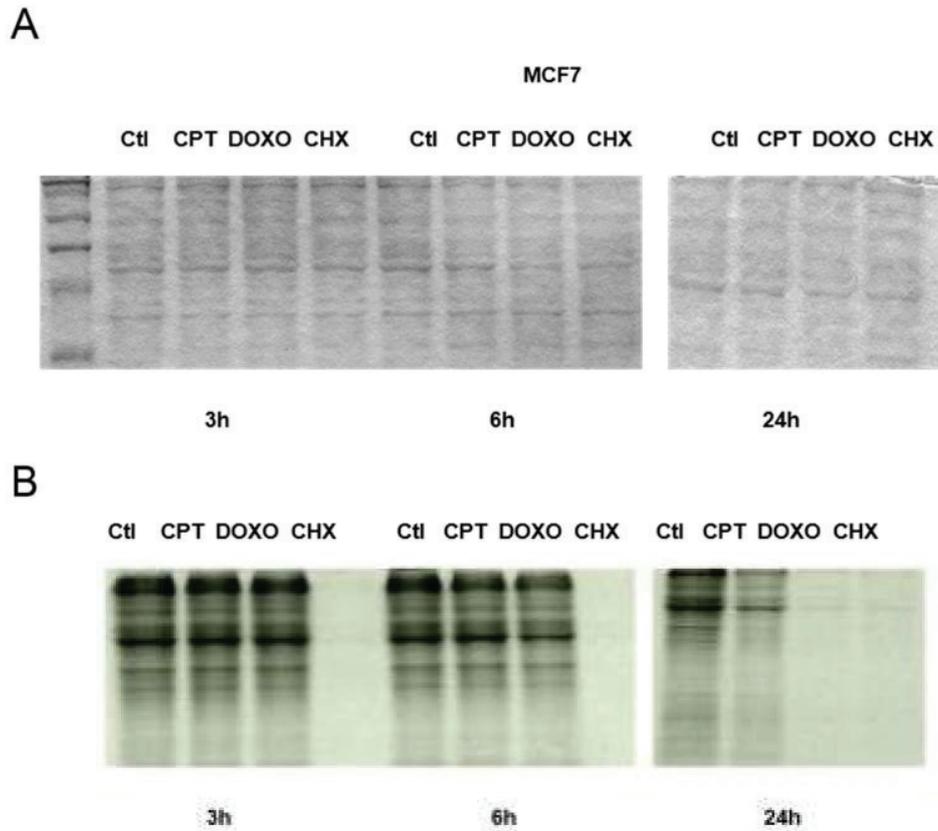


Figure. 21. *Effect of camptothecin and doxorubicin on protein synthesis of the breast cancer cell line MCF7. Cells were treated for 3h, 6h and 24h with 1 μ M camptothecin, 2 μ g/ml doxorubicin and 100 μ g/ml cycloheximide (positive control). All drugs were presented during metabolic labeling. A. SDS-polyacrylamide gel electrophoresis with Coomassie based stain, using 15 μ g of total protein per lane. B. The correspond profile for whole cell proteins visualized by autoradiography in SDS-polyacrylamide gel shows total labeled proteins during the last hour of 3h, 6h and 24h of treatment. Ctl, Control; CPT, camptothecin; Doxo, doxorubicin; CHX, cycloheximide.*

2. Optimization of 5-FU treatment conditions

2.1. Impact of 5-FU treatment on cell viability using MTS assay

5-FU is a cytotoxic drug. To optimize 5-FU treatment and thus identify a treatment condition allowing to harvest alive cells, we first evaluated the effect of different 5-FU concentrations on cell viability using MTS assay. Such assay measures reduction of tetrazolium agent (MTS) into formazan by dehydrogenase enzymes that occurs in the mitochondria of viable cells (Mosmann 1983). HCT116 cells were treated continuously with increasing concentrations of 5-FU (0, 1, 2, 5, 10, 20, 50, 100, 200, and 500 μ M) for 48h. Cell viability was assessed at 0, 6, 24 and 48h after addition of 5-FU by absorbance detection of tetrazolium agent reduction (Fig. 22). The control cells, corresponding to non-treated cells or 0 μ M treatment, exhibited a continuous increase in dehydrogenase activity, suggesting that in normal culture condition, cell viability increases continuously for 48h. This observation is compatible with proliferative cells, as expected in such condition.

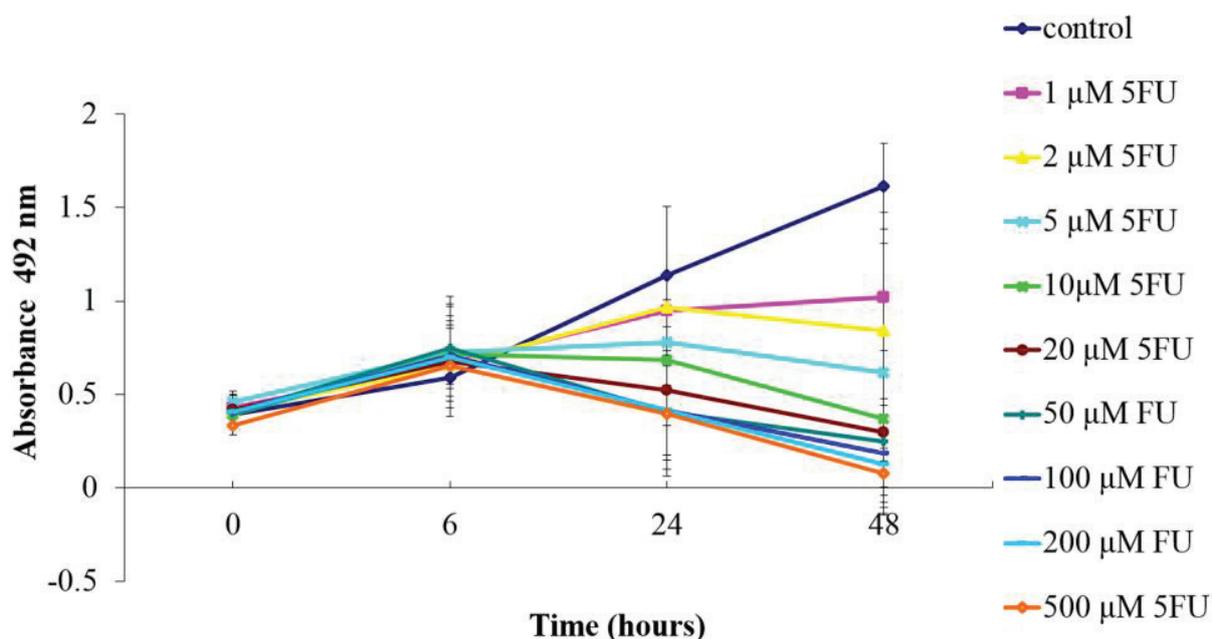


Figure .22. Effect of 5-FU treatment on cell proliferation. HCT116 cells were treated with 0, 10, 100 and 500 μ M of 5-FU, cell growth was measured by MTS at 0h, 6h, 24h and 48h post 5-FU administration. Means and SD present three independent experiments.

In response to 5-FU treatment, two different cell viability profiles were observed depending on 5-FU concentrations. At high 5-FU concentrations (20, 50, 100, 200 and 500 μ M), cell viability decreased from 24h post-treatment to reach a maximum at 48h, when dehydrogenase activity was almost inexistent for all treated cells. These data suggest that at high concentrations of 5-FU, no

more cells are viable, compatible with the cytotoxic effect of 5-FU. Moreover, this cytotoxic effect is dose and time dependent.

At low 5-FU concentrations (1, 2, 5, and 10 μ M), an increase in cell viability was observed until 24h when the cell viability became constant (1 μ M) or slightly decreased (2, 5 and 10 μ M). It has to be noted that at 10 μ M of 5-FU treatment, cell viability decreased until reaching the value of the starting cell viability that was observed at the beginning of the assay, suggesting that some cells are still alive. These data suggest that low concentrations of 5-FU also exhibits cytotoxic effect but in a different manner than high concentration of 5-FU. Interestingly, the two cell viability profiles can be distinguished on the basis of the half-maximal inhibitory concentration of 5-FU calculated at 24h [$IC_{(24h)50}=12.9\mu$ M]. These data showed that 5-FU treatment differentially affect cell viability, the IC_{50} being a good cut-off to distinguish the two pattern of cytotoxic effects of 5-FU treatment.

2.2. Real-time monitoring of cellular compartment during 5-FU treatment

To finely monitor the difference in the two cytotoxic effects of 5-FU observed in cell viability assay, we used the xCELLigence system. This method allows real-time monitoring of cell compartment throughout time using detection of electrical impedance. Cells are in direct contact with golden electronic sensors at the bottom of the wells and contacts between cells and sensors reduce the electrical current in a given well to produce impedance signals measured each 15 minutes. The impedance signals are plotted as cell index (CI) that directly reflect the cellular dynamic behavior throughout time. It has to be noted that CI thus corresponds to the integration of diverse cellular compartments, including changes in cell number, spreading, adhesion quality and morphologies. HCT116 cells were treated with 0, 10, 100 and 500 μ M of 5-FU and monitored for six days (Fig. 23). As shown by real time monitoring, the non-treated cells displayed a dynamic profile in which the CI increased with time until reaching a plateau at about 72h, corresponding to saturation of the surface well. Like MTS assay, this observation is in accordance with proliferative cells. Compared to non-treated cells, 5-FU treated cells exhibits two different real time monitoring profiles. Cells treated with high doses of 5-FU (100 and 500 μ M) exhibit the most drastic cytotoxic effect. Indeed, from 24h post-treatment, electrical impedance decreased drastically until reaching the CI background, indicating that no more cells were in contact with the well surface. These observations suggest that high concentration of 5-FU promotes either detachment of cells from the wells or high level of cell mortality. Since MTS assay showed that high doses of 5-FU abolished cell viability, we may expect that high concentrations of 5-FU promote cell mortality. Altogether, these data confirm the drastic cytotoxic effect of high concentration of 5-FU.

At low dose of 5-FU (10 μ M), cells displayed a dual real time monitoring profile. First, an increased CI was observed during the first 24h of treatment. Interestingly, the increased CI of 10 μ M treated cells during these first 24h was higher than the one of non-treated cells, suggesting that 10 μ M treated cells established more contacts with wells than non-treated cells. This observations indicate a change in either proliferative or morphological state. In MTS assay, 10 μ M treated cells exhibit a constant cell viability, suggesting that increase in CI corresponds to change in morphological state rather than in proliferative state. Second, at 24h post-treatment, 10 μ M

treated cells presented a decreased CI, which fallen down until its minimal values at 72h without reaching the CI background, suggesting that some cells are still in contact with the wells compared to cells treated with high doses of 5-FU. These observations are in accordance with MTS assay, since 10 μ M treated cells still exhibit metabolic activity compared to 500 μ M treated cells. Altogether, real-time monitoring reinforced the existence of two different cytotoxic effects of 5-FU. Low doses of 5-FU treatment indeed showed unexpected real time monitoring profiles that suggest a change in cell behavior in the first 24h compared to both non-treated cells and high dose-treated cells. Moreover, low concentrations of 5-FU allowed the survival of metabolically active cells at 72h post-treatment.

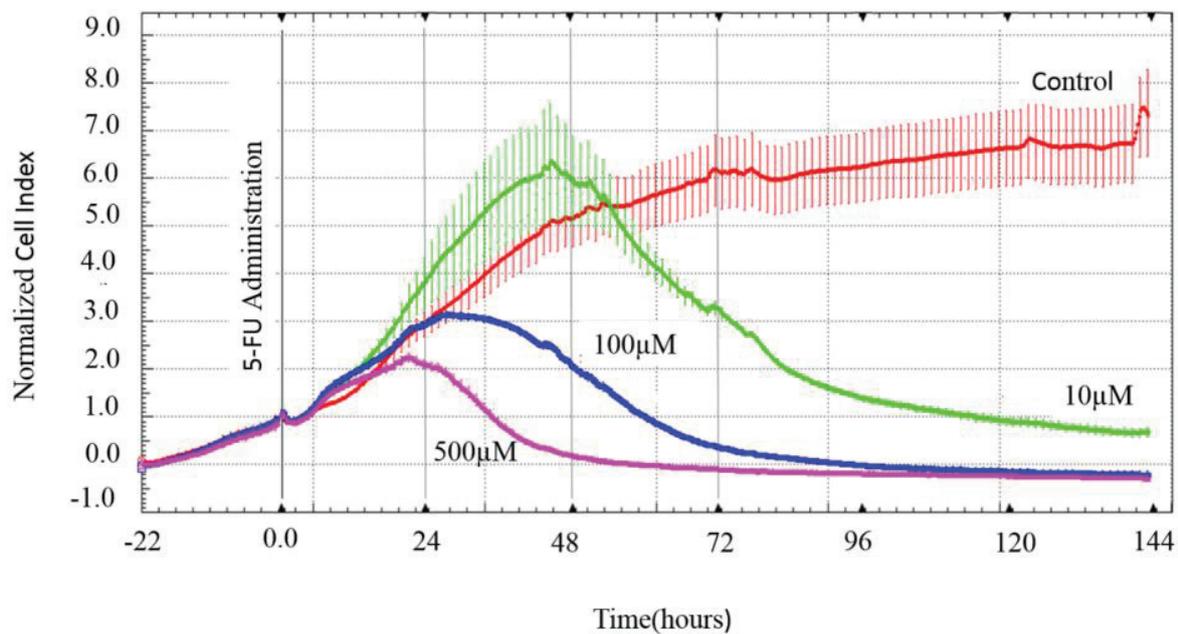


Figure. 23. Real-time monitoring analysis of HCT116 cells after 5-FU treatment. a. The real time curves of cell index (CI) during time (h) after treatment with different 5-FU doses (0, 10, 100 and 500 μ M). Data represent a presentative experiment repeated three times. The standard deviation of quadruplicates are displayed; CI was normalized to time point of 5-FU administration.

2.3. Determination of cell mortality in response to 5-FU treatment

To determine whether cytotoxicity effect of 5-FU involved cell mortality, we estimated the number of dead cells present at the bottom of the well using trypan blue method. This method is based on the fact that viable cells have integral membranes that exclude the dye, while the membranes in dead cells are damaged and can interact with blue trypan, which will inter and stain the cytoplasm. Therefore, only dead cells will get the blue colour (Strober 2001). HCT116 cells were continuously treated with increasing concentrations of 5-FU (0, 10, 100 and 500 μ M) then

counted at 24h and 48h post-treatment. In non-treated cells, an increase in total cell number was observed between 24h and 48h post-treatment, while no change in dead cell number was observed. In respect with MTS assay and real time monitoring of cell behavior, HCT116 cells exhibit proliferative state in normal cultured conditions (Fig. 24).

In contrast, 5-FU treatment at high concentration affected both total cell number and dead cell number. In response to high concentration of 5-FU, a significant reduction in total cell number was observed from 24h that was accentuated at 48h post-treatment. This reduction in total cell number was paralleled with a significant increase in dead cell number until reaching 42% of dead cells 48h after addition of 500 μ M 5FU. Thus, high concentrations of 5-FU treatment have drastic cytotoxic effects that promote cell mortality. This observation fully explained the lack of cell viability and attachment in response to high concentration of 5-FU.

Like high doses of 5-FU, 10 μ M 5-FU treatment significantly reduced total cell number at 24h posttreatment. However, no significant increase in dead cell number was observed, indicating that 10 μ M 5FU treatment reduced cell division rather than increasing cell death during the first 24h. This observation supports a change in morphological state of treated cells rather than increase in proliferation based on real time monitoring profile. At 48h post-treatment, amount of total cell remained unchanged compared to 24h post-treatment. However, a slight increase in dead cell number was observed at 48h post-treatment that can explain both the decrease in cell viability and in cellular index.

Overall, these data highlight the existence of two different cytotoxic effects of 5-FU drug. A drastic cytotoxic effect in response to high 5-FU concentration that promote massive cell mortality until eliminating all cells, and a medium cytotoxic effect in response to low doses of 5-FU that first alters cell behavior then promote selection of some resistant cells.

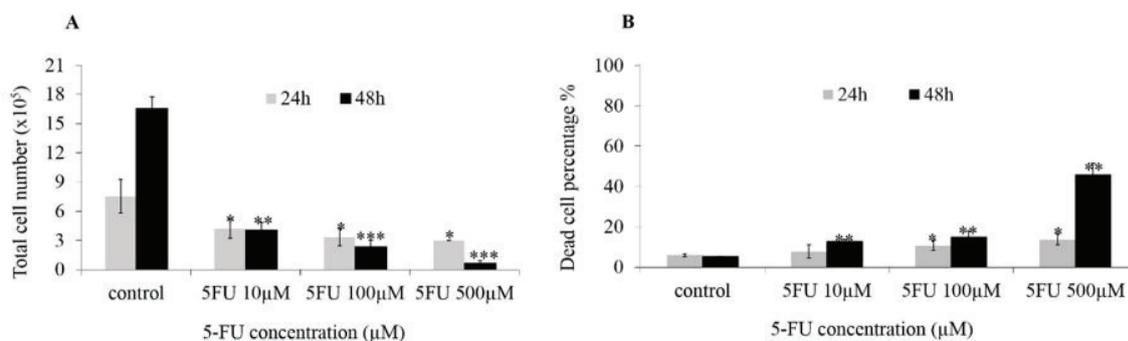


Figure. 24. Determining the effect of 5-FU on cell number and mortality. HCT116 cells were treated with 0, 10, 100 and 500 μ M 5-FU for 24h and 48 h. A. The total number of cells. B. The percentage of dead cells was calculated through counting cells excluded trypan blue. Mean values \pm s.d. were established based on the results from three independent experiments each with three replicates.

2.4. Effect of 5-FU on ribosome production

5-FU can disturb the rRNA processing and inhibit ribosome biogenesis (Greenhalgh and Parish 1990; Ghoshal and Jacob 1994; Burger, Muhl et al. 2010). To determine the effect of 5-FU on ribosome quantity, firstly we have quantified the neo-synthesized rRNAs. We have performed ^{32}P -orthophosphate 2 hours pulse labeling of the newly synthesized 18S and 28S rRNAs and quantified the incorporated ^{32}P after 3 hours chase. As shown in (Fig. 25A), HCT116 cells were treated for 12h, 24h, 48h and 72h by $10\mu\text{M}$ 5FU or by actinomycin D for 3h. Treatment with actinomycin D has completely abolished rRNAs synthesis comparing to non-treated cells while 5-FU treatment has partially affected the production of 28S and 18S. At 12h, 24h and 48h 5-FU post-treatment, the neo-synthesized 28S was decreased by $\sim 70\%$ while the production of 18S was less affected and reduced by only $\sim 40\%$. After 72h of 5-FU treatment, a more significant reduction of 80% and 60% has been observed for 28S and 18S, respectively.

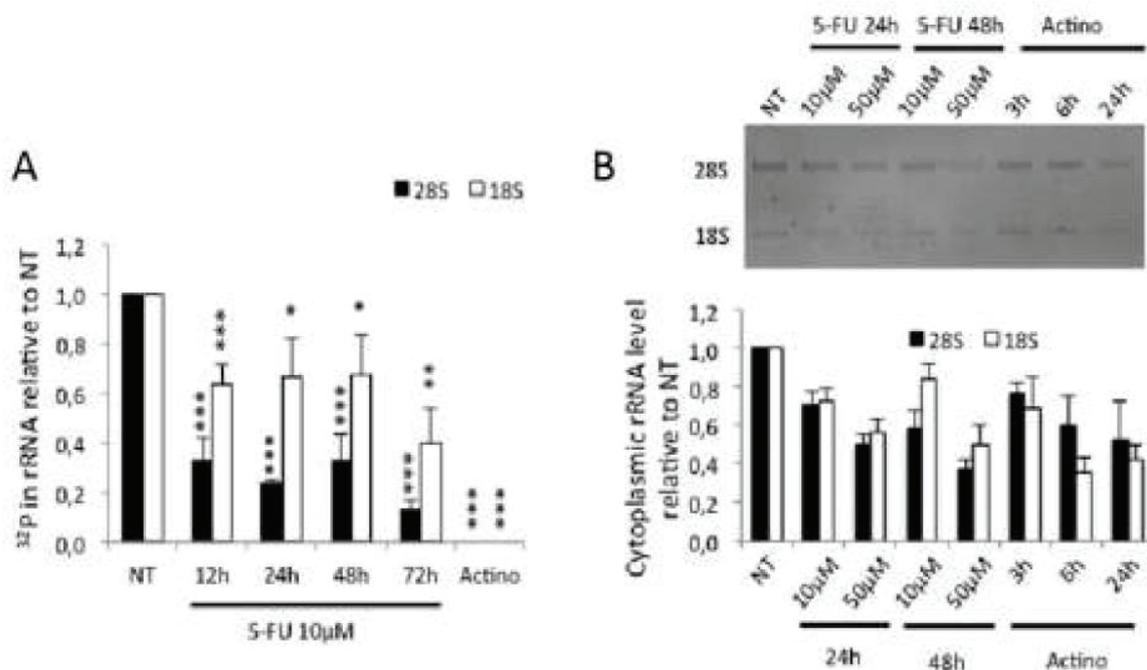


Figure. 25. The effect of 5-FU on ribosome biogenesis and ribosomes quantity. A. The rate of newly synthesized 28S and 18S rRNAs was monitored by 2h ^{32}P pulse labeling and 3h chase on HCT116 cells treated or not by $10\mu\text{M}$ 5-FU or actinomycin D for different time-points. B. The levels of 28S and 18S rRNAs were quantified by BET staining of the cytoplasmic RNAs extracted from the same number of cells which were treated or not by 5-FU (10 and $50\mu\text{M}$) for 24h and 48h and separated by denaturing agarose gel. Data shown present the average of at least three independent experiments after normalization vs the control. Error bars indicate the standard deviation of the average, significant differences from the non-treated cells (NT) condition are indicated by $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$.

Secondly, we have determined the effect of 5-FU on the total quantity of ribosomes. We have purified the cytoplasmic RNAs from an equal number of cells and quantified the 28S and 18S amounts. HCT116 cells were treated or not by (10 and 50 μ M) 5-FU for 24h and 48h or treated by actinomycin D for 3h, 6h and 24h. As shown in (Fig. 25B), low dose 5FU (10 μ M) has reduced 18S and 28S by \sim 30% at 24, this reducing effect has increased at 48h for only the 28S while the 18S has presented a small increase compared to 18S and 28S amounts in non-treated cells. The high 5-FU dose (50 μ M) has shown a more than 40% reduction in 18S and 28S that has continued to decrease at 48h. Cells treated with actinomycin D has more than 40% reduction in rRNA amount at 24h.

Together, these results show that cells under 5-FU treatment continue to produce the rRNAs: 18S and 28S. This production is sufficient to maintain the quantity of cytoplasmic ribosomes up to 50-80% of normal ribosome quantity produced in non-treated cells. These results suggest that 5-FU low dose is not sufficient to completely inhibit ribosome production.

3. Effect of 5-FU treatment on translation regulation

General experimental strategy. To identify the genes that are regulated at translational level, we have performed a genome-wide profiling for the translationally active and inactive mRNAs. We have used a sucrose gradient ultracentrifugation approach to isolate polysomes (active mRNAs) from free ribosomal subunits and monosomes (inactive mRNAs) from treated and non-treated cells. RNA were purified from these fractions and subjected to DNA microarray analysis. The translational status fold change (FC) was calculated as a double ratio and used to identify the translationally regulated genes (Fig. 26).

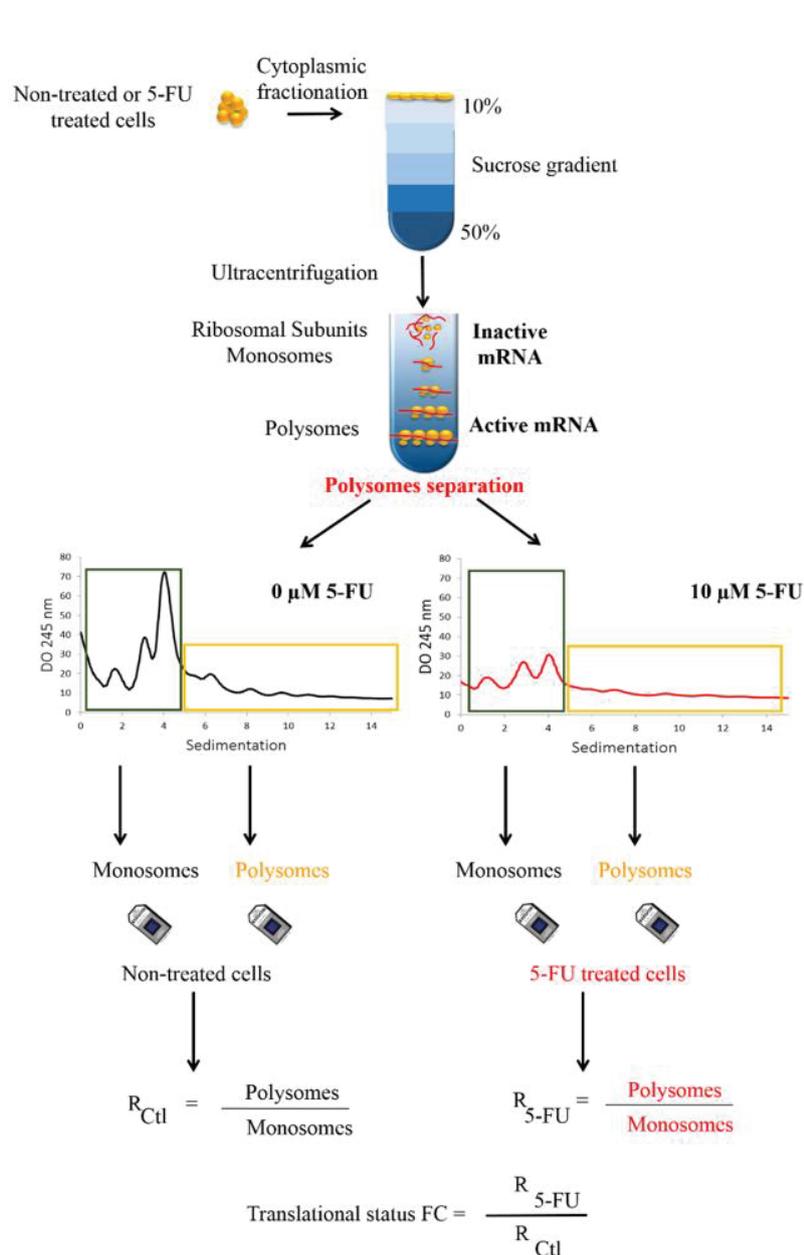


Figure. 26. Experimental design. Cytoplasmic cellular extracts from treated and non-treated cells were loaded onto sucrose gradients, monosomes and polysomes fractions were separated by ultracentrifugation. The absorbance profiles were generated at 245 nm. The monosome fractions (inactive mRNAs) and polysome fractions (active mRNAs) were collected and mRNA was extracted and used for microarray analysis. The alteration in the translational status for each mRNA was determined by calculating the “double ratio” or the ratio of ratio. It means calculating the ratio of active mRNA to inactive mRNA in treated cells (5-FU P/M) vs non-treated cells (control P/M).

3.1. Polysomes distribution after 5-FU treatment

HCT116 cells were treated with 0, 10, 100 and 500 μ M 5-FU for 24h. Cytoplasmic lysates containing free RNA, free ribosomal subunits as well as 80S ribosomes and polysomes were fractionated on sucrose gradients. Gradients were then collected and absorbance at 260 nm was determined during fraction collection to quantify the amount of RNA, ribosomes and/or ribosomal subunits present in each fraction of the gradients.

Polysome profile obtain from non-treated cells (Fig. 27A) displayed a typical profile with distinct peaks representing free RNA, 40S, 60S subunits, 80S monosome and polysomes. As shown in (Fig.27A, B, C, D), treatment with 5-FU induces an important modification of polysomes profiles.

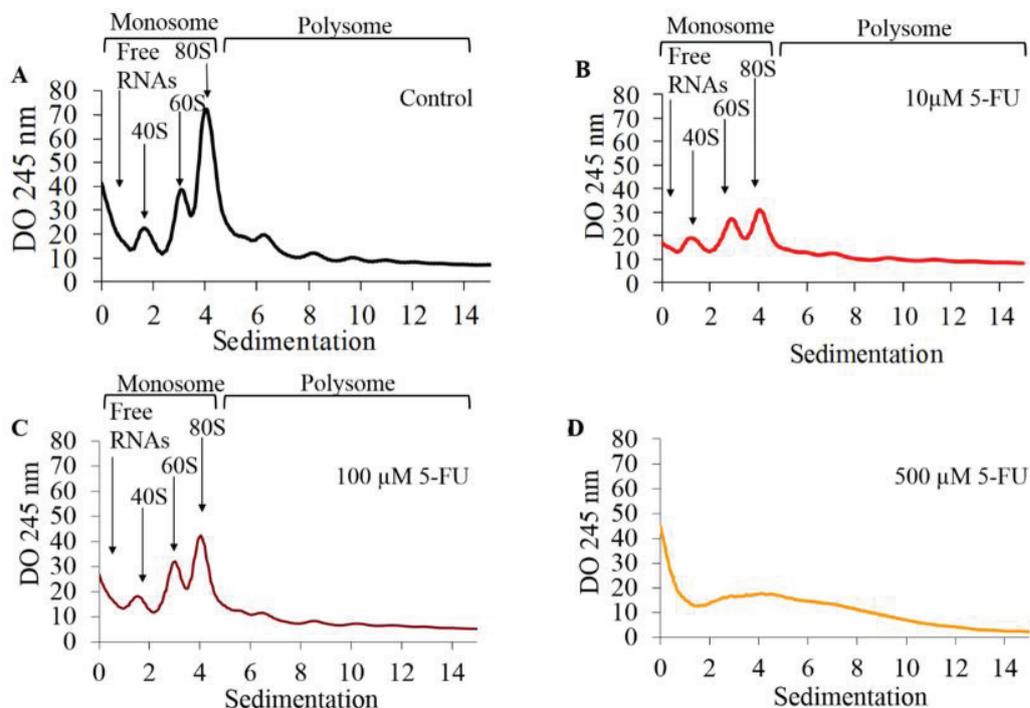


Figure. 27. Polysome profiles of ribosomes isolated from HCT116 cells and separated using sucrose gradients. (A-D) polysome profiles of samples that treated with 0, 10, 100 and 500 μ M of 5-FU for 24h. The 80S labels the monosome, 40S and 60S present the peaks of the small and large subunits, respectively.

To estimate accurately the effect of 5-FU treatment on ribosomal subunits and ribosome distribution within the different fractions of the gradient, the area under the curves (AUC) were measured for each of these profiles by weighting the profiles paper cutouts (Fig. 28A). Fractions containing more than one ribosome were collectively called the polysomal fraction (P). Fractions containing free RNA, 40S, 60S and 80S were collectively called the monosomal fraction (M). The ratio of ribosomes present in polysome fractions (P) vs ribosomes or ribosomal subunits present

in monosome fractions (M) were determined (Fig.28B). This allowed to determine that 10 μ M of 5-FU induces a decrease of the monosome fraction simultaneously with an increase of the polysomal fraction. The P/M ratio of cells treated with 100 μ M 5-FU was very similar to that of the non-treated cells. The polysome profile obtained from cells treated with high doses of 5-FU was dramatically altered and no ratio could be calculated.

These observations of a reduction in the monosome (80S) after treatment with 10 μ M 5-FU together with an increase of ribosomes in the polysome fraction strongly suggests that 5-FU induces a stimulation of translation.

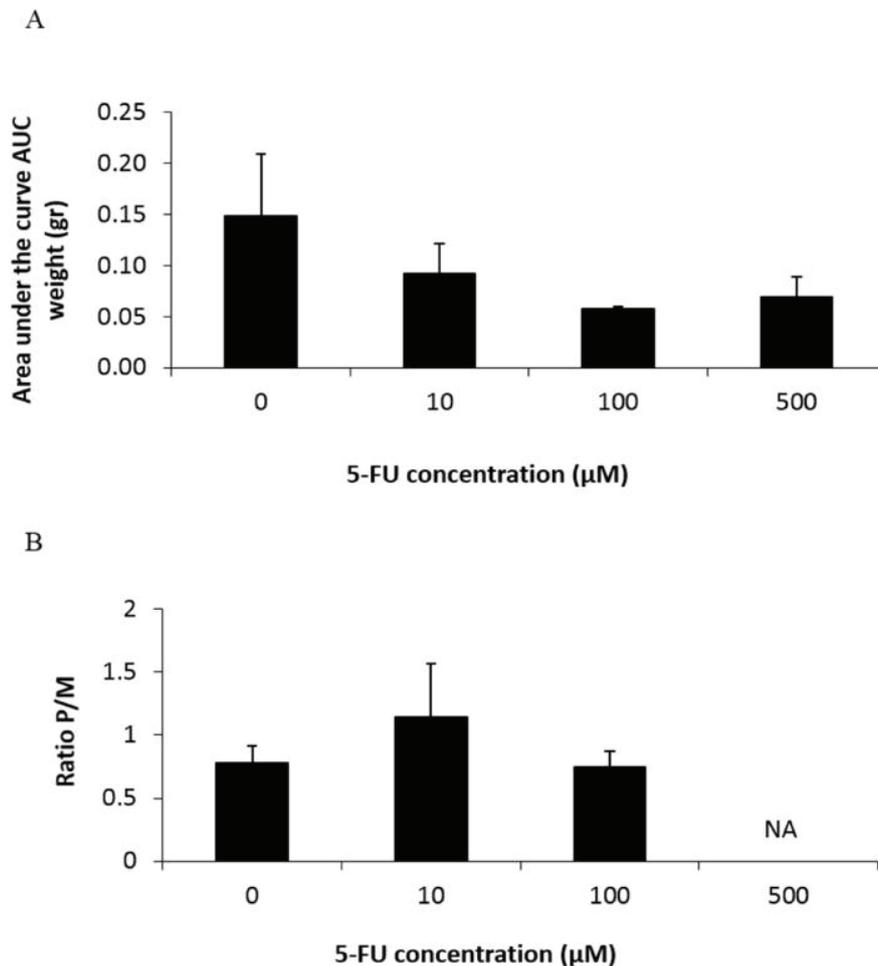


Figure. 28. Quantitative estimation of the overall mRNA translation. The polysomal profiles were quantitated by estimating the area under the curves (AUCs) and the ratios P/M were calculated for samples treated with 0, 10, 100 and 500 μ M of 5-FU.

3.2. 5-FU effect on translation

The next step was to identify the mRNA whose distribution varies between M and P fractions according to treatment with 10 μ M 5-FU. For this, RNA contained within M and P fractions were extracted, quantified and analyzed by Affymetrix Exon Arrays.

RNAs were extracted from two independent experiments and were subjected to bioanalyzer to determine RNA quality. RNA integrity number (RIN) allowed to evaluate RNA quality. RNA samples with a RIN above 6 are validated for this type of analysis (Table. 2).

Table. 2. Summary of the RNA integrity numbers (RINs). The RINs have been determined by the bioanalyzer for RNA extracted from monosomes (M) and polysomes (P) of control and (10 μ M) 5-FU treated cells. The quality control analysis of samples accepted RINs that were > 6.

Sample name	RIN	Sample name	RIN
Ctl/M (1)	6.60	10 μ M 5-FU/M (1)	7.60
Ctl/P (1)	6.50	10 μ M 5-FU/P (1)	7.70
Ctl/M (2)	9.30	10 μ M 5-FU/M (2)	9
Ctl/P (2)	8.60	10 μ M 5-FU/P (2)	8.80

Identification of translationally regulated genes after 5-FU treatment

The polysomes profiles (previously described in 3.1) allowed the separation of monosomes (inactive mRNAs) from polysomes (active mRNAs) for both the control and 5-FU treated cells (Scheme shown in Fig. 26). The isolated monosomal and polysomal mRNAs of control and treated cells were subjected to microarray analysis.

The ratio (P/M) of signal intensity of polysome (active mRNA) to the signal intensity of monosome (inactive mRNA) was calculated for each mRNA. This ratio determines the abundance of a unique mRNA in polysome versus monosome. Then, the P/M ratios that determine the mRNAs translational status in both treated and non-treated cells were calculated (*i.e.* P/M_(5-FU) and P/M_(ctl), respectively). Next, we have calculated the ratio of ratio R2 (ratio 5-FU/ ratio ctl) (as shown in scheme Fig. 26). The ratio of ratio R2 presents the translational status fold change for each mRNA. It allowed detecting the mRNAs that have different translational status upon 5-FU treatment.

In HCT116 cells, 14728 genes were expressed. Among these expressed genes, 1260 genes were translationally regulated after 5-FU treatment. Using cutoff values of $-1.3 \geq R2 \geq 1.3$, we have identified 855 genes with altered translational status, 642 (75.08 %) genes were translationally up regulated while 213 genes (24.91 %) were down translationally regulated. A complete list of translationally regulated (up and down) mRNAs with their translational status fold change is reported in (Table .S4).

Validation of predictive translationally regulated genes by quantitative RT-PCR

We have selected a subset of 13 genes for qRT-PCR validation depending on their fold change (FC) in microarray and their suitability for the designing of qRT-PCR primers. The ratio of ratio (FC) for qRT-PCR was calculated as demonstrated in (Fig. 26). Validation was carried out on monosomal and polysomal RNAs that were subjected to microarray experiments, and to RNAs extracted from independent experiments. In the qRT-PCR results, the FRK, HIVEP2, NUDT7, TAF1B, IL18 and HOXB9 mRNAs were translationally upregulated indicating that they were more associated with polysomes in 5-FU treated cells than in non-treated cells (Fig. 29A). The ASF1B, SRP68, LMNB1, STIP1, TBCD, CHMP5 and L3MBTL3 mRNAs were more presented in monosomes of treated cells than in control cells, which signify their translational down regulation (Fig. 29B). The quantitative PCR results are correlated with results obtained from microarrays and are presented in (Fig. 29C).

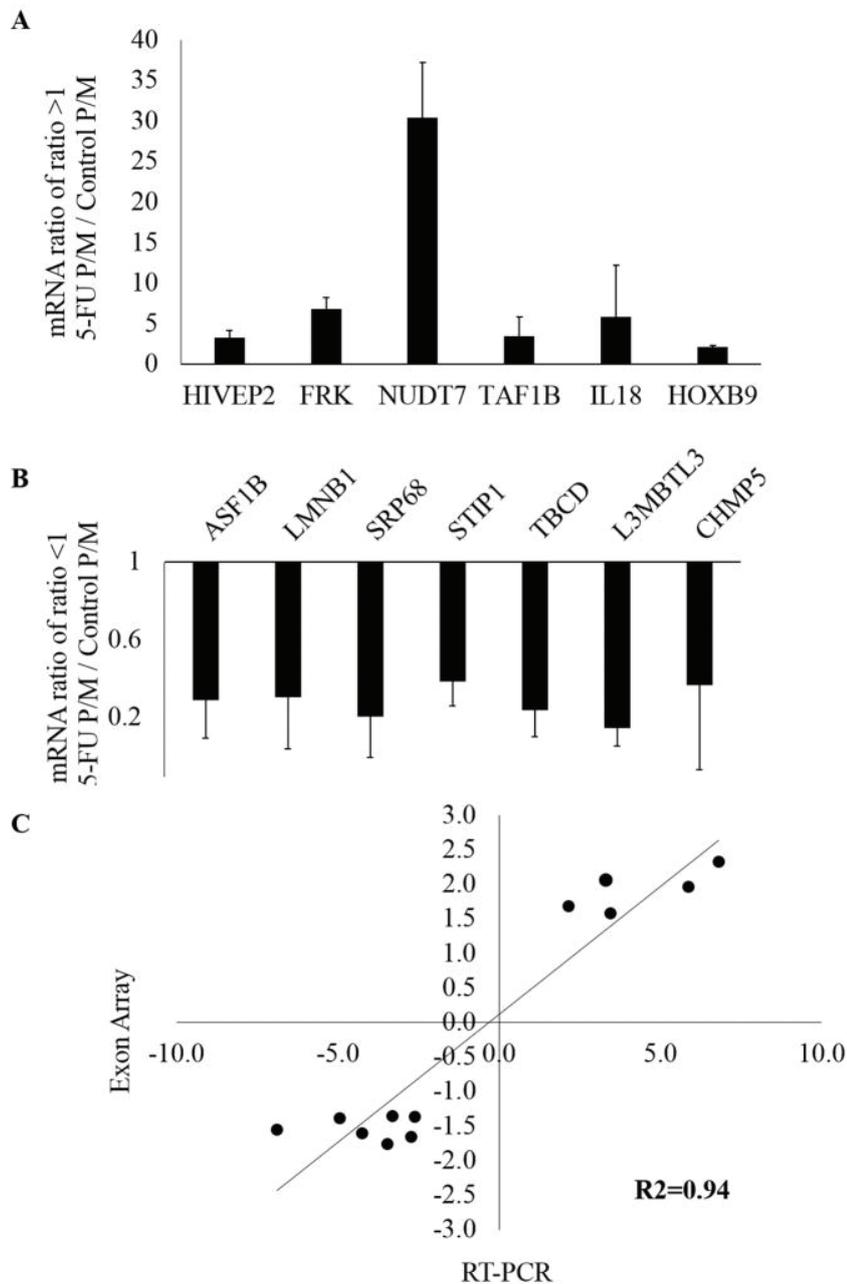


Figure. 29. RT-qPCR analyses of translationally upregulated mRNAs in HCT116 cells treated with 5-FU for 24h. (A-B) RT-qPCR analyses of translationally up- and downregulated mRNAs in HCT116 cells treated with 5-FU for 24h. C) Correlation between the average fold change of gene translational status determined by RT-qPCR and microarray after 10 μ M 5-FU treatment in HCT116. Mean values \pm s.d. were established based on the results from at least three independent experiments each with three replicates.

Functional analysis of the translationally regulated genes

The microarray gene expression data can be analyzed depending on distinct criteria. The genes can be classified basing on their different expressions under precise experimental conditions. In our assay, we have investigated the functional categories that are enriched as a response to 5-FU treatment after 24h. Our data concerning the 855 genes, which are translationally regulated, are processed using PANTHER GO annotation online software (<http://www.pantherdb.org/>). Panther GO can generate lists of genes according to their biological process, molecular function and protein class (Mi, Muruganujan et al. 2013; Mi, Muruganujan et al. 2013).

Biological processes

The biological functions analysis of the translationally-regulated genes indicates that 5-FU as antimetabolite anti-cancer drug influences many distinct biological themes that reflect its mechanism of action.

We have identified (350) genes involved in the primary metabolic process, (398) genes for metabolic process and (182) genes for nucleobase containing compound metabolic process. Genes responsible of biological process regulation counted (120) genes. Groups of genes implicated in protein metabolic process, translation, transcription, RNA metabolic and cellular protein modification were also identified (Fig. 30A). We have listed subsets examples of three biological categories in (Article 2: Table. S2A). Clearly, the translationally regulated-gene expression pattern after 5-FU application in HCT116 cells have two main features for the biological processes. Many sets of genes are responsible for various metabolic processes and/or DNA transcription. In addition, we have noted a set of genes involved in translation (Article 2: Table. S3A).

Molecular functions

We have characterized the functions of regulated genes at the molecular scale. A large numbers of genes were identified with nucleic acid binding activity (180), DNA binding transcription factor activity (102), DNA binding activity (114) and a small group structural elements of ribosomes (19) (Fig. 30B). The strongly presented gene expression signatures are nucleic acids binding activity and transcription. As well as, an enrichment in structural elements of the ribosomes, which signifies that the translation process is also implicated. A number of genes involved in different molecular functions have been reported in (Article 2: Table. S3B).

Protein classes

We have grouped the translationally regulated genes into functional protein classes as shown (Fig. 30C) and (Article 2: Table. 2SC and S3C). The first class of regulated proteins contains about (59) zinc fingers. Interestingly, transcriptional factors present a large class of regulated proteins, (40) for KRAB box transcription factors (40) and transcription factors (104). The nucleic acid binding function represent (126) proteins. In addition to (14) different kinase inhibitors and (20) ribosomal proteins.

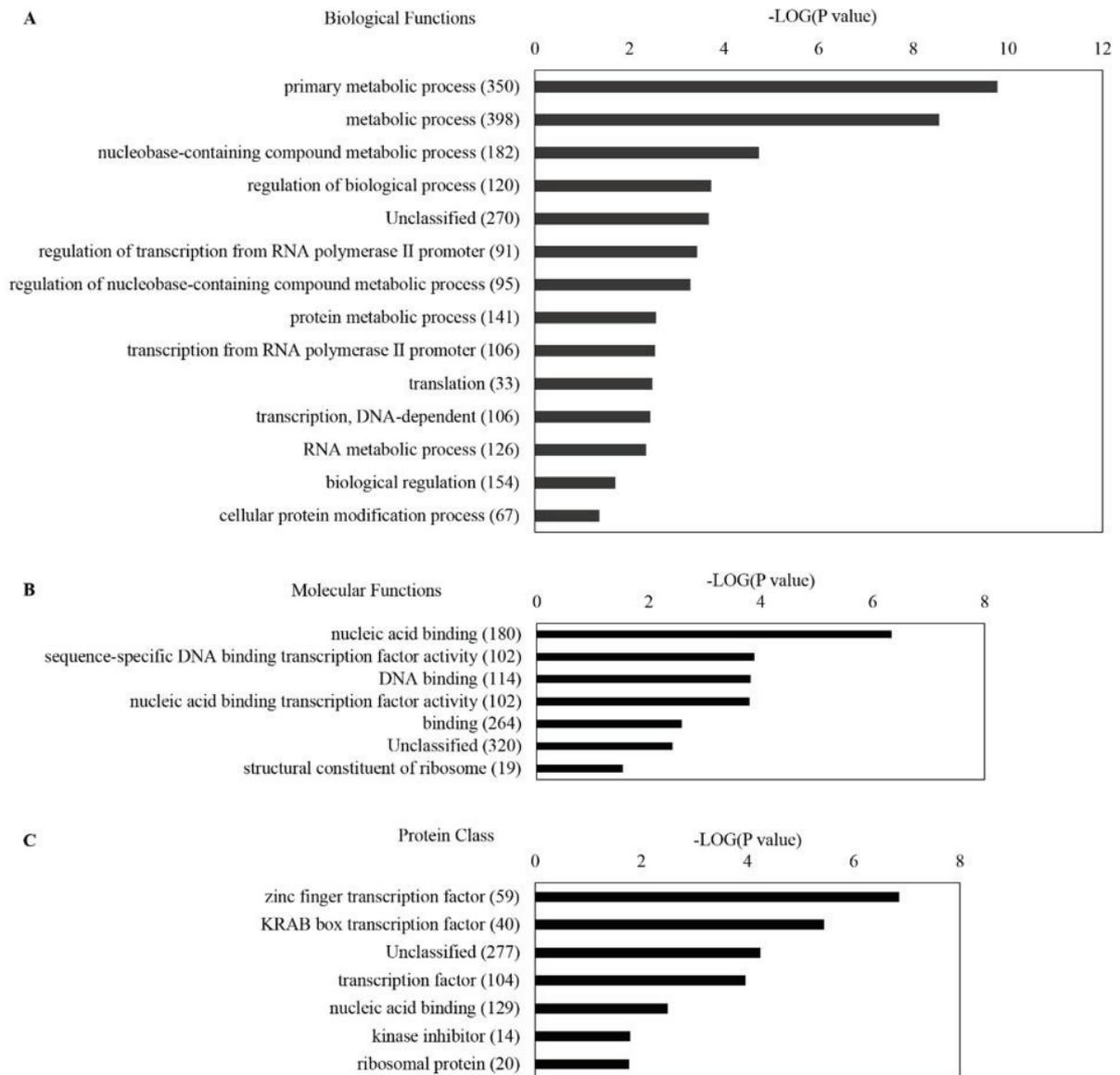


Figure. 30. Gene ontology (GO) analysis of translationally deregulated genes. Genes are distributed into groups according to their A) biological function, B) molecular function and C) protein class using PANTHER database.

4. 5-FU regulates HIVEP2 translation by miRNA-dependent mechanism

4.1. Effect of 5-FU on HIVEP2 expression

As exposed above determination of HIVEP2 mRNA polysomal distribution by microarray analysis and by RT-qPCR showed that 5-FU induces a translational upregulation of HIVEP2 gene. To have more comprehensive view of the effect of 5-FU on HIVEP2 gene's regulation, we examined the variation of HIVEP2 mRNA after treatment with 5-FU (Fig. 31A). A treatment of 10 μ M of 5-FU during 24 h induced a decrease of about 85% of the total amount of HIVEP2 transcripts, nuclear and cytoplasmic. However, this decrease was only about 40% for the cytoplasmic HIVEP2 transcripts. Therefore, these results showed that although 5-FU induced a decrease in the amount of HIVE2 mRNA, it induced in parallel a shift of the mRNA from monosome fractions to polysomal fractions suggesting an increase in the translational efficiency of the mRNA. The next step was therefore to determine by which molecular mechanism 5-FU could increase specifically translational efficiency of this particular mRNA.

4.2. miR-155 regulates HIVEP2 expression *via* its 3'UTR response element

The 3'UTR of HIVEP2 gene is an experimentally validated target of miR-155 (Yin, McBride et al. 2008; Yin, Wang et al. 2010). Therefore we hypothesized that the effect of 5-FU on translational activity of HIVEP2 mRNA could be mediated by miR155. To verify the effect of 5-FU on both miR-155 and HIVEP2, we have used the miR-155 vector and the appropriate HIVEP2 3'UTR reporter plasmid. First, we confirmed that, as expected, translation of a reporter mRNA harboring the corresponding miR155 response element in its 3'UTR region was inhibited by miR-155 in in our experimental system.

For this, we have used two reporter plasmids containing the miR-155 response element, BACH1 and HIVEP2. HCT116 cells were transfected with these two reporters BACH1, HIVEP2 and with the corresponding plasmid with no response element inserted in its 3'UTR together with either a miR-155 expression vector or a control vector. As shown in (Fig. 32A), miR-155 suppressed significantly the expression of 3'UTR of BACH1 and HIVEP2 containing the miR-155 response element compare to that of the control plasmid.

Then several experiments were carried out to determine the suitable concentration of HIVEP2 reporter plasmid allowing to identify expression variations when the system will be submitted to 5-FU treatments. For this, HCT116 cells were transfected with a panel of luciferase reporter plasmids, HIVEP2 and control (50, 100, 200, 300, 400, 500, 600, 700 and 800 ng) (Fig. 32B). The luciferase activity was measured and normalized to the protein activity in cells transfected with 50 ng of control reporter. The HIVEP2 3'UTR reporter concentration of 50 ng had the lowest luciferase activity, its activity increased steadily in parallel to increase of concentrations. The HIVEP2 reporter concentrations from 400 ng to 800 ng displayed relatively

stable luciferase activity. From these experiments it appeared that the concentration of 200 ng of HIVEP2 reporter plasmid was appropriate.

Next, we have evaluated the appropriate concentration of miR-155 vector. HCT116 cells were cotransfected with 3'UTR HIVEP2 luciferase reporter and different miR-155 vector concentrations (250, 500, 750 and 1000 ng), corresponding transfection controls were performed in parallel. Luciferase activity measurement of each miR-155 condition was normalized using the value of the control. As shown in (Fig. 32C), the lowest miR-155 concentration (250 ng) suppressed modestly HIVEP2 reporter expression. However, significant inhibition of HIVEP2 3'UTR reporter expression was observed with 500 ng and even more with 750 ng and 1000 ng of miR-155 vector.

At this stage, we had therefore defined the experimental system to determine whether the 5-FU induced increase of HIVEP2 translation was mediated by miR-155 and by the presence of its response element within the 3'UTR of mRNA. For this, HCT116 cells were co-transfected with miR-155 vector and HIVEP2 3'UTR luciferase reporter. Twenty-four hours after transfection treatment with 10 μ M 5-FU for 24h after was performed. The relative luciferase activity was normalized to non-treated cells co-transfected with miR control vector and control luciferase reporter. As expected, miR-155 suppressed the luciferase activity through HIVEP2 3'UTR compare to control. As shown in (Fig. 32D), 5-FU treatment increased the activity of luciferase activity of the HIVEP2 reporter vector and this increase was reduced by miR155 transfection to restore the normal expression found in non-treated control cells. Interestingly, this result demonstrate that 5-FU is able to up-regulate HIVEP2 translation by a miR-155 dependent mechanism.

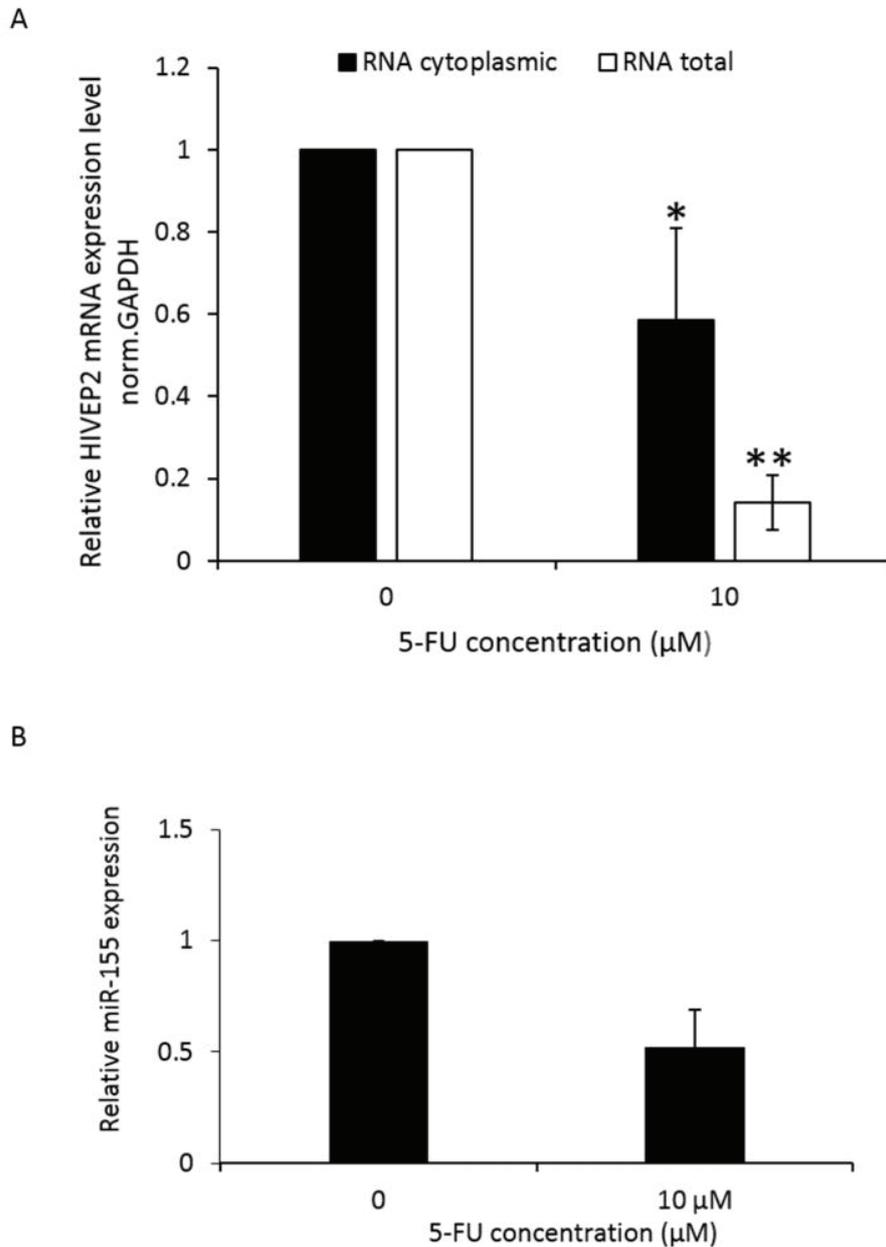


Figure. 31. Real time PCR analysis of HIVEP2 mRNAs and miR-155 in HCT116 cells. A. RT-qPCR analyses of HIVEP2 mRNA in both cytoplasmic and total mRNAs in control and 5-FU treated cells for 24h. B. The expression of miR-155 in control and 5-FU treated cells for 24h. Mean values \pm s.d. were established based on the results from three independent experiments each with 3 replicates.

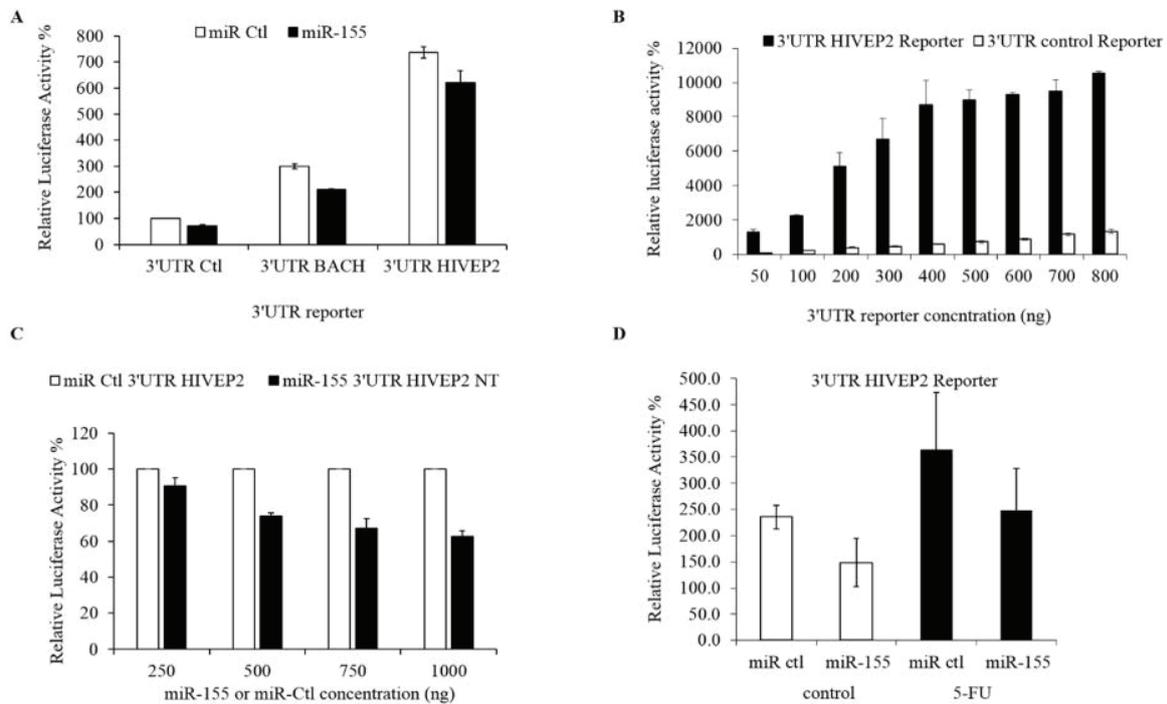


Figure. 32. Analysis of the effect of miR-155 on 3'UTR reporter targets in HCT116 cells. A. The analysis of HIVEP2 and BACH1 3'UTR luciferase reporters and the corresponding reporters with either miR-155 or miR control in both control and 5-FU treated cells for 24h. B. The expression of 3'UTR reporter of HIVEP2 in HCT116 cells using different concentrations: 50, 100, 200, 300, 400, 500, 600, 700 and 800 ng. C. The inhibitory effect of miR-155 on 3'UTR HIVEP2 reporter using different miR-155 concentrations: 250, 500, 750 and 1000 ng, all values were relative to the respective reporter cotransfected with the miR control expression vector. (A-C) the standard deviations were derived from triplicate transfections. D. The analysis of HIVEP2 3'UTR luciferase reporter expression with either miR-155 or miR control in both non-treated and 5-FU treated cells for 24h. Mean values \pm s.d. were derived from three independent experiments each with 3 replicates.

4.3. 5-FU inhibits miR-155 expression

Finally, to assess firmly that 5-FU increases HIVEP2 translation by a miR155 dependent mechanism it was necessary to determine whether 5-FU modulates expression of endogenous miR-155, as it has been shown for many microRNAs (Shah, Pan et al. 2011). The expression of miR-155 was measured by quantitative RT-qPCR in control and cells treated with 10 μ M 5-FU for 24h. As shown in (Fig. 31B), the expression of miR-155 is significantly reduced by approximately 50% in 5-FU treated cells compared to non-treated cells. Indeed, these results suggest that 5-FU inhibits the expression of miR-155 and this accordance with HIVEP2 translational upregulation.

4.4. HIVEP2 silencing impairs HCT-116 cell growth

To determine the effect of HIVEP2 on HCT-116 cell growth, various concentrations of HIVEP2-siRNA were transfected in HCT-116 cells and the real-time effect on cell proliferation (Fig. 33) was investigated using the XCELLigence technology (ACEA Biosciences).

A reduction in HIVEP2 expression of 55% was attained with 150 pM of transfected siRNA after 24 hours. We designed an RNA with no homology to any known mammalian gene and used it as an siRNA control (mock). Twenty-four hours after transfection, HCT-116 cells were seeded on E-plates and the real-time impedimetric signal generated by variations in cellular density was recorded every 15 minutes. We observed different patterns of cellular growth that discriminate between mock and HIVEP2-siRNA transfected cells. The HCT-116 control cells displayed a steady increase in cellular index (CI) characterized by an exponential growth starting 12 hours after seeding to reach a plateau at day 3 (Fig. 33A). The silencing of HIVEP2 gene expression in HCT-116 cells clearly enhances cellular growth as compared to control. The increase in impedimetric signal was detected 33 hours after seeding and the plateau could not be reached at day 3, the end of the experiment (Fig. 33A). Cell growth characteristic was estimated using a build-in software that calculates the slope coefficient between 0 and 48 hours after seeding. The analysis indicated that HCT-116 cells with reduced HIVEP2 expression have an increase of 70 % growth as compared to the HCT-116 control cells (Fig. 33B). The increase in cell growth calculated for the first 48 hours was maintained during the entire time of the experiment (Fig. 33B). Thus, HIVEP silencing alters HCT-116 cells functions leading to a robust increase in cellular growth.

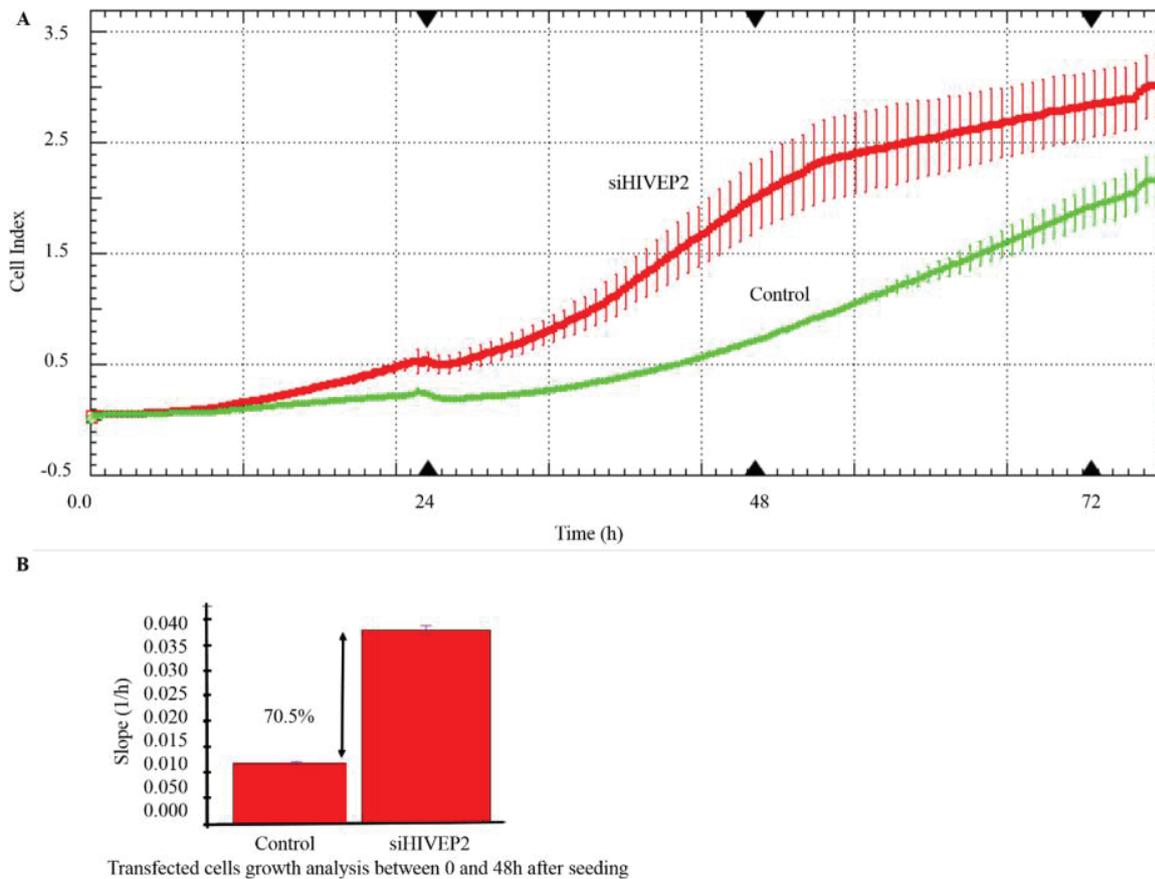


Figure. 33. Real-time monitoring analysis of HCT116 cells after HIVEP2 silencing. *A.* The real time curves of cell index (CI) during time (h) after 24h of HIVEP2 siRNA transfection. *B.* The diagram shows the slopes (cell index vs time (h)) during the 24-72h of HIVEP2 siRNA transfection. The slope values were calculated from xCELLigenec real-time measurements between 24-72h of transfection. Data represent a presentative experiment repeated two times. The standard deviation of quadruplicates are displayed; no normalization is needed.

DISCUSSION

One of the main objectives of this study was to evaluate the effect of low doses of 5-FU on translational regulation to identify the 5-FU mediated modification of gene network that could account, at least in part, for 5-FU treatment failure and/or cellular adaptation resulting in acquisition of 5-FU resistance. For this, the first step was to establish the experimental conditions allowing to analyze 5-FU effect during a time and dose window corresponding to this objective. Then, effect of 5-FU was analyzed on global protein synthesis efficiency and on translational activity of defined mRNA.

Effect of low doses of 5-FU on cell behavior

Optimization of 5-FU treatment conditions was performed before investigating the effect of 5-FU on translational control. Indeed, investigating the impact of 5-FU on translation regulation requires identification of concentration and time of treatment allowing harvesting of living cells. Moreover, the phenotype of treated cells at the identified condition has to be carefully described to compare functions of genes whose translation is deregulated in response to 5-FU and thus, potentially identify a gene expression/phenotype relationship in response to 5-FU.

To identify the optimal 5-FU doses for such approach, colorectal cancer cells HCT116 were treated with increasing concentrations of 5-FU and several approaches were used to analyze the 5-FU-related cellular phenotypes. In addition to the classical end point technics (MTS assays, trypan blue staining), we performed real-time monitoring to finely determine the optimal treatment duration (XCELLigence, Time-lapse). Using all these approaches, we identified two different 5-FU doses associated with two different phenotypes. First, the lethal doses with a 100% cytotoxicity correspond to doses higher than the calculated $IC_{50(24h)}=12.9\mu M$. Compared to non-treated cells, cells treated at high doses showed a rapid decrease in cell viability and cell contact associated with an increase in cell mortality. Moreover, lethal doses kill all the cells present in the dishes although the timing of 100% cytotoxicity appearance is dose-dependent. Due to the complete cell death resulting from lethal doses, the usage of high doses of 5-FU were thus excluded from the analysis. Second, sublethal doses were identified at doses lower than the calculated IC_{50} . Compared to lethal doses, cell treatment with low-doses 5-FU ($10\mu M$) did not cause a dramatic cell mortality. Indeed, only few dead cells were counted 24h post-treatment and cells maintained their viability. Similarly, studies using chick smooth muscle cells showed that low-dose 5-FU treatment induced a weak inhibition in proliferation and slight reduced viability, these effects were increased to 70% using high doses (Filgueiras Mde, Morrot et al. 2013). More importantly, a population of alive cells was observed long after 5-FU treatment (up to 140 h) suggesting that some cells have resisted to the cytotoxic effect of 5-FU. Overall, these observations demonstrated that in contrary to high-doses, low-doses of 5-FU did not induce death of all cell population. These results are in agreement with data obtained from human lung carcinoma epithelial cells A549 (Abassi, Xi et al. 2009), and HeLa cells treated with different 5-FU doses (Abassi 2010). In addition, it has to be noted that compared to non-treated cells, low doses of 5-FU increased the number cell contacts during the first 24 h post-treatment. Based on the time-lapse movies, this increase in cell contact may result from a change in cell morphology and/or in cell motility at $10\mu M$. Therefore, due to the sublethal phenotypes resulting from 5-FU treatment at $10\mu M$ and the unexpected increase in cell contact

revealed by real-time monitoring during the first 24h, the following analyses of 5-FU on translational control were performed at 24h post-treatment with that dose of 5FU.

Effect of 5-FU on protein synthesis and ribosomes production

We have first attempted to assess whether 5-FU could affect global translation by evaluation of [³⁵S] methionine incorporation into proteins following 5-FU treatment. This evaluation showed a weak reduction in protein synthesis when cells were treated with 100μM 5-FU or 200μM oxaliplatin during 24h. Unfortunately, at this stage, the corresponding experiment with a treatment of 10μM 5-FU during 24h has not been performed. Nevertheless, from this experiment it can be concluded that 5-FU induces only a slight inhibition of global protein synthesis rate, even at high doses known to induce a strong cytotoxic effect. These results are in concordance with data from literature showing that *in vitro* translation of mRNAs extracted from HT29 cells treated for 24h with 100μM 5-FU caused only a slight reduction in mRNA translation capacity. However, the treatment with 1000μM had generate no qualitative changes in translation after 2h. The treatment with lethal 5-FU doses has not produced any important quantitative or qualitative modifications in translational activity (Glazer and Hartman 1983). Furthermore, this conclusion is strengthened by our observation, albeit performed in a different experimental system with other cytotoxic drugs used in divers chemotherapeutic treatments, that doxorubicin induces a strong inhibition of protein synthesis.

Thus, we have investigated drug effect on ribosome quantity per cell that represents one of the feature of global protein synthesis. Low-dose 5-FU induces a light decrease of ribosomes quantity per cell, and reduced the monosomal fraction of polysome profile. As expected, high doses caused a strong decrease in ribosome quantities consistent with generally reduced or disfigured polysome profile. More precisely, pulse-chase experiments indicated that high and low doses of 5-FU has reduced neosynthesized rRNAs by 30%-80%. Additionally, total cytoplasmic rRNAs were partially decreased. These results show that 5-FU treated cells have always the capacity to produce ribosomes but less than non-treated cells, even though, at this stage of the study, it was unknown if these ribosomes were active and could maintain the translation process.

Therefore, we studied polysomal distribution after 5-FU treatment using sucrose gradient separation and we quantified the area under the curves (AUCs). As expected, both high and low 5-FU doses caused a global decrease in ribosomes quantity in accordance with the previous observations about the reductions in rRNAs. Interestingly, the calculation of P/M ratio showed a no dramatic changes in polysomal fraction after treatment with low dose 5-FU. In addition, *in vitro* experiments demonstrated that ribosomes produced under 5-FU could translate efficiently several reporters of different genes (Article 2, Fig. 5). Therefore, we have wondered whether the ribosomes present in the polysomal fraction selectively translate certain sets of mRNAs.

Effect of 5-FU on translation regulation

Because 5-FU induces a modulation of global translational efficiency, we have performed a microarray analysis of cytoplasmic mRNA localized within the sub-polysomal and within the polysomal fractions to identify the mRNA whose translational efficiency was changed following

5-FU treatment. Genes were classified according to three groups. Genes whose mRNA was more present within the polysomal fractions than in sub-polysomal fraction after 5-FU treatment compared to the non-treated condition (UP); genes whose mRNA was less present within the polysomal fractions after 5-FU treatment (DOWN) and genes whose mRNA distribution was unchanged after 5-FU treatment. Identity of the genes for each groups first allowed to show that about 65% of genes were subjected to change in translational control in response to 5-FU treatment. We also unexpectedly find out that low doses of 5-FU induced the translational upregulation of the majority of the translationally modified genes (~75%) whereas only a quarter of these genes (~25%) were translationally downregulated. The gene ontology analysis revealed the enrichment of translationally modified, which are involved in several biological functions, in particular regulation of gene expression (translation and transcription), cell proliferation, tumor suppression and metabolism.

5-FU regulates genes involved in translation

The functional analysis of genes regulated by 5-FU allowed to identify a group of translationally modified genes involved in the translation process. The majority of these genes encodes either ribosomal proteins (RPS15a, RPS16, RPL32 and RPL35a) or translation factors (eIF3E and eEF1A1) and exhibited translational upregulation.

This observation is concordant with a previous study, which investigated translational modifications occurring in response to 5-FU treatment using global approaches. Indeed, comparison of mRNA present under translationally active ribosomes purified using Hsp70 immunoprecipitation between non-treated HCT116 cells and cells treated with 5 μ M 5-FU for 24h revealed an enrichment of translationally modified genes encoding translational components or RNA-binding proteins that represented 40% of translationally modified genes in this study (Kudo, Xi et al. 2010). Among them, an upregulation of translation was observed for RPS15a, RPS16 and RPL35a, like in our study. The identification of genes whose translation is upregulated in response to 5-FU in HCT116 is similar to ours using a different strategy supports our data. In contrast, it has to be noted that analysis of RKO proteome cells in response to 5-FU treatment at 10 μ M for 12h showed a downregulation of proteins involved in translation (RPL18, eIF3H) (Marin-Vicente, Lyutvinskiy et al. 2013). This observation suggests that modulation of translation by 5-FU is dependent upon cell type and time of treatment.

Several of these genes involved in translation have shown a crucial survival role in cancer cells. Different studies have performed gene silencing experiments and have shown independently that downregulation of eIF3e in glioblastoma multiforme cells (Sesen, Cammas et al. 2014), eEF1A1 in Jurkat cells (Huang, Hu et al. 2012) and RPS15a in hepatocellular carcinoma cells (Xu, Wang et al. 2014) induce cell cycle arrest, inhibit proliferation and induce apoptosis (except for RPS15 no experiments have been performed to detect apoptosis). Furthermore, some of the genes that we have identified have been reported to be implicated in tumor progression or resistance. A genome-wide screening has been performed on prostate cancer cells at different progression stages. RPS16 and RPL32 were found to be upregulated when cells underwent transition from sensitive androgen-dependent phenotype to the androgen-independent phenotype, which is associated with poor prognosis and metastasis. These results demonstrate the implication of RPS16

and RPL32 in tumor pathogenesis (Karan, Kelly et al. 2002). Interestingly, the overexpression of RPL35a has increased survival and inhibited cell death in Jurkat cells submitted to different stress conditions that induce apoptosis such as UV irradiation and doxorubicin treatment (Lopez, Martinovsky et al. 2002). Because taken together these studies reveal the crucial and positive role of genes encoding ribosomal proteins and translation factors in tumor cells proliferation, progression and resistance it can be suggested that translational upregulation of these genes could participate to the maintenance of cell survival and inhibit cell death in cells treated with sub-lethal doses (10 μ M) of 5-FU.

5-FU regulates genes involved in metabolism

5-FU is an anti-metabolite anti-cancer drug and it is expected to affect the translation regulation of genes with metabolic functions.

Several kinases have also been translationally upregulated by 5-FU such as ABL2, PIK3C2B and FRK. ABL2 is a non-receptor tyrosine kinase that regulates cell growth and survival. ABL2 has been reported to be activated in leukemia, melanoma, colon and breast cancers. Additionally, it promoted proliferation in breast cancer cells under stress condition (nutrient deprivation) (Srinivasan, Sims et al. 2008; Greuber, Smith-Pearson et al. 2013). PIK3C2B is involved in PI3K/AKT signaling pathway that play an important role in regulating cell survival, metabolism and cell death. Experiments have demonstrated that PI3K2CB overexpression inhibits the cisplatin-induced apoptosis in oesophageal cell carcinoma via AKT signalling while PIK3C2B knockdown has sensitized cells to cisplatin and increased apoptosis (Liu, Sun et al. 2011). FRK is another upregulated kinase that belongs to src non-receptor kinase that was reported to have an oncogenic and tumor suppressor functions. A recent study performed on hepatocellular carcinoma patients' samples has demonstrated an increased in FRK expression by 52%. Moreover, the expression of FRK was correlated with the invasion capacities in hepatocellular carcinoma cell lines (Chen, Hung et al. 2013). However, FRK was reported to decrease proliferation and arrest cell cycle in breast cancer cell lines (Meyer, Xu et al. 2003). Again, upregulation of translation of the mRNA coding for these metabolic proteins could influence a number of process to maintain cell survival and keep the balance between proliferation and cell death and thus either explain the delays in cytotoxicity observed at 10 μ M 5-FU treatment compared to high doses or the escape of some cells to the cytotoxic 5-FU effect.

However, we cannot exclude that 5-FU addition constrains cell to metabolize this drug and thus promotes accumulation of dUTP. Thus, change in translation of genes involve in metabolism can only reflect the forced metabolism of 5-FU and is probably not involved in either the cytotoxic effects of 5-FU nor the survival of a cellular subpopulation at long terms. A proteomic study performed with uracil provided supporting this hypothesis (Marin-Vicente, Lyutvinskiy et al. 2013), although an increasing litterature show the role of metabolism in cell survival.

5-FU regulates genes involved in cell proliferation and tumor suppression

Our study revealed that 5-FU downregulated Asf1b at the translational level. Asf1b is an anti-silencing function 1B histone chaperon; it has been reported as a new proliferation marker with an important prognostic and diagnostic values for breast cancer patients (Corpet, De Koning et al. 2011). In both breast cancer cell lines and patients samples, it has been demonstrated that Asf1b was upregulated in highly proliferative cancer cells. The Asf1b was overexpressed by more than 2 folds at mRNA level, and more that 5 folds at protein levels in cancer cells compared to normal cells, these results indicate that Asf1b is regulated at translational level. Furthermore, Asf1b expression was highly correlated with proliferation status. In MCF7 breast cancer cells that has been rendered quiescent, Asf1b was down regulated at both mRNA and protein levels (Corpet, De Koning et al. 2011). Additionally, Asf1b expression was investigated in young and old human diploid primary fibroblasts which were different in their proliferation capability. Interestingly, it has been shown that Asf1b displayed a strong downregulation in senescent cells and is highly correlated to the proliferation status of cells (Corpet, De Koning et al. 2011). This suggests that the translational regulation of this gene observed in other biological conditions is key to target expression of this gene as a major actor cell response to 5-FU.

Another gene we have validated to be downregulated by 5-FU is STIP1. STIP1 stress induced phosphoprotein 1 is another biomarker with a prognosis interest. A study carried out on 330 samples of ovarian tumors revealed that STIP1 expression was associated with overall survival decrease, advanced cancer stage and cancer invasion. It has been demonstrated in ovarian cancer cells that recombinant STIP1 treatment induced cell proliferation and migration. However, STIP1 inhibition has withdrawn these effects (Chao, Lai et al. 2013). *CSN3* encodes the third component of COP9 signalosome complex (CSN). *CSN3* has reported to be upregulated in hepatocellular carcinoma (HCC) tissues samples. *CSN3* knockdown in HCC cell lines inhibits cell proliferation and induces apoptosis. Additionally, it has been proposed that *CSN3* knockdown could arrest cell cycle and induces quiescence. In vivo experiments showed that *CSN3* knockdown reduced considerably tumor growth in xenograft nude mice. These data indicate that *CSN3* acts as an oncogene and retain proliferation (Yu, Tang et al. 2012). Translational downregulation of these two genes at the translational level is in favor of an anti-proliferation action of 5-FU.

In addition, our study, revealed upregulation of genes acting as tumor suppressors such as *GNG7*, *RASSF6*, *RREB1* and *SOCS6* genes. This suggested that these genes could be involved in cytotoxic effect of 5-FU. The human G protein gamma 7 (*GNG7*) is reported to be frequently downregulated in many cancers such as pancreatic, gastrointestinal, head and neck cancers. In study on patients with oesophageal cancer, *GNG7* expression was strongly reduced and associated with poor prognosis and tumor invasion. The restoration of *GNG7* expression has reduced tumor invasion (Ohta, Mimori et al. 2008). The previous study has revealed the clinical importance of *GNG7* downregulation in patients. Another tumor suppressor gene is *RASSF6*, a new identified gene belongs to *RASSF* tumor suppressors' family, which has been reported to be downregulated during cancerogenesis. *RASSF6* has growth inhibitory and pro-apoptotic properties. *RASSF6* silencing has enhanced the tumorigenicity and its exogenous has promoted apoptosis (Allen,

Donninger et al. 2007). Ras responsive element binding protein 1 (RREBP1), is another tumor suppressor that can interact with p53 promoter and positively regulate p53 transcription. A study on osteosarcoma U2OS cells has demonstrated that RREBP1 suppression has decreased the expression of p53 and its target genes at both mRNA and protein levels (Liu, Hew et al. 2009). The suppressor of cytokine signalling 6 (SOCS6) has recently arisen as a tumor suppressor that reported to be frequently downregulated in different cancers such as stomach, prostate and colon cancers. In gastric tumors, it has been shown that SOCS6 had a suppressive function by inhibiting cell growth (Lai, Hsiao et al. 2010). The meta-analysis of previous microarrays data on colorectal cancer have reported that SOCS6 has low expression level in CRCs. SOCS6 was considered as a diagnostic biomarker, whereas the analysis of CRC patients samples has demonstrated that SOCS6 mRNA and protein levels was reduced (Letellier, Schmitz et al. 2014).

Altogether, these findings illustrate the critical role of translation regulation that clearly leads to both inhibition of cell proliferation and activate tumor suppression, which together support a kind of cellular cytostatic (steady state) that allows cells survival without proliferation, probably as long as being submitted to other stimuli inducing either cell death or restarting of cell proliferation. In this context, a possibility exists that this new 5-FU induced translational reprogramming provides to these cells new biological features; that in terms, will be deleterious for patients' outcome if cell restart to proliferate.

5-FU regulates genes involved in transcription activity

Finally functional analysis of genes whose translation was modulated by 5-FU allowed to point out a large number of genes with transcription factor activities. Some of them were translationally upregulated by 5-FU, such as FHL3, PDLIM2, HIVEP3 and HIVEP2.

HIVEP2 and HIVEP3 are two transcription factors implicated in differentiation processes of different cellular types such as osteocytes (Saita, Takagi et al. 2007; Liu, Madiari et al. 2011) and T cells (Kimura, Hosokawa et al. 2005; Allen, Richards et al. 2007). HIVEP2 and HIVEP3 belong to ZAS proteins family, which contains two zinc fingers that can bind to specific DNA sequences such as κ B motif. HIVEP2, for human immunodeficiency virus type I enhancer-binding protein 2, is also known as MIBP1 (c-myc intron 1 binding protein 1), Schnurri-2, MBP-2 (major histocompatibility complex MHC class 1 binding protein 2) and ZAS2. It encodes a large transcriptional factor that consists of 2437 amino acids containing two C2H2 zinc finger domains. The C-terminal zinc finger domain can bind to specific DNA sequences localized in the intron 1 of c-myc (Makino, Akiyama et al. 1994). Interestingly, a microarray analysis performed in HEK293 cells has reported that HIVEP2 overexpression resulted in downregulation of genes downstream NF κ B, MYC and TGF- β . Therefore, HIVEP2 was considered as an NF κ B modulator (Iwashita, Fukuchi et al. 2012). Additionally, it has also been demonstrated that HIVEP3 can inhibit NF κ B transcription in a κ B dependent mechanism and it is strongly possible that HIVEP3 can suppress promoters of NF κ B target genes via binding to κ B motif and alter the machinery of transcription (Hong and Wu 2010).

FHL3 contains four and a half LIM domain 3, which is a conserved double zinc finger. In glioma cells, FHL3 overexpression has suppressed proliferation and significantly increased apoptosis (Han, Xin et al. 2013). Similar results have been obtained in breast cancer cell lines;

FHL3 has inhibited cell growth and induced cell cycle arrest. This antiproliferative function of FHL3 can explain FHL3 downregulation in breast cancer patients which seems necessary for cell growth (Niu, Yan et al. 2011).

PDLIM2 (PDZ and LIM domain 2) controls cell polarity, epithelial differentiation and regulates the transcription activity of several transcription factors. It has the potential to inhibit cellular transformation and maintain the phenotype of epithelial to mesenchymal transition (EMT) (Deevi, Cox et al. 2014). In breast cancer cell lines, PDLIM2 suppression induces a reversed (EMT) associated with activation status alteration of several transcription factors such as SMAD4, TP53, SATA1 and KLF4 via COP9 signalosome (Bowe, Cox et al. 2014). Interestingly, KLF4 is one of the transcription factors identified by our gene function analysis to be upregulated by 5-FU. In gastric adenocarcinoma patients, KLF4 has displayed low expression levels which were correlated with poor prognosis (Hsu, Chan et al. 2013). However, the activation of KLF4 by demethylating agent in cervical cancer cells has inhibited proliferation and enhanced the sensitivity to chemotherapy.

Altogether, these observations showed, that 5-FU affects indirectly cell transcription by regulating the translation of transcription factor genes. These genes are involved in both anti-proliferation and differentiation. Therefore, we show that low doses of 5-FU induces a translational reprogramming that very probably results to a transcriptional reprogramming. At this stage it is tempting to speculate that this subsequent transcriptional reprogramming plays a major role in cell behavior and in acquisition of resistant phenotypes.

5-FU regulates HIVEP2 expression via a miR-155 dependent mechanism

The expression status and the role of HIVEP2 gene under 5-FU treatment have not been reported until now. In our study, we have demonstrated that HIVEP2 translation is activated after 5-FU treatment. However, although in our study we have not clearly defined the functional consequences of this activation, from the data of the literature, it can be postulated that the activation of HIVEP2 expression account for the mechanisms leading to establish a 5-FU induced cellular steady state status that we propose to be compared to a “quiescence-like or senescence-like” status. Indeed, HIVEP2 has been reported to be absent from proliferative cells and was shown to regulate differentiation. As such, HIVEP2 could be considered as a regulator of the balance between cell proliferation and/or transformation and differentiation. HIVEP2 was reported as a differentiation regulator in T cells differentiation and bone formation through its direct interaction with Smad1/4, which mediates some of bone morphogenetic signaling (Jin, Takagi et al. 2006). It can also interact with CLIC4 protein (chloride intracellular channel 4), which is critical for p53 and c-Myc mediated apoptosis. HIVEP2 and CLIC4 act as regulators of TGF β signaling by stabilizing the phosphorylation of Smad 2 and Smad 3 proteins (Shukla, Malik et al. 2009; Shukla and Yuspa 2010). In neuronal tissues, HIVEP2 is not expressed in proliferative zones while it is abundant in post-mitotic cells. In glioma, HIVEP2 was over-expressed in cells that express a differentiated phenotype (Sun, Chen et al. 2014). Interestingly, this hypothesis concerning the functional consequences of HIVEP2 as an inductor of cell quiescence is strongly supported by our

results showing that HIVEP2 silencing in HCT116 cells using RNA interference induces an activation of cell proliferation.

However, although the role of HIVEP2 is not yet clarified, our study clearly demonstrates that its expression is upregulated by 5-FU treatment at the translational level by a micro-RNA dependent mechanisms. Recently, it has been reported that HIVEP2 was regulated by miR-155 through a response element localize in the 3'UTR of the mRNA (Yin, McBride et al. 2008; Yin, Wang et al. 2010). We have transfected HCT116 cells by a luciferase reporter plasmid, which contained the 3'UTR HIVEP2 response element for miR155 inserted at the end of luciferase open reading frame. The cells were co-transfected by expression vectors coding for either miR-155 or for a control miRNA. As expected, these experiments showed that miR-155 repressed luciferase reporter expression containing the 3'UTR HIVEP2 response element and not that of the control vector. Conversely, treatment with low-dose of 5-FU (10 μ M) enhanced the expression of 3'UTR HIVEP2 containing luciferase reporter. These results indicated that 3'UTR HIVEP2 response element was sufficient to mediated the activation of expression observed by 5-FU treatment. Furthermore, experiments performed by co-expression of miR-155 together with 5-FU treatment showed that the increase of reporter gene expression induced by 5-FU was inhibited by co-expression of miR-155. This allows to firmly suggested that the 5-FU induced increase of reporter gene expression was mediated by presence of miR-155 with the HIVEP2 response element localized in the 3'UTR of the gene. These data with those from the literature suggested that 5-FU could modulate miR-155 expression. More particularly, because 5-FU stimulates reporter expression and because miRNA are generally involved in repression of mRNA translation, we postulated that 5-FU could induce a decrease of miR-155 expression. For this we have investigated the effect of 5-FU on mature miR-155. We demonstrated that 5-FU caused a significant reduction in mature miR-155 expression. In a study performed by others (Zhou, Zhou et al. 2010) also with HCT116 cells treated with 10 μ M 5-FU for 24h, the decrease of miR-155 was not pointed out. Many reasons can account for this discrepancy between the results, but the more probable is the fact that miR-155 displayed a low level of expression compare to other miRNAs and was removed during the normalization process since this study was dedicated to analyse the 5-FU induced expression variations of a very large panel of miRNAs (Zhou, Zhou et al. 2010).

Therefore, altogether our results support the notion that 5-FU induces an increase of HIVEP2 mRNA translation by inducing a decrease of miR-155 resulting in a reduction of translational inhibition through the 3'UTR response element contained within the mRNA.

Interestingly, miR-155 is considered as an oncomiR and its direct implication in cancer was demonstrated using transgenic mice with a miR-155 overexpression that leads to a neoplastic disease which can later develop lymphoblastic leukemia (Costinean, Zanasi et al. 2006). miR-155 is also involved in resistance against cancer therapies. In triple negative breast cancers treated with taxanes-based regimens, some patients exhibit worse outcome because of chemoresistance to taxanes due to the deregulation of specific miRs expression in particularly that of miR-155 (Gasparini, Cascione et al. 2014; Ouyang, Li et al. 2014). Moreover, the upregulation of miR-155 in human osteosarcoma cell line led to an induction of autophagy resulting in chemoresistance against doxorubicin and cisplatin (Chen, Jiang et al. 2014). Therefore, miR-155 has been

considered as an attractive target for anticancer drugs. Recently, specific anti-miR-155 (antisense oligomer) have been developed using a new delivery approach. The administration of anti-miR-155 on mice models with lymphoma has inhibited the expression of miR-155, as expected and resulted in tumor regression and survival advantage (Cheng, Bahal et al. 2014). Together, these studies suggest that regulation of miR-155 expression by 5-FU can modulate the cellular sensitivity and response to treatment. Therefore, we investigated the presence of other predicted or validated miR-155 target genes in our list of genes whose translation is modulated under 5-FU treatment. We found that 25% of miR-155 target genes were identified as translationally upregulated genes by 5-FU, while only 17% of miR-155 target genes were presented in 5-FU downregulated genes. This slight enrichment of genes whose translation is upregulated supports the finding that 5-FU induced modulation of miR-155 impact expression of several miR-155 targets as we have demonstrated for HIVEP2.

The effect of low dose (0.01 μ M) of 5-FU on miRNA profiles was also studied in MCF7 cells at 48h. This confirmed that 5-FU alters miRNAs expression profiles of miRNAs with many potential target genes with oncogenic, tumor suppressor and metabolic functions (Shah, Pan et al. 2011). Altogether, these results indicate that it is strongly possible that 5-FU translation regulation via miRNAs is a general process involving that implicates a large number of miRNAs.

The mechanisms by which 5-FU modulate the translational process, miRNAs production and/or function remain to be determined. The 5-FU induced modulation of translational ability could be due, at least in part, to ribosomes themselves since we have shown that 5-FU alter pre-rRNAs processing and is incorporated within mature rRNAs and, as a consequence modify their translational initiation capacities. Another possible mechanism is that 5-FU alters miRNA processing and the generation of mature miRNA either by affecting miRNA processing enzymes or by its direct incorporation into miRNA since it has been reported that downregulation of Dicer, one of the miRNA processing enzyme, is associated with 5-FU based-chemoradiotherapy resistance in oral squamous cell carcinoma (Kawahara, Nakayama et al. 2014). It is important to note that only miRNA mature form and not its expression abundance is sufficient to determine its polysome occupancy (Molotski and Soen 2012). In addition to modulating miRNA abundance, it is possible that 5-FU incorporates directly into miRNAs and affect their function in translation repression through altering miRNA polysome association. A recent study carried out on human embryonic stem cells (hESCs) and human foreskin fibroblasts (hFFs) demonstrated that miRNAs can be associated with polysomes (Molotski and Soen 2012). This miRNA-polysome association, which is also called miRNA polysome occupancy, is miRNA specific. It means that a given miRNA can have a low or high polysome association depending on miRNA sequence and the seed sequence of its mRNA target (Molotski and Soen 2012). Therefore, each miRNA has a distinct degree of polysome occupancy. For example, let7b and miR-21 were highly expressed in hFFs cells, even though they displayed different polysome associations. Furthermore, the energy required to form mRNA-miRNA duplex in high miRNA polysome occupancies was significantly different from that in low miRNA polysome occupancies (Molotski and Soen 2012). Consequently, it is possible that 5-FU incorporation into mRNAs and miRNAs can alter the energy of duplex formation, leading to changes in the miRNA polysome occupancy.

GENERAL CONCLUSION

5-FU is an anti-metabolic cytotoxic drug largely used in clinic to kill tumor cells. However, in this study, we have pointed out that a pool of cancer cells submitted to low-doses of 5-FU could escape to the cytotoxic effect of 5-FU. In addition, we have shown that low-doses of 5-FU does not induced a dramatic inhibition of translational activity but rather a translational reprogramming including translational activation of a large number of genes.

Interestingly, we have shown that 5-FU can induce translational activation of specific genes via miRNA dependent mechanism. The nature of the genes submitted to this translational activation allowed us to conclude that sub-lethal doses of 5-FU reprogram mRNA translation contributing to prevention cell proliferation but allowing long term cell survival and very probably inducing a subsequent transcriptional reprogramming accounting for initiation of a resistance mechanism. Indeed, from the results presented in this study, it will be important to investigate whether cells that have escaped to 5-FU cytotoxic effect are able to re-enter cell cycle, exhibiting an aggressive cancer phenotype eventually deleterious for the patients. This study provide data to help to continue to improve 5-FU based clinical protocols and to understand the mechanism of action of 5-FU and of resistance to this drug largely used for chemotherapeutic treatments.

ANNEXES

ARTICLE MANUSCRIPT I

Incorporation of 5-Fluorouracil into ribosomal RNA induces a modification of translational control

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ARTICLE MANUSCRIPT II

Effect of 5-Fluorouracil on translational regulation in colorectal cancer cells

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Abstract

5-Fluorouracil (5-FU) is an anti-metabolite intensely used in chemotherapeutic treatments in various cancers. It is active against a wide range of solid tumors, such as colorectal cancers (CRCs) and often used as a first line of treatment. However, the cellular and molecular mechanisms of action of this anti-cancer agent still remain to be determined. Because 5-FU is incorporated within all classes of RNA, knowledge of the different levels of gene expression regulation affected by 5-FU will help to decipher its mode of action.

We hypothesized that the translational control, one of the fundamental and highly regulated steps of gene expression, is altered by 5-FU treatment as a consequence of disrupted RNA metabolism.

In this study, the colorectal cancer cell line HCT116 has been treated or not by different doses of 5-FU for different periods of time to determine the time and dose window that induces striking modifications of cell behavior without leading to an extensive cell death. Translational reprogramming was then analyzed during this time and dose window of 10 μ M 5-FU for 24h treatment.

Cytoplasmic fractions were purified and separated through sucrose gradients to distinguish the actively translated mRNAs that are associated with more than one ribosome (polysomes) from the inactive mRNAs associated with no, or only one ribosome (monosomes). A microarray analysis was then performed to identify the mRNAs presented in monosome and polysome fractions with and without treatment. The ratio of a given mRNA between polysome and monosome fractions allowed to evaluate its translational activity. This polysome profiling approach reveals first that 5-FU treatment did not turn-off the global translation efficiency, but rather modulates translation efficiency of specific mRNAs. Secondly, more than 640 mRNAs were found to be up-translated following 5-FU treatment. These mRNAs encode for proteins involved in nucleic acids metabolism and binding, transcription regulation and translation. Some of these genes are involved in important biological processes such as cellular proliferation and premature senescence. Finally, we could demonstrate that 5-FU induced up-regulation of a newly identified target gene, named HIVEP2, also known as MIBP1 (*c-myc* intron-binding protein 1) could be mediated by a miRNA dependent mechanism and involved into HCT116 cell proliferation.

Altogether, our study will help to improve efficiency of 5-FU clinical usage that is generally administrated in combination with other anti-cancer molecules and to better understand the molecular mechanism underlying 5-FU resistance, particularly frequent in CRC of high grades and metastatic CRC.